THE *ZOSTERA MUELLERI* SEAGRASS MICROBIOME

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CERTIFICATE OF ORIGINAL AUTHORSHIP

I, Valentina Hurtado McCormick, declare that this thesis, is submitted in fulfilment of the requirements for the award of Doctor of Philosophy, in the Faculty of Science - School of Life Sciences at the University of Technology Sydney.

This thesis is wholly my own work unless otherwise referenced or acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis.

This document has not been submitted for qualifications at any other academic institution.

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'Absence of evidence is not evidence of absence' - Carl Sagan (Martin Rees)

THESIS FORMAT STATEMENT - THESIS BY COMPILATION

This thesis is formatted in accordance with the requirements of a thesis by compilation.

A thesis by compilation is structured as a single manuscript that comprises a combination of chapters and published/publishable works.

It should be noted that experimental chapters (Chapters 2 - 4) within this thesis are structured according to the requirements of each respective journal where the manuscripts have been published or submitted. However, formatting has been kept consistent throughout as much as possible.

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THESIS ABSTRACT

As many other terrestrial macroorganisms, marine benthic animals and plants establish tight relationships with a collection of microbes widely known as the microbiome. These interactions are maintained in a delicate ecological equilibrium that affects hosts' metabolism and health, and ultimately the biogeochemical processes and ecosystems that rely on them. Seagrasses are aquatic angiosperms that play critical ecological roles and provide very valuable ecosystem services. These plants are now considered holobiont models in the ocean, given the increasing evidence of the relevance of their associations with a wide range of microorganisms. However, seagrass microbiome research is still on its infancy, and our understanding of their dynamics at different spatial scales and the influence of the external environment is still very limited, particularly for the seagrass species *Zostera muelleri*. Despite the undeniable importance of microscale heterogeneity for marine microbial ecology, most investigations on the seagrass microbiome have identified microbial associates within the phyllosphere and rhizosphere, rather than at smaller microenvironmental scales that are likely most relevant to the organisms of interest.

The aims of this thesis were to identify the microbial communities that live in association with *Z. muelleri* and to investigate how changes in the environment or the host influence the seagrass microbiome. Microbial community structure, diversity and levels of conservation, and co-occurrence patterns of bacterial, microalgal and fungal members were explored at the regional and plant scales in a variety of marine locations characterised by different environmental conditions. The dynamics of the *Z. muelleri* microbiome were also investigated within the contexts of disease and environmentally-driven physiological changes of the host, to assess how and to what extent intrinsic features of the plant influence its associated microbiota.

Throughout the thesis, it was consistently demonstrated that the seagrass microbiome is highly dynamic and influenced by environmental and host-associated factors, and as a consequence significantly different microbial communities associate with disparate microenvironments or tissue types within a single plant. I also showed that certain core members within these communities are conserved at larger spatial scales, although this biogeographical signal can be lost in a diseased holobiont. Moreover, I demonstrated that substantial microbial changes in the phyllosphere, with potential detrimental effects, are concomitant with physiological responses of the host against climate stressors.

1 CHAPTER 1

General introduction

1.1 Seagrasses are fundamental to healthy coastal ecosystems

Seagrasses are the only group of monocotyledonous angiosperms that have adapted to the marine environment (Larkum et al., 2018). There are nearly 60 species of seagrass, phylogenetically organised across 11 genera and 4 families (Green and Short, 2003, Hartog and Kuo, 2006, Larkum et al., 2018), which typically occupy tropical and temperate regions of the coastal ocean (Short et al., 2007) and are globally distributed across 1.6 million km^2 (Jayathilake and Costello, 2018). Southern Australia hosts large numbers of temperate seagrass species (Larkum et al., 2018), with several *Posidonia* and *Zostera* species dominating extensive seagrass meadows in coastal regions of Queensland, New South Wales, Victoria, Tasmania, South Australia and Western Australia (Kirkman, 1997, Larkum et al., 2018, Short et al., 2007).

As one of the most ecologically important and productive biomes in the coastal ocean (Fourqurean et al., 2012, Jayathilake and Costello, 2018), seagrasses play an extensive number of ecological roles, including serving as important nurseries for many marine species (Heck Jr. et al., 2003). Moreover, seagrass meadows are socioeconomically important because they provide several ecosystem services (Campagne et al., 2015, Cullen-Unsworth et al., 2014, Nordlund et al., 2016), which have been valued at approximately $30,000 per hectare per year (Costanza et al., 2014). Despite our limited knowledge on the variability of these services within and among seagrass species and environments (Nordlund et al., 2018, Nordlund et al., 2016), seagrass ecosystems have also been recognised as a globally significant carbon stock (Fourqurean et al., 2012). It has been estimated that seagrass ecosystems could store as much as 19.9 Pg of organic carbon (Fourqurean et al., 2012), due to the high capacity of these plants to filter out particles from the water column and store them in soils (Duarte et al., 2013, Hendriks et al., 2008, Kennedy et al., 2010).

Due to the importance of seagrass-based ecosystems and their potential sensitivity to environmental changes (Duarte et al., 2018), a thorough understanding of the processes influencing the physiology and health of these important ecosystem engineers is critical. However, as we are learning from other organisms, including analogous benthic plants and animals and terrestrial plants, seagrasses do not live in isolation, yet host a microbiome, which can have a substantial effect on host physiology and health (Tarquinio

et al., 2019, Ugarelli et al., 2017). These seagrass-associated microorganisms are the focus of this thesis.

1.2 Marine benthic holobionts

The 'microbiome' is defined as the collection of microorganisms that live in association with a host organism (Marchesi and Ravel, 2015). A host organism and its associated microbiome collectively form a functional entity or 'holobiont' (Gordon et al., 2013), where complex inter-species relationships influence functions that govern host fitness (Matyssek and Lüttge, 2013). Both the concept of the microbiome and the holobiont have been increasingly considered in terrestrial plants and several marine benthic organisms, and have reshaped our thinking about how microbes influence host health, physiology and function.

The plant microbiome has received substantial attention in recent years, due to emerging evidence that microbial interactions maintained for millions of years (Heckman et al., 2001) are fundamental for plant health and productivity (Stone et al., 2018). Plant-associated microbes play critical roles in biogeochemical cycles (Philippot et al., 2009), plant resistance mechanisms (Balint-Kurti et al., 2010) and growth (Jeounchyun et al., 2006), and have been exploited to increase agricultural yields in crop species (Orozco-Mosqueda et al., 2018). It is now well-recognized that disparate compartments within a single plant harbour communities that differ substantially in composition and function and are involved in fundamental processes that influence plant physiology (Vandenkoornhuyse et al., 2015). These include, for instance, plant-growth-promoting rhizobacteria in the rhizosphere (Vacheron et al., 2013) and antagonists on the leaf surface (Ott et al., 2001).

The existence of holobionts within the marine realm is also very common, with corals (Thompson et al., 2015), sponges (Pita et al., 2018) and seaweeds (Egan et al., 2013) widely considered as model host organisms associated with complex holobionts. It is now believed that several important processes, including efficient cycling of nutrients (Ceh et al., 2013), acclimatisation and adaptation to climate warming (Ziegler et al., 2017) and protection from pathogens and disease (Bourne et al., 2009, Glasl et al., 2016, Krediet et al., 2013), rely on complex host-microbiome interactions. Similarly, the functional repertoire of sponge-associated microbes involves roles in stress response (Lesser et al., 2016) and nutrient cycling processes (Freeman et al., 2013), whereas microbes associated with seaweeds are required for the normal morphological development of their host

(Provasoli, 1958, Provasoli and Pintner, 1980) and also serve protective benefits to the algae by expressing antibacterial and antidiatom properties to regulate surface fouling of colonising organisms (Kumar et al., 2011). Although much less characterised than other benthic organisms like corals, sponges and seaweeds, it is also likely that seagrasses should be considered as holobionts, with an important association with the microbiome.

Both, the seagrass host and its resident and spatially proximal microorganisms benefit from the several fundamentally ecological links that exist between them (Seymour et al., 2018). The seagrass, for instance, takes advantage of a number of microbiological processes, such as nitrogen fixation by heterotrophic diazotrophic bacteria in the rhizosphere that enhances seagrass productivity (Bagwell et al., 2002, Sun et al., 2015, Welsh, 2000). On the other hand, the microbiome exploits chemically suitable seagrass microenvironments that are rich in organic carbon and other nutrients (Brodersen et al., 2018, Duarte, 1990, Evrard et al., 2005, Jensen et al., 2007). Ugarelli et al. (2017) summarised the general structure and functional potential of the seagrass microbiome in relation to biogeochemical transformations within the nitrogen, sulphur and carbon cycles, which directly influence large-scale processes, such as carbon sequestration (Fourqurean et al., 2012, Macreadie et al., 2015). Additional studies revealed an unexpected diversity of seagrass-colonising bacteria (Tarquinio et al., 2019) that are likely responsible for nutrient enhancement (Brodersen et al., 2017); plant growth and development (Celdran et al., 2012); and protection from toxic compounds (Küsel et al., 2006), oxidative stress (Sanchez-Amat et al., 2010) and harmful (micro) organisms (Marhaeni et al., 2011, Supaphon et al., 2013).These studies add to the emerging evidence of the importance of the microbiome and its ecological functions for seagrass fitness and global biogeochemical cycling. Given the variable nature of the environments that seagrass-associated microbes can occupy, very dynamic and complex communities are therefore expected to interact with the seagrass host.

1.3 Heterogeneity of the microbiome

Host-associated microbiomes exhibit large heterogeneity at multiple scales (Fahimipour et al., 2017, Marzinelli et al., 2015, Schmitt et al., 2012). Therefore, substantial research effort has focused on comparative studies to ascertain whether, and to what extent, the external environment and the host influence microbial associations. In marine systems, planktonic microbial community structure and distribution show robust geographical patterns mainly influenced by temperature and light (Brown et al., 2012, Ghiglione et al., 2012, Rusch et al., 2007). Similarly, microbes associated with benthic macroorganisms

often display biogeographical partitioning, sometimes indicating the interdependence of the microbiome on local environmental conditions (Taylor et al., 2005). For instance, intraregional variation is characteristic of both the micro- and patho-biomes (i.e. putatively pathogenic microbial assemblage) associated with corals (Kellogg et al., 2017, Rubio-Portillo et al., 2018). In the sponge microbiome, variable proportions of specific bacterial groups between inshore and offshore locations suggest that specialist microorganisms (i.e. present in either inshore or offshore locations) within these communities are driven by environmental factors (Luter et al., 2015). In seagrasses, microbial communities associated with different geographical locations differ strongly (Cucio et al., 2016), and this influence of the environment on the composition of the microbiome is also evident at smaller spatial scales (Ettinger et al., 2017). Also, despite the excellent dispersal potential of fungi, differences in habitat type are also responsible for the discrimination of fungal communities associated with the seagrass phyllosphere (Wainwright et al., 2018). Hence, it is now well-recognised that host-associated microbiomes can change according to different biogeographical locations in response to environmental variability.

In addition to environmental and biogeographical determinants of microbiome structure, there is also evidence for the strong influence of inherent features of the host on the composition of its microbiome, which can ultimately result in inter-individual or inter-species microbiome variability. For instance, in seaweeds, the stress condition of the host (i.e. bleached vs. healthy) is a critical determinant of the structure of its associated bacterial and archaeal communities; these inter-individual differences within a population are much stronger than those resulting from differences in geography and related environmental variables (Aires et al., 2016, Taylor et al., 2005), even at continental scales (Marzinelli et al., 2015). In other benthic organisms, such as the marine gastropod, each host species shows microbiome compositional specificity, even across a major environmental gradient (Neu et al., 2019). At higher taxonomic levels, in sponges, substantial variability of microbes has been observed across co-occurring host genera (Taylor et al., 2004).

Intrinsic characteristic of specific parts or microenvironments within the host can also influence microbiome structure. For example, fungal communities associated with mangrove trees exhibit a marked zonation in their distribution across aerial and intertidal parts of the plant (Arfi et al., 2012). Some studies in seagrasses have shown that the inherent characteristics of a plant host aid to select for particular bacteria within the

rhizosphere that differ from the communities associated with the surrounding environment, even though the same communities are shared between different seagrass genera (Cucio et al., 2016). Similarly, bacterial assemblages associated with leaves and roots do not differ between different *Zostera* species, but are different between each other (Crump et al., 2018). This indicates that although not host-specific, the seagrass microbiome is directly influenced by the plant at a number of taxonomic levels, with some microbial members conserved across different seagrass genera and others between different seagrass species. In all cases, conspicuous shifts in the microbiome are driven by particular morphological, physiological or metabolic changes in the host, but this is not exclusively determined by the degree of specificity of prokaryotes to host eukaryotes. Conclusively, the interdependence of macro- and micro-biota within holobionts is influenced by several environmental and host-associated factors, and this results in very dynamic microbiomes. However, some microbial members within these communities are consistently conserved, despite the highly variable conditions of the external (micro) environment (Rubio-Portillo et al., 2018), indicating the existence of a 'core' microbiome.

1.4 The core microbiome

A 'core microbiome' is comprised of members of the microbial community that are conserved within a specific habitat or environment across space and time, and its potential importance relies on the assumption that commonalities between samples are indicative of functional relevance (Shade and Handelsman, 2012). Moreover, this relevant functional attribute of the core microbiome might be mediated by vertical transmission of core taxa that outcompete occasional colonising microorganisms (Björk et al., 2018). In a range of benthic organisms including corals, sponges and seaweeds, taxonomically or functionally equivalent elements of the microbiome are conserved over very large biogeographical scales (Schmitt et al., 2012), time (Chu and Vollmer, 2016, Miranda et al., 2013) and depth (Hernandez-Agreda et al., 2016). Moreover, in oysters, jellyfish and seagrasses, there is evidence of host-driven selection of core microbiome members associated with disparate microhabitats within the host (King et al., 2012), with different host species (Stuij, 2018), and along different host's life stages (Lee et al., 2018). Considering the high variability of conditions across different locations, times and depths, as well as between host's microenvironments, species and life stages, the consistent maintenance of particular features of the microbiome shows that the host's inherent characteristics might have a stronger influence on the selection and maintenance of the core microbiome than the environment.

1.5 Seagrass responses to a changing ocean

The ocean is an extremely dynamic environment, with further environmental variability contributed to by the increasing influence of global climate change (Harley et al., 2006, Parmesan and Yohe, 2003) and other anthropogenic pressures (Crain et al., 2009, Grech et al., 2011, Halpern et al., 2007). Consequently, several critical marine ecosystems have been driven towards functional collapse due to the impacts of decreased water quality, overexploitation of key species, coastal development, marine heat waves and several other pressures (Bryndum-Buchholz et al., 2019, Hoegh-Guldberg et al., 2007, Kazanidis et al., 2019, Macreadie et al., 2019, Pergent et al., 2014). Seagrass meadows are among the most valuable yet threatened marine ecosystems (Hughes et al., 2009), and despite world-wide conservation and restoration efforts (Cullen-Unsworth and Unsworth, 2016, Cunha et al., 2012), meadows have declined globally at an accelerated rate of 7% per year (Waycott et al., 2009). There are many causes of such deterioration: sediment and nutrient runoff, physical disturbance, invasive species, commercial fishing practices, aquaculture, overgrazing, algal blooms and disease (Orth et al., 2006).

Many diseases in marine systems are likely the result of microbial dysbiosis and the rise of opportunistic or polymicrobial infections (Egan and Gardiner, 2016), which in some cases are linked to environmental change (Campbell et al., 2011) or favoured by shifts in the microbiome (Lloyd and Pespeni, 2018, Meyer et al., 2016). In terrestrial plants, these shifts also take place under variable physiological conditions of the host, and drastic changes in bacterial diversity and the predominance of aetiological agents (Doi et al., 1967, Kunkel, 1926) lead to pigmentation patterns on leaves of diseased plants (Bulgari et al., 2011, Christopher and Edgerton, 1930, Fránová et al., 2013). Within this context, disease-related changes or altered physiological states of the host can influence or be influenced by the microbiome.

'Dysbiosis' is broadly defined as a change in a host organism's microbiome relative to that found in healthy individuals (Petersen and Round, 2014) and can either precede the onset of a disease or syndrome (Schaubeck et al., 2016a), or occur following an initial infection that compromises host health (Oh et al., 2013). This is because dysbiotic effects usually take place in a host challenged by stress conditions, which results in the loss of beneficial microorganisms and/or the expansion of pathobionts (Minich et al., 2018, Petersen and Round, 2014). Upon disturbance, the dynamic equilibrium of the healthy holobiont is affected, and consequently the strength and outcome of the interactions among its members change, potentially yielding disease or intensifying already existing symptoms

(Pita et al., 2018). Several cases of microbial dysbiosis have been reported within marine ecosystems and are often related to diseases in benthic macroorganisms like corals (Quintanilla et al., 2018), seaweeds (Fernandes et al., 2012a), lobsters (Meres et al., 2012) and sea stars (Lloyd and Pespeni, 2018), whereby microbial imbalance leads to the rise of opportunistic pathogens and/or polymicrobial infections (Egan and Gardiner, 2016). Besides a restructured microbial assemblage, changes in diversity are also indicative of dysbiosis, with often substantially lower number of microbial species typically associated with diseased hosts or preceding dysbiosis events (Abrahamsson et al., 2014, Candela et al., 2012, Dobbler et al., 2017, Frank et al., 2007, Kusstatscher et al., 2019, Lloyd and Pespeni, 2018). Within the context of the external environment, there is emerging evidence across a range of hosts, that dysbiotic effects, or other less profound shifts in microbiome structure, can be triggered by shifts in environmental conditions, meaning that the state of an organism's microbiome is the sum of intrinsic host-governed processes and external forces.

1.6 The *Z. muelleri* microbiome remains poorly characterised

The importance of the microbiome for marine benthic macroorganisms like corals (Bernasconi et al., 2019), sponges (Thomas et al., 2016) and seaweeds (Egan et al., 2013) has been well recognised, and with ongoing technological advances (Crump et al., 2018, Ghosh et al., 2017, Parrot et al., 2019, Radax et al., 2012, Yang et al., 2019), our understanding of the roles of host-microbe associations in the ocean and their relevance for global processes have improved significantly. Moreover, there is good circumstantial evidence that the seagrass microbiome is fundamental for seagrass health and may play an important role in the survival of seagrass meadows that are experiencing increasing threats (reviewed by Tarquinio et al. (2019), Seymour et al. (2018) and Ugarelli et al. (2017)). However, relative to other organisms, we still have a very poor understanding of the structure of the seagrass microbiome and to what extent it is stable or conserved over different spatial scales, how it varies between disparate microenvironments within the plant and its surroundings, if and how it is affected by, or affects, seagrasses experiencing disease, and how it changes with different states of seagrass physiology.

A better understanding of the role and dynamics of the seagrass microbiome requires a fundamental expansion of focus from the above- versus below-ground discrimination of the seagrass holobiont to investigations at finer plant scales. Moreover, the incorporation of other diverse microbial taxa (i.e. beyond just bacteria) into the characterisation of seagrass-associated communities is essential for fully characterising the nature of the

seagrass holobiont and understanding the identity of the organisms that may influence seagrass health. The research conducted for this thesis was motivated by the relevance of seagrass-based ecosystems for the biogeochemical and ecological balance of marine environments and has the goal of defining the processes governing the structure and potential influence of the seagrass microbiome.

1.7 Aims of the thesis

The overarching aims of this thesis are to identify the microbial communities that live in association with the seagrass species *Z. muelleri*, and to investigate how changes in the external environment or the host influence the structure and conservation of the seagrass microbiome. We hypothesize that changes in both external environmental conditions and intrinsic features of the seagrass will contribute to shifts in seagrass-associated microbial assemblages, yet that a core microbiome will still be conserved. The findings of this research will contribute important new knowledge on the factors defining the nature of the microbiome of an important marine ecosystem engineer. To achieve this goal, amplicon sequencing targeting 16S rRNA and ITS genetic markers was used to characterise the diversity of seagrass-associated communities of bacteria, fungi and microalgae in three field-based studies that were conducted within the following specific aims:

i. **Investigate the biogeography and small-scale dynamics of the seagrass microbiome**

   There is evidence that microbial communities associated with a range of marine benthic hosts are heterogeneous over a continuum of spatial and temporal scales (Fahimipour et al., 2017, Marzinelli et al., 2015, Thomas et al., 2016), and that the patterns governing the composition of marine microbial assemblages range from abiotic factors such as temperature (Case et al., 2011), nutrients and salinity (Fraser et al., 2018), to the biotic features of the particular host (Chu and Vollmer, 2016). Understanding the contribution of different elements of the environment that seagrass-associated microbes occupy is important because these could potentially affect critical seagrass-microbe interactions, and the ecological functions and biogeochemical processes that rely on them. Here, we examined plant-scale and regional-scale variability (using putative levels of anthropogenic impact as one descriptor of environmental conditions) in the seagrass microbiome to understand how the external environment influences different components of the seagrass microbiome and the scales at which this happens in the ocean.

ii. **Investigate shifts in the seagrass microbiome during seagrass wasting disease**

The association between global declines of seagrass meadows and the impact of seagrass wasting disease (SWD) has been well established (Short and Wyllie-Echeverria, 1996), but there is still uncertainty about the specific mechanisms of disease and the potential involvement of an imbalanced microbiota (i.e. dysbiosis (Egan and Gardiner, 2016, Olesen and Alm, 2016, Petersen and Round, 2014)). An understanding of the microbial ecology of SWD is important because diagnostic and management strategies rely, to a large extent, on a causative agent, which in the case of SWD, might involve multiple pathogens and/or a dysbiotic effect.

iii. **Evaluate the influence of physiological changes in the seagrass host on the structure of the seagrass microbiome**

Seagrasses undergo a range of physiological responses to environmental stress (Marín-Guirao et al., 2019, Prange and Dennison, 2000, Ralph et al., 2007, York et al., 2013), including a leaf reddening phenomenon that is believed to be catalysed by the accumulation of anthocyanins in response to high solar radiation (Novak and Short, 2010). The prevalence, causes and function of leaf reddening in seagrasses have been widely addressed (Novak and Short, 2011b, Novak and Short, 2011a, Novak and Short, 2012), yet there is currently no knowledge of whether this phenotype leads to a shift in seagrass-microbe interactions. Here, we used seagrass leaf reddening as a model for addressing how microbial communities respond to physiological changes of the plant host.

1.8 Thesis structure

The thesis results (Chapters 2 - 4) are presented in manuscript form and are either published in peer-reviewed international journals or have been submitted for publication, with formatting of each chapter following the requirements for each of the journals targeted. The results presented in Chapter 2 describe the structure of bacterial, microalgal and fungal communities associated with *Z. muelleri*, and include a comparison of the variability of the seagrass microbiome across regional and plant microenvironment scales. These results have been published in ***Frontiers in Microbiology***. Chapter 3 examines patterns in the seagrass leaf microbiome in response to seagrass wasting disease (SWD) and reveal a shift in the microbiome and a loss of the conservation of the core microbiome as a consequence of disease onset. These results are presented in a manuscript that has been submitted to ***Marine and Freshwater Research***. To further investigate how shifts in

the physiology of the seagrass host influence the structure of the leaf microbiome, we examined the microbiological response to seagrass reddening, a phenomenon caused by the accumulation of anthocyanins in response to increased radiation. The results of this part of the study are presented in Chapter 4, which has been accepted for publication in ***Marine Ecology Progress Series***. Finally, Chapter 5 summarises the findings of the thesis, discusses the implications of these results within the context of the current knowledge on the seagrass microbiome, and provides suggestions for the direction of future work. To reduce redundancy and allow for brevity, the literature cited in each chapter has been presented within a single 'References' list at the end of the thesis.

2 CHAPTER 2

Regional and microenvironmental scale characterization of the *Zostera muelleri* seagrass microbiome

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Author contributions: VH-M conceived the study, designed the sampling strategy, conducted the fieldwork and lab work, developed the methodological approaches, analysed the data, drafted the manuscript, prepared the figures and tables and obtained the approval of the final submission. TK supported the bioinformatic analyses, developed the customised pipelines for data analysis, and provided important contributions to the results interpretation. KP assisted on the statistical analyses and provided critical contributions to the results interpretation. TJ participated in fungal sequencing and data analysis. PR supervised the study. JS conceived the study, designed the sampling strategy, provided the regular supervision of VH-M throughout the data analysis and interpretation, drafted the manuscript and substantially contributed to its intellectual content. All authors agreed to be accountable for the content of the work.

2.1 Preface

This Chapter explores and contrasts the ecological dynamics of microbial communities associated with six disparate microenvironments within the seagrass and its surrounding environments, across four different locations within the same biogeographical region. The focus of this work was to characterise bacterial, microalgal and fungal assemblages with the aim of capturing shifts at the community level that respond to the variability of the external environment and the host. The identification of core members within the seagrass microbiome allowed us to determine its level of conservation at large spatial scales.

2.2 Abstract

Seagrasses are globally distributed marine plants that represent an extremely valuable component of coastal ecosystems. Like terrestrial plants, seagrass productivity and health are likely to be strongly governed by the structure and function of the seagrass microbiome, which will be distributed across a number of discrete microenvironments within the plant, including the phyllosphere, the endosphere and the rhizosphere, all different in physical and chemical conditions. Here we examined patterns in the composition of the microbiome of the seagrass *Zostera muelleri*, within six plant-associated microenvironments sampled across four different coastal locations in New South Wales, Australia. Amplicon sequencing approaches were used to characterize the diversity and composition of bacterial, microalgal, and fungal microbiomes and ultimately identify "core microbiome" members that were conserved across sampling microenvironments. Discrete populations of bacteria, microalgae and fungi were observed within specific seagrass microenvironments, including the leaves and roots and rhizomes, with "core" taxa found to persist within these microenvironments across geographically disparate sampling sites. Bacterial, microalgal and fungal community profiles were most strongly governed by intrinsic features of the different seagrass microenvironments, whereby microscale differences in community composition were greater than the differences observed between sampling regions. However, our results showed differing strengths of microbial preferences at the plant scale, since this microenvironmental variability was more pronounced for bacteria than it was for microalgae and fungi, suggesting more specific interactions between the bacterial consortia and the seagrass host, and potentially implying a highly specialized coupling between seagrass and bacterial metabolism and ecology. Due to their persistence within a given seagrass microenvironment, across geographically discrete sampling locations, we propose that the identified "core" microbiome members likely play key roles in seagrass physiology as well as the ecology and biogeochemistry of seagrass habitats.

Keywords: *seagrass microbiome, diversity, core, bacteria, microalgae, fungi, amplicon sequencing*

2.3 Introduction

Seagrasses are the only group of flowering plants that have fully adapted to an underwater lifestyle (Hemminga and Duarte, 2000, Larkum et al., 2018). These marine plants are an extremely valuable component of coastal ecosystems (Orth et al., 2006, Costanza et al.,

1997, Beck et al., 2001), where they represent key habitat-forming species (Dayton, 1972) and ecosystem engineers (Wright and Jones, 2006). Furthermore, seagrass meadows are a globally significant carbon sink, accounting for about 10% (equivalent to 27.4 Tg C yr^{-1}) of marine organic carbon burial (Fourqurean et al., 2012). However, the health and survival of these organisms, which are ecologically important for the value of coastal ecosystems (Costanza et al., 2014), is likely to be reliant on, or fundamentally regulated by, their association with microorganisms (Brakel et al., 2014, Brodersen et al., 2015).

It is widely recognized that plant-microbe associations are essential for the function and health of terrestrial plants (Berendsen et al., 2012, Hirsch and Mauchline, 2012, Morgan et al., 2005, Schlaeppi and Bulgarelli, 2015, Vandenkoornhuyse et al., 2002, Vandenkoornhuyse et al., 2015), with many examples of both mutualistic and antagonistic plant-microbe interactions (Baker et al., 1997, Bourke, 1964, Mylona et al., 1995, Vincent, 1980). In the marine environment, similar close ecological associations exist between microbes and a wide range of marine benthic organisms, including corals (Bourne et al., 2009, Rohwer et al., 2002), sponges (Morrow et al., 2015, Taylor et al., 2007) and seaweeds (Egan et al., 2013, Marzinelli et al., 2015). Although less studied than terrestrial plants and other benthic eukaryotes, seagrasses also maintain intimate ecological interactions with microbial consortia living in association with the plant and within the surrounding seawater and sediments (Brodersen et al., 2018, Walters and Moriarty, 1993). For instance, microbes inhabiting seagrass leaves, roots, and rhizomes can mediate several metabolic exchanges and biogeochemical transformations that are essential for seagrass resource provision and plant growth (Cifuentes et al., 2003, Crump et al., 2018, Hansen et al., 2000, Harlin, 1973, Hemminga et al., 1991, Lehnen et al., 2016, Welsh, 2000).These include sulphide oxidation (Crump et al., 2018), sulphate reduction (Cifuentes et al., 2003, Hansen et al., 2000, Lehnen et al., 2016), nitrogen fixation and nitrification (Hansen et al., 2000, Hemminga et al., 1991, Lehnen et al., 2016, Welsh, 2000), urea turnover and ammonium production (Hansen et al., 2000), sedimentation and nutrient uptake by the leaves (Harlin, 1973, Hemminga et al., 1991), and microbial consumption of plant-derived organic exudates (Crump et al., 2018, Harlin, 1973). Collectively, the microorganisms comprising the seagrass microbiome have been increasingly recognised as pivotal players in seagrass ecology (Brodersen et al., 2018, Ugarelli et al., 2017).

Spatially and temporally stable associations between a host organism and specific members of its microbial consortia are characteristic of a 'core microbiome' (Astudillo-

Garcia et al., 2017), comprised of a conserved assemblage of microorganisms that likely impart critical ecological functions to the host (Shade and Handelsman, 2012). The concept of the core microbiome was initially developed to understand the dynamics of bacterial communities associated with humans (Turnbaugh et al., 2009), and has since been applied to a range of host organisms and ecosystems (Hernandez-Agreda et al., 2017). The composition of a host organism's core microbiome can be governed by both the intrinsic physiology of the host and external environmental factors (Marzinelli et al., 2015). In benthic marine organisms like sponges, core microbiomes can be both highly species-specific (Schmitt et al., 2012) and conserved across large biogeographical scales (Schmitt et al., 2012, Thomas et al., 2016). In many host organisms, discrete core microbiomes occur in association with different organs, tissues or other morphological features of the host (Huttenhower et al., 2012, Keenan and Elsey, 2013). For example, in corals, discrete core microbiomes are associated with the coral branches, the surface mucus layer, intracellular spaces within tissues and the skeletal matrix (Hernandez-Agreda et al., 2016, Rohwer et al., 2002). Similarly, in terrestrial plants discrete core microbiomes are associated with different plant features, including the phyllosphere (i.e., above-ground aerial surfaces of plants), endosphere (i.e., root interior), and rhizosphere (i.e., zone around the root that is influenced by the plant) (Coleman-Derr et al., 2016, Knief et al., 2012, Lindow and Brandl, 2003, Lundberg et al., 2012).

Microbial assemblages associated with seagrasses inhabit a number of discrete microenvironments within the plant, including the phyllosphere, the endosphere and the rhizosphere (Ugarelli et al., 2017). Levels of photosynthesis, oxygen and the diffusive exchange of organic substrates vary across the seagrass phyllosphere, from the upper leaf to the leaf sheath (Hogarth, 2015, Larkum et al., 2007, Rubio et al., 2017), creating marked small-scale spatial heterogeneity in microenvironmental conditions for leaf associated microorganisms. Below the sediment surface, the roots and rhizomes anchor the plant into the sediment and mediate nutrient uptake, while also mediating chemical exchanges with microorganisms through the exudation of dissolved organic material into the rhizosphere (Badri and Vivanco, 2009, Hemminga and Duarte, 2000, Hogarth, 2015, Koren et al., 2015, Kuzhiumparambil et al., 2017). Levels of oxygen and organic substrates within the rhizosphere are generally highly dissimilar to the surrounding sediments (Koren et al., 2015), promoting microscale heterogeneity in microbial abundance, activity and community composition (Brodersen et al., 2018). Hence, while often closely located, the different physical and chemical conditions within discrete seagrass microenvironments are likely to favour the growth of disparate microbial assemblages and underpin small-

scale partitioning in the composition and function of seagrass-associated microbial communities.

Seagrass microbiomes have previously been shown to differ above and below the sediment surface (Crump and Koch, 2008, Ettinger et al., 2017, Mejia et al., 2016), as well as between the seagrass and the adjacent seawater and sediment (Cucio et al., 2016, Fahimipour et al., 2017, Gordon-Bradley et al., 2014, Jensen et al., 2007, Martin et al., 2018). In addition to this small-scale heterogeneity, seagrass microbiomes have also been shown to vary across larger, regional scales, whereby microbiological properties are driven by local environmental conditions (Bengtsson et al., 2017, Crump et al., 2018, Ettinger et al., 2017, Fahimipour et al., 2017, Jiang et al., 2015, Uku et al., 2007). For instance, the microbial assemblages associated with *Zostera marina*, *Zostera noltii*, and *Cymodocea nodosa* have been shown to vary over continental scales (Cucio et al., 2016, Fahimipour et al., 2017). Observations to date indicate that the seagrass microbiome is a product of both localised intrinsic features of specific plant microenvironments and larger scale environmental drivers. However, a unified understanding of the factors determining the structure of the seagrass microbiome and the spatial and temporal scales over which these communities are governed by specific features of the seagrass environment is lacking.

Here, we aim to elucidate the significance of microenvironmental and regional forces in shaping the microbiome of the seagrass species *Zostera muelleri* (*Z. muelleri*). We compared bacterial, microalgal, and fungal communities associated with six different plant microenvironments, including the upper and lower leaf, the sheath, the roots and rhizomes, surficial sediment, and adjacent seawater across four spatially discrete locations, with the goal of understanding the nature and dynamics of the *Z. muelleri* microbiome.

2.4 Materials and methods

2.4.1 Field survey

Samples associated with the seagrass species *Z. muelleri* were collected from two coastal and two estuarine environments, across a region spanning 86 km of coastline in New South Wales (NSW), Australia (**Figure 1A**). These included, Palm Beach (33°35'15.8"S 151°19'25.0"E), Rose Bay (33°52'20.1"S 151°15'43.7"E), Lake Macquarie (33°09'29.4"S 151°31'54.9"E) and Narrabeen Lagoon (33°43'11.0"S 151°17'40.4"E). Our four sampling locations were chosen as distinct, yet representative environments colonized by seagrass

meadows in NSW (Green and Short, 2003), a region characterized by significant seagrass cover in both coastal and estuarine environments. Narrabeen Lagoon is a semi-enclosed lagoon and Lake Macquarie is an estuary, and both differed from our two open coastal locations (i.e., Palm Beach and Rose Bay) with respect to depth distribution, salinity, and seawater nutrient concentrations. Other differences among all sites include different extent of water inflows from the open-ocean, terrestrial runoff, and levels of anthropogenic impact due to human activities.

Sample collection took place between October and November 2015, with all sites surveyed during low-tide conditions (< 2 m depth). Water physicochemical properties (i.e., temperature and conductivity as indicative of salinity) were measured *in situ* using a multi-probe meter (WTW Multi 3430, Germany). At each site, samples were collected from six microenvironments associated with different features of the plant (**Figure 1B**). These included: (i) the upper and (ii) lower parts of the leaf, the (iii) sheath, (iv) roots and rhizomes, (v) surrounding sediment, and (vi) seawater. We considered these six microenvironments best represented seagrass morphology and anatomy, despite the wide phenotypic plasticity found between populations and species.

2.4.2 Sampling protocols

A highly standardized sampling protocol was used to collect samples from the seagrass (i.e., leaves and roots and rhizomes) and their surrounding microenvironments (i.e., surficial sediment and adjacent seawater). Individual specimens of *Z. muelleri* (i.e., total biomass) were collected with sterile-gloved hands from at least two physically separated meadows (i.e., well-defined area of a dense group of plants) per site, to account for potential differences between meadows. Sampled plants were homogeneously distributed across the meadows chosen and collected with a minimum distance of 20 cm between plants. Each shoot was pulled out from the substrate, ensuring all plant sections were intact and then placed onto a clean tray to separate surficial sediment (i.e., sediment adjacent to the roots and rhizomes). For each plant, 1 g of sediment was taken adjacent to the roots and rhizomes from 1-3 cm under the surface using a syringe, subsequently homogenized in a clean tray to ensure the detachment of plant material and other contaminants, and immediately placed into 1.8 mL Nunc® cryotubes. Once sediment was collected, each plant was rinsed with seawater collected on site, and placed into a Ziploc® plastic bag filled to 2/3 of its total volume with the same water. In addition, 10 L of seawater was collected from the surface waters of the sampling site using Nalgene bottles; all replicate seawater samples were obtained from within ~30 cm of the seagrass. After

collection, seagrass (*n* = 5), sediment (*n* = 5), and seawater samples (*n* = 3) were transported to the laboratory on ice and immediately processed upon arrival.

In the laboratory, each plant was gently rinsed free of adhering sediment with Milli-Q water (Millipore Corporation, Billerica, MS, USA) to avoid excess accumulated debris on the periphyton layer (i.e., mixture of microbes and detritus attached to submerged surfaces). Plant material was successively divided into four microenvironments (upper leaf, lower leaf, sheath, and roots and rhizomes) with sterile scissors and scalpels. For each tissue type, 5 biological replicates were collected, comprising a surface area of 2.5 cm^2 for leaves, 0.5 cm^2 for sheaths, and a volume of 2 mL for entire branched roots and rhizomes, in order to keep enough distance between leaf fractions and to collect the required 0.25g of sample for DNA extractions. Once processed and placed into 2 mL Nunc® cryotubes, seagrass and sediment samples were immediately snap frozen in liquid nitrogen and stored at -80 °C prior to analysis. Water samples were kept on ice until triplicate 2 L samples were immediately filtered onto 0.2 μm polycarbonate membrane filters (Millipore) using a peristaltic pump upon return to the laboratory. Filters were snap frozen and stored at -80°C.

2.4.3 DNA extraction

For the leaf, sheath, roots and rhizomes, and sediment samples, genomic DNA was extracted from 0.25 g of plant tissue or sediment, using a bead beating and chemical lysis-based DNA extraction kit (PowerSoil® DNA Isolation Kit, MoBio Laboratories, Carlsbad, CA, USA). Microbial DNA from water samples was extracted from filters using the PowerWater® DNA isolation Kit (MoBio Laboratories, Carlsbad, CA, USA). Both kits were used in accordance with the manufacturer's standard protocol. DNA quantity and purity were evaluated using a Nanodrop-1000 spectrophotometer (ThermoScientific, NanoDrop Products, Wilmington, DE, USA).

2.4.4 Bacterial community characterization

To examine bacterial community composition within all samples, the 16S rRNA gene was amplified with the universal forward primer 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and the universal reverse primer 519R (5'-CGGTTACCTTGTTACGACTT-3') (Weisburg et al., 1991). PCR reactions were performed in 25 μL volumes containing 12.5 μL GoTaq Green Master Mix, 0.4 μL of each primer (10 μM), and 2 μL of template DNA. PCR cycling conditions involved an initial activation step at 95 °C for 120 s, followed by 30 cycles of: denaturation at 95 °C for 30 s, annealing at 50 °C for 30 s and extension at 72 °C for 90 s,

followed by a holding stage at 72 °C for 10 min. The resultant amplicons were visualized on 1% agarose gel with GelRed (1:10000). Genomic DNA was used to prepare DNA libraries with the Illumina TruSeq DNA library preparation protocol. Sequencing was performed on the Illumina MiSeq platform (at Molecular Research LP, Shallowater, TX, USA) following the manufacturer's guidelines. Subsequently generated raw data files were deposited in the Sequence Read Archive (SRA) under BioProject number PRJNA342246.

2.4.5 Fungal community characterization

In order to characterize fungal community composition, we used Illumina Miseq profiling of internal transcriber spacer (ITS) markers. Specifically, the ITS2 region was amplified by targeting a site in the 5.8S encoding gene with the fITS7 (5'-GTGARTCATCGAATCTTTG-3')/ITS4 (5'-TCCTCCGCTTATTGATATGC-3') primer set (Ihrmark et al., 2012). PCR reactions were performed as follows: initial activation step of 94 °C for 5 min, followed by 35 cycles of: denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s, and extension at 72 °C for 30 s, followed by a holding stage at 72 °C for 7 min. Sequencing was performed on the Illumina MiSeq platform (at the Next Generation Genome Sequencing Facility of Western Sydney University). Raw data files in FASTQ format were deposited in the Sequence Read Archive (SRA) under BioProject number PRJNA493529.

2.4.6 Sequence data analysis

Bacterial 16S rRNA gene sequences were analysed using a customised pipeline (Kahlke, 2018). Briefly, paired-end DNA sequences were de-multiplexed using MOTHUR, v1.39.0 (Schloss et al., 2009), then joined using FLASH, v1.2.11 (Magoc and Salzberg, 2011), quality-filtered using MOTHUR, and finally de-replicated using VSEARCH, v2.3.2 (Rognes et al., 2016). Quality filtering involved both, trimming of ambiguous bases in each of the sequences, as well as removal of short fragments with low quality scores from the data set. Operational Taxonomic Units (OTUs) were defined at 97% sequence identity and subsequently clustered using VSEARCH. The same tool was also used to detect and remove chimera sequences based on curated sequences from the Greengenes database, released on 13/08/2013 (DeSantis et al., 2006), and to build the OTU table. Taxonomy assignments were performed using BLAST, vBLAST+ (Altschul et al., 1990), in QIIME, v1.9.1 (Caporaso et al., 2010) to generate a representative set of OTUs that was aligned against the Greengenes database. Sequences were rarefied to the same depth (2380 sequences per sample) to remove the effect of sampling effort upon analysis (**Supplementary Table 12**). Given the nature of this study's experimental design and the importance of replication in

complex data-sets, the rarefaction cut-off was chosen to include at least triplicates per sample type.

Microalgal communities were identified from a secondary taxonomic assignment performed on sequences classified as 'chloroplast' by the Greengenes classification obtained from the 16S rRNA analysis of bacterial communities (Needham and Fuhrman, 2016). A separate OTU table was generated by BLASTn search of the PhytoREF database, downloaded 01/07/2015 (Decelle et al., 2015), which was used to provide a phylogenetic characterization of chloroplast sequences. This OTU table was subsequently screened to exclude sequences classified as plants or macroalgae, and finally relative abundances of microalgae were re-calculated for each OTU from previously rarefied data (**Supplementary Table 13**).

