Dynamics of refractory carbon in seagrass meadows

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Certificate of Original Authorship

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Preface

This thesis has been prepared in publication format, whereby each chapter represents a manuscript ready for submission to a peer-reviewed journal. Therefore, there will be a degree of redundancy across chapter introductions and methodologies. As of yet, no individual chapter has been accepted for publication in a peer-reviewed journal. Therefore, the citations and manuscript follow ESA's Ecology Journal formatting for research articles, with the exception of Chapter 4, which is prepared as a note (results and discussion are combined).

Two published papers were produced in association with my PhD, but are not a part of this thesis. These two articles, which are sited within the thesis chapters, are attached as appendices at the end of the thesis.

> Trevathan-Tackett, S.M, P.I. Macreadie, P. Ralph, and J. Seymour. 2014. Detachment and flow cytometric quantification of seagrass-associated bacteria. Journal of Microbiological Methods **102**:23-25.
>
> Trevathan-Tackett, S. M., J.J. Kelleway, P.I. Macreadie, J. Beardall, P. Ralph and A. Bellgrove. 2015 (2015). Comparison of marine macrophytes for their contributions to blue carbon sequestration. Ecology **96**:3043-3057.

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Abstract

The protection and rehabilitation of natural landscapes in order to enhance their role in carbon sequestration is currently a hot topic for scientists and policymakers looking for solutions to reduce atmospheric CO_2 levels. Blue carbon ecosystems (seagrass, mangrove, saltmarsh) have recently been found to match or even exceed the capability of terrestrial ecosystems to sequester carbon. In seagrass habitats, seagrass carbon alone can account for half of the carbon in the top 10 cm of sediment. Litter quality, often measured as refractory carbon content, is one of the main factors that can influence the sequestration and storage of refractory carbon. Yet to-date, there has been little attempt to understand what factors help or hinder refractory carbon preservation in seagrass sediments.

The aim of this thesis was to unravel the processes and factors that influence, and even optimise, the preservation of refractory carbon in seagrass meadows beginning with the refractory carbon content in seagrass tissues, its persistence (or remineralisation) during decomposition and finally, its preservation in sediments and the mechanisms that provoke further remineralisation after burial. To accomplish these aims, a multi-variable approach was taken, which involved assessing the main and interaction effects of biological, chemical and environmental/physical variables on refractory carbon remineralisation and storage.

The results from this thesis revealed that the processes that affect refractory carbon dynamics in seagrass meadows are complex. It was shown that, while inherent refractory carbon content in the tissues can promote sequestration, decomposition was a strong influence on the persistence of refractory carbon. Anoxic conditions and structural complexity of the tissues promoted refractory carbon preservation and were dependent on the microbial communities present. Sheath and stem tissues were considered to be important carbon contributors due to their high refractory carbon content and chance of *in situ* burial. Temperature and the availability of labile organic matter and inorganic nutrients enhanced decay in the short-term under oxic conditions, while physical disturbance and habitat loss caused losses of sediment refractory carbon over the course of months to years depending on the type of disturbance.

In light of these results, a new conceptual model was developed for seagrass decomposition and have highlighted several important avenues of future blue carbon research, including the functional roles of microbes (bacteria, fungi and protists) in carbon remineralisation via bioinformatics and enzymes kinetics, as well as the conversion, or ‘up-cycling’, of labile carbon to refractory carbon within microbial biomass.

Thesis Introduction

Carbon sequestration and blue carbon habitats

Carbon dioxide (CO_2) production from fossil fuels and other industrial processes account for nearly 85% of greenhouse gases (GHG) emitted into the atmosphere (IPCC 2014). As a result of continued GHG emissions, ocean temperatures have been predicted to increase 0.6-2.0°C in the top 100 m by the end of this century, affecting both ocean biology and biogeochemistry (IPCC 2013, Wong et al. 2014). With CO_2 levels projected to reach 1400-3300 ppm CO_2 by 2100, scientists and policymakers are looking to solutions beyond emission reductions to mitigate the effects of GHGs on our climate (IPCC 2013). Some small-scale solutions have been proposed like the conversion of dead plant material into a more stable form of carbon via biocharing (i.e., pyrolysis of biomass in the absence of oxygen to ~550°C), thus preventing microbial remineralisation of carbon into CO_2 (Lehmann 2007). Larger-scale solutions are more popular, and include geoengineering methods, such as solar radiation management and subsurface CO_2 injection (Herzog et al. 2000, Vaughan and Lenton 2011), as well as ocean iron fertilisation to promote draw-down of CO_2 by photosynthesis (Blain et al. 2007). A more global approach to combating CO_2 emissions has been the protection and rehabilitation of natural landscapes in order to enhance their role in carbon (C) biosequestration (or sequestration). Terrestrial landscapes naturally remove CO_2 from the atmosphere through photosynthesis, and fix the C into their tissues (Herzog et al. 2000). To incentivise countries to use sequestration as a form of emission reductions, programs like REDD+ have been put in place to promote the reforestation, reduced deforestation and forest management of these ecosystems.

Coastal ecosystems have recently been found to match or even exceed the sequestration capability of terrestrial ecosystems (Laffoley and Grimsditch 2009). These ecosystems, including mangrove, saltmarsh and seagrass habitats, were named by the International Union for Conservation of Nature (IUCN) and partners as ‘blue carbon’ ecosystems (Laffoley and Grimsditch 2009). While the process of sequestration is similar for blue carbon ecosystems as it is for terrestrial or ‘green carbon’ ecosystems, there are a couple characteristics that set these coastal C-sequestering biomes apart. First, more than half of the C stores in terrestrial systems are locked up in living biomass (leaves, wood, roots), while the a significant portion of the C stored in blue

carbon systems, especially seagrass meadows, are in the sediments (Laffoley and Grimsditch 2009, Fourqurean et al. 2012). Sediment organic carbon from now on will be referred to as C_{org} to differentiate from inorganic carbon (C_{inorg}). The significant sediment C_{org} accumulation in blue carbon sediments is attributed to the particle-trapping characteristics of these macrophytes (Duarte et al. 2013b), and is one reason blue carbon habitats accumulate C_{org} ~40 times faster than terrestrial systems (McLeod et al. 2011). Secondly, blue carbon habitats are periodically or permanently inundated with seawater, which results in primarily anoxic sediments. Even though freshwater sediments are also anoxic, they are significant emitters of methane (Bastviken et al. 2011), a potent greenhouse gas. Conversely, coastal sediments are primarily anoxic sulphate-reducing systems, which have less microbial C_{org} remineralisation rates via methanogenesis (Mateo et al. 2006, Laffoley and Grimsditch 2009).

For seagrass habitats, living C stored globally within plants makes up a small fraction of the C_{org} pool due to their relatively low biomass, limited global habitat cover and high turn-over rate for some species (Mateo et al. 2006, Fourqurean et al. 2012). However, seagrass biomass is an important donor of C_{org} as they account for ~50% of the sediment C_{org} in the top 10 cm (Kennedy et al. 2010). This accumulation is linked to the enhanced preservation from anoxic conditions, as well as their low palatability to decomposers and the high proportion of refractory C compounds (e.g., lignin) relative to other marine macrophytes (Enríquez et al. 1993, Mateo et al. 2006). The remaining 50% of C_{org} in seagrass sediments comes from non-seagrass or allochthonous (outside C sources), such as terrestrial, mangrove or algal C (Kennedy et al. 2010). These allochthonous accumulations of C in seagrass habitats are enhanced by sedimentary inputs from land as well as the reduction in water flow and trapping of C provided by the leaf canopy (Duarte et al. 2013b).

While these characteristics of seagrass habitats enhance C_{org} sequestration, there are still gaps in our knowledge concerning other factors influencing C_{org} sequestration in blue carbon ecosystems. First, does the C_{org} sequestration capacity of seagrass habitats differ among different species (Macreadie et al. 2014a)? A recent survey noted an 18-fold difference is C_{org} stocks per m^2 across different species and habitat types (Lavery et al. 2013a). The question of what factors contribute to this variation still remains (e.g., plant refractory C, canopy structure, production rates and biomass; Macreadie et al. 2014a, Duarte et al. 2013b). Secondly, how are C_{org} stocks affected after habitat loss or

degradation? Seagrass habitat loss is occurring at 7% yr^{-1} (Waycott et al. 2009), and much of this is attributed to anthropogenic activities such as pollution and physical disturbance (Orth et al. 2006). Some research has assumed that seagrass loss will lead to the remineralisation of sediment C_{org} back into CO_2 up to 1 m depth (Fourqurean et al. 2012, Pendleton et al. 2012). However, these calculations are estimates based on lack of information on the magnitude of or mechanisms leading to CO_2 release after habitat loss (Macreadie et al. 2014a). Lastly, what role does refractory C play in C_{org} sequestration in seagrass meadows? Interestingly, refractory C, i.e., the C_{org} resistant to remineralisation into CO_2, has often been cited a primary attribute that enhances C_{org} sequestration in blue carbon habitats (Cebrián 1999, Mateo et al. 2006, Duarte et al. 2013a), yet to-date, there has been little attempt to understand what factors help or hinder refractory C_{org} preservation in blue carbon sediments (Macreadie et al. 2014a). For this reason, my thesis will address these unknowns with the aim to further our understanding of the factors that influence C_{org} sequestration in seagrass ecosystems.

Defining refractory carbon

First, refractory C should be defined, both in terms of cross-disciplinary classifications, as well as the use of the term within the context of this thesis. Defining organic carbon or organic matter[1] as labile, recalcitrant or refractory is relative and varies depending on the source or conditions in which it is found (Krull et al. 2003).

Plant macromolecules are often categorised as either labile or refractory based on how rapidly microbes are able to break them down, with refractory C being the form less easily remineralised by microbes (Benner et al. 1986). Labile compounds include soluble or non-structural carbohydrates like sugars and starches as well as proteins (Krull et al. 2003). The smaller sugar monomers, however, can also act as the precursors to the more refractory structural carbohydrates like hemicellulose and cellulose (Brett and Waldron 1996). The resulting cellulose in the cell wall forms into parallel microfibrils of β-1,4 glucan chains linked by hydrogen bonds that give it strength and rigidity within the plant (Brett and Waldron 1996). Lastly, lignin is considered the most

[1] Organic matter vs organic carbon: organic matter includes all parts of a particle inclusive of nitrogen-, sulphur- and phosphorus-containing compounds. In some cases, organic matter will be use instead of organic carbon when carbon content is not directly measured by an analysis. However, it is assumed that the organic matter is carbon-based and is associated with the organic carbon characteristics.

refractory form of plant carbon compounds. It is constructed from a combination of three phenolic acids, each made up of an aromatic hydrocarbon and at least one hydroxyl group (i.e., phenol) and forms in irregular patterns within the cell wall, i.e., lignocellulose (Brett and Waldron 1996). In the resulting plant cell wall, hemicellulose and lignin envelop the cellulose microfibrils adding structural support, as well as protection from microbial attack (Brett and Waldron 1996). Seagrasses benefitted from reduced lignin content in living underwater to cope with buoyancy and wave action during their evolution from xenophytes or aquatic macrophytes (Klap et al. 2000, Waycott et al. 2006). However, some lignin structures have been retained for lacunae and below-ground support or as a microbial barrier (Klap et al. 2000). Additionally, seagrasses are also high in phenolic (aromatic) compounds, which, in addition to acting as links between structural carbohydrate fibres (Harrison 1989), can serve various functions such as pathogen defence and herbivory deterrents (Vergeer and Develi 1997, Jensen et al. 1998, Arnold and Targett 2002).

Refractory C has also been defined as the C that escapes microbial remineralisation and subsequently accumulates in the sediment. The 'escaping remineralisation' definition does not define refractory C chemically, but inherently depends both on rates of detrital production, the refractility of macromolecules, nutrient content (commonly reported as C:N, C:P or lignin:N ratios) and environmental conditions during decay (Melillo et al. 1989, Enríquez et al. 1993, Cebrián 1999). A review on the production and utilisation of plant C found that the highest refractory C accumulation occurs in terrestrial or emergent habitats in the order of forests and shrubs followed by mangrove, grasses and saltmarshes (Cebrián 1999). Within the aquatic environment, seagrasses accumulate the highest amount of refractory C followed by freshwater macrophytes, macroalgae and microalgae/plankton (Cebrián 1999).

Once C accumulates and becomes a part of the sediment C_{org} stocks, the time-scales of refractory C preservation increases from years or decades (i.e., refractory C in plants) to centuries or millennia (i.e., refractory C_{org} in sediments) (Mateo et al. 2006). Following this shift, the characteristics of refractory C are typically defined differently. For example, abiotic conditions can promote condensation of C_{org} (e.g., geopolymerisation or humification) that increases the sediment refractility or stability (Burdige 2007). In soils, organic matter of < 50 μm in size is also considered refractory or 'stable' due to its association with clays/silts, which bind to the C_{org} and protect it

from remineralisation (Tiessen and Stewart 1983, Kiem et al. 2002, Kiem and Kögel-Knabner 2003). For mineral soils and sediments, protection and stabilisation of C_{org} is further enhanced via a C_{org} -mineral bond (Kramer et al. 2012, Lalonde et al. 2012, Johnson et al. 2015). Both labile and refractory C that escapes microbial degradation have the potential to form bonds with mineral aggregates, and this can increase the long-term, stable pools of C_{org} in sediments and soils (Ewing et al. 2006, Johnson et al. 2015).

Refractory C in blue carbon habitats

This thesis will consider both plant refractory C (lignocellulose) and sediment refractory C_{org} (ancient, stable) in seagrass ecosystems, and aims to investigate the factors that optimise the storage and sequestration of C_{org} in these blue carbon ecosystems. To do this, we take a holistic approach in answering the following questions by observing the interactions among biological (microbial community and function), chemical (refractory C, C:N content, C source) and environmental/physical (temperature, nutrient inputs, physical disturbance) variables (Fig. I1).

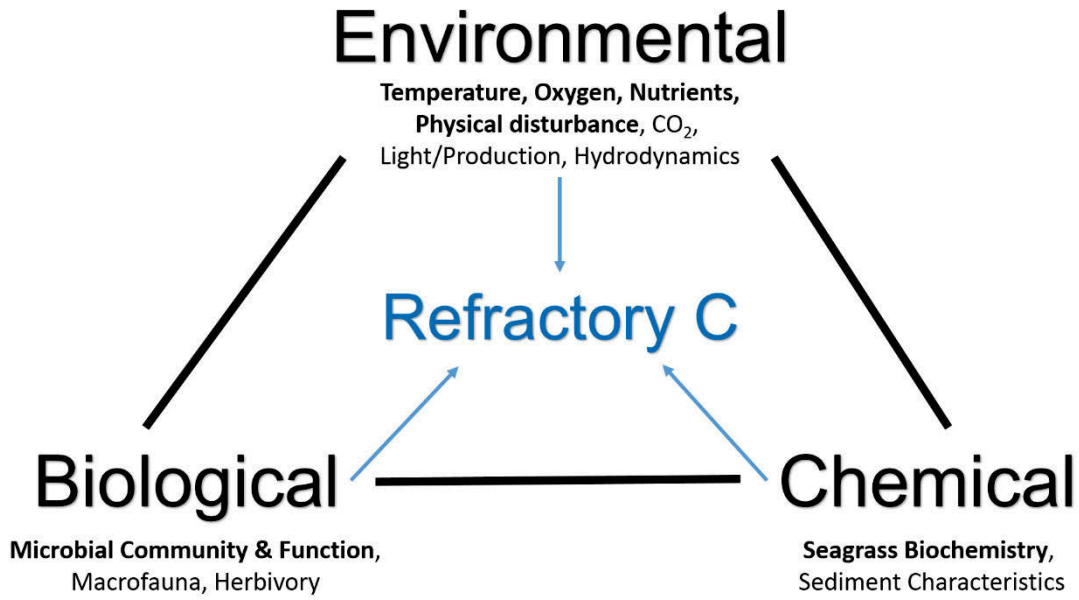


Figure I1: Conceptual model of the multi-factor approach used to investigate the dynamics of refractory carbon in seagrass meadows. The environmental, chemical and biological factors not only influence refractory carbon cycling, but the variables have the potential to influence each other. The bolded sub-factors will be investigated in this thesis.

1) To what degree does refractory carbon content vary across the range of seagrass morphologies and taxa, and how might that influence their roles as refractory carbon donors in seagrass meadows? (Chapter 1)

Seagrasses are important C donors to blue carbon systems, i.e., they contribute on average 50% of the sedimentary C_{org} in seagrass meadows (Kennedy et al. 2010). However, only three of the > 60 species of seagrass have been identified to having high C_{org} sink capacity due to their (a) large morphologies that maximise particle trapping and sediment stabilisation, (b) long life-span and low turnover rate and (c) high proportion of refractory C as lignin (*Thalassodendron ciliatum, Posidonia oceanica, P. australis*; Mateo et al. 2006). However, previous studies that have investigated the structural carbohydrate content as fibre content of seagrasses for nutritional studies suggest that many more seagrass species have the potential to contribute refractory C to

blue carbon systems (e.g., *Cymodocea*, *Enhalus*, *Thalassia*) (Lawler et al. 2006, de Los Santos et al. 2012). Additionally, much of the current data focuses on the leaves of tropical taxa, leaving a gap in what is known about refractory C in temperate seagrasses and below-ground tissues (rhizomes and roots). Therefore, the first thesis chapter will investigate the variation in refractory C content in a survey of global data. The aim is to categorise the taxa, regions and tissues that will most likely contribute to long-term C_{org} stocks.

2) What factors affect remineralisation of seagrass refractory carbon, and ultimately the carbon available for sequestration? (Chapters 2 and 3)

Godshalk and Wetzel (1978) formulated a model that represents the variables that drive seagrass decay: k = (T x O x N)/(R x S), where k = relative decay rates, T = temperature, O = dissolved oxygen, N = mineral nutrients necessary for microbial proliferation, R = tissue refractility and S = particle size of the organic matter (particle volume/surface area). By using relative values scaled from 0 to 1 and describing the variables as proportional or inversely proportional to k, they showed that as T, O and N increase, so does the decay rate, and as R and S increase, decay rates decline. Despite many decomposition studies having been performed since, there remain several knowledge gaps concerning seagrass remineralisation that may have important implication for C sequestration. First, besides Godshalk and Wetzel (1978), no single study has included more than two of these factors in the same experiment (Table I1), so there is very little information on if or how these five factors interact during seagrass decomposition. Second, most decay studies occur for less than a year, which makes quantifying detrital seagrass contributions to long-term C_{org} stocks impossible (Tables I1 and I2). Lastly, C remineralisation of seagrass detritus is dominated by microbes (Moriarty et al. 1986, Harrison 1989, Blum and Mills 1991, Pollard and Moriarty 1991), and ultimately microbial remineralisation determines the quantity and quality of C available for long-term sequestration. However, very few studies have combined the microbial response (M) with traditional decomposition response variables (Table I1). In order to fill these knowledge gaps, the next two chapters will employ a multi-variable experimental design to quantify the microbial response during short- and long-term

decomposition under both controlled laboratory and natural field conditions. The common litterbag experimental design was chosen to assess seagrass decomposition. While this method has limitations (e.g., loss of fine biomass through the mesh, exclusion of certain types of macrofauna, lack of natural turbulence or export; reviewed in Harrison 1989), this technique was the most appropriate and quantitative for a long-term field study and to assess microbe-dominated degradation of seagrass.

a) How does temperature and nutrient addition affect short-term leaf and rhizome/root decomposition? (Chapter 2, T x N x R + M), and
b) Do anthropogenically-impacted habitats affect long-term seagrass decomposition? (Chapter 3, N x O x R + M)

Table I1: Review of seagrass decomposition studies in chronological order and the variables and factors measured or analysed in each study. The bolded reference indicates the study that created the 5-factor decomposition model for seagrasses. C = carbon, N = nitrogen.

			Variables Measured							
Length of study (d)	Genus	Tissue	Element (C,N)	Oxygen	Labile/ Refractory C	Exogenous Nutrients	Temperature	Microbial Response	Sediment Type	References
180	***Zostera***	**Leaf**	**X**	**X**	**X**		**X**	**X**		**(Godshalk and Wetzel 1978)**
150	*Syringodium*	Leaf	X							(Rice and Tenore 1981)
150	*Thalassia*	Leaf	X							(Rice and Tenore 1981)
210	*Zostera*	Leaf	X							(Pellikaan 1982)
180	*Thalassia*	Leaf	X		X			X		(Rublee and Roman 1982)
170	*Zostera*	Rhizome	X		X				X	(Kenworthy and Thayer 1984)
247	*Thalassia*	Rhizome	X		X				X	(Kenworthy and Thayer 1984)
90	*Thalassia*	Leaf	X							(Newell et al. 1984)
51	*Zostera*	Leaf	X	X						(Pellikaan 1984)
42	*Thalassia*	Leaf	X		X					(Zieman et al. 1984)
42	*Syringodium*	Leaf	X		X					(Zieman et al. 1984)
42	*Halodule*	Leaf	X		X					(Zieman et al. 1984)

			Variables Measured							
Length of study (d)	Genus	Tissue	Element (C,N)	Oxygen	Labile/ Refractory C	Exogenous Nutrients	Temperature	Microbial Response	Sediment Type	References
340	*Halophila*	Leaf						X		(Wahbeh and Mahasneh 1985)
31	*Halophila*	Leaf	X	X						(Josselyn et al. 1986)
31	*Halophila*	Rhizome/ Root	X	X						(Josselyn et al. 1986)
170	*Zostera*	Rhizome	X					X		(Kenworthy et al. 1987)
247	*Thalassia*	Rhizome	X					X		(Kenworthy et al. 1987)
40	*Zostera*	Leaf						X		(Blum and Mills 1991)
121	*Cymodocea*	Leaf	X							(Hemminga and Nieuwenhuize 1991)
121	*Zostera*	Leaf	X							(Hemminga and Nieuwenhuize 1991)
16 & 230	*Cymodocea*	Leaf			X			X		(Peduzzi and Herndl 1991)
419	*Halodule*	Leaf			X					(Opsahl and Benner 1993)
30-180	*Posidonia*	Leaf	X							(Romero et al. 1992)
168	*Posidonia*	Leaf								(Pergent et al. 1994)
168	*Posidonia*	Leaf								(Pergent et al. 1994)

			Variables Measured							
Length of study (d)	Genus	Tissue	Element (C,N)	Oxygen	Labile/ Refractory C	Exogenous Nutrients	Temperature	Microbial Response	Sediment Type	References
60	*Ruppia*	Whole	X	X					X	(Kristensen et al. 1998)
66	*Zostera*	Leaf	X	X				X		(Bourguès et al. 1996)
150	*Posidonia*	Leaf								(Cebrián et al. 1997)
120	*Zostera*	Leaf								(Cebrián et al. 1997)
120	*Zostera*	Leaf								(Cebrián et al. 1997)
120	*Cymodocea*	Leaf								(Cebrián et al. 1997)
64-117	*Posidonia*	Leaf	X	X						(Mateo and Romero 1996)
54	*Thalassodendron*	Leaf		X						(Ochieng and Erftemeijer 1999)
65	*Enhalus*	Leaf	X							(Holmer and Olsen 2002)
65	*Enhalus*	Rhizome	X							(Holmer and Olsen 2002)
150	*Zostera*	Leaf		X			X			(Jedrzejczak 2002)
345	*Thalassia*	Leaf	X	X		X				(Fourqurean and Schrlau 2003)
345	*Thalassia*	Rhizome	X	X		X				(Fourqurean and Schrlau 2003)

			Variables Measured							
Length of study (d)	Genus	Tissue	Element (C,N)	Oxygen	Labile/ Refractory C	Exogenous Nutrients	Temperature	Microbial Response	Sediment Type	References
23	*Zostera*	Roots		X				X	X	(Neubauer et al. 2004)
168	*Zostera*	Leaf	X		X			X		(Vichkovitten and Holmer 2004)
168	*Zostera*	Rhizome	X		X			X		(Vichkovitten and Holmer 2004)
168	*Zostera*	Root	X		X			X		(Vichkovitten and Holmer 2004)
60	*Zostera*	Leaf	X							(Machás et al. 2006)
40	*Thalassia*	Leaf	X			X				(Vonk et al. 2008)
40	*Halodule*	Leaf	X			X				(Vonk et al. 2008)
40	*Cymodocea*	Leaf	X			X				(Vonk et al. 2008)
210	*Posidonia*	Matte	X				X			(Pedersen et al. 2011)
98	*Zostera*	Leaf	X		X	X				(Nicastro et al. 2012)
65	*Zostera*	Leaf				X	X			(Ainley and Bishop 2015)

Table I2: Review of the seagrass decay rates (percent per day) under oxic and anoxic conditions. Latitudes were reported from field decomposition studies only. Years until the biomass remaining at 50% and 1% was estimated using the final decay rate at the end of the study.

	Tissue	Final Decay Rate in % d^{-1}	Years until 50% mass lost	Years until 99% mass lost	Field Studies, Latitude	Hemisphere	Within-Study Factors	Reference
Aerobic Decay	Leaf	0.10	1.90	12.6			Temperature	(Godshalk and Wetzel 1978)
	Leaf	0.40	0.47	3.15			Temperature	(Godshalk and Wetzel 1978)
	Leaf	0.10	1.90	12.6			Species-variability	(Rice and Tenore 1981)
	Leaf	0.10	1.90	12.6			Species-variability	(Rice and Tenore 1981)
	Leaf	0.15	1.27	8.41	52	North		(Pellikaan 1982)
	Leaf	0.27	0.70	4.67	30	North		(Wahbeh and Mahasneh 1985)
	Leaf	0.19	1.00	6.64	20	North	Species-variability, intertidal	(Hemminga and Nieuwenhuize 1991)
	Leaf	0.62	0.31	2.04	20	North	Species-variability, subtidal	(Hemminga and Nieuwenhuize 1991)
	Leaf	0.60	0.32	2.10	20	North	Species-variability, intertidal	(Hemminga and Nieuwenhuize 1991)
	Leaf	0.81	0.23	1.56	20	North	Species-variability, subtidal	(Hemminga and Nieuwenhuize 1991)
	Leaf	0.11	1.72	11.5				(Peduzzi and Herndl 1991)
	Leaf	0.18	1.05	7.00	27	North		(Opsahl and Benner 1993)
	Leaf	0.62	0.31	2.04	41	North	Depth	(Pergent et al. 1994)
	Leaf	0.33	0.56	3.82	41	North	Depth	(Pergent et al. 1994)
	Leaf	0.91	0.21	1.39	42	North	Species-variability	(Cebrián et al. 1997)
	Leaf	1.46	0.13	0.86	42	North	Species-variability	(Cebrián et al. 1997)
	Leaf	2.36	0.08	0.53	42	North	Species-variability	(Cebrián et al. 1997)

	Tissue	Final Decay Rate in % d^{-1}	Years until 50% mass lost	Years until 99% mass lost	Field Studies, Latitude	Hemisphere	Within-Study Factors	Reference
	Leaf	1.89	0.10	0.67	42	North	Species-variability	(Cebrián et al. 1997)
	Leaf	1.70	0.11	0.74	4	South		(Ochieng and Erftemeijer 1999)
	Leaf	0.70	0.27	1.80	25	North		(Fourqurean and Schrlau 2003)
	Rhizome	0.12	1.58	10.5	25	North		(Fourqurean and Schrlau 2003)
	Leaf	1.60	0.12	0.79	37	North		(Machás et al. 2006)
	Leaf	1.10	0.17	1.15	33	South	Intertidal	(Nicastro et al. 2012)
	Leaf	1.39	0.14	0.91	33	South	Intertidal	(Nicastro et al. 2012)
	Leaf	1.475	0.13	0.86	33	South	Subtidal	(Nicastro et al. 2012)
	Leaf	2.15	0.09	0.59	30	South	Latitude, nutrient-loading	(Ainley and Bishop 2015)
	Leaf	1.65	0.12	0.76	30	South	Latitude, nutrient-loading	(Ainley and Bishop 2015)
	Leaf	1.95	0.10	0.65	35	South	Latitude, nutrient-loading	(Ainley and Bishop 2015)
	Leaf	2.00	0.10	0.63	35	South	Latitude, nutrient-loading	(Ainley and Bishop 2015)
min		*0.10*	*0.08*	*0.54*				
max		*2.36*	*1.90*	*12.6*				
mean		*0.93*	*0.59*	*3.92*				
median		*0.76*	*0.25*	*1.68*				

	Tissue	Final Decay Rate in % d^{-1}	Years until 50% mass lost	Years until 99% mass lost	Field Studies, Latitude	Hemisphere	Within-Study Factors	Reference
Anaerobic Decay	Leaf	0.10	1.90	12.6			Temperature	(Godshalk and Wetzel 1978)
	Leaf	0.20	0.95	6.31			Temperature	(Godshalk and Wetzel 1978)
	Sheath*	21.6	3.21	21.3	41	North	Depth	(Romero et al. 1992)
	Sheath*	8.60	8.06	53.5	41	North	Depth	(Romero et al. 1992)
	Leaf	1.90	0.10	0.66	45	North		(Bourguès et al. 1996)
	Rhizome	0.08	2.37	15.8				(Vichkovitten and Holmer 2004)
	Root	3.36	0.06	0.38				(Vichkovitten and Holmer 2004)
	Leaf	0.07	2.71	18.0				(Vichkovitten and Holmer 2004)
min		*0.07*	*0.06*	*0.38*				
max		*21.60*	*8.06*	*53.6*				
mean		*4.49*	*2.42*	*16.1*				
median		*1.05*	*2.14*	*14.2*				

* Decay rate in % $year^{-1}$

3) How do disturbances affect stored sediment refractory carbon? (Chapters 4 and 5)

As mentioned above, much of the habitat loss and degradation of seagrass meadows are attributed to anthropogenic causes, including sediment and nutrient runoff, physical disturbance, disease, algal blooms and global warming (Orth et al. 2006). There is currently a push in blue carbon research to understand how these anthropogenic pressures affect current C_{org} stocks and the ability of seagrass habitats to accumulate C_{org} (Jorda et al. 2012, Pendleton et al. 2012, Macreadie et al. 2014a). The last two chapters will address two of these pressures: nutrient inputs and habitat loss.

First, it has been repeatedly shown in terrestrial systems that the addition of fresh nutrients or labile C can lead to a 'microbial priming effect', which results in the stimulation of microbes to remineralisation refractory C_{org} that could not otherwise be utilised (Kuzyakov et al. 2000). Priming is thought to be a mechanism that increases the vulnerability of stable C_{org} to remineralisation in agricultural and terrestrial soils (Fontaine et al. 2004, Fontaine et al. 2007) by releasing large amounts of C-CO_2 in a short period of time (Kuzyakov et al. 2000). If this phenomenon is occurring in seagrass meadows, it could have a devastating effect on the sedimentary C_{org} stores, especially if old, deep sediment C_{org} is exposed as a result of a physical disturbance like dredging.

Secondly, the effects of seagrass loss on C_{org} stocks are still under debate in terms of the magnitude of C_{org} lost from the system as CO_2 (Macreadie et al. 2014a). Recent research suggests that large-scale losses of seagrass can reduce C_{org} sequestration by 11-52% (Marbà et al. 2014). Smaller-scale losses like those from boat scarring can be quite common, yet the research investigating how these disturbances affect sediment C_{org} pools is still growing (Macreadie et al. 2014b, Bourque et al. 2015). To better understand the consequences of labile C inputs and small-scale habitat loss on refractory C_{org} in sediment, the last two chapters of this thesis will address the following questions:

a) Does the microbial priming effect exist in coastal sediments, and is deep, aged refractory C_{org} susceptible to enhanced remineralisation as a result of the microbial priming effect? (Chapter 4), and
b) How does small-scale die-off, like those from boating activities, affect refractory C_{org} remineralisation in the sediments? (Chapter 5)

Chapter 1:

Assessing the organic composition of seagrasses for their capacity to contribute to long-term carbon sequestration: A global survey

Abstract

Seagrass ecosystems have recently been identified for their role in climate change mitigation due to their globally-significant carbon sinks; yet, the capacity of seagrasses to sequester carbon has been shown to vary greatly among species and their locations. We performed a global survey (23 species from 16 countries, representing 4 tissue types) to investigate how plant chemistry might influence the capacity of seagrasses to contribute to sediment carbon stocks. Thermogravimetric analysis was used to quantify the labile and refractory organic matter content, and solid-state ^{13}C-NMR and infrared spectroscopy were used to assess the main macromolecular compounds within seagrass tissues. We found a strong climate influence on carbon quality, whereby temperate seagrasses contained relatively more labile carbon (soluble carbohydrates), and tropical seagrasses contained relatively more refractory carbon (lignocellulose). Sheath/stem tissues were variable across taxa, with larger morphologies typically containing more refractory carbon. While roots were dominated by refractory carbon and rhizomes by labile carbon, high biomass production and preservation in anoxic sediments will likely enhance these below-ground tissues' contributions to long-term carbon stocks. Our study provides novel information on the variation in carbon quality across seagrasses that will be valuable for future modelling and management prioritisation of carbon sequestration in seagrass ecosystems.

Introduction

'Blue carbon' is defined as carbon that is sequestered by oceans and is a critical component in global greenhouse gas mitigation (Laffoley and Grimsditch 2009). Blue carbon ecosystems, including seagrass, saltmarsh, and mangroves, have disproportionately high organic carbon (C) burial rates compared to terrestrial habitats (McLeod et al. 2011). Seagrass meadows alone can bury organic C more rapidly than terrestrial forest and agricultural systems (138 vs 4.5-90 g C m^{-2} yr^{-1}, respectively) (Hutchinson et al. 2007, McLeod et al. 2011, Sanderman et al. 2014), and are globally significant sinks for sequestered C (Fourqurean et al. 2012). However, across-species organic carbon (C_{org}) stocks can vary up to 18-fold on an average basis (Lavery et al. 2013a). This heterogeneity can make predictions of C sequestration rates complex. Several factors that have been hypothesised to influence this among-meadow variation in C_{org} accumulation and preservation, including physical and hydrological conditions (Gacia et al. 1999, Duarte et al. 2013a, Lavery et al. 2013a), temperature (Godshalk and Wetzel 1978), oxygen environment (Mateo et al. 2006), microbial function and metabolism (Duarte et al. 2005) and C source (Mateo et al. 2006, Lavery et al. 2013a). In many ecosystems, litter quality is one of the main factors controlling global C_{org} remineralisation and cycling (Enríquez et al. 1993, Couteaux et al. 1995, Silver and Miya 2001).

In seagrass meadows, C may come from epiphytic, micro- and macroalgal, terrestrial and seagrass organic matter. It has been estimated that 16% of seagrass primary production is not remineralisation and subsequently accumulates in the sediment (Duarte and Cebrián 1996) and can account for on average 50% of the total stored sedimentary C_{org} (Kennedy et al. 2010). Therefore, the quality of C in seagrass organic matter is an important factor in determining the fate of C_{org}, remineralised or sequestered. While seagrasses are slightly more nutritious to decomposers than terrestrial substrates, they are relatively nutrient deplete (nitrogen, phosphorus) compared to other sources of marine organic matter and thus can decompose more slowly (Enríquez et al. 1993). Seagrasses are more resistant to microbial attack than other marine macrophytes because they retained some of their cell wall structural carbohydrates and lignin from their terrestrial origin (i.e., fibre or lignocellulose ≈ hemicellulose + cellulose + lignin) (Klap et al. 2000), and are the only marine vascular macrophytes, i.e., only source of lignocellulose formed in marine ecosystems (Lewis

and Yamamoto 1990). As a result, the relatively high C:N ratios and lignocellulose content increase seagrasses resistance to remineralisation and increase the importance of seagrass as contributors of refractory C into these blue carbon ecosystems (Trevathan-Tackett et al. 2015).

There is evidence that refractory carbon content varies with morphology and taxonomic diversity among seagrasses. Early histochemistry and microscopy studies on the lignocellulose content of seagrasses found certain tissues and cells to be more lignified than others, but these studies were limited to a handful of species, primarily *Posidonia* sp. For example, structural carbohydrates were found in leaf tissues, which were primarily comprised of cellulose, hemicellulose and pectin (Kuo and Cambridge 1978, Cambridge and Kuo 1982, Kuo et al. 1990, Barnabas 1994), while *Posidonia sinuosa* and *P. australis* had lightly lignified vascular tissue (Kuo 1978, Cambridge and Kuo 1982). Non-photosynthetic sheath tissues that protect leaf meristems have been shown to be more lignified than leaves and contained abundant phenolic compounds (Kuo and Cambridge 1978, Cambridge and Kuo 1982). Rhizomes, in addition to storing carbohydrates reserves, were more heavily lignified, particularly in epidermal cells, middle lamellae and vascular tissues (Kuo and Cambridge 1978, Cambridge and Kuo 1982). Similarly, roots had a higher degree of lignification than other tissues to provide better anchorage and support (Kuo and Cambridge 1978, Cambridge and Kuo 1982, Barnabas 1991, Kuo and Hartog 2006).

More recently, biochemical analyses have been used to quantify the lignocellulose content of seagrasses; however, the techniques and the seagrass taxa and tissue types analysed have been highly variable across studies (Appendix Table 1). For example, much of the quantitative analyses of lignocellulose content in seagrasses have focused on leaf fibre content (~ hemicellulose + cellulose + lignin) for nutritional herbivory studies (Appendix Table 1). As a result, there are sparse data on the structural carbohydrate content of the more lignified below-ground tissues (< 10 out of 43 studies; Table 1.1, Appendix Table 1). Furthermore, a summary of the available structural carbohydrate data suggest that lignin content is higher in above-ground than below-ground tissues, which is contradictory to histological observations (Table 1.1). Some of the common techniques used in previous studies such as proximate analyses to obtain neutral and acid detergent fibre (NDF, ADF) have limitations that may account for these disparate trends (Preston et al. 1997, Brinkmann et al. 2002, Hatfield and Fukushima

2005). ADF can result in impure separation (lignin-bound proteins; Brinkmann et al. 2002), or treatment of ADF with strong acid to quantify lignin (acid detergent lignin) may remove a significant proportion of the desired compound (i.e., terrestrial grasses can lose up to 50% of lignin in acid detergent solution; Hatfield and Fukushima 2005).

There are a few techniques for determining structural carbohydrates and lignin that do not rely on the modification of the cell wall matrix and require minimal preparation, i.e., drying and grinding. ^{13}C- Cross-Polarisation Magic-Angle Spinning (CPMAS) nuclear magnetic resonance (NMR) spectroscopy identifies the macromolecular content based on the functional groups to which the heavy carbon isotope (^{13}C) belongs. Although this technique can be time consuming, allocation of carbon to aromatic structures can be selectively quantified without prior removal of other biomolecular components or dissolution of aromatic materials (Hatfield and Fukushima 2005). ^{13}C-NMR does not differentiate between cellulose and simple monomeric carbohydrates (Nelson and Baldock 2005), but a mixing model was developed to quantitatively estimate the biochemical components in which each functional group belongs, including lignin, carbohydrates, protein and lipids (Baldock et al. 2004). In addition to ^{13}C-NMR, diffuse reflectance Fourier-transform infrared (FTIR) spectroscopy can be used as a rapid, non-invasive technique that identifies compound-specific bonds based on their wavelength absorption (Sackett et al. 2013); however, the technique does require parallel analysis using wet chemistry techniques to develop predicative algorithms that can be applied to acquired spectra to obtain quantitative data (Hatfield and Fukushima 2005). Another technique that has been recently used to quantify cell wall components is thermogravimetric analysis (TGA), which differentiates cell wall components based on the temperature at which they are pyrolysised (Trevathan-Tackett et al. 2015). TGA by pyrolysis is slightly different than combustion by muffled furnace in that the plant biomass conversion is absent of oxygen and thus is not totally converted to CO_2 and that much of the residues are converted to biochar at 600°C. TGA is most useful for the distinction and quantification of labile and refractory components (Capel et al. 2006, Carrier et al. 2011, Pasangulapati et al. 2012), whereby loss of mass at higher temperatures is associated with refractory components like lignocellulose. The limitation of this technique is that it cannot provide precise compound identification at each temperature interval since pyrolysis of some compounds can occur over a broad temperature range (Yang et al. 2006).

Table 1.1: Summary of structural carbohydrate and fibre content reported from the literature for seagrass tissue types. All averages are reported on a % dry weight basis. NDF = neutral detergent fibre, ADF = acid detergent fibre. The full list of species, tissues and references are in Appendix Table 1.

	Structural Carbohydrates			Fibre	
Tissue	Cellulose	Hemicellulose	Lignin	NDF	ADF
Leaf (All) *(n = 14 – 43)*	16.84 ± 1.29	12.70 ± 1.24	11.05 ± 1.46	37.59 ± 2.44	26.24 ± 1.62
Leaf Temperate *(n = 1 – 16)*	20.93 ± 3.95	17.07 ± 4.36	12.20 ± 4.82	49.65 ± 1.49	35.10 ± 0
Leaf Tropical *(n = 13 – 27)*	15.75 ± 1.20	11.76 ± 1.14	10.81 ± 1.52	30.44 ± 3.05	25.56 ± 1.58
Leaf + Stem *(n = 3-4)*	25.67 ± 2.64	18.18 ± 2.27	5.20 ± 0.90	46.80 ± 3.53	33.70 ± 3.62
Non-photosynthetic Above-ground *(n = 1 – 4)*	30.82 ± 7.18	21.00 ± 0	8.80 ± 6.15	N/A	N/A
Above-ground Grand Mean *(n = 18 – 47)*	19.12 ± 1.49	13.87 ± 1.17	10.17 ± 1.37	38.37 ± 2.28	27.90 ± 1.62
Rhizome (All) *(n = 4 – 8)*	17.77 ± 1.36	11.75 ± 4.55	3.03 ± 0.78	13.48 ± 3.61	17.83 ± 2.03
Rhizome Temperate *(n = 1 – 2)*	18.30 ± 0	28.90 ± 0	4.45 ± 0.95	N/A	N/A

Tissue	Structural Carbohydrates			Fibre	
	Cellulose	Hemicellulose	Lignin	NDF	ADF
Rhizome Tropical *(n = 4 – 8)*	17.68 ± 1.61	8.32 ± 3.66	2.56 ± 0.94	13.48 ± 3.61	17.83 ± 2.03
Root (All) *(n = 2 – 4)*	19.34 ± 3.12	34.80 ± 6.10	4.99 ± 0.99	3.09 ± 0.20	N/A
Root Temperate *(n = 1 – 2)*	21.30 ± 0	40.90 ± 0	6.10 ± 0.40	N/A	N/A
Root Tropical *(n = 1 – 4)*	18.37 ± 5.13	28.70 ± 0	3.88 ± 1.82	3.09 ± 0.20	N/A
Rhizome + Root *(n = 11 – 16)*	17.13 ± 2.14	8.02 ± 0.79	11.33 ± 1.19	38.31 ± 2.10	28.02 ± 1.58
Below-ground Grand Mean *(n = 16 – 28)*	17.64 ± 1.21	12.02 ± 2.41	8.05 ± 1.03	26.19 ± 3.16	25.47 ± 1.70
Whole Plant *(n = 5 – 10)*	22.31 ± 2.78	11.68 ± 1.62	10.18 ± 1.86	42.92 ± 2.65	29.77 ± 2.78

In this study, we surveyed the organic composition of seagrasses using each of these three non-invasive techniques to provide a multi-proxy approach to quantifying seagrass C quality. We hypothesise that the macromolecular content from the NMR data will help inform the mixed compounds with each of the TGA thermal intervals and will be based on lability and refractility. By collecting seagrasses from across the globe, we also aimed to resolve the variation in seagrass C quality across tissue types, taxa and geography. We hypothesise that some seagrass samples, such as below-ground tissues or seagrasses with a larger morphological structure, will have higher proportions of refractory C, which in turn, will make them superior C donors (i.e., by contributing to long-term C_{org} stocks) to blue carbon systems.

Methods

Sample Collection and Preparation

Between August 2012- March 2015, 23 seagrass species were collaboratively and opportunistically collected from 16 countries (Table 1.2; Fig 1.1). Collections occurred across all seasons. After being cleaned of epiphytes and attached infauna and sediments, samples were heat-dried until constant mass and sent to the University of Technology Sydney (UTS) for analysis. Tissue types were separated before being ground to a fine powder using a ball mill (Pulverisette 7, Fritsch, Germany) and stored in a desiccator to avoid moisture reabsorption.

Table 1.2: Summary of the seagrass samples collected and the variables explored: tissue type, taxa and climatic region. Climatic region was divided into temperate or tropical classifications and in some cases bioregions (Short et al. 2007). Temperate climates included bioregions 1 (Temperate Atlantic), 3 (Mediterranean), 4 (Temperate North Pacific) and 6 (Temperate Southern Ocean). Tropical climates included bioregions 2 (Tropical Atlantic) and 5 (Tropical Indo-Pacific).

Family	Species	Bioregion	Country	Leaf	Rhizome	Root	Non-Photosynthetic Above-ground		
							Sheath	Stem	Vertical Rhizome
Cymodoceaceae									
	Amphibolis antarctica	6	Australia*†	X					
	Cymodocea nodosa	1	Portugal*†	X	X	X			
		2	Mauritania	X	X	X	X		
	Cymodocea rotundata	5	Indonesia	X	X	X	X		
			Madagascar*	X	X	X			
			Thailand	X	X	X	X		
	Cymodocea serrulata	5	Madagascar*	X	X	X			
	Halodule uninervis	5	Australia*†	X	X	X			

Family	Species	Bioregion	Country	Leaf	Rhizome	Root	Non-Photosynthetic Above-ground		
							Sheath	Stem	Vertical Rhizome
			Indonesia	X	X	X	X		
			Madagascar	X	X	X			
	Halodule wrightii	2	Mauritania	X	X	X			
			USA	X	X	X			
	Syringodium isoetifolium	5	Indonesia	X	X	X			
			Madagascar*†	X	X	X			
	Thalassodendron ciliatum	5	Madagascar*†	X	X	X			X
Hydrocharitaceae	*Enhalus acoroides*	5	Australia*†	X	X	X	X		
			Thailand*	X			X		
	Halophila australis	6	Australia	X	X			X	
	Halophila ovalis	5	Australia*†	X	X				
			Indonesia	X	X	X			

Family	Species	Bioregion	Country	Leaf	Rhizome	Root	Non-Photosynthetic Above-ground		
							Sheath	Stem	Vertical Rhizome
			Madagascar	X	X	X			
			Thailand	X	X				
	Halophila spinulosa	5	Australia	X	X	X		X	
	Thalassia hemprichii	5	Indonesia	X	X	X	X		
			Madagascar*†	X	X	X			
			Thailand*	X	X	X	X		
	Thalassia testudinum	2	USA	X	X	X	X		
Posidoniaceae	*Posidonia australis*	6	Australia*†	X	X	X	X		
	Posidonia oceanica	3	Corsica*†	X	X	X	X		
Ruppiaceae	*Ruppia maritima*	1	Sweden*†	X	X	X	X		
Zosteraceae	*Phyllospadix iwatensis*	4	Japan	X	X	X	X		

Family	Species	Bioregion	Country	Leaf	Rhizome	Root	Non-Photosynthetic Above-ground		
							Sheath	Stem	Vertical Rhizome
	Zostera capensis	6	South Africa	X	X	X			
	Zostera chilensis	6	Chile*	X	X	X		X	
	Zostera marina	1	Sweden*	X	X	X	X		
			Denmark	X	X	X	X		
			Finland	X	X	X	X		
			Portugal	X	X	X			
			Germany	X	X	X	X		
		4	Japan*	X	X	X	X		
			USA	X	X	X			
	Zostera muelleri	5	Australia†	X	X	X			
		6	Australia*†	XX					
	Zostera nigricaulis	6	Australia*†	X					

Family	Species	Bioregion	Country	Leaf	Rhizome	Root	Non-Photosynthetic Above-ground		
							Sheath	Stem	Vertical Rhizome
	Zostera noltii	1	Netherlands	X	X	X	X		
			Germany	X	X	X	X		
		2	Mauritania	X	X	X			

* FTIR analysis; † NMR analysis

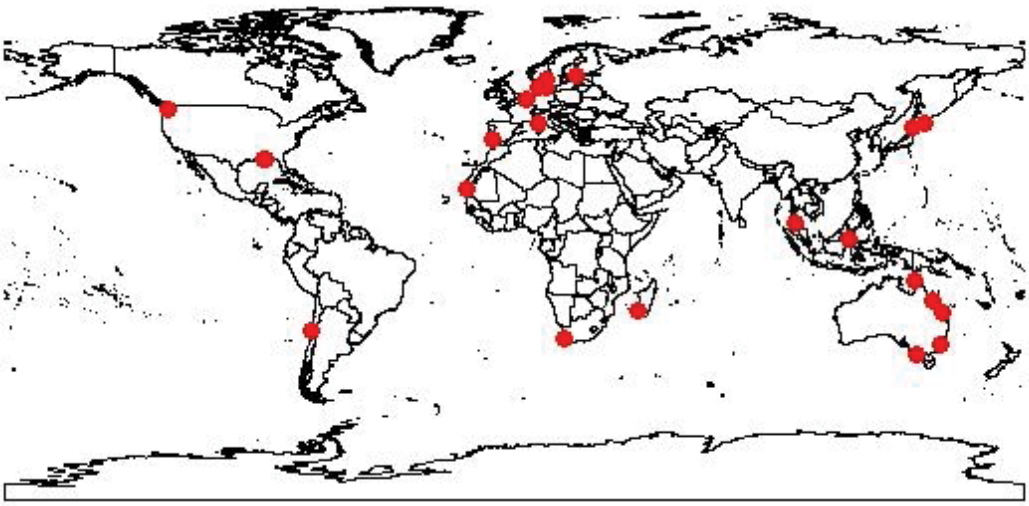


Figure 1.1: Map of seagrasses collected.

Thermogravimetric Analysis (TGA)

At UTS, all samples were analysed using thermogravimetry (SDT Q600, TA Instruments, New Castle, DE, USA) with a 0.1 μg balance sensitivity. An aliquot of ground sample (10 mg) was place in a platinum cup and heated under N_2 (gas flow 100 mL min^{-1}) at 10°C min^{-1} (Yang et al. 2006, Ncibi et al. 2009) to 600°C. Universal Analysis software (TA Instruments) was used to aid the identification and quantification of mass loss within specific temperature intervals. Delineation of thermal intervals (TI) was based on the rate-of-change derivative (% mass loss $°C^{-1}$), which indicated distinct temperatures of mass loss (Yang et al. 2006). Mass loss occurring from 30°C to 180°C was associated with the loss of moisture. The first temperature interval (TI_1: labile; hemicellulose) ranged from 180°C to 220°C, followed by TI_2 (labile; hemicellulose) from 220°C to 300°C. TI_3 (refractory; cellulose) extended from 300°C to 400°C, then TI_4 (refractory; lignin and insoluble polysaccharide residues) from 400-600°C. TI_{1-4} were recalculated as a gravimetric proportion of total organic matter (OM) from 180°C to 600°C.

Fourier Transform Infrared (FTIR) Spectroscopy

FTIR analyses were performed on a subset of untreated samples (Table 1.2) using FTIR instrumentation described in Baldock et al. (2013b). Diffuse reflectance mid/near-infrared spectra were acquired over 6000–400 cm^{-1} with a resolution of 8 cm^{-1}.

The background signal intensity was quantified by collecting 240 scans on a silicon carbide disk before analysing any samples. In total, 60 scans were acquired and averaged to produce a reflectance spectrum for each individual sample. The Omnic software (Version 8.0; Thermo Fisher Scientific Inc.) was used to convert the acquired reflectance spectra into absorbance spectra (log-transform of the inverse of reflectance).

FTIR spectra were pre-processed for statistical analysis using The Unscrambler 10 (Camo, Oslo) software package. Second derivatives were generated for the spectra using the Savitsky-Golay function with 9 smoothing points, before removing scattering effects using the Extended Multiplicative Scatter correction algorithm. The spectra were then split into two groups: a calibration set consisting of about two thirds of the data, and a validation set made up of the remaining third. These were then used to build PCA models in order to reveal groupings within the data that would be otherwise hidden within the complex spectra using a review of FTIR spectroscopy for biological tissues (Movasaghi et al. 2007).

^{13}C-CPMAS Solid State Nuclear Magnetic Resonance (NMR) Spectrometry

A subset of samples (Table 1.2) analysed with FTIR spectroscopy were also analysed using solid-state ^{13}C- Cross-Polarisation Magic-Angle Spinning (CPMAS) nuclear magnetic resonance (NMR) spectroscopy (Baldock et al. 2013a, with slight modification). Briefly, a 200 Avance spectrometer (Bruker Corporation, Billerica, MA, USA) equipped with a 4.7 T, wide-bore superconducting magnet operating at a resonance frequency of 50.33 MHz was used to obtain the spectra. Weighed samples (200-400 mg) were packed into 7 mm diameter zirconia rotors with Kel-F® end caps and spun at 5 kHz. A cross-polarisation ^{13}C-NMR (CP) analysis using a 90° pulse of 3.2 µs at 195 W, a contact time of 1 ms, and a recycle time ranging from 1 to 5 s was used for all samples with a total of 5000 scans being collected for each sample. The duration of the recycle delay was set to greater than 5 times sample specific T_1H values determined using an inversion recovery pulse sequence. All spectral processing was completed using the Bruker TopSpin 3.1 software (Baldock et al. 2013a). The acquired total signal intensity was divided into a series of chemical shift regions: amide/carboxyl/ketone (215–165 ppm), O-aromatic (165–145 ppm), aromatic (145–110 ppm), di-O-alkyl (110–95 ppm), O-alkyl (95–60 ppm), N-alkyl/methoxy (60–45 ppm), and alkyl (45 to 10 ppm). The NMR spectral intensities were entered into a molecular

mixing model (MMM) for terrestrial soils (Baldock et al. 2004) in order to predict macromolecule content (carbohydrate, lignin, lipid and protein) of the seagrass tissue. The original terrestrial protein component of the MMM was altered to be reflective of the amino acid composition of seagrasses using data from *Posidonia australis*, *Thalassia testudinum*, *Halodule wrightii* and *Syringodium filiforme* (Zieman et al. 1984, Torbatinejad et al. 2007). The model was constrained with the elemental carbon (C) and nitrogen (N) content of the tissues. Elemental C:N data was obtained from bulk tissue using an elemental analyser (LECO TruSpec, St. Joseph, MI, USA). If there was not enough material for CN analysis, estimates were obtained from the literature (Duarte 1990, Hansen et al. 2000, Lavery et al. 2009).

Statistical Analysis

Multi-factorial permutational analysis of variance (PERMANOVA) was used to investigate differences in the size of the four temperature intervals (TI_1, TI_2, TI_3, TI_4 normalised to total organic mass loss and total organic matter, OM) between taxa, tissue type and climatic zone for TGA and MMM analyses. Preliminary 1-factor analyses comparing TGA intervals for *tissue types* (6 levels: leaf, rhizome, root, sheath, stem, and vertical rhizome) identified that there were significant differences amongst most tissue types ($Pseudo\text{-}F_{(5, 145)} = 9.75$ $P\text{-}perm = 0.001$). There was no significant difference between sheath, stem and vertical rhizome. Although they represent diversity in morphology, chlorophyll content and location across the water-sediment interface, they were combined into a tissue category that represented most of the samples: 'non-photosynthetic above-ground' tissue. A 3-factor PERMANOVA further examined differences in pyrolysis dynamics for tissue types, independently, comparing *climatic zone* (temperate or tropical), *family nested within climatic zone* and *genus nested within family* (Table S1.1). Since *Zostera* was the most well sampled taxonomic group, finer-scale variation in tissues and individual bioregions from the climatic zones (Temperate Atlantic, Tropical Atlantic, Mediterranean, Temperate North Pacific, Tropical Indo-Pacific, Temperate Southern Ocean; Short et al. 2007) were investigated within the *Zostera* genus with a 2-way PERMANOVA (*tissue* and *bioregion*). For NMR/MMM analyses, data was not as deeply sampled as TGA, so *tissue type* was not separated for statistical analysis. Instead, 3-way PERMANOVA was used to analyse macromolecular content between *tissues* and *climatic zone* and *family nested in climatic zone*.

Pairwise comparisons of tissue types, climatic zone, families and genera were made. Monte Carlo approximated P-values (*P(MC)*) were used to interpret comparisons with low numbers of unique permutations (i.e., < 100) for all analyses. Similarity percentage (SIMPER) analysis was used to identify the contributions of each temperature interval or MMM component to observed differences amongst tissue types, climatic zone and taxa. Principal components analysis (PCA) plots provided explanation of variation linked to the factors. PCA plots were also made between the molecular mixing model output (MMM) and TGA thermal intervals. C:N ratios were not included since they were accounted for in the MMM. Only the data that were analysed for both NMR and TGA were used. Analyses were based on untransformed data and Euclidean distance resemblance matrices calculated by PRIMER v6 for Windows (PRIMER-E; Clark 1993). Analyses were also performed using PRIMER v6 with PERMANOVA+ add on.

Results

TGA

A principal components analysis (PCA) of all samples showed that climatic region driven by OM (56.7%) and tissue type driven by TI_3 (25.6%) explained most of the variation in TGA results across the seagrass samples as PC1 and PC2, respectively (Fig. 1.2, Tables S1.1, S1.2). Seagrass tissues were further investigated, and were shown to be significantly different from each other ($Pseudo\text{-}F_{(3, 147)} = 15.964$ $P\text{-}perm = 0.001$; pairwise tests $P\text{-}perm \leq 0.018$; Figs. 1.2b, 1.3, 1.4; SIMPER values in Table S1.1). Leaf tissues had the highest amount of cellulose-associated refractory OM (TI_3, ~47%), while the roots had the highest amount of lignin-associated refractory OM (TI_4, ~24%) and lowest overall OM content (35%). Rhizomes were highest in overall OM content (51%) and proportion of labile OM (TI_1, ~16%). Non-photosynthetic above-ground tissue quality was intermediate between leaf and rhizome tissues (Fig. 1.2b).

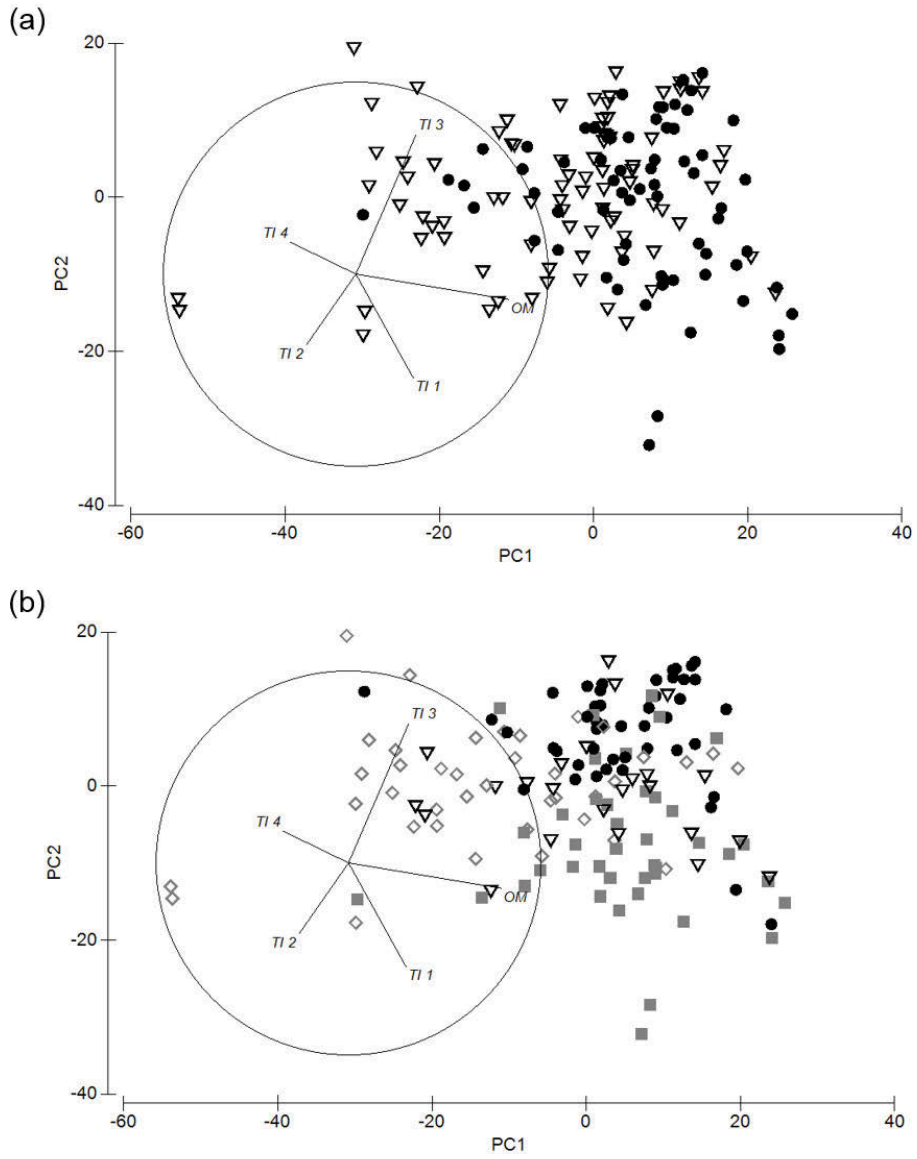


Figure 1.2: Principal components analysis of seagrass organic matter quality using thermogravimetric analyses by (a) climatic zone (● = temperate and ▽ = tropical) and (b) tissue type (● = leaf, ▽ = non-photosynthetic above-ground tissue, ■ = rhizome, ◊ = root). PC1 = 56.8% variation, PC2 = 25.9% variation. Eigenvectors are reported in Table S1.2. Thermal intervals (TI) represent distinct organic matter components from TGA normalised to total organic matter (TI_1: labile, carbohydrates, hemicellulose; TI_2: labile, carbohydrates, hemicellulose; TI_3: refractory, cellulose; TI_4: refractory, lignin and residues).

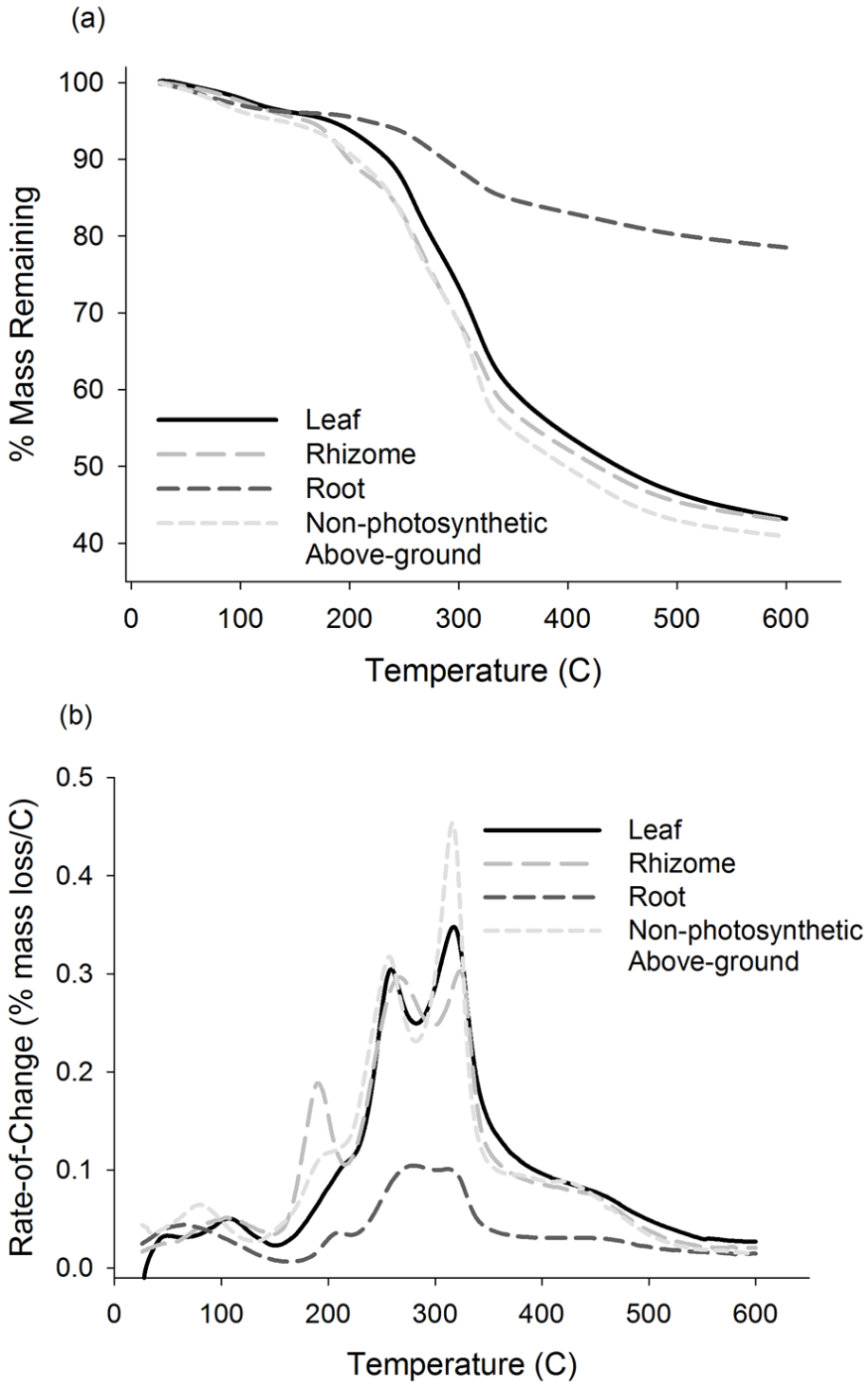


Figure 1.3: Representative thermograms for each tissue type. (a) % mass remaining with increasing temperature. (b) Derivative rate-of-change (% mass loss per °C) with increasing pyrolysis temperature.

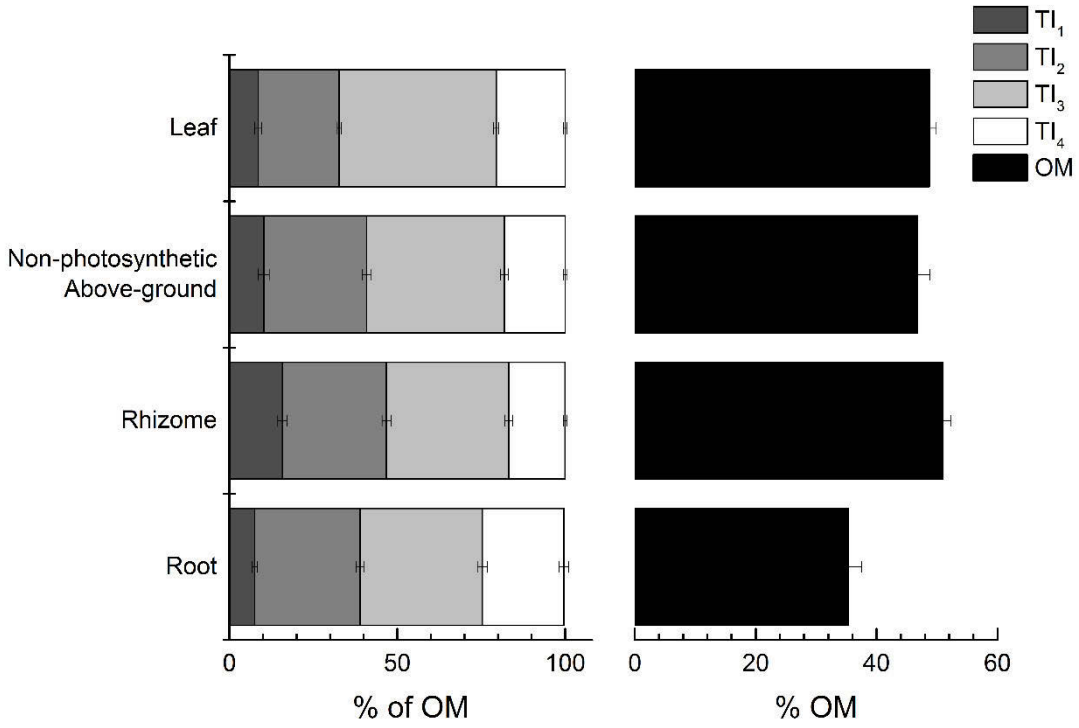


Figure 1.4: Thermal intervals (TI) as percent of total organic matter (OM) and OM as percent of the total mass across all tissue types. TIs represent distinct organic matter components from TGA normalised to total organic matter (TI_1: labile, carbohydrates, hemicellulose; TI_2: labile, carbohydrates, hemicellulose; TI_3: refractory, cellulose; TI_4: refractory, lignin and residues).Values represent mean ± 1 S.E.M.

Investigation of the variation in climatic zone and taxa for each tissue type revealed that differences in temperate and tropical TGA signatures were consistent and explained most of the variation across all tissue types, which was driven by OM content (PC1 = 46.2-70.1%; Figs. S1.1, S1.2; Table S1.2, Appendix Table 1). Temperate regions had highest total OM in all tissue types (Fig. S1.1, Table S1.1). For leaf (*Pseudo-*$F_{(1, 32)}$ = 7.01 *P-perm* = 0.006), rhizome (*Pseudo-*$F_{(1, 27)}$ = 3.65, *P-perm* = 0.015) and non-photosynthetic above-ground tissues (*Pseudo-*$F_{(1, 13)}$ = 5.26, *P-perm* = 0.009), labile TI_1 content was 1.5 - 2.5-times higher than their tropical counterparts (Table S1.1). Refractory OM content was higher in the tropics for leaf and rhizome tissues as TI_3, while roots (*Pseudo-*$F_{(1, 26)}$ = 3.44, *P-perm* = 0.048) had higher TI_4, but lower TI_3 in the tropics (Table S1.1). Linear regressions of OM and the thermal

intervals from all tissues across latitudes revealed that increased % OM content had a strong, highly significant positive relationship with higher latitudes (Adj. $R^2 = 0.1294$, $F = 23.2969$, $P < 0.0001$; Fig. 1.5a). The first three temperature intervals showed a similar positive relationship, which was highly significant for TI_1 (Adj. $R^2 = 0.1155$, $F = 20.5817$, $P < 0.0001$; Fig. 1.5b), a weaker, but still significant relationship with TI_2 (Adj. $R^2 = 0.0280$, $F = 5.3256$, $P < 0.0224$) and an insignificant relationship for TI_3 (Adj. $R^2 < 0.001$, $F = 0.8782$, $P = 0.3502$). Even though lignin-associated (TI_4) content did not contribute to similarities in SIMPER analysis (Table S1.1), TI_4 content had a highly significant inverse relationship with increasing latitude (Adj. $R^2 = 0.0926$, $F = 16.3020$, $P < 0.0001$; Fig. 1.5c). When northern and southern latitudes were compared with OM and TIs separately, the OM and TI_4 relationship became stronger and remained highly significant for the Northern Hemisphere samples (Adj. $R^2 = 0.1891$, $F = 23.8478$, $P < 0.0001$ and Adj. $R^2 = 0.1829$, $F = 22.9296$, $P < 0.0001$, respectively). Conversely, the OM and TI_4 relationship within the Southern Hemisphere samples were insignificant (Adj. $R^2 = 0.0266$, $F = 2.3942$, $P = 0.1281$ and Adj. $R^2 = 0.0061$, $F = 1.3114$, $P = 0.3576$, respectively), while the positive, significant relationship between latitude and TI_1 was maintained (Adj. $R^2 = 0.1378$, $F = 9.1508$, $P = 0.0039$).

There was no influence of taxa on leaf, rhizome or root thermograms (Table S1.1, Fig. S1.3). Non-photosynthetic above-ground tissues had significant differences among families (*Pseudo-*$F_{(4, 13)} = 3.05$, *P-perm* = 0.017) and genera (*Pseudo-*$F_{(5, 13)} = 3.76$, *P-perm* = 0.007). Pairwise tests showed that significant differences between temperate Posidoniaceae and Zosteraceae ($t = 2.83$, *P-perm* = 0.010) were driven by a higher amount of OM and TI_1 in Zosteraceae. Temperate Hydrocharitaceae and Zosteraceae were also significantly different ($t = 2.15$, *P(MC)* = 0.034), driven equally by higher OM, TI_1 and TI_3 content in Zosteraceae. Within the Hydrocharitaceae family, *Enhalus* contained higher TI_3 and OM than *Thalassia*, which was higher in TI_2 ($t = 2.91$, *P(MC)* = 0.045).

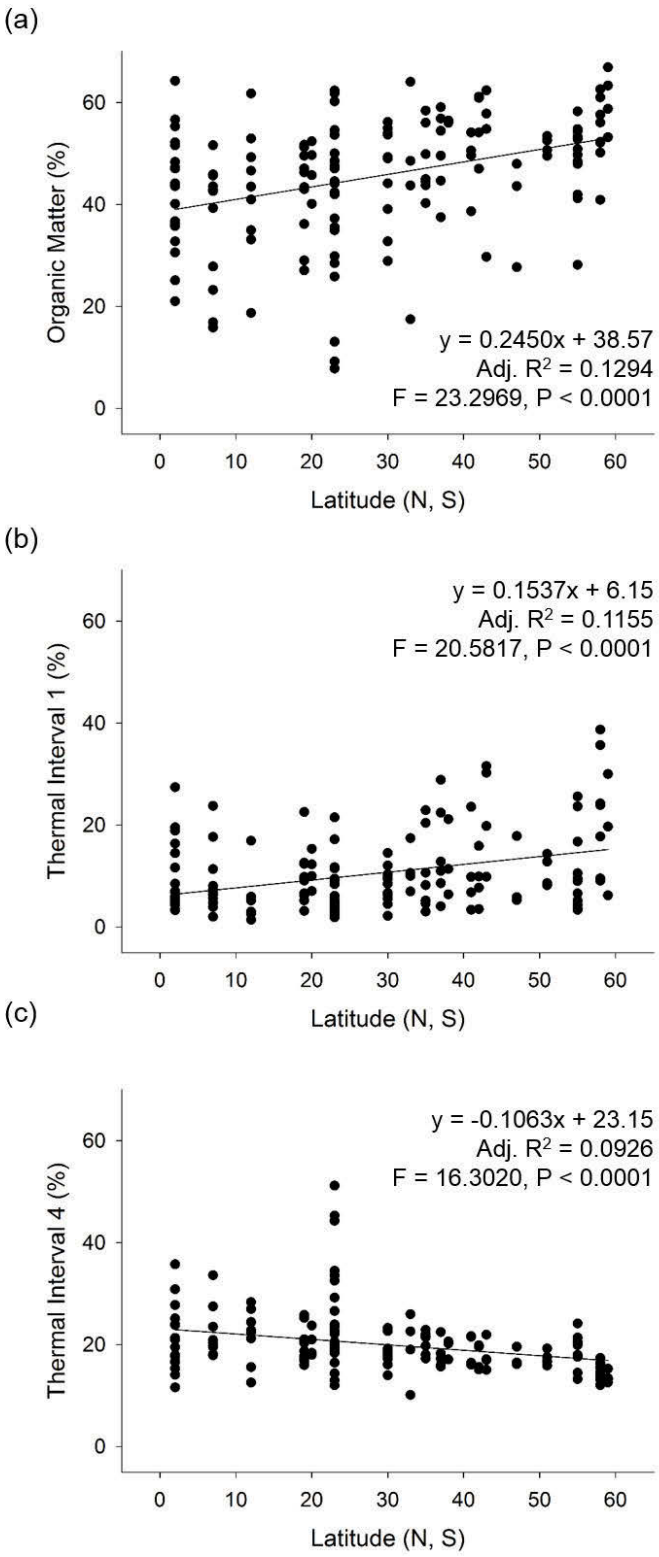


Figure 1.5: Linear regressions of the strongest relationships (adjusted $R^2 > 0.09$) between TGA (a) organic matter, (b) thermal interval 1 and (c) thermal interval 4 with latitudes for all tissue types.

Within-*Zostera* analyses showed similar trends as the overall tissue (*Pseudo-*$F_{(3, 32)}$ = 7.52, *P-perm* = 0.001) and climatic region analyses (*Pseudo-*$F_{(4, 32)}$ = 2.29, *P-perm* = 0.026; Fig. S1.4, Tables S1.1), e.g., OM drove the variation in PC1, while TI_3 drove variation in PC2 (Table S1.2). Non-photosynthetic above-ground tissues were not significantly different between leaves and rhizomes, and TI_4 did not contribute to the differences among tissue types. Instead, roots contained higher proportions of TI_2 and TI_3, and rhizomes contained higher OM and TI_1 than leaves (Fig. S1.4). Temperate Atlantic and Pacific *Zostera* species had consistently more OM and higher proportions of TI_3 and TI_1, while Tropical Atlantic and the Indo-Pacific *Zostera* had more TI_2 (Table S1.1). *Zostera* samples from the Temperate Southern Ocean were not significantly different from the other four bioregions.

NMR Molecular Mixing Model (MMM)

The NMR spectral intensity regions of each tissue type are summarised in Table S1.3 and Figure S1.5. The 4-component model (carbohydrate, protein, lignin, lipid) was chosen to predict the macromolecular content of the seagrasses. The root mean squared error (RMSE) of this model was higher than the 5-component model that included a pure carbonyl component (1.8 - 4.5 vs. 0.8 - 1.5). However, the carbonyl component typically represents a decomposition by-product, which would not be present in fresh samples and not appropriate for this study.

PCA revealed that both PC1 (55.3%) and PC2 (42.7%) were explained by variation in tissues (Fig. 1.6). PC1 was due to variation between protein-dominated leaves and carbohydrate-rich below-ground tissues. The variation in PC2 was driven by lignin-rich roots. Non-photosynthetic above-ground tissue data points were diffuse across PC2 (Fig. 1.6). PERMANOVA analysis revealed that the significantly different macromolecular content across tissue types (*Pseudo-*$F_{(3, 15)}$ = 6.09, *P-perm* = 0.001; Fig. 1.6, Table S1.1) was driven by differences between leaves and non-photosynthetic above-ground tissues (t = 4.47, *P-perm* = 0.001), leaves and rhizomes (t = 3.69, *P-perm* = 0.002), and leaves and roots (t = 2.71, *P-perm* = 0.01). SIMPER analysis showed that lignin content was highest in roots (30.4%) and lowest in leaf tissues (21.3%). Protein was highest in leaves (23.3%) and very low in non-photosynthetic above-ground tissues (7.9%) and root tissues (10.8%). Carbohydrate content was highest in rhizome tissues (64.4%).

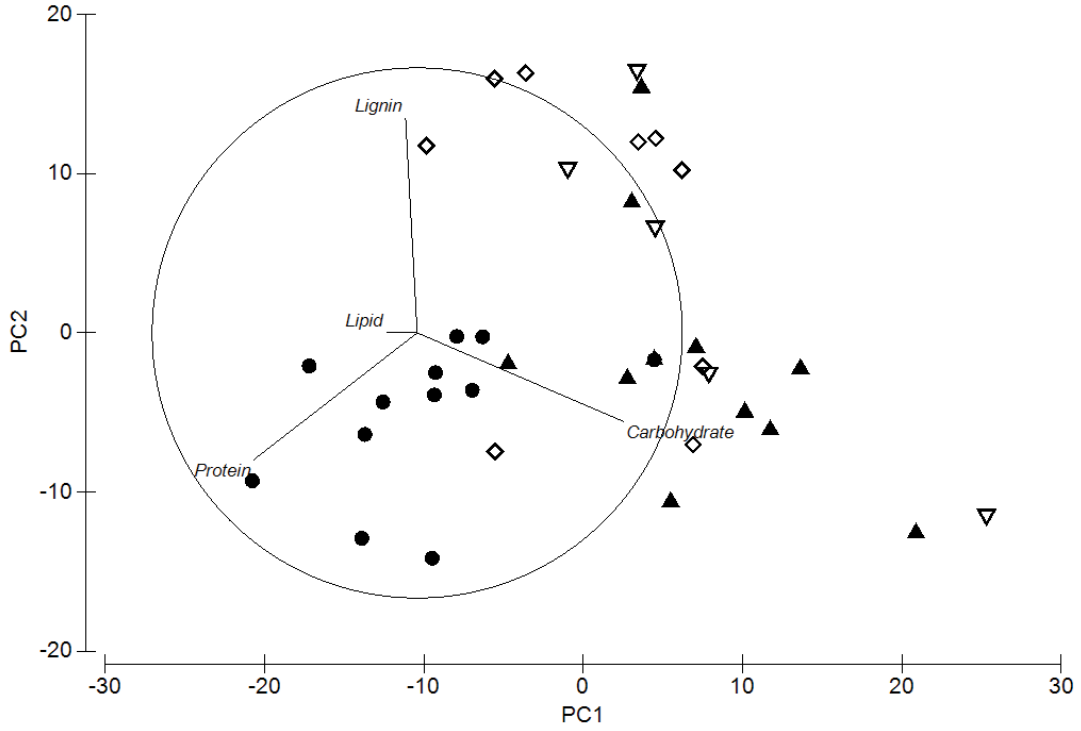


Figure 1.6: Principal components analysis of molecular mixing model predictions from NMR analysis. PC1 = 55.3% variation, PC2 = 42.7% variation. Eigenvectors are reported in Table S1.2. ● = leaf, ▽ = non-photosynthetic above-ground tissue, ▲= rhizome, ◊ = root.

There were marginally significant differences between temperate and tropical MMM predictions (*Pseudo-*$F_{(1, 15)}$ = 3.03, *P-perm* = 0.052; Table S1.1), primarily due to higher carbohydrate content in the tropical samples and more protein in the temperate samples. Lignin content was similar between climatic zones, though tropical samples contained slightly higher aromatic content (Table S1.1). Additionally, significant differences were found between a few seagrass families (*Pseudo-*$F_{(5, 15)}$ = 2.95, *P-perm* = 0.009): tropical Hydrocharitaceae and Cymodoceaceae (t =2.18, *P-perm* = 0.021) and temperate Cymodoceaceae and Posidoniaceae (t =3.09, *P-perm* = 0.006). The differences in the two tropical families were driven by higher carbohydrate and protein content in Hydrocharitaceae and higher lignin content in Cymodoceaceae (Table S1.1).

Alternatively, temperate Cymodoceaceae contained more protein and slightly more carbohydrates than Posidoniaceae, which had higher lignin content (Table S1.1).

FTIR Spectroscopy

Climatic zone explained most of the variation in FTIR data (PC1 = 33%), followed by tissue type that explained a further 20% of the variation (PC2; Figs. 1.7, 1.8). PC1 was driven by tropical seagrasses having higher lipid (~2985 cm^{-1}, lipid carbonyl stretching) and glucose (~1040 cm^{-1}), while temperate seagrass contained higher phosphorus (~1080 cm^{-1}, P=O stretching) content (Fig. 1.7a). PC2 loadings showed that leaf tissues had higher lipid content as lipid carbonyl stretching (~2860 cm^{-1}) as well as protein content indicated by higher Amide I (~1670 cm^{-1}) and Amide II (~1560 cm^{-1}) levels (Fig. 1.7b). Rhizome and root tissues were characterised by higher glucose and ribose (1020-995 cm^{-1}) and phosphorus levels (~1080 cm^{-1}) levels (Fig. 1.7b). Cellulose content (~1150 cm^{-1}) did not vary greatly among tissue types. Non-photosynthetic above-ground tissues showed intermediate biochemical qualities between leaf and rhizome tissues (Fig. 1.8).

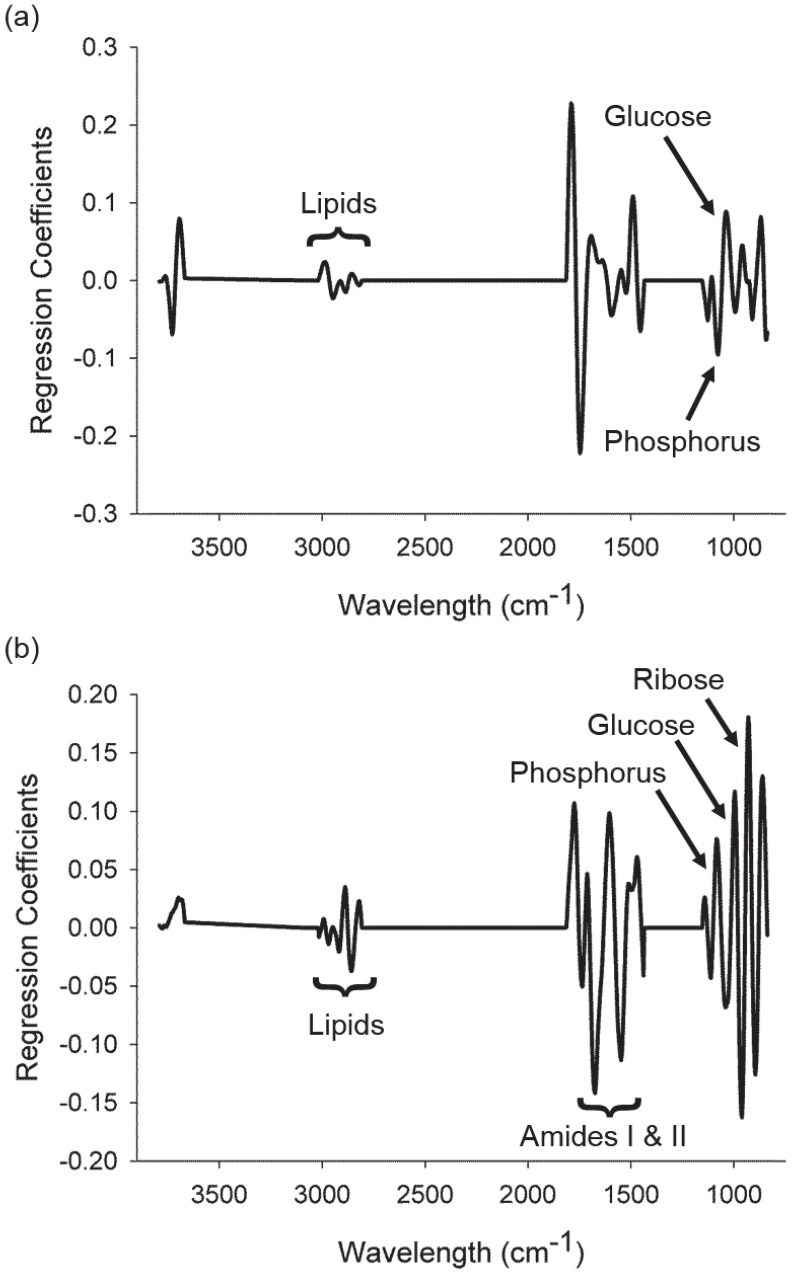


Figure 1.7: FTIR spectroscopy loadings. (a) Climatic zone (PC1 = 33% variation) where positive regressions coefficients correspond with greater signals in tropical regions and negative coefficients with temperate regions. (b) Tissue type (PC2 = 20% variation) where positive regressions coefficients correspond with greater signals in rhizomes and roots and negative coefficients with leaves. PCA scores can be found in Figure 1.8.