Initial sequence processing for fungal ITS genes was conducted using QIIME, v1.9.1 (Caporaso et al., 2010). Briefly, low-quality regions were trimmed from the 5' end of sequences, and paired ends were joined with fastq-join (Aronesty, 2011, Aronesty, 2013) and de-multiplexed. Sequences containing ambiguous bases were removed from the dataset along with low-quality reads and chimeric sequences. Referenced-based chimera detection (Nilsson et al., 2015) was performed using the UCHIME algorithm from the USEARCH package (Edgar, 2010, Edgar et al., 2011) implemented within VSEARCH, v2.3.2 (Rognes et al., 2016). OTUs were defined as clusters of 97% sequence similarity using UCLUST (Edgar, 2010), and taxonomy was assigned against the UNITE database, v6.9.7 (Koljalg et al., 2013) using BLAST, vBLAST+ (Altschul et al., 1990). The resultant OTU table was filtered to remove singletons and rarefied to an even number of sequences per samples to ensure equal sampling depth (i.e., lower number of sequences per sample = 1456). Given the nature of this study's experimental design and the importance of replication in complex data-sets, the rarefaction cut-off was chosen to include at least triplicates per sample type (**Supplementary Table 14**).

2.4.7 Post-sequencing analyses

Alpha diversity was estimated by calculating the Chao1 and Shannon's diversity indices in QIIME, v1.9.1 (Caporaso et al., 2010). The exponential function was applied to the Shannon's diversity index to calculate the true Shannon's diversity (i.e. effective number of OTUs) in accordance to the approaches used by Lundberg et al. (2012) to estimate alpha diversity of bacterial communities associated with the rhizosphere (including surrounding sediments) and the endophytic compartment of the model, terrestrial plant *Arabidopsis*

thaliana (*A. thaliana*). Permutational Multivariate Analysis of Variance (PERMANOVA) was used to test the statistical significance of the differences between and within microenvironments and sites, separately, in a nested design. These statistical analyses were performed in PRIMER-E, v7 (Clarke, 1993, Clarke and Gorley, 2015, Clarke et al., 2014).

Differences in community composition (i.e. beta diversity) were characterized using non-parametric multi-dimensional scaling (nMDS). PERMANOVA was used to test for statistical significance of the differences between and within microenvironments and sites. In order to further characterize the significant differences observed between sites within each microenvironment, we performed hierarchical CLUSTER analyses (Timm, 2002). Each of these analyses were performed in PRIMER-E, v7 (Clarke, 1993, Clarke and Gorley, 2015, Clarke et al., 2014).

To identify 'discriminatory OTUs' between microenvironments, we coupled pair-wise analyses of Similarity Percentages (SIMPER) (Clarke, 1993) performed in PRIMER-E, v7 (Clarke, 1993, Clarke and Gorley, 2015, Clarke et al., 2014), with extensive hypothesis testing of taxonomic profiles using Kruskal-Wallis-H and Tukey-Kramer statistical tests performed in STatistical Analysis of Metagenomic Profiles (STAMP, v2.1.3) (Parks et al., 2014). Significantly over-represented OTUs with the highest contributions to the differences between microenvironments were defined as 'discriminatory OTUs', with exceptions including non-significantly over-represented OTUs with consistent high contributions.

A custom script was used for the selection of core microbiomes (Kahlke, 2017). Core microbiomes were defined for each microenvironment and for the entire leaf (i.e. pooling the three phyllosphere microenvironments) in accordance with the approaches used by Lundberg et al. (2012) to define the core microbiome of the endophitic compartment within the bacterial communities in the rhizosphere of *A. thaliana*. In order to account for possible outliers in the data, any OTU present (relative abundance > 0%) in two out of three biological replicates within a given site (occurrence ≥ 67%), across all four sites, was classified as a core OTU. Abundant (greater than 1%) pelagic microbes were removed from the phyllosphere core microbiomes to eliminate the influence of possible sampling artefacts.

2.4.8 Study site characterization using *intI1*

To characterize the putative level of anthropogenic influence experienced by seagrasses in each of the four study environments, quantitative PCR (qPCR) was used to quantify the relative abundance of the clinical class 1 integron-integrase gene (*intI1*), which has previously been demonstrated to be a good proxy for anthropogenic pollution (Gillings et al., 2015). Serial dilutions of a plasmid harboring the *intI1* gene amplified from an environmental sample (seawater collected at Botany Bay, NSW, Australia) were used as a template to generate a standard curve. All samples and the standard curve were run in the same plate, which was prepared by an epMotion® 5075l Automated Liquid Handling System and conducted on a BIO-RAD CFX384 Touch™ Real-Time PCR Detection System™ (Bio-Rad Laboratories, Inc., Hercules, CA, USA). We used the BIO-RAD CFX Manager software to estimate *intI1* gene copies for triplicate reactions per sample (n = 12). Each 5 µL reaction consisted of 2.5 µL of iTaq UniverSYBR Green SMX 2500® (Bio-Rad Laboratories, Inc., Hercules, CA, USA), 0.1 µL of nuclease free water, 0.2 µM of the forward primer int1.F (5'-GGGTCAAGGATCTGGATTTCG-3'), 0.2 µM of the reverse primer int1.R (5'-ACATGCGTGTAAATCATCGTCG-3') (Mazel et al., 2000) and 2 µL of diluted (1:5) DNA template. The qPCR was subsequently run under the following thermal cycling conditions: initial denaturation for 3 min at 95°C, followed by 39 cycles of denaturation for 15 s at 95°C and annealing and extension for 1 min at 60°C. Coupling the results of this analysis with the measured environmental parameters and habitat feature data, allowed us to categorize our sampling locations into four levels of anthropogenic impact.

2.5 Results and discussion

2.5.1 Characterization of sampling sites

We coupled measurements of physicochemical parameters (**Supplementary Table 1**) and quantification of a genetic marker for anthropogenic pollution (*intI1*, **Supplementary Figure 1**) (Gillings et al., 2015) to categorize our four study sites based on an anthropogenic impact ranking (**Supplementary Table 1**), highlighting the disparate conditions of the sampled locations. Based on our categorization, Narrabeen Lagoon was the most impacted site (i.e., highest level of influence from human activities), followed by Rose Bay and Lake Macquarie, whereas Palm Beach was the most pristine site. However, given the highly dynamic nature of coastal/estuarine environments, where conditions can change markedly on short time periods, our sampling events represent discrete snap-shots in time that lack historical information about the prior conditions of the

environment, and therefore we suggest caution regarding the use of this information to infer the drivers of microbiome structure.

2.5.2 The seagrass bacterial microbiome

We investigated bacterial community composition and diversity in six discrete seagrass microenvironments associated with *Z. muelleri* across the four different sampling locations (**Figure 1**), in order to: i) characterize the seagrass microbiome, ii) determine the variability and/or level of conservation of the seagrass microbiome across different spatial scales (i.e., plant microenvironments and the region), and iii) identify persistent, or 'core' microorganisms within the seagrass microbiome. Using 16S rRNA gene sequencing, we contrasted patterns in alpha- and beta- diversity of bacterial assemblages among seagrass microenvironments and sites differing in physicochemical properties of the seawater (i.e., temperature and salinity), exposure to the open ocean (coastal vs. estuarine environments) and anthropogenic impact (**Supplementary Table 1** and **Supplementary Figure 1**).

Alpha diversity, as measured by Chao1 and Shannon's diversity index, varied significantly both between sampling locations (p_{Chao1} = 0.0017, $p_{Shannon's}$ = 0.0001) and seagrass microenvironments (p = 0.0001). However, post-hoc analyses for both diversity indices (**Supplementary Table 2** and **Supplementary Table 3**) indicated that the between site differences were solely driven by differences between Rose Bay and all other sites ($p <$ 0.05), with the exception of Lake Macquarie (p_{Chao1} = 0.1495). Alpha diversity levels within the three microenvironments within the phyllosphere (i.e., upper leaf, lower leaf, and the sheath) did not differ statistically from one another or between sites ($p > 0.05$), with the exception of the upper leaf and the sheath at Narrabeen Lagoon (p_{Chao1} = 0.0465). The bacterial assemblages inhabiting the roots and rhizomes and the sediments were the most diverse microenvironments (**Figure 2**), which might be a consequence of higher levels of microscale heterogeneity and persistence of biogeochemical gradients within this zone (Brodersen et al., 2018, Evrard et al., 2005, Fraser et al., 2018, Jensen et al., 2007, Stapel and Hemminga, 1997).

Significant variability (p = 0.0001) in bacterial assemblage structure occurred between seagrass microenvironments (**Figure 3**), which was apparent in both multi-dimensional scaling plots (nMDS, **Figure 4**) and dendograms (**Supplementary Figure 2**), whereby clear clustering of bacterial community composition between specific seagrass microenvironments was evident within each sampling site, except for the three

microenvironments within the leaf. Similar to the patterns in alpha diversity, significant differences in bacterial assemblage structure were also observed between study sites (p = 0.0001), supporting the influence of local environmental forces on the seagrass microbiome. However, the differences in bacterial assemblage structure between microenvironments were greater than those between the sampling regions. Despite the spatial separation of just a few centimetres across an individual plant, the microbial communities from the different microenvironments showed the greatest variability, with only 42% shared bacterial taxa, whereas at the regional scale, where plants were separated by up to 52 km (i.e., largest distance between sites) and subject to differing local environmental conditions, sampling locations shared a higher proportion of 58% of bacterial taxa (ECV, **Supplementary Table 4**).

To further explore the key drivers of the variability within bacterial structures across different seagrass microenvironments and geographical locations, we coupled similarity percentages community analysis (SIMPER) (Clarke, 1993, Clarke and Gorley, 2015, Clarke et al., 2014) with extensive hypothesis testing of taxonomic profiles using Kruskal-Wallis-H and Tukey-Kramer statistical tests (Parks et al., 2014). Using this combined approach, we found 8 discriminatory OTUs that were i) significantly enriched in a given microenvironment ($p < 0.03$), and/or ii) among the top five contributors to the observed dissimilarities between microenvironments as determined by SIMPER. These OTUs spanned three bacterial phyla, including four classes of the Proteobacteria (**Figure 5**).

The clear clustering of seawater samples on nMDS (**Figure 4**) was principally driven by the dominant bacteria within these samples, corresponding to the families Pelagibacteraceae (12 unique OTUs), Rhodobacteraceae (139 unique OTUs), and Cryomorphaceae (20 unique OTUs), which made up 26%, 17% and 11% of these communities, respectively (**Figure 3**). These families, along with a member of the Halomonadaceae (*Candidatus* Portiera sp., 7 unique OTUs), discriminated seawater samples from the other microenvironments (**Figure 5**). This is consistent with *Pelagibacter,* and other members of the SAR11 clade, being the dominant bacteria in seawater communities (Bowman et al., 2012, Brown et al., 2012, Giovannoni, 2017, Morris et al., 2002), and members of the Rhodobacteraceae, Halomonadaceae and Cryomorphaceae often dominating pelagic microbial assemblages in coastal and estuarine environments (Buchan et al., 2014, Jeffries et al., 2016, Pinhassi et al., 2004, Prabagaran et al., 2006).

The bacterial assemblages inhabiting the seagrass sediments also represented a clearly distinguished cluster from the other microenvironments on the nMDS, with conservation of the bacterial assemblage structure within this microenvironment across all four sampling sites (**Figure 4** and **Supplementary Figure 2**). Within this microenvironment, OTUs matching the Flavobacteriaceae (116 unique OTUs), the order Chromatiales (39 unique OTUs), and the Desulfobacteraceae (*Desulfococcus* sp., 26 unique OTUs) dominated these communities, accounting for 13%, 13%, and 8% of the sequences, respectively (**Figure 3**). The relative enrichment of these organisms within the sediment was also most responsible for the differences in bacterial assemblage structure relative to the other five microenvironments (**Figure 5**). Members of these three taxa have previously been shown to dominate the sediments associated with seagrasses (Cucio et al., 2016, Cucio et al., 2018, Ettinger et al., 2017, Sun et al., 2015) and salt marsh plants (Thomas et al., 2014), where Chromatiales and Desulfobacteraceae play important roles in nutrient cycling, given their sulphur-oxidizing and sulphate-reducing capabilities, respectively (Kleindienst et al., 2014, Varon-Lopez et al., 2014). Members of the Flavobacteriaceae are also often abundant in coastal marine sediments when sufficient oxygen is available (Raulf et al., 2015b, Sun et al., 2015), where they can play a prominent role in the degradation of complex polymeric substrates (i.e., organic matter decomposition) (Bowman et al., 2012).

Like the communities associated with the surrounding microenvironments, bacterial assemblages within the roots and rhizome samples collected across the four sites generated a discrete cluster, discriminated from the other microenvironments on nMDS (**Figure 4**). The bacterial community in the roots and rhizomes was significantly different from the microbiomes associated with the surrounding sediments and seawater across all four sampling locations ($p < 0.05$, **Supplementary Table 4**). There were also statistically significant differences between these samples and each of the three phyllosphere microenvironments at all sites ($p < 0.05$ for 10 comparisons), with only two exceptions at Palm Beach ($p_{sh} = 0.0776$) and Narrabeen Lagoon ($p_{lol} = 0.0510$) (**Supplementary Table 4**). Relative to the other five microenvironments, the roots and rhizomes were characterized by a higher proportion of unclassified members of the orders Chromatiales (39 unique OTUs) and Bacteroidales (63 unique OTUs) and the Spirochaetaceae (*Spirochaeta* sp., 41 unique OTUs) (**Figure 3**). The same OTUs from the Chromatiales that dominated sediment communities were also enriched in the roots and rhizomes relative to the phyllosphere and surrounding seawater, and along with the spirochaetes, these bacteria drove the differences between this community and the other five microenvironments (**Figure 5**). Although not often found directly in association with the

roots and rhizomes, members of the Spirochaetaceae are often found within seagrass sediments (Cifuentes et al., 2000, Doty, 2015, Trevathan-Tackett et al., 2017), while members of the Bacteroidales have elsewhere been shown to dominate communities attached to roots of aquatic angiosperms (Crump and Koch, 2008).

In contrast to the clear discrimination of bacterial assemblages in the other microenvironments, the three microenvironments within the phyllosphere (i.e., upper leaf, lower leaf, and sheath) overlapped with one another on the nMDS plot (**Supplementary Figure 3**), but were clearly discriminated from the bacterial assemblages from the roots and rhizomes and the surrounding seawater and sediments (**Figure 4**). Furthermore, there were no statistical differences in bacterial community structure between these three compartments of the phyllosphere at Palm Beach and Rose Bay (p > 0.05), whereas only the upper leaf and the sheath differed from each other at Narrabeen Lagoon (p = 0.0411) and Lake Macquarie (p = 0.0282) (**Supplementary Table 4**). It should be noted, however, that the lack of statistical differences between the three microenvironments within the phyllosphere could have resulted from either a more homogenous distribution of bacteria across the entire phyllosphere or from the high level of heterogeneity observed across replicates within each site (**Figure 3**). Therefore, while no statistically significant differences were observed between the three different compartments of the phyllosphere, there remains the possibility that a higher degree of replication may have resolved significant differences, given the spatially variable photosynthetic rates and nutrient contents throughout the leaf (Borum et al., 2006, Duarte, 1990, Hemminga et al., 1991, Koren et al., 2015, Larkum et al., 2007, Stapel and Hemminga, 1997), and the dissimilar oxic conditions between the sheath and the upper leaf (Tyerman et al., 1984).

Across all sampling locations, a single family, the Burkholderiaceae (2 unique OTUs), dominated all three microenvironments within the phyllosphere, representing an average of 30% of these communities (**Figure 3**). Some OTUs, however, were exclusively dominant in a single phyllosphere microenvironment. These included OTUs matching the Rhodobacteraceae in the upper leaf (including *Loktanella* sp., 139 unique OTUs, average relative abundance = 13%), the Comamonadaceae in the lower leaf (9 unique OTUs, relative abundance = 6%), and the Paenibacillaceae in the sheath (*Paenibacillus* sp., 1 unique OTU, relative abundance = 6%). The assemblage structure of phyllosphere-associated bacteria differed from the other microenvironments primarily due to an enrichment of the Burkholderiaceae (2 unique OTUs) in both the lower leaf and the

sheath, the Rhodobacteraceae (*Loktanella* sp., 14 unique OTUs) in the upper leaf, and the Pseudomonadaceae (*Pseudomonas* sp., 4 unique OTUs) in the lower leaf. Together, these bacteria drove the differences between the phyllosphere to the rhizosphere and the adjacent seawater (**Figure 5**).

Overall, these results show that while the nature of the seagrass microbiome is influenced to some extent by local environmental conditions that can vary with biogeography, intrinsic differences between the discrete microenvironments associated with the host have a larger effect on shaping the seagrass microbiome structure (**Figure 3**, **Figure 4** and **Supplementary Figure 2**). Some regional differences in the overall bacterial assemblage structure between sampling locations (**Supplementary Table 4**) were potentially governed by environmental characteristics at each site, such as physicochemical conditions, exposure to the open ocean and anthropogenic impact (**Supplementary Table 1** and **Supplementary Figure 1**). However, the observed conservation of some bacterial OTUs within specific microenvironments across all sites, is highly suggestive of the existence of universally important members of the microbiome across all environments (Astudillo-Garcia et al., 2017, Shade and Handelsman, 2012). To explore this pattern more directly, we next determined the existence of core microbiome members within the specific seagrass microenvironments.

2.5.3 The bacterial core microbiomes

No single OTU was observed across all seagrass microenvironments, which both indicates that there is not an overall 'core seagrass bacterial microbiome' and confirms that the seagrass microenvironments examined here represent markedly different microbial niches. However, core microbiome members were found in each of the microenvironments, whereby there was evidence of maintenance of specific core members across the four discrete sampling regions. The size of core microbiomes varied substantially between microenvironments, ranging from one core OTU within the sheath microenvironment, up to 102 core OTUs within the surrounding sediments (**Figure 6A**). The core microbiome members of the six seagrass microenvironments cumulatively spanned more than 39 bacterial families, across 14 phyla (**Supplementary Table 5**).

The upper leaf microenvironment was characterized by a core community including two OTUs from the Alpha- and Gamma- Proteobacteria, which together made up 75% of the core within this microenvironment. This is consistent with previous observations, whereby members of these groups represented ≥50% of bacterial communities associated

with the seagrass phyllosphere (Jiang et al., 2015, Weidner et al., 2000), and both classes are widely recognised as abundant bacteria within the *Zostera* microbiomes (Bengtsson et al., 2017, Crump et al., 2018, Cucio et al., 2016, Ettinger et al., 2017, Fahimipour et al., 2017). More specifically, these two core OTUs were classified as the Pseudomonadaceae and Rhodobacteraceae families and made up 46% and 29% of the upper leaf core, respectively (**Figure 6A** and **Supplementary Table 5**). Notably, the Pseudomonadaceae include pathogens and leaf epiphytes of terrestrial angiosperms (Hirano and Upper, 2000) and have also been shown to dominate the microbiomes of seagrass leaves from geographically linked coastal locations (Jiang et al., 2015). The Rhodobacteraceae are common pelagic and surface-associated marine bacteria that incorporate a broad suite of metabolisms, including chemoorganotrophy and photoheterotrophy (Dixon and Kahn, 2004, Haselkorn and Kapatral, 2005), with some members known to produce antibacterial compounds that may influence leaf surface colonization by other microbes, including pathogens (Dang et al., 2008). Members of this family are commonly observed on the leaves of seaweeds (Fernandes et al., 2012b) and seagrasses (Crump and Koch, 2008, Ettinger et al., 2017, Mejia et al., 2016), and particularly in *Zostera marina* (Crump and Koch, 2008, Ettinger et al., 2017). Their relative abundance previously demonstrated to be linked to specific features of the host (i.e., different compartments and health status) or environmental conditions (i.e., water turbidity, nutrients and geomorphological features). Members of this group have also been implicated in macrophyte pathogenesis due to their increased abundance in aged and bleached macroalgal phenotypes (Fernandes et al., 2012b, Mancuso et al., 2016, Zozaya-Valdes et al., 2015).

The core bacterial assemblage inhabiting the lower leaf exhibited similarities to that of the upper leaf and sheath microenvironments, with an OTU from the Pseudomonadaceae overlapping with the upper leaf core and another OTU from the Propionibacteriaceae coinciding with the only core member of the sheath microbiome (**Figure 6A** and **Supplementary Table 5**). The classical propionibacteria have been traditionally isolated from dairy products, but there are also strains isolated from soils and terrestrial plants (Stackebrandt et al., 2006), and even from different areas of the human body (McGinley et al., 1978). These microorganisms are known as a ubiquitous family within coral- (Kuang et al., 2015) and cone snail-associated microbiomes (Valliappan et al., 2014). While not previously reported in seagrasses, other bacteria within the higher taxonomic rank, the Actinobacteria, have been repeatedly observed dominating the communities associated with the seagrass leaf and the rhizosphere (Bengtsson et al., 2017, Crump et al., 2018, Cucio et al., 2016, Fahimipour et al., 2017, Mejia et al., 2016, Ugarelli et al., 2019). When

firstly defining the three phyllosphere cores, we observed that shared members across these three groups (i.e., phyllosphere microenvironments) included OTUs from the Pelagibacteraceae and Synechococcaceae families. Given that *Pelagibacter* and *Synechococcus* are both ubiquitous and dominant members of pelagic microbial assemblages (Giovannoni and Stingl, 2005, Li, 1998), it is probable that their consistent occurrence on leaf surfaces represented a sampling artefact. This was also supported by our observations of OTUs from the Pelagibacteraceae and Synechococaceae dominating the core associated with the surrounding seawater, and therefore we removed abundant pelagic microbes from the phyllosphere datasets in order to analyse the phyllosphere core microbiomes.

The core microbiome of the roots and rhizomes included 61 bacterial OTUs (**Figure 6A** and **Supplementary Table 5**). Among these were a large number of core OTUs from the Chromatiales (18% of total core OTUs in the roots and rhizomes), Desulfobacteraceae (7% of total core OTUs in the roots and rhizomes), and Rhodobacteraceae (7% of total core OTUs in the roots and rhizomes). Members of the Desulfobacteraceae family are anaerobic, chemolithotrophic microorganisms, commonly involved in sulphate reduction and nitrogen fixation processes in seagrass environments, particularly near to the roots and rhizomes (Bagwell et al., 2002, Crump et al., 2018, Cucio et al., 2016, Devereux, 2013, Ettinger et al., 2017, Lehnen et al., 2016, Lovell, 2002, Sun et al., 2015, Welsh, 2000, Welsh et al., 1996). Moreover, these bacteria are well-known abundant microorganisms within *Zostera* microbiomes, where they discriminate communities associated with roots from those associated with the leaf and surrounding sediments (Ettinger et al., 2017). Therefore, we suggest that core members from the Desulfobacteraceae are nitrogen fixers within the rhizosphere of *Z. muelleri.* The Chromatiales are members of a large group of purple sulphur bacteria (Overmann, 1997, Storelli et al., 2013) that are commonly observed in sediments surrounding *Zostera* meadows and salt marshes (Thomas et al., 2014). Co-habitation of sulphate reducing and sulphur oxidising bacteria within seagrass rhizomes has been observed elsewhere (Cifuentes et al., 2000, Crump and Koch, 2008, Cucio et al., 2016, Cucio et al., 2018), whereby sulphur oxidizing bacteria are likely to play an essential role in the detoxification of sulphides produced by the sulphate reducing bacteria (Cifuentes et al., 2000, Crump and Koch, 2008, Cucio et al., 2016, Cucio et al., 2018, Fahimipour et al., 2017).

Together, these results provide evidence of a clear differentiation of core bacterial communities across the different microenvironments within the seagrass, instead of a

unified seagrass core microbiome. The phyllosphere core microbiome mainly consisted of Alpha- and Gamma- Proteobacterial OTUs exploiting the oxic conditions and high levels of labile organic substrates within the leaf surface microenvironment, whereas the core microbiome of the roots and rhizomes included, likely sulphate reducing, members of the Deltaproteobacteria. The persistence of core microbiomes across the seagrass microenvironments has not previously been explored at this level of detail. However, our demonstration of discrete core microbiomes across the different seagrass microenvironments is consistent with patterns in terrestrial plants, whereby the rhizosphere, the phyllosphere and the root and leaf endospheres host communities that are both distinct from one other and the surrounding soils (Coleman-Derr et al., 2016). The patterns observed here are also consistent with other benthic marine organisms including corals, where distinct microbial communities colonize different microenvironments within the coral colony, coral polyps and coral tissue (Ainsworth et al., 2010).

2.5.4 The seagrass microalgal microbiome

Microalgal communities within seagrass meadows collectively provide crucial ecosystem services, including the contribution of considerable levels of primary production and energy transfer to higher trophic levels (Stafford-Bell, 2016). Consistent with the patterns observed for bacteria, levels of alpha diversity among microalgal communities varied significantly between both seagrass microenvironments (p = 0.0001) and sites (p_{Chao1} = 0.0327, $p_{Shannon's}$ = 0.0001) (**Supplementary Table 6** and **Supplementary Table 7**). However, post-hoc analyses for both diversity indices indicated that the between site differences were solely driven by differences between Palm Beach and Lake Macquarie (p_{Chao1} = 0.0018, $p_{Shannon's}$ = 0.0001). Although no consistent patterns were observed across locations, several significant differences in alpha diversity were observed between seagrass microenvironments within each site (**Supplementary Table 6** and **Supplementary Table 7**). In general, microalgal diversity within the seagrass microenvironments was lower ($p < 0.05$) than in the surrounding seawater (**Figure 2**), which might be attributed to competitive interactions between microalgae and other epiphytes on the seagrass leaves, and/or regulatory mechanisms whereby microalgae are suppressed by metabolic products from the host (Harlin, 1975, Pinckney and Micheli, 1998).

Similar to the bacteria, the composition of microalgal assemblages varied significantly between both seagrass microenvironments (p = 0.0001) and sampling sites (p = 0.0001).

Although statistically significant differences in microalgal composition were observed between sampling sites ($p < 0.05$), the differences between microenvironments within each sampling location were greater (ECV, *Mi(Si)* = 488.81, **Supplementary Table 8**). However, the clear partitioning within the assemblage structure that was observed for bacteria across the different seagrass microenvironments on nMDS plots was not as evident for microalgae, with only the surrounding seawater and sediment associated communities generating clearly discrete clusters (**Figure 7** and **Supplementary Figure 4**). Furthermore, unlike the seagrass-associated bacterial communities, the nature of the variability in microalgal structure across microenvironments differed between locations. Explicitly, there were no significant differences between microalgal communities associated with different plant microenvironments (i.e., phyllosphere and roots and rhizomes, **Supplementary Figure 5**) at Rose bay and Lake Macquarie ($p > 0.05$), whereas the roots and rhizomes assemblages differed significantly from the lower leaf communities at Palm Beach and Narrabeen Lagoon ($p < 0.05$) and also from the upper leaf at Narrabeen Lagoon ($p = 0.0482$) (**Supplementary Table 8**).

A single and ubiquitous order of green microalgae, namely the Chlorellales (3 unique OTUs), dominated the microalgal community across all microenvironments and sites, representing on average 23% of these assemblages, with the exception of the surrounding sediments (**Figure 8**). The sediments, on the other hand, were exclusively dominated by the Cymatosirales (2 unique OTUs) and the Cymbellales (1 unique OTU), comprising 14% and 16% of the sequences within all sediment samples, respectively.

In the upper leaf microenvironment, members of the Bacillariophyceae (4 unique OTUs) were the dominant microalgae, comprising groups known to contain both benthic and pelagic representatives (Crosby and Wood, 1958a, Crosby and Wood, 1958b). This family represented an average of 33% of sequences and was responsible for the greatest differentiation from the other microenvironments, where it made up < 9% of the microalgal assemblage (**Figure 9**). The Bacillariophyceae include diatoms, commonly among the most abundant and productive phototrophic microbes associated with seagrasses (Ambo-Rappe, 2016, Govindasamy and Anantharaj, 2013, Jacobs and Noten, 1980). Compositional changes of epiphytic diatoms, including members of the Bacillariophyceae, are closely related to morphological changes of the seagrass leaf (Chung and Lee, 2008). These differences in species composition and the specific modifications of the blade surface itself might alter competitive interactions between major algal groups (Pinckney and Micheli, 1998).

The roots and rhizomes were dominated by OTUs affiliated with the Vaucheriales (2 unique OTUs) and Triceratiales (1 unique OTU) orders, which represented 29% and 24% of the sequences across all locations, respectively (**Figure 8**). The Triceratiales include benthic and epontic diatom species, with representatives previously isolated from corals, fossil beds, marine mud, seagrasses, and similar aquatic plants (Crosby and Wood, 1958b). Notably, this order, along with several members of the Bacillariophyceae, have been shown to be major components of the epiphytic diatom community in other seagrass species (López-Fuerte et al., 2013). The Vaucheriales are yellow-green algae that have also been widely observed as epiphytes in salt marshes, seagrass meadows and mangroves (Gallagher and Humm, 1981, Saifullah et al., 2003).

Our results provide evidence for microenvironmental partitioning of the seagrass microalgal microbiome, with often clear differences in the identity of microalgal OTUs dominating different microenvironments. However, and in contrast to our observations for bacterial assemblages, no core microalgal members of the seagrass microbiome were observed for any of the plant-associated microenvironments, indicating a lower level of geographic conservation of these patterns. Core microalgal microbiomes were only identified for the sediment and seawater microenvironments (**Figure 6B**). The sediment-associated microbiome included three core members belonging to the orders Cymbellales (1 OTU) and Cymatosirales (2 OTUs), whereas the seawater-associated microbiome comprised eight core members matching the orders Chlorellales (1 OTU), Pyrenomonadales (4 OTUs), Mamiellales (2 OTUs), and Triceratiales (1 OTU). The absence of any clear 'core microalgal microbiome' within *Z. muelleri* perhaps implies a weaker ecological coupling between seagrasses and specific microalgal taxa, relative to that observed for the bacterial component of the seagrass microbiome.

2.5.5 The seagrass mycobiome

Although less studied in seagrasses, several fungi have been demonstrated to be highly beneficial for aquatic and terrestrial plant fitness while establishing intimate relationships with their host (i.e., mycorrhizal associations) to facilitate nutrient uptake or compete against other potentially pathogenic microbes (Azcon-Aguilar et al., 1999, Kohout et al., 2012, Raghukumar, 2012). In this study, fungal communities associated with *Z. muelleri* displayed significantly different levels of alpha diversity for both measured indices (Chao1 and Shannon's Index) between seagrass microenvironments (p = 0.0001), but only Shannon's index varied between sites (p = 0.0011) (**Supplementary Table 9** and

Supplementary Table 10). Similar to microalgal assemblages, no consistent patterns were observed across locations. However, several significant differences in alpha diversity were observed between seagrass microenvironments within each site (**Supplementary Table 9** and **Supplementary Table 10**). In general, and similar to the patterns we observed for microalgal assemblages, seagrass-associated microenvironments had lower levels of fungal diversity than the surrounding seawater and sediments (**Figure 2**), possibly due to antifungal chemical defences and physiological responses from the host against co-occurring marine fungi, which have been well described for other seagrass species (Ross et al., 2008).

Consistent with the patterns observed for bacterial and microalgal assemblages, fungal community structure varied significantly across both seagrass microenvironments (p = 0.0001) and sampling sites (p = 0.0001). Similar to the patterns observed for microalgae, there was not a clear separation of fungal communities between the different plant-associated microenvironments (**Supplementary Figure 6**) and only sediment and seawater communities formed discrete, separated clusters that could be discriminated from the other seagrass-associated microenvironments (**Figure 10** and **Supplementary Figure 7**). Despite this lack of clear graphical evidence, the differences between microenvironments within each sampling location explained a greater level of variation between mycobiomes compared to the differences between sites (ECV, *Mi(Si)* = 953.23, **Supplementary Table 11**).

Fungal OTUs identified within five taxonomic groups consistently dominated fungal assemblages across all microenvironments and sites (**Figure 11** and **Figure 12**). This is consistent with the hypothesis of extreme ecological flexibility acclaimed for obligate marine fungal species (Nicoletti and Andolfi, 2018). Explicitly, OTUs matching the order Pleosporales (468 unique OTUs) and the species *Wallemia ichthyophaga* (58 unique OTUs) represented the most abundant fungi across all six microenvironments, making up an average of 59% and 15% of these communities, respectively (**Figure 11** and **Figure 12**). Many freshwater and marine species of Pleosporales have been described to date, including several endophytes and saprophytes of plants, as well as symbionts, parasites and pathogens of seagrasses and marine macroalgae (Boonmee et al., 2012, Hashimoto et al., 2017, Hyde et al., 2013, Suetrong et al., 2009, Zhang et al., 2009). Some species are also dominant members of microbiomes associated with mangroves, showing a microenvironmental preference for intertidal parts of the host, which occur above the water level (Raghukumar, 2012). This preference might explain the higher relative

abundance of the Pleosporales that we observed in periodically exposed phyllosphere microenvironments, such as the upper leaf, where this order represented 91% of the sequences, compared to the totally submerged roots and rhizomes, where it only represented 58% of the sequences. These patterns are highly consistent with previous reports of the dominance of a single marine fungus from the Pleosporales, probably representing a new genus, associated with the roots of the seagrass species *Posidonia oceanica* (Vohník et al., 2016). While, to our knowledge, the other dominant fungal species, *Wallemia ichthyophaga,* has not previously been reported in seagrasses, it has previously been found to occur in association with other benthic marine organisms, including corals (Raghukumar, 2012).

Besides the Pleosporales and *Wallemia ichthyophaga,* we observed additional OTUs that were consistently present in all plant microenvironments, with the exception of the upper leaf. These included members of the Glomeraceae family (133 unique OTUs), which were consistently found in the lower leaf, sheath, and roots and rhizomes, where they represented an average of 9% of these assemblages (**Figure 11** and **Figure 12**). The Glomeraceae are arbuscular mycorrhizal fungi, known for their obligate, symbiotic association with the roots of vascular plants (Schubler et al., 2001). While the lack of mycorrhizal symbioses in seagrasses has been previously proposed (Nielsen et al., 1999), our observations of the consistent presence of the Glomeraceae within the mycobiomes associated with lower, achlorophyllous parts of the seagrass, across all sampling locations, suggest a potentially important role of this fungus in the *Z. muelleri* mycobiome.

OTUs belonging to the Rhytismataceae family (53 unique OTUs) were consistently present only in the upper leaf and accounted for 4% of these communities (**Figure 11** and **Figure 12**). While we only observed Rhytismataceae in the leaf, and not in the roots and rhizomes or the surrounding sediments, members of this group have been previously isolated from the rhizosphere in other seagrass species (Gnavi et al., 2014, Panno et al., 2013). As many endophytes of the foliar communities in wood plants (Ganley et al., 2004), they may represent substantial, unknown biodiversity with functional novelties.

Here we chose to use a 97% similarity criteria for defining fungal OTUs characterized using our ITS sequencing approach, which we consider a suitable conservative approach given the lower levels of taxonomic diversity covered in fungal ITS data-bases (relative to e.g., bacteria) and is consistent with values previously used to characterize the mycobiome associated with terrestrial plants (Giordano et al., 2009) and coastal grasses (Sánchez-

Márquez et al., 2008). The overall dominance of five taxonomic groups across all microenvironments is in line with previous observations of very narrow mycobiomes associated with seagrasses (Devarajan and Suryanarayanan, 2002, Vohník et al., 2016), plants from saltmarshes (Al-Nasrawi and Hughes, 2012), mangroves (Xing and Guo, 2011) and other aquatic plants (Kohout et al., 2012). Nevertheless, and similar to microalgae, we did not observe a conserved 'core' of fungal associates within any of the seagrass microenvironments, which is indicative of a weaker ecological coupling between seagrasses and fungal taxa, relative to that observed for seagrass-bacterial interactions. We propose that, relative to bacteria, which appear to display highly specific interactions with different components of the plant due to a stronger influence of the conditions at the microscale, seagrass-associated fungi appear to establish more generalistic relationships with their host.

2.6 Conclusion

Our results indicate that the seagrass species *Z. muelleri* harbours specific microbial assemblages that differ significantly from the adjacent seawater and sediments. Our data also indicate that discrete bacterial, microalgal, and fungal communities occur within specific key seagrass microenvironments, and that the identity of members of these microenvironment-specific communities are often conserved across geographically disparate locations (**Figure 13**). Indeed, for all three microbial taxa, differences in community composition between the specific seagrass microenvironments, which were generally separated by just a few centimetres, were significantly greater than the differences observed between geographical locations spanning 86 km of coastline. These results indicate, that as with many other organisms, seagrasses host several discrete microbial assemblages that are each adapted to local environmental conditions.

In the case of bacteria, for example, members of the Pseudomonadaceae, Rhodobacteraceae and Comamonadaceae are dominant features of the microbiome inhabiting the *Z. muelleri* phyllosphere, where they exploit the oxic conditions and high levels of dissolved organic carbon on the leaf surface (Dixon and Kahn, 2004, Haselkorn and Kapatral, 2005, Hirano and Upper, 2000, Jorgensen et al., 2009, Juárez-Jiménez et al., 2010). On the other hand, sulphate reducing and sulphur oxidizing bacteria from the Desulfobacteraceae and Chromatiales are dominant core microbiome members within the roots and rhizomes, where they likely regulate the carbon and sulphur cycling processes that influence the decomposition of organic material and ultimately the health of the host

(Kleindienst et al., 2014, Lehnen et al., 2016, Storelli et al., 2013, Thomas et al., 2014, Varon-Lopez et al., 2014).

Overall, our study demonstrates that while the seagrass microbiome is highly heterogeneous at small-scales, specific microbial assemblages are organised according to local environmental conditions, with this structure maintained across broad geographic scales. These patterns are indicative of highly specialised, and likely ecologically important, roles of the seagrass microbiome, with bacterial, microalgal and fungal assemblages shifting according to the changing conditions across the disparate microhabitats within the plant and its surroundings. Our findings provide fundamental, baseline information of the composition and structure of microbial communities associated with *Z. muelleri*. Future work defining the seagrass microbiome function by using, for instance, metagenomics approaches will be critical in evaluating the relevance of particular seagrass-microbe association.

2.7 Figures

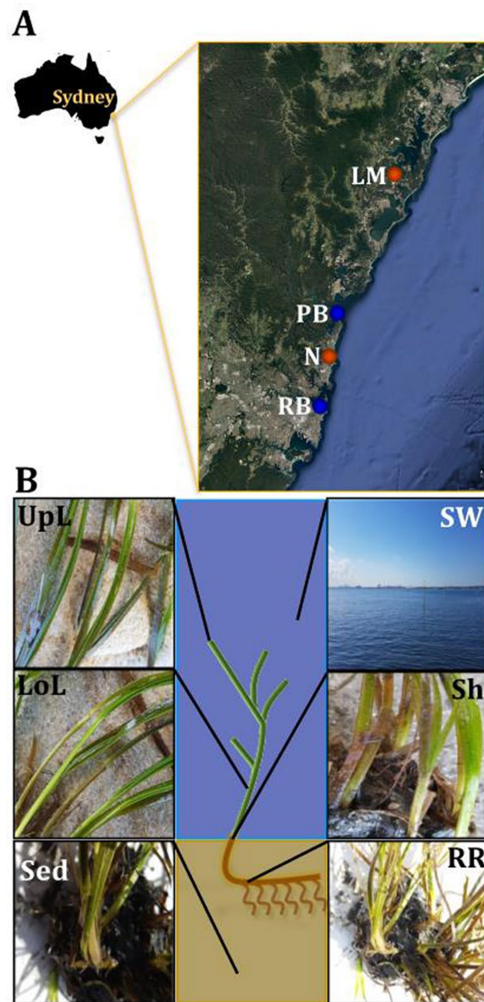


Figure 1: Study sites and sampling strategy.

Samples were collected between October and November 2015 across a region spanning 86 km of coastline in NSW, Australia (**A**). Study sites included coastal (blue) and estuarine (orange) environments, which were selected in accordance to habitat feature data (e.g., proximity to contamination sources and human activities) that was subsequently coupled with environmental parameters (e.g., water temperature and salinity) and genetic markers of anthropogenic pollution (i.e., *intI1*) to rank sites according to their specific conditions and level of impact. For details of the study sites selection criteria and categorization, see **Supplementary Figure 1** and **Supplementary Table 1.** At each site, samples from six different microenvironments within the plant (black fonts) and its surroundings (white fonts) were collected, based on the variety of conditions offered by these different niches (**B**). UpL, upper leaf; LoL, lower leaf; Sh, sheath; RR, roots and rhizomes; Sed, sediment; SW, seawater; PB, Palm Beach; RB, Rose Bay; N, Narrabeen Lagoon; LM, Lake Macquarie.

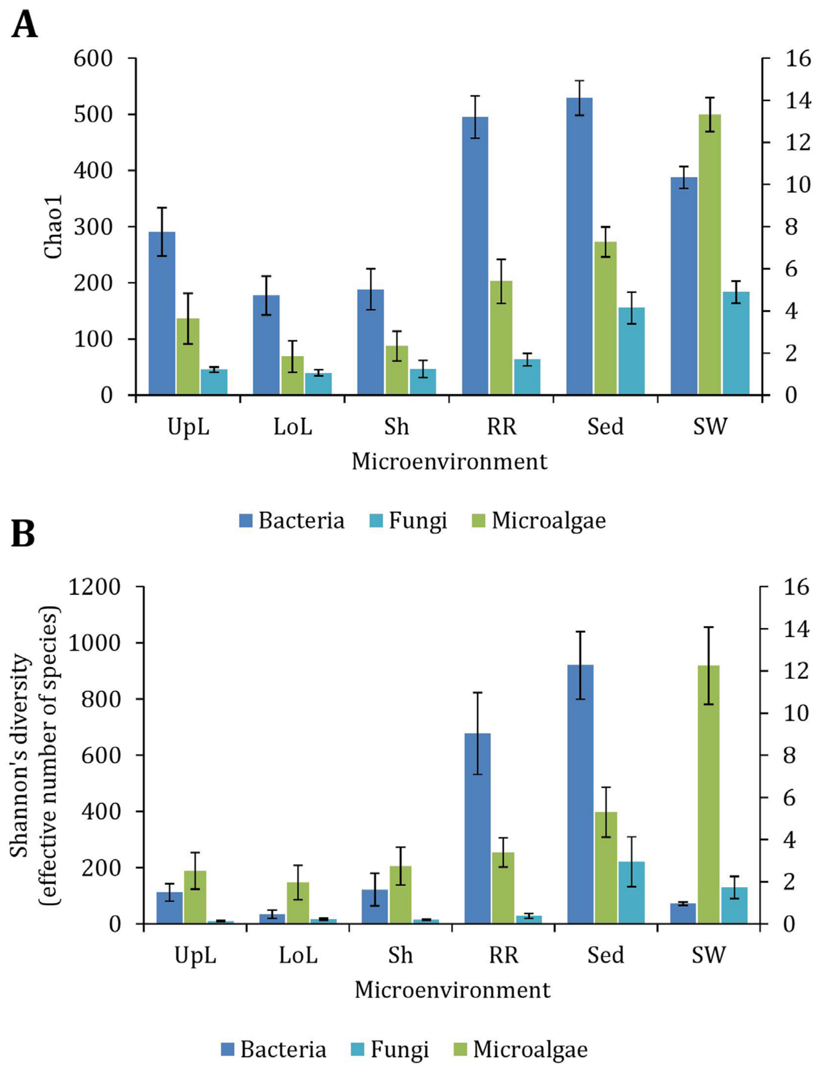


Figure 2: Microbial mean alpha diversity across seagrass microenvironments. Multiple comparisons between Chao1 diversity (**A**) and Shannon's diversity index (**B**), calculated for each taxa and microenvironment separately, were tested for statistical significance with Permutational Multivariate Analysis of Variance (PERMANOVA, Minkowski metric distance matrix and nested design). Mean values for each microenvironment are shown, and error bars reflect the standard error of the mean.