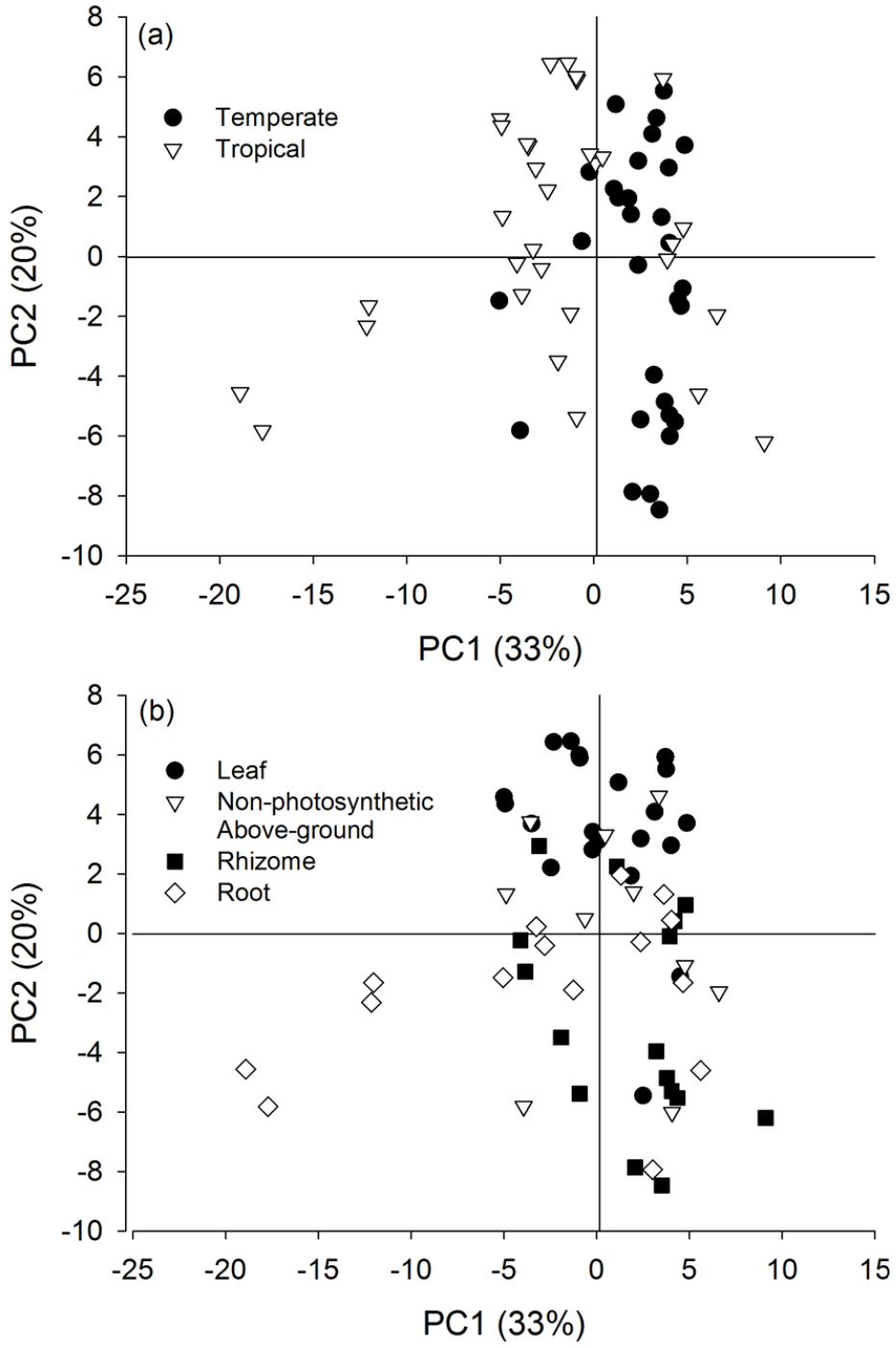


Figure 1.8: Principal components analysis of FTIR analysis shown by (a) latitudinal region and (b) tissue type.

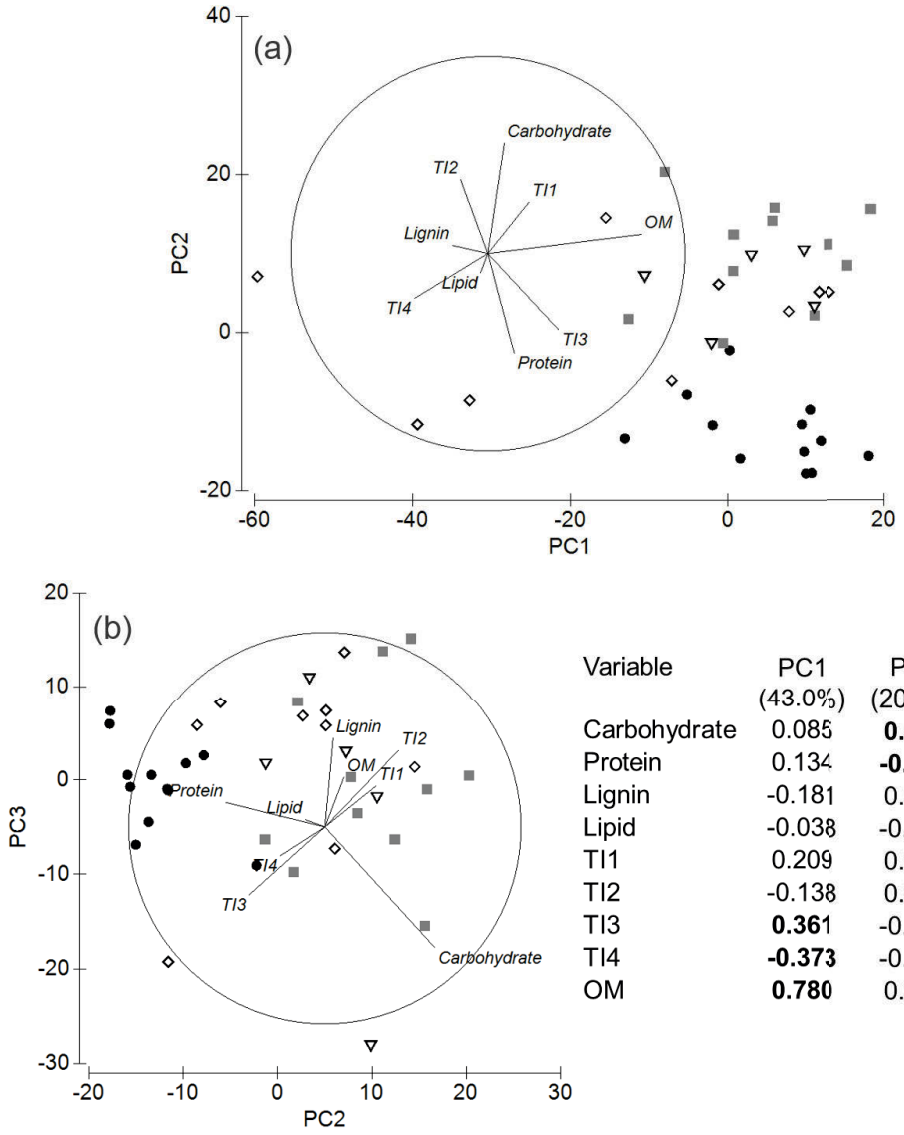
Comparison of MMM and TGA data

A Principal Components Analysis was used to quantitatively compare the NMR molecular mixing model (MMM) signatures with the TGA thermal intervals (39 samples total; Fig. 1.9). The variability in PC1 (43.0%) was driven by higher OM in most samples, while the high TI_4 content was driven by three root samples. PC2 (20.6%) was driven by higher protein in the leaf tissues and carbohydrate content in the rhizomes. Along the PC1 and PC2 axes, the orientation of protein and TI_3 were similar, while lignin was orientated between TI_4 and TI_2 (Fig. 1.9a). PC3 (13.1%) variation was driven by lignin and carbohydrate content (Fig. 1.9b). The lignin looked to be linked to root and rhizome tissues, however, plotting of the variables by genus (data not shown), suggested *Posidonia* and *Thalassodendron* were driving the higher lignin content. The carbohydrate content of *Enhalus* was driving the negative axis if PC3.

Discussion

Tissue Biochemistry and Comparison with Previous Estimates

Tissue type explained most of the variation in macromolecule content predicted by the NMR mixing model (MMM), primarily as the difference between protein content of the leaves and carbohydrate content of the below-ground tissues (PC1 = 55.3%), followed by the high lignin content of the roots (PC2 = 42.7%; Fig. 1.6). Although, lipids did not influence differences among samples (Table S1.1), concentrations were highest in leaf tissues (Figs. 1.6 & 1.7) and corresponded with previously reported concentrations for all tissues (≤ 4% DW) (Lowe and Lawrence 1976, Dawes and Lawrence 1980, 1983, Lawrence et al. 1989, Dawes and Guiry 1992, Touchette and Burkholder 2002).



Variable	PC1 (43.0%)	PC2 (20.6%)	PC3 (13.1%)
Carbohydrate	0.085	**0.561**	**-0.614**
Protein	0.134	**-0.504**	0.125
Lignin	-0.181	0.042	**0.452**
Lipid	-0.038	-0.099	0.038
TI1	0.209	0.260	0.208
TI2	-0.138	0.375	0.390
TI3	**0.361**	-0.387	-0.346
TI4	**-0.373**	-0.228	-0.146
OM	**0.780**	0.097	0.255

Figure 1.9: PCA plots for molecular mixing model and thermogravimetry variables. Plot (a) shows PC1 and PC2, and plot (b) shows PC2 and PC3. The table represents the Eigenvalues and Eigenvectors for each PC and variable. The highest correlations in each PC are bolded. Samples are presented as tissue type (● = leaf, ▽ = non-photosynthetic above-ground tissue, ■ = rhizome, ◊ = root).

The average leaf protein content (23%) predicted by MMM was higher than most studies have recorded (10-12% DW), although some have reported concentrations upwards of 20% DW (Dawes and Lawrence 1979, 1980, 1983, Dawes et al. 1987, Dawes and Guiry 1992, Siegal-Willott et al. 2010). The potential over-estimation of protein may have resulted from (a) previous underestimation of amino acid or protein content from wet chemistry methodologies that use strong chemicals to break apart molecules (e.g., Lawrence et al. 1989, Zieman et al. 1984), or (b) performing the MMM analyses under the constraint of maintaining sample-specific C:N molar ratios. When run in this manner, the MMM has no option but to allocate all sample N to protein. In samples with appreciable contents of inorganic N or non-protein forms of organic N, the allocation of C to protein within the MMM will be over-predicted. Conversely to leaf tissues, rhizome and root protein content in this study was comparable to the literature (4-17% and 4-7%, respectively) (Dawes and Lawrence 1979, 1980, 1983, Dawes et al. 1987, Dawes and Guiry 1992). Both above- and below-ground seagrass tissues take up inorganic N from the water-column and sediments, respectively (Touchette and Burkholder 2000). Unless samples were collected from areas of high nutrient loading or enrichment, most of the N in the tissues in this study would be expected to be in primarily organic forms.

Carbohydrates made up more than half of the macromolecule content in all samples and likely represented both soluble (oligosaccharide, starch, hemicellulose; Dubois et al. 1959) and insoluble (remainder by subtraction, e.g., cellulose) carbohydrate content. Studies that reported both soluble and insoluble carbohydrate content of *Zostera*, *Thalassia* and *Halophila* plants reported very similar ranges of carbohydrate content to this study for leaves (38-60% DW), rhizomes (54-70% DW) and roots (30-60% DW) (Dawes and Lawrence 1983, Dawes et al. 1987, Dawes and Guiry 1992). Data on sheath and stem components are not as common, but reported values for *Thalassia* and *Posidonia* coincided with carbohydrate (31-70% DW) and protein (2.5-10% DW) concentrations as found in this study (Appendix Table 1; Lawrence 1989, Dawes and Lawrence 1983).

The non-invasive, non-destructive solid-state NMR method and subsequent mixing model predicted much higher lignin concentrations for all tissues (21-30%) compared to previously reported values, which were, on average, ~13% DW for any given tissue (Appendix Table 1), and only occurred at such high levels in a few

individual reports for *Posidonia* and *Cymodocea* (Appendix Table 1; Bettai et al. 2015, Lawler et al. 2006, Ncibi et al. 2009). This could be a result of one or a combination of methodology effects: (a) an underestimation from using techniques that rely on chemical cell wall modification, and could result in losing material in the process (Hatfield and Fukushima 2005) and (b) an overestimation of lignin content from MMM (Baldock et al. 2004). In this latter scenario, comparing the NMR spectra to the MMM output, there was an over allocation to the N-Alkyl/Methoxyl region (60-45 ppm) suggesting the model was over-predicting the sum of lignin+protein. Although the model parameters were modified to fit the amino acid profile of seagrasses, the lignin component includes both gymnosperm and angiosperm lignin (Baldock et al. 2004). Since seagrasses are angiosperms, this may partly explain the poor relationship between the spectra and the MMM. Additionally, seagrasses contain other compounds that include aromatics other than phenolics and lignin, such as suberin and chlorophyll (Enríquez et al. 1992, Papenbrock 2012), which may further explain the deviation of the MMM.

Thermogravimetric data were compared quantitatively with the macromolecular data from MMM and qualitatively with FTIR. While TGA is useful for understanding the quality of OM of plants or sediments in terms of the labile or refractory nature of the substrate and how those qualities may influence decomposability (Capel et al. 2006, Trevathan-Tackett et al. 2015), individual compounds may be lost over broad temperature intervals making it difficult to infer absolute concentrations of such components (Yang et al. 2006). Cellulose content was not significantly different among tissues according to FTIR analysis (Fig. 1.7), yet TGA analyses showed that cellulose-associated TI_3 was variable among tissues types (Fig. 1.4), which suggests that other components may contribute to TI_3. TGA studies on vascular plants are often linked to biochar or biofuel applications, which assess lignocellulose content of plant residues or by-products (Yang et al. 2006, Wang et al. 2007, Carrier et al. 2011, Chadwick et al. 2014), and therefore, do not take into account other components like soluble carbohydrates, proteins or lipids found in whole plant tissues. In the comparison between the MMM and TGA data, protein and TI_3 showed similar orientations along the PC1 and PC2 axes (Fig. 1.9a). Pyrolysis of protein- and lipid-rich microalgae has shown that these components can be lost at temperatures $\geq 300°C$ (Kapusniak and Siemion

2007, Kebelmann et al. 2013); therefore, it is possible that the relatively high protein and lipid levels found in leaves are linked with the TI_3 mass loss between 300-400°C.

Rhizomes had the highest OM content, which was dominated by labile OM from TI_1 (Fig. 1.4). Since rhizomes contain the energy reserves for seagrasses, the abundance of labile OM was likely due to storage of simple carbohydrates, and was potentially masking the relatively large lignocellulose content from TI_3 and TI_4 that would be expected in these rigid tissues. More than a quarter of the mass of pure sucrose is lost before 275°C in thermogravimetric analyses (Eggleston et al. 1996), and links with the large mass loss of TI_1 between 150-220°C for the rhizome tissues (Fig. 1.3), as well as to the similarity with MMM carbohydrates and TI_1 and TI_2 along the PC2 axis in the comparative PCA (Fig. 1.9a). In contrast to the rhizomes, roots had the least amount of total OM with more than half the OM content as refractory OM (TI_3 and TI_4; Fig. 1.4). The significantly higher proportion of TI_4 coincides with significantly higher lignin content predicted by the MMM compared to the other tissues, although this was depicted in only 3 of the 10 root samples analysed by NMR (Fig. 1.9a). As hypothesised, increased lignin content would support better anchorage into sediments (Barnabas 1991), but also could be involved in protection against microorganism invasions or water-logging (Kuo and Cambridge 1978). Interestingly, FTIR analysis indicated that both rhizome and root tissues also had higher amounts of phosphorus compared to leaves (Fig. 1.7b). Rhizomes and roots do take up inorganic P from sediment porewater (Touchette and Burkholder 2000), and so higher P signals may represent this stored or in-transit inorganic P in the below-ground tissues.

Climatic Variation in Seagrass Organic Composition

Trends in climatic variation are likely strongly influenced by taxa since most genera are strictly found in either tropical or temperate regions. This confounding factor needs to be considered when making any general inference between tropical and temperate seagrasses. Therefore, we chose to analyse the data in three ways in order to draw meaningful conclusions about the data within these limitations. First, both tropical and temperate groups of seagrass contain both small, ephemeral genera and large, persistent genera. While the number of samples obtained for the large taxa were higher for the tropical (9) compared to the temperate (3) samples due to limitations in collecting protected, endemic Australian *Posidonia*, we were otherwise satisfied that the

range of morphology-based functional groups were represented in both climatic regions (Orth et al. 2006, Carruthers et al. 2007). Second, when we analysed taxonomic variation in organic composition, we accounted for confounding factors by normalising for climatic region and tissue type (i.e., genera nested within family, family nested in climatic region for each tissue type). Lastly, *Zostera* was the most well sampled genus and contained both temperate and tropical samples. We took advantage of this broad sampling and used *Zostera* as a case-study for climatic influence within a taxonomic group.

The location of seagrasses, whether temperate or tropical, had a strong influence on TGA thermograms and FTIR spectra when all samples were analysed together (PC1 = 33-57%; Figs. 1.2a, 1.8a). The influence of climatic region was also maintained once tissue types were separated for statistical analysis (PC1 = 42.6-70.1%; Fig. S1.1) and when analysing the *Zostera* genus alone (PC1 = 53%; Fig. S1.4). Temperate seagrasses were characterised by higher OM content, primarily as TI_1 but also TI_3, across all tissue types (Table S1.1), with a positive correlation between OM and higher latitudes (Fig. 1.5a). This suggests that temperate seagrasses consistently have more labile storage carbohydrates in their tissues compared to tropical seagrasses. This is consistent with increasing biomass and production of above-ground tissues with increasing latitude found by Duarte and Chiscano (1999), although no latitudinal trends were previously found with below-ground biomass and production (Duarte and Chiscano 1999). Lastly, some of the variability between *Zostera* from temperate and tropical regions could be due to differences in physiochemical conditions, which has been shown to influence seagrass morphology and phenotypic plasticity in the same species (Peralta et al. 2005, Peralta et al. 2006, Bricker et al. 2011).

The differences in labile OM between temperate and tropical seagrasses in this study were not likely related to light regimes since seagrasses are able to acclimate to light levels without affecting productivity (Lee et al. 2007). Time of collection was also unlikely to be an influence since similar proportions of temperate seagrasses were collected in summer, autumn and winter and tropical seagrasses in summer and winter. While, the grazing pressures and nutrients regimes of the samples collected in this study are unknown or at best can only be estimated, the greater occurrences of nutrient limitations and grazing of tropical seagrasses may limit their biomass and production (Duarte and Chiscano 1999). Furthermore, grazing has been shown to be a strong

influence on carbohydrate reserves in both above- and below-ground tissues due to the export of soluble carbohydrates to support new leaf growth (Moran and Bjorndal 2007, Eklöf et al. 2008). It is also possible that in response to grazing, seagrasses could upregulate production of aromatics like tannins, phenolics or lignin as a defence mechanism (Arnold and Targett 2002, Arnold et al. 2008, Vergés et al. 2008), leading to proportionally lower labile OM compared to temperate seagrasses.

Biochemical Variation among Seagrass Taxa

Variation among taxa was not consistent across families (grouped within climatic zones) or genera (grouped within families) for NMR or TGA analyses. MMM analysis inclusive of all tissue types revealed that there was significantly higher lignin content in tropical Cymodoceaceae than tropical Hydrocharitaceae (Table S1.1). This observation is in line with the previous reports of lignin content in these families (review in Appendix Table 1). The MMM predicted lower lignin content in temperate Cymodoceaceae compared to temperate Posidoniaceae (Table S1.1). While Posidoniaceae had the highest lignin content across all temperate seagrasses and tissue types (mean of 22.2% DW; Appendix Table 1), this study is the first to report lignin content for temperate Cymodoceaceae seagrasses.

Within-tissue type comparison of thermogravimetric data revealed that taxa did not influence the OM quality in leaf, rhizome or root tissues (Table S1.1). This suggests that while some taxa may be more robust and have more above- and below-ground biomass than others, the overall OM quality is consistent across all seagrass species included in this study. OM quality of non-photosynthetic above-ground tissues had the most variation across taxa (Fig. S1.3b, Table S1.1). At the family-level, temperate Posidoniaceae had more refractory OM as TI_4 and TI_3 than Zosteraceae suggestive of higher lignocellulose content, while Zosteraceae contained proportionally more storage products (i.e., OM and TI_1). While there is no data on lignocellulose content of *Zostera* sheaths or stems, *Posidonia* sheaths are known to have high lignocellulose content (Ncibi et al. 2009). There were also significant differences between temperate Hydrocharitaceae (*Halophila* stem) and Zosteraceae (sheaths and stems) driven by the higher total OM content of Zosteraceae seagrasses as TI_1 and TI_3. Again, very little is known about the organic composition of these tissues, but *Halophila* stems may be more abundant in hemicelluloses (TI_2) than storage products (TI_1) and cellulose (TI_3).

Within the tropical Hydrocharitaceae family, the more robust *Enhalus* had more TI_3 and OM than *Thalassia*, which is indicative of cellulose and possibly protein. *Thalassia* sheaths had more of its OM as hemicellulose-associated TI_2. These results indicate that sheath and stem biomass can significantly vary across taxa, and that more morphologically robust taxa typically have higher refractory OM content. The diversity in OM quality of the sheaths may also be related to their protective function of the leaf meristem and new shoots (Hemminga and Duarte 2000, Kuo and Hartog 2006). This protective function could be more important for the morphologically robust taxa, or *K*-strategists, characterised by slower production and turnover rates (Duarte and Chiscano 1999).

Seagrass Contribution to as Blue Carbon Stocks

The quality of organic C is often a major factor in ecosystem C-cycling since the remineralisation by microbes relies both on stoichiometry (C:N:P:S ratios) and production of specific exo-enzymes by microbes and decomposers for the breakdown of complex C such as lignin (Enríquez et al. 1993, Couteaux et al. 1995, Silver and Miya 2001, Sinsabaugh et al. 2002). It is likely that nutrient-rich leaves containing higher lipid and protein content will more likely be consumed by herbivores before it enters the detrital pool (Cebrián 1999) or will be rapidly used during decomposition (Canuel and Martens 1996, Mateo et al. 2006). The holocellulose polysaccharides and non-lignin phenolic compounds, on the other hand, may persist and contribute to C stocks as they are difficult to remineralise in coastal environments (Godshalk and Wetzel 1978, Enríquez et al. 1993). Non-photosynthetic tissues, which were relatively deplete in labile OM, lipids and proteins, contained more aromatic compounds than leaves and thus have potential to contribute refractory C in blue carbon systems, e.g., a large component of *Posidonia* mattes (Kuo and Cambridge 1978, Romero et al. 1992, Mateo et al. 1997). It is possible in meadows with high sedimentation rates and rapid burial, the amount of above-ground detritus would be more slowly remineralised under anoxic conditions and thus would enhance blue carbon stocks (Kuo and Cambridge 1978).

As hypothesised, below-ground biomass is likely to be the highest and most consistent contributor of refractory C in seagrass meadows for several reasons. First, root tissues were found to contain the highest amount of refractory macromolecules in the current study, and therefore have potential for significant contributions to blue

carbon stocks in seagrass habitats. Although, rhizomes had a high proportion of labile C, the remaining lignocellulose (TI_3 + TI_4 = 50-60% DW or lignin/phenolics = 23-30% DW) will be slowly, anaerobically decomposed until the substrate is no longer able to be utilised by microbes through enzymatic or physiochemical limitations (e.g., adsorption) (Pellikaan 1982, Burdige 2007, Conant et al. 2011). In addition to the living C stocks rhizome and roots tissues being typically higher per area than above-ground tissues (Fourqurean et al. 2012), these tissues are subjected to less grazing and exportation and are protected from physical removal or disturbances, which would promote further C accumulation. Lastly, it has been shown that below-ground tissues had a high percent (~15%) of biomass pyrolysised > 600°C not present in above-ground tissues (Trevathan-Tackett et al. 2015). This fraction of thermally stable biomass was hypothesised to be attributed to sulphation of polysaccharides and a mechanism for enhanced C contribution and sequestration (Trevathan-Tackett et al. 2015).

Preliminary data from another global survey, indicated that temperate seagrasses have more organic C on an areal basis in their living biomass compared to tropical seagrasses (~8.6x; Fourqurean et al. 2012). While we have shown that this variation from autochthonous seagrass C could be due to greater carbohydrate reserves (labile C) in temperate seagrasses, we would expect much of the labile C would be quickly remineralised by microbes *in situ* (Harrison 1989, Vichkovitten and Holmer 2004). Preliminary data from Fourqurean et al. (2012) also showed that there is more sediment C_{org} per area in temperate than tropical habitats (5.1x), but deeper sampling by latitude and species is needed to make inferences concerning climatic influences on seagrass C and C_{org} stocks (i.e., sedimentation, particle trapping). In tropical habitats, grazing may be an important factor that affects OM content and quality by increasing relative refractory OM content (and sequestration potential) via production of cell wall components or aromatic anti-herbivory compounds (Vergés et al. 2008, Steele and Valentine 2012).

Overall, there was very little variation in the quality of OM content across seagrass taxa within a climatic zone or tissue type. In some cases, i.e., sheaths and stems, morphologically larger taxa, like *Posidonia* and *Enhalus*, have potential to contribute more refractory organics to blue carbon habitats in addition to providing other functions that can enhance C-sequestration like sediment stabilisation and particle capture (Duarte and Chiscano 1999, Orth et al. 2006). However, this is likely to be

highly dependent upon the environment, particularly hydrodynamics and allochthonous inputs (Lavery et al. 2013a), which could ultimately affect sedimentation rates and vertical growth of the seagrass and thus the amount of organic carbon available for sequestration (Macreadie et al. 2014a).

We have provided a conceptual model detailing the variation in seagrass carbon/OM quality (Fig. 1.10). For the sake of simplicity, we focused on using the TGA results to rank the C quality, while understanding that many factors (e.g., microbial remineralisation, production and biomass, sediment type and burial rates, etc.) will strongly influence the ultimate fate of seagrass-C sequestration. In summary, we highlighted the importance of considering the location of a seagrass (temperate or tropical) as a driver of C quality, in addition to demonstrating the potential contributions that sheaths and stems can make to C stocks. We encourage the data produced herein to be incorporated in future modelling of C-sequestration processes, and to be used for considerations of blue carbon ecosystem management.

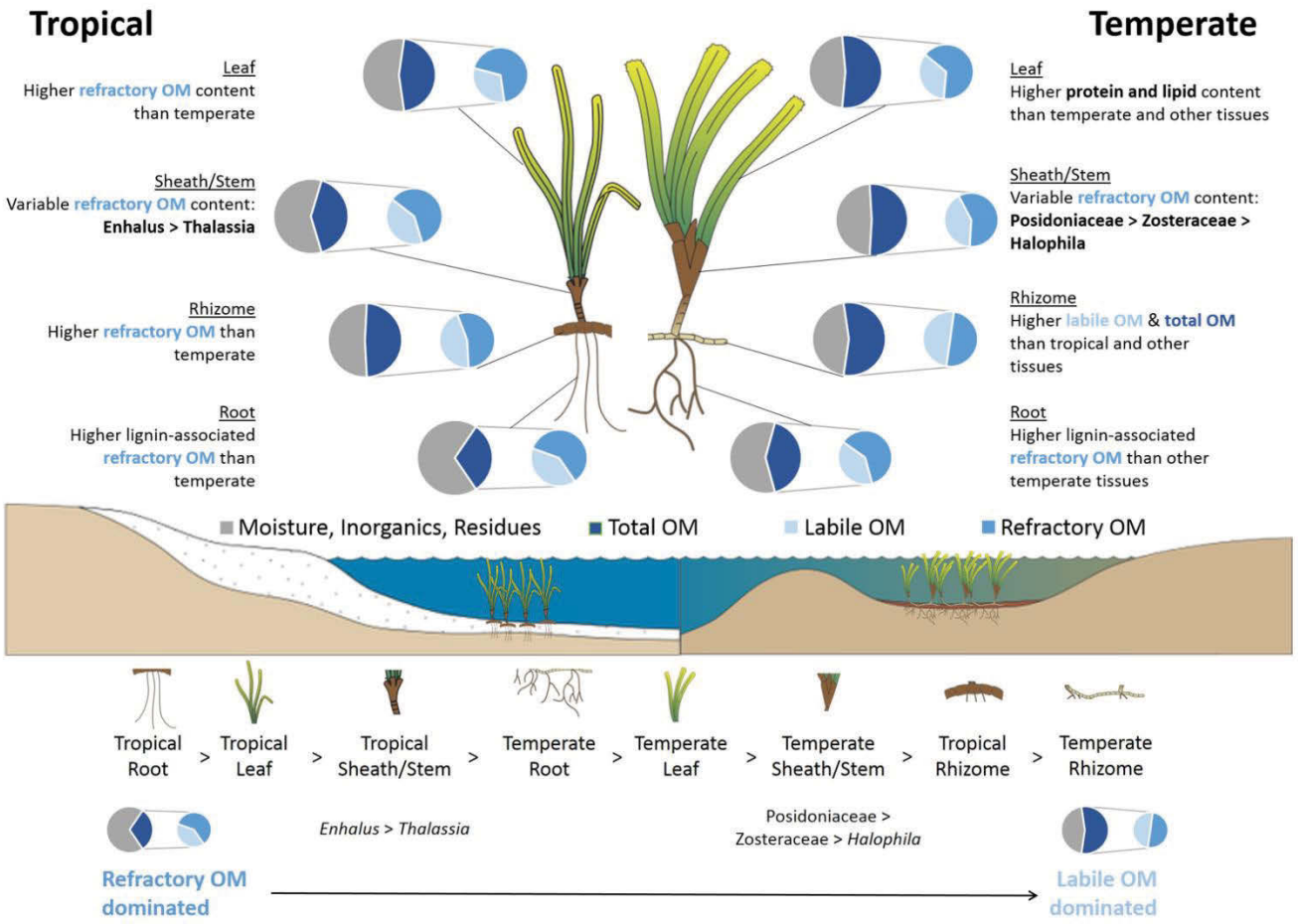


Figure 1.10: Conceptual model describing the organic matter (OM) quality of seagrasses in different climatic regions, tissue types and taxa based on the thermogravimetric analyses. The model also includes a ranking of seagrass tissues based on refractory OM-dominated to labile OM-

dominated characteristics. Total OM is represented by the proportion of total mass that was pyrolysised from 180-600°C, while moisture, inorganics and residues are the proportions lost below 180°C and that remained after pyrolysis to 600°C, respectively. Labile OM is the proportion of total OM primarily as soluble carbohydrates and hemicellulose ($TI_1 + TI_2$; 180-300°C), and refractory OM is the proportion of total OM as cellulose and lignin (including possible char residues from pyrolysis, $TI_3 + TI_4$; 300-600°C). Symbols used here are courtesy of the Integration and Application Network, University of Maryland Center for Environmental Science (ian.umces.edu/symbols/).

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This project would not have happened without all the fellow seagrass researchers and their lab groups who volunteered their time and efforts in collecting and sending the seagrass samples used in this study: Pemika Apichanangkool, Alecia Bellgrove, Marjolijn Christianen, Laura Govers and Jim de Fouw, Dominik Kneer, Cloverley Lawrence, Gilles Lepoint, Peter Macreadie, Masahiro Nakaoka and Minako Ito, Lina Nordlund, Michael Rasheed and Jac Davies, Lina Rasmusson, Tiina Salo and Camillia Gustafson, Tim Sherman, João Silva, Brooke Sullivan, Yoshiyuki Tanaka and Paul York. We want to acknowledge the contributions of Janine McGowan and Bruce Hawke for providing training and assistance in the acquisition and processing of NMR and FTIR spectra.

Chapter 1 Supplementary Tables and Figures

Table S1.1: SIMPER table of significant PERMANOVA pairwise comparisons of thermogravimetric analysis (TGA) and molecular mixing model (MMM). Values represent average abundance of each variable followed by the percent contribution in parentheses. NP = Non-photosynthetic above-ground tissue, Temp. = temperate, Trop. = tropical, Atl. = Atlantic. Thermal intervals (TI) represent distinct organic matter components from TGA normalised to total organic matter (TI_1: labile, carbohydrates, hemicellulose, 180°C to 220°C; TI_2: labile, carbohydrates, hemicellulose, 220°C to 300°C; TI_3: refractory, cellulose, 300°C to 400°C; TI_4: refractory, lignin and residues, 400-600°C).

		TGA					MMM			
Pairwise Comparison		Avg. Sq. Dist.	OM	TI_1	TI_2	TI_3	TI_4	Avg. Sq. Dist.	Carbo-hydrates	Lignin	Protein
Tissue											
	Leaf - Rhizome	674	48.8-51.0 (20.2)	8.58-15.8 (25.8)	24.1-31.0 (19.2)	46.8-36.4 (28.7)		474	51.8-64.4 (45.7)	21.3-23.4 (12.2)	23.3-11.3 (39.6)
	Leaf - Root	930	48.8-35.3 (45.5)		24.1-31.4 (13.5)	46.8-36.4 (23.1)	20.5-24.3 (10.7)	443	51.8-56.0 (17.7)	21.3-30.4 (33.7)	23.3-10.8 (45.9)
	Rhizome - Root	1079	51.0-35.5 (46.4)	15.8-7.55 (15.8)	31.0-31.4 (11.9)	36.4-36.4 (12.6)	16.8-24.3 (13.3)				
	Leaf - NP	496	48.8-46.8 (33.4)	8.58-10.3 (21.1)	24.1-30.6 (20.3)	46.8-41.0 (19.6)		657	51.8-63.1 (37.3)	21.3-28.0 (19.7)	23.3-7.93 (41.6)
	Rhizome - NP	614	51.0-46.8 (31.2)	15.8-10.3 (27.4)	31.0-30.6 (18.6)	36.4-41.0 (18.7)					
	Root - NP	866	35.3-46.8 (48.0)	7.55-10.3 (10.4)	31.4-30.6 (12.2)	36.4-41.0 (15.7)	24.3-18.1 (13.8)				

		TGA						MMM		
Pairwise Comparison		Avg. Sq. Dist.	OM	TI_1	TI_2	TI_3	TI_4	Avg. Sq. Dist.	Carbo-hydrates	Lignin	Protein
Latitudinal Region											
Temperate-Tropical								384	55.7-59.9 (37.9)	25.4-24.8 (27.44)	16.7-13.0 (32.45)
	Leaf	371	52.9-45.4 (38.7)	11.9-5.94 (29.7)	22.4-25.5 (8.47)	46.7-46.8 (15.3)					
	Rhizome	649	54.6-48.5 (23.9)	20.6-12.4 (30.4)	28.8-32.5 (22.9)	35.4-37.2 (18.4)					
	Root	912	41.4-31.1 (45.6)		31.5-31.3 (11.6)	41.1-33.0 (19.6)	19.6-27.7 (17.9)				
	NP	643	51.9-40.7 (42.5)	14.2-5.61 (23.3)	26.9-35.0 (19.4)	41.8-40.0 (11.7)					
Taxa											
Family											
Hydrocharitaceae - Zosteraceae	NP (Temp.)	417	45.0-54.4 (27.7)	10.6-15.7 (21.1)	34.5-26.0 (20.9)	33.3-42.0 (23.0)					
Posidoniaceae - Zosteraceae	NP (Temp.)	493	43.6-54.4 (31.3)	4.36-15.7 (38.9)	30.1-26.0 (12.5)	45.7-42.0 (14.4)					
Cymodoceaceae - Posidoniaceae	(Temp.)							454	58.3-55.3 (12.2)	17.4-30.8 (48.3)	23.0-12.4 (38.7)

		TGA					MMM			
Pairwise Comparison		Avg. Sq. Dist.	OM	TI_1	TI_2	TI_3	TI_4	Avg. Sq. Dist.	Carbo-hydrates	Lignin	Protein
Hydrocharitaceae - Cymodoceaceae	(Trop.)							467	61.8-57.4 (40.1)	21.1-29.5 (33.6)	15.4-10.2 (24.3)
Genus											
Enhalus-Thalassia	NP	746	44.6-32.5 (23.4)		25.9-40.9 (34.2)	47.3-31.2 (39.0)					
Zostera											
Tissue	Leaf - Rhizome	758	50.7-52.4 (14.7)	11.0-18.3 (28.5)	24.3-32.2 (22.0)	45.5-34.4 (31.0)					
	Leaf - Root	660	50.7-38.2 (47.9)	11.0-6.01 (15.8)	24.3-34.1 (18.0)	45.5-39.6 (15.1)					
	Rhizome - Root	939	52.4-38.2 (42.1)	18.3-6.01 (26.1)	32.2-34.1 (11.5)	34.4-39.6 (15.6)					
	Root - NP	633	38.2-53.4 (59.4)	6.01-13.7 (15.7)	34.1-27.2 (9.87)	39.6-42.6 (10.3)					
Bioregion	Temp. Atl. - Trop. Atl.	827	51.8-36.2 (41.7)	14.3-9.14 (17.0)	27.6-37.3 (18.5)	41.2-33.1 (18.8)					
	Temp. Atl.- Indo-Pacific	668	51.8-40.0 (32.4)	14.3-8.78 (21.3)	27.6-37.3 (22.7)	41.2-34.4 (20.3)					

		TGA					MMM			
Pairwise Comparison		Avg. Sq. Dist.	OM	TI_1	TI_2	TI_3	TI_4	Avg. Sq. Dist.	Carbo-hydrates	Lignin	Protein
	Trop. Atl. - Temp. Pacific	489	36.2-44.6 (35.7)	9.14-10.4 (12.0)	37.3-29.7 (21.8)	33.1-41.6 (26.0)					
	Temp. Pacific - Indo-Pacific	383	44.6-40.0 (26.2)	10.4-8.78 (14.9)	29.7-37.3 (27.6)	41.6-34.4 (27.9)					

Table S1.2: Summary of Principal Components Analyses (PCA) Eigenvectors from Chapter 1. NP-AG = Non-Photosynthetic Aboveground Tissues.

TGA PCA Eigenvectors	Fig. 1.1: All Samples		Fig. S1.1a: Leaves		Fig. S1.2a: NP-AG Tissue		Fig. S1.1c: Rhizome		Fig. S1.1d: Root		Fig. S1.4: *Zostera*	
	PC1 (56.8%)	PC2 (25.9%)	PC1 (57.9%)	PC2 (31.8%)	PC1 (67.1%)	PC2 (22.1%)	PC1 (46.2%)	PC2 (30.2%)	PC1 (70.1%)	PC2 (18.1%)	PC1 (52.7%)	PC2 (35.0%)
TI_1	0.298	-0.54	0.514	0.599	0.452	0.627	0.655	-0.382	0.149	-0.053	0.56	0.404
TI_2	-0.257	-0.369	-0.239	0.116	-0.443	0.18	-0.537	-0.474	-0.097	-0.838	-0.29	0.445
TI_3	0.313	0.726	0.047	-0.724	0.151	-0.742	0.087	0.78	0.422	0.428	-0.053	-0.764
TI_4	-0.339	0.165	-0.322	0.008	-0.161	-0.065	-0.195	0.08	-0.443	0.293	-0.218	-0.096
OM	0.795	-0.132	0.757	-0.321	0.742	-0.137	0.487	-0.118	0.77	-0.162	0.743	-0.213

MMM PCA Eigenvectors	Fig. 1.6: All Samples	
	PC1 (55.3%)	PC2 (42.7%)
Carbohydrate	0.777	0.334
Protein	-0.617	0.479
Lignin	-0.044	-0.812
Lipid	-0.117	-0.001

Table S1.3: Raw spectral intensities of main ^{13}C-CPMAS NMR functional groups expressed as % of total spectral intensity for selected samples subsample. The spectral ranges integrated for each functional group were amide/carboxyl/ketone (215–165 ppm), O-aromatic (165–145 ppm), aromatic (145–110 ppm), di-O-alkyl (110–95 ppm), O-alkyl (95–60 ppm), N-alkyl/methoxy (60–45 ppm), and alkyl (45 to 10 ppm).