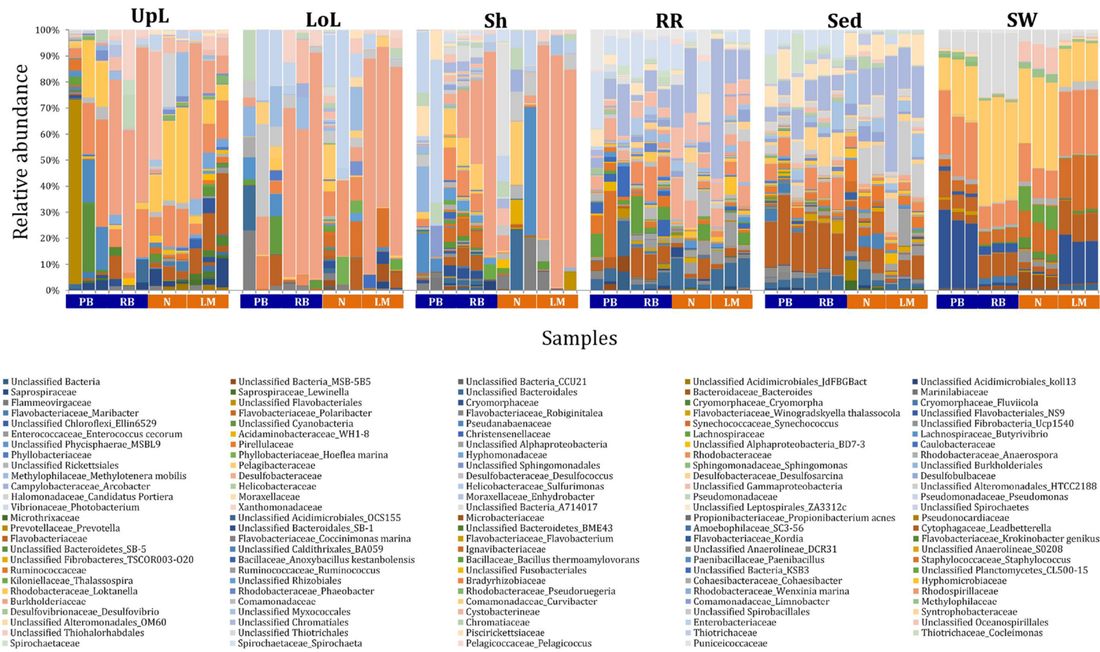


Figure 3: Bacterial community composition across seagrass microenvironments. Beta diversity of bacterial microbiomes across the six microenvironments within the plant and its surroundings. Triplicate samples per microenvironment within each of the four sampling sites (n = 72) are coloured by the highest assigned taxonomic level. Unique OTUs were summarized at the species level, and the representation of taxonomic groups within each sample are plotted. Only representative species with a relative abundance > 1% in all samples are shown to help remove visual clutter. UpL, upper leaf; LoL, lower leaf; Sh, sheath; RR, roots and rhizomes; Sed, sediment; SW, seawater; PB, Palm Beach; RB, Rose Bay; N, Narrabeen Lagoon; LM, Lake Macquarie.

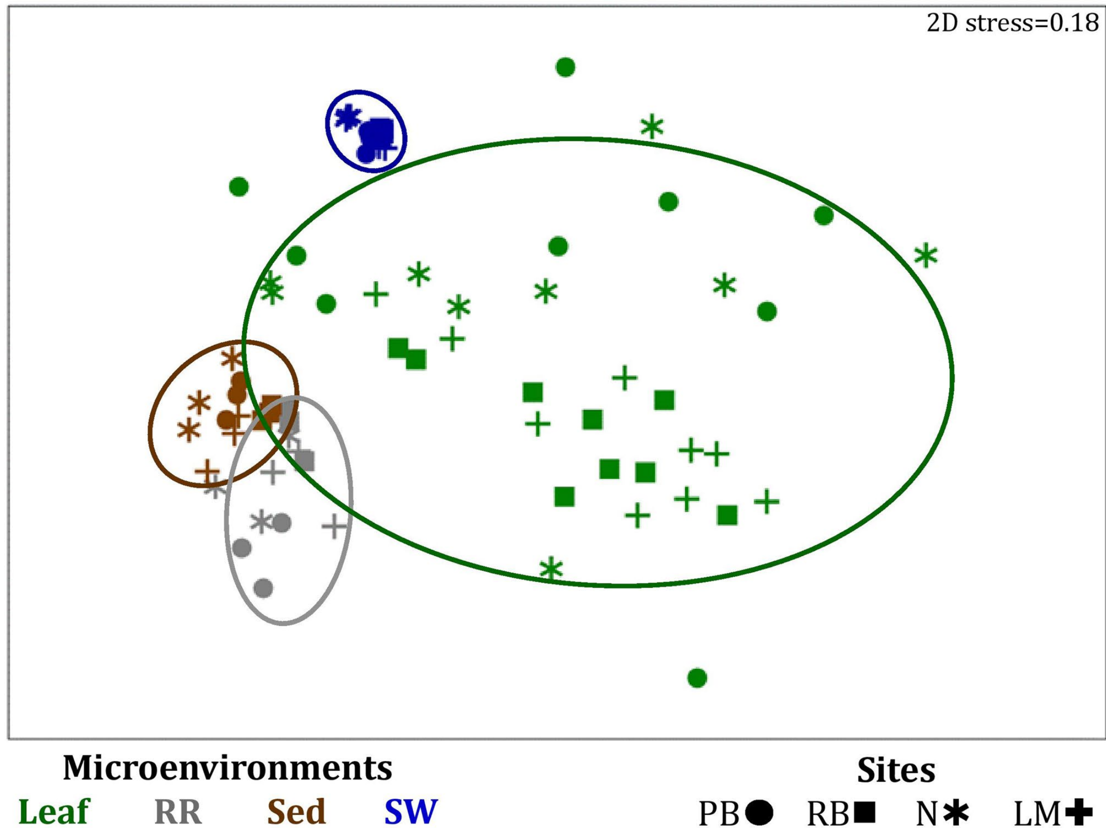
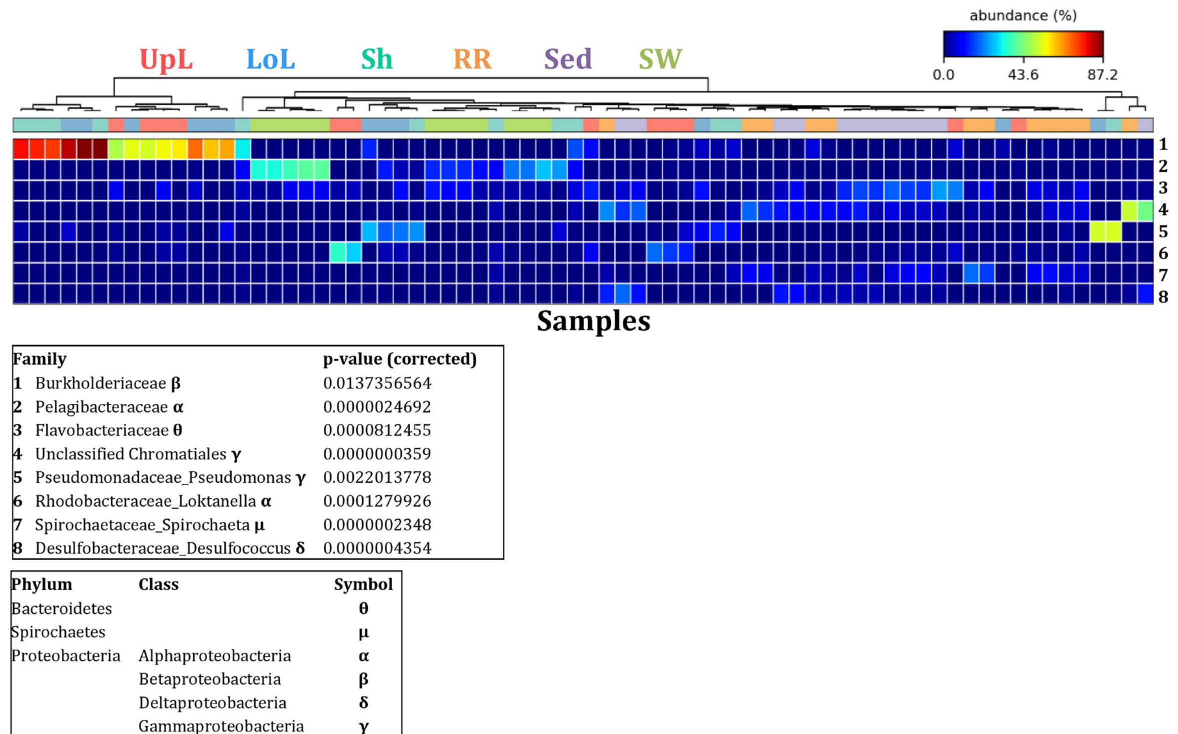


Figure 4: Microenvironmental and regional partitioning of the seagrass bacterial microbiome.

Non-parametric multidimensional scaling (nMDS) of bacterial microbiomes (n = 72), based on a lower triangular resemblance calculated with the S17 Bray-Curtis similarity measure from relative abundances of OTUs (high values down-weighted with square root). Samples are coloured by microenvironment (Leaf: upper and lower sections, RR, roots and rhizomes; Sed, sediment; SW, seawater), with different shapes for sites (PB, Palm Beach; RB, Rose Bay; N, Narrabeen Lagoon; LM, Lake Macquarie). Sample clustering patterns by microenvironment are shown in ellipses in the nMDS plot, representing the level of similarity between samples based on the degree to which OTUs are shared between them. The 2D stress is shown in the upper right corner of the nMDS plot (Kruskal stress formula = 1, minimum stress = 0.01). The nMDS for the three microenvironments within the phyllosphere is provided in **Supplementary Figure 3** and a hierarchical cluster analysis (CLUSTER) for all samples is provided in **Supplementary Figure 2**.



Family	p-value (corrected)
1 Burkholderiaceae β	0.0137356564
2 Pelagibacteraceae α	0.0000024692
3 Flavobacteriaceae θ	0.0000812455
4 Unclassified Chromatiales γ	0.0000000359
5 Pseudomonadaceae_Pseudomonas γ	0.0022013778
6 Rhodobacteraceae_Loktanella α	0.0001279926
7 Spirochaetaceae_Spirochaeta μ	0.0000002348
8 Desulfobacteraceae_Desulfococcus δ	0.0000004354

Phylum	Class	Symbol
Bacteroidetes		θ
Spirochaetes		μ
Proteobacteria	Alphaproteobacteria	α
	Betaproteobacteria	β
	Deltaproteobacteria	δ
	Gammaproteobacteria	γ

Figure 5: Bacterial discriminatory OTUs at the microenvironmental scale.
Extensive hypothesis testing of taxonomic profiles was coupled with similarity percentages analyses (SIMPER) for bacterial microbiomes across the six microenvironments. The proportion of sequences (mean frequency %) of OTUs significantly enriched (Kruskal-Wallis H test, $\alpha = 0.05$, effect sizes: η^2) and consistently contributing to the differences between microenvironments is indicated by varying colour intensities. Corrected p-values were calculated using the Benjamini-Hochberg's approach. Two-way crossed SIMPER analyses were performed with site and microenvironment variables as factors (S17 Bray-Curtis similarity matrix). High contributors were selected from the top-5 contributors of each pair-wise comparison between microenvironments, and those OTUs consistently accounting for the dissimilarities between any given microenvironment and at least three other microenvironments were chosen as high contributors to couple with the statistical results. High contributors that were significantly enriched were classified as discriminatory OTUs (i.e., 1-8). OTUs are sorted by decreasing mean abundance, and samples are clustered by average neighbour distance (UPGMA, distance threshold = 0.75) and coloured by microenvironment. Different symbols represent the distribution of enriched phyla. UpL, upper leaf; LoL, lower leaf; Sh, sheath; RR, roots and rhizomes; Sed, sediment; SW, seawater.

A

Bacterial core microbiome												
RA (%)	UpL (2)		LoL (3)		Sh (1)		RR (61)		Sed (102)		SW (76)	
min=0.0001	Pseudomonadaceae_Pseudomonas_1150	0.0061	Pseudomonadaceae_Pseudomonas_1150	0.0143	Propionibacteriaceae_Propionibacterium-acnes_1828	0.0054	Unclassified-Chromatiales_546	0.0533	Unclassified-Alteromonadales_HTCC2188_HTCC_713	0.0173	Pelagibacteraceae_2265	0.8605
50th pctl=0.0023	Rhodobacteraceae_2791	0.0039	Propionibacteriaceae_Propionibacterium-acnes_1828	0.0099			Unclassified-Chromatiales_460	0.0452	Flavobacteriaceae_694	0.0168	Rhodobacteraceae_2345	0.3895
max=0.8605			Comamonadaceae_1435	0.0082			Desulfobacteraceae_124	0.0411	Flavobacteriaceae_690	0.0142	Synechococcaceae_Synechococcus_2428	0.2739
							Unclassified-Chromatiales_729	0.0387	Unclassified-Chromatiales_632	0.0120	Cryomorphaceae_992	0.2347
							Cystobacterineae_125	0.0315	Desulfobacteraceae_Desulfosarcina_18	0.0086	Halomonadaceae_Candidatus-Portiera_894	0.2225
							Cohaesibacteraceae_Cohaesibacter_2378	0.0291	Desulfobacteraceae_Desulfococcus_25	0.0083	Rhodobacteraceae_2687	0.1977
							Unclassified-Chromatiales_485	0.0282	Unclassified-Bacteroidales_1459	0.0073	Cryomorphaceae_1371	0.1666
							Lachnospiraceae_1626	0.0276	Myxococcales_583	0.0073	Flavobacteriaceae_1369	0.1225
							Unclassified-Anaerolineae_DRC31_1861	0.0261	Myxococcales_506	0.0071	Synechococcaceae_Synechococcus_2638	0.0865
							Unclassified-Gammaproteobacteria_702	0.0228	Unclassified-Chromatiales_656	0.0066	Unclassified-Oceanospirillales_352	0.0829
							Unclassified-Chromatiales_519	0.0228	Piscirickettsiaceae_740	0.0064	Unclassified-Planctomycetes_OM190_CL500-15_247	0.0731
							Unclassified-Gammaproteobacteria_677	0.0173	Piscirickettsiaceae_785	0.0059	Halomonadaceae_Candidatus-Portiera_1161	0.0698
							Ruminococcaceae_1779	0.0153	Unclassified-Chromatiales_2186	0.0056	Microbacteriaceae_1785	0.0644
							Hyphomicrobiaceae_2377	0.0137	Unclassified-Chromatiales_930	0.0053	Flavobacteriaceae_913	0.0563
							Ruminococcaceae_1781	0.0133	Flavobacteriaceae_832	0.0052	Halomonadaceae_Candidatus-Portiera_1178	0.0559
							Unclassified-Bacteroidales_1148	0.0127	Piscirickettsiaceae_791	0.0049	Pelagibacteraceae_2363	0.0555
							Unclassified-Thiotrichales_624	0.0117	Hyphomicrobiaceae_2702	0.0044	Synechococcaceae_Synechococcus_2512	0.0518
							Methylophilaceae_Methylotenera-mobilis_905	0.0110	Piscirickettsiaceae_858	0.0043	Methylophilaceae_682	0.0470
							Unclassified-Chromatiales_492	0.0094	Piscirickettsiaceae_749	0.0042	Flavobacteriaceae_Polaribacter_1370	0.0445
							Unclassified-Chromatiales_480	0.0093	Piscirickettsiaceae_762	0.0041	Unclassified-Cyanobacteria_2340	0.0443

B

Microalgal core microbiome				
RA (%)	Sed (3)		SW (8)	
min=0.0098	Cymbellales_Cymbellaceae_2248	0.1167	Chlorellales_Chlorellaceae_2050	1.5917
50th pctl=0.1167	Cymatosirales_Cymatosiraceae_2227	0.1095	Pyrenomonadales_2261	0.6935
max=1.5917	Cymatosirales_Cymatosiraceae_2241	0.0183	Mamiellales_Bathycoccaceae_2134	0.6153
			Mamiellales_Mamiellaceae_2173	0.3383
			Pyrenomonadales_2309	0.1186
			Pyrenomonadales_2274	0.0880
			Pyrenomonadales_2276	0.0756
			Triceratiales_Triceratiaceae_2225	0.0098

Figure 6: The seagrass core microbiomes.

Bacterial (**A**) and microalgal (**B**) core OTUs associated with the seagrass and surrounding microenvironments were identified based on their predominance (i.e., occurrence ≥ 67%, relative abundance > 0%) across the four sampling sites. Cores are listed under columns for each microenvironment, and their sizes are shown in brackets. Core OTUs were identified at the family (bacteria) and order (microalgae) levels and are coloured by their relative abundance within each microenvironment. Abundant pelagic microbes were removed from the phyllosphere core microbiomes, and only the 20 most abundant bacterial core OTUs within each microenvironment are plotted to help remove visual clutter. The full version of the table is provided in **Supplementary Table 5**. UpL, upper leaf; LoL, lower leaf; Sh, sheath; RR, roots and rhizomes; Sed, sediment; SW, seawater; RA, relative abundance.

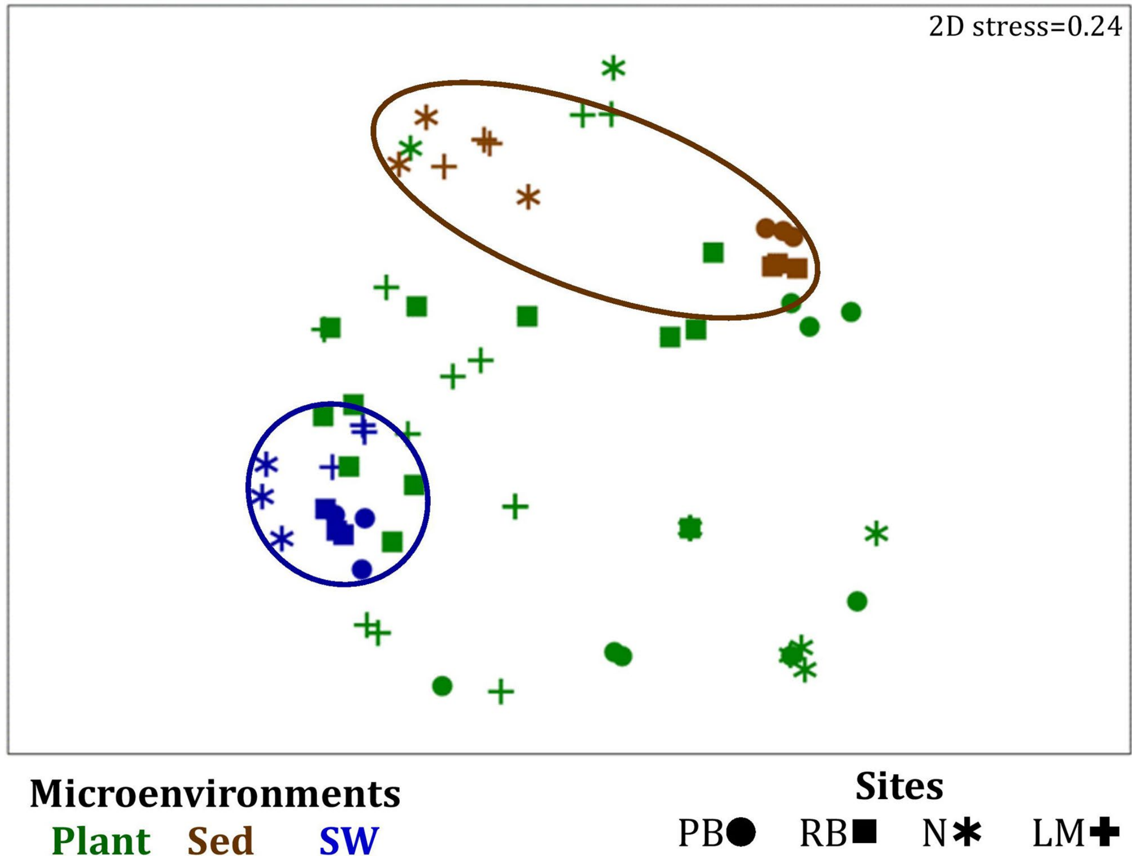


Figure 7: Microenvironmental and regional partitioning of the seagrass microalgal microbiome.

Non-parametric multidimensional scaling (nMDS) of microalgal microbiomes (n = 72), based on a lower triangular resemblance calculated with the S17 Bray-Curtis similarity measure from relative abundances of OTUs (high values down weighted with square root). Samples are coloured by microenvironment (Plant: upper leaf, lower leaf, sheath and roots and rhizomes; Sed, sediment, SW, seawater), with different shapes for sites (PB, Palm Beach; RB, Rose Bay; N, Narrabeen Lagoon; LM, Lake Macquarie). Sample clustering patterns by microenvironment are shown in ellipses in the nMDS plot, representing the level of similarity between samples based on the degree to which OTUs are shared between them. The 2D stress is shown in the upper right corner of the nMDS plot (Kruskal stress formula = 1, minimum stress = 0.01). A hierarchical cluster analysis (CLUSTER) for all samples is provided in **Supplementary Figure 4** and the nMDS for the four microenvironments associated with the plant is provided in **Supplementary Figure 5**.

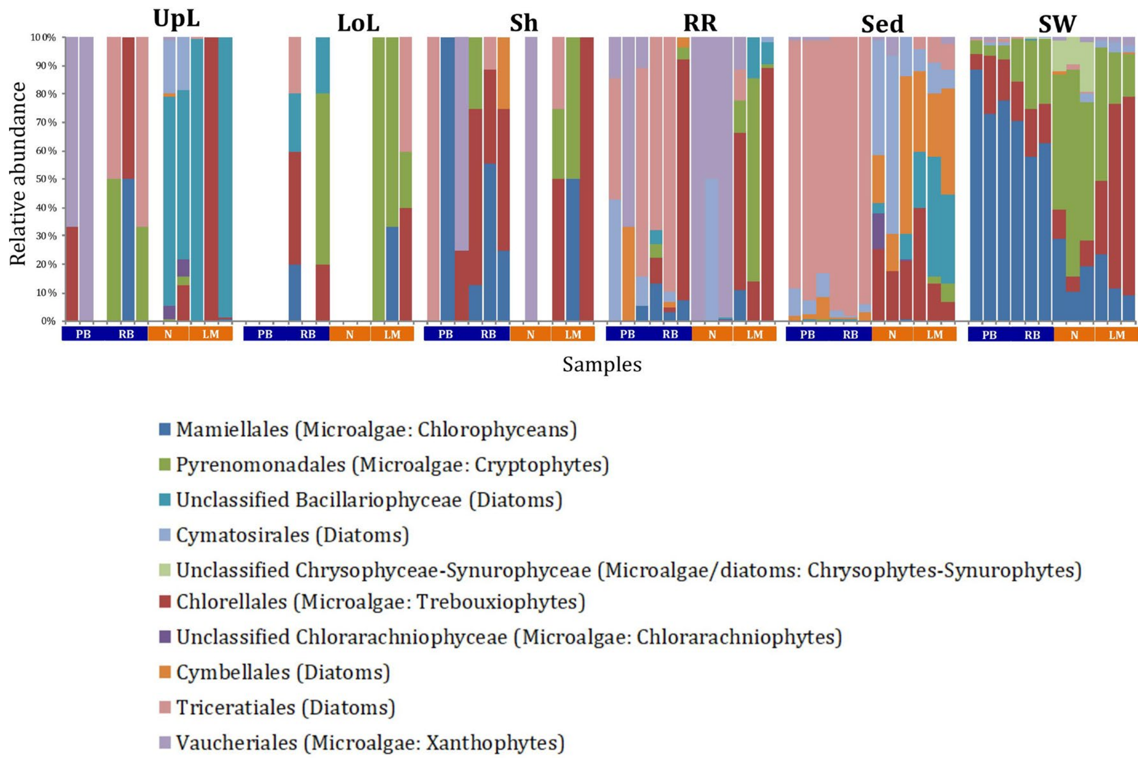
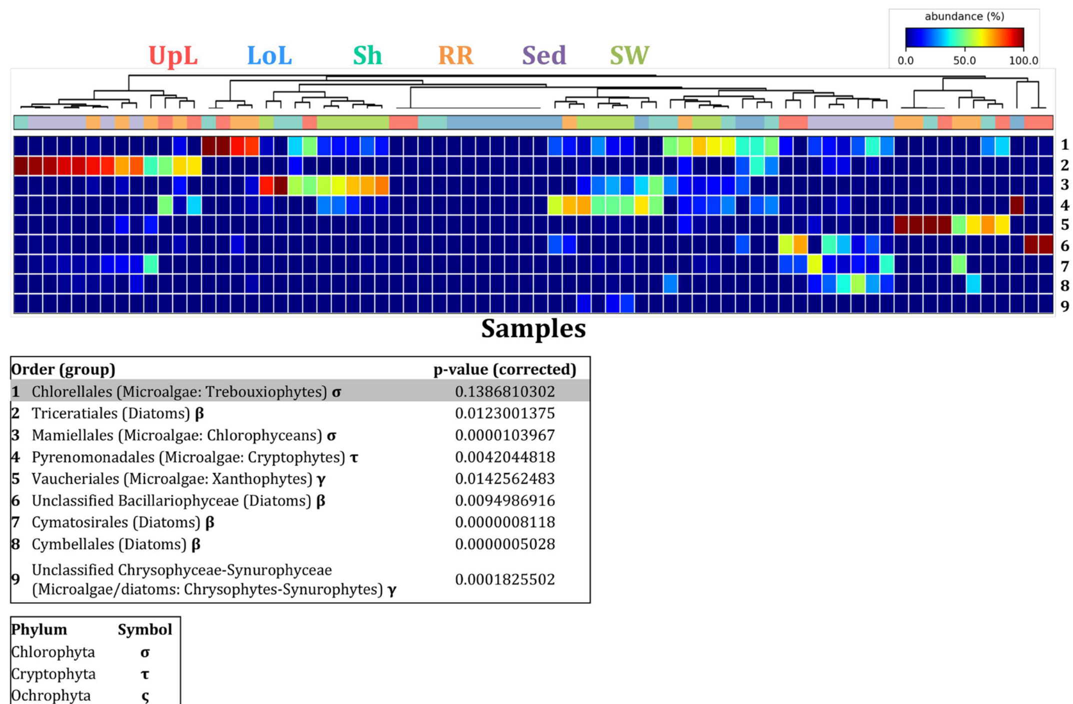


Figure 8: Microalgal community composition across seagrass microenvironments. Beta diversity of microalgal microbiomes across the six microenvironments within the plant and its surroundings. Triplicate samples per microenvironment within each of the four sampling sites (n = 72) are coloured by taxonomic order. Unique OTUs were summarized at the species level, and the representation of taxonomic groups within each sample are plotted. Only representative species with a relative abundance > 1% in all samples are shown to help remove visual clutter. UpL, upper leaf; LoL, lower leaf; Sh, sheath; RR, roots and rhizomes; Sed, sediment; SW, seawater; PB, Palm Beach; RB, Rose Bay; N, Narrabeen Lagoon; LM, Lake Macquarie.



	Order (group)	p-value (corrected)
1	Chlorellales (Microalgae: Trebouxiophytes) σ	0.1386810302
2	Triceratiales (Diatoms) β	0.0123001375
3	Mamiellales (Microalgae: Chlorophyceans) σ	0.0000103967
4	Pyrenomonadales (Microalgae: Cryptophytes) τ	0.0042044818
5	Vaucheriales (Microalgae: Xanthophytes) γ	0.0142562483
6	Unclassified Bacillariophyceae (Diatoms) β	0.0094986916
7	Cymatosirales (Diatoms) β	0.0000008118
8	Cymbellales (Diatoms) β	0.0000005028
9	Unclassified Chrysophyceae-Synurophyceae (Microalgae/diatoms: Chrysophytes-Synurophytes) γ	0.0001825502

Phylum	Symbol
Chlorophyta	σ
Cryptophyta	τ
Ochrophyta	ς

Figure 9: Microalgal discriminatory OTUs at the microenvironmental scale. Extensive hypothesis testing of taxonomic profiles was coupled with similarity percentages analyses (SIMPER) for microalgal microbiomes across the six microenvironments. The proportion of sequences (mean frequency %) of OTUs significantly enriched (Kruskal-Wallis H-test, $\alpha = 0.05$, effect sizes: η^2) and consistently contributing to the differences between microenvironments is indicated by varying colour intensities. Corrected p-values were calculated using the Benjamini-Hochberg's approach. Two-way crossed SIMPER analyses were performed with site and microenvironment variables as factors (S17 Bray-Curtis similarity matrix). High contributors were selected from the top-5 contributors of each pair-wise comparison between microenvironments, and those OTUs consistently accounting for the dissimilarities between any given microenvironment and at least three other microenvironments were chosen as high contributors to couple with the statistical results. High contributors that were significantly enriched were classified as discriminatory OTUs (i.e., 1-9). OTUs are sorted by decreasing mean abundance, and samples are clustered by average neighbour distance (UPGMA, distance threshold = 0.75) and coloured by microenvironment. Different symbols represent the distribution of enriched phyla. High contributors that were not significantly enriched (grey) were also classified as discriminatory OTUs if their contribution to the differences between microenvironments was consistent. UpL, upper leaf; LoL, lower leaf; Sh, sheath; RR, roots and rhizomes; Sed, sediment; SW, seawater.

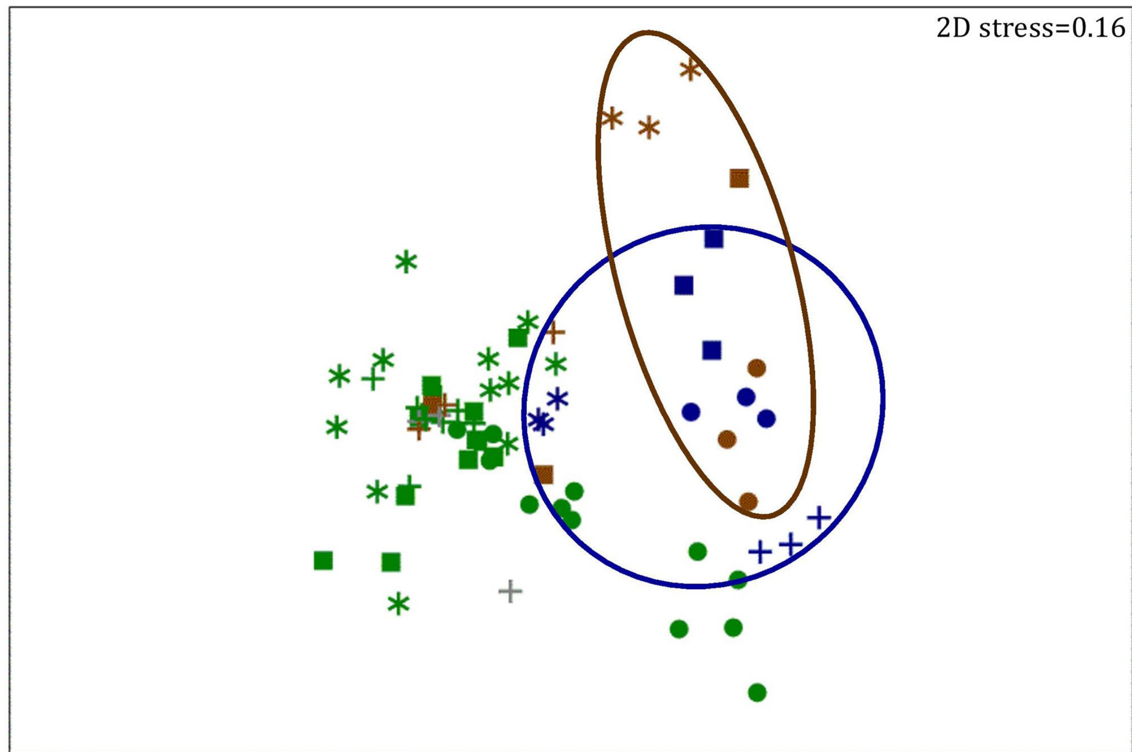


Figure 10: Microenvironmental and regional partitioning of the seagrass fungal microbiome.

Non-parametric multidimensional scaling (nMDS) of fungal microbiomes (n = 72), based on a lower triangular resemblance calculated with the S17 Bray-Curtis similarity measure from relative abundances of OTUs (high values down-weighted with square root). Samples are coloured by microenvironment (Plant: upper leaf, lower leaf, sheath and roots and rhizomes; Sed, sediment, SW, seawater), with different shapes for sites (PB, Palm Beach; RB, Rose Bay; N, Narrabeen Lagoon; LM, Lake Macquarie). Sample clustering patterns by microenvironment are shown in ellipses in the nMDS plot, representing the level of similarity between samples based on the degree to which OTUs are shared between them. The 2D stress is shown in the upper right corner of the nMDS plot (Kruskal stress formula = 1, minimum stress = 0.01). The nMDS for the four microenvironments associated with the plant is provided in **Supplementary Figure 6** and a hierarchical cluster analysis (CLUSTER) for all samples is provided in **Supplementary Figure 7**.

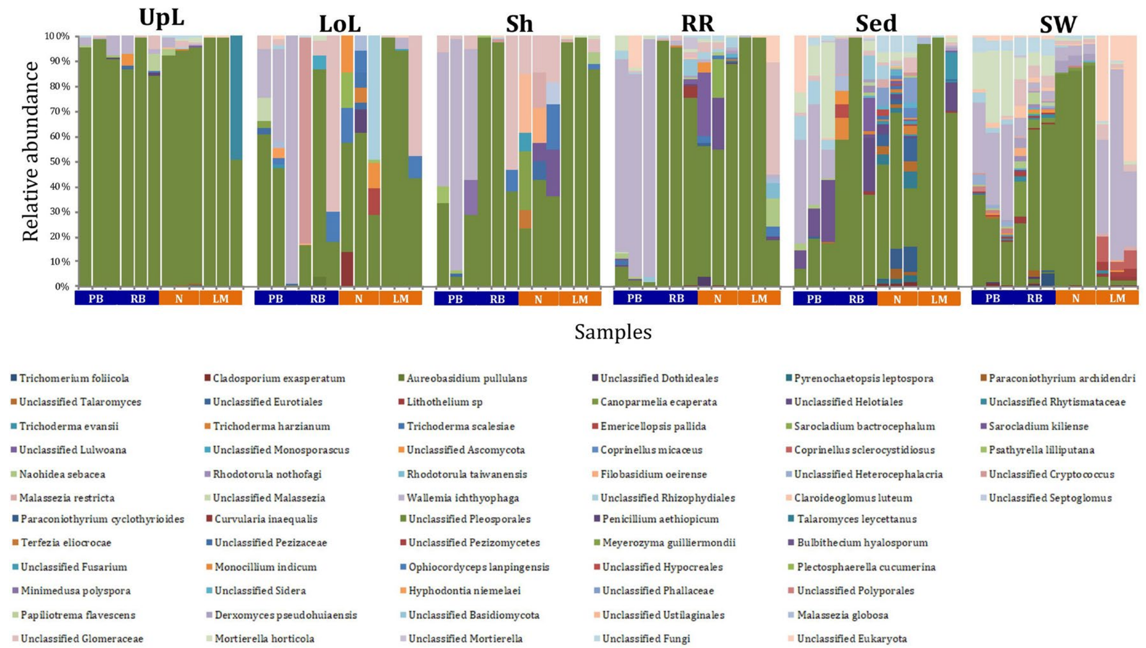
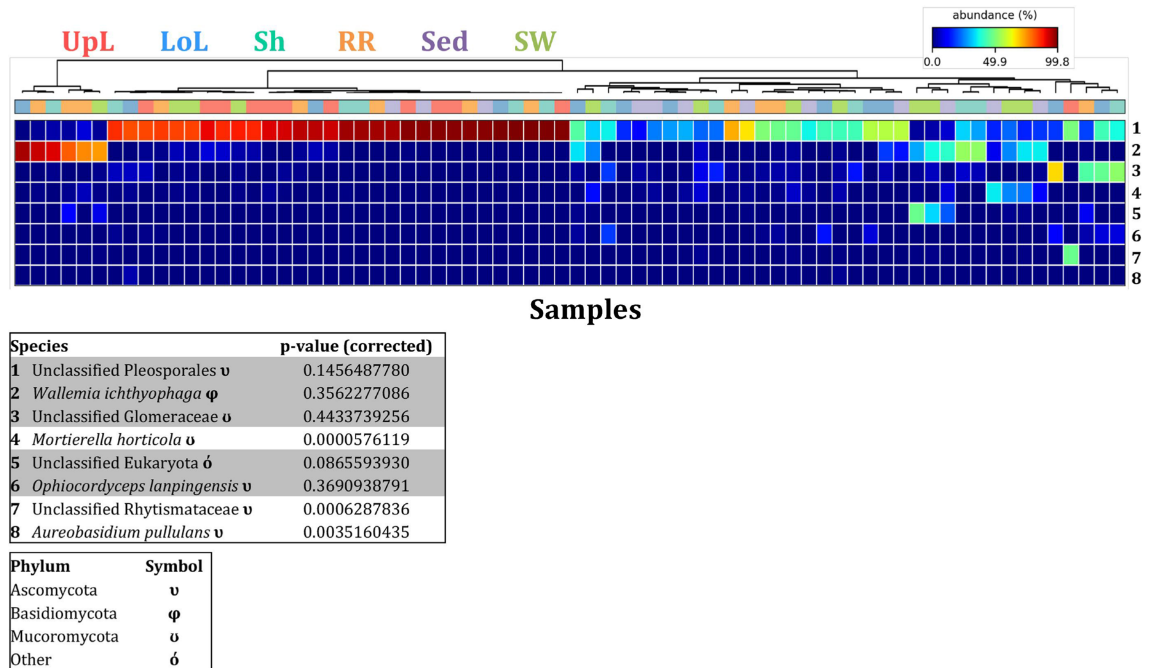


Figure 11: Fungal community composition across seagrass microenvironments. Beta diversity of fungal microbiomes across the six microenvironments within the plant and its surroundings. Triplicate samples per microenvironment within each of the four sampling sites (n = 72) are coloured by the highest assigned taxonomic level. Unique OTUs were summarized at the species level, and the representation of taxonomic groups within each sample are plotted. Only representative species with a relative abundance > 1% in all samples are shown to help remove visual clutter. UpL, upper leaf; LoL, lower leaf; Sh, sheath; RR, roots and rhizomes; Sed, sediment; SW, seawater; PB, Palm Beach; RB, Rose Bay; N, Narrabeen Lagoon; LM, Lake Macquarie.



	Species	p-value (corrected)
1	Unclassified Pleosporales υ	0.1456487780
2	*Wallemia ichthyophaga* φ	0.3562277086
3	Unclassified Glomeraceae ʋ	0.4433739256
4	*Mortierella horticola* ʋ	0.0000576119
5	Unclassified Eukaryota ό	0.0865593930
6	*Ophiocordyceps lanpingensis* υ	0.3690938791
7	Unclassified Rhytismataceae υ	0.0006287836
8	*Aureobasidium pullulans* υ	0.0035160435

Phylum	Symbol
Ascomycota	υ
Basidiomycota	φ
Mucoromycota	ʋ
Other	ό

Figure 12: Fungal discriminatory OTUs at the microenvironmental scale.
Extensive hypothesis testing of taxonomic profiles was coupled with similarity percentages analyses (SIMPER) for fungal microbiomes across the six microenvironments. The proportion of sequences (mean frequency %) of OTUs significantly enriched (Kruskal-Wallis H-test, $\alpha = 0.05$, effect sizes: η^2) and consistently contributing to the differences between microenvironments is indicated by varying colour intensities. Corrected p-values were calculated using the Benjamini-Hochberg's approach. Two-way crossed SIMPER analyses were performed with site and microenvironment variables as factors (S17 Bray-Curtis similarity matrix). High contributors were selected from the top-5 contributors of each pair-wise comparison between microenvironments, and those OTUs consistently accounting for the dissimilarities between any given microenvironment and at least three other microenvironments were chosen as high contributors to couple with the statistical results. High contributors that were significantly enriched were classified as discriminatory OTUs (i.e., 1-8). OTUs are sorted by decreasing mean abundance, and samples are clustered by average neighbour distance (UPGMA, distance threshold = 0.75) and coloured by microenvironment. Different symbols represent the distribution of enriched phyla. High contributors that were not significantly enriched (grey) were also classified as discriminatory OTUs if their contribution to the differences between microenvironments was consistent. UpL, upper leaf; LoL, lower leaf; Sh, sheath; RR, roots and rhizomes; Sed, sediment; SW, seawater.

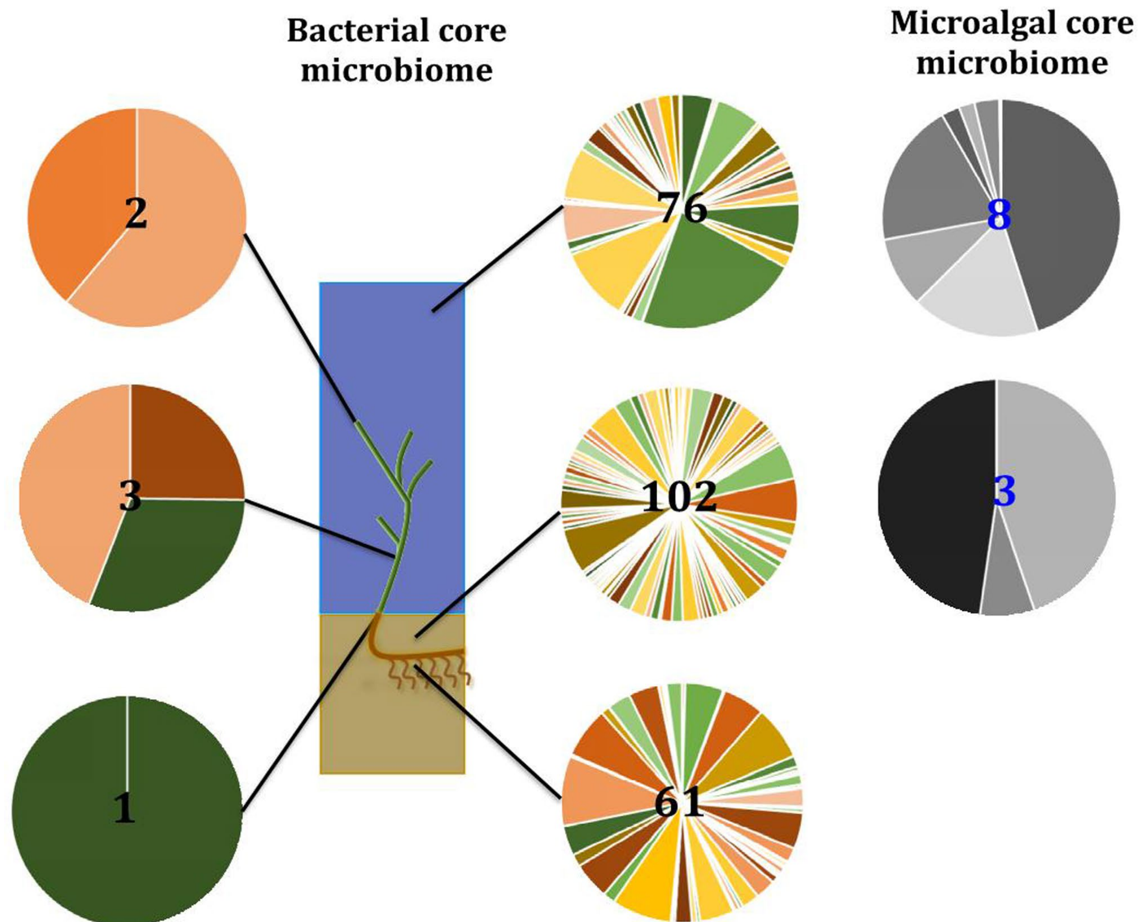
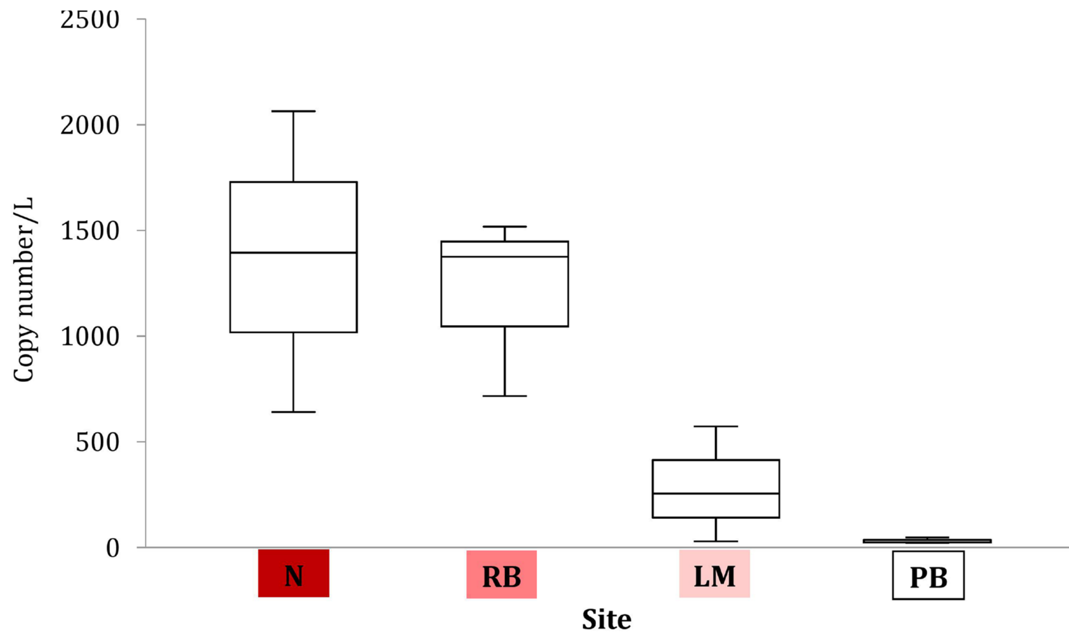


Figure 13: Ecological dynamics of the seagrass microbiomes.

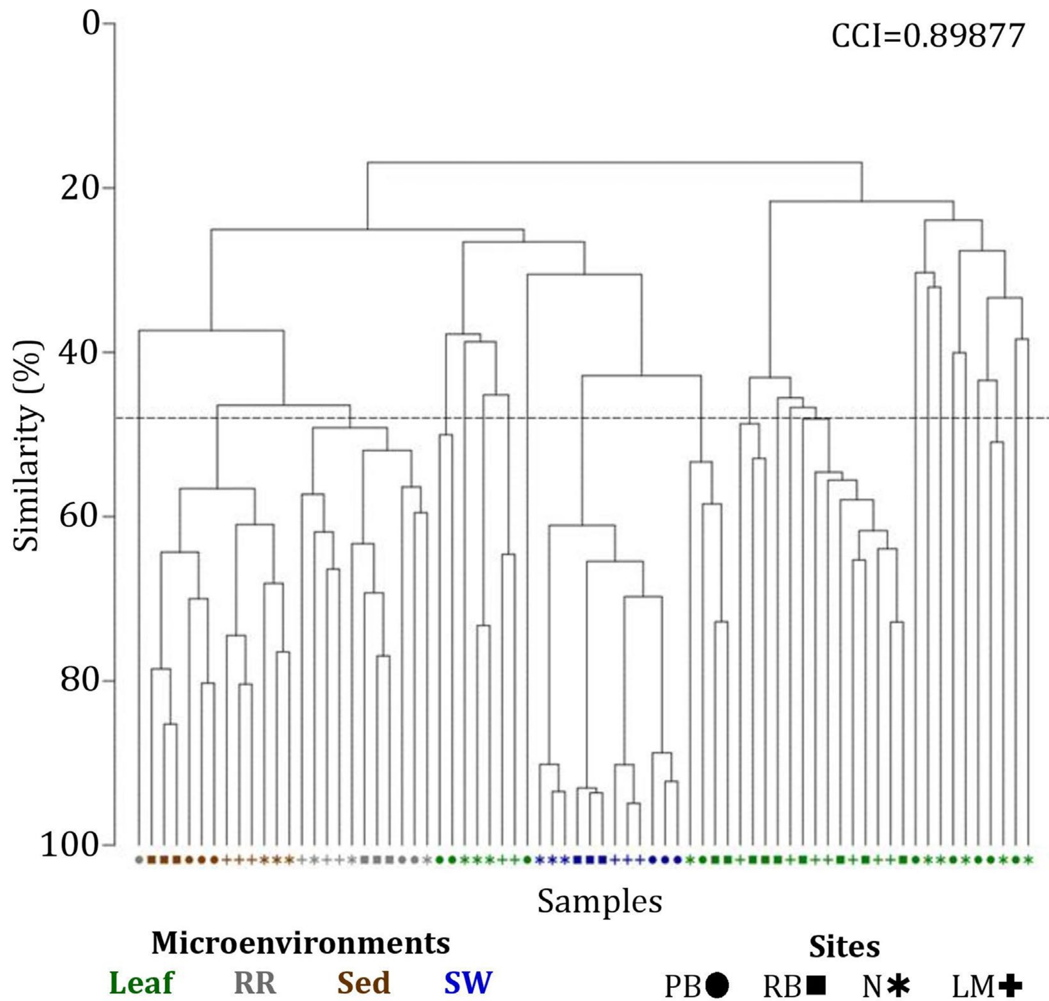
Distinct microbial communities live in association with disparate sections of the plant (i.e., upper leaf, lower leaf, sheath, and roots and rhizomes) and its surroundings (i.e., surficial sediment and adjacent seawater). Their composition and structure are strongly shaped by the varying conditions offered within each microenvironment and also influenced by the environment. Therefore, specific bacterial (full colour) and microalgal (grey) members make up core microbiomes that are different from each other and constitute up to 4% of the entire microbiome. This variability at the microscale is well conserved within each site, and despite the biogeographical changes of microbial communities, there are some microorganisms that consistently occur within microenvironment types. Numbers in the middle represent total number of core members.

2.8 Supplementary figures

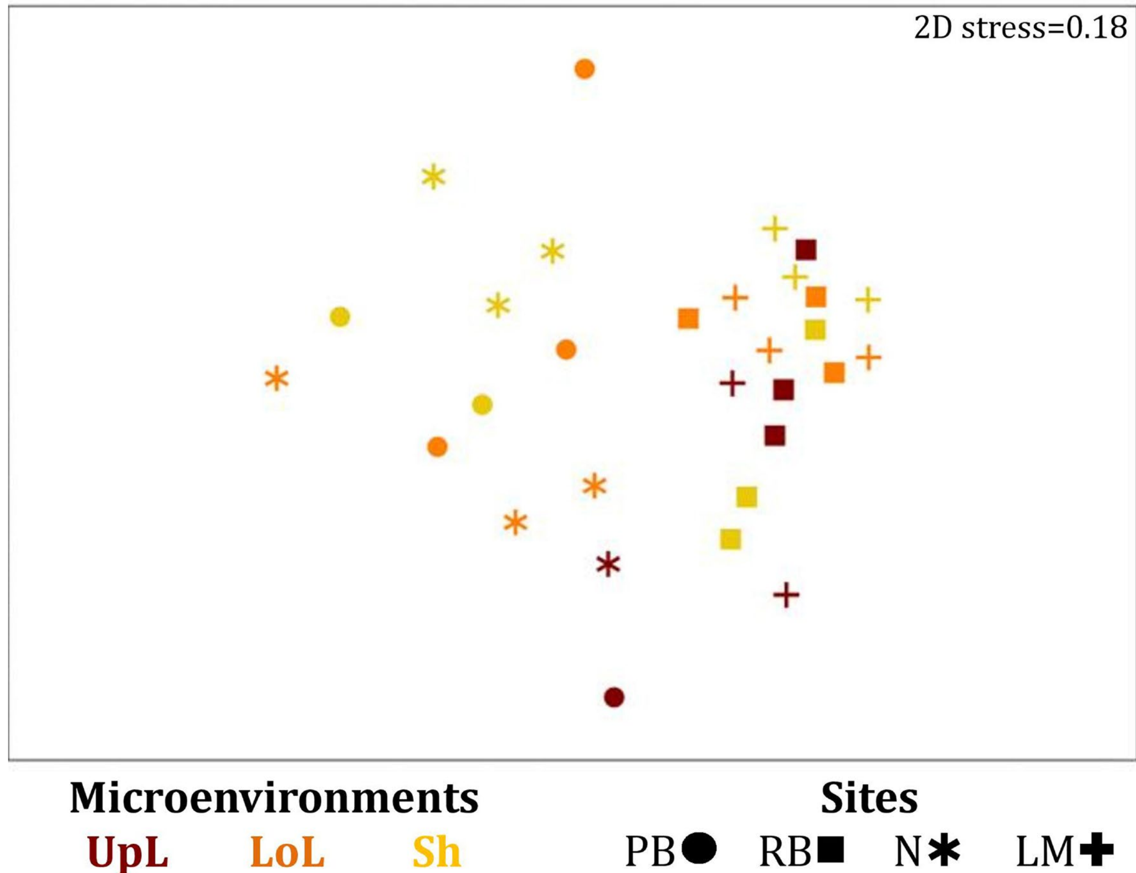


Supplementary Figure 1: Quantification of the clinical class 1 integron-integrase gene intI1 as a proxy for anthropogenic pollution.

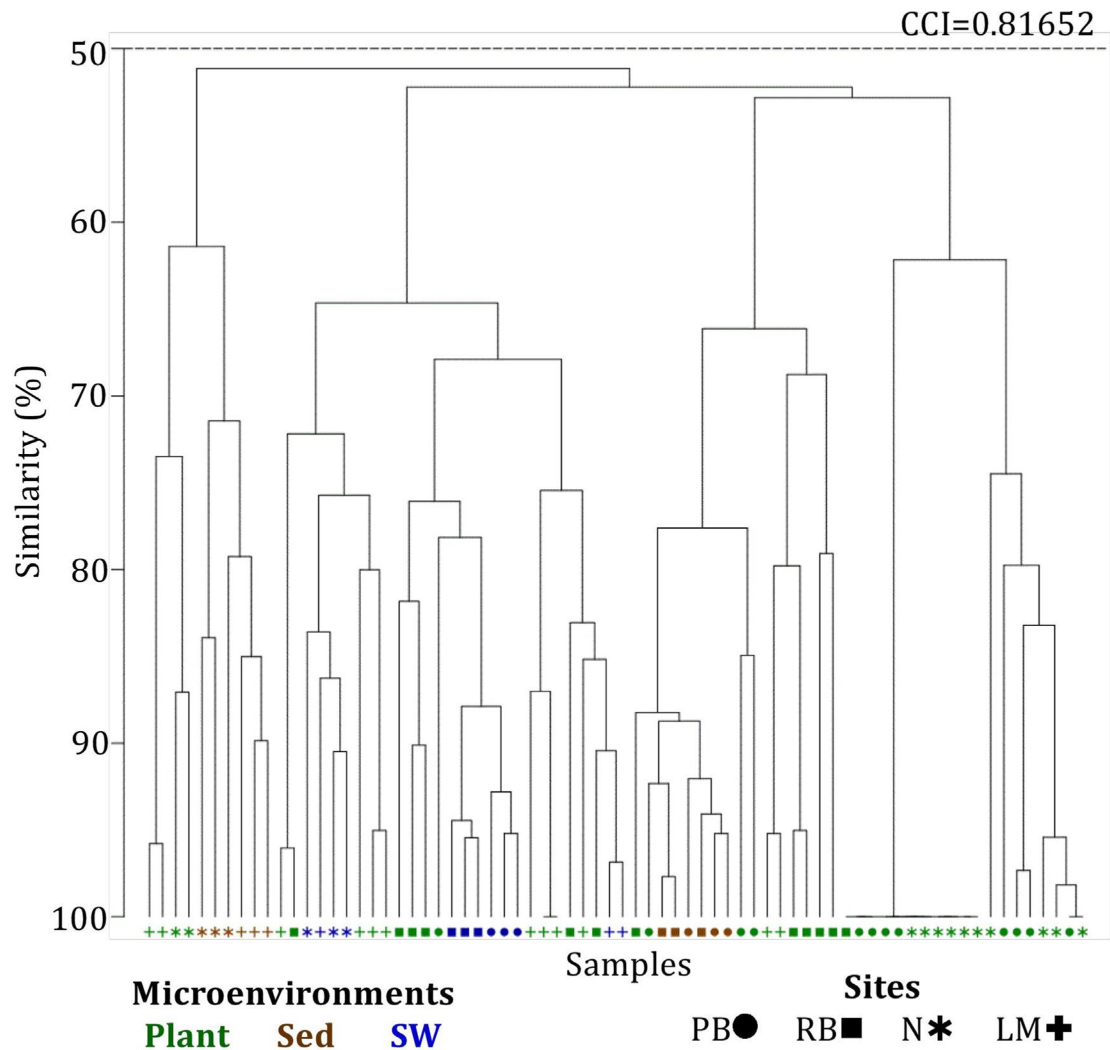
The relative abundance of the gene intl1 was estimated by quantitative real-time polymerase chain reaction (qPCR). Normalized values and the corresponding standard deviation (n = 3) are shown for each site (N: Narrabeen Lagoon, RB: Rose Bay, LM: Lake Macquarie, PB: Palm Beach). Sites are colored by decreasing levels of anthropogenic impact from left to right.



Supplementary Figure 2: Clustering patterns of the seagrass bacterial microbiome. Hierarchical cluster analysis (CLUSTER) of bacterial microbiomes, based on a lower triangular resemblance calculated with the S17 Bray-Curtis similarity measure from relative abundances of OTUs (high values down-weighted with square root). Samples are coloured by microenvironment (Leaf, RR: roots and rhizomes, Sed: sediment, SW: seawater), with different shapes for sites (PB: Palm Beach, RB, Rose Bay, N: Narrabeen Lagoon, LM: Lake Macquarie). Group average linkage was used to calculate distances between clusters and generate a dendogram and a cophenetic distance matrix (unsupervised learning method). The cophenetic correlation index (CCI) is provided in the upper right corner of the dendogram, and it was used to assess the faithfulness of the dendogram by computing cophenetic correlation coefficients.

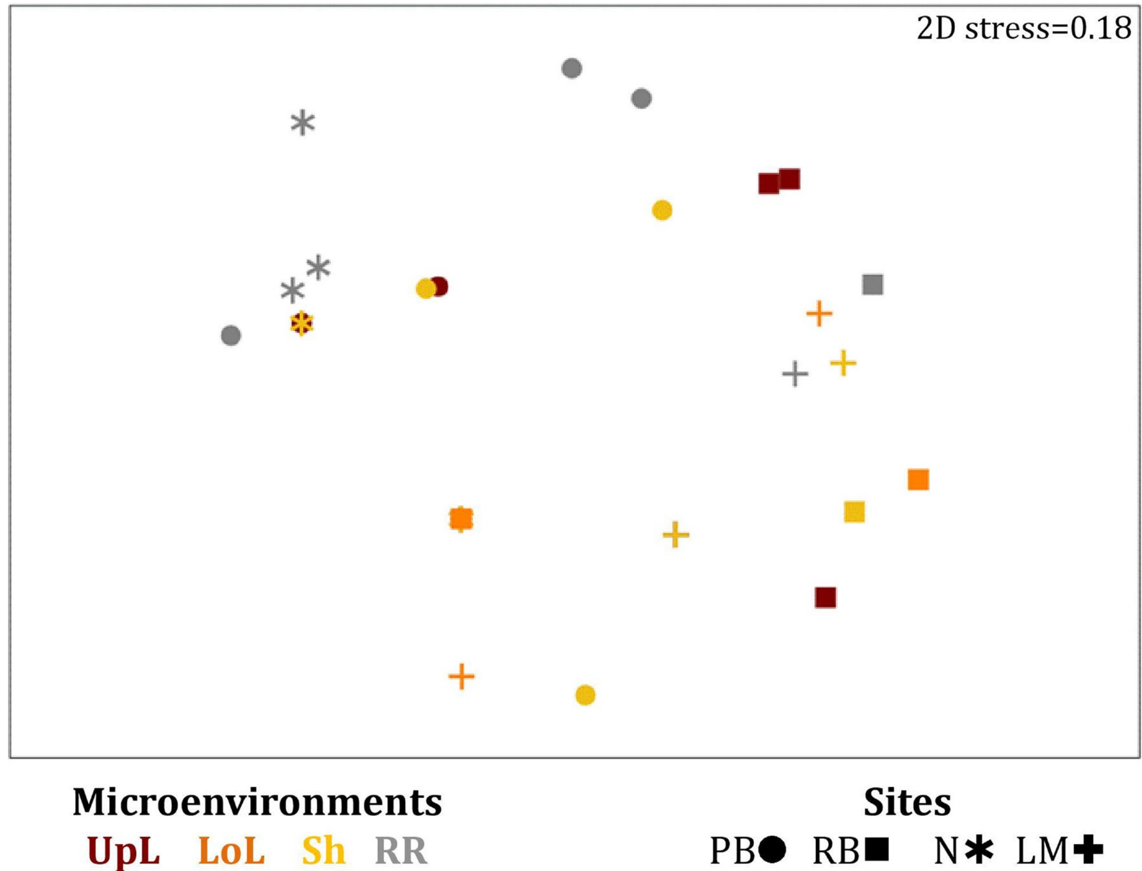


Supplementary Figure 3: The seagrass leaf-associated microbiomes (Bacteria). Non-parametric multidimensional scaling (nMDS subset) of bacterial microbiomes associated with the seagrass phyllosphere (n = 9), based on a lower triangular resemblance calculated with the S17 Bray-Curtis similarity measure from relative abundances of OTUs (high values down-weighted with square root). Samples are coloured by microenvironment within the leaf (UpL: upper leaf, LoL: lower leaf, Sh: Sheath), with different shapes for sites (PB: Palm Beach, RB, Rose Bay, N: Narrabeen Lagoon, LM: Lake Macquarie). Clustering patterns represent the level of similarity between samples based on the degree to which OTUs are shared between them. The 2D stress is shown in the upper right corner of the nMDS plot (Kruskal stress formula = 1, minimum stress = 0.01).

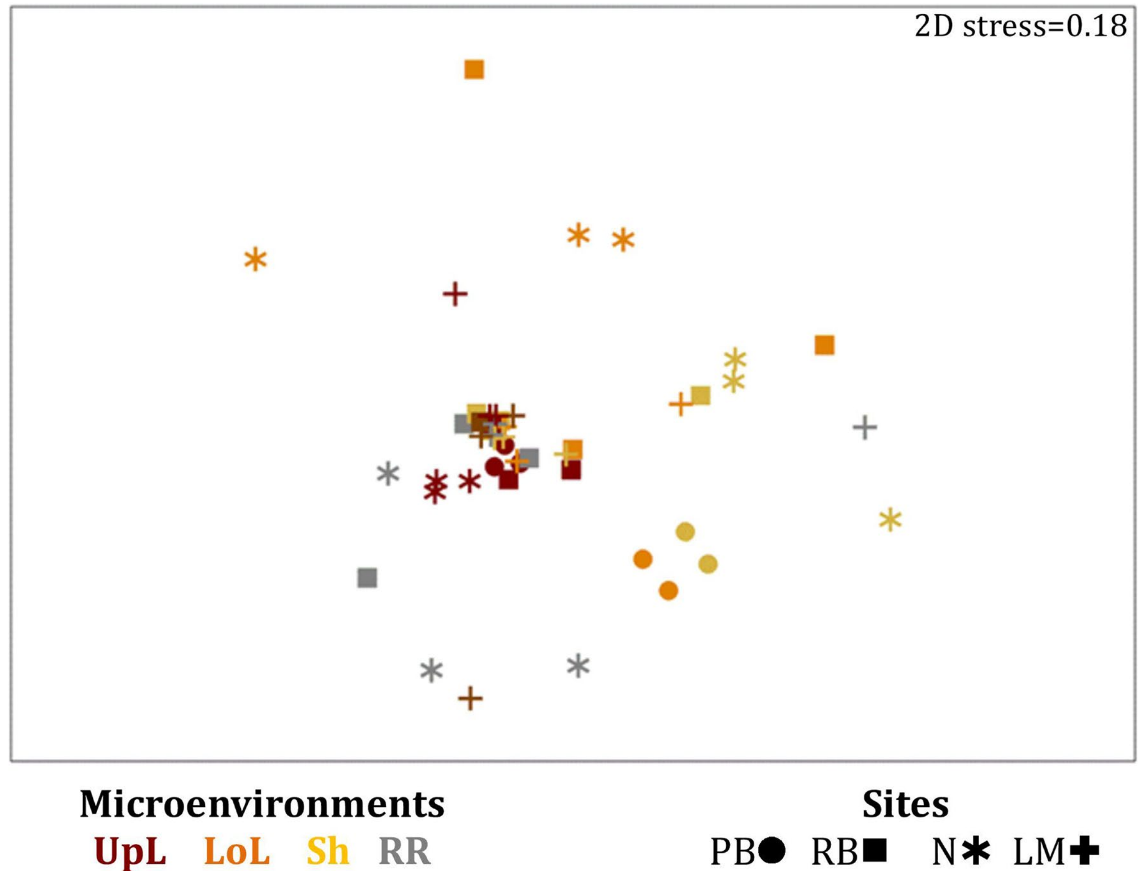


Supplementary Figure 4: Clustering patterns of the seagrass microalgal microbiome.

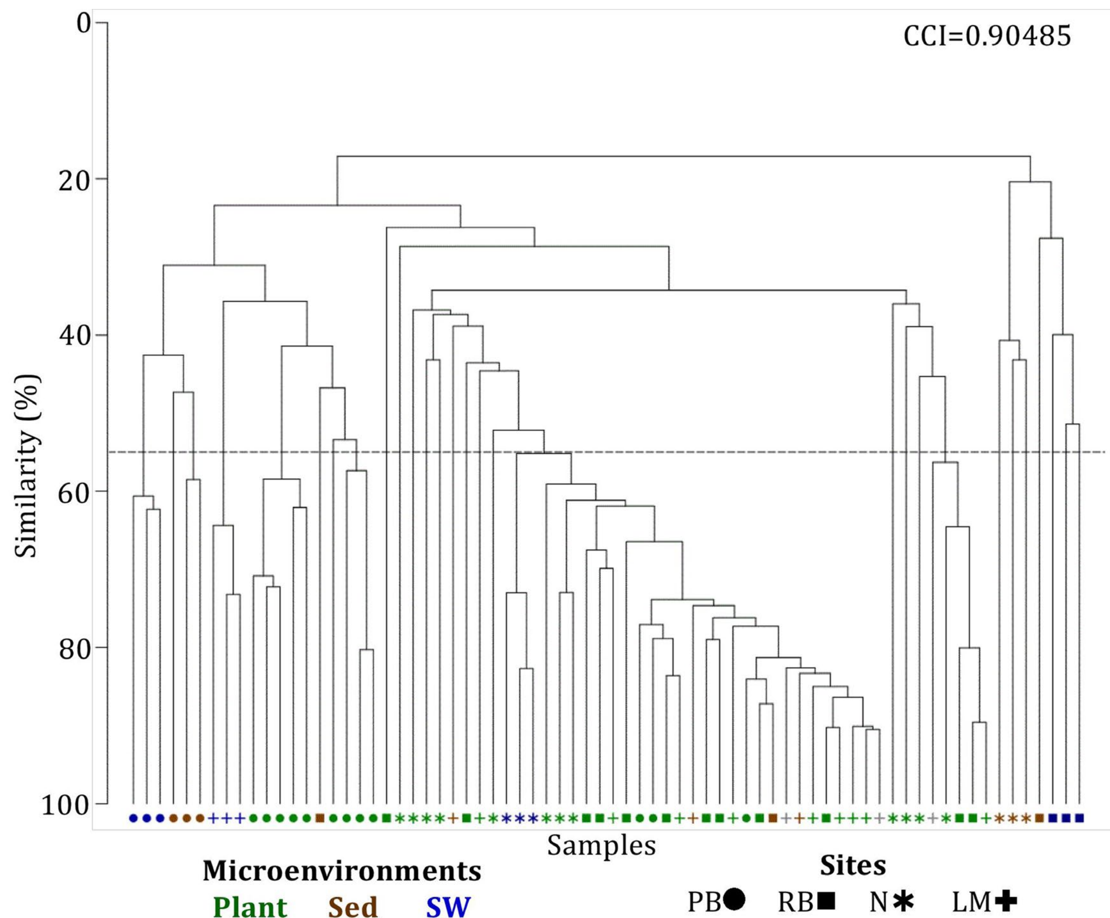
Hierarchical cluster analysis (CLUSTER) of microalgal microbiomes, based on a lower triangular resemblance calculated with the S17 Bray-Curtis similarity measure from relative abundances of OTUs (high values down-weighted with square root). Samples are coloured by microenvironment (Plant: upper leaf, lower leaf, sheath and roots and rhizomes, Sed: sediment, SW: seawater), with different shapes for sites (PB: Palm Beach, RB, Rose Bay, N: Narrabeen Lagoon, LM: Lake Macquarie). Group average linkage was used to calculate distances between clusters and generate a dendogram and a cophenetic distance matrix (unsupervised learning method). The cophenetic correlation index (CCI) is provided in the upper right corner of the dendogram, and it was used to assess the faithfulness of the dendogram by computing cophenetic correlation coefficients.



Supplementary Figure 5: The seagrass plant-associated microbiomes (Microalgae). Non-parametric multidimensional scaling (nMDS subset) of microalgal microbiomes associated with the seagrass phyllosphere (*n* = 9), based on a lower triangular resemblance calculated with the S17 Bray-Curtis similarity measure from relative abundances of OTUs (high values down-weighted with square root). Samples are coloured by microenvironment within the plant (UpL: upper leaf, LoL: lower leaf, Sh: Sheath, RR: roots and rhizomes), with different shapes for sites (PB: Palm Beach, RB, Rose Bay, N: Narrabeen Lagoon, LM: Lake Macquarie). Clustering patterns represent the level of similarity between samples based on the degree to which OTUs are shared between them. The 2D stress is shown in the upper right corner of the nMDS plot (Kruskal stress formula = 1, minimum stress = 0.01).



Supplementary Figure 6: The seagrass plant-associated microbiomes (Fungi). Non-parametric multidimensional scaling (nMDS subset) of fungal microbiomes associated with the seagrass phyllosphere (n = 9), based on a lower triangular resemblance calculated with the S17 Bray-Curtis similarity measure from relative abundances of OTUs (high values down-weighted with square root). Samples are coloured by microenvironment within the plant (UpL: upper leaf, LoL: lower leaf, Sh: Sheath, RR: roots and rhizomes), with different shapes for sites (PB: Palm Beach, RB, Rose Bay, N: Narrabeen Lagoon, LM: Lake Macquarie). Clustering patterns represent the level of similarity between samples based on the degree to which OTUs are shared between them. The 2D stress is shown in the upper right corner of the nMDS plot (Kruskal stress formula = 1, minimum stress = 0.01).



Supplementary Figure 7: Clustering patterns of the seagrass fungal microbiome. Hierarchical cluster analysis (CLUSTER) of fungal microbiomes, based on a lower triangular resemblance calculated with the S17 Bray-Curtis similarity measure from relative abundances of OTUs (high values down-weighted with square root). Samples are coloured by microenvironment (Plant: upper leaf, lower leaf, sheath and roots and rhizomes, Sed: sediment, SW: seawater), with different shapes for sites (PB: Palm Beach, RB, Rose Bay, N: Narrabeen Lagoon, LM: Lake Macquarie). Group average linkage was used to calculate distances between clusters and generate a dendogram and a cophenetic distance matrix (unsupervised learning method). The cophenetic correlation index (CCI) is provided in the upper right corner of the dendogram, and it was used to assess the faithfulness of the dendogram by computing cophenetic correlation coefficients.

2.9 Supplementary tables

Supplementary Table 1: Study sites selection criteria and categorization based on anthropogenic impact ranking.

Study site name	Region	Coordinates (latitude longitude)	T (°C) a	S (mS/cm) b	Reference site c	Reference site type c	Reference sanitary inspection category c	Reference microbial assessment category c	Reference suitability grade c	Distance to closest contamination source (m)d	Relative abundance of the clinical class 1 integron-integrase gene *intI1* (gene copy number/L) e	Overall categorization f
Narrabeen Lagoon	Northern Sydney	33°43'11.0"S 151°17'40.4"E	24.1	54.3	Narrabeen Lagoon	Lagoon	Moderate	Category B	Good	308 to Pittwater Road (A8)	1366.43	4
Rose Bay	Central Sydney	33°52'20.1"S 151°15'43.7"E	24.0	52.9	Rose Bay Beach	Estuarine	Moderate	Category B	Good	927 to Point Piper Marina	1203.36	3
Lake Macquarie	Hunter Region	33°09'29.4"S 151°31'54.9"E	30.3	50.4	Sunshine	Lagoon/lake	Moderate	Category B	Good	1323 to the Vales Point Power Station	285.78	2
Palm Beach	Northern Sydney	33°35'15.8"S 151°19'25.0"E	21.1	52.7	Palm Beach	Ocean Beach	Low	Category A	Very Good	1063 to Palm Beach Ferry Wharf	31.29	1

a Temperature
b Salinity (conductivity)
c According to State of the Beaches 2014-2015 Report (BeachWatch, Office of Environment & Heritage)
d From environmental survey observations (distances measured in Google Earth)
e Quantified by qPCR (average for n=3 per site). See details in **Figure 2.8-1.**
f Final assessment of anthropogenic impact level (1=very low risk, 6=very high risk)

Supplementary Table 2: Statistical analyses for bacterial mean alpha diversity (Chao1 diversity).

Differences in alpha diversity as measured by Chao1 diversity across sites and microenvironments (main test), between sites and between microenvironments within sites (pair-wise) were tested for statistical significance in Permutational Multivariate Analysis of Variance (PERMANOVA, Minkowski metric distance matrix, nested design).

Main test

Factor	P(perm)
Site	0.0017
Microenvironment (Site)	0.0001

ECV

Source	Estimate	Sq.root
S(si)	3096.80	**55.65**
S(mi(si))	**22715.00**	150.72
V(Res)	10933.00	104.56

Pair-wise (Site)

P(perm)	PB	RB	N	LM
PB				
RB	0.0021			
N	0.8536	0.0019		
LM	0.0664	0.1495	0.0553	

Pair-wise (Micenv. PB)

P(MC) **i**	UpL	LoL	Sh	RR	Sed	SW
UpL						
LoL	0.2761					
Sh	0.8144	0.2423				
RR	0.1652	0.0356	0.2564			
Sed	0.0099	0.0002	0.0270	0.1442		
SW	0.0231	0.0014	0.0638	0.4560	0.0770	

Pair-wise (Micenv. RB)

P(MC) **i**	UpL	LoL	Sh	RR	Sed	SW
UpL						
LoL	0.5277					
Sh	0.6042	0.8607				
RR	0.1556	0.0238	0.0280			
Sed	0.1533	0.0282	0.0317	0.8830		
SW	0.8966	0.2542	0.3359	0.0432	0.0577	

Pair-wise (Micenv. N)

P(MC) **i**	UpL	LoL	Sh	RR	Sed	SW
UpL						
LoL	0.0650					
Sh	0.0484	0.4732				
RR	0.0465	0.0002	0.0001			
Sed	0.2357	0.0014	0.0011	0.0629		
SW	0.9587	0.0025	0.0002	0.0011	0.0372	

Pair-wise (Micenv. LM)

P(MC) **i**	UpL	LoL	Sh	RR	Sed	SW
UpL						
LoL	0.6931					
Sh	0.2997	0.3728				
RR	0.1850	0.0705	0.0382			
Sed	0.0989	0.0479	0.0287	0.4854		
SW	0.2222	0.0470	0.0274	0.5525	0.2319	

i Monte Carlo p-values used when there were not enough possible permutations (unrestricted permutation method, 9999 permutations)

Significant values at the 0.05 level are shown in grey
ECV: estimates of components of variation
UpL: upper leaf, LoL: lower leaf, Sh: sheath, RR: roots and rhizomes, Sed: sediment, SW: seawater
PB: Palm Beach, RB: Rose Bay, N: Narrabeen Lagoon, LM: Lake Macquarie

Supplementary Table 3: Statistical analyses for bacterial mean alpha diversity (Shannon's diversity index).

Differences in alpha diversity as measured by Shannon's diversity index across sites and microenvironments (main test), between sites and between microenvironments within sites (pair-wise) were tested for statistical significance in Permutational Multivariate Analysis of Variance (PERMANOVA, Minkowski metric distance matrix, nested design).

Main test

Factor	P(perm)
Site	0.0001
Microenvironment (Site)	0.0001

ECV

Source	Estimate	Sq.root
S(si)	14990.00	**122.43**
S(mi(si))	**176980.00**	420.70
V(Res)	34447.00	185.60

Pair-wise (Site)

P(perm)	PB	RB	N	LM
PB				
RB	0.0022			
N	0.9303	0.0004		
LM	0.6282	0.0006	0.4958	

Pair-wise (Micenv. PB)

P(MC) **i**	UpL	LoL	Sh	RR	Sed	SW
UpL						
LoL	0.0969					
Sh	0.4639	0.3676				
RR	0.0135	0.0068	0.9387			
Sed	0.0068	0.0064	0.0454	0.0148		
SW	0.1324	0.0027	0.5523	0.0190	0.0082	

Pair-wise (Micenv. RB)

P(MC) **i**	UpL	LoL	Sh	RR	Sed	SW
UpL						
LoL	0.1516					
Sh	0.1727	0.1481				
RR	0.0004	0.0004	0.0017			
Sed	0.0120	0.0118	0.0291	0.6959		
SW	0.0679	0.0024	0.2027	0.0002	0.0118	

Pair-wise (Micenv. N)

P(MC) **i**	UpL	LoL	Sh	RR	Sed	SW
UpL						
LoL	0.4809					
Sh	0.0707	0.3325				
RR	0.0116	0.0090	0.0022			
Sed	0.0075	0.0061	0.0029	0.0646		
SW	0.1776	0.7298	0.0022	0.0030	0.0035	

Pair-wise (Micenv. LM)

P(MC) **i**	UpL	LoL	Sh	RR	Sed	SW
UpL						
LoL	0.0938					
Sh	0.0873	0.1957				
RR	0.1410	0.0253	0.0257			
Sed	0.1855	0.0506	0.0458	0.9076		
SW	0.2155	0.0007	0.0001	0.0379	0.0693	

i Monte Carlo p-values used when there were not enough possible permutations (unrestricted permutation method, 9999 permutations)

Significant values at the 0.05 level are shown in grey
ECV: estimates of components of variation
UpL: upper leaf, LoL: lower leaf, Sh: sheath, RR: roots and rhizomes, Sed: sediment, SW: seawater
PB: Palm Beach, RB: Rose Bay, N: Narrabeen Lagoon, LM: Lake Macquarie

Supplementary Table 4: Statistical analyses for multidimensional scaling (Bacteria).

Differences between bacterial communities across sites and microenvironments (top), between sites (middle), and between microenvironments within sites (bottom) were tested for statistical significance in Permutational Multivariate Analysis of Variance (PERMANOVA, Bray-Curtis dissimilarity matrix, nested design).

Main test

Factor	P(perm)
Site	0.0001
Microenvironment (Site)	0.0001

ECV

Source	Estimate	Sq.root
S(Si)	248.33	**15.76**
S(Mi(Si))	**1763.90**	42.00
V(Res)	1128.50	33.59

Pair-wise (Site)

P(perm)	PB	RB	N	LM
PB				
RB	0.0001			
N	0.0332	0.0001		
LM	0.0001	0.0048	0.0001	

Pair-wise (Micenv. PB)

P(MC) **i**	UpL	LoL	Sh	RR	Sed	SW
UpL						
LoL	0.1444					
Sh	0.1465	0.4527				
RR	0.0331	0.0404	0.0776			
Sed	0.0290	0.0296	0.0586	0.0242		
SW	0.0247	0.0270	0.0454	0.0045	0.0005	

Pair-wise (Micenv. RB)

P(MC) **i**	UpL	LoL	Sh	RR	Sed	SW
UpL						
LoL	0.3820					
Sh	0.1210	0.1154				
RR	0.0086	0.0055	0.0296			
Sed	0.0072	0.0032	0.0194	0.0202		
SW	0.0061	0.0032	0.0200	0.0009	0.0003	

Pair-wise (Micenv. N)

P(MC) **i**	UpL	LoL	Sh	RR	Sed	SW
UpL						
LoL	0.1194					
Sh	0.0411	0.3954				
RR	0.0353	0.0510	0.0286			
Sed	0.0184	0.0349	0.0143	0.0343		
SW	0.0098	0.0428	0.0143	0.0022	0.0003	

Pair-wise (Micenv. LM)

P(MC) **i**	UpL	LoL	Sh	RR	Sed	SW
UpL						
LoL	0.0552					
Sh	0.0282	0.5277				
RR	0.0164	0.0087	0.0057			
Sed	0.0107	0.0047	0.0034	0.0220		
SW	0.0091	0.0043	0.0022	0.0017	0.0005	

i Monte Carlo p-values used when there were not enough possible permutations (unrestricted permutation method, 9999 permutations)

Significant values at the 0.05 level are shown in grey
ECV: estimates of components of variation
UpL: upper leaf, LoL: lower leaf, Sh: sheath, RR: roots and rhizomes, Sed: sediment, SW: seawater
PB: Palm Beach, RB: Rose Bay, N: Narrabeen Lagoon, LM: Lake Macquarie

Supplementary Table 5: The seagrass bacterial core microbiome (full version).