Family	Species	Country	Tissue	Alkyl	N-Alkyl/ Methoxyl	O-Alkyl	Di-O-Alkyl	Aromatic	O-Aromatic	Amide/Ketone /Carboxyl
Cymodoceaceae	*Amphibolis antarctica*	Australia, VIC	Leaf	7.53	4.08	51.34	9.82	11.40	4.67	11.17
	Cymodocea nodosa	Portugal	Leaf	12.60	5.83	47.26	9.38	9.74	3.85	11.33
			Rhizome	6.01	4.99	57.10	11.54	7.53	3.76	9.08
			Root	9.74	5.66	49.73	10.62	9.43	4.90	9.93
	Halodule uninervis	Australia, QLD	Rhizome	3.31	2.47	57.64	13.55	9.39	4.07	9.56
			Root	12.08	5.52	38.45	9.36	10.68	6.10	17.81
	Syringodium isoetifolium	Madagascar	Leaf	11.11	4.75	47.38	10.27	10.89	4.40	11.18
			Rhizome	9.38	3.50	50.99	11.01	8.77	3.63	12.72
			Root	5.61	2.32	48.38	11.24	11.65	4.90	15.90
	Thalassodendron ciliatum	Madagascar	Leaf	10.73	4.48	45.46	10.27	11.85	5.12	12.09
			Vertical Rhizome	8.92	5.56	45.75	11.35	13.59	6.33	8.51
			Rhizome	5.08	4.89	47.34	12.01	14.91	7.44	8.32
			Root	5.70	5.63	48.45	11.97	14.51	6.35	7.41

Family	Species	Country	Tissue	Alkyl	N-Alkyl/ Methoxyl	O-Alkyl	Di-O-Alkyl	Aromatic	O-Aromatic	Amide/Ketone /Carboxyl
Hydrocharit-aceae	*Enhalus acoroides*	Australia, QLD	Leaf	12.92	5.04	48.98	10.87	7.98	3.18	11.04
			Sheath	1.72	1.43	67.02	14.43	5.87	2.11	7.42
			Rhizome	1.98	2.45	65.52	14.43	6.16	3.09	6.37
			Root	7.32	3.86	55.15	11.88	7.69	3.49	10.61
	Halophila ovalis	Australia, QLD	Leaf	10.08	4.50	43.92	10.00	9.96	4.13	17.41
			Rhizome	5.34	2.93	52.86	11.90	8.86	3.53	14.58
	Thalassia hemprichii	Madagascar	Leaf	11.12	4.64	44.98	10.51	11.54	4.55	12.65
			Rhizome	5.27	2.71	52.72	12.62	11.15	4.87	10.65
			Root	7.77	4.05	41.30	9.96	13.02	5.83	18.07
Posidoniaceae	*Posidonia australis*	Australia, NSW	Leaf	10.47	3.85	46.51	11.38	12.32	5.40	10.07
			Sheath	6.73	4.42	49.71	11.67	11.96	5.64	9.87
			Rhizome	6.86	4.47	49.41	11.62	13.17	6.65	7.82
			Root	10.98	5.15	41.69	11.02	15.93	7.38	7.84
	Posidonia oceanica	France	Leaf	11.16	4.20	47.26	10.57	11.69	4.45	10.67
			Sheath	4.15	4.51	47.94	10.83	18.59	6.74	7.24
			Rhizome	5.32	4.50	49.81	12.11	14.70	6.85	6.70
			Root	6.33	4.72	49.55	10.83	16.28	6.12	6.16

Family	Species	Country	Tissue	Alkyl	N-Alkyl/ Methoxyl	O-Alkyl	Di-O-Alkyl	Aromatic	O-Aromatic	Amide/Ketone /Carboxyl
Ruppiaceae	*Ruppia maritima*	Sweden	Leaf	10.52	5.53	46.23	9.69	12.01	4.97	11.06
			Sheath	5.05	2.97	55.30	11.96	10.17	4.74	9.80
			Rhizome	6.36	3.71	56.50	11.65	8.24	4.12	9.42
			Root	8.60	4.27	51.42	11.33	9.85	4.01	10.52
Zosteraceae	*Zostera muelleri*	Australia, VIC	Leaf	17.01	7.10	41.26	8.27	11.03	3.58	11.75
		Australia, QLD	Leaf	11.39	4.94	46.27	9.75	12.16	3.70	11.79
			Rhizome	5.75	3.35	57.87	12.11	8.59	2.99	9.35
			Root	7.43	3.60	53.45	11.38	9.14	3.44	11.55
	Zostera nigricaulis	Australia, VIC	Leaf	15.95	6.41	39.73	8.95	11.00	3.66	14.30

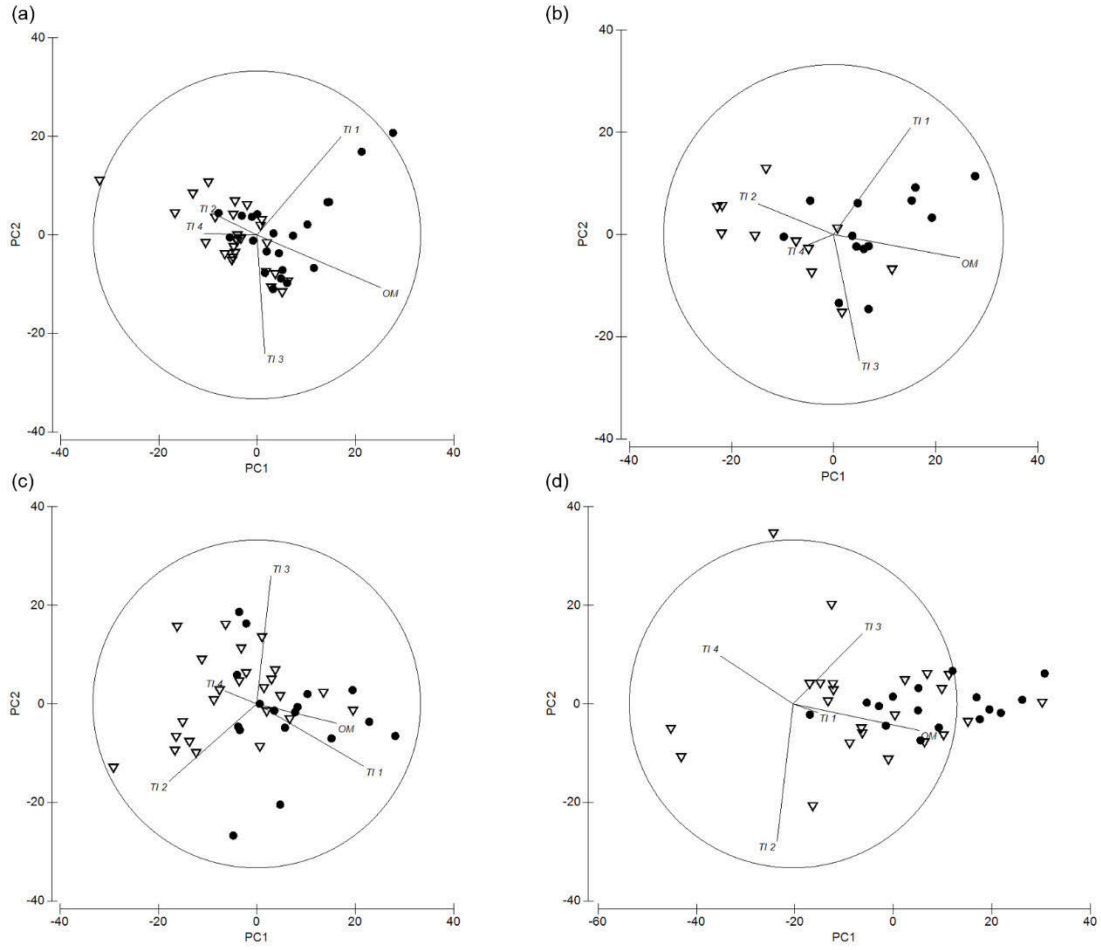


Figure S1.1: Principal components analysis of seagrass organic matter quality using thermogravimetric analyses for (a) leaves (PC1 = 57.9% variation, PC2 = 31.8% variation), (b) non-photosynthetic above-ground tissues (PC1 = 67.1% variation, PC2 = 22.1% variation), (c) rhizome (PC1 = 46.2% variation, PC2 = 30.2% variation), (d) root (PC1 = 70.1% variation, PC2 = 18.1% variation) by latitudinal region (● = temperate and ▽ = tropical). Eigenvectors are reported in Table S1.2. Thermal intervals (TI) represent distinct organic matter components from TGA normalised to total organic matter (TI_1: labile, carbohydrates, hemicellulose, 180°C to 220°C; TI_2: labile, carbohydrates, hemicellulose, 220°C to 300°C; TI_3: refractory, cellulose, 300°C to 400°C; TI_4: refractory, lignin and residues, 400-600°C).

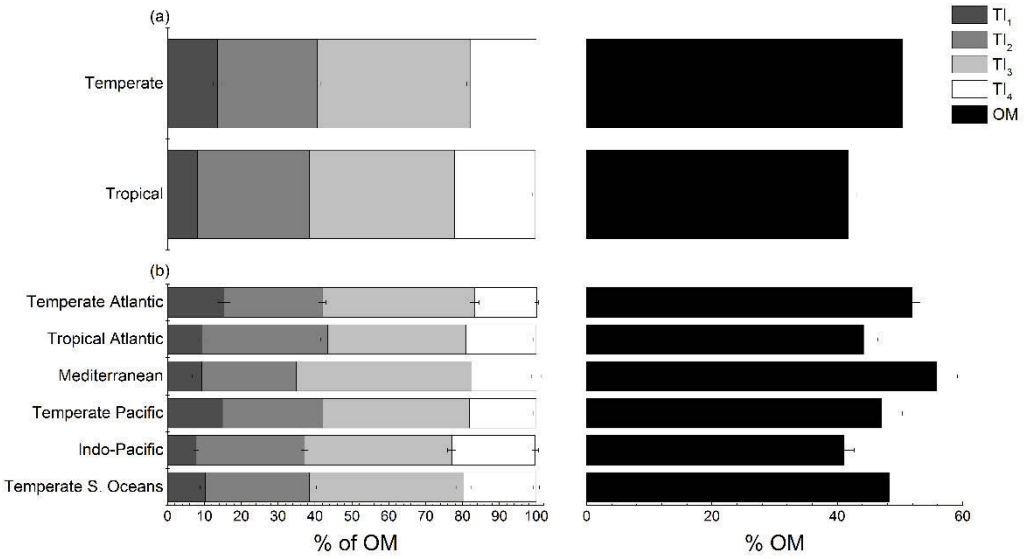


Figure S1.2: Seagrass organic matter quality for all tissues types from thermogravimetric analyses between (a) temperate and tropical regions and (b) across all bioregions. Thermal intervals (TI) represent distinct organic matter components from TGA normalised to total organic matter (TI_1: labile, carbohydrates, hemicellulose, 180°C to 220°C; TI_2: labile, carbohydrates, hemicellulose, 220°C to 300°C; TI_3: refractory, cellulose, 300°C to 400°C; TI_4: refractory, lignin and residues, 400-600°C). Values represent mean ± 1 S.E.M.

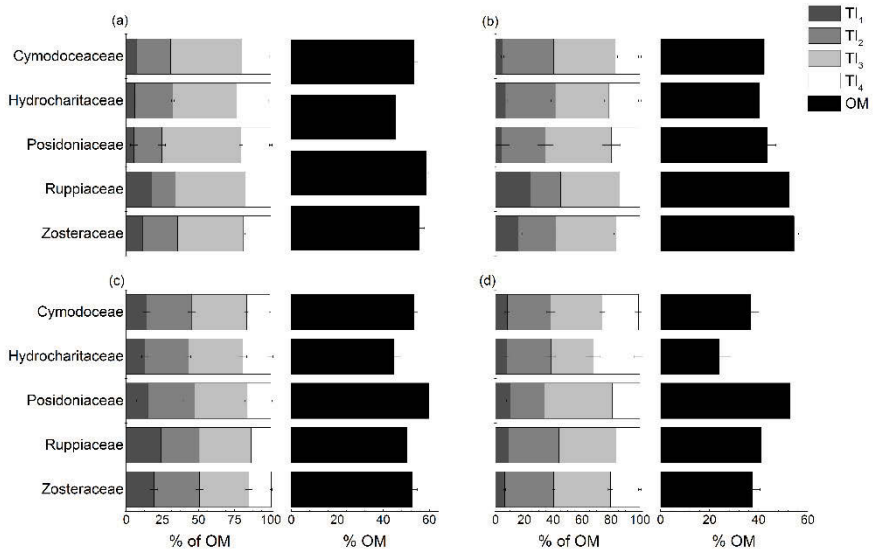


Figure S1.3: Seagrass organic matter quality from thermogravimetric analyses across families for (a) leaf, (b) non-photosynthetic above-ground tissue, (c) rhizome and (d) root tissues. Thermal intervals (TI) represent distinct organic matter components from TGA normalised to total organic matter (TI_1: labile, carbohydrates, hemicellulose, 180°C to 220°C; TI_2: labile, carbohydrates, hemicellulose, 220°C to 300°C; TI_3: refractory, cellulose, 300°C to 400°C; TI_4: refractory, lignin and residues, 400-600°C).Values represent mean ± 1 S.E.M.

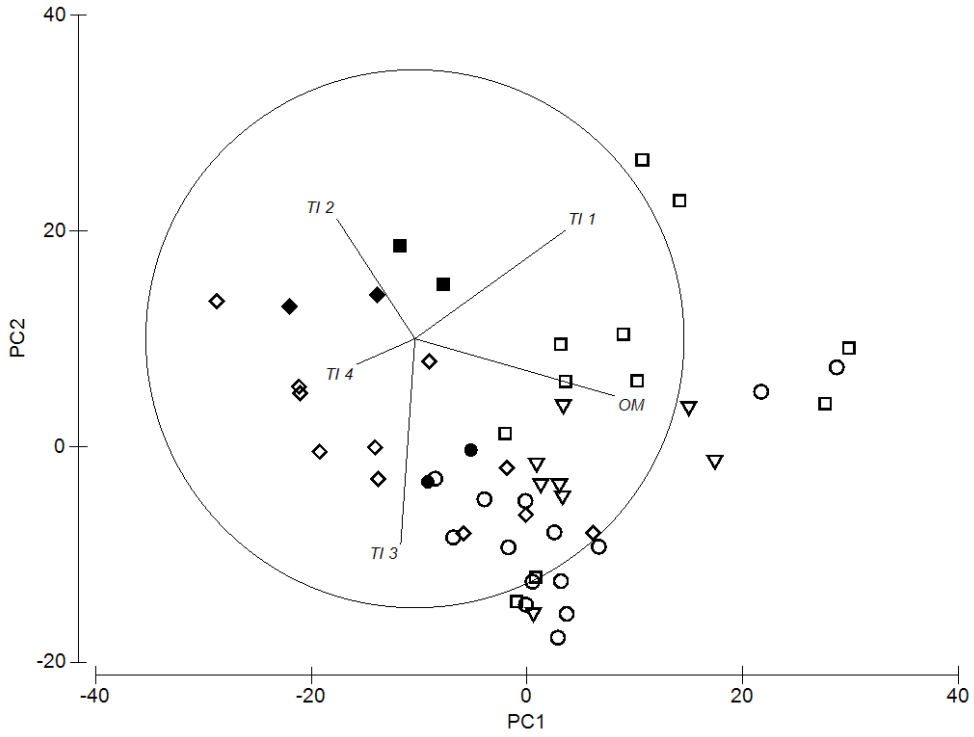


Figure S1.4: Principal components analysis of TGA for *Zostera* samples. Open symbols represent temperate seagrass and solid symbols represent tropical seagrass. ○ = leaf, ▽ = non-photosynthetic above-ground tissue, □ = rhizome, ◊ = root). PC1 = 52.7% variation, PC2 = 35.0% variation. Eigenvectors are reported in Table S1.2.

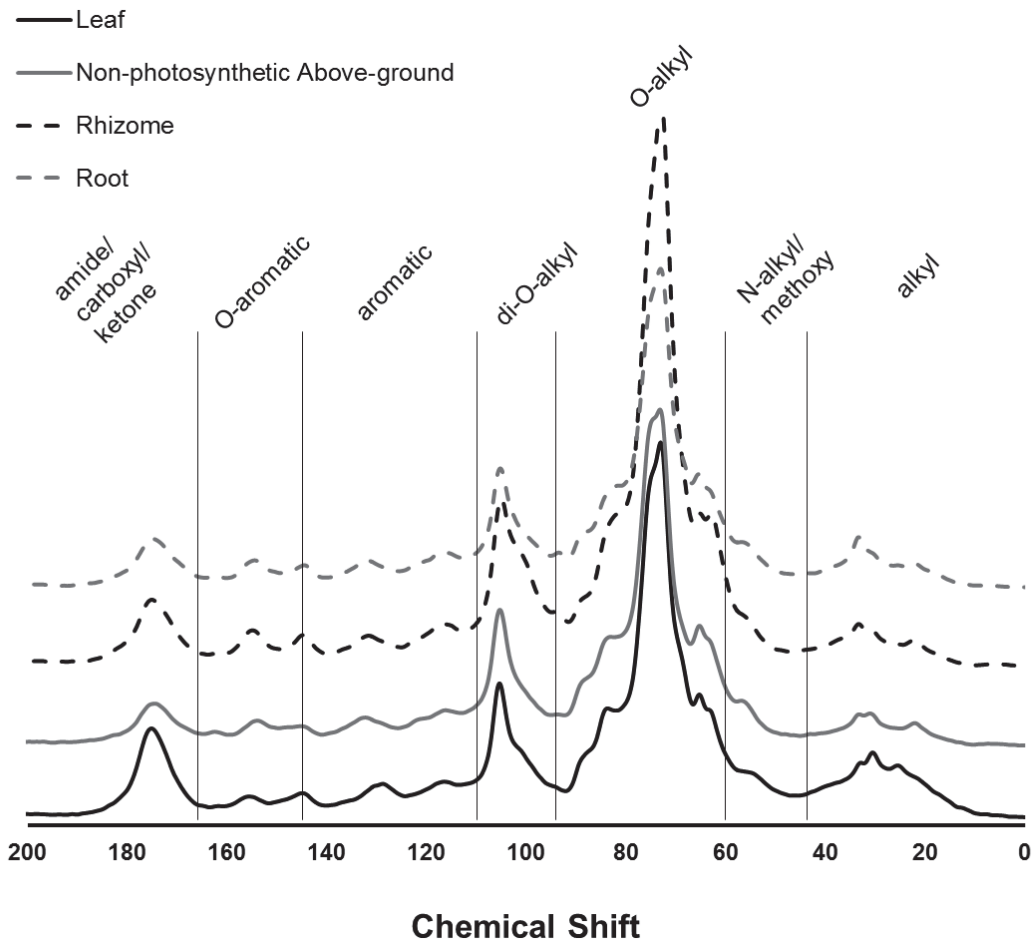


Figure S1.5: Overlay of the average solid-state ^{13}C-CPMAS NMR spectra of each tissue type. Lines indicate the spectral ranges integrated for each functional group: amide/carboxyl/ketone (215–165 ppm), O-aromatic (165–145 ppm), aromatic (145–110 ppm), di-O-alkyl (110–95 ppm), O-alkyl (95–60 ppm), N-alkyl/methoxy (60–45 ppm), and alkyl (45 to 10 ppm).

Chapter 2:
Microbial-driven seagrass remineralisation influenced by temperature and sediment chemistry

Abstract

Seagrass habitats are significant sinks for organic carbon (C), and microbial communities are instrumental in determining the quantity and quality of C sequestered. However, environmental perturbations are predicted to affect microbial-driven seagrass decomposition and subsequent C sequestration. To assess the potential effects of increasing seawater temperatures and eutrophication on seagrass decomposition, we incubated seagrass detritus under conditions of elevated temperatures and inorganic nutrient additions. 16S rDNA amplicon sequencing of seagrass-associated bacterial communities revealed that the early stages of decomposition of leaves and rhizome/roots were driven by *r*-strategists, including members of the Alpha- and Gammaproteobacteria, followed by a shift to *K*-strategists, such as Cytophagia and Actinobacteria after 3 months. Nutrient additions had a negligible effect on decomposition, indicating an absence of inorganic nutrient limitation. However, under elevated temperatures, aerobic decay rates of seagrass leaves nearly doubled and coincided with enhanced lignocellulose degradation. Conversely, anaerobic decomposition of rhizome/root tissues was unaffected by temperature. These observations suggest oxygen availability constrains the stimulatory effects of temperature increases on bacterial remineralisation of refractory C. Consequently, under future scenarios of rising seawater temperature, C storage within seagrass habitats may be diminished if the sediments are also subjected to disturbances that cause reoxygenation and subsequently support elevated rates of aerobic remineralisation.

Introduction

Seagrass habitats fundamentally influence the biogeochemistry of coastal habitats due to their high productivity and rates of organic carbon (C_{org}) burial (Duarte and Chiscano 1999, Marbà et al. 2006, McLeod et al. 2011), and are consequently important within the context of global C_{org} sequestration (Duarte et al. 2010, McLeod et al. 2011). Within these habitats, microbes play pivotal roles in several biogeochemical cycling processes, including nitrogen-fixation by leaf- and root-associated bacteria (Borowitzka et al. 2006) and oxygen, iron and sulphur cycling within the rhizosphere and surrounding sediments (Duarte et al. 2005). Bacteria are also the primary decomposers of seagrass detritus in these habitats and thus drive the transformation and cycling of seagrass-derived C (Harrison 1989), which ultimately influences the rates and efficiency of C_{org} sequestration. While bacteria have been shown to oxidise 30-80% of seagrass-derived substrate back into carbon dioxide (CO_2) (Blum and Mills 1991, Pollard and Moriarty 1991), a significant proportion of the detritus escapes remineralisation and is sequestered into the sediment (Burdige 2007).

On average, half of the C sequestered in a seagrass meadow (20 - 101 Tg C_{org} $year^{-1}$ globally) originates from seagrass detritus (Duarte et al. 2013b), with the remaining C donated by algal or terrestrial C sources and captured within the seagrass meadow (Kennedy et al. 2010). Seagrass meadows sequester ~40 times more C_{org} on an area basis than terrestrial ecosystems (McLeod et al. 2011), and are therefore considered a 'blue carbon' (blue C) habitat for their role in natural, long-term sequestration of CO_2 (Laffoley and Grimsditch 2009). However, environmental perturbations, linked to climate change and coastal eutrophication, may influence the rates and amounts of C sequestered in seagrass meadows, making it difficult to predict the conditions for optimal sequestration or identify threats to the preservation of stored C_{org}.

In terrestrial environments, temperature and litter quality (i.e., nitrogen and lignocellulose content) influence the microbial metabolism of plant litter, which subsequently controls organic C cycling and sequestration in these systems (Couteaux et al. 1995, Silver and Miya 2001, Cleveland et al. 2014). In general, higher temperatures enhance microbial metabolism and growth, resulting in higher CO_2 production, until temperatures increase beyond an optimum range or microbes become acclimated (Pendall et al. 2004, Pietikäinen et al. 2005, von Lützow and Kögel-Knabner 2009). In marine ecosystems, elevated temperatures have also been shown to enhance labile and

refractory C remineralisation in coastal and marine sediments, as well as the pelagic ocean (Weston and Joye 2005, Matsui et al. 2013, Bendtsen et al. 2015). As such, increases in marine microbial remineralisation of organic C as a result of elevated seawater temperatures can be expected to drive future reductions in the efficiency of marine C burial, due to either the liberation of stored C_{org} or enhanced CO_2 release. Therefore, given their large role in marine C_{org} sequestration, it is fundamentally important to resolve the potential effects of predicted seawater temperature increases on seagrass C remineralisation.

Due to the relatively low nutrient content of seagrasses (high C:N:P), bacterial metabolism of seagrass detritus is typically slower than of other sources of marine detritus (e.g., macroalgae; Rice and Tenore 1981, Enríquez et al. 1993). However, seagrass habitats are often located in proximity to the mouths of rivers, estuaries, or lagoons where the input of external nutrient sources (i.e., runoff of inorganic nutrients or labile organic matter) can occur (Ralph et al. 2006). These allochthonous additions may 'kick-start' microbial remineralisation of refractory C, by providing previously limiting nutrients or organic matter, a process that has been described as a 'microbial priming effect' (Kuzyakov et al. 2000, Cloern 2001, Guenet et al. 2010). A priming effect may result in not only a shift in sediment biogeochemistry, but a reduction in the C_{org} sequestration and storage potential of seagrass meadows (López et al. 1998).

With global warming processes projected to drive substantial increases in both global (Stocker et al. 2013) and local (Astles and Loveless 2012) seawater temperature and increases in eutrophication caused by terrestrial runoff into coastal habitats (Cloern 2001, Orth et al. 2006), the future C_{org} sequestration capacity of seagrass meadows may be threatened (Pendall et al. 2004, Guenet et al. 2010). Using the temperate seagrass *Zostera muelleri,* we followed seagrass decay and shifts in seagrass macromolecular chemistry, while examining microbiological, biogeochemical and physical processes in a short-term laboratory decomposition experiment. We hypothesise that elevated temperatures and nutrient additions to the sediments will enhance microbial remineralisation of seagrass decomposition and lead to enhanced loss of labile OM. We aim to understand how seagrass-associated bacterial communities and metabolism of seagrass detritus are affected by increased seawater temperature and inorganic nutrient levels and determining how these shifts could alter future C cycling in seagrass habitats.

Methods

Experimental Design

We conducted a laboratory seagrass decomposition incubation using brown leaf wrack, fresh rhizome/root biomass and sediments collected from a *Zostera muelleri* Irmisch ex Ascherson meadow in Fagans Bay (33.4306 S, 151.3210 E) on the east coast of Australia. Leaf material was collected as fresh wrack along the shoreline. Live plants were collected for rhizome/root material and subsequently cleaned of sediments and attached animals. All seagrass material was stored frozen prior to start of experiment, so that microbial degradation stopped completely. For the decay experiment, *Z. muelleri* biomass was added to 1.0 mm nylon mesh litter bags (whole leaves ~ 12 g DW, 15 cm x 15 cm; 2-4 cm segmented rhizome/roots ~6 g DW 12 cm x 12 cm; Miami Aqua-culture, Inc., Florida, USA). While using the litterbag technique doesn't allow for natural movement of litter and can prevent macrofaunal influence on decay, it is the most common method to precisely track seagrass mass loss (Harrison 1989). The sediments had high organic matter content (OM ≥ 9%) in the top 10 cm with half of the grain size in the clay and slit fraction (≤ 62.5 μm; see Chapter 3). C_{org} was 2-3% and total N was approximately 0.4%. To replicate natural decomposition conditions, rhizome/root bags were buried under 1 cm of sediment near the natural rhizosphere (personal observation), and leaf bags were kept on top of the sediment. Each incubation container had an area of 225 cm^2 with a total sediment depth of 3.5-4.0 cm. Water pumps (600 L hr^{-1}) and airstones were used to maintain oxygenated, circulating water. A 6 mm diameter hole was punched through all the bags prior to filling with seagrass material so microprofiles could be made safely through the litter bags. The holes were marked with a zip tie for the buried rhizome/root samples.

The temperature and inorganic nutrient treatments were designed orthogonally with 4 replicates. The temperatures were chosen based on current annual temperatures at Fagans Bay. The ambient temperature of 23°C is the annual mean temperature, while 30°C is approximately the average maximum summer temperatures (Fig. S2.1). Under predicted warming seawater temperatures, it would be expected that decomposing seagrass would spend longer periods of time under the elevated temperature. The ambient temperature treatment (23°C) was controlled with the test room's automatic thermostat, while the high temperature treatment (30°C) was maintained with aquatic

heaters. Temperature loggers reported temperatures ranging 23.5 ± 2°C and 30 ± 1°C for the 23°C and 30°C treatments, respectively. The nutrient treatments were applied by adding controlled-release fertiliser pellets, which is a common method in simulating nutrient enrichment and fertilisation in coastal sediments (López et al. 1998, Leoni et al. 2008). Coarse particulates were removed from all sediments with a 3.0 mm sieve prior to adding 1 g per L of Grow! Controlled-Release Fertiliser Pellets to the nutrient addition treatments (+NP; NSW, Australia). The addition of nutrients pellets increased the nutrients porewater levels by 1.3-1.5-fold for NH_4 and 1.8-4.4-fold for PO_4 after 1 week. The nutrient addition treatments maintained higher porewater nutrient levels throughout the experiment, except for PO_4 in the leaf and rhizome/root samples, which declined to control levels for the 23°C treatment by Day 84.

Microsensor profiling

Oxygen (O_2), hydrogen sulphide (H_2S) and pH profiles of the decomposing seagrass samples were measured with Clark-type electrochemical O_2 (tip size 100 µm, 0.5% stirring sensitivity, 6 s response time), H_2S (tip size 100 µm, 2% stirring sensitivity, <10 s response time) and pH (tip size 500 µm, <10 s response time) sensors, respectively (Revsbech 1989, Kühl and Jørgensen 1992) (Unisense A/S, Aarhus, Denmark). The profiles were taken for the control sediments (no seagrass) and litter bags for control temperature and no nutrient added treatments only due to time limitations. The profiling will provide information of the oxic or anoxic conditions within the litter bags and how the addition and decomposition of seagrass within sediments will influence microbial metabolism.

Each microsensor profile was performed in triplicate except for the leaf H_2S profiles due to high risk of breakage of the sensor tip through the litter bag. For litter bag samples, the profiles were measured through pre-made holes in the centre of the litter bags, while profiles in control sediments were measured in the centre of the sample container. For leaf samples sitting atop the sediment, all three profiles were taken at 1 mm intervals beginning at 3 mm above the litter bag surface and ending at 35 mm (~5-7 mm below the sediment surface). For control sediment and rhizome/root samples, oxygen profiles were taken at 100 µm intervals starting at 500 µm above the sediment surface to anoxia. Data for H_2S and pH profiles for rhizome/root and sediment samples were taken at every 1 mm beginning at 3 mm above the sediment surface to 25

mm depth to ensure a complete profile through the bag. Oxygen profiles were adjusted so that the surface of the sediment (0 mm) was directly below the linear section of the profile, representing the diffusion boundary layer (Jørgensen and Revsbech 1985). Triplicate profiles were averaged to obtain a mean profile for each analyte for each sample. The mean profiles were then averaged for the true replicate samples (n = 3, control sediment, leaf and rhizome/root).

For H_2S profiles, total sulphides ($S_{tot}^{2-} = H_2S + HS^- + S^{2-}$) were calculated as per manufacturers guidelines as $S_{tot}^{2-} = [H_2S] * (1 + k1/H_3O^+)$ where $H_3O^+ = 10^{-pH}$, $k1 = 10^{-pk1}$, pk1= -98.08 + (5765.4/T) + (15.04555*lnT) + (-0.157* S0.5) + 0.0135*S , T= temperature in Kelvin and S= salinity in % (Unisense A/S H2S Manual). Diffusive flux for S_{tot}^{2-} were calculated using Fick's First Law: flux = -D * porosity * $\Delta C/\Delta T$, where D is the diffusion coefficient (calculated based on the temperature and salinity dependent diffusivity of O_2, D_{oxygen}, obtained from unisense.com, where $D_{H2S} = D_{oxygen}*0.7573$), porosity = 0.8, ΔC is the change in concentration over the linear region of the profile (nmol cm^{-3}), and ΔT is change in distance over the linear section (cm). Average sulphide reduction rates (mmol m^{-2} d^{-1}) were calculated per sampling event for each sample type. Sulphide reduction rates were the sum of the upward and downward sulphide flux for rhizome samples (Kühl and Jørgensen 1992). For leaf and control sediment samples, only the upward flux was captured in the profiles, and thus represents minimum sulphide reduction rate estimates.

Seagrass biochemical analysis

Destructive sampling of the litter bags occurred at Days 2, 7, 14, 28 and 84 (n = 4, total N = 160). After sampling for bacterial analyses (below), the remaining bulk litter was washed of sediments, weighed before and after oven drying at 60°C, and then mixed before grinding a representative subsample into a fine powder (Pulvisette 7, Fritsch, Germany) for elemental and biochemical analyses. Decay rates were calculated by fitting the proportion of dry mass remaining to single-component ($W_t/W_o = e^{-kt}$) and double-component ($W_t/W_o = \alpha e^{-k_1 t} + (\alpha - 1)e^{-k_2 t}$) exponential decay models (Fourqurean and Schrlau 2003) (tolerance = 0.001, 1000 iterations, Dynamic Fit Wizard, Sigma-Plot, Systat Software, Inc., San Jose, CA, USA).

A LECO elemental analyser was used to quantify the bulk carbon (C) and nitrogen (N) content of the seagrass tissue. The standard for plant samples was rice flour

(LECO TruSpec, St. Joseph, MI, USA). Solid-state ^{13}C-CPMAS nuclear magnetic resonance (NMR) and diffuse reflectance mid-infrared (MIR) spectrometry were performed to investigate how seagrass organic components changed with decay. MIR spectra were attained with the instrumentation described in Baldock et al. (2013b). Spectra were acquired over 6000–400 cm^{-1} with a resolution of 8 cm^{-1}. The background signal intensity was quantified by collecting 240 scans on a silicon carbide disk before analysing. In total, 60 scans were acquired and averaged to produce a reflectance spectrum for each individual sample, and the Omnic software (Version 8.0; Thermo Fisher Scientific Inc.) was used to convert the acquired reflectance spectra into absorbance spectra (log-transform of the inverse of reflectance). For MIR data processing, second derivatives were generated for the spectra using the Savitsky-Golay function with 15 smoothing points, before removing scattering effects using the Standard Normal Variate (SNV) correction algorithm. The spectra were then split into two groups: a calibration set consisting of about two thirds of the data, and a validation set made up of the remaining third. These were then used to build PCA models in order to reveal groupings within the data that would be otherwise hidden within the complex spectra.

Solid-state ^{13}C-Cross-Polarisation Magic-Angle Spinning (CPMAS) NMR spectra were acquired for one representative sample of each tissue type per treatment per sampling day, selected by applying a Kennard-Stone algorithm to the MIR spectra acquired for all samples (Baldock et al. 2013b). All NMR were acquired according to Baldock et al. (2013a) with slight modification. Briefly, a 200 Avance spectrometer (Bruker Corporation, Billerica, MA, USA) equipped with a 4.7 T, wide-bore superconducting magnet operating at a resonance frequency of 50.33 MHz was used to obtain the spectra. Weighed samples (200-400 mg) were packed into 7-mm-diameter zirconia rotors with Kel-F® end caps and spun at 5 kHz. A cross-polarisation ^{13}C NMR (CP) analysis using a 90° pulse of 3.2 µs at 195 W, a contact time of 1 ms, and a recycle ranging from 1 to 5 s was used for all samples with a total of 5000 scans being collected for each sample. The duration of the recycle delay was set to greater than 5 times sample specific T_1H values determined using an inversion recovery pulse sequence. All spectral processing was completed using the Bruker TopSpin 3.1 software (Baldock et al. 2013a). The acquired total signal intensity was divided into a series of chemical shift regions: amide/carboxyl/ketone (215–165 ppm), O-aromatic (165–145 ppm), aromatic

(145–110 ppm), di-O-alkyl (110–95 ppm), O-alkyl (95–60 ppm), N-alkyl/methoxy (60–45 ppm), and alkyl (45 to 10 ppm).

The loss of C-containing compounds over the decomposition process was assessed based on changes in the distribution of NMR signal intensity (Smernik and Oades 2000b, a). First, the differences between the initial and final spectra were calculated based on C observed by the NMR. These 'loss' spectra were then normalised to the mass loss and the elemental C loss experienced between the initial and final samples during decomposition. The signal intensity associated with the resulting loss spectra was integrated across the spectral regions, and then entered in a molecular mixing model (MMM) for terrestrial soils to predict molecular composition (Baldock et al. 2004). The original terrestrial protein component of the MMM was altered to be reflective of the amino acid composition of *Posidonia australis*, *Thalassia testudinum*, *Halodule wrightii* and *Syringodium filiforme* (Zieman et al. 1984, Torbatinejad et al. 2007). Preliminary tests indicated that the 5-component MMM including contributions from carbohydrates, lignin, protein, lipids and carbonyls had the best fit between measured and predicted composition for both tissue types (root mean squared error ≤ 1.31), and therefore was used for all samples.

Microbial Community Analyses

Samples for bacterial abundance and community characterisation were subsampled immediately after litter bags were removed from the incubation chamber on Days 0, 7, 28 and 84 (n = 4). Seagrass litter was sampled by cutting 1-2 cm sections from the bulk sample, and sediments were sampled from the top 1 cm of sediment as a microbial community reference by using a 3 mL cut-off syringe, before storing in cryovials at -80°C. Samples for abundance data were fixed in 0.01% glutaraldehyde and stored at -80°C before quantification using flow cytometry (Trevathan-Tackett et al. 2014). Briefly, four samples from each treatment were quick-thawed before adding 150 µL of the surfactant Tween 80 (Sigma-Aldrich, MO, USA). Samples were briefly vortexed and then incubated in the solvent for 15 min at room temperature before vortexing for 10 min. Samples were centrifuged at 800 x g for 1 min then filtered through a 20 µm mesh to remove any particulates. SYBR Green DNA stain was added (25 µL, 1:10,000 final concent.; Invitrogen, CA, USA) and incubated in the dark at room temperature for at least 15 min. Finally, 1 µm fluorescent beads were added to the

samples in a final concentration of 3.6 x 10^6 beads mL^{-1} as an internal standard (Invitrogen, CA, USA). All samples were analysed using a LSRII (Becton Dickson, NJ, USA) flow cytometer and analysed using Flowing Software v. 2.5 (www.flowingsoftware.com).

Samples collected for analysis of seagrass-associated bacterial community composition were stored at -80°C before genomic DNA was extracted from each sample using the PowerSoil DNA Isolation Kit (MoBio Technologies, CA, USA). Extracted DNA was amplified using the V4 variable regions of 16S rDNA gene with the Eubacterial primers 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5′GGACTACVSGGGTATCTAAT-3′) prior to sequencing using the Illumina MiSeq platform following the manufacturer's guidelines (www.mrdnalab.com, TX, USA). Sequenced 16S rDNA data was processed using the QIIME package (Caporaso et al. 2010). Following de-multiplexing, barcodes were removed and sequences shorter than 200 bp, containing ambiguous base calls (Ns) and bases with a quality score < Q20 were removed. Paired ends were joined using fastq-join (Aronesty 2011), and operational taxonomic units (OTUs) were defined *de novo* as sequences sharing 97% similarity using UCLUST (Edgar 2010). Representative sequences of each OTU were compared against the Greengenes database (DeSantis et al. 2006) using BLAST (E < 0.001) (Altschul et al. 1997) to assign taxonomy. Chimeras were removed using ChimeraSlayer (Haas et al. 2011), and samples were rarefied to 3795 sequences to ensure even sampling depth. Weighted UNIFRAC was performed to take into account community differences associated with different lineages in a phylogenetic tree (Lozupone and Knight 2005). QIIME was also used to calculate alpha diversity indices chao 1 and Simpson's evenness on *de novo*-picked genus-level taxa (Caporaso et al. 2010) to provide more information on changes on the microbial communities as a whole. Finally, we employed the PICRUSt analysis package to predict microbial functional capacity from the 16S rDNA profiles (Langille et al. 2013). For this analysis, closed-reference picking using 97% similarity from the Greengenes database was employed. After rarefication at 2706 sequences, OTUs were normalised by copy number, and functions were predicted for levels 2 and 3 of KEGG pathways.

Statistical Analysis

A 2-way Analysis of Covariance (ANCOVA) was used to analyse the effects of temperature and nutrient addition (independent variables) on mass loss, elemental content, bacterial abundance and bacterial and archaeal alpha diversity (dependent variables) with time as a covariate. A Sidak test was used for post-hoc analysis. A 1-way ANOVA was used to analyse the differences in sulphide reduction rates over the course of the experiment. As initial seagrass samples did not receive temperature or nutrient manipulation, they were considered a separate treatment. All treatment and interaction effects with $\alpha < 0.05$ were considered statistically significant. Samples were log-transformed to achieve homogeneity of variance where required. ANCOVA and ANOVA analyses were performed using SPSS (v19, IBM).

Multivariate statistics were used to statistically analyse the weighted UNIFRAC bacterial and archaeal taxa using Primer (version 6.1.11, Primer-E; Clarke and Gorley 2006). Bacterial and archaeal OTU data were square-root transformed prior to creating a Bray-Curtis similarity resemblance matrix. Data were visualised using multidimensional scaling (MDS). Analysis of Similarities (ANOSIM) was applied to determine if differences between treatments were significantly different from a randomly permutated distribution. Similarity percentage analysis (SIMPER), with 90% cut off for low contributions, was used to identify the microbial taxa driving the shift between the clusters observed in the MDS plots. The predicted PICRUSt metagenome functions were analysed using the Statistical Analysis of Metagenomic Profiles (STAMP) program (Parks et al. 2014). All STAMP analyses were performed with a two-sided Welch's t-test and Bonferroni correction to minimise Type I errors.

Results

Seagrass chemistry and the abundance and composition of the associated microbial communities changed significantly throughout the three month study and were primarily influenced by tissue type, time and temperature (ANCOVA and ANOSIM Statistics Tables S2.1, S2.2). The effects of added nutrients were minimal on both seagrass decay and microbial community dynamics and limited to an increase in rhizome/root %N (pairwise tests, $P = 0.022$) and a slight increase in tissue-associated

bacterial abundance (pairwise tests, P = 0.014). Henceforth, we primarily focus on the influence of tissue type and temperature on seagrass decay.

Seagrass decay and biochemistry

In general, NMR, MIR and elemental analyses revealed that leaf tissues consistently contained higher amounts of elemental N, protein and sucrose than below-ground tissues (Figs. S2.2, S2.4). Rhizome/root tissues had higher carbohydrate content as glucose and cellulose (Fig. S2.4). Each tissue type underwent distinct decomposition patterns during the three month incubation (Fig. 2.1). A single exponential decay model described leaf wrack decomposition better than the double-component model (Adj. R^2 > 0.95 vs Adj. R^2 = 0.80-0.86). During the three months of decomposition, leaf decay was dominated by loss of carbohydrates, which closely resembled pure cellulose (Kobayashi et al. 2011), but also other compounds including lipids and proteins (Table 2.1, Figs. S2.3a, S2.4). Under elevated temperatures, the rate of leaf decay nearly doubled relative to decay rates at ambient temperature (k = 0.0071 vs. 0.0038 d^{-1}, respectively; Fig. 2.1). This enhanced mass loss coincided with significant loss in elemental N and increased C:N ratio (Fig. S2.2), as well as a larger decrease in lignin (4.6%), lipids (3.0%) and carbonyls (2.8%; Table 2.1).

Mass loss and elemental and macromolecular content of the rhizome/root samples was altered throughout the decomposition process but were not affected by elevated temperature (Table 2.1, Fig. 2.1, Fig. S2.3a). The single exponential decay rate fit poorly for the rhizome/root samples due to the large initial loss in ~50% mass in the first two days (Adj. R^2 = 0.03-0.13). The double-component model had a better fit, though still weak, and predicted high k-values (Fig. 2.1). When taking into account only the mass loss between Day 2 and Day 84, the decay rate ranged from 0.0022-0.0031 d^{-1}. According to the MMM derived from the NMR spectra, the large mass loss consisted of approximately 69% carbohydrates, 13.0% lignin, 8% protein, 7% carbonyls and 3% lipids. During the remainder of the experiment, rhizome/root detritus decay occurred more slowly (Fig. 2.1) and resulted in an increase in %N (Fig. S2.2) and a loss of carbohydrates, closely resembling pure cellulose (Table 2.1, Figs. S2.3a, S2.4; Kobayashi et al. 2011).