Bacterial core microbiome												
RA (%)	UpL (2)		LoL (3)		Sh (1)		RR (61)		Sed (102)		SW (76)	
min=0.0001	Pseudomonadaceae_Pseudomonas_1150	0.0061	Pseudomonadaceae_Pseudomonas_1150	0.0143	Propionibacteriaceae_Propionibacterium-acnes_1828	0.0054	Unclassified-Chromatiales_546	0.0533	Unclassified-Alteromonadales_HTCC2188_HTCC_713	0.0173	Pelagibacteraceae_2265	0.8605
50th pctl=0.0023	Rhodobacteraceae_2791	0.0039	Propionibacteriaceae_Propionibacterium-acnes_1828	0.0099			Unclassified-Chromatiales_460	0.0452	Flavobacteriaceae_694	0.0168	Rhodobacteraceae_2345	0.3895
max=0.8605			Comamonadaceae_1435	0.0082			Desulfobacteraceae_124	0.0411	Flavobacteriaceae_690	0.0142	Synechococcaceae_Synechococcus_2428	0.2739
							Unclassified-Chromatiales_729	0.0387	Unclassified-Chromatiales_632	0.0120	Cryomorphaceae_992	0.2347
							Cystobacterineae_125	0.0315	Desulfobacteraceae_Desulfosarcina_18	0.0086	Halomonadaceae_Candidatus-Portiera_894	0.2225
							Cohaesibacteraceae_Cohaesibacter_2378	0.0291	Desulfobacteraceae_Desulfococcus_25	0.0083	Rhodobacteraceae_2687	0.1977
							Unclassified-Chromatiales_485	0.0282	Unclassified-Bacteroidales_1459	0.0073	Cryomorphaceae_1371	0.1666
							Lachnospiraceae_1626	0.0276	Myxococcales_583	0.0073	Flavobacteriaceae_1369	0.1225
							Unclassified-Anaerolineae_DRC31_1861	0.0261	Myxococcales_506	0.0071	Synechococcaceae_Synechococcus_2638	0.0865
							Unclassified-Gammaproteobacteria_702	0.0228	Unclassified-Chromatiales_656	0.0066	Unclassified-Oceanospirillales_352	0.0829
							Unclassified-Chromatiales_519	0.0228	Piscirickettsiaceae_740	0.0064	Unclassified-Planctomycetes_OM190_CL500-15_247	0.0731
							Unclassified-Gammaproteobacteria_677	0.0173	Piscirickettsiaceae_785	0.0059	Halomonadaceae_Candidatus-Portiera_1161	0.0698
							Ruminococcaceae_1779	0.0153	Unclassified-Chromatiales_2186	0.0056	Microbacteriaceae_1785	0.0644
							Hyphomicrobiaceae_2377	0.0137	Unclassified-Chromatiales_930	0.0053	Flavobacteriaceae_913	0.0563
							Ruminococcaceae_1781	0.0133	Flavobacteriaceae_832	0.0052	Halomonadaceae_Candidatus-Portiera_1178	0.0559
							Unclassified-Bacteroidales_1148	0.0127	Piscirickettsiaceae_791	0.0049	Pelagibacteraceae_2363	0.0555
							Unclassified-Thiotrichales_624	0.0117	Hyphomicrobiaceae_2702	0.0044	Synechococcaceae_Synechococcus_2512	0.0518
							Methylophilaceae_Methylotenera-mobilis_905	0.0110	Piscirickettsiaceae_858	0.0043	Methylophilaceae_682	0.0470
							Unclassified-Chromatiales_492	0.0094	Piscirickettsiaceae_749	0.0042	Flavobacteriaceae_Polaribacter_1370	0.0445
							Unclassified-Chromatiales_480	0.0093	Piscirickettsiaceae_762	0.0041	Unclassified-Cyanobacteria_2340	0.0443
							Desulfobacteraceae_127	0.0083	Desulfobacteraceae_Desulfococcus_41	0.0041	Rhodobacteraceae_2684	0.0441
							Flavobacteriaceae_690	0.0078	Desulfobacteraceae_Desulfococcus_53	0.0037	Unclassified-Rickettsiales_2865	0.0428
							Unclassified-Chromatiales_753	0.0066	Flavobacteriaceae_959	0.0035	Unclassified-Flavobacteriales_NS9_1163	0.0422
							Rhodobacteraceae_Loktanella_2698	0.0051	Unclassified-Chromatiales_771	0.0032	Unclassified-Acidimicrobiales_OCS155_1802	0.0357
							Pseudomonadaceae_Pseudomonas_1150	0.0039	Piscirickettsiaceae_773	0.0031	Pelagibacteraceae_2385	0.0339
							Unclassified-Bacteria_KSB3_589	0.0039	Unclassified-Chromatiales_546	0.0030	Flavobacteriaceae_1402	0.0336
							Spirochaetaceae_Spirochaeta_403	0.0035	Desulfobacteraceae_Desulfosarcina_90	0.0029	Flavobacteriaceae_917	0.0288
							Hyphomicrobiaceae_2603	0.0034	Pirellulaceae_1817	0.0027	Unclassified-Bacteria_SAR406_AB16_Arctic96B-7_A714017_ZA3312c_262	0.0283
							Desulfobacteraceae_Desulfosarcina_49	0.0031	Ignavibacteriaceae_1129	0.0027	Flavobacteriaceae_Coccinimonas-marina_408	0.0253
							Unclassified-Pelagibacteraceae_2265	0.0029	Myxococcales_557	0.0027	Unclassified-Alteromonadales_OM60_1529	0.0235
							Saccharospirillaceae_1497	0.0028	Unclassified-Caldithrixales_BA059_159	0.0026	Pelagibacteraceae_2431	0.0207
							Pirellulaceae_1765	0.0028	Desulfobacteraceae_Desulfococcus_105	0.0025	Rhodobacteraceae_2408	0.0205
							Campylobacteraceae_Arcobacter_1836	0.0023	Unclassified-Chloroflexi_Ellin6529_1973	0.0025	Unclassified-Alphaproteobacteria_2839	0.0178
							Unclassified-Anaerolineae_S0208_2013	0.0023	Pirellulaceae_1851	0.0023	Flavobacteriaceae_1094	0.0163
							Flavobacteriaceae_832	0.0020	Unclassified-Chromatiales_806	0.0023	Synechococcaceae_Synechococcus_2647	0.0157
							Desulfobacteraceae_Desulfosarcina_90	0.0019	Unclassified-Gammaproteobacteria_351	0.0022	Unclassified-Alphaproteobacteria_2400	0.0152
							Rhodobacteraceae_2345	0.0019	Desulfobacteraceae_Desulfosarcina_87	0.0021	Sphingomonadaceae_Sphingomonas_2876	0.0143
							Unclassified-Bacteroidales_1485	0.0018	Unclassified-Bacteroidales_1510	0.0021	Cryomorphaceae_1385	0.0141
							Unclassified-Sphingomonadales_2444	0.0017	Unclassified-Chromatiales_2145	0.0021	Pelagibacteraceae_2838	0.0136
							Unclassified-Phycisphaerae_MSBL9_1672	0.0015	Unclassified-Chromatiales_2087	0.0021	Cryomorphaceae_938	0.0128
							Hyphomicrobiaceae_2472	0.0015	Piscirickettsiaceae_779	0.0021	Unclassified-Acidimicrobiales_OCS155_1808	0.0097
							Rhodobacteraceae_Dinoroseobacter_2629	0.0012	Desulfobacteraceae_Desulfococcus_64	0.0020	Rhodobacteraceae_Nautella-italica_2394	0.0092
							Rhodobacteraceae_2687	0.0012	Desulfobacteraceae_Desulfococcus_77	0.0020	Flavobacteriaceae_1203	0.0090
							Flavobacteriaceae_1248	0.0011	Unclassified-Bacteroidales_1485	0.0019	Rhodobacteraceae_2413	0.0085
							Piscirickettsiaceae_740	0.0011	Flammeovirgaceae_1252	0.0019	Rhodobacteraceae_2711	0.0078
							Phyllobacteriaceae_2503	0.0010	Unclassified-Gammaproteobacteria_759	0.0019	Methylophilaceae_754	0.0077
							Unclassified-Chromatiales_656	0.0009	Flammeovirgaceae_1534	0.0019	Unclassified-Acidimicrobiales_wb1_P06_1928	0.0066
							Unclassified-Chromatiales_2186	0.0009	Unclassified-Thiohalorhabdales_182	0.0018	Flavobacteriaceae_1588	0.0053
							Synechococcaceae_Synechococcus_2428	0.0008	Pirellulaceae_2065	0.0018	Unclassified-Sphingobacteriales_995	0.0052
							Unclassified-Chromatiales_632	0.0007	Unclassified-Chromatiales_355	0.0018	Halomonadaceae_Candidatus-Portiera_1558	0.0047
							Halomonadaceae_Candidatus-Portiera_894	0.0007	Unclassified-Anaerolineae_DRC31_1953	0.0018	Unclassified-Flavobacteriales_NS9_941	0.0043
							Cryomorphaceae_992	0.0006	Unclassified-Acidimicrobiales_1873	0.0017	Flavobacteriaceae_Flavobacterium_948	0.0042
							Piscirickettsiaceae_773	0.0006	Rhodobacteraceae_2598	0.0017	Verrucomicrobiaceae_Verrucomicrobium_1000	0.0038
							Cryomorphaceae_1371	0.0005	Unclassified-Anaerolineae_S0208_2013	0.0016	Unclassified-Alphaproteobacteria_2659	0.0035
							Unclassified-Bacteroidales_1510	0.0005	Unclassified-Alteromonadales_OM60_1415	0.0016	Unclassified-Actinomycetales_1863	0.0033
							Unclassified-Alteromonadales_HTCC2188_HTCC_713	0.0004	Pirellulaceae_1845	0.0016	Flavobacteriaceae_1419	0.0032
							Unclassified-Cyanobacteria_2340	0.0003	Pirellulaceae_2075	0.0016	Pelagibacteraceae_2347	0.0028
							Unclassified-Gammaproteobacteria_759	0.0003	Unclassified-Anaerolineae_SHA-20_2006	0.0016	Pelagibacteraceae_2628	0.0024
							Synechococcaceae_Synechococcus_2512	0.0002	Piscirickettsiaceae_710	0.0016	Unclassified-Cryomorphaceae_Fluviicola_577	0.0021
							Halomonadaceae_Candidatus-Portiera_1161	0.0002	Pirellulaceae_1765	0.0016	Halomonadaceae_Candidatus-Portiera_1693	0.0021
							Unclassified-Alphaproteobacteria_2400	0.0001	Piscirickettsiaceae_900	0.0015	Unclassified-Betaproteobacteria_1362	0.0021
									Unclassified-Chloroflexi_Ellin6529_1975	0.0015	Unclassified-Alphaproteobacteria_2437	0.0021
									Pirellulaceae_1727	0.0014	Flavobacteriaceae_1075	0.0020
									Chromatiaceae_1122	0.0013	Unclassified-Alphaproteobacteria_2843	0.0020
									Pelagibacteraceae_2265	0.0013	Unclassified-Alphaproteobacteria_2554	0.0017
									Rhodobacteraceae_2703	0.0013	Unclassified-Alphaproteobacteria_2474	0.0015
									Pirellulaceae_1719	0.0012	Cryomorphaceae_1285	0.0014
									Unclassified-Chromatiales_2073	0.0012	Piscirickettsiaceae_1678	0.0014
									Flavobacteriaceae_1248	0.0012	Pirellulaceae_1737	0.0012
									Desulfobulbaceae_160	0.0011	Unclassified-Cryomorphaceae_Fluviicola_605	0.0012
									Pirellulaceae_1685	0.0011	Pelagibacteraceae_2282	0.0011
									Spirochaetaceae_1668	0.0010	Unclassified-Rhizobiales_2754	0.0010
									Rhodobacteraceae_Dinoroseobacter_2629	0.0010	Halomonadaceae_Candidatus-Portiera_2146	0.0010
									Unclassified-Gammaproteobacteria_HTCC2188_HTCC2089_330	0.0010	Rhodospirillaceae_2364	0.0007
									Unclassified-Acidobacteria_OS-K_166	0.0010	Flavobacteriaceae_690	0.0004
									Spirochaetaceae_1616	0.0010	Unclassified-Rickettsiales_2367	0.0004
									Piscirickettsiaceae_770	0.0009		
									Rhodobacteraceae_Anaerospora_2584	0.0009		
									Rhodobacteraceae_Loktanella_2698	0.0009		
									Unclassified-Phycisphaerae_MSBL9_1767	0.0008		
									Rhodobacteraceae_2345	0.0007		
									Flavobacteriaceae_836	0.0007		
									Hyphomicrobiaceae_2847	0.0007		
									Hyphomicrobiaceae_2603	0.0007		
									Rhodobacteraceae_2687	0.0006		
									Flavobacteriaceae_1038	0.0006		
									Flavobacteriaceae_Coccinimonas-marina_408	0.0006		
									Unclassified-Bacteroidales_1148	0.0006		
									Pirellulaceae_1725	0.0005		
									Desulfobacteraceae_75	0.0005		
									Pirellulaceae_1774	0.0005		
									Synechococcaceae_Synechococcus_2428	0.0005		
									Unclassified-Anaerolineae_S0208_1995	0.0004		
									Unclassified-Anaerolineae_DRC31_1913	0.0004		
									Cryomorphaceae_992	0.0003		
									Unclassified-Acidobacteria-6_CCU21_274	0.0003		
									Unclassified-Gammaproteobacteria_677	0.0002		
									Cryomorphaceae_1371	0.0002		
									Halomonadaceae_Candidatus-Portiera_1161	0.0002		
									Unclassified-Cyanobacteria_2340	0.0002		
									Rhodobacteraceae_2684	0.0001		
									Unclassified-Oceanospirillales_352	0.0001		

Supplementary Table 6: Statistical analyses for microalgal mean alpha diversity (Chao1 diversity).

Differences in alpha diversity as measured by Chao1 diversity across sites and microenvironments (main test), between sites and between microenvironments within sites (pair-wise) were tested for statistical significance in Permutational Multivariate Analysis of Variance (PERMANOVA, Minkowski metric distance matrix, nested design).

Main test

Factor	P(perm)
Site	0.0327
Microenvironment (Site)	0.0001

ECV

Source	Estimate	Sq.root
S(si)	0.86	**0.93**
S(mi(si))	**19.51**	4.42
V(Res)	7.05	2.65

Pair-wise (Site)

P(perm)	PB	RB	N	LM
PB				
RB	0.0621			
N	0.4247	0.5507		
LM	0.0018	0.1531	0.0744	

Pair-wise (Micenv. PB)

P(MC) **i**	UpL	LoL	Sh	RR	Sed	SW
UpL						
LoL	0.1628					
Sh	0.6367	0.0159				
RR	0.0695	0.0056	0.0670			
Sed	0.0029	0.0004	0.0025	0.0168		
SW	0.0004	0.0001	0.0005	0.0004	0.0012	

Pair-wise (Micenv. RB)

P(MC) **i**	UpL	LoL	Sh	RR	Sed	SW
UpL						
LoL	0.7859					
Sh	0.2706	0.4692				
RR	0.0062	0.3052	0.9768			
Sed	0.0882	0.2143	0.5951	0.4220		
SW	0.0058	0.0243	0.0636	0.0178	0.1055	

Pair-wise (Micenv. N)

P(MC) **i**	UpL	LoL	Sh	RR	Sed	SW
UpL						
LoL	0.1328					
Sh	0.1414	0.3692				
RR	0.4372	0.0042	0.0090			
Sed	0.9736	0.0129	0.0151	0.1341		
SW	0.4939	0.0003	0.0007	0.0050	0.1834	

Pair-wise (Micenv. LM)

P(MC) **i**	UpL	LoL	Sh	RR	Sed	SW
UpL						
LoL	0.5769					
Sh	0.7176	0.3996				
RR	0.1414	0.2367	0.1155			
Sed	0.0570	0.1435	0.0322	0.8260		
SW	0.0015	0.0055	0.0007	0.1766	0.0344	

i Monte Carlo p-values used when there were not enough possible permutations (unrestricted permutation method, 9999 permutations)

Significant values at the 0.05 level are shown in grey
ECV: estimates of components of variation
UpL: upper leaf, LoL: lower leaf, Sh: sheath, RR: roots and rhizomes, Sed: sediment, SW: seawater
PB: Palm Beach, RB: Rose Bay, N: Narrabeen Lagoon, LM: Lake Macquarie

Supplementary Table 7: Statistical analyses for microalgal mean alpha diversity (Shannon's diversity index).

Differences in alpha diversity as measured by Shannon's diversity index across sites and microenvironments (main test), between sites and between microenvironments within sites (pair-wise) were tested for statistical significance in Permutational Multivariate Analysis of Variance (PERMANOVA, Minkowski metric distance matrix, nested design).

Main test

Factor	P(perm)
Site	0.0001
Microenvironment (Site)	0.0001

ECV

Source	Estimate	Sq.root
S(si)	2.19	**1.48**
S(mi(si))	**22.61**	4.75
V(Res)	5.80	2.41

Pair-wise (Site)

P(perm)	PB	RB	N	LM
PB				
RB	0.0001			
N	0.0001	0.7814		
LM	0.0001	0.8242	0.6405	

Pair-wise (Micenv. PB)

P(MC) **i**	UpL	LoL	Sh	RR	Sed	SW
UpL						
LoL	0.1884					
Sh	0.7752	0.0273				
RR	0.0685	0.0027	0.0369			
Sed	0.2638	0.0015	0.2031	0.1050		
SW	0.0155	0.0023	0.0130	0.0778	0.0183	

Pair-wise (Micenv. RB)

P(MC) **i**	UpL	LoL	Sh	RR	Sed	SW
UpL						
LoL	0.6696					
Sh	0.1863	0.4089				
RR	0.7667	0.7556	0.2329			
Sed	0.0002	0.3184	0.0980	0.1169		
SW	0.0008	0.0081	0.0295	0.0014	0.0008	

Pair-wise (Micenv. N)

P(MC) **i**	UpL	LoL	Sh	RR	Sed	SW
UpL						
LoL	0.1806					
Sh	0.2120	0.3789				
RR	0.3423	0.0427	0.1193			
Sed	0.5285	0.0076	0.0093	0.0193		
SW	0.0280	0.0022	0.0021	0.0025	0.0165	

Pair-wise (Micenv. LM)

P(MC) **i**	UpL	LoL	Sh	RR	Sed	SW
UpL						
LoL	0.1441					
Sh	0.1783	0.4952				
RR	0.1161	0.6512	0.3122			
Sed	0.0047	0.0639	0.0153	0.1810		
SW	0.0022	0.1315	0.0183	0.4128	0.2881	

i Monte Carlo p-values used when there were not enough possible permutations (unrestricted permutation method, 9999 permutations)

Significant values at the 0.05 level are shown in grey
ECV: estimates of components of variation
UpL: upper leaf, LoL: lower leaf, Sh: sheath, RR: roots and rhizomes, Sed: sediment, SW: seawater
PB: Palm Beach, RB: Rose Bay, N: Narrabeen Lagoon, LM: Lake Macquarie

Supplementary Table 8: Statistical analyses for multidimensional scaling (Microalgae).

Differences between microalgal communities across sites and microenvironments (top), between sites (middle), and between microenvironments within sites (bottom) were tested for statistical significance in Permutational Multivariate Analysis of Variance (PERMANOVA, Bray-Curtis dissimilarity matrix, nested design).

Main test

Factor	P(perm)
Site	0.0001
Microenvironment (Site)	0.0001

ECV

Source	Estimate	Sq.root
S(Si)	188.98	**13.75**
S(Mi(Si))	**488.81**	22.11
V(Res)	436.77	20.90

Pair-wise (Site)

P(perm)	PB	RB	N	LM
PB				
RB	0.0003			
N	0.0033	0.0001		
LM	0.0001	0.0189	0.0001	

Pair-wise (Micenv. PB)

P(MC) **i**	UpL	LoL	Sh	RR	Sed	SW
UpL						
LoL	0.1251					
Sh	0.5737	0.5241				
RR	0.2001	0.0289	0.5339			
Sed	0.0072	0.0001	0.1330	0.2144		
SW	0.0085	0.0001	0.2231	0.0142	0.0001	

Pair-wise (Micenv. RB)

P(MC) **i**	UpL	LoL	Sh	RR	Sed	SW
UpL						
LoL	0.7958					
Sh	0.2682	0.2692				
RR	0.6803	0.3314	0.2047			
Sed	0.2205	0.0386	0.0014	0.0999		
SW	0.2205	0.1172	0.0883	0.0516	0.0001	

Pair-wise (Micenv. N)

P(MC) **i**	UpL	LoL	Sh	RR	Sed	SW
UpL						
LoL	0.1152					
Sh	0.1304	0.3753				
RR	0.0482	0.0024	0.1022			
Sed	0.0989	0.0008	0.0061	0.0061		
SW	0.0155	0.0003	0.0043	0.0012	0.0020	

Pair-wise (Micenv. LM)

P(MC) **i**	UpL	LoL	Sh	RR	Sed	SW
UpL						
LoL	0.0641					
Sh	0.1927	0.5429				
RR	0.2231	0.2780	0.7963			
Sed	0.0707	0.0128	0.7963	0.0376		
SW	0.0391	0.1278	0.5700	0.3410	0.0010	

i Monte Carlo p-values used when there were not enough possible permutations (unrestricted permutation method, 9999 permutations)

Significant values at the 0.05 level are shown in grey
ECV: estimates of components of variation
UpL: upper leaf, LoL: lower leaf, Sh: sheath, RR: roots and rhizomes, Sed: sediment, SW: seawater
PB: Palm Beach, RB: Rose Bay, N: Narrabeen Lagoon, LM: Lake Macquarie

Supplementary Table 9: Statistical analyses for fungal mean alpha diversity (Chao1 diversity).

Differences in alpha diversity as measured by Chao1 diversity across sites and microenvironments (main test), between sites and between microenvironments within sites (pair-wise) were tested for statistical significance in Permutational Multivariate Analysis of Variance (PERMANOVA, Minkowski metric distance matrix,

Main test

Factor	P(perm)
Site	0.6676
Microenvironment (Site)	0.0001

ECV

Source	Estimate	Sq.root
S(si)	-57.60	**-7.59**
S(mi(si))	**4952.10**	70.37
V(Res)	2232.20	47.25

Pair-wise (Site)

P(perm)	PB	RB	N	LM
PB				
RB	0.5801			
N	0.7793	0.7748		
LM	0.1134	0.6185	0.3384	

Pair-wise (Micenv. PB)

P(MC) **i**	UpL	LoL	Sh	RR	Sed	SW
UpL						
LoL	0.3199					
Sh	0.0002	0.4528				
RR	0.0185	0.5553	0.7828			
Sed	0.0019	0.0031	0.0010	0.0014		
SW	0.0004	0.0008	0.0001	0.0001	0.0745	

Pair-wise (Micenv. RB)

P(MC) **i**	UpL	LoL	Sh	RR	Sed	SW
UpL						
LoL	0.8771					
Sh	0.7668	0.7893				
RR	0.3787	0.3370	0.3255			
Sed	0.1974	0.1891	0.1892	0.4024		
SW	0.0201	0.0207	0.0157	0.0527	0.2925	

Pair-wise (Micenv. N)

P(MC) **i**	UpL	LoL	Sh	RR	Sed	SW
UpL						
LoL	0.0883					
Sh	0.0212	0.0902				
RR	0.3206	0.1168	0.0653			
Sed	0.0391	0.0303	0.0238	0.0788		
SW	0.0039	0.0011	0.0010	0.1441	0.1568	

Pair-wise (Micenv. LM)

P(MC) **i**	UpL	LoL	Sh	RR	Sed	SW
UpL						
LoL	0.3741					
Sh	0.2400	0.3605				
RR	0.0845	0.3646	0.5670			
Sed	0.3918	0.7326	0.4926	0.7634		
SW	0.0003	0.0103	0.6583	0.0321	0.0829	

i Monte Carlo p-values used when there were not enough possible permutations (unrestricted permutation method, 9999 permutations)

Significant values at the 0.05 level are shown in grey
ECV: estimates of components of variation
UpL: upper leaf, LoL: lower leaf, Sh: sheath, RR: roots and rhizomes, Sed: sediment, SW: seawater
PB: Palm Beach, RB: Rose Bay, N: Narrabeen Lagoon, LM: Lake Macquarie

Supplementary Table 10: Statistical analyses for fungal mean alpha diversity (Shannon's diversity index).

Differences in alpha diversity as measured by Shannon's diversity index across sites and microenvironments (main test), between sites and between microenvironments within sites (pair-wise) were tested for statistical significance in Permutational Multivariate Analysis of Variance (PERMANOVA, Minkowski metric distance matrix,

Main test

Factor	P(perm)
Site	0.0011
Microenvironment (Site)	0.0001

ECV

Source	Estimate	Sq.root
S(si)	1862.90	**43.16**
S(mi(si))	**18649.00**	136.56
V(Res)	6861.80	82.84

Pair-wise (Site)

P(perm)	PB	RB	N	LM
PB				
RB	0.1043			
N	0.0012	0.3854		
LM	0.0027	0.0215	0.0010	

Pair-wise (Micenv. PB)

P(MC) **i**	UpL	LoL	Sh	RR	Sed	SW
UpL						
LoL	0.3641					
Sh	0.9316	0.3770				
RR	0.3936	0.2155	0.3207			
Sed	0.0348	0.0876	0.0342	0.0263		
SW	0.0009	0.0019	0.0007	0.0005	0.0065	

Pair-wise (Micenv. RB)

P(MC) **i**	UpL	LoL	Sh	RR	Sed	SW
UpL						
LoL	0.1925					
Sh	0.3974	0.5622				
RR	0.3699	0.3955	0.4548			
Sed	0.3628	0.3676	0.3794	0.4657		
SW	0.0079	0.0091	0.0079	0.0166	0.4163	

Pair-wise (Micenv. N)

P(MC) **i**	UpL	LoL	Sh	RR	Sed	SW
UpL						
LoL	0.2886					
Sh	0.0427	0.7707				
RR	0.0304	0.2229	0.0756			
Sed	0.0144	0.0172	0.0163	0.0191		
SW	0.7965	0.2560	0.0051	0.0305	0.0163	

Pair-wise (Micenv. LM)

P(MC) **i**	UpL	LoL	Sh	RR	Sed	SW
UpL						
LoL	0.8743					
Sh	0.8583	0.9750				
RR	0.4722	0.4887	0.4898			
Sed	0.4099	0.4362	0.4375	0.9676		
SW	0.3373	0.3433	0.3537	0.5970	0.5789	

i Monte Carlo p-values used when there were not enough possible permutations (unrestricted permutation method, 9999 permutations)

Significant values at the 0.05 level are shown in grey
ECV: estimates of components of variation
UpL: upper leaf, LoL: lower leaf, Sh: sheath, RR: roots and rhizomes, Sed: sediment, SW: seawater
PB: Palm Beach, RB: Rose Bay, N: Narrabeen Lagoon, LM: Lake Macquarie

Supplementary Table 11: Statistical analyses for multidimensional scaling (Fungi).

Differences between fungal communities across sites and microenvironments (top), between sites (middle), and between microenvironments within sites (bottom) were tested for statistical significance in Permutational Multivariate Analysis of Variance (PERMANOVA, Bray-Curtis dissimilarity matrix, nested design).

Main test

Factor	P(perm)
Site	0.0001
Microenvironment (Site)	0.0001

ECV

Source	Estimate	Sq.root
S(Si)	423.34	**20.58**
S(Mi(Si))	953.23	30.87
V(Res)	**1266.20**	35.58

Pair-wise (Site)

P(perm)	PB	RB	N	LM
PB				
RB	0.0001			
N	0.0001	0.0133		
LM	0.0001	0.0846	0.0002	

Pair-wise (Micenv. PB)

P(MC) **i**	UpL	LoL	Sh	RR	Sed	SW
UpL						
LoL	0.0593					
Sh	0.0074	0.5612				
RR	0.0031	0.2192	0.0748			
Sed	0.0057	0.0650	0.0135	0.0352		
SW	0.0024	0.0350	0.0050	0.0106	0.0531	

Pair-wise (Micenv. RB)

P(MC) **i**	UpL	LoL	Sh	RR	Sed	SW
UpL						
LoL	0.2440					
Sh	0.5072	0.5433				
RR	0.3773	0.2558	0.3836			
Sed	0.4272	0.4933	0.4212	0.5444		
SW	0.0147	0.0574	0.0198	0.0242	0.1798	

Pair-wise (Micenv. N)

P(MC) **i**	UpL	LoL	Sh	RR	Sed	SW
UpL						
LoL	0.0903					
Sh	0.0338	0.1400				
RR	0.2375	0.2564	0.0875			
Sed	0.0132	0.0307	0.0192	0.0336		
SW	0.0537	0.0370	0.0169	0.1197	0.0097	

Pair-wise (Micenv. LM)

P(MC) **i**	UpL	LoL	Sh	RR	Sed	SW
UpL						
LoL	0.4808					
Sh	0.3180	0.4958				
RR	0.4637	0.8140	0.5668			
Sed	0.4185	0.4604	0.5104	0.6347		
SW	0.0039	0.0036	0.0016	0.0188	0.0068	

i Monte Carlo p-values used when there were not enough possible permutations (unrestricted permutation method, 9999 permutations)

Significant values at the 0.05 level are shown in grey
ECV: estimates of components of variation
UpL: upper leaf, LoL: lower leaf, Sh: sheath, RR: roots and rhizomes, Sed: sediment, SW: seawater
PB: Palm Beach, RB: Rose Bay, N: Narrabeen Lagoon, LM: Lake Macquarie

Supplementary Table 12: Amplicon sequencing information (16S rRNA, Bacteria).

Sample	Site*	Microenvironment**	Description	FW primer sequence***	Rv primer sequence***	Number of sequences
PB22	PB	LoL	II32	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	2380
N31	N	Sh	N3	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	2393
PB13	PB	UpL	II36	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	2397
N11	N	UpL	N1	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	2402
PB11	PB	UpL	II26	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	2429
PB32	PB	Sh	II33	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	2458
N22	N	LoL	N7	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	2466
RB53	RB	Sed	V15	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	2626
N32	N	Sh	N8	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	3550
N52	N	Sed	N10	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	4712
PB23	PB	LoL	II37	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	4861
PB21	PB	LoL	II27	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	4989
PB53	PB	Sed	II40	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	5026
PB51	PB	Sed	II30	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	5763
LM52	LM	Sed	IV10	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	6039
N33	N	Sh	N13	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	6358
LM51	LM	Sed	IV5	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	6384
LM53	LM	Sed	IV15	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	6614
N51	N	Sed	N5	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	7814
N53	N	Sed	N15	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	7874
RB52	RB	Sed	V10	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	8490
RB22	RB	LoL	V7	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	8754
N23	N	LoL	N12	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	8854
RB51	RB	Sed	V5	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	8903
PB52	PB	Sed	II35	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	9835
PB42	PB	RR	II34	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	10669
LM33	LM	Sh	IV13	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	10764
N42	N	RR	N9	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	11539
LM11	LM	UpL	IV1	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	12710
RB13	RB	UpL	V11	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	13148
LM32	LM	Sh	IV8	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	13556
LM21	LM	LoL	IV2	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	14126
N13	N	UpL	N11	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	14496
PB31	PB	Sh	II28	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	14589
N12	N	UpL	N6	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	14769
RB42	RB	RR	V9	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	15079
RB41	RB	RR	V4	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	15359
N43	N	RR	N14	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	15727
RB21	RB	LoL	V2	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	17600
LM31	LM	Sh	IV3	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	18234
PB12	PB	UpL	II31	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	18431
LM41	LM	RR	IV4	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	19041
RB33	RB	Sh	V13	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	19490
LM22	LM	LoL	IV7	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	20265
N21	N	LoL	N2	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	20278
LM12	LM	UpL	IV6	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	20467
RB23	RB	LoL	V12	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	20985
PB43	PB	RR	II39	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	22429
N41	N	RR	N4	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	22883
LM23	LM	LoL	IV12	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	24197
PB41	PB	RR	II29	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	24851
LM42	LM	RR	IV9	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	25226
LM13	LM	UpL	IV11	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	25505
RB12	RB	UpL	V6	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	25679
RB11	RB	UpL	V1	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	25770
RB43	RB	RR	V14	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	26479
PB61	PB	SW	IISW1	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	27357
LM43	LM	RR	IV14	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	28831
PB62	PB	SW	IISW2	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	29065
PB63	PB	SW	IISW3	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	29115
RB62	RB	SW	VSW2	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	29279
N62	N	SW	NSW2	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	30510
N63	N	SW	NSW3	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	31272
N61	N	SW	NSW1	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	32130
RB61	RB	SW	VSW1	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	41382
RB63	RB	SW	VSW3	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	44703
LM61	LM	SW	IVSW1	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	44705
RB31	RB	Sh	V3	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	45858
RB32	RB	Sh	V8	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	46027
PB33	PB	Sh	II38	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	47043
LM63	LM	SW	IVSW3	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	61425
LM62	LM	SW	IVSW2	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	62527

* PB: Palm Beach, RB, Rose Bay, N: Narrabeen Lagoon, LM: Lake Macquarie
** UpL: upper leaf, LoL: lower leaf, Sh: sheath, RR: roots and rhizomes, Sed: sediment, SW: seawater
*** Direction: 5' - 3'

Supplementary Table 13: Amplicon sequencing information (16S rRNA-cloroplast, Microalgae).

Sample	Site*	Microenvironment**	Description	FW primer sequence***	Rv primer sequence***	Number of sequences
LM51	LM	Sed	IV5	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	72
RB53	RB	Sed	V15	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	74
N52	N	Sed	N10	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	83
LM52	LM	Sed	IV10	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	105
LM53	LM	Sed	IV15	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	119
N42	N	RR	N9	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	137
N61	N	SW	NSW1	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	139
N62	N	SW	NSW2	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	157
N63	N	SW	NSW3	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	184
N53	N	Sed	N15	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	202
N51	N	Sed	N5	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	203
LM41	LM	RR	IV4	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	250
PB53	PB	Sed	II40	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	300
PB62	PB	SW	IISW2	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	332
PB63	PB	SW	IISW3	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	334
PB22	PB	LoL	II32	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	394
N31	N	Sh	N3	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	405
PB61	PB	SW	IISW1	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	436
RB62	RB	SW	VSW2	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	453
RB61	RB	SW	VSW1	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	555
N11	N	UpL	N1	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	560
PB11	PB	UpL	II26	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	599
RB42	RB	RR	V9	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	609
RB63	RB	SW	VSW3	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	641
LM11	LM	UpL	IV1	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	776
PB21	PB	LoL	II27	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	819
N13	N	UpL	N11	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	843
PB13	PB	UpL	II36	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	868
RB43	RB	RR	V14	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	929
RB52	RB	Sed	V10	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	973
RB51	RB	Sed	V5	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	992
LM61	LM	SW	IVSW1	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	1080
PB42	PB	RR	II34	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	1156
RB22	RB	LoL	V7	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	1164
PB32	PB	Sh	II33	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	1239
N41	N	RR	N4	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	1499
LM62	LM	SW	IVSW2	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	1547
LM63	LM	SW	IVSW3	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	1635
LM33	LM	Sh	IV13	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	1681
N22	N	LoL	N7	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	1700
N12	N	UpL	N6	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	1897
LM42	LM	RR	IV9	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	2067
PB23	PB	LoL	II37	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	2163
PB51	PB	Sed	II30	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	2210
N43	N	RR	N14	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	2335
LM43	LM	RR	IV14	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	2369
RB12	RB	UpL	V6	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	2393
RB41	RB	RR	V4	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	2426
RB13	RB	UpL	V11	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	2742
N32	N	Sh	N8	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	3127
LM12	LM	UpL	IV6	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	3399
LM13	LM	UpL	IV11	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	3726
PB43	PB	RR	II39	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	3732
N33	N	Sh	N13	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	5256
PB52	PB	Sed	II35	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	5565
N23	N	LoL	N12	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	6345
RB11	RB	UpL	V1	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	6366
LM32	LM	Sh	IV8	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	7516
LM21	LM	LoL	IV2	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	8186
PB41	PB	RR	II29	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	9659
PB12	PB	UpL	II31	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	11416
LM22	LM	LoL	IV7	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	11557
LM31	LM	Sh	IV3	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	12194
PB31	PB	Sh	II28	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	13896
RB21	RB	LoL	V2	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	14189
RB23	RB	LoL	V12	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	14753
RB33	RB	Sh	V13	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	15248
N21	N	LoL	N2	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	19574
LM23	LM	LoL	IV12	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	20704
RB31	RB	Sh	V3	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	45098
PB33	PB	Sh	II38	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	45181
RB32	RB	Sh	V8	AGAGTTTGATCMTGGCTCAG	CGGTTACCTTGTTACGACTT	45193

* PB: Palm Beach, RB, Rose Bay, N: Narrabeen Lagoon, LM: Lake Macquarie
** UpL: upper leaf, LoL: lower leaf, Sh: sheath, RR: roots and rhizomes, Sed: sediment, SW: seawater
*** Direction: 5' - 3'

Supplementary Table 14: Amplicon sequencing information (ITS rRNA, Fungi).

Sample	Site*	Microenvironment**	Description	FW primer sequence***	Rv primer sequence***	Number of sequences
LM13	LM	UpL	IV11	GTGARTCATCGAATCTTTG	TCCTCCGCTTATTGATATGC	1456
RB61	RB	SW	VSW1	GTGARTCATCGAATCTTTG	TCCTCCGCTTATTGATATGC	2850
LM61	LM	SW	IVSW1	GTGARTCATCGAATCTTTG	TCCTCCGCTTATTGATATGC	6360
LM63	LM	SW	IVSW3	GTGARTCATCGAATCTTTG	TCCTCCGCTTATTGATATGC	7242
LM62	LM	SW	IVSW2	GTGARTCATCGAATCTTTG	TCCTCCGCTTATTGATATGC	8225
RB62	RB	SW	VSW2	GTGARTCATCGAATCTTTG	TCCTCCGCTTATTGATATGC	9454
RB53	RB	Sed	V15	GTGARTCATCGAATCTTTG	TCCTCCGCTTATTGATATGC	9518
RB13	RB	UpL	V11	GTGARTCATCGAATCTTTG	TCCTCCGCTTATTGATATGC	9813
RB33	RB	Sh	V13	GTGARTCATCGAATCTTTG	TCCTCCGCTTATTGATATGC	10183
RB43	RB	RR	V14	GTGARTCATCGAATCTTTG	TCCTCCGCTTATTGATATGC	13931
N61	N	SW	NSW1	GTGARTCATCGAATCTTTG	TCCTCCGCTTATTGATATGC	15695
LM43	LM	RR	IV14	GTGARTCATCGAATCTTTG	TCCTCCGCTTATTGATATGC	15924
LM23	LM	LoL	IV12	GTGARTCATCGAATCTTTG	TCCTCCGCTTATTGATATGC	17685
RB63	RB	SW	VSW3	GTGARTCATCGAATCTTTG	TCCTCCGCTTATTGATATGC	17836
LM53	LM	Sed	IV15	GTGARTCATCGAATCTTTG	TCCTCCGCTTATTGATATGC	20452
RB11	RB	UpL	V1	GTGARTCATCGAATCTTTG	TCCTCCGCTTATTGATATGC	21336
PB21	PB	LoL	II27	GTGARTCATCGAATCTTTG	TCCTCCGCTTATTGATATGC	23042
RB31	RB	Sh	V3	GTGARTCATCGAATCTTTG	TCCTCCGCTTATTGATATGC	24413
PB52	PB	Sed	II35	GTGARTCATCGAATCTTTG	TCCTCCGCTTATTGATATGC	25106
RB51	RB	Sed	V5	GTGARTCATCGAATCTTTG	TCCTCCGCTTATTGATATGC	26339
LM31	LM	Sh	IV3	GTGARTCATCGAATCTTTG	TCCTCCGCTTATTGATATGC	26598
LM51	LM	Sed	IV5	GTGARTCATCGAATCTTTG	TCCTCCGCTTATTGATATGC	28862
N11	N	UpL	N1	GTGARTCATCGAATCTTTG	TCCTCCGCTTATTGATATGC	28864
PB61	PB	SW	IISW1	GTGARTCATCGAATCTTTG	TCCTCCGCTTATTGATATGC	31429
LM41	LM	RR	IV4	GTGARTCATCGAATCTTTG	TCCTCCGCTTATTGATATGC	31796
N13	N	UpL	N11	GTGARTCATCGAATCTTTG	TCCTCCGCTTATTGATATGC	32428
LM33	LM	Sh	IV13	GTGARTCATCGAATCTTTG	TCCTCCGCTTATTGATATGC	34964
N51	N	Sed	N5	GTGARTCATCGAATCTTTG	TCCTCCGCTTATTGATATGC	35223
PB22	PB	LoL	II32	GTGARTCATCGAATCTTTG	TCCTCCGCTTATTGATATGC	35526
RB22	RB	LoL	V7	GTGARTCATCGAATCTTTG	TCCTCCGCTTATTGATATGC	36221
PB51	PB	Sed	II30	GTGARTCATCGAATCTTTG	TCCTCCGCTTATTGATATGC	36317
N22	N	LoL	N7	GTGARTCATCGAATCTTTG	TCCTCCGCTTATTGATATGC	37627
RB21	RB	LoL	V2	GTGARTCATCGAATCTTTG	TCCTCCGCTTATTGATATGC	37698
RB23	RB	LoL	V12	GTGARTCATCGAATCTTTG	TCCTCCGCTTATTGATATGC	37793
N53	N	Sed	N15	GTGARTCATCGAATCTTTG	TCCTCCGCTTATTGATATGC	38158
PB63	PB	SW	IISW3R	GTGARTCATCGAATCTTTG	TCCTCCGCTTATTGATATGC	38978
N62	N	SW	NSW2	GTGARTCATCGAATCTTTG	TCCTCCGCTTATTGATATGC	42161
RB12	RB	UpL	V6	GTGARTCATCGAATCTTTG	TCCTCCGCTTATTGATATGC	42275
N41	N	RR	N4	GTGARTCATCGAATCTTTG	TCCTCCGCTTATTGATATGC	44851
N42	N	RR	N9	GTGARTCATCGAATCTTTG	TCCTCCGCTTATTGATATGC	45240
PB42	PB	RR	II34	GTGARTCATCGAATCTTTG	TCCTCCGCTTATTGATATGC	46687
LM42	LM	RR	IV9	GTGARTCATCGAATCTTTG	TCCTCCGCTTATTGATATGC	49243
LM11	LM	UpL	IV1	GTGARTCATCGAATCTTTG	TCCTCCGCTTATTGATATGC	49748
PB12	PB	UpL	II31	GTGARTCATCGAATCTTTG	TCCTCCGCTTATTGATATGC	52265
N23	N	LoL	N12	GTGARTCATCGAATCTTTG	TCCTCCGCTTATTGATATGC	52763
PB33	PB	Sh	II38	GTGARTCATCGAATCTTTG	TCCTCCGCTTATTGATATGC	53056
PB43	PB	RR	II39	GTGARTCATCGAATCTTTG	TCCTCCGCTTATTGATATGC	53431
N31	N	Sh	N3	GTGARTCATCGAATCTTTG	TCCTCCGCTTATTGATATGC	53953
RB52	RB	Sed	V10	GTGARTCATCGAATCTTTG	TCCTCCGCTTATTGATATGC	54557
PB13	PB	UpL	II36	GTGARTCATCGAATCTTTG	TCCTCCGCTTATTGATATGC	55665
LM32	LM	Sh	IV8	GTGARTCATCGAATCTTTG	TCCTCCGCTTATTGATATGC	55998
PB53	PB	Sed	II40	GTGARTCATCGAATCTTTG	TCCTCCGCTTATTGATATGC	56242
N32	N	Sh	N8	GTGARTCATCGAATCTTTG	TCCTCCGCTTATTGATATGC	57220
N12	N	UpL	N6	GTGARTCATCGAATCTTTG	TCCTCCGCTTATTGATATGC	58414
PB62	PB	SW	IISW2	GTGARTCATCGAATCTTTG	TCCTCCGCTTATTGATATGC	61432
RB42	RB	RR	V9	GTGARTCATCGAATCTTTG	TCCTCCGCTTATTGATATGC	61644
RB41	RB	RR	V4	GTGARTCATCGAATCTTTG	TCCTCCGCTTATTGATATGC	61707
N33	N	Sh	N13	GTGARTCATCGAATCTTTG	TCCTCCGCTTATTGATATGC	61732
N52	N	Sed	N10	GTGARTCATCGAATCTTTG	TCCTCCGCTTATTGATATGC	61791
PB32	PB	Sh	II33	GTGARTCATCGAATCTTTG	TCCTCCGCTTATTGATATGC	62774
RB32	RB	Sh	V8	GTGARTCATCGAATCTTTG	TCCTCCGCTTATTGATATGC	64506
LM21	LM	LoL	IV2	GTGARTCATCGAATCTTTG	TCCTCCGCTTATTGATATGC	67346
N63	N	SW	NSW3	GTGARTCATCGAATCTTTG	TCCTCCGCTTATTGATATGC	69447
LM52	LM	Sed	IV10	GTGARTCATCGAATCTTTG	TCCTCCGCTTATTGATATGC	69538
N43	N	RR	N14	GTGARTCATCGAATCTTTG	TCCTCCGCTTATTGATATGC	72103
PB23	PB	LoL	II37	GTGARTCATCGAATCTTTG	TCCTCCGCTTATTGATATGC	72514
LM22	LM	LoL	IV7	GTGARTCATCGAATCTTTG	TCCTCCGCTTATTGATATGC	74046
PB31	PB	Sh	II28	GTGARTCATCGAATCTTTG	TCCTCCGCTTATTGATATGC	76281
LM12	LM	UpL	IV6	GTGARTCATCGAATCTTTG	TCCTCCGCTTATTGATATGC	82332
PB11	PB	UpL	II26	GTGARTCATCGAATCTTTG	TCCTCCGCTTATTGATATGC	82949
PB41	PB	RR	II29	GTGARTCATCGAATCTTTG	TCCTCCGCTTATTGATATGC	95872
N21	N	LoL	N2	GTGARTCATCGAATCTTTG	TCCTCCGCTTATTGATATGC	118255

* PB: Palm Beach, RB, Rose Bay, N: Narrabeen Lagoon, LM: Lake Macquarie
** UpL: upper leaf, LoL: lower leaf, Sh: sheath, RR: roots and rhizomes, Sed: sediment, SW: seawater
*** Direction: 5' - 3'

3 CHAPTER 3

Shifts in the seagrass leaf microbiome associated with seagrass wasting disease in *Zostera muelleri*

Submitted as: Hurtado-McCormick, V., Tschitschko, B., Ralph, P.J., Seymour, J.R. (n.d.). Shifts in the seagrass leaf microbiome associated with seagrass wasting disease in *Zostera muelleri*. *Marine and Freshwater Research* (under review).