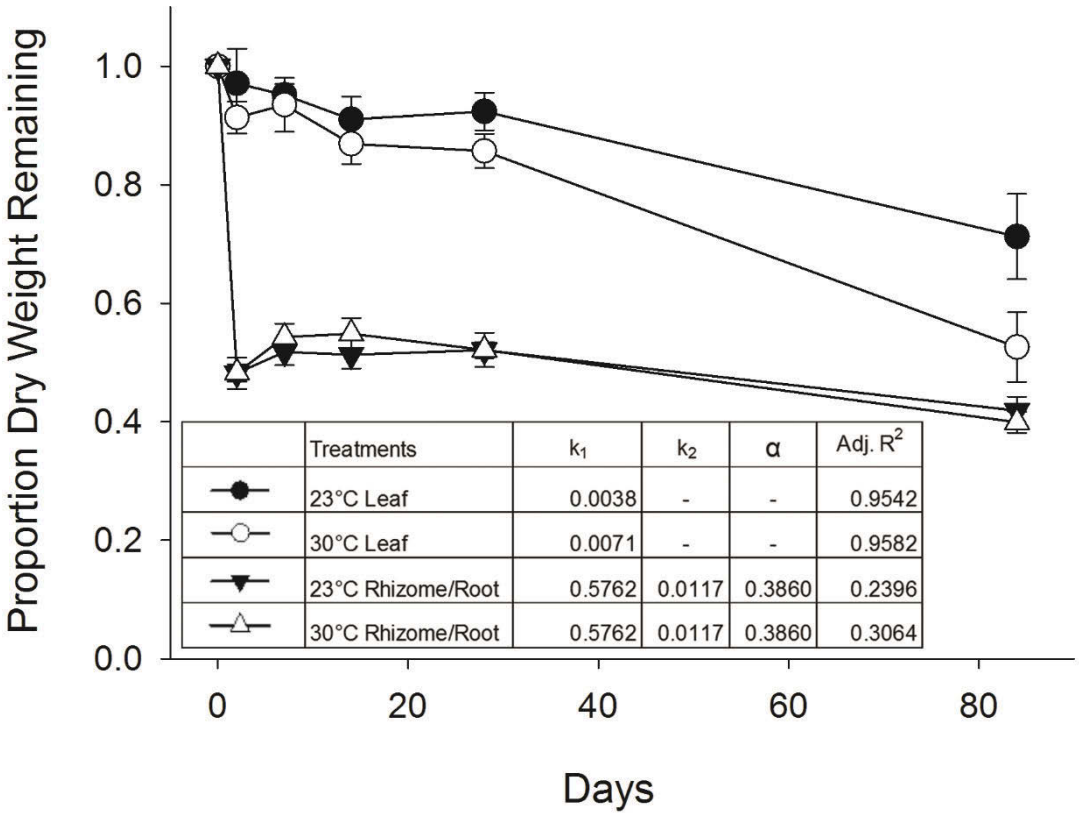


Figure 2.1: Seagrass mass loss throughout decomposition. Decay rates were calculated using a single- or double-component exponential decay function. Alpha represents the proportion of decay under k_1. Data represent mean ± S.E.M.

Table 2.1: Percent of macromolecular compound losses from decaying seagrasses predicted from ^{13}C-CPMAS NMR spectra between Day 0 and Day 84 (i.e., net changes) and between Day 28 and Day 84 (i.e., progressed decomposition) at both temperature treatments. Data represent mean ± S.E.M.

	Losses between Day 0 and Day 84			
	Leaf, 23°C	Leaf, 30°C	Rhizome/Root, 23°C	Rhizome/Root, 30°C
Carbohydrate	81.7 ± 6.9	69.8 ± 1.5	74.5 ± 2.5	71.1 ± 0.7
Protein	2.3 ± 2.3	3.8 ± 1.1	6.8 ± 2.4	8.0 ± 0.8
Lignin	7.3 ± 2.4	11.9 ± 0.4	10.3 ± 0.2	11.5 ± 0.9
Lipid	2.9 ± 1.7	5.8 ± 0.3	2.0 ± 0.3	2.4 ± 0.1
Carbonyl	5.9 ± 0.5	8.6 ± 0.3	6.5 ± 0.3	7.0 ± 0.6
	Losses between Day 28 and Day 84			
	Leaf, 23°C	Leaf, 30°C	Rhizome/Root, 23°C	Rhizome/Root, 30°C
Carbohydrate	69.1 ± 2.7	64.4 ± 1.3	95.3 ± 4.8	100
Protein	5.3 ± 5.3	4.0 ± 2.9	0	0
Lignin	14.4 ± 0.9	16.1 ± 0.2	2.2 ± 2.2	0
Lipid	2.7 ± 0.9	4.0 ± 0.3	0	0
Carbonyl	8.7 ± 2.5	11.5 ± 1.7	2.6 ± 2.6	0

Sediment chemistry

Microsensor profiles of the control treatments (23°C, no NP) illustrated that leaf decomposition occurred under largely oxic conditions at the sediment surface with oxygen levels consistently above 50 μM (Fig. S2.5e). Conversely, rhizome/root decay occurred while buried in highly reduced, anoxic sediments with oxygen penetrating no deeper than 2 mm (Figs. S2.5c, d). The maximum total sulphide concentrations always occurred within the rhizome/root litter bag (Fig. S2.5d). Sulphide reduction was the dominant form of metabolism for rhizome/root remineralisation reaching a maximum rate of 50 mmol m^{-2} d^{-1} during the first week of decomposition followed by a decrease to ~22 mmol m^{-2} d^{-1} (Fig. S2.5d).

Microbial Dynamics

The number of bacterial cells significantly increased in both leaf and rhizome/root samples over the length of the study (Fig. S2.6), but there was no effect of temperature on bacterial abundance in either tissue type. During the first week, there was a rapid increase in bacterial cells on both the leaf (4-5-fold) and rhizome/root (6-7-fold) tissues. Relatively slower increases in cell abundances occurred during the remainder of the experiment (Fig. S2.6).

Genus-level community evenness significantly increased with decomposition and elevated temperature for leaf samples ($P < 0.001$ and $P = 0.028$, respectively), but there was no effect on community richness (chao 1; Fig. S2.7). In rhizome/root samples, both community richness and evenness more than doubled during the first week of decay (Fig. S2.7). Richness subsequently changed very little, and evenness significantly declined by Day 84 ($P = 0.001$). Increased temperatures also led to increased evenness for rhizome/root communities ($P = 0.034$). The sediment samples had similar bacterial community evenness, but higher community richness than either of the detrital communities.

The initial (Day 0) bacterial community composition did not significantly differ between the leaf and rhizome/root litter. However, the bacterial communities associated with these tissue types became increasingly dissimilar as they shifted during the decomposition process. Pairwise analyses indicated that significantly different community signatures formed after the first week of the experiment for each substrate type ($P = 0.010$; Fig. 2.2). These bacterial communities associated with each substrate

also changed significantly during early (Day 7) and later (Day 84) decomposition (P = 0.004). Additionally, within each time point, there was a highly significant effect of elevated temperature on the bacterial community composition, except in the case of sediment communities at Day 84 (P = 0.001; Fig. 2.2).

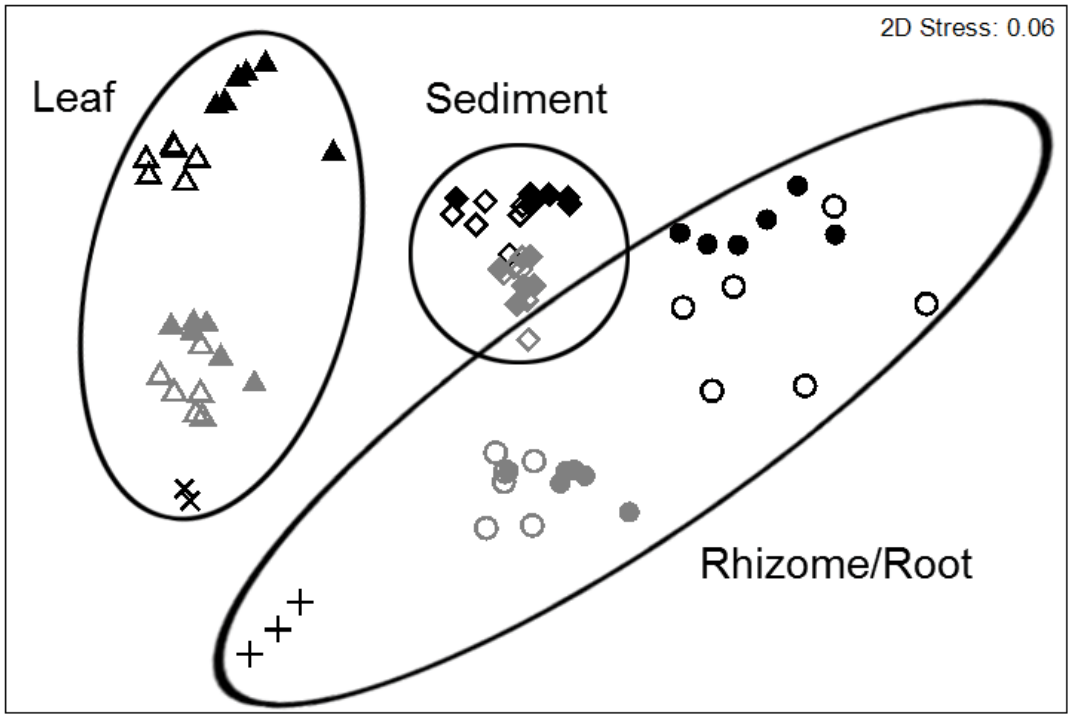


Figure 2.2: MDS plot of bacterial OTUs grouped by tissue, time and temperature. Leaves = triangle, Rhizome/root = circles, Sediment = diamonds, Day 0 = crosses, Day 7 = grey, Day 84 = black, 23°C = open symbols, 30°C = closed symbols.

Further investigation with SIMPER analyses revealed which taxa contributed to the dissimilarity between these significantly different microbial assemblages (Figs. 2.3, 2.4). Sediment bacterial communities remained relatively stable during the course of the experiment, with Delta- and Gammaproteobacteria making up ~40% of the population (Figs. 2.3c, 2.4c). Both initial leaf and rhizome/root litter were dominated by the Gammaproteobacteria, with the *Vibrio* genus comprising the largest fraction of the group. Over the course of the experiment, however, the abundance of Gammaproteobacteria consistently declined (Figs. 2.3, 2.4). Leaf samples became dominated by aerobic Alphaproteobacteria, mainly including members of the *Erythrobacter* and Rhodobacteraceae (Figs. 2.3a, 2.4a). Conversely, rhizome/root

tissues became dominated by anaerobic bacteria including Clostridia, Bacteroidia and the Deltaproteobacteria, which were primarily composed of *Desulfovibrio* and Desulfobacteraceae (Figs. 2.3b, 2.4b).

Within the distinct developing communities associated with each tissue type, the abundance of several groups increased during decomposition. In the leaf samples, over the course of the 3-month experiment, there were increases in Alphaproteobacteria such as Rhizobiales and Hyphomicrobiaceae, as well as Actinobacteria, Myxococcales and Cytophagia (Figs. 2.3a, 2.4a). In the rhizome/root samples there was an increase in Clostridia, Bacteroidia and the Deltaproteobacteria during the first week of the study. After three months, there was a marked increase in Spirochaetes and Deltaproteobacteria (Figs. 2.3b, 2.4b). Among the Deltaproteobacteria, there was a large decline in Desulfovibrionaceae between Days 7 and 84 (~65% to 20%) with a concomitant diversification of other sulphate reducers (Fig. 2.4b).

Increased temperature led to further shifts in the bacterial communities. In the leaf samples, the 30°C treatment increased the abundance of *Erythrobacter*, *Marinobacter* and Clostridia during the first week. After three months, increased temperatures promoted higher abundances of leaf-associated ammonia-oxidising Archaea *Nitrosopumilus,* purple non-sulphur bacteria from the Rhodospiralles and Rhizobiales, but also a decline in some Alphaproteobacteria including *Loktanella* and N-fixing Phyllobacteriaceae (Figs. 2.3a, 2.4a). On the other hand, higher temperature in the rhizome/root samples led to an increase in abundances of already dominant anaerobic classes, such as Clostridia, Bacteroidia and Fusibacteria during the first week, and Deltaproteobacteria by Day 84 (Fig. 2.3b). While the Deltaproteobacteria assemblages were nearly identical between temperature treatments in the first week, by the end of the experiment higher temperatures promoted an increase in sulphate reducers including Desulfobacteraceae and Desulfarculaceae at the expense of other groups including *Sulfurimonas* and Bacteroidia (Figs. 2.3b, 2.4b).

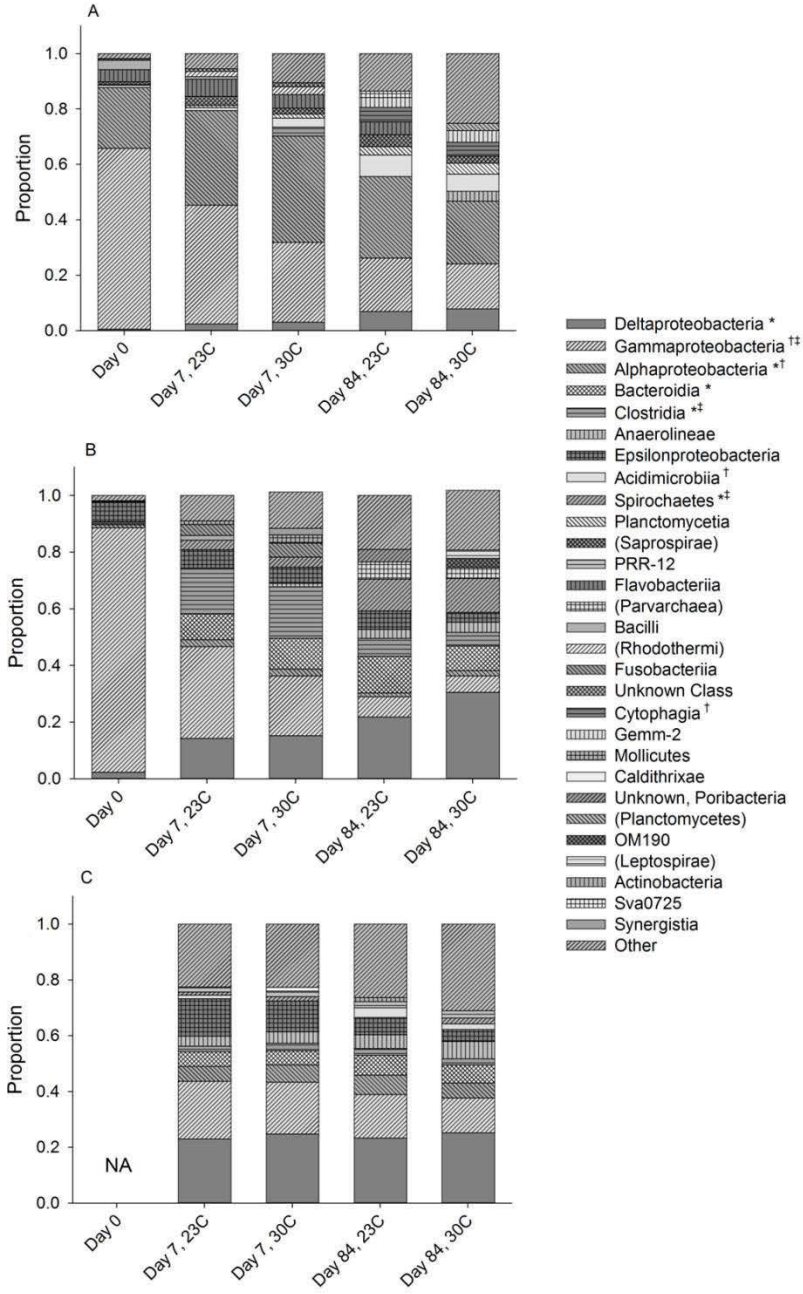


Figure 2.3: Taxonomic shifts of bacterial community at class-level related to decomposition time and temperature. Proportions of each class represents average (n = 3) of each taxa for (a) leaf tissue, (b) rhizome/root tissue and (c) bulk sediment. Symbols represent taxa contributing to top 5% dissimilarity in SIMPER analysis between leaf and rhizome/root tissue types (*) and through time for leaf (†) and rhizome/root (‡) samples.

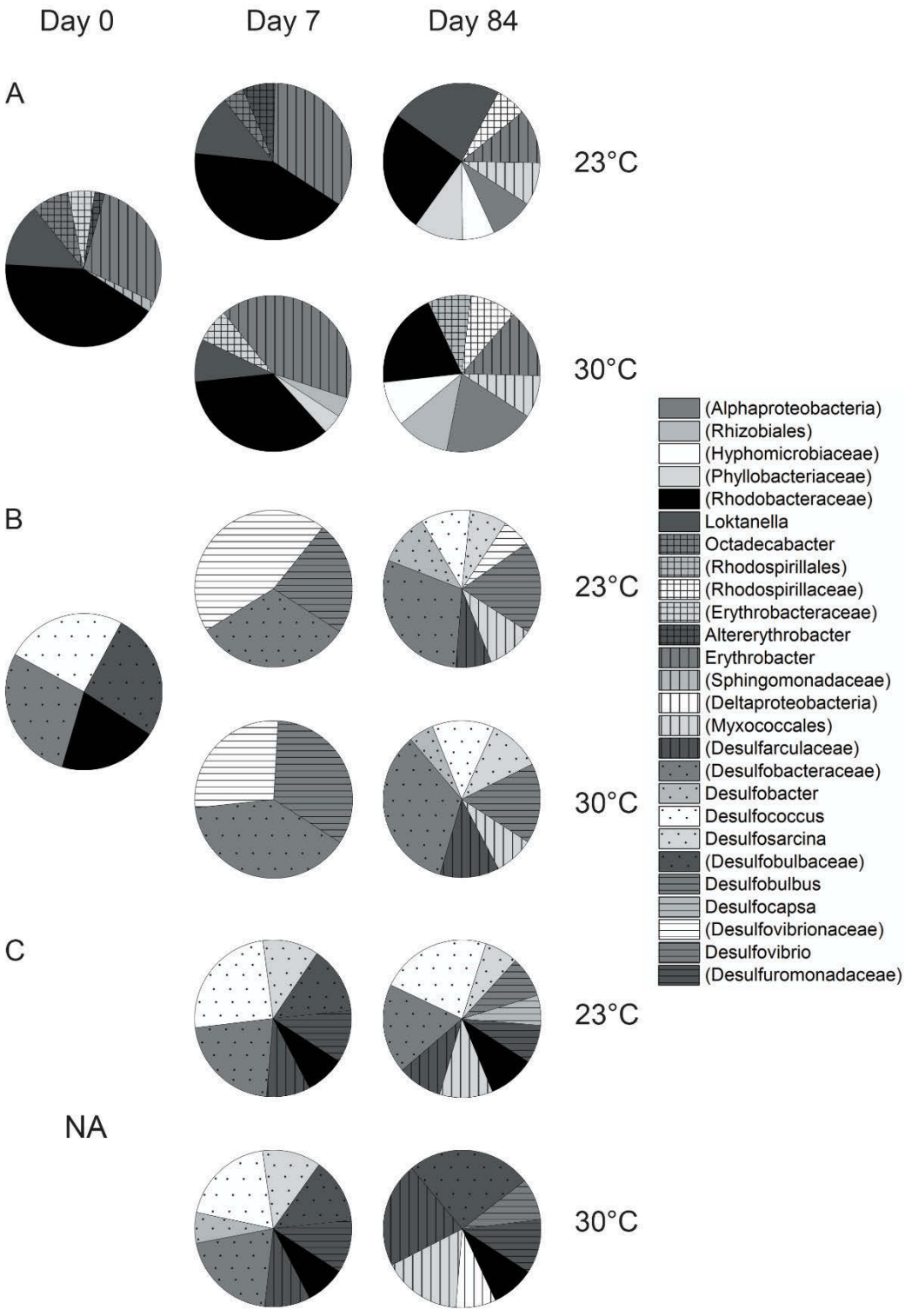


Figure 2.4: Proportion of genera within Alpha- and Deltaproteobacteria for (a) leaves, (b) rhizome/root, and (c) sediment through time and with temperature treatments. Parentheses indicate the lowest taxonomic assignment if the genus was unknown. Values represent means.

PICRUSt analysis was used to predict the functional capacity of the bacterial communities based on known metagenomics data of related taxa (Langille et al. 2013). Leaf-associated bacterial communities showed over-representation in several KEGG categories relative to rhizome/root samples. These included aromatic and non-aromatic amino acid metabolism and xenobiotics metabolism, i.e., degradation of aromatic pollutants (Fig. 2.5). Alternatively, bacterial communities associated with rhizome/roots displayed an over-representation of genes involved in glycan metabolism and nitrogen and methane metabolism (i.e., energy metabolism; Fig. 2.5a). During the course of the experiment, leaf bacteria showed an increased in their capacity for energy metabolism (Fig. S2.8a), including sulphur and methane metabolism. Among rhizome/root-associated bacteria, there was a relative increase in several metabolic functions including carbohydrate metabolism (i.e., glucose, starch and other sugars) and nitrogen and methane (energy) metabolism during the first week of the study (Fig. S2.8b). Following the first week, genes involved in lipid biosynthesis and the production of other secondary metabolites, including some antibiotics and flavonoids, significantly increased (Fig. S2.8c). Somewhat surprisingly, given the taxonomic changes observed, neither the temperature nor nutrient treatment had a significant effect on predicted community function.

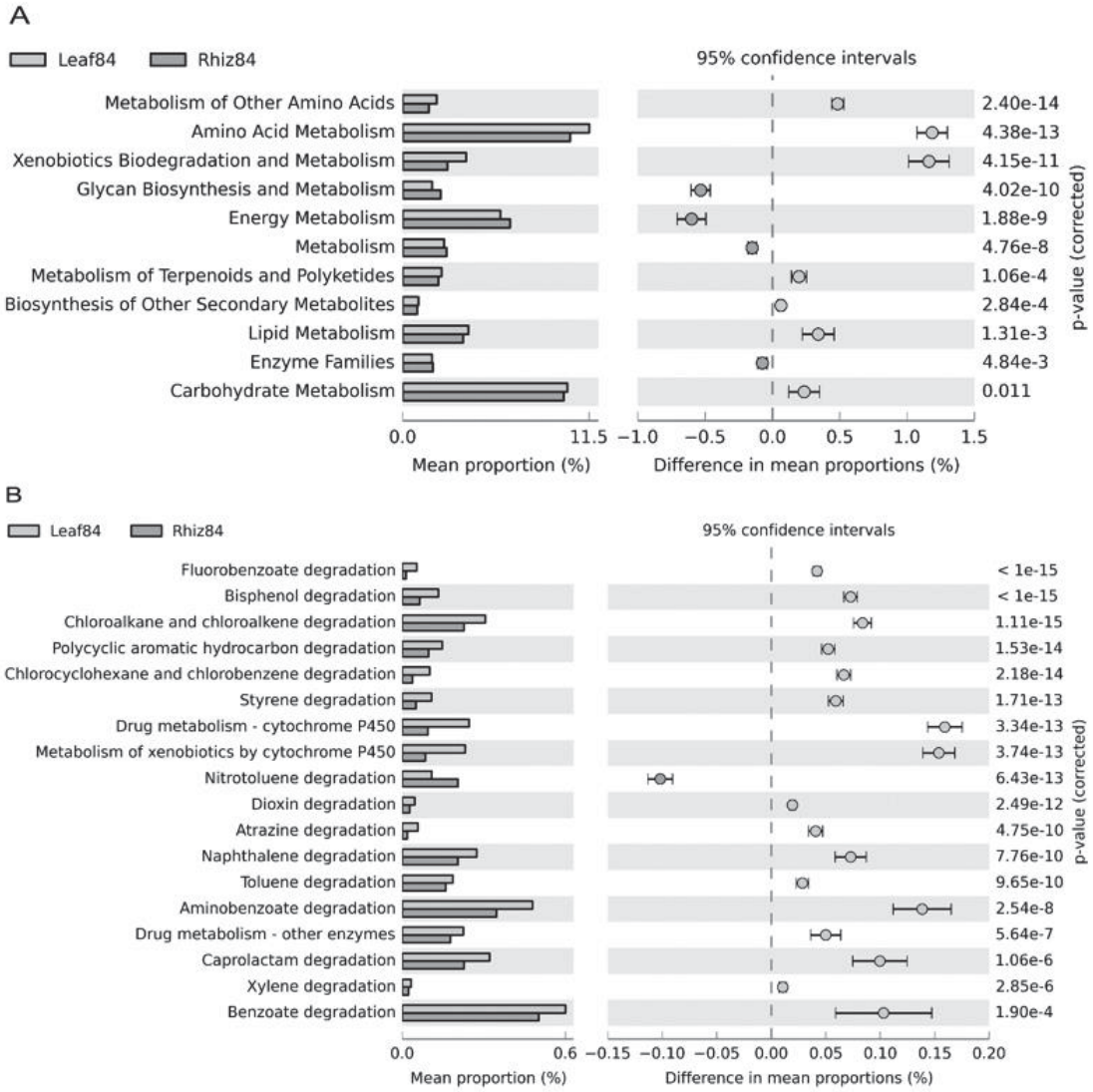


Figure 2.5: Statistical Analysis of Metagenomic Profiles (STAMP) of predicted metagenomes from PICRUSt analysis for differences in metabolic KEGG Pathways between leaf and rhizome/root tissues on Day 84 for (a) Level 2 and (b) xenobiotics metabolism at Level 3.

Discussion

Our goal was to assess the effects of elevated seawater temperatures and nutrient concentrations on bacterial remineralisation of seagrass detritus to determine how these parameters may ultimately affect seagrass contributions to C sequestration in blue carbon habitats. While microbial priming did not occur as a response to inorganic nutrient additions in this experiment, there was a significant enhancement of microbial remineralisation of seagrass under elevated temperatures as evidenced by changes in seagrass biomass and bacterial dynamics. However, the temperature-induced enhanced remineralisation only occurred under oxic conditions suggesting the existence of an important oxygen-temperature interaction involved in C remineralisation in seagrass habitats.

No effect of nutrients

In contrast to our hypothesis that nutrient inputs will kick-start microbial remineralisation of seagrass detritus, the addition of inorganic nutrients to the sediments did not influence seagrass decay. Mixed conclusions about the influence of exogenous nutrients on seagrass decomposition have been derived from previous studies. In a temperate *Posidonia oceanica* meadow, nutrient addition led to an increase in sediment bacterial activity, and it was predicted that the sediment's capacity to store C would subsequently be decreased (López et al. 1998). However, another study that tracked sediment C and N cycling during 33 days of *Zostera marina* decomposition showed that bacteria utilised N from the surrounding sediments, rather than seagrass N, while breaking down seagrass C-based molecules (Pedersen et al. 1999). Since unamended sediments in this study had a C:N ratio of 7.52 ± 0.48, which is low but not abnormal for terrigenous sediments rich in clays and silts (Holmer et al. 2004, Kennedy et al. 2010), it is likely that the bacteria had sufficient access to N in the sediments or porewater to supplement the breakdown of seagrass C in this short-term experiment (Kenworthy and Thayer 1984). Our results support a hypothesis related to the microbial priming effect, which under non-nutrient-limiting conditions, there is smaller effect of additional nutrients on microbial metabolism (Kuzyakov et al. 2000, Guenet et al. 2010).

Microbial degradation of seagrass through time

Typical plant decomposition occurs in three stages. The first is the passive leaching of soluble compounds, which benefits fast-growing *r*-strategist microbes, or copiotrophs, which rapidly utilise labile C (Fierer et al. 2007, Berg and McClaugherty 2008b). Next, active microbial decomposition occurs, followed by the very slow microbial breakdown of refractory compounds, or refractory stage (Wilson et al. 1986a, Wilson et al. 1986b). These latter two processes are dominated by slower-growing *K*-strategists, or oligotrophs, that can breakdown more refractory compounds under low-nutrient conditions, including hemicellulose and cellulose polysaccharides and lignin (Sinsabaugh et al. 2002, Fierer et al. 2007).

In the current study, leaf and rhizome/root detritus underwent significant microbial degradation dominated by bacterial taxa, which involved shifts in biochemistry and microbiology. The decay rates for both tissue types were within the ranges previously reported for seagrass material (k = 0.001-0.110, d^{-1}; Enríquez et al. 1993; Harrison 1989) . However, the leaf decay was slower than previously shown for fresh *Zostera* over similar time periods (k = 0.011-0.019; Nicastro et al. 2012; Bourguès et al. 1996). This is potentially a result of using dead, leaf wrack for the starting material in this study. Dead seagrass leaves have less soluble components and organic matter than green leaves (Harrison 1989) due to leaching, which when not incorporated in the decay rates, results in lower k-values (Harrison and Mann 1975).

Despite the absence of a significant leaching phase, leaf detritus supported high abundances of putative *r*-strategists, including members of the Alpha- and Gammaproteobacteria (DeAngelis et al. 2013). The later stages of decomposition involved increased abundances of known *K*-strategists, such as Cytophagia and Actinobacteria, which can breakdown complex organic matter (Pasti et al. 1990, Rosselló-Mora et al. 1999). During this time, there was also an increase in capacity for methane and sulphur metabolism (Figs. 2.3, S2.8a). Despite oxygen profiles showing that the leaf litter bags were generally oxic throughout the experiment, increase in anaerobic metabolism could be a result of increasing presence of anoxic microniches within the bags, resulting from the lowered oxygen concentrations and collapse of the leaf material during degradation.

Rhizome/root decomposition involved both leaching and microbial degradation phases. The leaching phase resulted in the rapid loss of up to 50% of the biomass during

the first two days of the study (Fig. 2.1). While this degradation was faster than what is typically reported for belowground biomass (4 days - 2 weeks; Josselyn et al. 1986, Vichkovitten and Holmer 2004), rhizome/roots from the smaller seagrass *Halophila* have been shown to lose ~ 70% wet weight after 4 days under anoxic conditions (Josselyn et al. 1986). The alpha coefficient in the decay equation, here representing the leaching stage of decay, was similar to *Thalassia testudinum* rhizome alpha of ~0.3 (Fourqurean and Schrlau 2003). Since the leaching phase lasted only two days in this study, the 0.39 estimate of the alpha coefficient is likely a mathematical overestimation and explains the relatively low fit of the 2 component model.

The mass loss spectra during the first week of rhizome/root decay indicated that the biomass lost during this phase was composed of macromolecules typical of seagrass leachate (Vichkovitten and Holmer 2004, Maie et al. 2006, Torbatinejad et al. 2007), and was matched by an increased capacity for the bacteria to degrade carbohydrates and amino acids (Fig. S2.8b). This period of leaching also supported high metabolism via sulphate reduction similar to those reported near seagrass rhizospheres (Marbà et al. 2006), as well as an increase in bacterial abundances and diversity. Additionally, there was also a notable presence of Bacteroidetes, which have been recognised as important *r*-strategists during degradation of phytoplankton-derived organic matter (Fig. 2.3; Mayali et al. 2011, Teeling et al. 2013) .

During the last two months of rhizome/root decomposition, a significant breakdown of carbohydrates and cellulose had occurred, resulting in a relative increase in elemental N (Figs. S2.1c, S2.3b). This is characteristic of preferential consumption of non-N compounds in seagrass detritus (Harrison 1989). The associated bacterial community in these later stages of decay declined in evenness and showed a strong shift toward *K*-strategist anaerobes like Spirochaetes, *Desulfosarcina* and Clostridia, which have been shown to degrade cell wall components in saltmarsh (Darjany et al. 2014) and seagrass habitats (Roth and Hayasaka 1984). This pattern suggests degradation of the cellulose component of lignocellulose during earlier stages of seagrass decay by select bacteria capable of this kind of enzymatic breakdown, and thus we predict preferential lignin degradation would occur during advanced stages of remineralisation beyond the timeframe of this work (Benner et al. 1986, Benner et al. 1987).

Effects of temperature and oxygen availability on seagrass remineralisation

Bacterial remineralisation of leaf detritus was enhanced by elevated temperatures, while rhizome/root remineralisation remained unaffected. We hypothesise that these differences may be related to the availability of oxygen during microbial degradation, whereby seagrass leaves decompose under oxic conditions and below-ground tissues under anoxic conditions. These dissimilar conditions led to the development of unique microbial assemblages and subsequently different capacity to degrade the seagrass substrate (Crump and Koch 2008, Fuchs et al. 2011) under differential temperature regimes.

This temperature-enhanced rate of seagrass decomposition has been demonstrated previously when *Zostera marina* leaves experienced enhanced mass loss and decline in fibre content (hemicellulose, cellulose, lignin) over 90 days of aerobic decomposition at elevated temperatures (10°C vs. 25°C) (Godshalk and Wetzel 1978). We also showed that oxic conditions and elevated temperatures caused a doubling of decay rates (Fig. 2.1). Elevated temperatures also heightened degradation of lipid, carbonyl and aromatic compounds predicted from the NMR model (Table 2.1). However, low aromatic signals in the loss spectra (Fig. S2.3) but relatively high aromatic content predicted in the model (Table 2.1) suggest the predicted amount of lignin lost needs to be interpreted with caution. Bacteria have shown to be capable of degrading refractory lignocellulose in marine sediments (Benner et al. 1984, Benner and Hodson 1985, Benner et al. 1986). While we only observed a slight increase in aromatic lignin degradation under increased temperature, we found evidence for increased potential for bacterial metabolism of aromatics in leaf samples, including the degradation of simple aromatics like xylene and metabolism by the enzyme cytochrome P450 (Fig. 2.5). Notably, this latter enzyme is believed to be involved in a key pathway for bacterial lignin degradation (Brown and Chang 2014). Although genes involved in lignin metabolism are not included among the KEGG categories, the data obtained from our analysis of predicted bacterial function using PICRUSt (Langille et al. 2013) implies that seagrass-degrading bacteria have the functional capacity to degrade refractory, aromatic compounds under oxic conditions.

In contrast to the oxic degradation of leaf tissue, we found that the anaerobic metabolism of rhizome and root material was not significantly affected by elevated

temperatures (Fig. 2.1). Since rhizome/roots were not subjected to oxic conditions, it is difficult to distinguish if decomposition of the rhizome/roots was influenced by its greater structural complexity and physical rigidity rather than anoxic conditions alone. However, it has been shown that the easily degradable, interior portions of seagrass rhizomes are preferentially consumed by bacteria, leaving only a hollowed cylinder of organic matter during later stages of decay (Kenworthy and Thayer 1984). Since the microbes were only limited by the rigid, lignified outer walls during advanced stages of decay (170-250 days; Kenworthy and Thayer, 1984), the structural differences between leaf and rhizome/root detritus were not likely to significantly influence early decay of *Z. muelleri* in this short-term experiment.

For rhizome/root decay, there may be an important interaction between oxygen and temperature, whereby the absence of oxygen constrains the effects of temperature-enhanced microbial metabolism. Few studies have looked at this interaction on seagrass remineralisation, but have shown that either the effects of elevated temperatures on remineralisation were negligible under anaerobic conditions compared to aerobic conditions (Godshalk and Wetzel 1978), or that elevated temperatures enhance anaerobic remineralisation to a limit, after which remineralisation rates declined (Pedersen et al. 2011). Specifically, Pedersen et al. (2011) hypothesised that the maintenance and metabolism of slower-growing microbes may be limited at higher temperatures and linked to the time for adaptation to increasing temperatures. Here, we found that elevated temperature did not hinder, but in fact, enhanced aerobic decomposition. This highlights the need for more research to be done on the limitations on anaerobic metabolism under different temperatures and acclimation periods. In either case of enzymatic or growth rate limitations, anoxic conditions may be providing some 'protection' from remineralisation under elevated temperatures. This protection is particularly pertinent within seagrass meadows, since anaerobic respiration dominates C remineralisation in these habitats (Marbà et al. 2006).

The implications of this oxygen-temperature relationship are significant for future C_{org} stocks in blue carbon habitats. For one, the reduced remineralisation capacity of bacteria under anoxic conditions, which are fundamental and ubiquitous to seagrass habitats, may provide a degree of C_{org} protection under warming seawater temperatures, giving these systems a sequestering advantage. On the other hand, as seagrass meadows are becoming increasingly lost or degraded through physical disturbances and sediment

resuspension (Orth et al. 2006), previously anoxic pools of stored C_{org} may be introduced to oxic conditions, resulting in enhanced microbial remineralisation of lignocellulose (refractory C). In this scenario, the projected increased temperatures combined with additional aerobic remineralisation, will amplify the risk for loss of refractory C. These observations suggest that bacterial remineralisation of seagrass C is a complex process influenced by potentially interacting abiotic factors. Consequently, blue carbon sequestration may shift as a result of differential microbial communities and processes influenced by changing environmental conditions and physical disturbances.

Chapter 2 Supplementary Tables and Figures

Table S2.1: Summary of Analysis of Covariance (ANCOVA) statistics. Temp = temperature treatment, Nutr = nutrient treatment.

Tissue	Response Variable	Residual MS Error	*F*-value[a]			
			Time	Temp	Nutr	Temp X Nutr
Leaf	% weight loss	0.015	73.976***	7.069*	0.014	0.261
	% carbon	5.945	2.250	0.206	1.343	4.163*
	% nitrogen	0.011	0.287	16.273***	0.980	2.660
	C:N ratio	7.090	1.626	14.768***	0.020	0.000
	Bacterial abundance	2.641E+12	9.044**	1.624	1.883	0.137
	Chao1	2277.813	0.181	1.428	0.003	0.486
	Simpson's Evenness	< 0.001	129.695***	8.240**	0.719	0.664
	Sulphide reduction rate	249.173	1.437	n/a	n/a	n/a
Rhizome/Root	% weight loss	0.004	25.172**	0.350	0.580	0.111
	% carbon	5.474	2.147	2.549	0.000	0.569
	% nitrogen	0.005	56.649***	0.002	8.118**	0.004
	C:N ratio	20.411	42.228***	1.250	4.915*	0.589
	Bacterial abundance	1.649E+12	12.798***	0.024	7.553**	3.968
	Chao1	1388.442	3.840	2.748	0.070	2.558
	Simpson's Evenness	< 0.001	16.370**	7.645*	0.422	0.424
	Sulphide reduction rate	214.916	1.969	n/a	n/a	n/a
Sediment Only	Chao1	2189.373	3.068	0.264	1.304	0.729
	Simpson's Evenness	0.001	4.625*	2.954	0.016	2.481
	Sulphide reduction rate	0.120	84.224***	n/a	n/a	n/a

[a] *** $p \leq 0.001$; ** $0.001 < p \leq 0.01$; * $0.01 < p \leq 0.05$; no asterisk $p > 0.05$

Table S2.2: Summary table of Analysis of Similarity (ANOSIM) statistics.

	Bacterial Community	
	R-statistic	P-value
Tissue	**0.690**	**0.001**
Time	**0.342**	**0.001**
Temperature	**0.119**	**0.002***
Nutrient	**0.090**	**0.003***
Tissue x Time	**0.945**	**0.001***
Tissue x Temperature	**0.682**	**0.001**
Tissue x Nutrient	**0.649**	**0.001**
Temperature x Time	**0.265**	**0.001***
Temperature x Nutrient	**0.070**	**0.013***
Tissue x Time x Temperature	**0.919**	**0.001**
Tissue x Time x Nutrient	**0.874**	**0.001***

* Pairwise tests indicate that overall significance is due to differences related to initial bacterial community only.

Table S2.3: Raw spectral intensities of main ^{13}C- CPMAS NMR functional groups expressed as % of total spectral intensity for each tissue and treatment throughout incubation period. The spectral ranges integrated for each functional group were amide/carboxyl/ketone (215–165 ppm), O-aromatic (165–145 ppm), aromatic (145–110 ppm), di-O-alkyl (110–95 ppm), O-alkyl (95–60 ppm), N-alkyl/methoxy (60–45 ppm), and alkyl (45 to 10 ppm).

Tissue	Treatment	Sampling Day	Alkyl	N-Alkyl/ Methoxyl	O-Alkyl	Di-O-Alkyl	Aromatic	O-Aromatic	Amide/Carboxyl / Ketone
Leaf		Day 0	7.98	3.46	57.50	11.92	8.24	1.92	8.98
Leaf	23°C, no NP	Day 7	7.51	3.16	54.13	11.41	10.34	3.15	10.31
		Day 28	7.10	3.19	55.47	11.53	9.90	2.75	10.06
		Day 84	9.02	4.03	50.82	10.53	10.70	3.37	11.53
Leaf	23°C, + NP	Day 7	6.44	2.74	53.77	11.50	11.11	3.33	11.11
		Day 28	7.97	3.53	54.43	11.20	9.79	2.99	10.09
		Day 84	7.72	3.35	53.46	10.92	10.28	3.28	10.99
Leaf	30°C, no NP	Day 7	8.09	3.41	55.84	11.42	8.94	2.73	9.57
		Day 28	7.35	3.36	56.34	11.53	8.95	2.71	9.77
		Day 84	7.68	3.49	55.79	11.08	9.41	2.58	9.97

Tissue	Treatment	Sampling Day	Alkyl	N-Alkyl/ Methoxyl	O-Alkyl	Di-O-Alkyl	Aromatic	O-Aromatic	Amide/Carboxyl / Ketone
Leaf	30°C, + NP	Day 7	7.30	3.25	55.44	11.48	9.75	2.77	10.02
		Day 28	7.83	3.54	55.96	11.41	9.22	2.58	9.46
		Day 84	6.77	3.17	56.54	11.46	9.19	2.80	10.06
Rhizome /root		Day 0	6.98	3.32	58.10	12.12	7.85	2.64	8.99
Rhizome /root	23°C, no NP	Day 7	6.53	3.21	56.29	12.20	8.10	2.99	10.67
		Day 28	5.99	2.73	57.62	12.42	8.07	3.02	10.16
		Day 84	7.38	3.22	52.37	11.50	9.62	3.69	12.22

Tissue	Treatment	Sampling Day	Alkyl	N-Alkyl/ Methoxyl	O-Alkyl	Di-O-Alkyl	Aromatic	O-Aromatic	Amide/Carboxyl / Ketone
Rhizome /root	23°C, + NP	Day 7	7.07	2.97	56.20	12.10	8.15	3.06	10.44
		Day 28	7.39	3.46	58.49	12.27	6.94	2.31	9.16
		Day 84	9.55	4.28	48.42	10.58	9.79	4.15	13.24
Rhizome /root	30°C, no NP	Day 7	7.36	3.50	59.03	12.36	6.22	2.45	9.08
		Day 28	6.63	2.89	57.51	12.26	7.71	2.72	10.28
		Day 84	7.17	3.26	51.23	11.44	10.03	4.04	12.83
Rhizome /root	30°C, + NP	Day 7	6.46	2.87	53.81	11.95	9.35	3.43	12.13
		Day 28	6.87	2.78	58.46	12.40	7.27	2.58	9.64
		Day 84	7.71	3.17	53.50	11.69	8.94	3.29	11.69

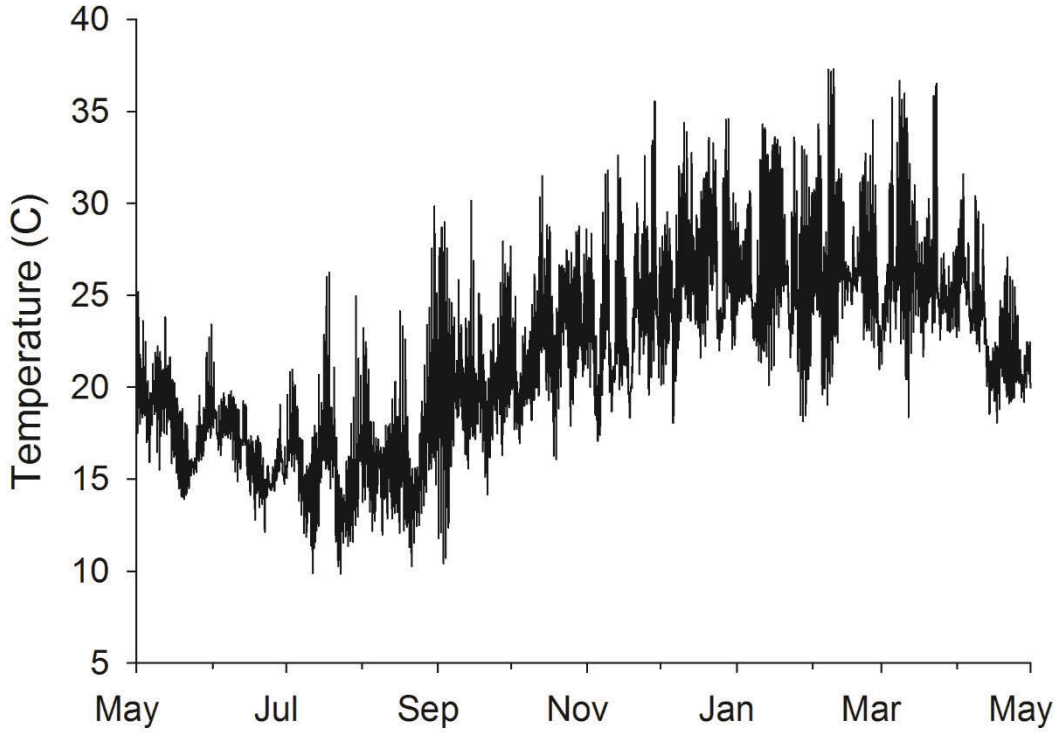


Figure S2.1: Water temperature recorded over the course of 1 year (May 2013 – May 2014) in a *Zostera muelleri* meadow in Fagans Bay, Central Coast, Australia.