Author contributions: VH-M conceived the study, designed the sampling strategy, conducted the fieldwork and lab work, developed the methodological approaches, analysed the data, drafted the manuscript, prepared the figures and tables and obtained the approval of the final submission. BT provided substantial contributions to initial data curation and quality control processes and to the editing of the manuscript. PR supervised the study. JS conceived the study, designed the sampling strategy, provided the regular supervision of VH-M throughout the data analysis and interpretation, drafted the manuscript and substantially contributed to its intellectual content. All authors agreed to be accountable for the content of the work.

3.1 Preface

In Chapter 2, we demonstrated that the *Z. muelleri* microbiome is extremely complex and variable, and as a consequence, different microbial communities associate with disparate parts of the plant. We also showed that this microenvironmental partitioning of the seagrass microbiome is biogeographically conserved, and that bacterial members within these communities likely stablish more specific interactions with the seagrass host. This indicates that intrinsic features of the plant strongly influence the composition and structure of its associated microbiota. In this Chapter, we performed a more focused analysis on the dynamics of the bacterial consortia associated with leaves exhibiting symptoms of seagrass wasting disease, a well-characterised infection possibly related to a dysbiotic behaviour of the phyllosphere microbiome.

3.2 Abstract

Significant seagrass declines are occurring globally and in many regions have been linked to seagrass wasting disease (SWD), which is believed to be caused by *Labyrinthula zosterae* (*L. zosterae*). There is, however, still uncertainty about the mechanisms of disease and the potential involvement of other opportunistic colonizing microorganisms. We examined the microbiome associated with diseased specimens of the seagrass species *Zostera muelleri* (*Z. muelleri*) using 16S rRNA amplicon sequencing and compared it with adjacent (asymptomatic) and healthy tissues. *Pseudomonas* and *Burkholderia* dominated healthy and adjacent microbiomes, whereas *Rubidimonas* and *Saprospiraceae* were prominent within the diseased and adjacent tissues, respectively. Regional conservation of the leaf microbiome structure was found among healthy plants but lost within SWD lesions and adjacent tissues. Our functional prediction revealed a shift from heterotrophy to autotrophy, but no evidence for an increase in bacterial pathogenicity or virulence was observed in the adjacent or SWD microbiomes. The patterns observed here indicate that SWD coincides with pronounced changes in the seagrass microbiome, which may either be caused by post-infection colonization by opportunistic bacteria, or the involvement of a dysbiosis effect before or during the infection process. Using a PCR assay specific for the detection of L. zosterae, we demonstrated the presence of *L. zosterae* in SWD tissues, but only in one of the two sampled locations. This suggests that other potential microbiological factors may be involved in the initiation and/or development of the SWD-like symptoms that we observed in samples were *L. zosterae* was not detected. These results suggest that the dynamics of the seagrass microbiome should be considered within the diagnosis and management of SWD.

Keywords: *Seagrass wasting disease, microbiome, dysbiosis, 16S.*

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3.9 Figures

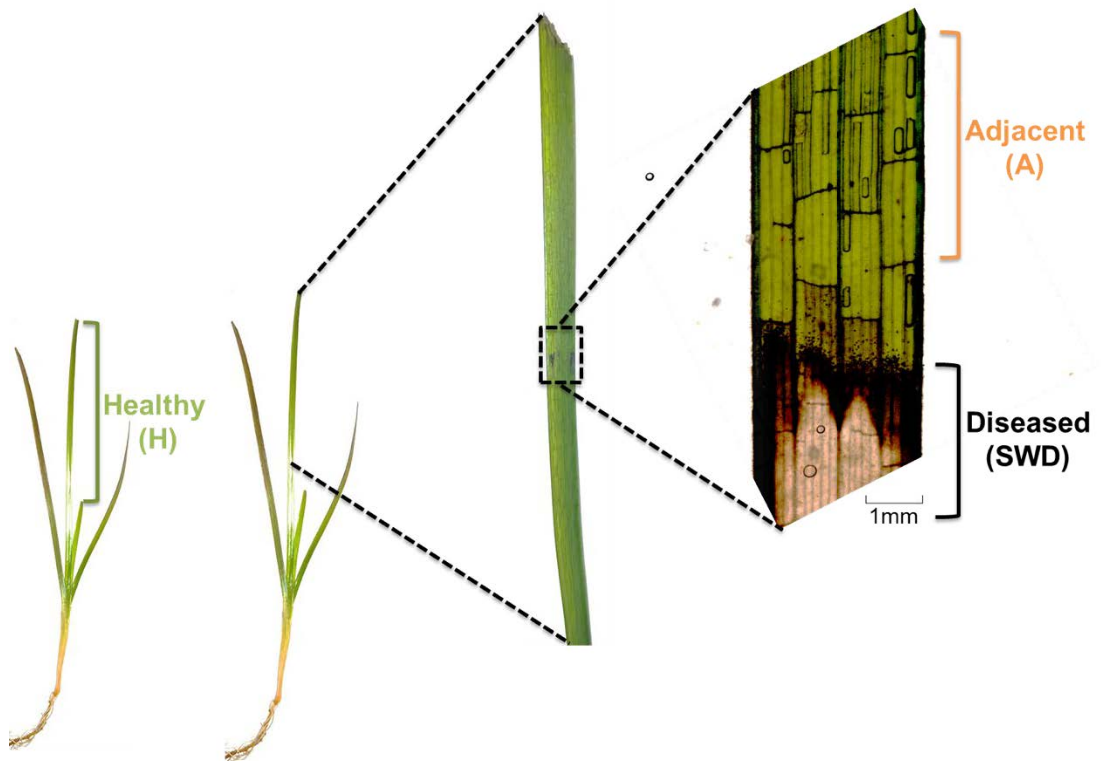


Figure 1: Sampling strategy.

Leaf samples from healthy (i.e., completely asymptomatic, *n* = 12) specimens of Z. muelleri were collected and compared with diseased (i.e., presenting single or multiple necrotic lesions typical of SWD, *n* = 10) and asymptomatic leaf tissues immediately adjacent to active lesions (*n* = 10). Healthy tissues were collected from a different plant. Sample collection took place between October and November 2015 at Rose Bay and Lake Macquarie (New South Wales, Australia), which are two locations separated by 120 km that offer different environmental conditions. Seagrass leaf images were generated using the Precipoint's M8 dual microscope and scanner.

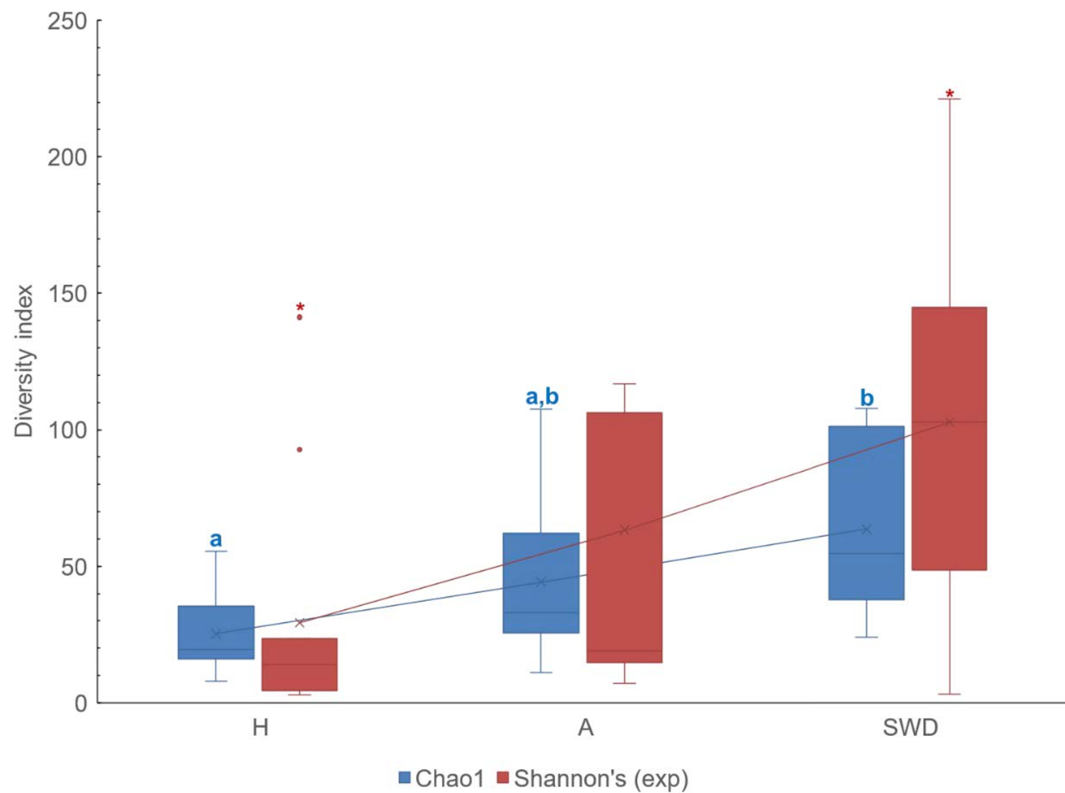


Figure 2: Bacterial richness and diversity across seagrass tissues.
Chao1 diversity index (i.e., bacterial richness, blue) and Shannon's diversity index (i.e., bacterial diversity, red), were calculated for each tissue type. The true Shannon's diversity (i.e., effective number of OTUs) was subsequently calculated by applying the exponential function to the Shannon's diversity index. Multiple comparisons between metrics were tested for statistical significance. Analysis of Variance (ANOVA) and the Tukey HSD test were used to test inter- and intra- tissue differences, respectively. P-values were corrected for multiple testing using the Benjamini-Hochberg's FDR approach. Mean values and quartiles are shown for each sample type: healthy (H), adjacent (A) and diseased (SWD) tissues. Homogeneous subsets (i.e., groups with the same mean, $p > 0.05$) are shown with letters above the bars, and asterisks represent a significant mean difference ($p < 0.05$). For detailed statistical results, see **Supplementary Table 1**.

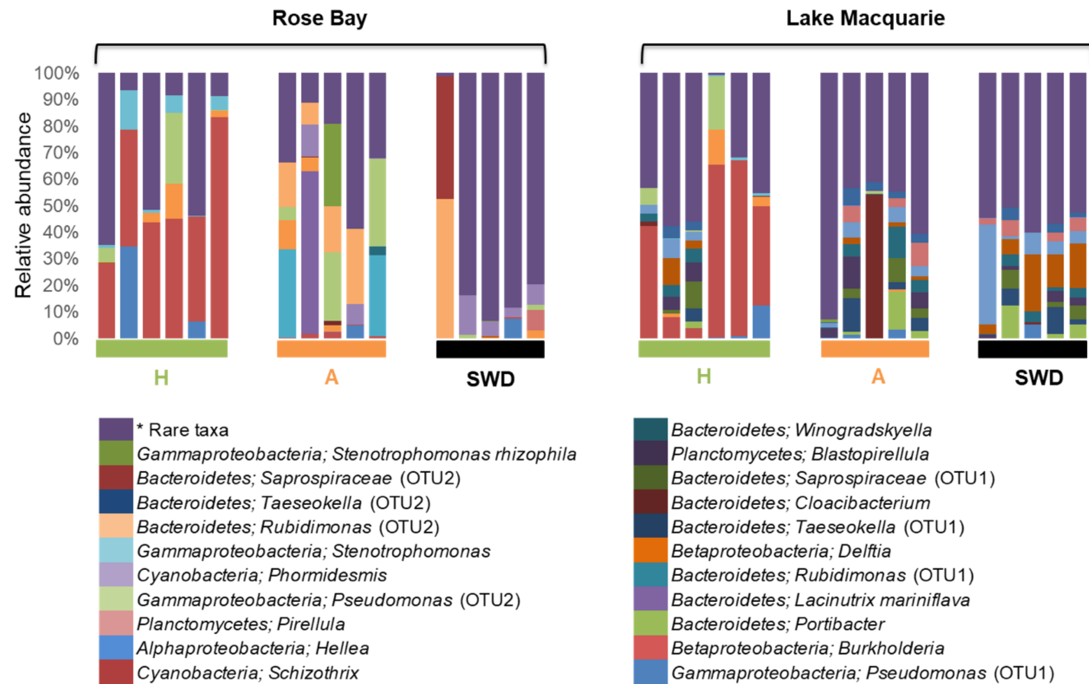


Figure 3: Bacterial community composition across seagrass tissue types.
Beta diversity of bacterial microbiomes associated with healthy (H), adjacent (A) and diseased (SWD) seagrass tissues. Samples (n = 32) were compared across two sampling locations (i.e., Rose Bay and Lake Macquarie) and unique OTUs within each sample are coloured by the highest assigned taxonomic level. Rare members of the microbiome (relative abundance < 0.01% in all samples) were excluded to help remove visual clutter.

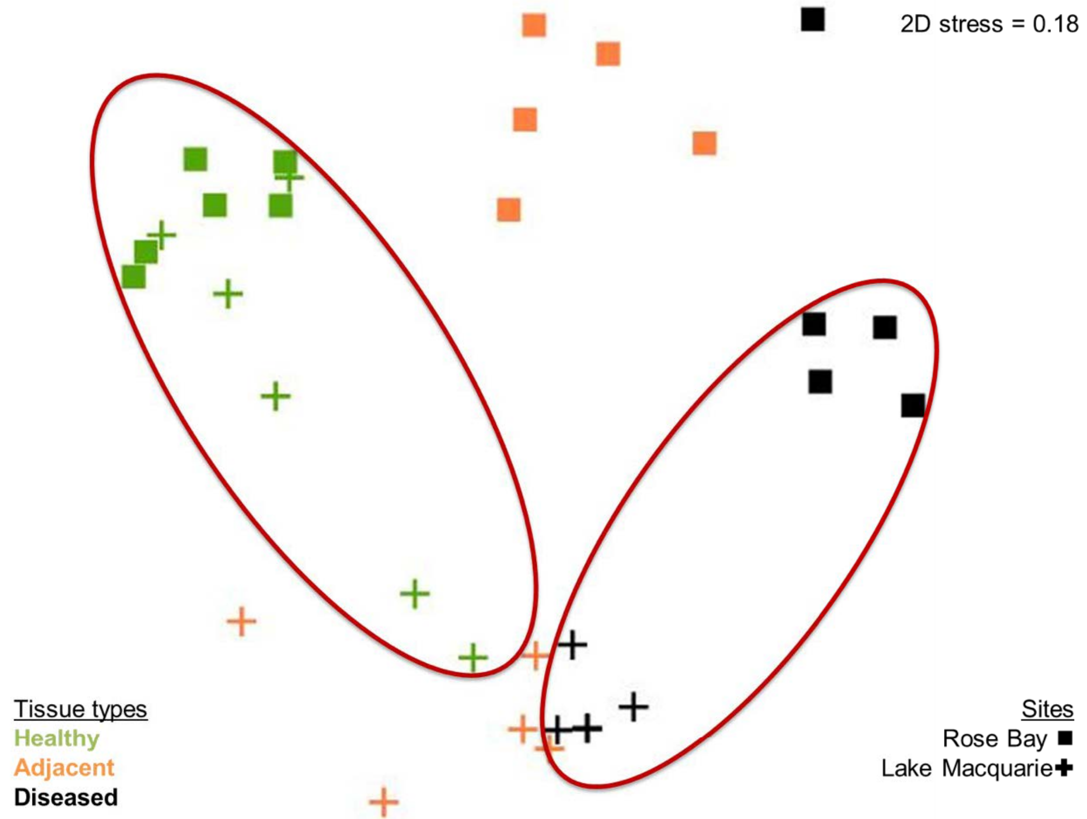


Figure 4: Clustering patterns across seagrass tissue types.

Non-parametric multidimensional scaling (nMDS) of bacterial microbiomes (n = 32), based on a lower triangular resemblance calculated with the S17 Bray-Curtis similarity measure from normalised abundances of OTUs (square-root transformed values). Samples are coloured by tissue type (i.e., healthy, adjacent and diseased) with different shapes for sites (i.e., Rose Bay and Lake Macquarie). Sample partitioning by tissue type are shown in ellipses in the nMDS plot and represent the level of similarity between samples, based on the degree to which OTUs are shared between them. Based on Permutational Multivariate Analysis of Variance (PERMANOVA, Bray-Curtis dissimilarity matrix, simple design), statistically different tissue types (α = 0.05) are outlined in red. The 2D stress is shown in the upper right corner of the nMDS plot (Kruskal stress formula = 1, minimum stress = 0.01).

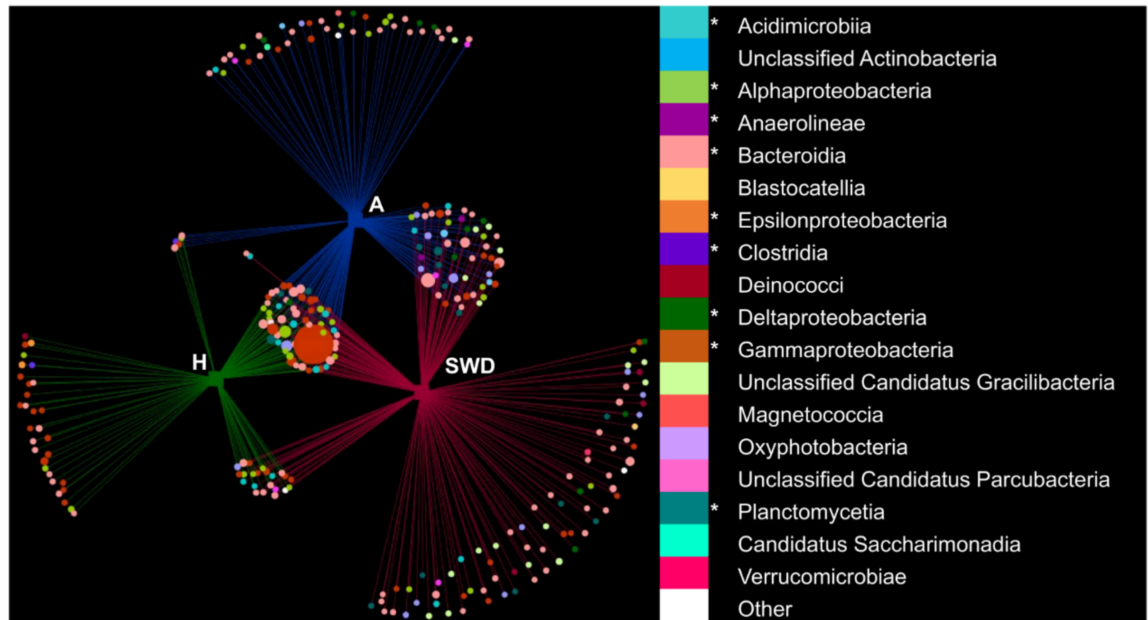


Figure 5: Bacterial OTUs co-occurrence network.

OTUs (nodes as small circles) are linked to each tissue type that they are associated with (coloured squares) by lines (edges) in the bipartite network. Unique (outer clusters of nodes) and shared (nodes in the centre) members of bacterial communities associated with healthy (H), adjacent (A) and diseased (SWD) seagrass tissues are coloured by taxonomic class, with the size of nodes representing OTUs relative abundance (range = 1.0 - 5196.0, min. size = 20, max. size = 132).). For visual purposes only, no replicate threshold was used for 'unique' members of the microbiome. Asterisks represent OTUs previously reported as seagrass associates, as reported by (Ugarelli et al., 2019).

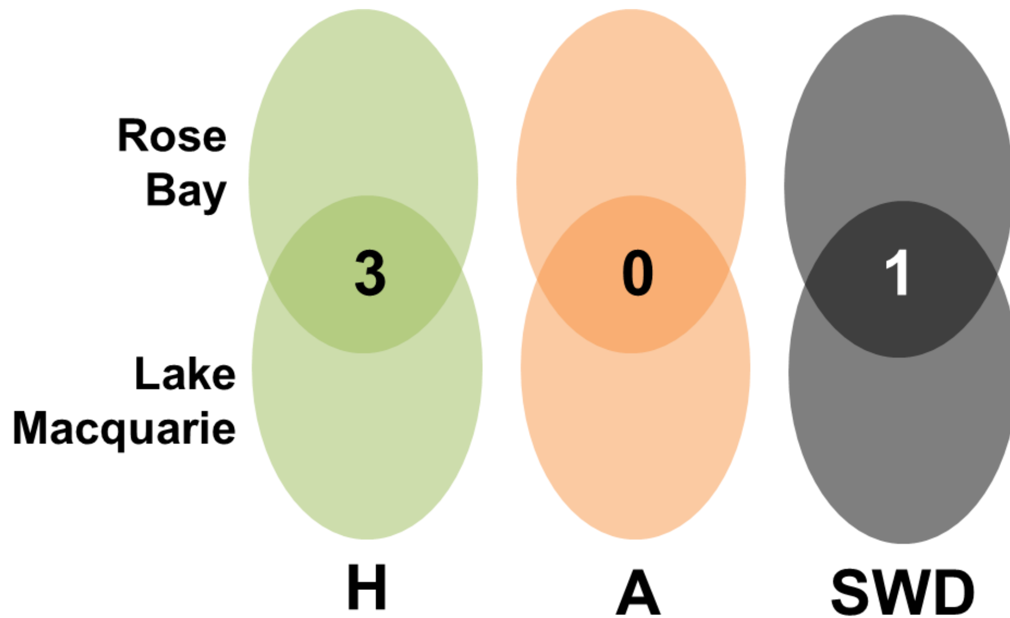


Figure 6: Loss of the conservation of the seagrass leaf microbiome.

Bacterial core OTUs maintained within each seagrass tissue type were identified as those microorganisms consistently present (relative abundance > 0) in most of the samples (n - 1), across both sites within each pigmentation category. Numbers in the middle represent the amount of core OTUs identified for each tissue type (i.e. core sizes). Core members known to be biogeographically conserved in healthy leaves (H) are lost in response to the changing conditions caused by seagrass wasting disease (SWD). Adjacent asymptomatic tissues (A) are characterized by transitional microbiomes with no core members.

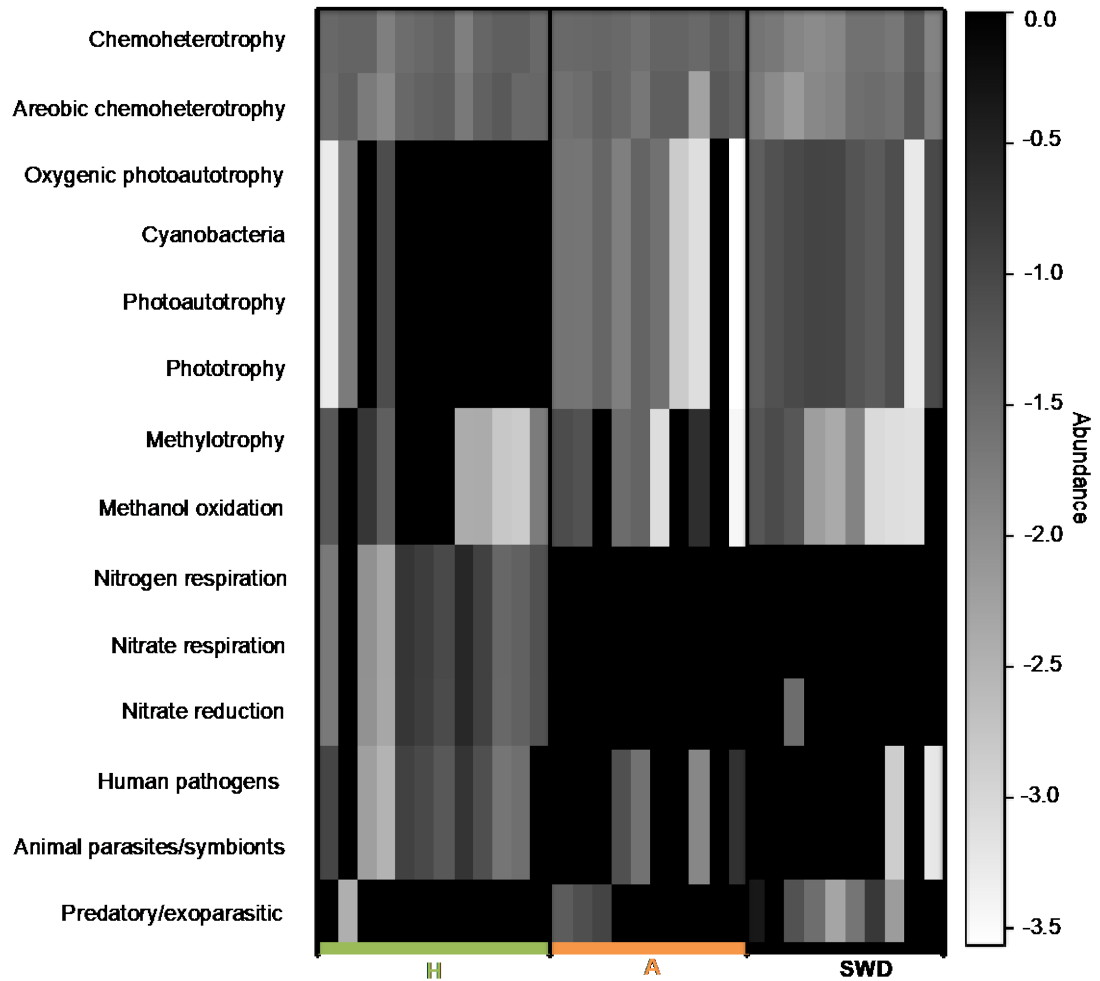


Figure 7: Tissue-associated predicted functional profiles.

Similarity percentages analysis (SIMPER, two-way crossed analyses) was used to identify predicted functional categories (rows) discriminating between tissue types (i.e., samples in columns). Putative functional categories are clustered by UPGMA hierarchical clustering, and their relative abundances (log-transformed) are indicated by varying colour intensities. Predicted functional profiles were generated from 16S rRNA sequencing data using an annotation database created on the basis of genomic compliment of sequenced genomes and the Functional Annotation of Prokaryotic Taxa (FAPROTAX) pipeline. Each taxonomically annotated OTU was compared against each FAPROTAX annotation rule. High contributors (contribution > 70%) were selected from pair-wise comparisons between tissues and putative functions discriminating between healthy (H) and diseased (SWD) samples are plotted.

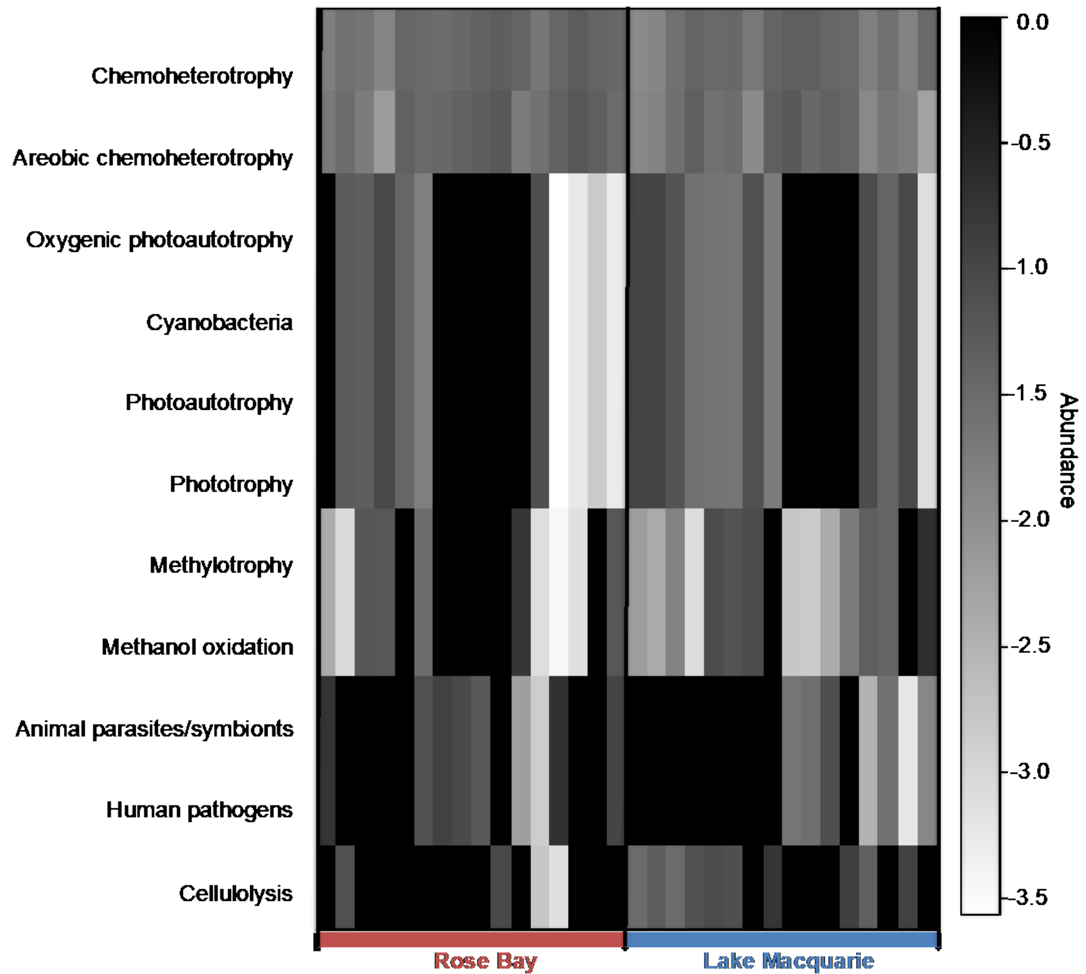
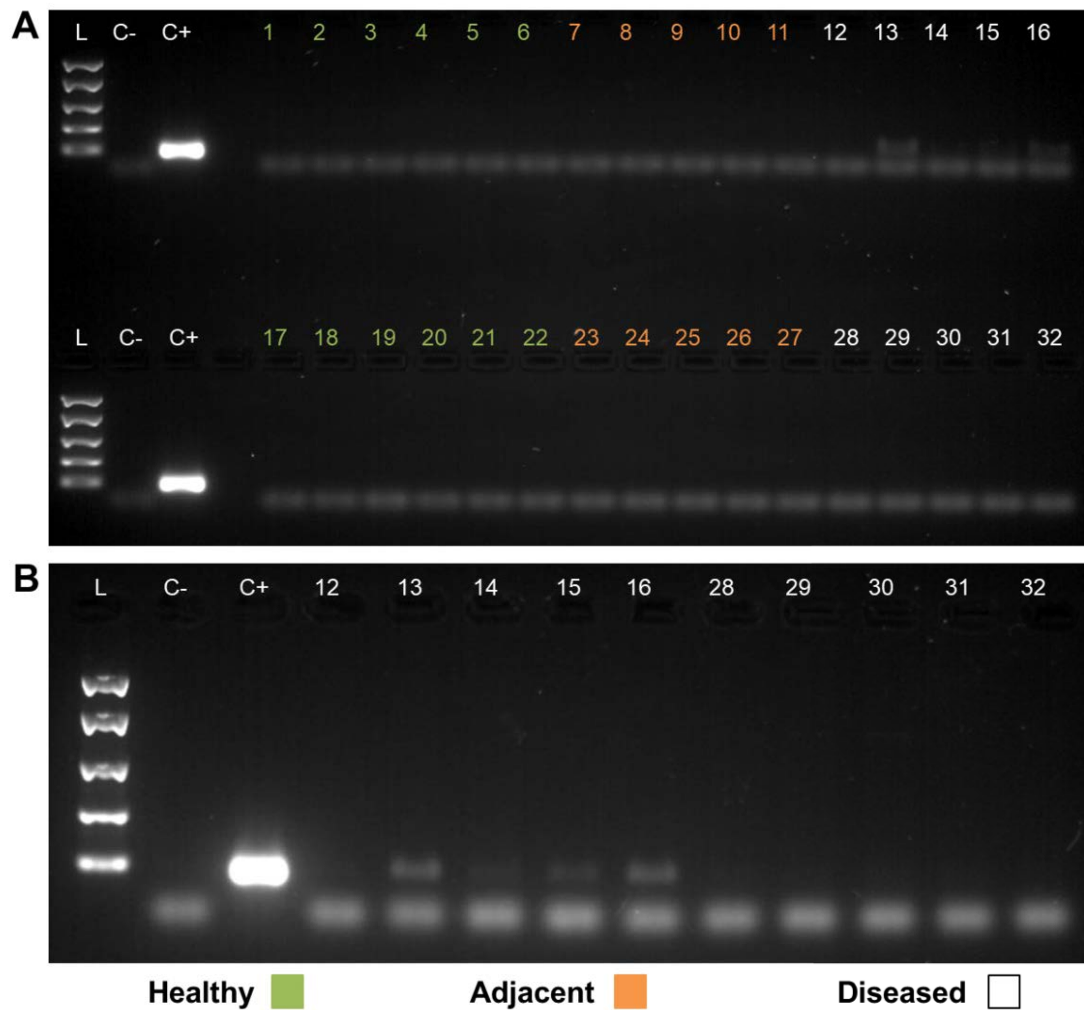


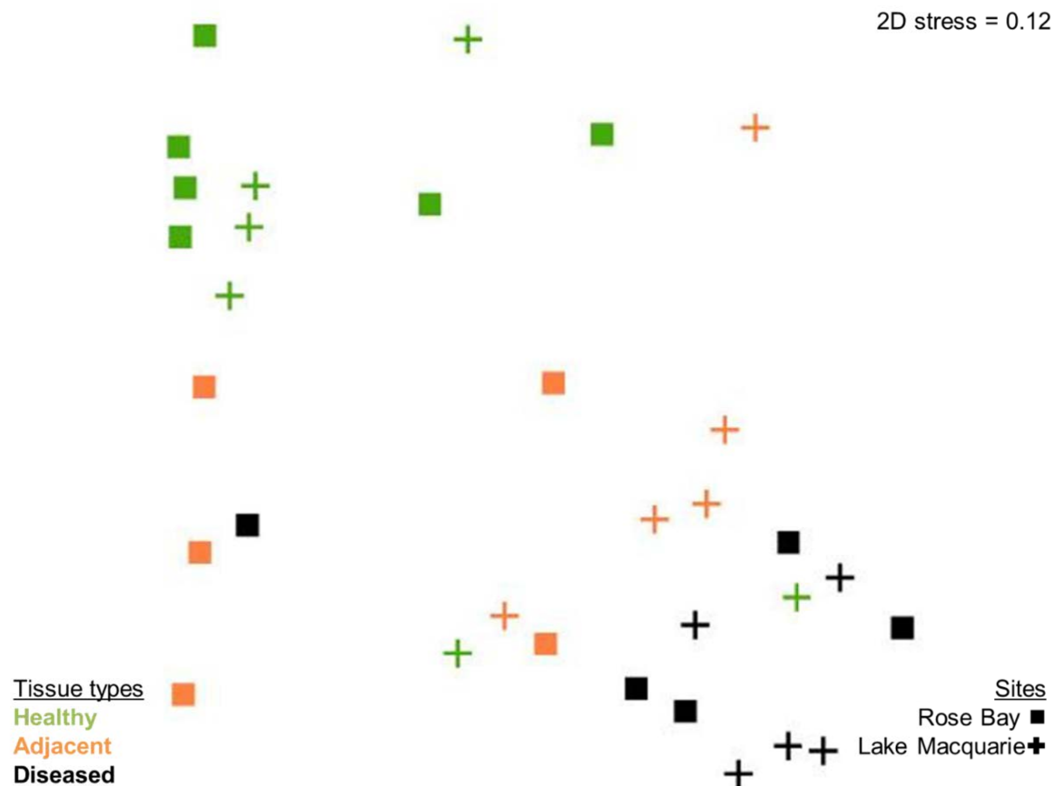
Figure 8: Site-associated predicted functional profiles.

Similarity percentages analysis (SIMPER, two-way crossed analyses) was used to identify predicted functional categories (rows) discriminating between sampling sites (i.e. samples in columns). Putative functional categories are clustered by UPGMA hierarchical clustering, and their relative abundances (log-transformed) are indicated by varying colour intensities. Predicted functional profiles were generated from 16S rRNA gene sequencing data using an annotation database created on the basis of genomic compliment of sequenced genomes and the Functional Annotation of Prokaryotic Taxa (FAPROTAX) pipeline. Each taxonomically annotated OTU was compared against each FAPROTAX annotation rule. High contributors (cumulative contribution > 70%) were selected from the pair-wise comparison between the two sites and putative functions most responsible for the differences between sites are plotted.

3.10 Supplementary figures

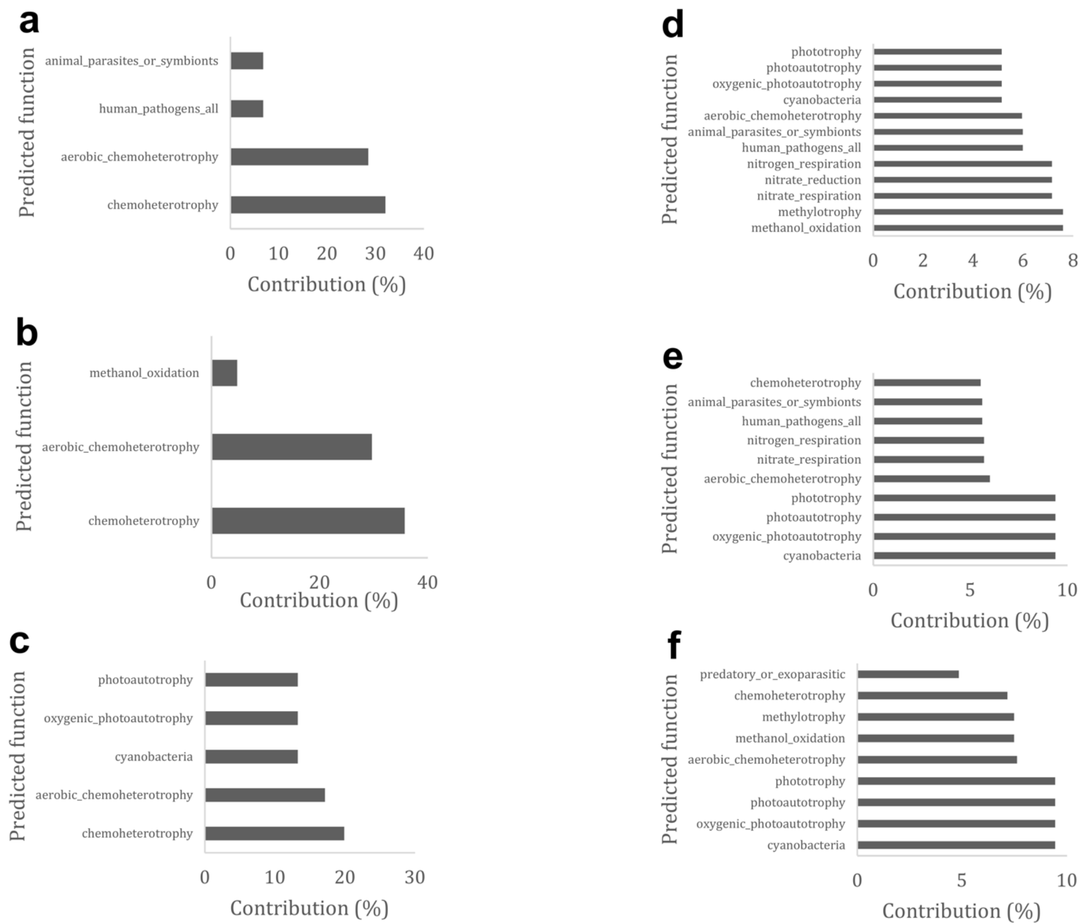


Supplementary Figure 1: PCR detection of genomic DNA from *L. zosterae.*
Presence of the putative pathogen involved in seagrass wasting disease-the protozoan *Labryinthula zosterae,* was detected by polymerase chain reaction (PCR) with primers specifically designed for *L. zosterae* detection on seagrass leaves. All samples were initially tested (**A**) and positive amplifications of the 80 bp region between the internal transcribed (ITS) spacers 1 and 2 were subsequently confirmed by running the same detection assay only in SWD samples (**B**). L: molecular weight ladder (EasyLadder I, Bioline); C-, negative control (Mili-Q water instead of DNA template); C+, synthetized positive control designed with the consensus sequence of *L. zosterae* (GenBank accession number JN121409.1); lines 1-32: *Z. muelleri* leaves gDNA (Rose Bay: 1-16, Lake Macquarie: 17-32). Samples are coloured by tissue type.



Supplementary Figure 2: Clustering patterns across seagrass tissue types (functional predictions).

Non-parametric multidimensional scaling (nMDS) of putative functional profiles (n = 52), based on a lower triangular resemblance calculated with the S17 Bray-Curtis similarity measure from normalized abundances of predicted functional categories (square-root transformed values). Predicted functional profiles were generated from 16S rRNA sequencing data using an annotation database created on the basis of genomic compliment of sequenced genomes and the Functional Annotation of Prokaryotic Taxa (FAPROTAX) pipeline. Each taxonomically annotated OTU was compared against each FAPROTAX annotation rule. Samples are coloured by tissue type (i.e. healthy, adjacent and diseased) with different shapes for sites (i.e. Rose Bay and Lake Macquarie). The 2D stress is shown in the upper right corner of the nMDS plot (Kruskal stress formula = 1, minimum stress = 0.01).



Supplementary Figure 3: Typical and discriminatory predicted functional categories across tissue types (SIMPER analysis).

Relative contribution of predicted functional categories that make up 70% of the dissimilarities between tissue types (n = 14). Putative functional categories exclusively represented in a single tissue type were classified as "typical" functions, and those differentially enriched in more than one sample type were classified as "discriminatory" functions. Typical functions within the healthy (**a**), adjacent (**b**) and diseased (**c**) tissues, and functional groups discriminating between adjacent and healthy (**d**), diseased and healthy (**e**) and adjacent and diseased (**f**) sample types are shown.

3.11 Supplementary tables

Supplementary Table 1: Statistical analyses for alpha diversity.

One-way ANOVA (Chao1)

factor	ti
p-value	0.006

One-way ANOVA (Shannon_exp*)

factor	ti
p-value	0.028

Tukey HSD tests (Chao 1)

p-value	h	a	swd
h	ns	ns	0.004
a	0.217	ns	0.205
swd	ns	ns	

Tukey HSD tests (Shannon_exp*)

p-value	h	a	swd
h	ns	ns	<0.05
a	ns	ns	ns
swd	ns	ns	

Differences between bacterial communities across tissue types were tested for statistical significance in Analysis of Variance (ANOVA).
P-values > 0.05 are shown for homogeneous subsets resulting from Tukey HSD tests.
Significant values at the 0.05 level are shown in grey.
h, healthy; a, adjacent; swd, diseased.
ns: no significant, in the absence of homogeneous subsets.
* The true Shannon's diversity was calculated by applying the exponential function to the Shannon's diversity index.
P-values were corrected for multiple testing using the Benjamini-Hochberg's FDR approach.

Supplementary Table 2: Statistical analyses for multidimensional scaling.

Main tests

factor	si	ti	sixti	siti*
p-value [P(perm)]	0.0001	0.0098	0.0221	0.0001
		P(MC) i		

ECV

Source	Estimate	Sq.root
V(si)	941.480	30.684
S(ti)	764.320	27.646
V(sixti)	375.470	19.377
V(Res)	2687.500	51.841

ECV*

Source	Estimate	Sq.root
S(siti)	1438.300	37.925
V(Res)	2687.500	51.841

Pair-wise (si)

p-value [P(perm)]	rb	lm
rb		
lm	0.0001	

Average similarity between/within sites ii

si	rb	lm
rb	13.220	
lm	7.701	22.764

Pair-wise (ti)

p-value P(MC) i	h	a	swd
h			
a	0.0574		
swd	0.0292	0.3398	

Average similarity between/within tissues ii

ti	h	a	swd
h	29.439		
a	9.127	13.800	
swd	5.880	12.014	16.076

Pair-wise: siti

p-value P(MC) i	rb-h	rb-a	rb-swd	lm-h	lm-a	lm-swd
rb-h						
rb-a	0.0028					
rb-swd	0.0029	0.0494				
lm-h	0.1845	0.0104	0.0060			
lm-a	0.0004	0.0075	0.0115	0.0366		
lm-swd	0.0005	0.0027	0.0044	0.0117	0.2138	

Average similarity between/within sites ii

siti	rb-h	rb-a	rb-swd	lm-h	lm-a	lm-swd
rb-h	35.671					
rb-a	10.100	22.391				
rb-swd	3.995	9.255	17.321			
lm-h	28.516	9.591	2.910	25.421		
lm-a	2.348	5.211	4.350	14.470	26.683	
lm-swd	1.349	3.284	5.517	15.266	31.169	41.229

Rose Bay (only)

Main test

factor	ti
p-value [P(perm)]	0.0001

ECV

Source	Estimate	Sq.root
S(ti)	1422.000	37.710
V(Res)	2836.500	53.258

Pair-wise (ti)

p-value P(MC) i	h	a	swd
h			
a	0.0040		
swd	0.0029	0.0455	

Lake Macquarie (only)

Main test

factor	ti
p-value [P(perm)]	0.0001

ECV

Source	Estimate	Sq.root
S(ti)	857.570	29.284
V(Res)	2538.500	50.384

Pair-wise (ti)

p-value P(MC) i	h	a	swd
h			
a	0.0408		
swd	0.0141	0.2205	

Differences between bacterial communities across tissue types and sites were tested for statistical significance in Permutational Multivariate Analysis of
Significant values at the 0.05 level are shown in grey
i Corrected Monte Carlo p-values used when there were not enough possible permutations (<500 out of 9999 permutations, unrestricted permutation method)
ii Values displayed in percentages.
* Substantiates the use of pair-wise comparisons between 6 independent samples types (i.e., levels of combined sites and tissues)
ECV: estimates of components of variation
si, site(s); ti, tissue(s); siti, site(s) and tissue(s)
h, healthy; a, adjacent; swd, diseased
rb, Rose Bay; lm, Lake Macquarie

Supplementary Table 3: SIMPER analysis (taxonomy).

A - SWD

OTUs*	Contrib%
Rubidimonas	5.15
Pseudomonas	3.49
Saprospiraceae	3.02
Hellea	2.09
Lacinutrix mariniflava	1.57
Phormidesmis sp.	1.55
Schizothrix	1.46
Cellvibrionaceae	1.35
Delftia	1.32
Cloacibacterium	1.31
Algitalea	1.25
Micrococcus luteus	1.19
Pirellula	1.17
Candidatus *Megaira*	1.11
Stenotrophomonas rhizophila	1.10
Microtrichaceae	1.08

A - H

OTUs*	Contrib%
Burkholderia	9.82
Pseudomonas	5.90
Rubidimonas	5.74
Stenotrophomonas	3.66
Cloacibacterium	2.72
Delftia	2.30
Lacinutrix mariniflava	1.82
Phormidesmis sp.	1.54
Cryomorphaceae	1.49
Candidatus *Megaira*	1.36
Winogradskyella	1.26
Moraxella osloensis	1.20
Alphaproteobacteria (SAR11)	1.12
Micrococcus luteus	1.12
Sphaerotilus	1.01

SWD - H

OTUs*	Contrib%
Burkholderia	8.85
Pseudomonas	3.96
Saprospiraceae	2.85
Stenotrophomonas	1.94
Rubidimonas	1.90
Schizothrix	1.74
Delftia	1.66
Phormidesmis sp.	1.62
Pirellula	1.32
Hellea	1.30
Cryomorphaceae	1.24
Cellvibrionaceae	1.22
Algitalea	1.15
Pleurocapsa sp.	1.00

a Discriminatory OTUs with the highest contribution (Contrib% □ 1%) to the differences between seagrass tissue types. Two way-crossed similarity percentages analysis (SIMPER , site x tissue). Top-3 contributor OTUs are shown in grey.

* Pooled at genus level (cumulative contributions).

H, healthy; A, adjacent; SWD, diseased.

Supplementary Table 4: SIMPER analysis (functional prediction compared between tissue types).

Functional category	OTUs *	Av.Abund (SWD)	Av.Abund (H)	Over-rep.	Av.Diss	Diss/SD	Contrib%	Cum.%	Levene's i	Test ii	Over-rep.	p-value iii
Cyanobacteria	15	0.33	0.05	SWD	5.27	2.09	9.38	9.38	>0.05	t-test	SWD	4.92E-05
Oxygenic photoautotrophy	15	0.33	0.05	SWD	5.27	2.09	9.38	18.76	>0.05	t-test	SWD	4.92E-05
Photoautotrophy	15	0.33	0.05	SWD	5.27	2.09	9.38	28.14	>0.05	t-test	SWD	4.92E-05
Phototrophy	15	0.33	0.05	SWD	5.27	2.09	9.38	37.52	>0.05	t-test	SWD	4.92E-05
Aerobic chemoheterotrophy	55	0.41	0.53	H	3.37	1.59	6.00	43.51	>0.05	t-test	H	0.050
Nitrate respiration	2	0.00	0.17	H	3.20	1.58	5.70	49.21	<0.05	Welch's	H	6.38E-03
Nitrogen respiration	2	0.00	0.17	H	3.20	1.58	5.70	54.91	<0.05	Welch's	H	6.38E-03
Human pathogens	3	0.01	0.17	H	3.15	1.41	5.61	60.53	<0.05	Welch's	H	5.79E-03
Animal parasites/symbionts	3	0.01	0.17	H	3.15	1.41	5.61	66.14	<0.05	Welch's	H	5.79E-03
Chemoheterotrophy	67	0.45	0.57	H	3.11	1.77	5.53	71.67	>0.05	t-test	H	0.014

a Predicted functional categories responsible for 56% average dissimilarity between healthy (H) and diseased (SWD) samples, based on similarity percentages analysis (two way-crossed, site x tissue).
* Total records assigned to FAPROTAX functional categories.
i Levene's test was used to assess equality of variances.
ii Two-groups statistic test (STAMP).
iii Statistical significance at α = 0.05. P-values were corrected for multiple testing using the Benjamini-Hochberg's FDR approach.
Av, average; Diss, dissimilarity; Contrib, contribution; Cum, cumulative; Over-rep, over-representation; NA, not applicable.

Supplementary Table 5: SIMPER-STAMP analysis (functional predictions compared between sites).

Functional category	OTUs *	Av.Abund (RB)	Av.Abund (LM)	Over-rep.	Av.Diss	Diss/SD	Contrib%	Cum.%	Levene's i	Test ii	Over-rep.	p-value iii
Methanol oxidation	9	0.11	0.16	LM	3.41	1.13	9.22	9.22	>0.05	t-test	NA	NA
Methylotrophy	9	0.11	0.16	LM	3.41	1.13	9.22	18.44	>0.05	t-test	NA	NA
Aerobic chemoheterotrophy	55	0.52	0.46	LM	2.88	1.18	7.81	26.25	>0.05	t-test	NA	NA
Cellulolysis	8	0.03	0.14	LM	2.43	1.08	6.59	32.83	<0.05	Welch's	LM	0.015
Cyanobacteria	15	0.12	0.21	LM	2.18	0.88	5.91	38.74	>0.05	t-test	NA	NA
Oxygenic photoautotrophy	15	0.12	0.21	LM	2.18	0.88	5.91	44.65	>0.05	t-test	NA	NA
Photoautotrophy	15	0.12	0.21	LM	2.18	0.88	5.91	50.55	>0.05	t-test	NA	NA
Phototrophy	15	0.12	0.21	LM	2.18	0.88	5.91	56.46	>0.05	t-test	NA	NA
Human pathogens	3	0.13	0.05	RB	2.14	0.96	5.79	62.25	<0.05	Welch's	RB	0.033
Animal parasites/symbionts	3	0.13	0.05	RB	2.14	0.96	5.79	68.05	<0.05	Welch's	RB	0.033
Chemoheterotrophy	67	0.56	0.52	LM	1.79	0.99	4.84	72.88	>0.05	t-test	NA	NA

a Predicted functional categories responsible for 73% average dissimilarity between Rose Bay (RB) and Lake Macquarie (LM) samples, based on similarity percentages analysis (SIMPER, two-way crossed, site x tissue).
i Levene's test was used to assess equality of variances.
ii Two-groups statistic test (STAMP).
iii Statistical significance at α = 0.05. P-values were corrected for multiple testing using the Benjamini-Hochberg's FDR approach.
* Total records assigned to FAPROTAX categories.
Av, average; Diss, dissimilarity; Contrib, contribution; Cum, cumulative; Over-rep, over-representation; NA, not applicable.

4 CHAPTER 4
Seagrass leaf reddening alters the microbiome of *Zostera muelleri*

To be published as: Hurtado-McCormick, V., Kahlke, T., Krix, D., Larkum, T., Ralph, P., Seymour, J.R. (in press). Seagrass leaf reddening alters the microbiome of *Zostera muelleri*. *Marine Ecology Progress Series*. doi: 10.3354/meps13409.

Author contributions: VH-M conceived the study, designed the sampling strategy, conducted the fieldwork and lab work, developed the methodological approaches, analysed the data, drafted the manuscript, prepared the figures and tables and obtained the approval of the final submission. TK supported the bioinformatic analyses, developed the customised pipelines for data analysis, and provided substantial contributions to the results interpretation and the intellectual content of the manuscript. DK assisted statistical analyses with Generalized Lineal Models and data visualization in R. TL assisted the microscopy work and provided important contributions to the results interpretation. PR supervised the study. JS conceived the study, designed the sampling strategy, provided the regular supervision of VH-M throughout the data analysis and interpretation, drafted the manuscript and substantially contributed to its intellectual content. All authors agreed to be accountable for the content of the work.

4.1 Preface

In Chapter 3, we provided evidence that SWD coincides with pronounced changes in the *Z. muelleri* microbiome, which may either be caused by post-infection colonisation by opportunistic bacteria, or the involvement of a dysbiosis effect before or during the infection process initiated by *Labyrinthula zosterae*. We also showed that these changes involve the loss of the leaf microbiome structure within SWD lesions and adjacent tissues, as well as a potential functional shift from heterotrophy to autotrophy. These results proved the strong influence that the seagrass host has on its associated bacteria, which probably respond to the changing conditions caused by SWD. In Chapter 4, we tested the hypothesis of similar microbial responses to other physiological changes in the host by characterising bacterial communities associated with leaves showing atypical symptoms

of seagrass leaf reddening, an environmentally-driven response to increased radiation.

4.2 Abstract

Seagrasses host an extremely diverse microbiome that plays fundamental roles in seagrass health and productivity but may be sensitive to shifts in host physiology. Here, we observed a leaf reddening phenomenon in *Zostera muelleri* and characterized bacterial assemblages associated with green and reddened leaves to determine whether this change in leaf pigmentation stimulates a shift in the seagrass microbiome. Using 16S rRNA gene amplicon sequencing, we observed that the microbiome associated with four different leaf pigmentation categories (i.e., green, white, purple and black) differed significantly, with substantial changes in microbiome composition when the tissue is whitened (non-pigmented). *Actinobacteria*, *Rhodobacteraceae*, *Erythrobacter*, *Sulfitobacter* and *Granulosicoccus* were enriched in black and/or purple tissues and discriminated these microbiomes from those associated with green leaves. Contrastingly, all "discriminatory" zero-radius Operational Taxonomic Units (zOTUs) were depleted within the communities associated with white samples. While 40% of the abundant zOTUs identified were exclusively associated with a single pigmentation category, only 3% were shared across all categories, indicating partitioning of the phyllosphere microbiome. However, a significant proportion of the "normal" (green) leaf core microbiome was also retained in the core communities associated with black (70%) and purple (70%) tissues. Contrastingly, no core zOTUs were maintained in the white tissues. These results indicate that environmentally-driven physiological shifts in seagrasses, such as leaf reddening expressed in response to high irradiance, can impact the seagrass leaf microbiome, resulting in significant shifts in the microbiome of reddened leaves with the most extreme expression (in white tissue of reddened leaves).

Keywords: *Seagrass, microbiome, 16S rRNA genes, diversity-ecosystem functioning, pigmentation, irradiance.*

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4.9 Figures

Figure 1: Leaf reddening in *Z. muelleri*.

Samples from three distinctively pigmented parts of the leaf (i.e., black, purple and white) showing symptoms of leaf reddening were collected and compared with green, asymptomatic leaves (pooled tissues) not displaying obvious changes in pigmentation. All *Z. muelleri* specimens (n = 6) were collected from the same meadows in Bonna Point, New South Wales, Australia, which were surveyed on November 2016. Seagrass leaf images were generated using the Precipoint's M8 dual microscope and scanner.

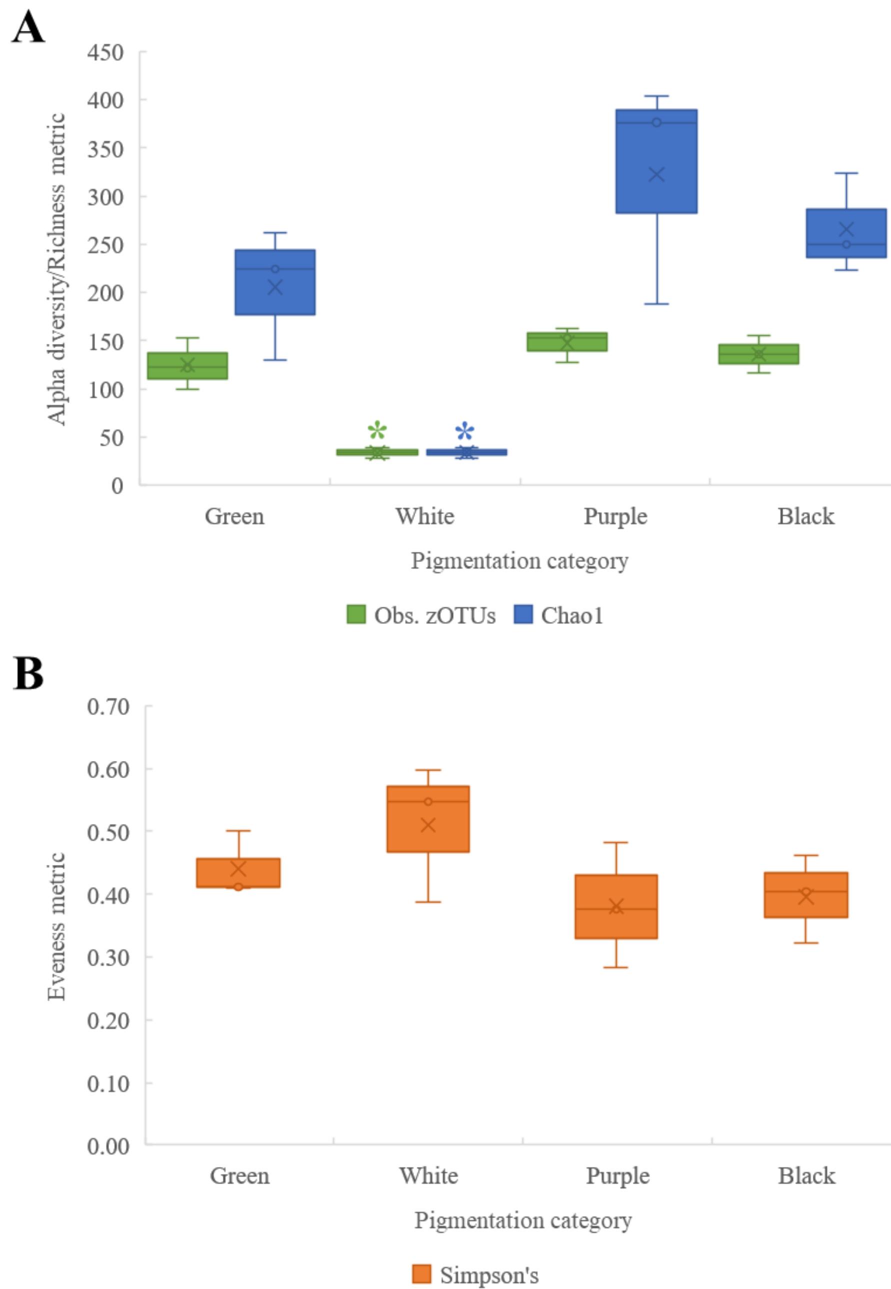


Figure 2: Alpha diversity and richness across pigmentation types (rarefied data). Chao1 diversity index and observed zOTUs (**A**), and the Simpson's diversity index (**B**) were compared across samples (n = 12), and the between-tissues differences were tested for statistical significance (α = 0.05) using Analysis of Variance (ANOVA). The differences between green leaves and reddened tissues (i.e., white, purple and black) were tested separately for statistical significance using unpaired-t tests. Mean values and quartiles are shown for each pigmentation category. * = Significant mean differences with respect to green leaves ($p < 0.05$).

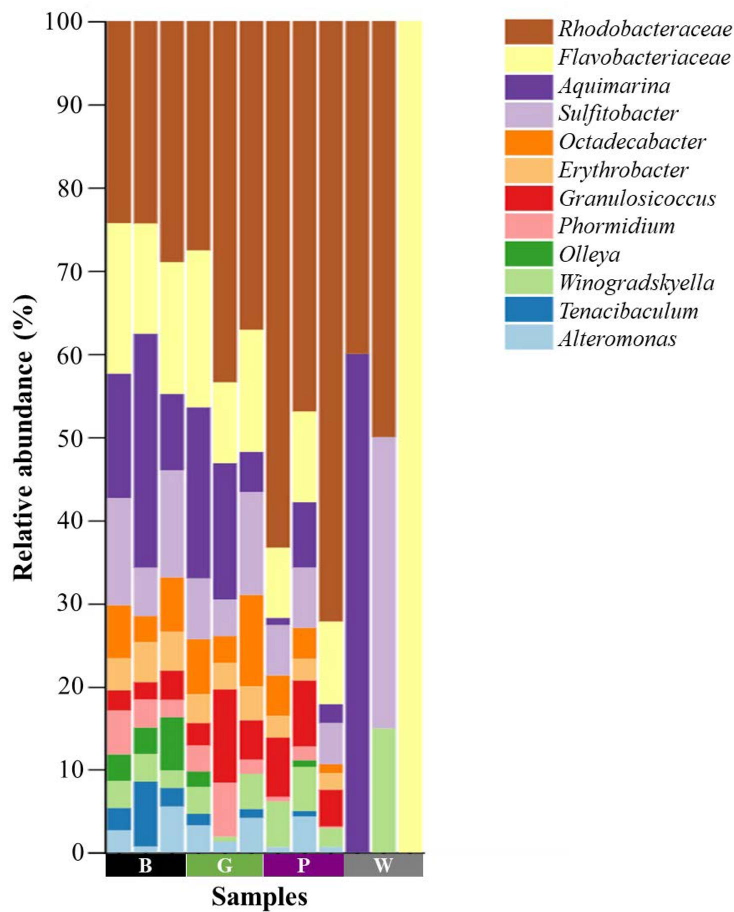


Figure 3: Beta diversity across pigmentation types (unrarefied data).

Beta diversity of bacterial microbiomes associated with black (B), green (G), purple (P) and white (W) seagrass leaf tissues is plotted, with unique zOTUs within each sample coloured by the highest assigned taxonomic level. Rare members of the microbiome were excluded to help remove visual clutter, and thus only representative zOTUs with a relative abundance > 1% in all samples (*n* = 12) are shown.

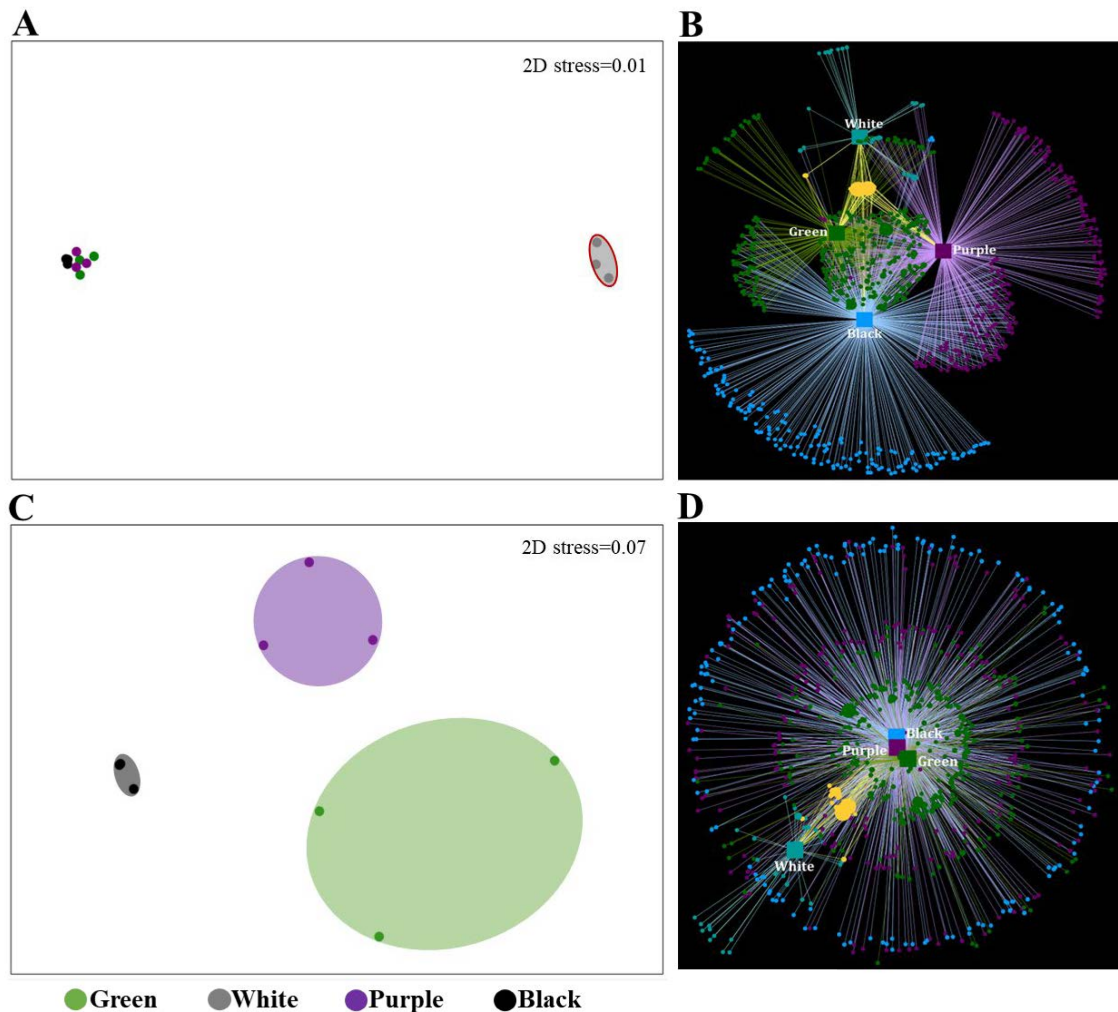


Figure 4: Bacterial community structure (unrarefied data).

Non-parametric multidimensional scaling (nMDS, **A**) and bipartite network (**B**) of bacterial assemblages associated with seagrass green leaves and reddened tissues (i.e., white, purple and black; n = 12). The nMDS plot was generated from a lower triangular resemblance calculated with the S17 Bray-Curtis similarity measure from normalized abundances of zOTUs (high values down-weighted with square root). Samples are coloured by pigmentation category and clustering patterns are shown in ellipses, representing the level of similarity between samples based on the degree to which zOTUs are shared between them. The 2D stress is shown in the upper right corner of the nMDS plot (Kruskal stress formula = 1, minimum stress = 0.01). Based on Permutational Multivariate Analysis of Variance (PERMANOVA, Bray-Curtis dissimilarity matrix, simple design), pigmentation categories that are statistically different from all other sample types (α = 0.05) are outlined in red. Clustering patterns of the reddened tissues only (**C**) were generated using the same approach. In the co-occurrence network, zOTUs (nodes as small circles) are linked to each pigmentation type that they are associated with (coloured

squares) by lines (edges). “Unique” (outer clusters of nodes) and “shared” (yellow nodes in the centre) members of microbiomes associated with green leaves (green), and white (aquamarine), purple (magenta) and black (blue) tissues are displayed, with the size of nodes representing zOTUs absolute abundance (range = 23.0 - 12536.0). The same network with an alternative edge-weighted spring embedded layout is provided (**D**) to highlight “shared” zOTUs between the green, purple and black tissues (3%, statistically similar in clustering analyses).

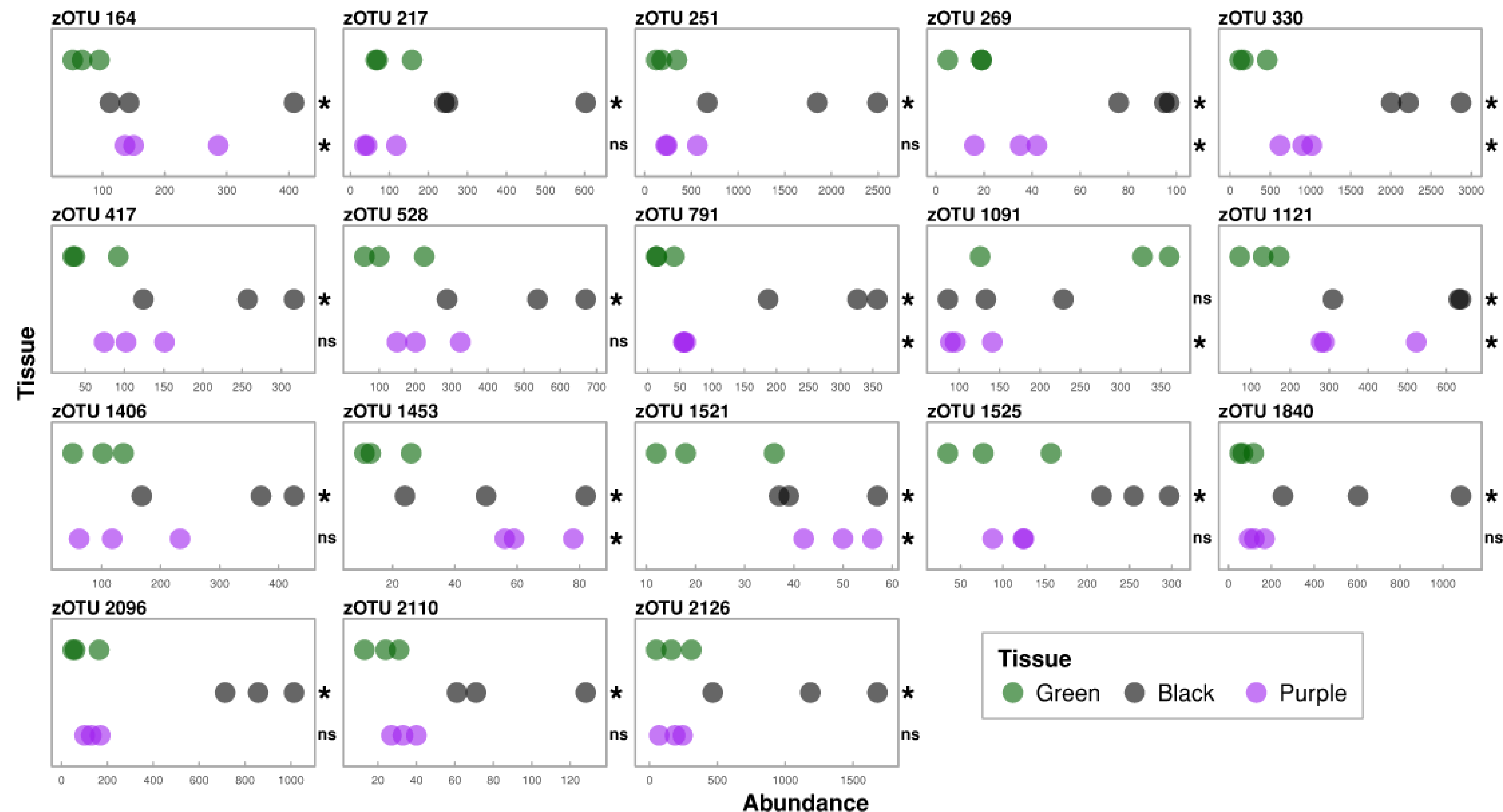


Figure 5: Bacterial taxonomical changes between healthy and reddened leaves (unrarefied data).

The model-based approach mvabund (main overall test and adjustments for multiple testing), individual GLMs and contrasts in emmeans were used to identify bacterial discriminatory zOTUs that differed significantly ($p < 0.05$) between green and reddened tissues. Sequencing reads counts (i.e., abundance) for each of the 18 significant zOTUs (panels) are shown, and the statistical significance at $\alpha = 0.05$ of the contrasts comparing green leaves with black or purple tissues (contrast family: "trt.vs.ctrl") are indicated on the right side of each panel. Samples are coloured by tissue type. * = significant p-value, ns = non-significant p-value. For detailed information on these statistical results, please refer to **Supplementary Table 4**.

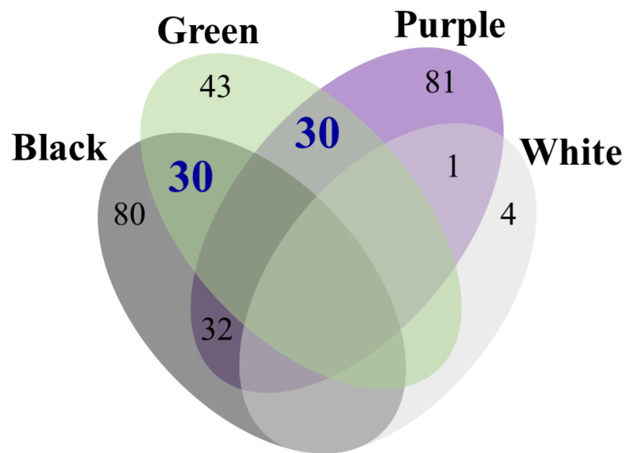


Figure 6: Core microbiomes and retained zOTUs (unrarefied data).

Members of bacterial microbiomes maintained across all samples within each pigmentation category and their possible combinations are shown. Core zOTUs were identified as those microorganisms consistently present (relative abundance > 0.1%) in all samples (*n*) of a given pigmentation category. The abundance threshold was established to account for the high proportion of rare microbiome zOTUs. Numbers in the middle represent core sizes > 0, with core members of the green leaves-associated communities that were retained as core zOTUs across the other three pigmentation categories shown in blue font. See **Supplementary Table 5** and **Supplementary Table 6** for detailed information on lost and retained core members, respectively.

4.10 Supplementary material

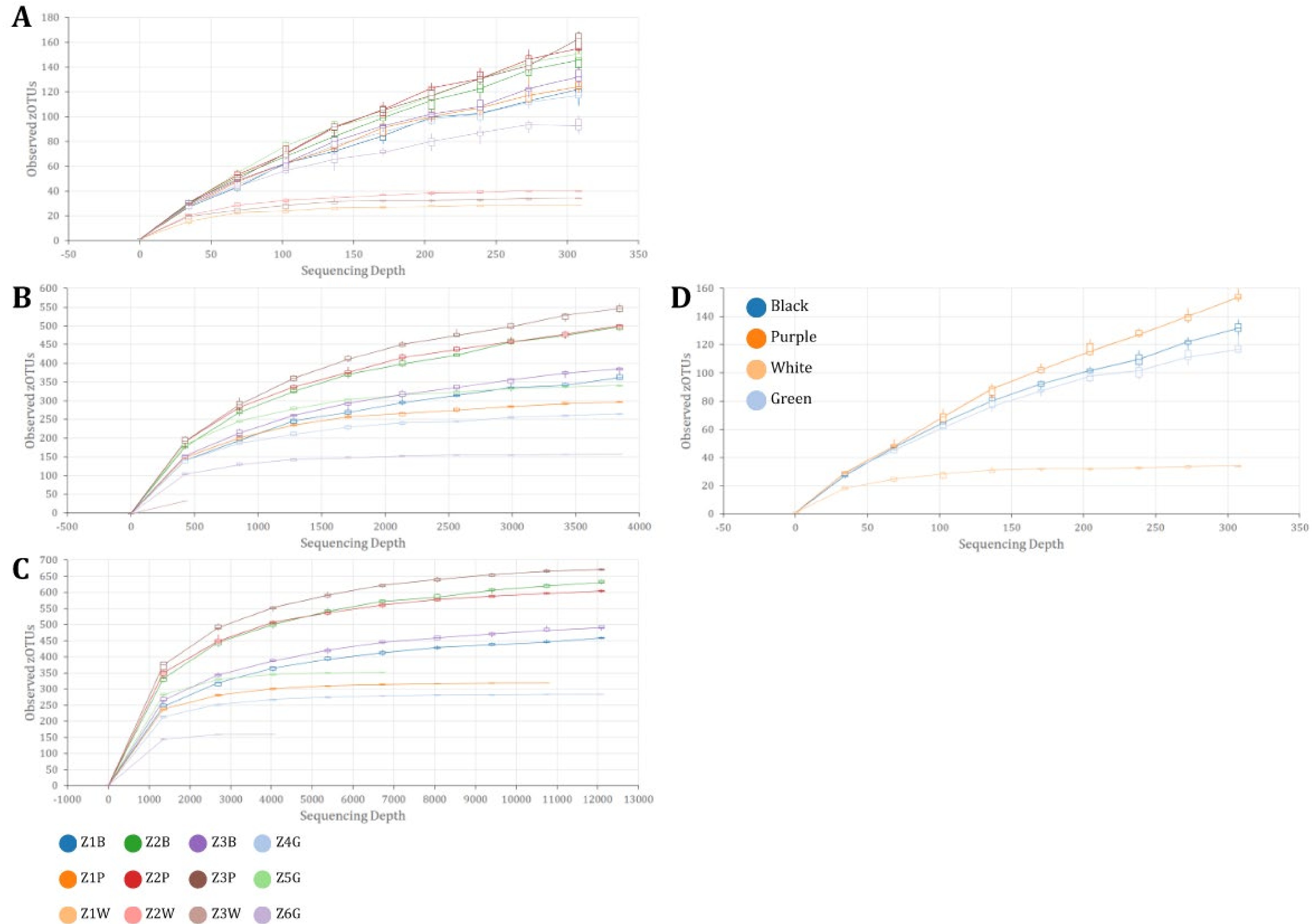
4.10.1 Low bacterial abundance in white leaf tissues (detailed)

After removal of contaminant, non-bacterial (i.e., chloroplast, mitochondrion and seagrass) sequences, the sequence counts ranged from as low as 308 to 60,409 sequences per sample. Notably, the samples with the lowest read counts exclusively corresponded to samples taken from the white tissues. This could potentially be attributed to a number of technical issues, including differing amounts of starting material, differences in DNA extraction methods and differences in DNA yield for this particular tissue type, or to low bacterial abundance in this (i.e., white tissues) environment. However, the amount of plant tissue used as starting material for DNA extractions was consistent across all samples and the extraction methods were performed using standardized procedures. Moreover, all tissue types showed comparable library sizes before removal of non-bacterial sequences, but the ratio of bacterial reads in the white tissue samples was substantially lower than in the other three tissue types (**Supplementary Table 1**). We also analyzed rarefaction curves of all environments to determine whether the sequencing depth sufficiently covered the microbial diversity of each sample and observed that even the rarefaction curves of the white tissue converged at approximately 100 sequences despite the low number of reads (**Supplementary Figure 1**). Moreover, a detailed analysis of zOTUs that recorded zero abundance in green tissues showed that most of the zOTUs that are absent within the microbiomes associated with green leaves are also absent within those associated with white tissues (Supplementary Figure 2
Supplementary Figure 2), which supports our rationale of a lower number of bacterial sequences in the white tissue type. In our opinion, these results indicate that the low number of bacterial reads in the white tissue is not a sampling/sequencing artefact, but instead reflect the low abundance of bacteria in the white parts of the leaf. Based on this observed biological signal, we did not rarefy the data for subsequent taxonomic analyses, as it would have artificially equalized very different environments. To support this, we confirmed general trends between the environments by assessing the extent to which unrarefied and rarefied data sets were comparable (see **Section 4.10.2**). Sequencing reads were rarefied to the same depth (308 sequences per sample) for alpha diversity analyses and corresponding statistical community analyses.

Supplementary Table 1: Library size summary.

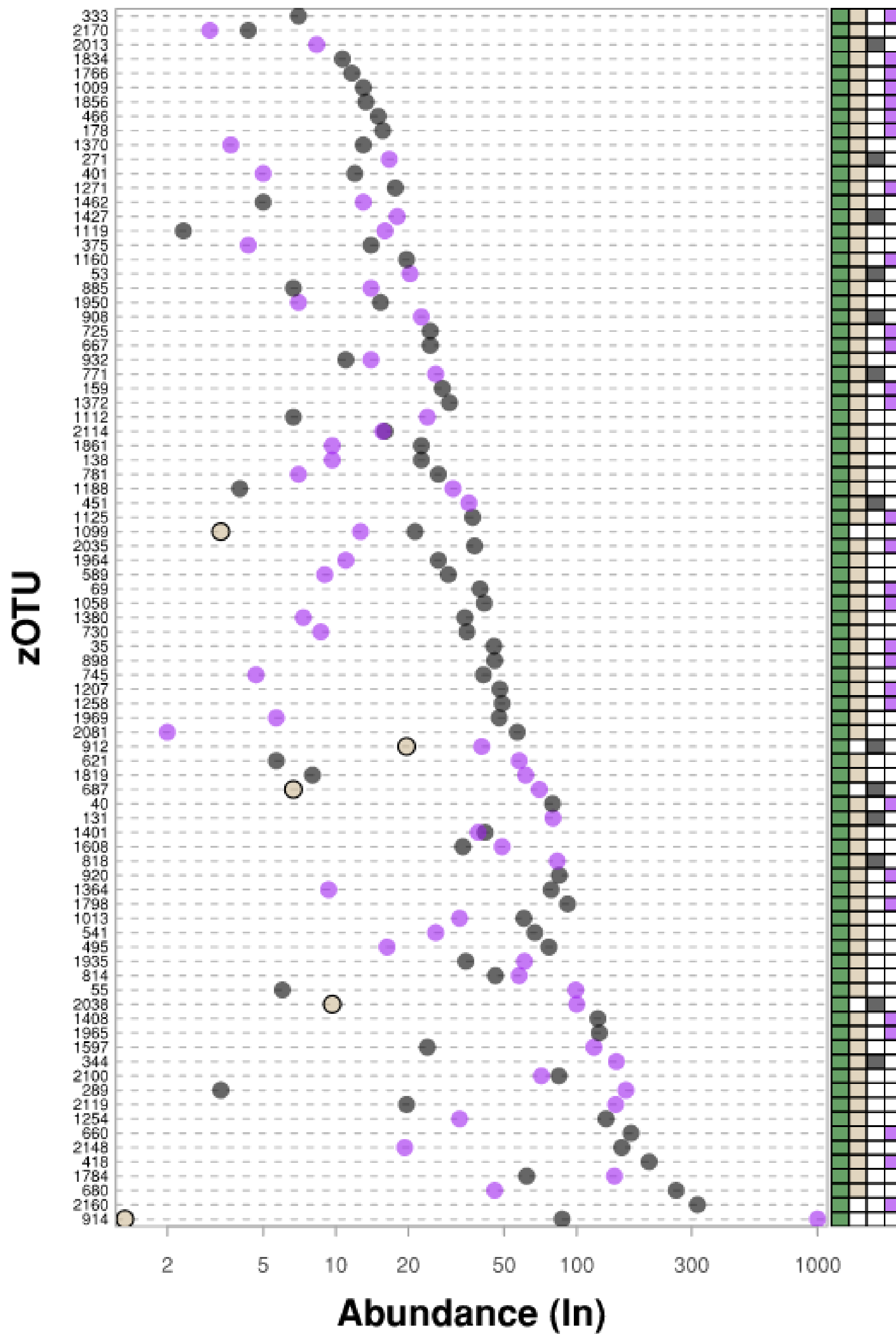
Sample ID	Sequence count *	Sequence count **
Z4G	235,941	12,705
Z5G	274,454	7,698
Z6G	289,852	4,458
Z1W	315,092	308
Z2W	359,233	423
Z3W	315,762	556
Z1P	293,070	11,495
Z2P	255,257	21,565
Z3P	277,230	22,597
Z1B	233,531	60,409
Z2B	252,356	46,073
Z3B	288,058	45,211
Total	**3,389,836**	**233,498**
Minimum	233,531	308
Median	282,644	12,100
Mean	**282,486**	**19,458**
Maximum	359,233	60,409

Z1-Z6: biological replicates
G, green; W, white; P, purple; B, black
* Raw sequencing data
** Filtered non-bacterial sequences



Supplementary Figure 1: Rarefaction curves.

Average alpha diversity values (number of zOTUs) were computed for each sample and plotted as a function of sampling depth. Rarefied tables (n = 10 per sampling depth) were generated at maximum depths of 308 (minimum frequency, **A**), 3,482 (first quartile, **B**), and 12,100 (median frequency, **C**). Samples were grouped by pigmentation category (B, black; P, purple; W, white; G, green) at the chosen rarefaction threshold of 308 sequences per sample (**D**). Z1 - Z6: biological replicates.