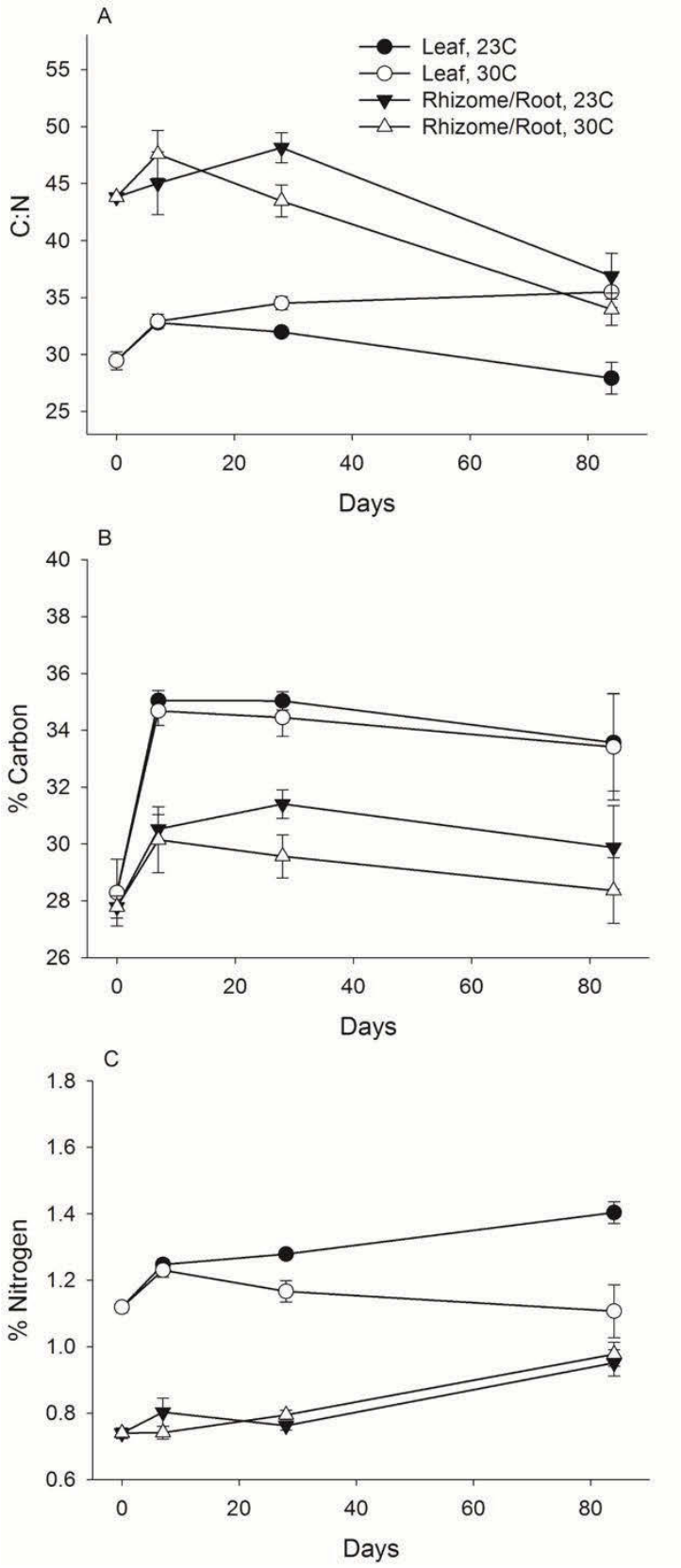


Figure S2.2: Elemental (a) C:N ratio (b) carbon and (c) nitrogen content. Values represent mean ± S.E.M.

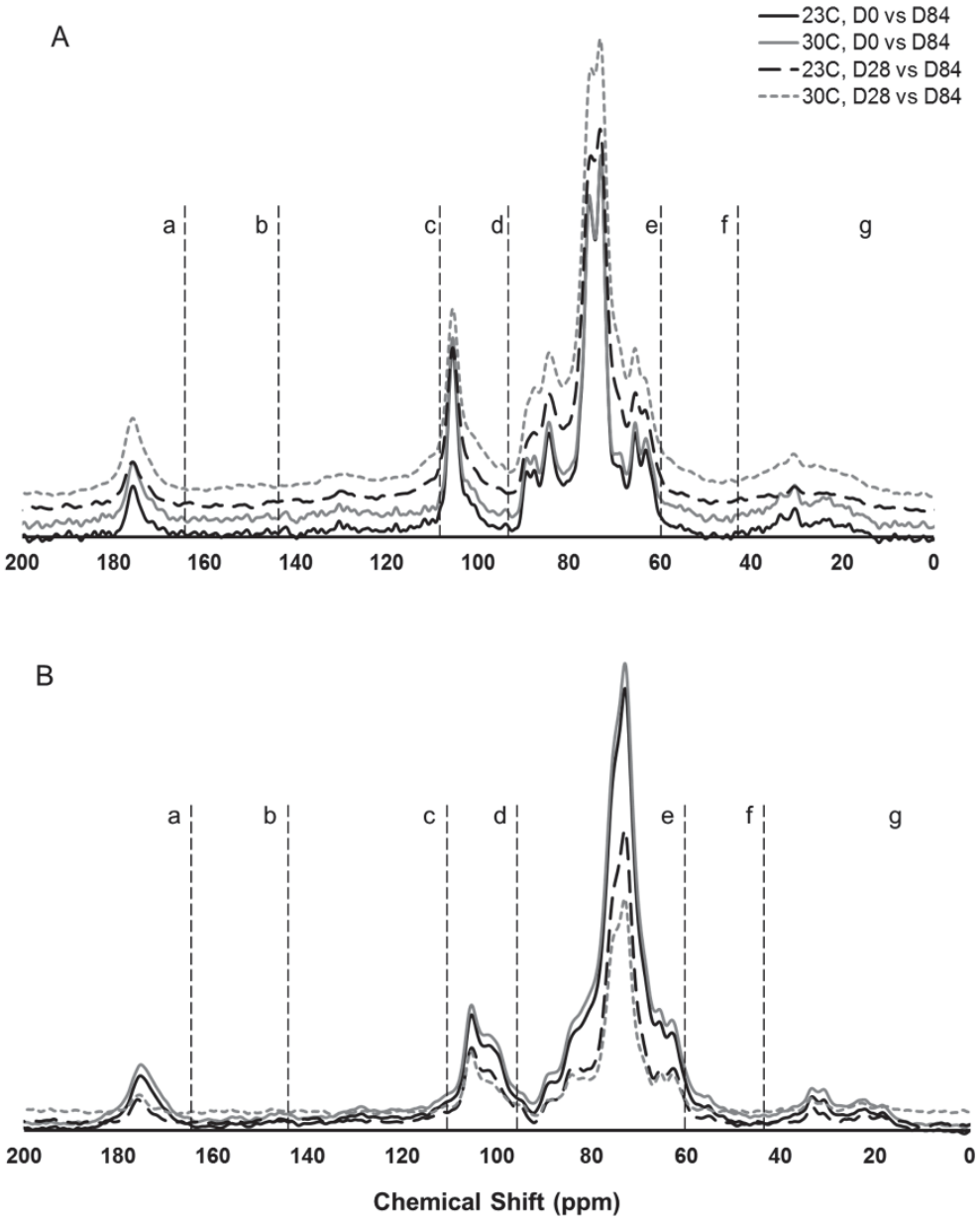


Figure S2.3: Solid-state ^{13}C- CPMAS NMR spectra of mass loss through decomposition normalised to organic C loss for (a) leaves and (b) rhizome/roots over the entire experiment (Day 0 vs. Day 84) and during the last two months (Day 28 vs. Day 84) under each temperature treatment. Letters indicate the functional groups: (a) Amide/Carboxyl/Ketone (165-215 ppm), (b) O-Aromatic (145-165 ppm), (c) Aromatic (110-145 ppm), (d) Di-O-Alkyl (95-110 ppm), (e) O-Alkyl (60-95 ppm), (f) N-Alkyl/Methoxy (45-60 ppm), (g) Alkyl (0-45 ppm).

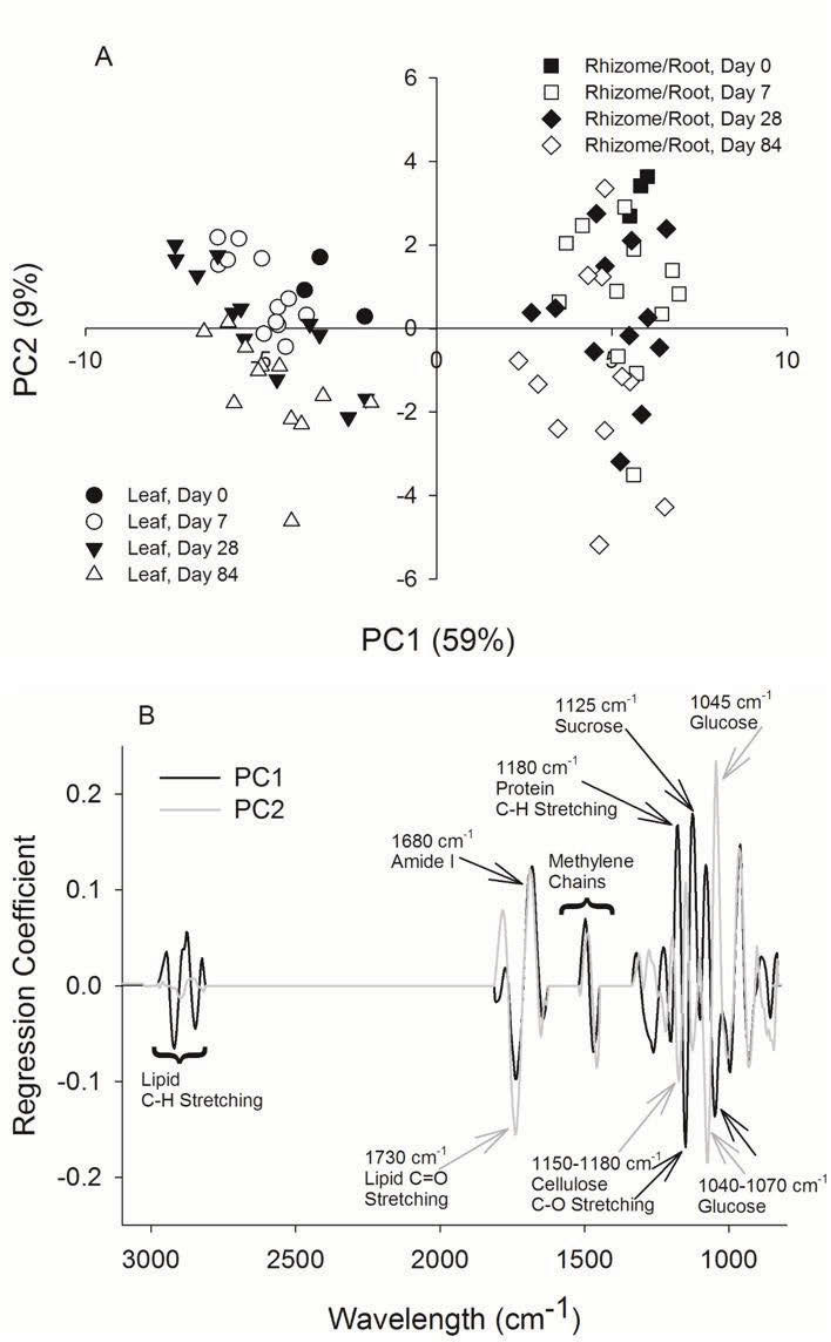


Figure S2.4: Diffuse reflective mid-infrared spectroscopy. (a) Principal components analysis with PC1 representing differences among tissue types and PC2 representing shifts through time. (b) Spectra loadings based on PCA scores. Positive PC1 regression coefficients (black) correspond with greater signals in leaf tissues and negative coefficients for rhizome/root tissues. Positive PC2 regression coefficients (grey) correspond with greater signals in late stages of decay (Day 28, Day 84) and negative coefficients for early stages of decay (Day 0, Day 7).

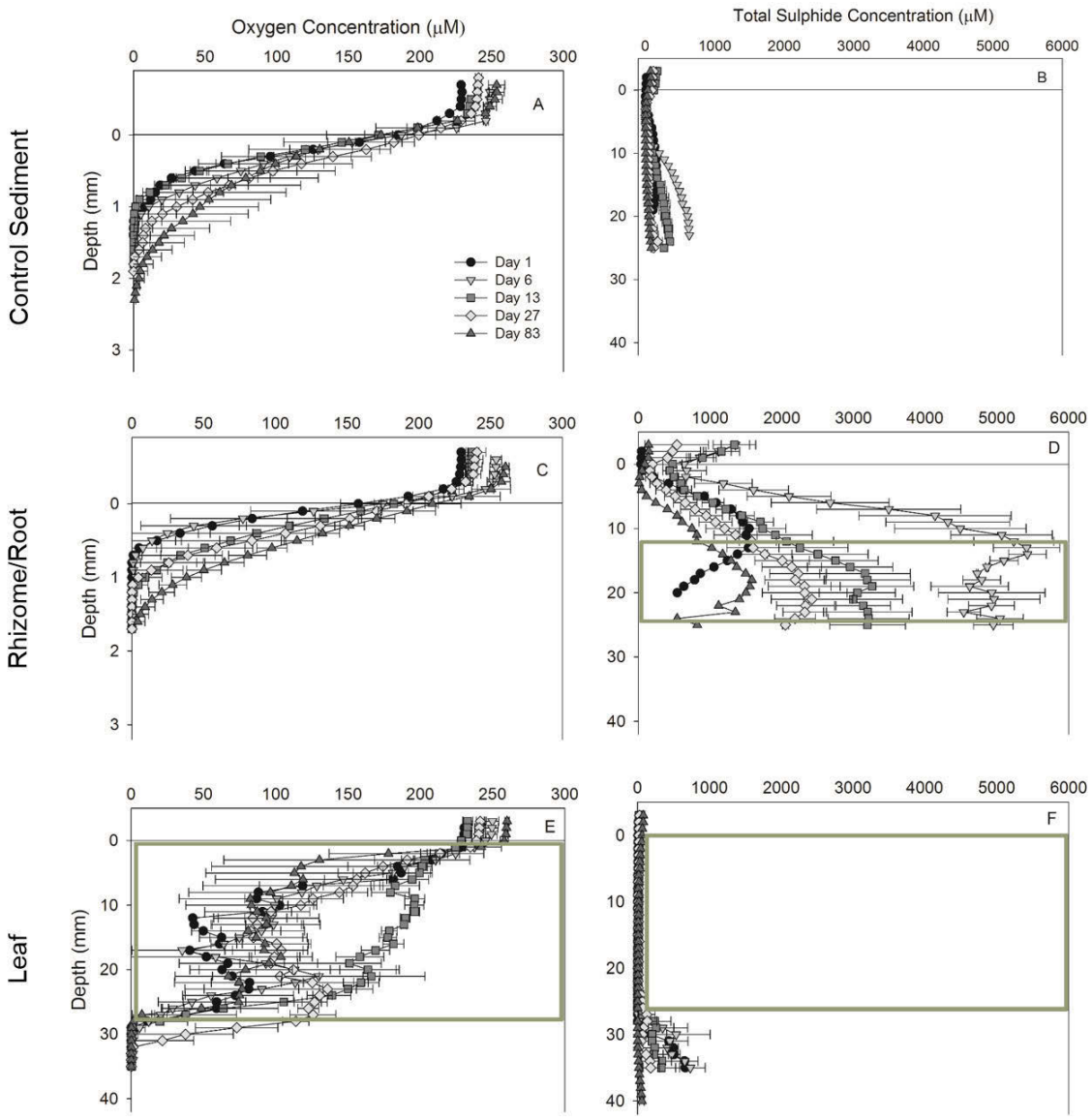


Figure S2.5: Oxygen and total sulphide microsensor profiles for 23°C, no nutrient treatments. (a-b) Control, sediment samples, (c-d) rhizome/root samples, and (e-f) leaf samples. Boxes indicated the location of the litter bags for leaf and for rhizome/root samples. Zero on the y-axis represents the sediment surface for control and rhizome/root samples and the litter bag surface for leaf samples. Note the y-axis for (a) and (c) focus only on the top 3 mm of the sediment surface. Values represent mean ± S.E.M.

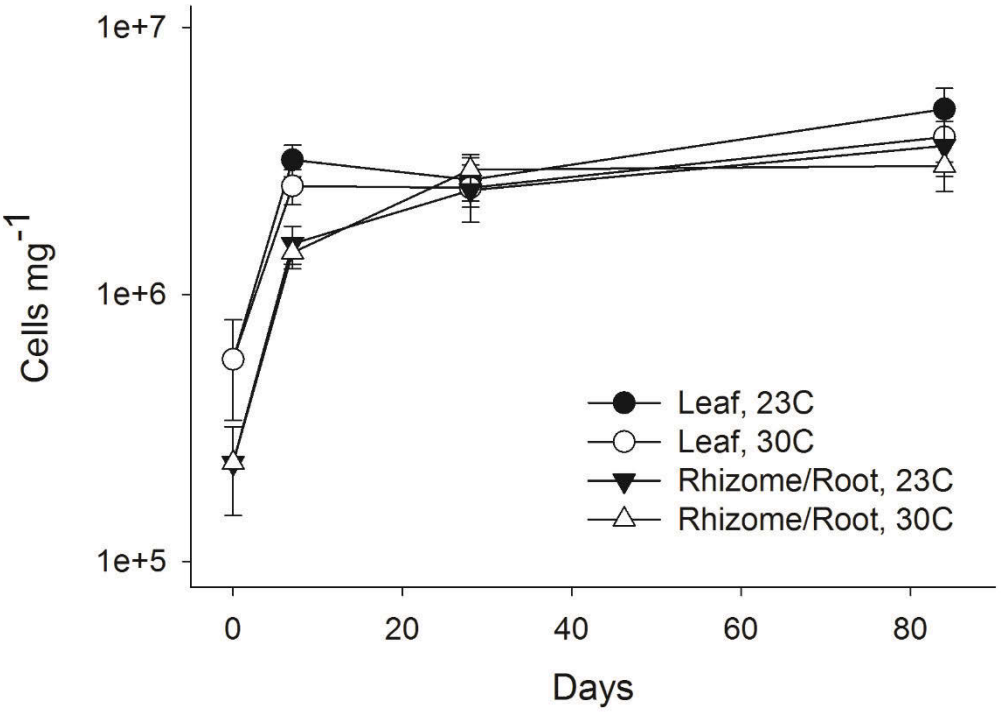


Figure S2.6: Abundance of bacterial cells associated with decaying seagrass tissue. Values represent mean ± S.E.M. Note the y-axis is on a logarithmic scale.

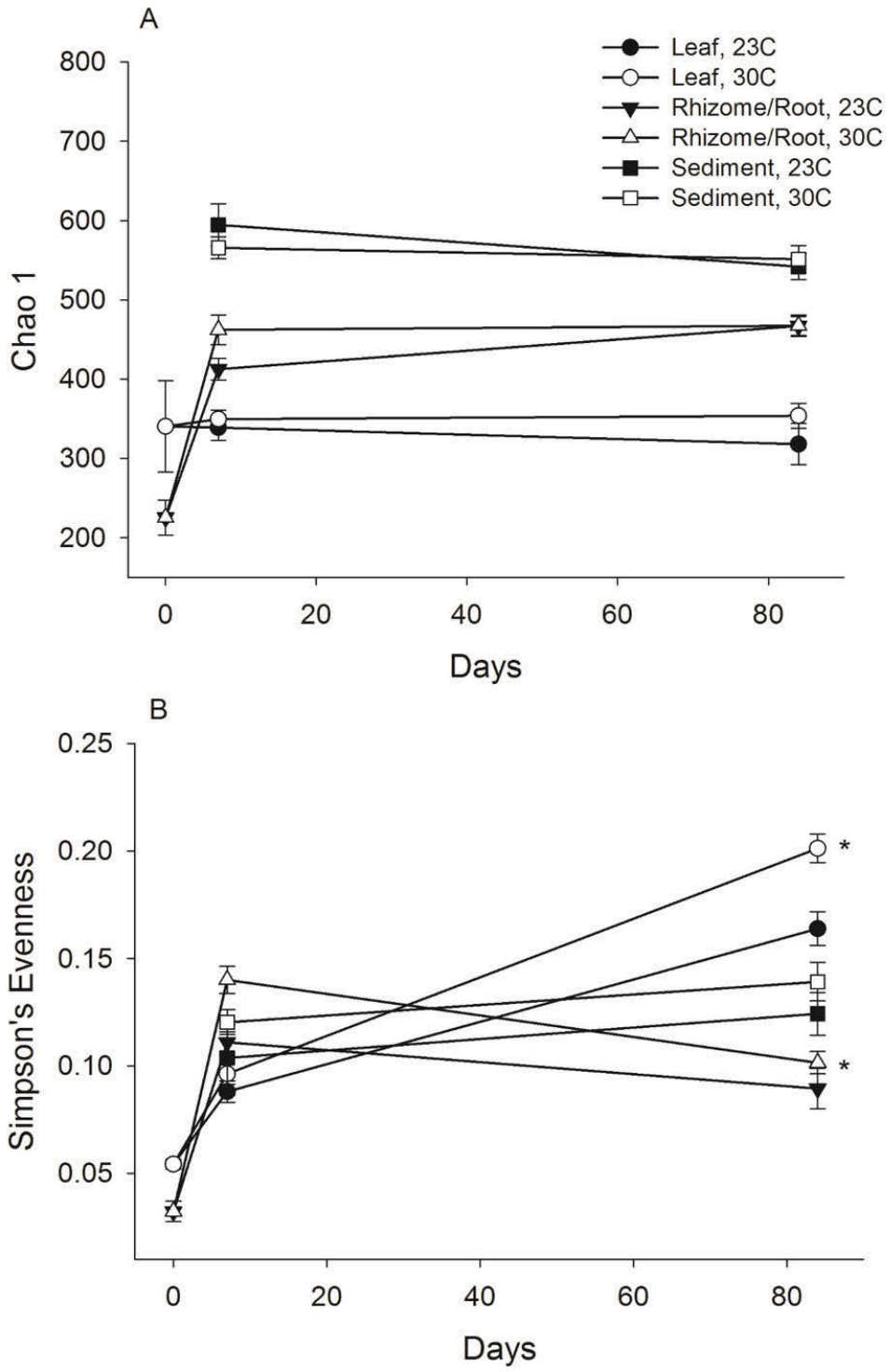


Figure S2.7: Alpha diversity statistics (a) Chao 1 richness and (b) Simpson's Evenness, for the bacterial and archaeal communities at the genus-level. Chao 1 did not significantly vary through time for any substrate type, while evenness varied significantly for all substrates through time. Asterisks indicate significant differences due to elevated temperature. Values represent mean ± S.E.M.

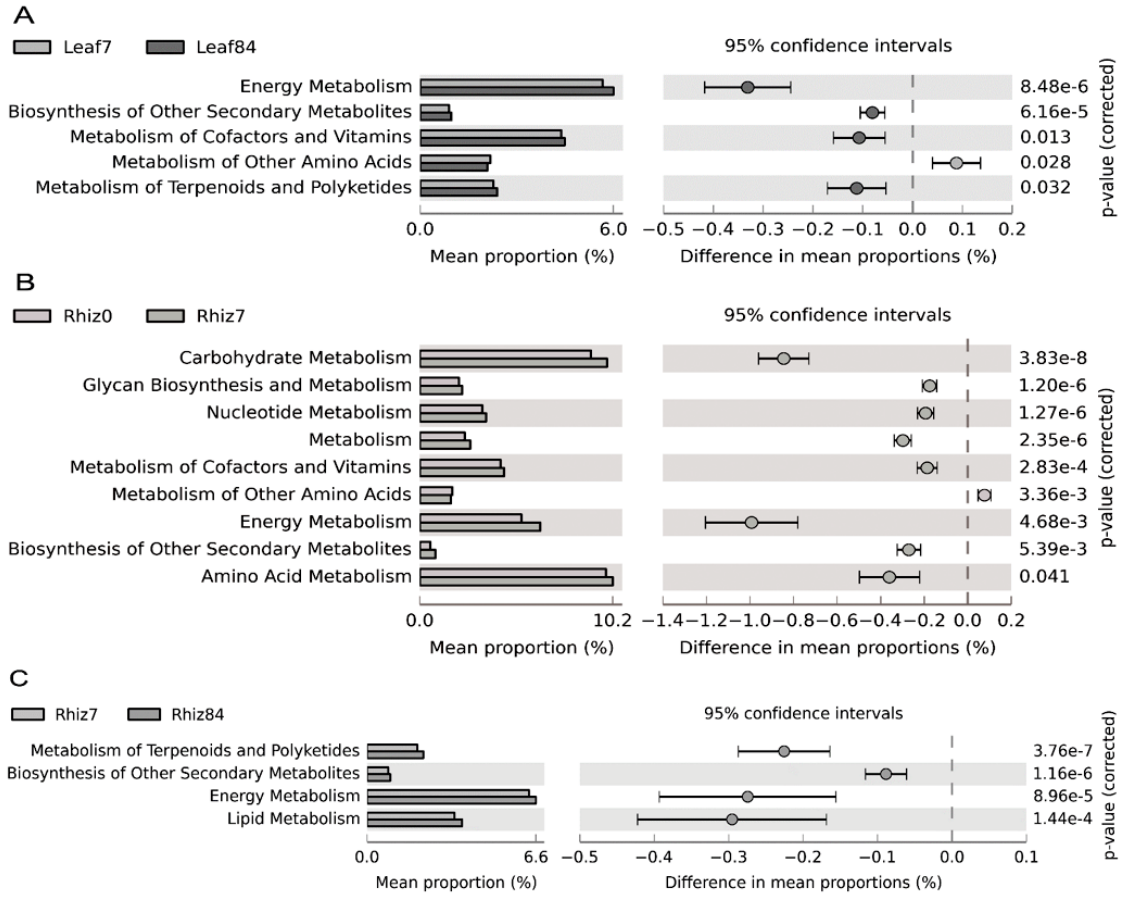


Figure S2.8: Statistical Analysis of Metagenomic Profiles (STAMP) of predicted metagenomes from PICRUSt analysis for differences in metabolic KEGG Pathways throughout time at Level 2 for (a) leaf tissue between Days 7 and 84, and for rhizome/root tissue (b) metabolism between Days 0 and 7, and (c) metabolism between Days 7 and 84.

Chapter 3:

Long-term microbial remineralisation of seagrass under natural field conditions

Abstract

Seagrass biomass represents a significant source of carbon that can contribute to long-term sediment carbon stocks in coastal habitats. Microbial remineralisation of seagrass detritus is one of the main drivers of carbon-cycling in seagrass meadows and ultimately influences the amount and quality of carbon available for sequestration. Here, our goal was to better understand how litter quality (leaf vs. rhizome/root), nutrients (nutrient-enriched vs. unenriched sites) and oxygen availability (linked to microbial communities) affect the long-term microbial decomposition of the seagrass *Zostera muelleri*. Site nutrient condition influenced sediment biogeochemistry, hydrodynamics and the seagrass-associated bacterial communities, but had little influence on long-term decomposition rates. The results showed a clear succession of bacterial communities for both tissues types from *r*-strategists like Alphaproteobacteria to *K*-strategies such as Deltaproteobacteria, Spirochaetes, Cytophagia and Acidobacteriia. We were also able to detect for the first time the refractory, or stable, phase of decomposition in seagrasses. The persistence of rhizome/root biomass after two years of decay were attributed to the anoxic conditions and the accumulation refractory organic matter in the later stages of decay. While we predict that rhizome/root biomass will contribute more to the long-term sediment carbon stocks, the preservation of leaf carbon may be enhanced at locations were sedimentation is high and burial to anoxic conditions occurs quickly.

Introduction

Seagrass ecosystems represent a globally significant sink of organic carbon (C_{org}), and accordingly, their biosequestration of the greenhouse gas CO_2 is critical for climate change mitigations efforts (Laffoley and Grimsditch 2009, McLeod et al. 2011). Seagrass meadows derive C from autochthonous sources (plant-produced through the photosynthetic fixation of dissolved CO_2 from the water column), as well as allochthonous sources (externally-produced from phytoplankton, terrestrial plants, macroalgae) (Duarte et al. 2013b, Macreadie et al. 2014a). Seagrass biomass alone provides on average half of the C_{org} to the top 5-10 cm of sediment C_{org} stocks (Kennedy et al. 2010), and depending on burial rate and microbial remineralisation, can persist for decades or centuries (Macreadie et al. 2012, Greiner et al. 2013).

Microbial remineralisation is the primary process of plant carbon (C) transformation and cycling of seagrass biomass (Harrison 1989), and thus ultimately determines the amount of C available for sequestration into sediments. The process of microbial remineralisation of plant matter typically occurs in three phases. The leaching phase is characterised by the passive loss of labile compounds, which benefits fast-growing *r*-strategist microbes, or copiotrophs, (Fierer et al. 2007, Berg and McClaugherty 2008a). Next, microbes colonise and actively decompose the substrate through enzymatic processes, which is followed by a refractory phase characterised by little to no loss in detrital mass (Wilson et al. 1986a). These latter two phases are dominated by slower-growing *K*-strategists, or oligotrophs, that can breakdown more refractory compounds under low-nutrient conditions, including (holo)cellulose polysaccharides and lignin (Sinsabaugh et al. 2002, Fierer et al. 2007). Seagrass decomposition has been hypothesised to follow a similar sequence of three phases (Pellikaan 1982), while a more complex 5-factor model was created to help explain the factors that influence seagrass decomposition (Godshalk and Wetzel 1978). In this second model, elevated temperatures and oxygen and exogenous nutrient availability were predicted to increase decay rates, while increased refractory C content and larger detritus particle size decreased the rate of seagrass decay (Godshalk and Wetzel 1978).

Despite subsequent studies utilising these two decomposition models, there still remains several knowledge gaps concerning the factors that influence microbial remineralisation of seagrass C, having important implications for C_{org} sequestration. First, the leaching and microbial remineralisation phases of decay have been shown to

occur for leaves, rhizomes and roots (Pellikaan 1982, Josselyn et al. 1986, Harrison 1989), but the third refractory phase of decay has never been quantified. This is likely attributed to most decay studies occurring for less than a year (Table I1), under the hypothesis that much of the seagrass biomass does not persist after a year of microbial remineralisation (Mateo et al. 2006). However, there is in fact evidence that leaf and below-ground biomass can persist for longer than a year of decomposition (Wahbeh and Mahasneh 1985, Opsahl and Benner 1993, Fourqurean and Schrlau 2003). Secondly, no single seagrass decay study has included more than two of the five factors from the Godshalk and Wetzel (1978) model in the same experiment (Table I1). As a result, we have very little information on if or how these five factors interact during seagrass decomposition. Lastly, although microbial remineralisation drives seagrass decomposition, only a third of the decay studies have quantified a microbial response in conjunction with traditional decomposition response variables (e.g., decay rates, elemental content; Table I1). Furthermore, much of what is known about the bacterial response during seagrass decomposition centres on bacterial growth rates, abundance and metabolic pathways (Wahbeh and Mahasneh 1985, Blum and Mills 1991, Peduzzi and Herndl 1991), but little is known about the microbial taxonomic communities driving seagrass decay (Wahbeh and Mahasneh 1985).

This study will address each of these knowledge gaps by assessing the microbial decomposition of leaf and rhizome/root detritus from the seagrass *Zostera muelleri*. First, we aimed to capture the beginning stages of the refractory phase of decomposition by extending the decomposition experiment over a two-year period, as well as evaluating the microbial communities associated with the decaying seagrass via 16S rDNA amplicon sequencing. Additionally, we included three factors in the Godshalk and Wetzel (1978) model (refractory C, exogenous nutrient availability and oxygen availability) to assess their effects and potential interactions on seagrass decomposition. The refractory component was represented by litter type: *Z. muelleri* leaf and rhizome/root tissues. Leaf tissues typically are more nutritious due to their lower C:N ratios (Vichkovitten and Holmer 2004), and would be expected to decompose more quickly than rhizome/root tissues, which contain more refractory C in the form of lignocellulose (Trevathan-Tackett et al. 2015). The nutrient factor was represented by the field locations used for the incubations, i.e., eutrophic vs non-eutrophic conditions. We hypothesise that the higher supply of exogenous nutrients at the eutrophic sites will

provide non-nutrient limiting conditions for the microbial populations and lead to enhance seagrass remineralisation (López et al. 1998). Lastly, the oxygen conditions used for each tissue type reflected the conditions the detritus would experience under natural conditions, i.e., rhizome/roots were buried in the sediment and leaves were placed on top of the sediment and allowed to be buried by sediments over time. We hypothesised that anoxic conditions will reduce the microbial decomposition of the detritus (Godshalk and Wetzel 1978). By using this multi-variable approach, we aim to further inform the seagrass decomposition models and re-evaluate the factors influencing the contribution of seagrass C to sediment C_{org} stocks.

Methods

Site selection and sediment characterisation

Litter bag incubations took place at 3 sites in the Brisbane Waters Estuary, Central Coast, New South Wales, Australia from 1 May 2013- 28 April 2015 (729 days). The sites were chosen based on their nutrient loads and presence of *Zostera muelleri* Irmisch ex Ascherson habitat. Overall, the estuary experiences medium anthropogenic pressure, primarily attributed to high nutrient and sediment inputs (Roper et al. 2011). The highly impacted sites chosen for this study were in Fagans Bay, one within a *Z. muelleri* meadow ('Impacted'; 33.4306S, 151.3211E) and one that was currently bare ('Bare'; 33.4347S, 151.3228E). The bay has the slowest flushing rate in the catchment and receives input from Narara Creek that provides nearly a third of the total N and total P load for the entire Brisbane Waters catchment (Collier and Mackenzie 2008). The non-impacted site, located near Saratoga, NSW ('Pristine'; 33.4732S, 151.3357E), is approximate 4.5 km south of Fagans Bay and 7.5 km from the estuary inlet. This site also contains extensive *Z. muelleri* beds but is located next to a natural reserve. While no data was available on direct loads for the Saratoga site, there is no nearby waterway that could provide point-source nutrient inputs. Additionally, water column nitrogen concentrations at the Saratoga (Pristine) site are approximately half of what is experienced in Fagans Bay (Collier and Mackenzie 2008).

Cores were taken to characterise the sediments at each site. Ten cores (10 cm diameter, 30 cm length) were taken along a transect parallel to the shore at 1 m intervals in ~0.25 m water depth. Cores were refrigerated at 4°C, while processing occurred over

the following 10 days. Cores were divided at 0-3 cm, 3-10 cm, 10-15 cm and 15+ cm, weighed then dried at 60°C. Dried subsamples were taken for particle size analysis, elemental content (total C, C_{org}, nitrogen) and stable isotope content ($\delta^{13}C$). All subsamples, except those for particle size analysis, were ground to a fine powder (Pulvisette 7, Fritsch, Germany). Stable isotope and C_{org}:N data were used for estimating the OM sources contributing to the sediments, so plant references were also collected from each site: *Z. muelleri* wrack, *Avicennia marina* mangrove leaves, epiphytes from live seagrass blades and seston/plankton. Seston/plankton was collected by filtering 1 L of estuarine water on pre-combusted GFF filters. Filters and plant material were dried at 50°C and ground by mortar and pestle (epiphytes) or ball mill (wrack, mangrove; Pulvisette 7, Fritsch, Germany) prior to analysis.

Decomposition experimental design and sampling procedure

Z. muelleri leaf and rhizome/root material for the decomposition experiment were collected from Fagans Bay from October 2012 - January 2013. Leaf material was collected as fresh wrack along the shoreline. Live plants were collected for rhizome/root material and subsequently washed of sediments and attached infauna. All seagrass material was stored at -20°C prior to start of experiment. *Z. muelleri* leaf wrack (~500 g FW, 70 g DW; 30 cm x 30 cm) and fresh rhizome/root biomass (~60 g FW, 10 g DW; 15 cm x 15 cm), were weighed and added to 1.0 mm polyester mesh litter bags (Miami Aqua-culture, Inc., Miami, FL, USA). To replicate natural decomposition conditions, rhizome/root bags were buried under at least 2 cm of sediment, and leaf bags were anchored down on top of the sediment. The 2 cm burial depth is within the rhizosphere range and is fully anoxic based on previous microsensor work done on NSW estuaries, which have shown that the anoxic zone begins after ~2 mm (data unpublished). Bags were assigned to random positions in a 15 bag x 6 row grid (i.e., 45 bags for each tissue type at each site). Each row was kept in place by rope and anchors. Temperature was monitored by loggers attached to the anchors at each site (n = 3; UA-002-08 HOBO Pendant®, Onset Computer Corp., Bourne, MA, USA). Destructive sampling occurred at 14, 42, 98, 168, 389, 519 and 729 days (n = 5 for each tissue type per site). Samples for bacterial community characterisation were subsampled from the seagrass litter (~ 1-2 cm sections of tissue) within 24 hrs after returning to the laboratory and stored at -80°C until processing. The remaining bulk litter was washed of sediments and weighed

before and after drying at 60°C. Seagrass litter samples were subsequently mixed before grinding a representative subsample into a fine powder for biochemical analyses (Pulvisette 7, Fritsch, Germany).

Biogeochemical analyses

Sediment particle size was analysed from 4 representative cores from each site (Mastersizer 2000, Malvern Instruments Ltd., Worcestershire, UK). Samples were first roughly broken apart by mortar and pestle before sieving at 2 mm to remove large shells. Samples were sonicated in a bath for 5 min to break apart any remaining non-natural aggregates caused by drying, then rested for 5 min to cool and allow for reformation of any natural aggregates (Elliott and Cambardella 1991, Buurman et al. 1997, Sperazza et al. 2004). The particle size of each sample was scanned five times then averaged for a final distribution under the following specifications: refractive index = 1.522, absorbance = 0.1-1.0. Sediments were categorised as coarse sand = 500-2000 μm, fine to medium sand = 62.5-500 μm, clay and silt = 0.01-62.5 μm.

Total carbon (C) and nitrogen (N) were quantified for sediments and plants using an elemental analyser (LECO TruSpec, St. Joseph, MI, USA). Prior to C_{org} and ^{13}C isotope analysis, inorganics were removed by acidification using 1.0 M HCl. Samples were acidified overnight, then washed with ultrapure water to remove excess acid (final pH > 5.5). Sediment C_{org} and $\delta^{13}C$ isotope content were analysed at the Australian Nuclear Science and Technology Organisation (ANSTO) by isotope-ratio mass spectrometry (Thermo Fisher Delta V Plus IRMS linked to Thermo Fisher Flash 2000 HT Elemental Analyser, Thermo Fisher Scientific Inc, Waltham, MA USA). Plant references were analysed by IsoEnvironmental (Europa Scientific 20-20 IRMS linked to ANCA SL Prep Unit, Sercon, Cheshire, UK).

Organic matter (OM) quality of the decaying seagrass was analysed using thermogravimetric analysis (TGA; SDT Q600, TA Instruments, New Castle, DE, USA). One representative sample from each tissue type at each site and sampling time were analysed. Approximately 10 mg of sample was placed in a platinum cup and heated under N_2 (pyrolysis; gas flow 100 mL min^{-1}) at 10°C min^{-1} to 600°C (Yang et al. 2006, Ncibi et al. 2009). Delineation of thermal intervals was based on the rate-of-change derivative (% mass loss $°C^{-1}$), which indicated distinct temperature intervals (TI) of mass loss (Yang et al. 2006, Trevathan-Tackett et al. 2015). Mass loss occurring from

30°C to 180°C is associated with the loss of moisture. Preliminary analysis showed that much of the TI_1 (labile; soluble carbohydrates, hemicellulose) mass from 180°C to 220°C (Chapter 1) was not present, likely due to degradation. For simplicity, both labile (soluble carbohydrates, hemicellulose) $TI_{1\ \&\ 2}$ were combined to create $TI_{\boldsymbol{1}}$ from 180°C to 300°C. $TI_{\boldsymbol{2}}$ (refractory; cellulose) extended from 300°C to 400°C, then $TI_{\boldsymbol{3}}$ (refractory; lignin and insoluble polysaccharide residues) from 400-600°C. $TI_{\boldsymbol{1\text{-}3}}$ were recalculated as a proportion of total organic matter (OM) from 180°C to 600°C.

Microbial community analysis

In addition to seagrass-associated bacterial samples collected from the litter, bacterial samples were also collected from the top 1 cm of the bulk sediment at each site and sampling time as a microbial community reference using a 3 mL syringe corer. Genomic DNA was extracted from seagrass and sediment samples using the PowerSoil DNA Isolation Kit (MoBio Technologies, CA, USA). Extracted DNA was amplified using the V4 variable region of 16S rDNA gene with the Eubacterial primers 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5′GGACTACVSGGGTATCTAAT-3′) prior to sequencing using the Illumina MiSeq platform and processed following the manufacturer's guidelines (www.mrdnalab.com, TX, USA). Briefly, paired ends were joined using fastq-join (Aronesty 2011), barcodes were removed and then sequences <150 bp and ambiguous base calls were removed. Operational taxonomic units (OTUs) were generated and chimeras were removed. OTUs were defined at 97% similarity *de novo* and annotated against the Greengenes database (DeSantis et al. 2006).

Data and Statistical Analyses

Seagrass decay rates were calculated by fitting proportion dry weight remaining to single-component ($Wt/Wo = e^{-kt}$), double-component ($Wt/Wo = \alpha e^{-k_1 t} + \beta e^{-k_2 t}$) and triple-component ($Wt/Wo = \alpha e^{-k_1 t} + \beta e^{-k_2 t} + \delta e^{-k_3 t}$) exponential decay models using 2-, 4- and 6-parameter models, respectively (1000 iterations, 0.001 tolerance; Dynamic Fit Wizard; Sigma-Plot 12, Systat Software, Inc., San Jose, CA, USA). The k parameter represented the decay rate for each component, and each component represented a varying lability, which can be divided into different phases or rates of decay (Valiela et al. 1985): α = leaching phase, β = microbial colonisation and remineralisation phase, δ = refractory phase. The sum of all components in a model equalled one.

A mixing model using the $\delta^{13}C$ data specific to each site was used to estimate the plant sources contributing to the sediment OM (IsoSource, Phillips and Gregg 2003). Models were run at 1% increments and a 0.01 tolerance using three sources (seagrass wrack, mangrove leaf, seston/plankton) and one constraint ($\delta^{13}C$). Box plots (25/75 percentile, minimum and maximum) were used to visualise the distribution of possible mixing model solutions.

The effects of site, tissue type and decomposition time on sediment elemental content, and the effects tissue type and decomposition on seagrass response variables were analysed with analysis of variance (ANOVA, SPSS v19, IBM, Armonk, NY, USA). If data did not meet normality or homogeneity of variance assumptions after arcsine transformation, non-parametric Kruskal-Wallis analyses were used. Analyses were performed with $\alpha = 0.05$, and a Bonferroni adjustment was applied for all pairwise comparisons. Multi-factorial permutational analysis of variance (PERMANOVA) were performed on stable isotope/C:N content of the sediments and the bacterial community data (PRIMER-E, Clark and Gorley 2006). A Euclidean resemblance matrix on non-transformed sediment isotope and elemental data was created prior to running a 2-way PERMANOVA test (*site* x *depth*). The TGA data was limited by analysing one replicate, so a 2-way PERMANOVA (*tissue* x *time*) performed to identify the shifts in seagrass OM quality over time (TI_{1-3}) after a Euclidean resemblance matrix on non-transformed data was created. Bacterial class data were square-root transformed prior to creating a Bray-Curtis similarity resemblance matrix, followed by a 3-way PERMANOVA analysis (*site* x *tissue type* x *time*). Monte Carlo approximated P-values (*P(MC)*) were used in all analyses to interpret comparisons with low numbers of unique permutations (i.e., <100). All data were visualised using multidimensional scaling (MDS), and included variable vectors based on Pearson's Correlations of the average values. Similarity percentage analysis (SIMPER), with 90% cut off for low contributions, was used to identify the factors driving the shift between the clusters observed in the MDS plots.

Results

Sediment Biogeochemistry

The sediments at all three sites primarily consisted of fine to medium sand (45-65%) with ≤ 15% coarse sand (Table 3.1). At the Bare site, the 0-3 cm and 3-10 cm depths had the highest amount of clay and silt (50-55%), which decreased to ~40% below 10 cm (Table 3.1). Elemental content (N and C_{org}) significantly varied across sites with Impacted and Bare sediments having significantly higher N ($F_{2,104} = 4.959$, $P = 0.009$) and C_{org} ($F_{2,107} = 35.545$, $P < 0.001$) than the Pristine site (Fig. 3.1a, b). Elemental content did not change with depth for the Pristine sediments (Fig. 3.1). N decreased exponentially with depth for Impacted ($F_{3,34} = 14.043$, $P < 0.001$) and Bare ($F_{3,34} = 40.605$, $P < 0.001$) sediments, and C_{org} decreased linearly with depth for Impacted ($F_{3,34} = 31.171$, $P < 0.001$) and Bare ($F_{3,36} = 20.802$, $P < 0.001$) sediments (Fig. 3.1a, b). The cumulative C_{org} stocks in the top 15 cm was highest in the Bare and Impacted sites, while the Pristine site, comparably, had approximately a third of the C_{org} stocks (Table 3.1).

Stable isotope and elemental signatures of the sediments were compared with those of the plant reference sources (Figs. 3.1, S3.1). The polygon created by the elemental C_{org}:N ratio and $\delta^{13}C$ signature of the plant references contained 60% of the sediment samples (Fig. 3.1c). The references contained the entire span of $\delta^{13}C$ signatures, so the remaining 40% of the samples had C_{org}:N ratios outside the range of the references (C_{org}:N = 15-25; Fig. 3.1c). Although Pristine samples were more enriched than the Impacted and Bare sites (Fig. 3.1c), PERMANOVA analysis indicated that there was no difference in isotope/element signatures across sites. There were significant differences across depths (*Psuedo-*$F_{3,95} = 3.1303$, *P(perm)* = 0.033) and a site x depth interaction (*Psuedo-*$F_{6,95} = 2.892$, *P(perm)* = 0.018). Pairwise comparisons and SIMPER analyses showed that the significant differences among depths were driven by the increased C_{org}:N ratios with increasing depth. The significant site x depth interaction further revealed that the increasing C_{org}:N ratios were driven by changes in the Bare site only, e.g., 6.7 at 0-3 cm to 12.1 at 3-10 cm to 18.4 at 10-15 cm to 37.4 below 15 cm.

Table 3.1: Grain size and C_{org} stocks of each site from the Brisbane Waters Estuary. Grain sizes are reported as a range across all depths. C_{org} values are the cumulative means (± S.E.M.) from the top 15 cm.

Site	Clay/ Silt (0.01 - 63.5 μm)	Fine-Medium Sand (62.5 – 500 μm)	Coarse Sand (500 -2000 μm)	C_{org} Stocks (g cm^{-3})
Pristine	17.4 - 31.0	60.5 - 68.8	7.5 - 17.4	14.8 ± 1.1
Impacted	25.0 - 45.9	45.3 - 71.7	4.0 - 22.6	41.2 ± 2.8
Bare	29.2 - 59.9	31.4 - 67.4	2.9 - 19.6	46.3 ± 1.7

Mixing model analyses were used to predict the OM contributions of the sediments. Since the C:N ratio the plant materials typically increase through the course of decomposition, e.g., preferential use of N, including them in the mixing model without accounting for this shift would not be appropriate (Fourqurean and Schrlau 2003). Similarly, epiphyte signatures were not included in the mixing model since *Zostera* wrack is typically devoid of epiphytes, either due to grazing or decomposition after leaf senescence (pers. observation). The Pristine site had little down-core variation in OM contributions with seston/plankton having the highest contribution to sediment OM for all depths (medians ranged from ~41-50%; Fig. 3.2). Mangroves contributed higher proportions at the Impacted site (medians 34-44%), while seagrass had a quarter to a third of the contribution across all sites (Fig. 3.2). For Impacted and Bare sediments, mangrove contributions increased ~10-20% with increasing depth at the expense of reduced seagrass contributions (Fig. 3.2).

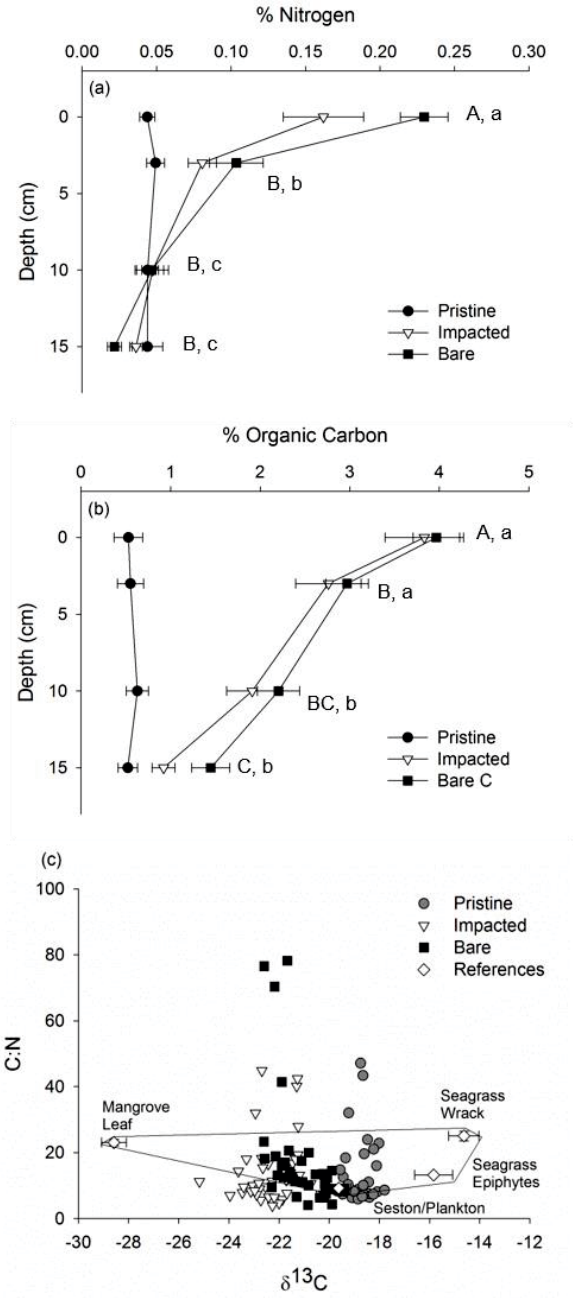


Figure 3.1: Elemental and stable isotope characteristics of the sediments from the Brisbane Waters Estuary. Down-core nitrogen (a) and organic carbon (b) percentages represent means ± 1 S.E.M. Letters indicate significant changes in down-core elemental content from Impacted (capital) and Bare (lower case) sites according to Bonferroni pairwise comparisons after ANOVA statistical analyses. Stable isotope ($\delta^{13}C$) and C:N signatures (c) for sediment samples and the plant reference material. The mixing polygon was created using the plant reference data (means ± 1 S.E.M).

Seagrass decomposition

The effect of site nutrient condition on seagrass decay, C, N, C:N and OM quality by site were not statistically analysed since there nutrient loading condition could not be replicated with multiple sites, e.g., n = 1 per site condition. Descriptively, there was very little variation in these variables across sites throughout the decomposition experiment (Table 3.2, Figs. 3.3, 3.4). Therefore, the results will focus on the differences of these variables observed between the tissue types and the changes occurring throughout the decomposition process. During the two-year decomposition process, the temperatures were similar at all sites, with the winter lows ~12°C, summer highs ~35°C and biannual mean at 21.2 ± 2°C (1 S.E.M). Leaf bags became buried by sediment between the 6 and 12 month sampling. During this same period, some leaf samples had increased abundances of invertebrate and filamentous algae biomass within the litter bags. Additionally after the 6 month sampling, new vegetative seagrass growth began to colonise the top of the leaf litter bags and resulted in increased growth of root mass inside the bag. A section of the Bare site also experienced seagrass colonisation between 18 and 24 months. In the above cases, samples were omitted due to the uncertainty that all root, animal or algae contaminations were entirely removed prior to biomass and chemical analyses.

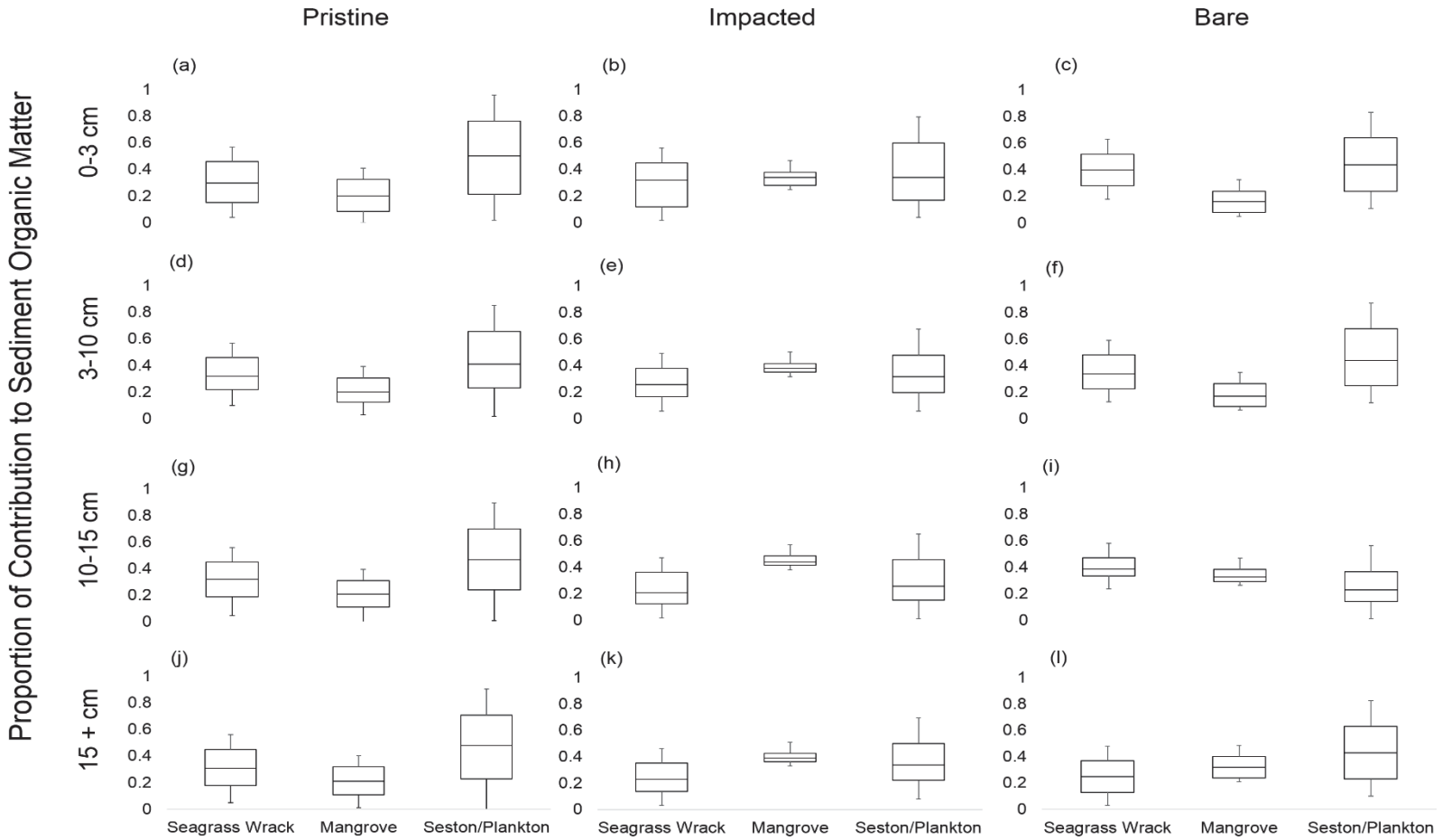


Figure 3.2: Box plots of organic matter contribution predictions for the top 20 cm of sediments. IsoSource mixing model predictions were based on $\delta^{13}C$ signatures.

After two years of decay, leaf litter bags contained slightly less biomass (10 ± 2%) compared to the rhizome/root bags (15 ± 2%), and each tissue type had slightly different patterns of decay (Fig. 3.3). For leaf tissues, Pristine samples fit best with the single-component model and had 11-12% weaker fit for the double- and triple-component models (Table 3.2). The Impacted and Bare samples, however, had strong fits for all three models ($R^2 > 0.90$; Table 3.2). During the first two weeks, the leaf mass increased 20% and had slightly lower decay rates for k_1 in the double- and triple-component models compared to k_2. Leaf tissue mass was continually lost over the remainder of the experiment and did not show signs of a stable refractory phase (Fig. 3.3a). For rhizome/root samples, on the other hand, increasing the number of components in the model only had a slight effect on the model fits (< 5%; Table 3.2). Rhizome/root tissues experienced a 20-30% loss in mass during the first few weeks of decay (Fig. 3.3), which was indicated as a higher decay rate for the double- and triple-component decay rates (k_1 = 0.0209-0.0213 d^{-1} and 0.0067-0.0069 d^{-1}, respectively) for Impacted and Bare sites (Table 3.2). There was no difference in k_1 and k_2 for the Pristine double-component model, but the triple-component model had a comparable k_1 to the other sites (Table 3.2). After the initial loss in the first few weeks of decay, rhizome/root tissue mass increased ~10%, but after 98 days experienced mass loss until 12 -18 months, after which the mass loss stabilised (Fig. 3.3b). The triple-component model the predicted the refractory decay rate to occur at k_3 = 0.0014-0.0028 d^{-1} (Table 3.2).

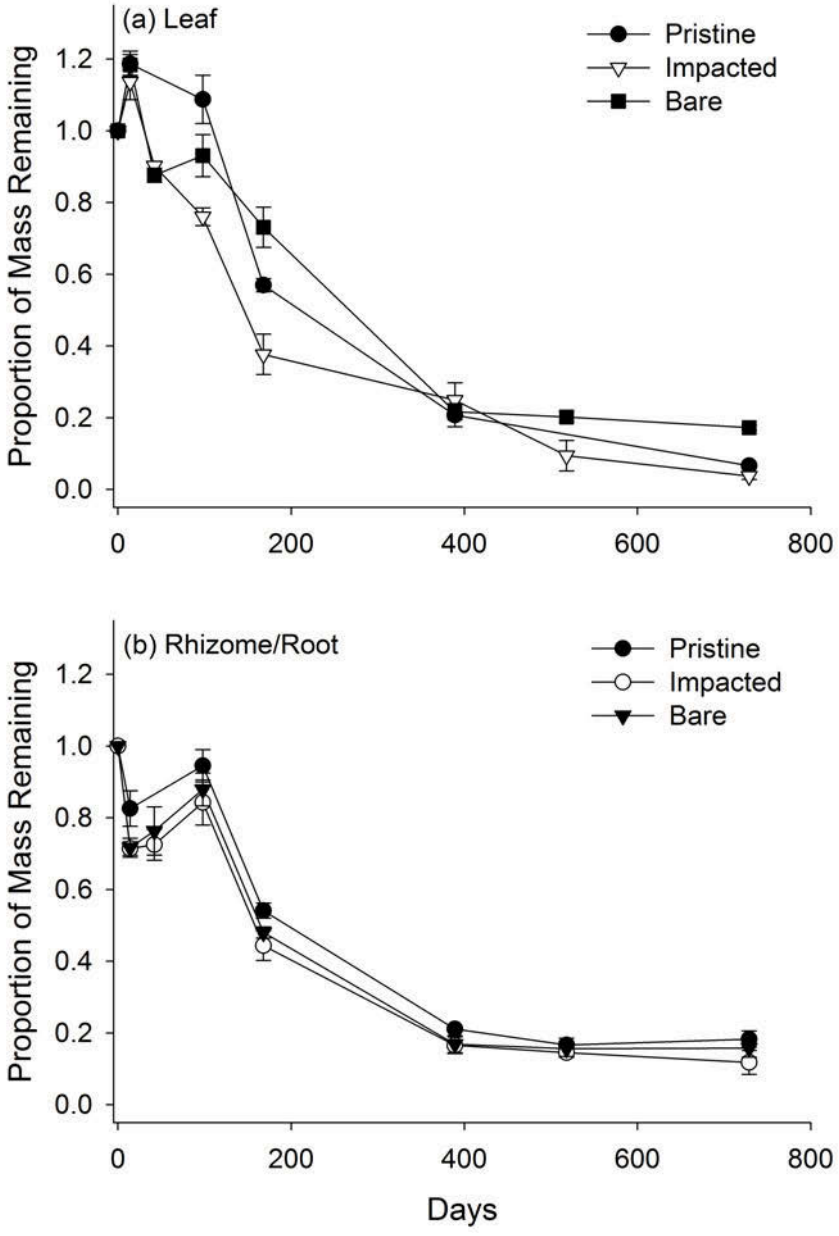


Figure 3.3: Proportion of mass remaining of (a) leaf and (b) rhizome/root biomass during two years of decomposition. Values represent means ± 1 S.E.M.

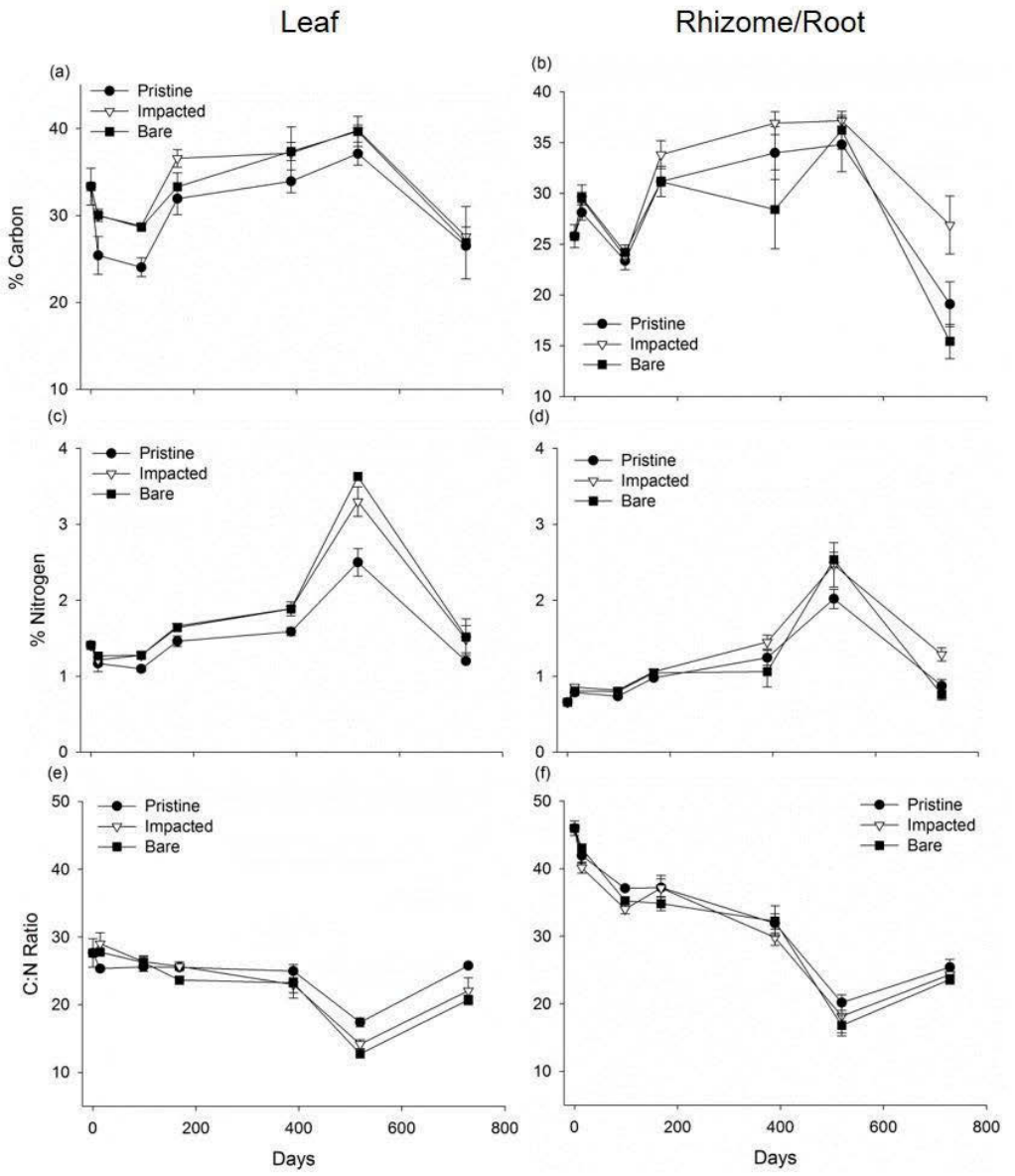


Figure 3.4: Elemental content of leaf (a, c, e) and rhizome/root (b, d, f) biomass over the period of decomposition. Letters represent significant changes in carbon, nitrogen and C:N ratios according to Bonferroni pairwise comparisons after ANOVA or Kruskal-Wallis statistical analysis for leaf and rhizome/root samples, respectively, with sites pooled together. Values represent means ± 1 S.E.M.

Table 3.2: Decay rates ($k = d^{-1}$) predicted by single-, double- and triple-component decay models for leaves and rhizome/roots from each site. Alpha (α) represents the portion of mass lost during the leaching phase at rate k_1, β represents the portion of mass lost during the microbial colonisation/remineralisation phase (k_2) and δ represents the portion of mass lost during the refractory phase (k_3). Models fitted to the data are presented in Figs. S3.2 and S3.3.

		Leaf			Rhizome/root		
		Pristine	Impacted	Bare	Pristine	Impacted	Bare
Single	k	0.004	0.0046	0.0033	0.0032	0.0036	0.0034
	R^2	0.953	0.9735	0.9409	0.9107	0.9267	0.9041
Double	k_1	0.0029	0.0053	0.0031	0.0032	0.0213	0.0209
	k_2	3.14E-06	0.0041	0.0024	0.0032	0.0035	0.0034
	α	0.9999	7.57E-05	0.3639	0.8157	0.0676	0.0676
	β	5.1E-05	0.9999	0.6361	0.1843	0.9324	0.9324
	R^2	0.8402	0.9481	0.9083	0.9056	0.8765	0.8685
Triple	k_1	0.0032	0.008	0.0027	0.0065	0.0069	0.0067
	k_2	0.0038	0.0059	0.0032	0.0032	0.0046	0.0045
	k_3	0.0023	0.0032	0.002	0.0028	0.0014	0.0014
	α	0.2704	8.28E-05	0.2703	0.2011	0.2859	0.2858
	β	0.3813	0.3648	0.3806	0.2462	0.4064	0.4064
	δ	0.3483	0.6351	0.3491	0.5527	0.3077	0.3078
	R^2	0.8352	0.9463	0.9071	0.8984	0.8686	0.8661

The elemental content of the seagrass litter significantly changed over the course of decomposition. For C content, both tissue types had an initial decrease in C during the first 3 months, but increased and stabilised to 30-35% until 18 months, after which there was a significant loss in %C (Fig. 3.4a, b). For both tissue types, nitrogen increased throughout decomposition and led to a decrease in molar C:N content for both tissues throughout the decomposition process (Fig. 3.4e-f).

The OM quality analysed by thermogravimetry was significantly different between tissue types (*Psuedo-*$F_{1,24}$ = 57.076, *P(perm)* = 0.001), driven primarily by the lower labile OM (TI_1) content in the leaves (SIMPER: 56.58% contribution to dissimilarity). There was also a significant change in OM quality over time (*Psuedo-*$F_{6,24}$ = 13.269, *P(perm)* = 0.001), as well as a significant tissue x time interaction (*Psuedo-*$F_{6,24}$ = 11.559, *P(perm)* = 0.001). The pairwise tests of the interaction effect indicated that the significant differences were driven by changes in rhizome/root OM quality only (Fig. 3.5). During the first two weeks, there was a slight but non-significant 5% decrease in labile OM (TI_1), followed by a significant 8.3% increase in labile OM by 168 days (*t* = 4.1717, *P(perm)* = 0.008). Subsequently, there was a significant loss in labile OM and relative increase in cellulose-associated OM (TI_2, 17%) during the summer months between 168 and 389 days (*t* = 9.5106, *P(perm)* = 0.001). After the first year, there was little change in labile OM, but significant losses in TI_2 occurred between 389 and 519 days (*t* = 4.3113, *P(perm)* = 0.005) and 389 and 789 days (*t* = 4.7085, *P(perm)* = 0.003). SIMPER analyses revealed that these losses in cellulose-associated OM coincided with nearly equal relative increases in lignin-associated OM (TI_3). By the end of the experiment when the mass loss had stabilised, the rhizome/roots consisted of ~25% TI_3 and 41% TI_2.

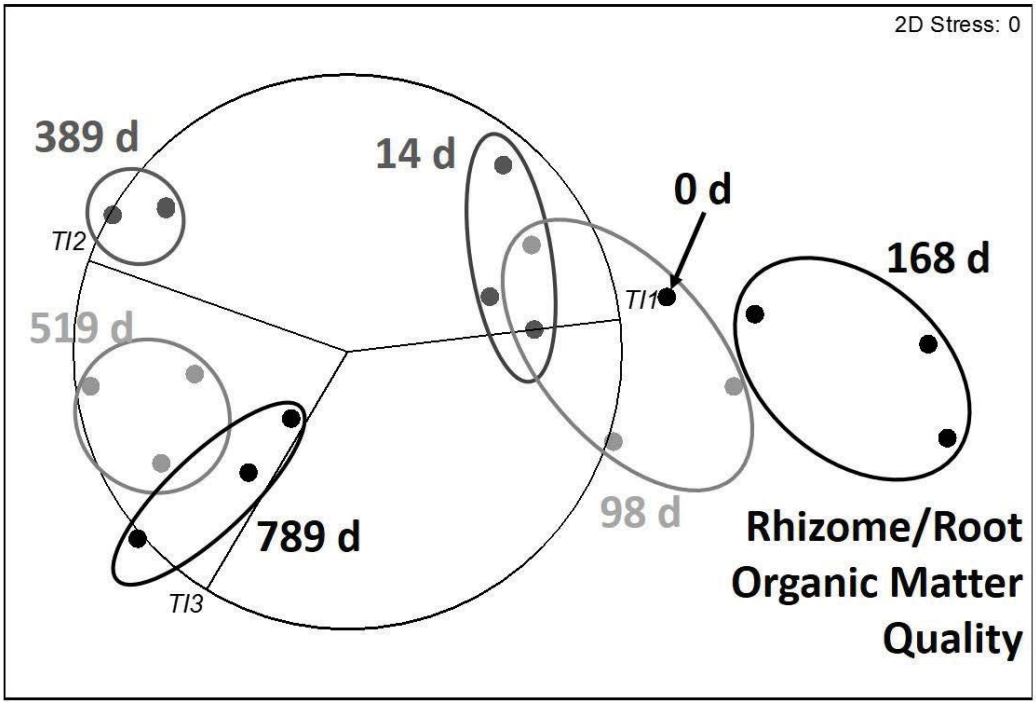


Figure 3.5: MDS plot of the shifts in rhizome/root organic matter (OM) quality over the period of decomposition. Thermal intervals (TI) indicate the OM quality based on the thermal stability during pyrolysis: TI_1 = labile OM, hemicellulose, soluble compounds (180-300°C), TI_2 = cellulose-associated refractory OM (300-400°C), TI_3 = lignin-associated refractory OM (400-600°C).

Microbial Communities

The microbial communities (class-level taxa) showed significant differences among sites (*Psuedo-*$F_{2,70}$ = 10.681, *P(perm)* = 0.001), tissue type (*Psuedo-*$F_{1,70}$ = 27.738, *P(perm)* = 0.001) and time (*Psuedo-*$F_{3,70}$ = 86.623, *P(perm)* = 0.001). There were also significant interaction effects for tissue type and site (*Psuedo-*$F_{4,70}$ = 5.2798, *P(perm)* = 0.001), tissue type and time (*Psuedo-*$F_{6,70}$ = 12.843, *P(perm)* = 0.001) and site and time (*Psuedo-*$F_{6,70}$ = 2.0159, *P(perm)* = 0.001) (Fig. 3.6). Pairwise comparisons of the tissue and site interaction effect indicated that the communities associated with the decaying leaves and rhizome/roots and the bulk sediments were significantly different within each site (*P(Perm)* = 0.001). SIMPER analysis revealed that these differences were fairly consistent across sites (Fig. 3.7a). For example, at all sites, leaf-

associated communities had higher abundances of the Alpha- and Gammaproteobacteria as well as Acidobacteriia and Actinobacteria than rhizome/root communities. Conversely, rhizome/root-associated microbes were dominated by Deltaproteobacteria, Spirochaetes, Clostridia, Cytophagia (only at Pristine), Bacteroidia and methanogen archaea. Seagrass-associated microbial communities were also different than bulk sediment communities driven by higher proportions of Deltaproteobacteria, Dehalococcoidia, Anaerolinae and unassigned taxa in the sediments, while bacteria associated with the seagrass tissues had higher proportions of Spirochaetes, Alphaproteobacteria and Cytophagia.

Leaf-associated communities were significantly different among all sites (Fig. 3.7a). These differences were driven by several taxa: Alphaproteobacteria (Impacted (I) > Pristine (P) > Bare (B)), Gammaproteobacteria (I > P > B), Deltaproteobacteria (B > P > I), Dehalococcoidia (B > P > I), Spirochaetes (B > P > I), Actinobacteria (I > P > B) and Methanobacteria (B > P > I) (Fig. 3.7a). Rhizome/root-associated bacteria were only different between Impacted and Bare sites due to higher Alpha- and Gammaproteobacteria at the Impacted sites, and higher Bacteroidia, Cytophagia, Dehalococcoidia and Planctomycetia at Bare sites (Fig. 3.7a). The bacterial communities in the bulk sediments were also different among sites (data not shown). Namely, Actinobacteria and Gammaproteobacteria were most abundant in Pristine sediments. Impacted and Bare sites both had higher abundances of Bacteroidia, unclassified bacteria, Dehalococcoidia, and methanogen archaea. Impacted sediments had the highest abundances of Nitrospira.

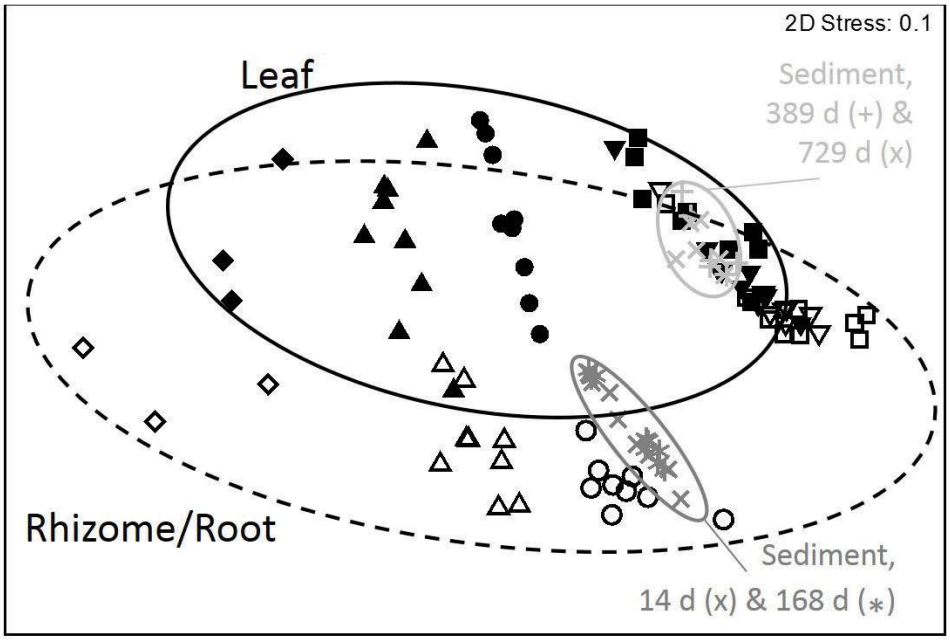


Figure 3.6: MDS plot of microbial community shifts for each tissue type and sediment over time. Open symbols represent rhizome/root tissues, and closed symbols represent leaf tissues. Day 0 = ♦, Day 14 = ▲, Day 168 = ●, Day 389 = ■, Day 729 = ▼.

The microbial communities associated with the decaying seagrass also underwent significant shifts throughout the decomposition process (tissue type x time interaction, *Psuedo-*$F_{6,70}$ = 12.843, *P(perm)* = 0.001; Fig. 3.7b). For leaf detritus, there was a substantial increase in Alphaproteobacteria, Sphingobacteriia and Actinobacteria between days 0, 14 and 168. After 6 months, there was marked increase in Cytophagia, Spirochaetes, and methanogen archaea. Both Deltaproteobacteria and Acidobacteriia consistently increased throughout the two years, while the proportion of Gammaproteobacteria consistently declined. For rhizome/root substrates, the initial stages of decay (0-168 days) were characterised by at least a 2-fold increase of Clostridia, Bacteroidia, Spirochaetes, Alphaproteobacteria and unclassified taxa (Fig. 3.7b). After 6 months, there was a diversification of taxa including increased abundances of Deltaproteobacteria, Cytophagia, Methanobacteria, Acidobacteriia, Dehalococcoidia and nitrite-oxidising Planctomycetia.

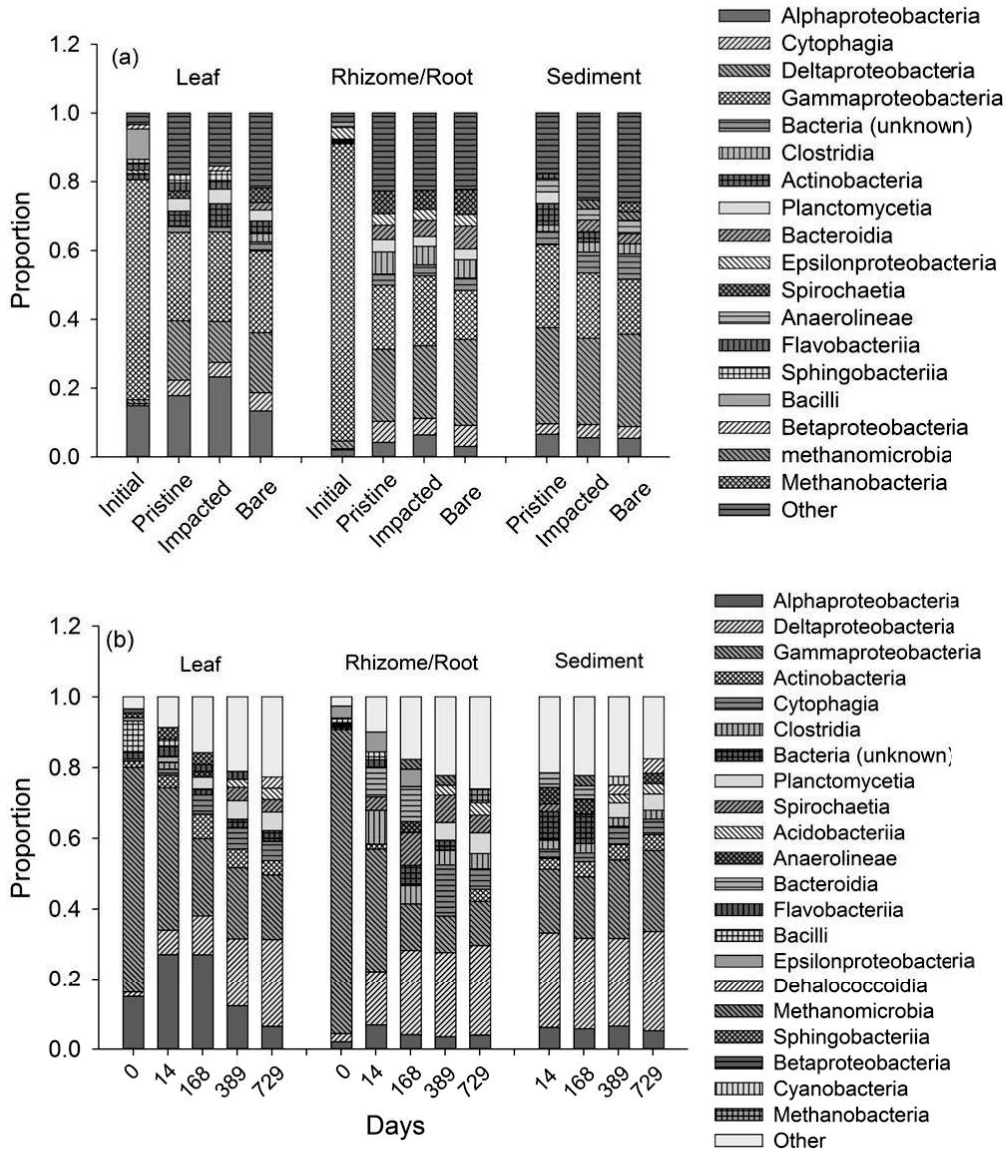


Figure 3.7: Bacterial communities from seagrass and sediments (a) at different sites and (b) over the period of decomposition. Values represent means of the top 10 most abundant class-level taxa for each sample. Day 0 (Initial) sediment bacterial communities were not sampled.

Discussion

Effects of nutrient conditions

Sediment characteristics were significantly different across the sites and reflected the differences in nutrient and sediment loads experienced at each site. The two sites at

Fagans Bay (Impacted and Bare) had very high silt/clay content and 8-times more C_{org} and 3-4.5-times more N compared to the Pristine site (Fig 3.1). There was no difference in % C_{org} between Impacted (with seagrass) and Bare (without seagrass) sites, which suggests that sedimentation rate and hydrodynamics in the bay may be influencing C_{org} burial (Lavery et al. 2013a) and may be more influential than the particle-trapping functions seagrasses provide. C_{org} was lost linearly with increasing depth, possibly through one or more mechanisms, i.e., microbial remineralisation, humification or adsorption to minerals (Burdige 2007). Interestingly, even though N accumulation was very high at the nutrient-loaded sites, it exponentially decayed with depth suggesting preferential N utilisation within the sediments, either through microbial processes, such as denitrification, or humification (Kristensen 1994, Rivera-Monroy and Twilley 1996, Cornwell et al. 1999).

Comparing the stable isotope and C_{org}:N ratio signatures of the sediments with selected plant references provided some insight into the sources contributing to the C_{org} in the sediments (Fig. 3.1c). The lighter $\delta^{13}C$ signatures of the sediments suggested a greater mangrove/terrestrial input at the Impacted and Bare sites, but higher seagrass contribution to Pristine sites (Fig. 3.1b) (Kennedy et al. 2010). Further analysis with the mixing model showed there was little change down-core in source contributions for the Pristine site, indicating little $\delta^{13}C$ diagenesis occurred and is possibly linked to the lack of variation in C_{org} down-core. Conversely, Impacted and Bare site signatures shifted toward higher mangrove signatures with increasing depth suggesting as decomposition occurred (evident as decreasing C_{org} content; Fig. 3.1c), mangrove refractory C persistence of in sediment C_{org} was greater relative to seagrass refractory C (Trevathan-Tackett et al. 2015). Across all three sites, the relatively abundant seston/plankton contributions to C_{org} was somewhat unexpected since it has been shown that mangrove and seagrass contributions are typically higher due to their abundance in NSW estuaries and their higher refractory C (lignocellulose) content compared to phytoplankton (Enríquez et al. 1993, Cebrián 1999, Ainley and Bishop 2015). We suspect that these results are related to the high runoff inputs, including sources of labile C and exogenous N (Fig. 3.1), accumulating in the sediments, particularly for the Impacted and Bare sites, and are skewing the data toward the seston/plankton references. Using $\delta^{15}N$ as a model constraint may have provided more information on the source of N as it has been previously used to identify nutrient-polluted systems (Schlacher et al. 2005).

Site was also an important factor for seagrass- and sediment-associated microbial community composition, and reflected the differential conditions between high- and low-loading conditions. Impacted and Bare sediments had higher proportions of bacteria like Dehalococcoidia that can anaerobically degrade pollutants like organo-halides (Löffler et al. 2013), as well as a diverse array of C-sources including aromatic compounds (Wasmund et al. 2014). Methanogen archaea were also higher at these sites, suggesting that in addition to sulphate reduction, which is the dominant form of anaerobic respiration in seagrass sediments (Sørensen et al. 1979), methanogenesis was also occurring at these sites. Interestingly nitrite-oxidising Nitrospira were highest at the Impacted site, which have been shown to be important for N-cycling in coastal sediments and for wastewater treatment (Bartosch et al. 2002, Dionisi et al. 2002).