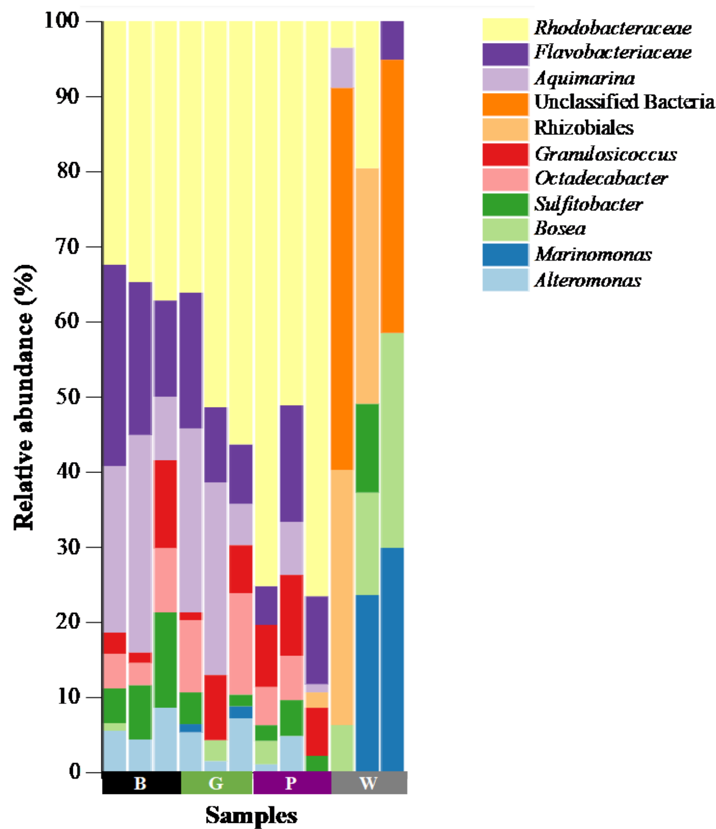


Supplementary Figure 2: Consistent patterns of bacterial occurrence across tissue types (unrarefied data).

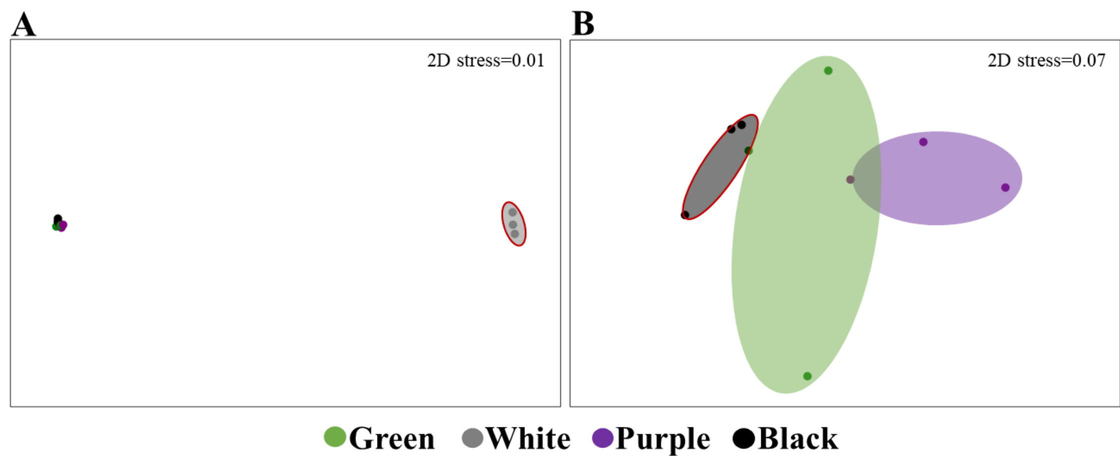
zOTUs absent within green leaves-associated microbiomes that recorded abundances higher than zero in all samples of at least one reddened tissue type are plotted as natural log-transformed means (n = 3 for each tissue type) for a given zOTU. The coloured matrix on the right indicates tissue types were a given zOTU was absent. "Discriminatory" zOTUs were excluded from this analysis.

4.10.2 Comparison between unrarefied and rarefied data

Rarefication is a common normalization technique intended to enable meaningful comparison of sequencing data by standardizing the library size across samples (Brewer and Williamson, 1994, Weiss et al., 2017). `However, it can reduce statistical power and it is not suitable for compositional data like the relative abundance of taxa in a given sample (Weiss et al., 2017). To confirm general trends between the environments, we assessed the extent to which unrarefied and rarefied data sets were comparable by rarefying reads to the minimum depth and performing the same diversity, ordination and statistical analyses for both rarefied and unrarefied data. Both data sets provided equivalent diversity levels for each pigmentation category (**Figure 3** and **Supplementary Figure** 3), as well as comparable clustering patterns (**Figure 4A** and **Figure 4C** and **Supplementary Figure 4**). The overall trends and statistical results were consistent between unrarefied and rarefied data (**Supplementary Table 2** and **Supplementary Table 3**) and showed significant differences between tissue types ($p_{unrarefied}$ = 0.002, $p_{rarefied}$ = 0.001). However, the differences in beta diversity between green leaves and white tissues were significant with unrarefied data (p = 0.045) and just above the significant level when the data was not rarefied (p = 0.067). Altogether, our results from the comparisons between these two data sets, rarefied and unrarefied, demonstrate that these led to the same biological interpretations.



Supplementary Figure 3: Beta diversity across pigmentation types (rarefied data). Beta diversity of bacterial microbiomes associated with black (B), green (G), purple (P) and white (W) seagrass leaf tissues is plotted, with unique zOTUs within each sample coloured by the highest assigned taxonomic level. Rare members of the microbiome were excluded to help remove visual clutter, and thus only representative zOTUs with a relative abundance > 1% in all samples (n = 12) are shown. Sequencing reads were rarefied to the same depth (308 sequences per sample), in order to asses biased effects for differences in sampling effort upon analysis.



Supplementary Figure 4: Bacterial community structure (rarefied data).
Non-parametric multidimensional scaling (nMDS) of bacterial assemblages associated with seagrass green leaves and reddened tissues (i.e., white, purple and black; *n* = 12). The nMDS plot was generated from a lower triangular resemblance calculated with the S17 Bray-Curtis similarity measure from normalized abundances of zOTUs (high values down-weighted with square root). Samples are coloured by pigmentation category and clustering patterns are shown in ellipses, representing the level of similarity between samples based on the degree to which zOTUs are shared between them. The 2D stress is shown in the upper right corner of the nMDS plot (Kruskal stress formula = 1, minimum stress = 0.01). Based on Permutational Multivariate Analysis of Variance (PERMANOVA, Bray-Curtis dissimilarity matrix, simple design), pigmentation categories that are statistically different from all other sample types (α = 0.05) are outlined in red. Clustering patterns of the reddened tissues only (**B**) were generated using the same approach. Sequencing reads were rarefied to the same depth (308 sequences per sample), in order to asses biased effects for differences in sampling effort upon analysis.

Supplementary Table 2: Statistical analyses for multidimensional scaling (unrarefied data).

Differences between bacterial communities across pigmentation categories were tested for statistical significance in Permutational Multivariate Analysis of Variance (PERMANOVA, Bray-Curtis dissimilarity matrix, one-way design).

A) Main tests

factor	P(perm)
ti	0.0002

B) ECV

Source	Estimate	Sq.root
S(ti)	1603.200	40.040
V(Res)	1955.800	**44.225**

C) Pair-wise tests

tissue (P(MC)) i	b	p	w	g
b				
p	0.0368			
w	0.0194	0.0319		
g	0.0538	0.1295	0.0449	

Significant values at the 0.05 level are shown in grey
i Monte Carlo p-values were used when there were not enough possible permutations (<500 out of 9999 permutations, unrestricted permutation method)
ECV: estimates of components of variation
ti, tissue; b, black; p, purple; w, white, g, green

Supplementary Table 3: Statistical analyses for multidimensional scaling (rarefied data).

Differences between bacterial communities across pigmentation categories were tested for statistical significance in Permutational Multivariate Analysis of Variance (PERMANOVA, Bray-Curtis dissimilarity matrix, one-way design). Sequencing reads were rarefied to the same depth (308 sequences per sample) in order to assess the extent to which unrarefied and rarefied data sets were comparable.

A) Main tests

factor	P(perm)
ti	0.0001

B) ECV

Source	Estimate	Sq.root
S(ti)	1116.200	33.410
V(Res)	2289.600	**47.850**

C) Pair-wise tests

tissue (P(MC)) i	b	p	w	g
b				
p	0.1089			
w	0.0411	0.0513		
g	0.2899	0.2324	0.0698	

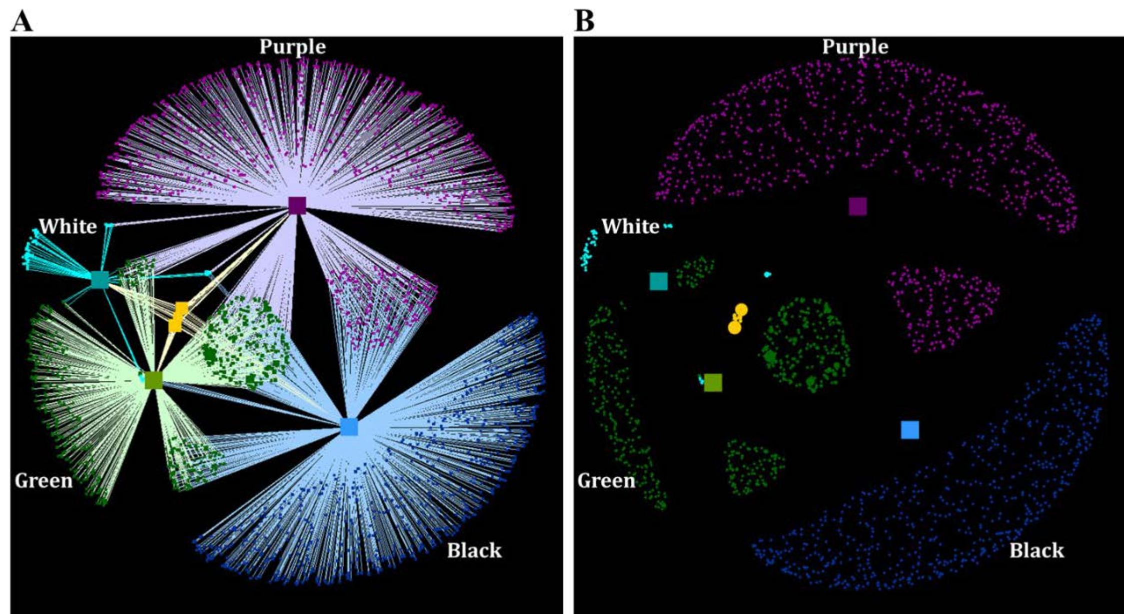
Significant values at the 0.05 level are shown in grey
i Monte Carlo p-values were used when there were not enough possible permutations (<500 out of 9999 permutations, unrestricted permutation method)
ECV: estimates of components of variation
ti, tissue; b, black; p, purple; w, white, g, green

Supplementary Table 4: GLMs analyses.

Discriminatory zOTUs that differed significantly between green leaves and reddened tissues. Given that zOTUs making the differences in mvabund were at zero for the white tissues, this level was removed from the data set for contrast analyses.

zOTU	Taxonomy*	P-value**	Contrast	P-value***	Depletion	Enrichment
otu_164	*Granulosicoccus*	3.10E-03	black - green	1.65E-03		black
			purple - green	7.26E-03		purple
otu_217	*Litoreibacter*	7.00E-04	black - green	1.75E-02		black
			purple - green	7.85E-01		
otu_251	*Rhodobacteraceae*	3.09E-08	black - green	8.26E-08		black
			purple - green	3.60E-01		
otu_269	*Rhodopirellula*	2.49E-12	black - green	1.18E-11		black
			purple - green	1.10E-02		purple
otu_330	*Rhodobacteraceae*	7.37E-12	black - green	7.90E-14		black
			purple - green	7.61E-05		purple
otu_417	*Rhodobacteraceae*	2.21E-05	black - green	6.17E-06		black
			purple - green	5.07E-02		
otu_528	*Rhodobacteraceae*	2.36E-04	black - green	9.79E-05		black
			purple - green	1.70E-01		
otu_791	*Actinobacteria*	2.20E-16	black - green	1.55E-14		black
			purple - green	1.48E-03		purple
otu_1091	*Rhodobacteraceae*	6.27E-03	black - green	8.12E-02		
			purple - green	3.89E-03	purple	
otu_1121	*Granulosicoccus*	8.64E-07	black - green	1.03E-07		black
			purple - green	1.06E-04		purple
otu_1406	*Rhodobacteraceae*	1.88E-03	black - green	1.47E-03		black
			purple - green	5.12E-01		
otu_1453	*Rhodobacteraceae*	3.45E-05	black - green	3.94E-04		black
			purple - green	1.74E-05		purple
otu_1521	*Schleiferia*	3.37E-04	black - green	2.35E-03		black
			purple - green	3.29E-04		purple
otu_1525	*Loktanella*	3.20E-04	black - green	4.37E-04		black
			purple - green	6.44E-01		
otu_1840	*Sulfitobacter*	8.83E-11	black - green	4.84E-10		black
			purple - green	2.54E-01		
otu_2096	*Erythrobacter*	2.20E-16	black - green	1.49E-14		black
			purple - green	2.89E-01		
otu_2110	*Rhodobacteraceae*	1.42E-08	black - green	6.04E-08		black
			purple - green	2.29E-01		
otu_2126	*Octadecabacter*	6.55E-06	black - green	1.04E-04		black
			purple - green	9.93E-01		

Significant values at $\alpha = 0.05$ are shown un grey.
* Highest assigned taxonomic level.
** Individual GLMs, main ANOVA. P-values adjusted by the dunnettx method.
*** Individual GLMs, contrasts. P-values adjusted by the dunnettx method.



Supplementary Figure 5: Bacterial community structure (unrarefied data, rare microbiome included).

Bipartite network of bacterial assemblages associated with seagrass green leaves and reddened tissues (*n* = 12). zOTUs (nodes as small circles) are linked to each pigmentation category that they are associated with (coloured squares) by lines (edges). "Unique" (outer clusters of nodes) and "shared" (inner nodes in the centre) members of microbiomes associated with green leaves (green), and white (aquamarine), purple (magenta) and black (blue) tissues are displayed, with the size of nodes representing zOTUs absolute abundance (range = 1.0 - 12536.0). The same network without edges is also provided (**D**) to highlight the low proportion of zOTUs (1%) that are shared between all four pigmentation categories (yellow nodes).

Supplementary Table 5: Lost core microbiomes.

Core members of the green leaves-associated communities (**A**) not maintained as core zOTUs within black (**B**), purple (**C**) and white (**D**) tissue samples.

A) Green core microbiome

Taxonomy*	# zOTUs**
Actinobacteria	1
Flavobacteriaceae	1
Aquimarina (ambiguous taxa)	1
Croceitalea	1
Nonlabens (uncultured)	1
Winogradskyella (ambiguous taxa)	1
Schleiferia	1
Saprospiraceae	2
Rubidimonas (uncultured)	1
Pleurocapsa (ambiguous taxa)	1
Phormidium	1
Rhodopirellula	1
Hyphomonadaceae (uncultured, ambiguous taxa)	1
Phyllobacteriaceae	2
Pseudahrensia (uncultured)	1
Rhodobacteraceae	12
Litoreibacter (ambiguous taxa)	1
Loktanella	2
Octadecabacter (ambiguous taxa)	1
Sulfitobacter	1
Erythrobacter (ambiguous taxa)	1
Methylotenera	1
Alteromonas	1
Pseudoalteromonas	1
Granulosicoccus	4
Granulosicoccus (uncultured)	1
Total	**43**

B) Lost in black tissue samples

Taxonomy*	# zOTUs**
Croceitalea	1
Schleiferia	1
Saprospiraceae	1
Rubidimonas (uncultured)	1
Phyllobacteriaceae	2
Rhodobacteraceae	4
Methylotenera	1
Granulosicoccus	2
Total	**13**

C) Lost in purple tissue samples

Taxonomy*	# zOTUs**
Croceitalea	1
Nonlabens (uncultured)	1
Saprospiraceae	1
Rubidimonas (uncultured)	1
Pleurocapsa (ambiguous taxa)	1
Phormidium	1
Hyphomonadaceae (uncultured, ambiguous taxa)	1
Phyllobacteriaceae	2
Pseudahrensia (uncultured)	1
Rhodobacteraceae	1
Pseudoalteromonas	1
Granulosicoccus	1
Total	**13**

D) Lost in white samples

Taxonomy*	# zOTUs**
Actinobacteria	1
Flavobacteriaceae	1
Aquimarina (ambiguous taxa)	1
Croceitalea	1
Nonlabens (uncultured)	1
Winogradskyella (ambiguous taxa)	1
Schleiferia	1
Saprospiraceae	2
Rubidimonas (uncultured)	1
Pleurocapsa (ambiguous taxa)	1
Phormidium	1
Rhodopirellula	1
Hyphomonadaceae (uncultured, ambiguous taxa)	1
Phyllobacteriaceae	2
Pseudahrensia (uncultured)	1
Rhodobacteraceae	12
Litoreibacter (ambiguous taxa)	1
Loktanella	2
Octadecabacter (ambiguous taxa)	1
Sulfitobacter	1
Erythrobacter (ambiguous taxa)	1
Methylotenera	1
Alteromonas	1
Pseudoalteromonas	1
Granulosicoccus	4
Granulosicoccus (uncultured)	1
Total	**43**

* Highest-resolution taxonomic assignment
** Pooled zOTUs with the same taxonomic assignment

Supplementary Table 6: Retained core microbiomes.

Core members of the green leaves-associated communities (**A**) also consistently maintained as core zOTUs across all black (**B**) and purple (**C**) leaf tissue samples.

A) Green core microbiome

Taxonomy*	# zOTUs**
Actinobacteria	1
Flavobacteriaceae	1
Aquimarina (ambiguous taxa)	1
Croceitalea	1
Nonlabens (uncultured)	1
Winogradskyella (ambiguous taxa)	1
Schleiferia	1
Saprospiraceae	2
Rubidimonas (uncultured)	1
Pleurocapsa (ambiguous taxa)	1
Phormidium	1
Rhodopirellula	1
Hyphomonadaceae (uncultured, ambiguous taxa)	1
Phyllobacteriaceae	2
Pseudahrensia (uncultured)	1
Rhodobacteraceae	12
Litoreibacter (ambiguous taxa)	1
Loktanella	2
Octadecabacter (ambiguous taxa)	1
Sulfitobacter	1
Erythrobacter (ambiguous taxa)	1
Methylotenera	1
Alteromonas	1
Pseudoalteromonas	1
Granulosicoccus	4
Granulosicoccus (uncultured)	1
Total	**43**

B) Retained in all black tissue samples

Taxonomy*	# zOTUs**
Actinobacteria	1
Flavobacteriaceae	1
Aquimarina (ambiguous taxa)	1
Nonlabens (uncultured)	1
Winogradskyella (ambiguous taxa)	1
Saprospiraceae	1
Pleurocapsa (ambiguous taxa)	1
Phormidium	1
Rhodopirellula	1
Hyphomonadaceae (uncultured, ambiguous taxa)	1
Pseudahrensia (uncultured)	1
Rhodobacteraceae	8
Litoreibacter (ambiguous taxa)	1
Loktanella	2
Octadecabacter (ambiguous taxa)	1
Sulfitobacter	1
Erythrobacter (ambiguous taxa)	1
Alteromonas	1
Pseudoalteromonas	1
Granulosicoccus	2
Granulosicoccus (uncultured)	1
Total	**30**

C) Retained in all purple tissue samples

Taxonomy*	# zOTUs**
Actinobacteria	1
Flavobacteriaceae	1
Aquimarina (ambiguous taxa)	1
Winogradskyella (ambiguous taxa)	1
Schleiferia	1
Saprospiraceae	1
Rhodopirellula	1
Rhodobacteraceae	11
Litoreibacter (ambiguous taxa)	1
Loktanella	2
Octadecabacter (ambiguous taxa)	1
Sulfitobacter	1
Erythrobacter (ambiguous taxa)	1
Methylotenera	1
Alteromonas	1
Granulosicoccus	3
Granulosicoccus (uncultured)	1
Total	**30**

* Highest-resolution taxonomic assignment
** Pooled zOTUs with the same taxonomic assignment

5 CHAPTER 5

Synopsis of results and general discussion

5.1 Summary

Providing several valuable ecosystem services (Costanza et al., 2014, Nordlund et al., 2016), seagrass meadows are recognised as one of the most productive ecosystems in the coastal zone (Deyanova et al., N.A.). However, seagrass declines of up to 40% have been recorded in recent years (Evans et al., 2018), due to the synergistic impacts of multiple poorly understood stressors (Unsworth et al., 2015, Grech et al., 2011), including climate change (Duarte et al., 2018) and seagrass syndromes or diseases (Sullivan et al., 2013, Unsworth et al., 2015). The research presented in this thesis was motivated by our limited understanding of the role of the seagrass microbiome in seagrass health and sustainability, and was designed to address questions about the main drivers of microbial community dynamics that could ultimately influence the health, fitness and overall function of seagrasses.

My work demonstrates that the seagrass microbiome is diverse, complex and very dynamic, and that it responds to both environmental conditions and intrinsic characteristics of the seagrass host (**Figure 1**). These features are consistent with those found in analogous marine holobionts such as corals (Bourne et al., 2016), sponges (Schmitt et al., 2012) and seaweeds (van der Loos et al., 2019), and are predicted to affect the delicate balance of the interactions between the organisms involved, and the ecological and biogeochemical processes that they mediate. Within this final chapter, the results from each experimental chapter are addressed within the context of three research questions that are in line with the thesis specific aims detailed in Chapter 1. The significance and implications of the thesis findings and future directions of the research related to the contributions of this thesis are also proposed.

5.2 Synthesis of results

5.2.1 Is the structure of the seagrass microbiome retained over biogeographical scales?

There is evidence from a wide range of marine organisms that host-associated microbiomes often display a high level of species-specificity and spatial and temporal conservation (Aires et al., 2016, Grossart et al., 2005, Hardoim and Costa, 2014, Lima et al.,

2012, Neu et al., 2019, Reveillaud et al., 2014) - that is, the same, or similar, microbes inhabit the microbiomes of hosts regardless of time or place of sampling. However, there are also microbiomes that are more strongly influenced by the local environmental conditions and consequently show biogeographic variation (Kellogg et al., 2017, Luter et al., 2015, Meiser et al., 2014, Neu et al., 2019, Rubio-Portillo et al., 2018, Taylor et al., 2005, Roder et al., 2015). In Chapter 2, I demonstrated that the microbiome associated with different parts of the seagrass is conserved between environments separated by up to 52 km (i.e. largest distance between sampling locations). Interestingly, the extent of this regional conservation differed among the three microbial taxa studied here, with microalgal and fungal members of the microbiome demonstrating higher inter-site variability, perhaps indicative of a weaker ecological coupling with the seagrass host. Notably, similar patterns, whereby host-associated fungal communities display greater biogeographical heterogeneity than bacterial members of the microbiome have been observed in terrestrial plants (Coleman-Derr et al., 2016, Shakya et al., 2013, Talbot et al., 2014).

Several bacterial 'core microbiome' members were consistently associated with specific seagrass microenvironments across disparate locations spanning 86 km of coastline, indicating the maintenance of specific associations between the seagrass and these microorganisms irrespective of differing conditions in the external environment. In Chapters 3 and 4, I further demonstrated this spatial conservation of the seagrass leaf microbiome, but showed that disturbances such as Seagrass Wasting Disease and leaf reddening can disrupt the structure of the microbiome, indicating a fundamental shift in the physiological state of the seagrass and its associations with its microbiota. This demonstrates that the interplay between multiple environmental and host-associated factors shapes the community composition of the seagrass microbiome. The results of this thesis are consistent with recent studies in switchgrasses (Singer et al., 2019), seaweeds (Aires et al., 2016, Marzinelli et al., 2015) and marine invertebrates (Lo Giudice et al., 2019, Menezes et al., 2010, Taylor et al., 2004, Yarden, 2014) that similarly show that differences in bacterial, archaeal and fungal community composition and or diversity are strongly influenced by both, the external environment and the host, and potentially highlight common principles underpinning seagrass-microbe interactions. Factors considered in these and related investigations include, for instance, the specific compartment, genotype or ecotype (Aires et al., 2016, Menezes et al., 2010, Singer et al., 2019), health status (Marzinelli et al., 2015) and vigour of the host (Yarden, 2014); the origin of its associated microbes and the presence of other resident or invasive

microorganisms in the host (Yarden, 2014); water physicochemical properties (Taylor et al., 2004, Yarden, 2014) and anthropogenic pollution (Aires et al., 2016, Marzinelli et al., 2015). Here, I provide baseline information for future functional approaches to interrogate the maintenance of seagrass-microbe interactions at different scales.

5.2.2 Does the seagrass microbiome vary at the plant scale?

While I observed conservation of the seagrass microbiome across broad regional scales, in contrast, I observed marked microbial shifts across different microenvironments associated with an individual seagrass-these including both seagrass tissues and the external environment. This pattern is consistent with the proposition that different processes influence bacterial community assembly in different seagrass compartments (Fahimipour et al., 2017) as a consequence of very heterogeneous microenvironmental conditions. In Chapter 2, I observed distinct bacterial, microalgal and fungal populations across different compartments within the seagrass host (i.e. upper and lower leaf, sheath, and roots and rhizomes) and its surrounding microenvironments (i.e. sediment and seawater), even though the assemblages associated with the three fractions leaf were generally similar. Notably, these patterns were generally conserved across disparate geographic locations. This small-scale spatial heterogeneity is typical of both, the phyllosphere and rhizosphere, given varying levels of photosynthesis, oxygen and the diffusive exchange of organic substrates along the leaf (Hogarth, 2015, Larkum et al., 2007, Rubio et al., 2017, Borum et al., 2006), and a range of chemical exchanges with microorganisms through the exudation of dissolved organic material from the roots and rhizomes (Badri and Vivanco, 2009, Hemminga et al., 1991, Hogarth, 2015, Koren et al., 2015, Kuzhiumparambil et al., 2017, Devereux, 2013). I observed that, as a consequence, aerobic heterotrophs dominate the phyllosphere where they exploit the oxic conditions and high levels of dissolved organic carbon on the leaf surface (Hirano and Upper, 2000, Jorgensen et al., 2009), whereas sulphate reducing and sulphur oxidising bacteria inhabit the rhizosphere due to high levels of organic material and anoxic conditions characteristic of these microenvironments, where these microorganisms likely regulate the carbon and sulphur cycling processes (Kleindienst et al., 2014, Lehnen et al., 2016, Storelli et al., 2013, Thomas et al., 2014, Varon-Lopez et al., 2014). I also found that substantial microbial shifts occur in response to changing microenvironmental conditions in the host due to two specific circumstances: disease (Chapter 3) and physiological responses to environmental stress (Chapter 4). I observed that changes in microbial community composition from healthy to diseased-associated seagrass tissues were linked to a predicted functional shift from heterotrophy to autotrophy (Chapter 3). Functional changes of this kind are typical

of microbial imbalance and might be indicative of an increasing predominance of potentially harmful bacteria (Egan and Gardiner, 2016, Littman et al., 2011).

I also found drastic changes of the microbiome associated with normal green leaves when exhibiting red coloration (Chapter 4). As an environmentally-driven physiological response (Novak and Short, 2011b), seagrass leaf reddening represents a suitable model to assess if and to what extent inherent characteristics of the seagrass (e.g. leaf pigmentation) influence its associated microbiota, given that further morphological, physiological and chemical changes are concomitant with the reddening. My observations of significantly lower levels of bacterial diversity and the loss of potentially beneficial associates are indicative of the role of seagrass physiology, driven by environmental processes, in structuring microbial communities in the phyllosphere.

Studying microscale distributions of the microbial assemblage across different seagrass tissues (i.e. healthy vs. diseased plants; green leaves vs. reddened tissues) provided clear insights into the factors influencing microbial diversity and community structure at the plant-scale. This scale is presumably more relevant than larger scenarios in the ocean, because it is at the level of centimetres along an individual leaf that substantial shifts take place to favour (or not) microbes with putative functional relevance for the entire seagrass holobiont. For instance, the transitional communities observed in tissues adjacent to active SWD lesions may not have been evident if the entire leaf, that showed symptoms of disease, had been sampled. The results described in Chapters 2, 3 and 4 emphasised that microscale heterogeneity is a prominent feature of the seagrass microbiome, and that the plant host is mainly, but not solely, responsible for this variation.

5.2.3 How does disease or shifts in seagrass physiology alter the structure of the seagrass microbiome?

Despite extensive research to determine the specific causes of SWD (Graham et al., 2018b, Hughes et al., 2018, Jakobsson-Thor et al., 2018, Nienhuis, 1994, Trevathan-Tackett et al., 2018), there is still uncertainty about the mechanisms of disease and the potential involvement of opportunistic colonising microorganisms in disease onset. The results described in Chapter 3 revealed that pronounced changes in the seagrass microbiome take place under the specific microenvironmental conditions associated with SWD. These patterns were most clearly manifest in the substitution of healthy tissue-associated *Burkholderia* OTUs by OTUs classified as members of the *Arenicella* genus within microbiomes from SWD lesions, which may have substantial implications for seagrass

metabolism and health due to the roles that these microorganisms play in Nitrogen cycling (Elliott et al., 2007, Govindarajan et al., 2008) and disease (Feinman et al., 2017, Whitten et al., 2014), respectively.
There are several examples of host-associated microbiome shifts within the context of marine diseases (King et al., 2019, Lloyd and Pespeni, 2018, Mohammed and Arias, 2015, Roder et al., 2014b, Webster et al., 2008, Sunagawa et al., 2009), and most of these studies highlight that such severe changes result in disease, not necessarily because of a single etiological agent, but instead due to the cumulative and/or synergistic effect of multiple opportunistic microorganisms. On the other hand, shifts in microbiome composition also often occur as a consequence of disease, rather than the cause (Meyer et al., 2014, Roder et al., 2014b, Sunagawa et al., 2009). Both of these different scenarios can be described within the context of microbial imbalance generally known as 'dysbiosis' (Egan and Gardiner, 2016, Olesen and Alm, 2016, Petersen and Round, 2014), although it is often difficult to establish whether its correlation with marine diseases represents causality. Hence, rather than being indicative of other putative causal agents different from *L. zosterae*, the dominance of potentially pathogenic microbes, such as *Arenicella* described in Chapters 3, could alternatively be a direct consequence of colonisation by opportunistic microbes, supported by changing microenvironmental conditions, rather than "infection" by these organisms.

Another community feature indicative of dysbiosis as a consequence of disease, or physiological changes in the host, were changes in alpha diversity. The increased bacterial diversity and richness observed within disease-associated leaf tissues, relative to healthy leaves (Chapter 3) supports the proliferation of a diverse collection of opportunistic microbes, rather than the predominance of a single etiological agent. These patterns have been observed in other marine diseases in sponges (Angermeier et al., 2011, Webster et al., 2008) and corals (Cooney et al., 2002, Pantos and Bythell, 2006, Sunagawa et al., 2009), and indicate that the occurrence of microbial imbalances in *Z. muelleri* might follow, rather than precede or accompany, an initial infection by *L. zosterae*.

Conversely to the changes in alpha diversity observed in seagrass tissues exhibiting signs of SWD, the results described in Chapter 4 demonstrate that abnormal colouration patterns can both increase and decrease bacterial diversity levels, whereby a substantially higher microbial richness occurs within the tissues where anthocyanins are accumulated, while significantly lower diversity occurs within the tissues not exhibiting red pigmentation. This could hypothetically suggest that physiological changes in the

phyllosphere trigger microbial diversity changes by the provision of secondary metabolites for nutrient acquisition to the reddened tissues-associated microbiomes, or the lack of such nutritional offerings to the communities associated with non-coloured tissues.

Conclusively, the absence of clear evidence for the involvement of bacterial pathogens in the development of SWD is not supportive of a bacterial-mediated infection. Therefore, I propose that substantial shifts observed in disease-associated microbiomes (Chapter 3) might result from the opportunistic colonisation by bacteria after infection by *L. zosterae*, and are indicative of a dysbiotic effect concomitant to substantial changes in the infected host that may have important implications for seagrass metabolism. Similarly, the drastic shifts I observed between different leaf pigmentation categories in Chapter 4 demonstrate that other physiological changes (simultaneously occurring in leaves with high contents of anthocyanins) in the host, can alter the structure of seagrass phyllosphere microbiome.

5.3 Relevance of findings: ecological and biochemical perspectives

The distinct nature of microbial assemblages associated with different seagrass tissue types or geographical locations is likely governed by multiple factors, including environmental parameters (Chapter 2), the health status of the plant (Chapter 3) and its physiological responses to environmental stressors (Chapter 4). Patterns of microbial heterogeneity at both regional- and plant-scales could have important consequences for the way that different microbial populations interact with each other and with the host, which ultimately could impact seagrass metabolism and global biochemical processes mediated by the entire holobiont. Co-occurrence networks have been broadly used to examine putative ecological interactions between microorganisms and the influence of the environment on coexistence within biological communities (Williams et al., 2014). I used this approach to characterise the level of specificity of the microbes associated with different leaf tissue types, and showed that compositional changes of the microbiome, such as the ones persistently observed in this thesis, led to high proportions of unique microbial associations (Chapter 3). Notably, these patterns are strongly influenced by the most abundant microorganisms within the phyllosphere, which play a more important role in discriminating different leaf tissues (Chapter 4).

Altered microbial profiles with detrimental functions to the host have been increasingly reported in a number of macroorganisms, ranging from humans (Sobhani et al., 2011) to corals (Littman et al., 2011) and sponges (Fan et al., 2013), highlighting that host-microbe

relationships are critical for the health, fitness, adaptability and overall function of any holobiont. In seagrasses, most recent research has focused on elucidating where, when and how these changes happen purely from taxonomic profiles (Bengtsson et al., 2017, Cucio et al., 2016, Ettinger et al., 2017, Fahimipour et al., 2017, Ugarelli et al., 2019), whereas formal functional approaches (Crump et al., 2018, Cucio et al., 2018, Wojahn, 2016, Fraser et al., 2018) are still limited. The results of this thesis constitute a deep, comprehensive characterisation of the seagrass *Z. muelleri* microbiome based on the identity of its associated microbiota. Although non-empirical data is provided to assess the functional roles of these microorganisms, their functional capacity was inferred from the literature and by sequence-homology. This allowed me to determine putative functional roles for the most predominant and persistent members within the communities studied. Among those, the involvement of several microorganisms in nutrient cycling processes, especially in the Nitrogen, Sulphur and Carbon cycles was clearly evident. Consequently, microbial variability at both the regional- and plant-scales, with expected metabolic shifts such as those predicted in SWD-associated microbiomes (Chapter 3), could influence biogeochemical transformations in the ocean, with implications at the global-scale.

5.4 Implications of microbiome changes for seagrass-based ecosystems

The research conducted in this thesis supports the proposition that the seagrass microbiome is very important to the host, since several microorganisms are retained by the plant despite the differing environmental conditions offered by disparate locations within the same region (Chapters 2 and 3). Moreover, the occurrence of distinct microbial communities within different microenvironments in a single plant implies a highly specialised coupling between the seagrass host and its microbiome (Chapter 2). These tight and important relationships are, however, subject to change when the plant experiences disease (Chapter 3) or physiological changes (Chapter 4), with potentially negative, but as yet undefined implications due to the loss of the healthy seagrass-microbiome associations. Several of the functions that these microorganisms are believed to play within seagrass ecosystems might be altered or even lost (e.g. heterotrophy in SWD) during the development of disease/reddening. However, given the entirely predictive nature of the functional approaches used in this thesis (Section 5.5), only potential implications of taxonomic changes in the microbiome can be inferred from this work.

Taxonomic and/or (putative) functional changes might ultimately influence seagrass-based ecosystems by altering marine macroorganisms distributions (Hovel and Lipcius, 2001), or increasing risk of disease (Lamb et al., 2017), and disturbing global nutrient cycling processes (Fourqurean et al., 2012, Lavery et al., 2013). Given that these habitat-forming plants perform numerous ecological functions in coastal environments (Cullen-Unsworth et al., 2014, Hemminga and Duarte, 2000, Larkum et al., 2018, Zieman et al., 1984) and provide several valuable ecosystem services (Campagne et al., 2015, Costanza et al., 2014, Cullen-Unsworth and Unsworth, 2013, Dewsbury et al., 2016, Nordlund et al., 2018, Nordlund et al., 2016, Unsworth et al., 2019)shifts in the microbiome with detrimental effects to the host may represent triggers for large-scale phenomena.

5.5 Future directions

Here, I provided a comprehensive characterisation of patterns in the taxonomic composition of the *Z. muelleri* microbiome. However, these patterns do not always directly reflect functional characteristics of a microbial community (Fuhrman et al., 2015), and therefore future research using "omic" approaches will shed more light on how the spatial shifts in seagrass microbiome structure affect the functional capacity of the microbiome and the resultant metabolic and biogeochemical connections with the host. Shot gun metatranscriptomics, for example, could be used in future studies to assess the existence of "functional cores" within the seagrass-associated microbiomes, especially in microenvironments like the phyllosphere, where stochastic microbial colonisation is very likely. Functional profiling of microbial communities has been used in several marine hosts (Dinsdale et al., 2008, Fan et al., 2013, Kimes et al., 2010, Pfister et al., 2010), including seagrasses (Crump et al., 2018, Cucio et al., 2018, Wojahn, 2016, Fraser et al., 2018), and although not entirely predictive, most of these genome-based methodologies should be validated by functional assays that test each gene product or protein separately. Alternatively, a time-series approach could complement this more reductionist single gene/protein validation (Fuhrman et al., 2015).

The results presented in this thesis represent discrete snapshots in time that lack historical information about the prior conditions of the (micro) environment, and this should be considered when using this information to infer the drivers of microbiome structure.

5.6 Conclusions

This thesis provides robust evidence that, like many other benthic organisms, seagrasses develop dynamic and potentially ecologically important relationships with microorganisms. My research demonstrates synergistic impacts of ecosystem-level drivers and intrinsic characteristics of the seagrass host on microbiome structure, which operate across a continuum of spatial scales - from plant microenvironment to the geographical scale. However, it is notable that, particularly among the bacterial compartment of the microbiome, key elements of the microbiome (e.g. the core microbiome) are conserved over regional scales (Chapter 2). This work has also demonstrated that shifts in the physiology and health (i.e. impact of SWD) of the seagrass host govern microbiome structure. As observed in similar terrestrial (Clemmensen et al., 2015, Shen et al., 2018) and marine (Lloyd and Pespeni, 2018, Meyer et al., 2016, Mohammed and Arias, 2015, Webster et al., 2008) systems, these microbial shifts have potentially negative, but as yet undefined implications for the seagrass host. The work presented in this thesis has added to the growing field of host-microbiome research by providing a comprehensive characterisation of microbial communities associated with one of the most dominant meadow-forming species in Australia, at two different spatial scales, and within the context of two physiological conditions of the plant. By demonstrating apparently strong ecological coupling between *Z. muelleri* and its microbiome, the findings presented here have relevance for the ecology of seagrass-based coastal ecosystems.

5.7 General schematic

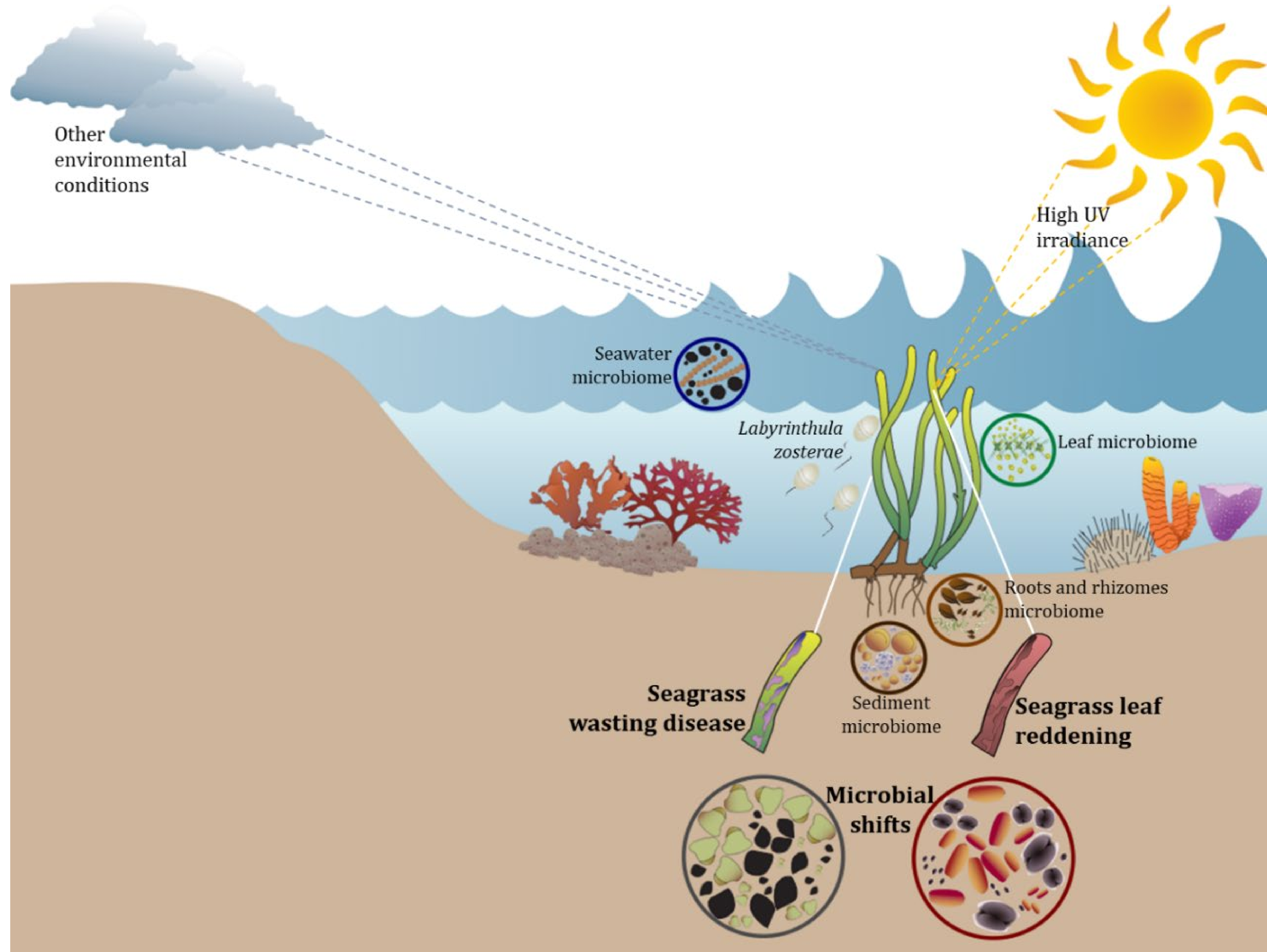


Figure 1: The *Zostera muelleri* seagrass microbiome responds to its host and the environment.

Different microbial assemblages associate with disparate microenvironments within the seagrass host and its surroundings, including the leaf, roots and rhizomes, surficial sediment and adjacent seawater. Even though *Z. muelleri* associates are also influenced by the particular conditions of the external environment, this microenvironmental partitioning is conserved at larger spatial scales. Therefore, intrinsic features of the plant host are major drivers of microbial community composition and structure. Substantial shifts within the seagrass associated microbiota are concomitant with physiological changes in the host resulting from disease or in response to environmental changes and might disturb the ecological balance of host-microbe interactions that are potentially critical for seagrass-based ecosystems.

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