The bacterial communities associated with leaf tissues were significantly different among the sites, while rhizome/root samples had less variation. For all the taxa that drove the inter-site variation in the leaf communities, the Pristine site had intermediate abundances between the Bare and Impacted sites. Impacted sites had higher abundances of aerobic taxa like Alphaproteobacteria and Actinobacteria, while anaerobic bacteria and archaea were more prevalent at the Bare site (e.g., Deltaproteobacteria, Spirochaetes, Dehalococcoidia, methanogens). These data suggest that the leaf litter bags at the Impacted site may have maintained oxygenated conditions for longer or more frequently than the Bare sites, which may also explain the higher variability, though insignificant, in leaf mass loss patterns between Impacted and Bare samples prior to 12 months (Fig. 3.3a).

Despite the differential sediment characteristics and microbial community composition, the seagrass decomposition patterns and shifts in elemental content showed little variation across sites (Figs. 3.3, 3.4). There was some variation in the oxic decomposition of leaf tissues across sites, but due to the lack of replication across nutrient regimes, it is impossible to tell if this was noise or true variation due to nutrient regimes. However, a recent study has shown that higher nutrient loads, in estuaries in a similar region to this study, led to a 25% greater loss of mass during aerobic *Z. muelleri* decay (n = 2) (Ainley and Bishop 2015). This indicates that that exogenous nutrient availability can induce higher microbial remineralisation of seagrass detritus during the first few months of aerobic decay. Exogenous nutrient availability also affected saltmarsh plant decomposition, but primarily during the earlier leaching phase (Valiela

et al. 1985, Wilson et al. 1986a). Furthermore, long-term decomposition studies have shown that the later stages of decomposition were limited by other factors like N recalcitrance, desiccation and litter quality rather than nutrient availability (Valiela et al. 1985, Fourqurean and Schrlau 2003). Therefore, nutrient availability may be an important factor influencing decomposition rates and C-cycling in the early stages of decay under aerobic conditions, rather than throughout the entire decomposition process.

Microbial degradation of seagrass

The microbial degradation of seagrass leaf and rhizome/root tissues occurred with different patterns over the course of two years. While the single-component model had the best fit and produced similar k-values for both tissues types (Table 3.2, Fig. S3.2, S3.3), the two- and three- component models likely had a reduced fit due to the increase in the number of parameters with the addition of each component to the model. The multiple component models did provide information on the variable patterns of decay between the two tissue types. For leaf tissues, the leaching phase was absent as a result of using wrack biomass, and likely lost most of its soluble compounds prior to being collected (Harrison 1989, Opsahl and Benner 1993). Without a leaching phase, there was still preferential utilisation of C in the first few months (Fig. 3.4a) and the colonisation of the litter by *r*-strategist bacteria indicating there were still easily degradable substrates available. Following this stage of decay, mass loss began after the first few weeks and was characterised by increasing %N, which is indicative of bacteria utilising exogenous N during the remineralisation process, i.e., N immobilisation (Peduzzi and Herndl 1991, Tremblay and Benner 2006, Vonk et al. 2008). The leaf TGA signatures did not change throughout the decomposition process despite mass loss. It could be possible that the microbial colonisation masked the shifts from labile-dominant to refractory-dominant OM that we would expect to see during decay. However, the TGA signature of microbial biomass has never been quantified to know definitively.

There was no refractory phase for leaf decomposition since leaf mass loss did not stabilise by the end of the two years. Rather, the double-component decay model may be capturing two phases of the microbial remineralisation phase: (1) more rapid aerobic decay performed by *r*-strategist microbes followed by (2) slower degradation by

anaerobic *K*-strategist microbes. A similar succession of bacterial communities occurred during a 1-year decomposition study on *Halophila stipulaceae* wrack (Wahbeh and Mahasneh 1985). Likewise, we showed a shift in bacterial communities between 6-12 months, but in our study, this shift also coincided with the burial of the litter bags. The seagrass-associated bacterial communities in the first 6 months were dominated by aerobic, *r*-strategic bacteria like Sphingobacteriia and Alpha- and Gammaproteobacteria (Wahbeh and Mahasneh 1985, DeAngelis et al. 2013). Actinobacteria were also abundant at the 6-month sampling in this study suggesting the beginning of refractory C degradation (Wahbeh and Mahasneh 1985, Pasti et al. 1990). Following the shift to anoxic conditions, microbes that specialise in sulphate, methane and nitrogen metabolism became more abundant (e.g., Deltaproteobacteria, methanogen archaea, nitrite-oxidising Planctomycetia; Fig. 3.7b). During the last year of decay, there was an increase in *K*-strategist taxa that have been previously shown to degrade lignocellulose, e.g., Spirochaetes, Cytophagia, Deltaproteobacteria, Acidobacteriia (Roth and Hayasaka 1984, Pasti et al. 1990, Rosselló-Mora et al. 1999, Ward et al. 2009, Darjany et al. 2014).

Seagrass rhizomes and roots typically have very different decay patterns, i.e., roots leach 40-60% of its mass in the first 2 months of decay, and rhizomes have significantly slower decay rates and sometimes lack a leaching phase (see Chapter 2) (Kenworthy and Thayer 1984, Holmer and Olsen 2002, Vichkovitten and Holmer 2004). In using a rhizome/root composite in this study, the phases of decay captured in this study reflect the decay patterns of both tissue types. The leaching phase lasted for 2-6 weeks and was also characterised by a loss of 20-30% mass and a 4% decrease in labile OM (TI_1) (Figs. 3.3 & 3.5). The leaching likely consistent of mostly non-structural carbohydrates and occurred at a similar rates found for *Zostera* roots (k_1 = 0.03 vs. 0.02, respectively) (Vichkovitten and Holmer 2004). We found that the leaching phase supported fast-growing, *r*-strategist bacteria like Bacteroidia and Alphaproteobacteria (Mayali et al. 2011, Teeling et al. 2012). Some *K*-strategists like Clostridia and Spirochaetes also colonised the rhizome/root tissues during this phase and could have indicated the beginning of cellulose polysaccharide degradation (9% loss of TI_2, Fig. 3.5) (Roth and Hayasaka 1984, Darjany et al. 2014). After 6 months, C:N ratios continued to decrease, indicating N-immobilisation was occurring under strictly anaerobic conditions (Holmer and Olsen 2002, Fourqurean and Schrlau 2003).

There was also a consistent decrease in both TI_1 and TI_2 content during this time period, which caused a relative increase in lignin-associated TI_3 at the end of the experiment (Fig. 3.5). This period after extensive microbial degradation and the stabilisation of mass marked the beginnings of the refractory phase of decay. The refractory phase also coincided with increased abundances of *K*-strategic bacteria that have the capability to breakdown celluloses (e.g., Cytophagia, Acidobacteriia) (Rosselló-Mora et al. 1999, Ward et al. 2009) and aromatic compounds (e.g., Deltaproteobacteria, Spirochaetes, Dehalococcoidia) (Darjany et al. 2014, Wasmund et al. 2014).

Perspectives on long-term seagrass decomposition

The results found in this study suggest that the long-term microbial decomposition of seagrass tissues is a complex process involving interactions among environmental conditions and litter quality, and may affect the current paradigms on C-cycling and sequestration in coastal systems. First, nutrient-and sediment-loading can significantly affect C_{org} and N accumulation in sediments independent of particle-trapping and plant CN contributions. The loss of C_{org} and N down-core suggest these accumulations are not permanent and are subject to biotic and abiotic processes than influence their sequestration. While exploring the fate of sediment C_{org} and N was not a direct goal of this study, we hypothesise that these data could be useful for predicting century- or millennia-long shifts in C_{org} storage in blue carbon habitats. For example, if age-data were obtained for the sediment cores in this study, time could replace depth allowing for C_{org} and N decomposition to be modelled over hundreds or thousands of years.

Secondly, we found evidence that there may be a nutrient-oxygen interaction on the microbial decomposers, whereby anoxic conditions and predominantly anaerobic microbial communities limit the enhanced remineralisation that is expect to occur under high nutrient conditions (López et al. 1998). This is likely also related to the quality of litter (labile vs. refractory), as well as the functional capacity of the bacteria present. While we found the presence of refractory C-degrading bacterial taxa under both oxic and anoxic conditions, their function and metabolic and enzymatic capacities still need to be quantified for various substrates under different oxygen availabilities (Arnosti et al. 2014). However, based on the results of this study, rhizome/roots have more potential to contribute to sediment C_{org} stocks. We also suggest that hydrological

conditions are especially important for the preservation of leaf-based C, as rapid burial of leaf detritus would provide a greater degree of C protection under anoxic and physically protected conditions (Duarte et al. 2013b).

In light of these results, previous decay models may be over-estimating seagrass remineralisation and underestimating the degree of preservation of seagrass detritus in sediment stocks. We propose that bacterial succession may be the key component to C sequestration, and is linked to their functional capacity and how their functions change with (a) shifts from aerobic to anaerobic conditions and (b) shifts in substrate quality (e.g., refractory carbon). Therefore, the second phase of decay, microbial remineralisation, should be divided into two rates in order to reflect the oxic and anoxic conditions. In the oxic phase, nutrients may influence remineralisation. In contrast, the anoxic microbial remineralisation and the refractory phases of decay (assuming anoxic conditions) are predominantly influenced by microbial function and the refractory C content of the detritus. We encourage future research to centre around, or at the very least include, the microbial response when investigating C_{org} sequestration dynamics in blue carbon ecosystems.

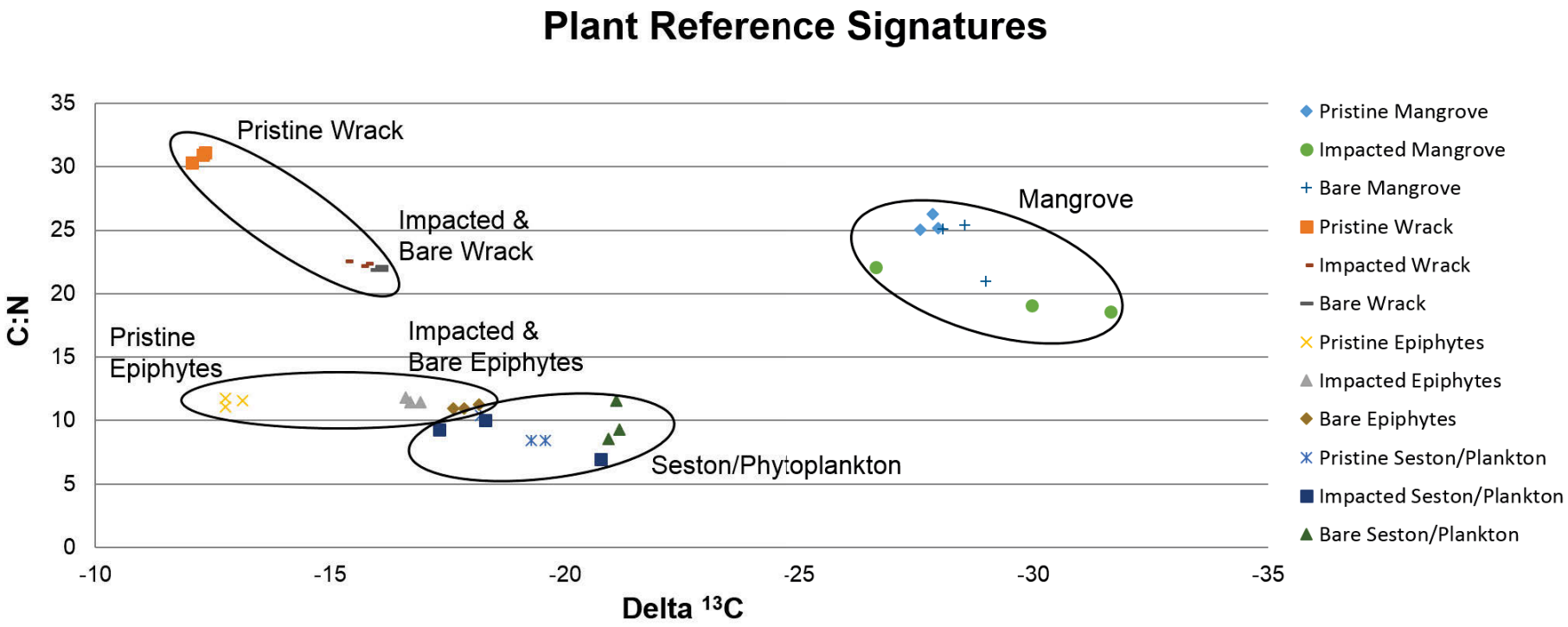


Figure S3.1: C:N ratios and $\delta^{13}C$ signatures for the plant reference collected at each of the three sites from this study. The site variation was greatest between Pristine and Impacted/Bare for the seagrass wrack and seagrass epiphytes sources.

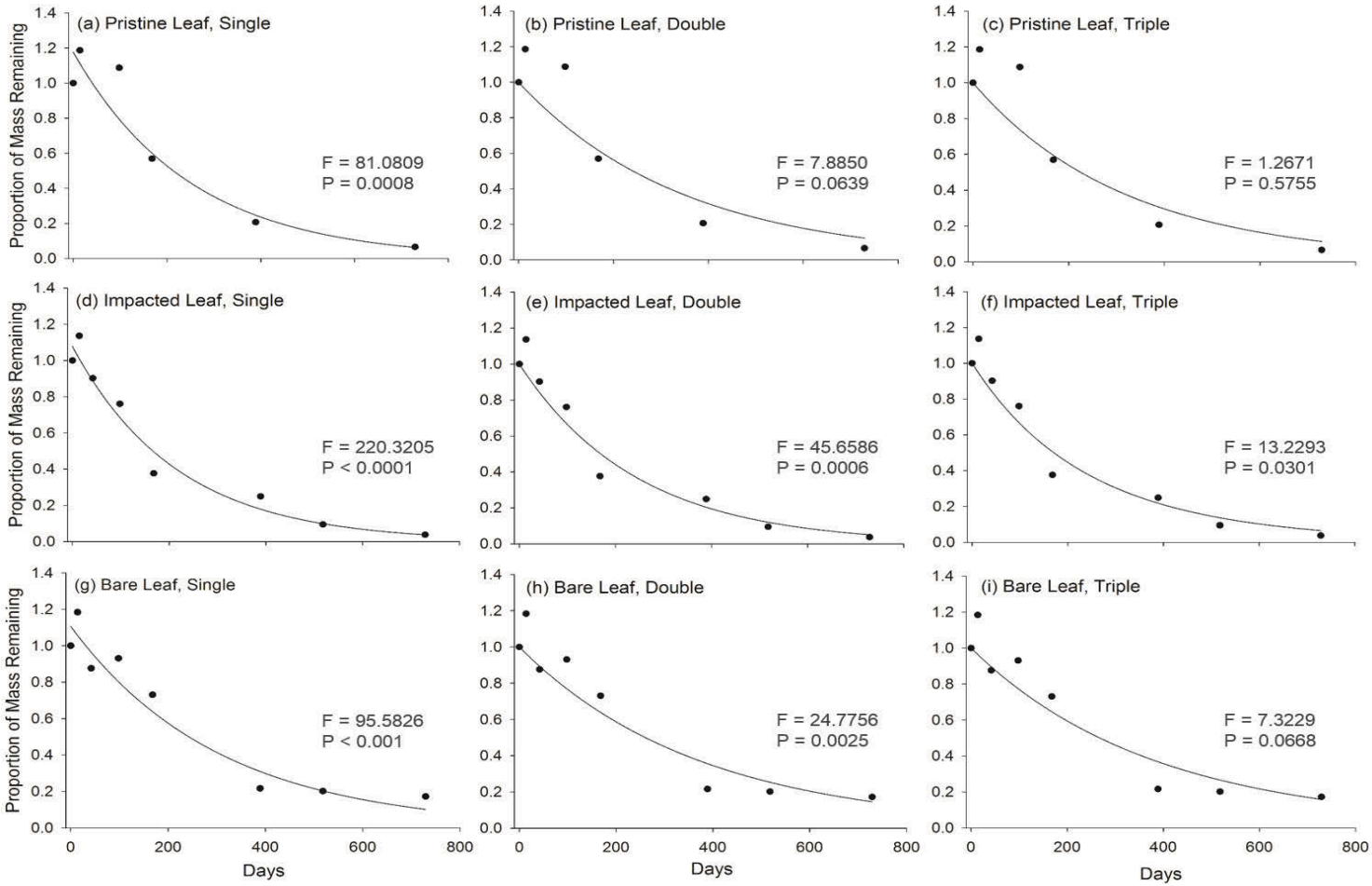


Figure S3.2: Decay models fitted to leaf mass loss data. Each site presented separately for single-, double- and triple component exponential decay models. ANOVA statistics are also presented.

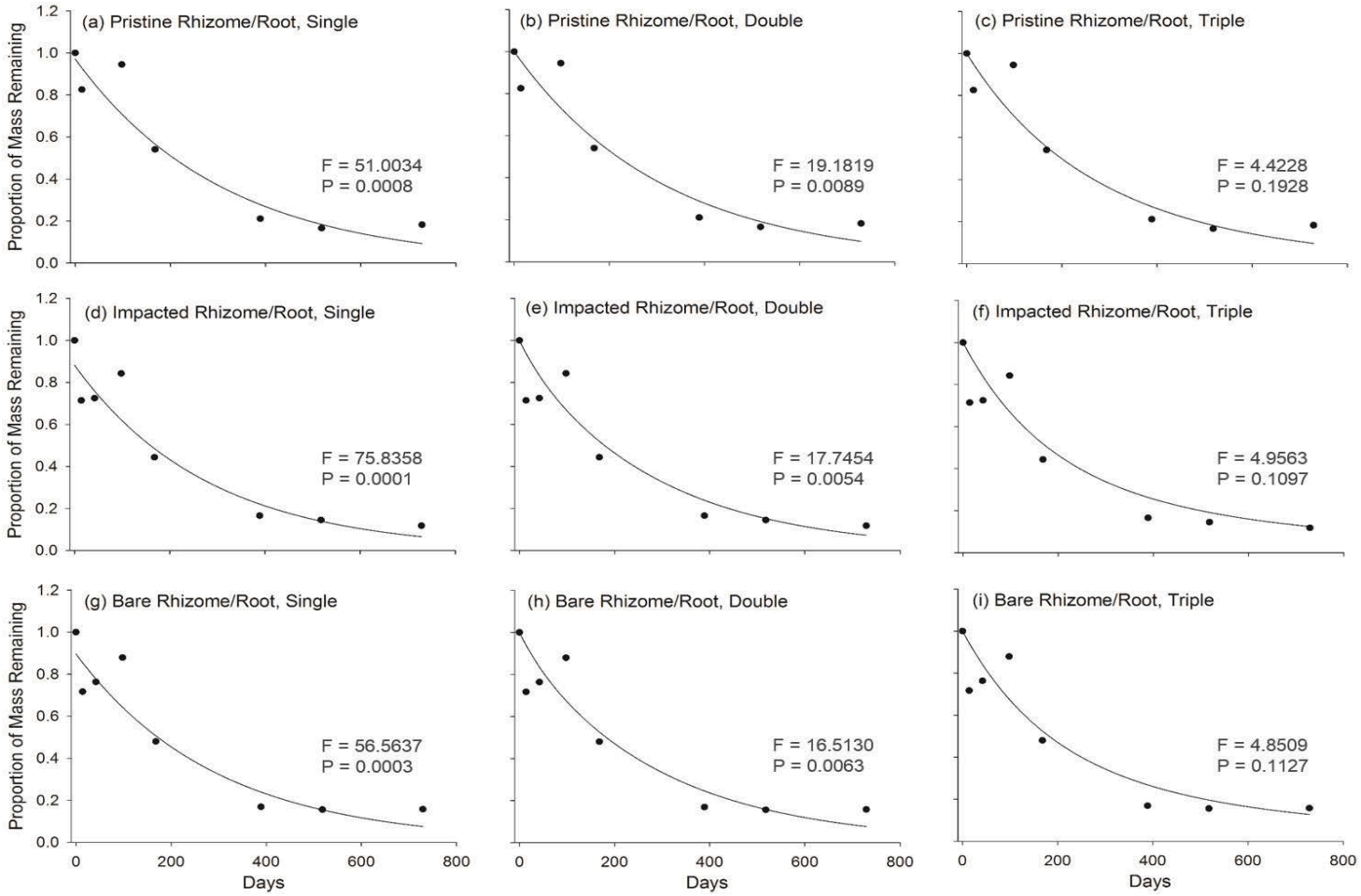


Figure S3.3: Decay models fitted to rhizome/root mass loss data. Each site presented separately for single-, double- and triple component exponential decay models. ANOVA statistics are also presented.

Chapter 4:
Microbial priming effect as a mechanism for enhanced CO_2 release in coastal sediments

Abstract

Blue carbon ecosystems (mangrove, saltmarsh, seagrass) are powerful tools for climate change mitigation due to their ability to naturally sequester carbon in their sediments for millennia. The 'microbial priming effect', defined as the enhanced remineralisation of stable sediment carbon in response to stimulated microbial activity following the input of fresh carbon, has been proposed as a process that can diminish sequestered carbon stocks. Priming is a well-established concept in terrestrial ecosystems, and in this study, our aim was to quantify the microbial priming effect in blue carbon systems. Here we showed that the addition of fresh carbon led to a 1.7-2.7-fold increase in CO_2 released from both recent and ancient sediments. The presence of a negative priming effect, i.e., fresh carbon additions decreasing sediment carbon remineralisation, suggested that the intensity of priming was influenced by the quality of both the fresh inputs and the sediment carbon. We propose that microbial priming occurring at the sediment surface is a natural occurrence, and the initial loss of carbon may be offset by refractory components of the fresh inputs (i.e., lignocellulose). However, the occurrence of microbial priming for deep sediments, in scenarios where physical disturbance reintroduces sediments to the water column, can stimulate the loss of millennial carbon from blue carbon ecosystems.

Introduction

The continuously increasing atmospheric carbon dioxide (CO_2) levels have resulted in a global push to develop carbon-sequestering solutions to mitigate CO_2 emissions (Herzog et al. 2000). Recently, blue carbon ecosystems have been highlighted as systems that naturally bury organic carbon into their sediments at rates approximately 40-times faster than terrestrial systems (McLeod et al. 2011). These habitats, which include seagrass meadows, saltmarshes and mangroves, sequester this carbon by fixing CO_2 within live photosynthetic biomass and by accumulating organic carbon (OC) into their sediments (Laffoley and Grimsditch 2009, Macreadie et al. 2014a). Seagrass meadows alone have been shown to store OC in their sediments for millennia (Mateo et al. 1997), and consequently act as efficient sinks of atmospheric CO_2 (Laffoley and Grimsditch 2009, Duarte et al. 2013b). This enhanced, prolonged storage has been attributed to processes including rapid burial in predominantly anoxic conditions that limit microbial remineralisation rates, the adsorption of OC onto minerals making them inaccessible to microbes, and the existence of nutrient-limited, refractory OC (ROC) (e.g., humic substances) (Burdige 2007).

Although blue carbon habitats are ideal for rapid, permanent carbon sequestration, preservation of these ecosystems has been challenged by high rates of habitat losses recent decades (Laffoley and Grimsditch 2009). Seagrass habitat loss, alone, is occurring at a rapid rate (110 km^2 yr^{-1}) (Waycott et al. 2009), much of which has been attributed to anthropogenic activities, such as eutrophication and physical disturbances (Orth et al. 2006). Habitat destruction not only diminishes the C-sequestering capacity of blue carbon habitats, but increases the risk of losing stored ROC (Fourqurean et al. 2012, Marbà et al. 2015). As a result of habitat loss and degraded environmental conditions, the loss of stored ROC has been hypothesised to occur up to a metre deep, i.e., 63-297 Tg ROC yr^{-1} returned to the atmosphere as CO_2, globally (Fourqurean et al. 2012).

In addition to direct losses, there may be an additional mechanism that can exacerbate ROC loss via the stimulation of microbial remineralisation, i.e., the 'microbial priming effect'. The priming effect is a phenomenon in which access to fresh, labile organic carbon (LOC) or inorganic nutrients 'kick-starts' sediment microbes to metabolise ROC that would not have been otherwise utilised (Kuzyakov et al. 2000). This concept is widely accepted for terrestrial soils systems, and priming has

been shown in some instances to double the amount of ROC metabolised by microbes (Fontaine et al. 2007, Guenet et al. 2010, Guenet et al. 2012).

The microbial priming effect has yet to be directly quantified in marine systems, but there are some mechanisms that may potentially stimulate priming, such as injection of LOC into deep sediments by bioturbation (Kristensen et al. 2011), addition of inorganic nutrients causing changes to porewater dynamics and bacterial nutrient limitations (López et al. 1998) and the displacement of deep sediments (Kuzyakov et al. 2000) to the water column or sediment surface (and fresh nutrients or LOC) via physical disturbances like dredging and boating activities. Already, there is some evidence that the priming phenomenon can occur in coastal habitats (Guenet et al. 2010). For example, the addition of LOC in the form of microalgae or seagrass detritus has also been shown to increase bacterial respiration and biomass in coastal sediments (Danovaro et al. 1994, Farjalla et al. 2009), indicating the potential for enhanced ROC remineralisation. Since the quality of LOC provided to microbes by microalgae and seagrass detritus produces differential decomposition patterns (Enríquez et al. 1993), dissimilar LOC sources could likewise induce different degrees of priming.

This study aims to quantify the existence of the microbial priming effect in coastal seagrass sediments, as well as to elucidate its potential role in weakening ROC stocks in these blue carbon habitats. To do this, we added two common LOC sources: microalgae to represent an algae bloom and seagrass detritus. Both LOC sources were enriched with ^{13}C before adding to sediments from two depths, which will represent undisturbed (surface) and disturbed (deep) sediments. To assess the microbial priming effect, we quantified the amount of CO_2 respired after LOC additions compared to controls. We also traced the OC source (LOC or ROC) being metabolised by microbes by analysing $\delta^{13}C$ values. We hypothesise that the addition of both forms of LOC will induce a priming effect, but algal LOC would be utilised more rapidly than detrital seagrass LOC. We also hypothesise that surface sediments may produce a larger priming effect due to a higher OC content available for remineralisation.

Methods

Sediment Characterisation and Sample Collection

The sediment (ROC) used in this project was from Fagans Bay in Gosford, New South Wales, Australia (33.43 S, 151.32 E). The bay is characterised as a high-input environment, with patchy *Zostera muelleri* Irmisch ex Ascherson meadows along the shore. We chose to study the priming effect on surface sediments (0-1 cm), which routinely come in contact with LOC, and deep sediments (29-30 cm) to simulate the reintroduction to the surface after a physical disturbance (Walker et al. 1989). Previous age-dating (^{210}Pb- and ^{14}C) analyses of the Fagans Bay sediments confirmed that the sediment depths used in this study represent recent and ancient deposited sediments (Macreadie et al. unpublished data). Using Constant Rate of Supply (CRS) model for ^{210}Pb-dating, the mean calculated ages of the shallow sediment slices were 1.7 yrs for 0-0.5 cm of the profile. Radiocarbon dating aged 25-27 cm at 1,820 ± 30 yrs BP. While the deep depths of the other study and this study differ by 2 cm, we estimated that the 29-30 cm sediment samples millennial deposits (>1000 yr).

Sediment sampling occurred in September 2014 along the northern shore of the bay. The PVC cores were 5 cm in diameter and at least 50 cm in length. Coring occurred along a transect parallel to the shore with each core at least 1 m apart, then were capped and stored at 4°C for a maximum of 4 days until processing. Cores were haphazardly assigned to the following treatments (n = 3): control (unamended), *Chlorella vulgaris* addition (+ *Chlorella*), *Zostera muelleri* detritus addition (+ *Zostera*). An additional five cores were used to calculate approximate bulk density and for isotopic and elemental analysis for the surface and deep samples used in the incubation. Once the core met refuse in the sediments, measurements of the inner and outer core lengths (top of sediment to top of core) were taken. These measurements were used to correct for compaction (% compaction applied equally across the score) before obtaining the 0-1 cm and 29-30 cm slices using a core extruder. Live seagrass leaves were removed from the surface slices, and wet weights were taken for the sediments before adding them to Petri dishes in the incubation chamber.

Isotope Labelling

Pre-enriched model alga, *Chlorella vulgaris*, was used for the microalgal incubations (97.2% atm; IsoLife, Wageningen, Netherlands). For the seagrass treatment,

whole *Z. muelleri* plants were collected from Fagans Bay and labelled on the same day. Epiphytes were gently removed before seagrass leaves were labelled with 99% atm ^{13}C-enriched sodium bicarbonate (Novachem, VIC, Australia) in the laboratory. The labelled sodium bicarbonate (0.6 g) was mixed with 20 L of water collected from Fagans Bay (Kaldy et al. 2013). The labelled seawater was transferred to a clean 80 L plastic bin holding the submerged seagrass leaves (Kaldy et al. 2013). The leaves were incubated in the labelling solution under 150 μmol photons m^{-2} s^{-1} of light for 1-1.5 hours before washing excess label and salts with ultrapure water (Sartorius AG, Goettingen, Germany). Labelled seagrass leaves were removed from the below-ground tissue, dried at 60°C and shredded with a handheld blender. Only fragments < 3 mm were used in the study to remove size bias. Preliminary analyses revealed the labelled seagrass ranged from 53-72 $^{o}/_{oo}$ (mean ± 1 S.E.M. = 65.9 ± 6.2 $^{o}/_{oo}$).

Incubation Experiment and Sampling

The experimental design was fully-orthogonal with three LOC treatments (control, + *Chlorella*, + *Zostera*) and two ROC sediment depths (0-1 cm, 29-30 cm) with 5 replicates. A procedural control (i.e., blank) was included to account for the background CO_2 concentrations within the chamber (no sediment). LOC was added to the sediments immediately before closing the incubation chamber at the start of the experiment. For the + *Chlorella* treatments, 30 mg DW (46.5% C) of lypholised *Chlorella* was mixed with 2 mL of sterile CO_2-free seawater at a salinity of ~22 before adding to the surface of the sediment. The concentration of algae used was based on benthic microalgal bloom concentrations in coastal sediments (Sun and Dai 2005). The + *Zostera* treatments received 200 mg DW (30% C) of labelled seagrass, which represented low detritus concentrations in NSW *Z. muelleri* meadows (Rossi and Underwood 2002). 2 mL of sterile CO_2-free seawater was added to moisten the seagrass LOC.

The incubation chambers consisted of sterilised 1.4 L plastic containers with a silicon seal to ensure an air-tight closure. Each chamber held sediment + treatment, 40 mL 0.1 M sterile, CO_2-free sodium hydroxide (NaOH) to trap the CO_2 being respired and 20 mL sterile CO_2-free ultrapure water to maintain moisture within the chamber (Townsend et al. 1997, Cleveland et al. 2002, Kindler et al. 2006, Keith et al. 2011, Wang et al. 2014). Each chamber was randomly placed into an incubator and

maintained in the dark at 20°C (ICC50, Labec, Laboratory Equipment, Pty. Ltd, NSW, Australia). Repeated sampling occurred at 1, 3, 7, 14, 28, 56 and 84 days. At each sampling day, the NaOH was removed, sealed with Parafilm M® and replaced with fresh NaOH. The NaOH samples containing the dissolved inorganic carbon (DIC) was stored at 4°C until shipped for analysis.

Thermogravimetry and Elemental and Stable Isotope Analyses

Initial sediment quality was also assessed using thermogravimetric analysis following Lopez-Capel et al. (2006). Finely ground sediments at each depth from a representative initial core were placed in a platinum cup and heated under air (gas flow 50 mL min^{-1}) at 20°C min^{-1} to 900°C (SDT Q600, TA Instruments, New Castle, DE, USA). Universal Analysis software (TA Instruments, New Castle, DE, USA) was used to aid in the identification and quantification of mass loss within specific exotherms (Exo) (Lopez-Capel et al. 2006). Delineation of thermal intervals was based on the rate-of-change derivative (% mass loss $°C^{-1}$), which indicated distinct temperature mass loss. Mass loss occurring from the start to 180°C was associated with the loss of moisture. According to Lopez-Capel et al. (2006), the first exotherm interval (labile OM corresponding to aliphatics and carbohydrates) ranged from 200°C to 400°C, followed by recalcitrant + refractory OM (polycondensed forms of lipids, aromatics and organic residues) from 400°C to 650°C. Inorganics (e.g., carbonates) ranged from 650-900°C. Labile and recalcitrant + refractory were recalculated as a proportion of total organic matter from 200°C to 650°C.

DIC samples, initial sediments and labelled *Zostera* reference leaves were sent to West Australian Biogeochemistry Centre at the University of Western Australia for elemental and stable isotopes analyses. Prior to analysis, sediments were acidified with 1 M HCl to remove inorganic carbon. The sediments were washed with ultrapure water to remove residual acid and subsequently dried and ground to a find powder. DIC samples were analysed for $\delta^{13}C$ DIC and C content (mg L^{-1}) using a GasBench II coupled with Delta XL Mass Spectrometer (Paul and Skrzypek 2007) (Thermo-Fisher Scientific). Sediment and *Zostera* leaf samples were analysed for $\delta^{13}C$ and %C content using an Automated Nitrogen Carbon Analyser system consisting of a Sercon 20-22 mass spectrometer an elemental analyser (SERCON, UK). $\delta^{13}C$ were given in per mil (‰, VPDB) according to delta notation and as ^{13}C atm% (Skrzypek 2013). International

standards from IAEA were used to normalise the data (Coplen et al. 2006, Skrzypek et al. 2010).

Calculations and Statistical Analyses

The average C captured as CO_2 (mg C-CO_2 L^{-1}) of the blank samples from each collection day was subtracted from the C-CO_2 content of the control and amended samples from the corresponding day. The resulting C-CO_2 content from each time point was normalised to the area of the sediment slice (g C-CO_2 m^{-2}). The priming effect was estimated by subtracting the C-CO_2 content in the control sediments from amended sediments (Fontaine et al. 2007). A mixing model was then used to incorporate the stable isotope values (%atm for + *Chlorella* to accommodate for the highly enriched samples and per mil for + *Zostera*) to quantify the proportion of respired CO_2 from LOC and ROC: $P_{ROC} = (\delta_{Mixture} - \delta_{LOC})/(\delta_{ROC} - \delta_{LOC})$, where P is the proportion respired as C-CO_2. These proportions were applied to the total g C respired over the 84 day experiment to estimate g C respired as a result of the priming effect. A 2-way ANCOVA (*treatment*, *depth*) with repeated measures and *time* as covariate was used to analyse the cumulative g C-CO_2 m^{-2} respired and respiration rates (mmol C-CO_2 m^{-2} d^{-1}) (Guenet et al. 2012). A Bonferroni adjustment was used for pairwise comparisons. SPSS was used for all statistical analyses (IBM, Armonk, NY, USA).

Results and Discussion

Incubation of the control sediments (ROC) resulted in 2-5-times greater respiration of CO_2 compared to CO_2 collected in the blank chambers, indicating there were active microbial populations in both surface and deep sediments (Fig. 4.1). The addition of LOC significantly increased the amount ($P \leq 0.001$) and rate ($P \leq 0.022$) of CO_2 respired for all samples (Figs. 4.1 and 4.2, respectively). The respiration rates for + *Chlorella* samples were significantly higher than control sediments ($P = 0.022$), however, very little net priming occurred over the 84-day experiment (Fig. 4.1a). When the sources of CO_2 respired were assessed, the average total g C-CO_2 respired in the + *Chlorella* treatment was less than the control sediments, with 17% of that CO_2 derived from *Chlorella* (Fig. 4.3). This is evidence for a negative priming effect, which can occur in non-nutrient-limited conditions (Guenet et al. 2010). In such conditions,

microbes preferentially utilise LOC over ROC, resulting in less ROC lost from the system (Kuzyakov et al. 2000, Guenet et al. 2010). This is also evidence for an apparent priming effect in which the CO_2 captured reflects microbial stimulation by the LOC rather than enhanced remineralisation of ROC (Blagodatskaya and Kuzyakov 2008).

Zostera-amended sediments showed a very different response compared to + *Chlorella* treatments. Both surface and deep sediments had significantly higher respiration rates than control sediments ($P = 0.001$), which were similar to rates from *in situ* seagrass sediments (Holmer and Olsen 2002). *Zostera*-amended sediments also had the higher priming effect between the two LOC treatments (Fig. 4.1b, d). The priming effect lasted for approximately 8 weeks for surface sediments (Fig. 4.1b). The time-frame of priming for these samples were within the range found of terrestrial soils and a real priming effect (Fontaine et al. 2007, Blagodatskaya and Kuzyakov 2008, Guenet et al. 2012), and suggest that after two months, easily accessible LOC became deplete and resulted in a reduced rate of remineralisation, e.g., enzymatic degradation of more recalcitrant compounds. Due to an unaccounted for ~10-fold enrichment of the labelled *Zostera* LOC, the proportion of *Zostera*-CO_2 respired could only be estimated. By making a conservative estimate of over-enrichment, approximately 5% of CO_2 came from the remineralisation of the newly introduced *Zostera* detritus, and 1.7-times more CO_2 was respired as a result of priming (Fig. 4.3). Much of the *Zostera* biomass remained after the three-month experiment suggesting there is potential for a shift in microbial communities and function throughout decomposition (Chapter 2 and 3), and *Zostera*-based ROC inputs in the future, likely in the form of lignocellulose (Trevathan-Tackett et al. 2015).

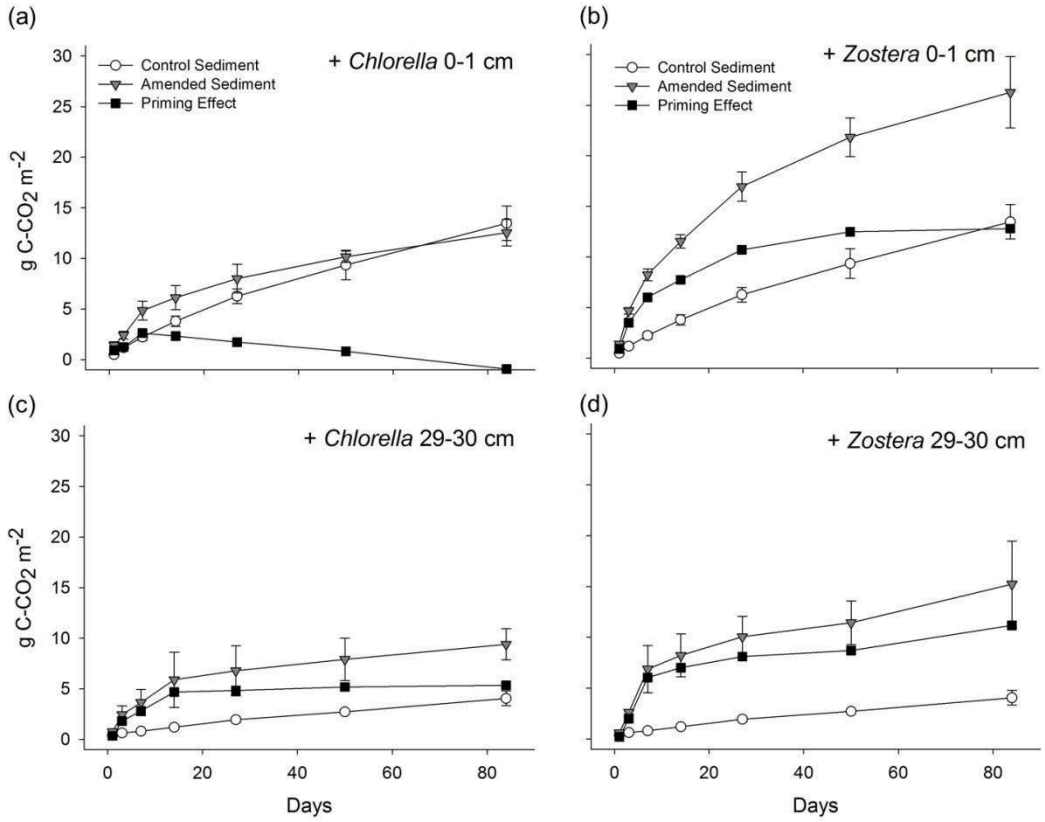


Figure 4.1: Cumulative microbial respiration from surface and deep sediments. Priming effect was calculated as the difference in cumulative CO_2 respired between amended and control sediments. Sediment values represent mean ± S.E.M.

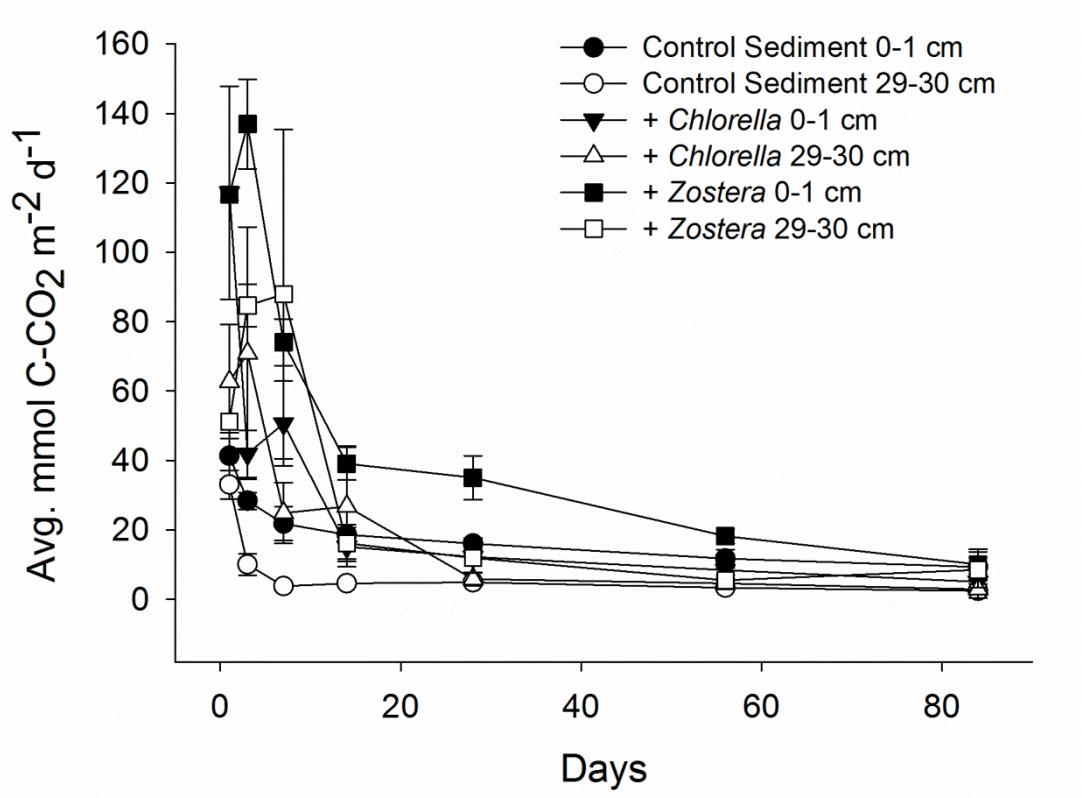


Figure 4.2: Average respiration rates for control and amended sediments for surface (open symbols) and deep (closed symbols) sediments. Values represent mean ± S.E.M.

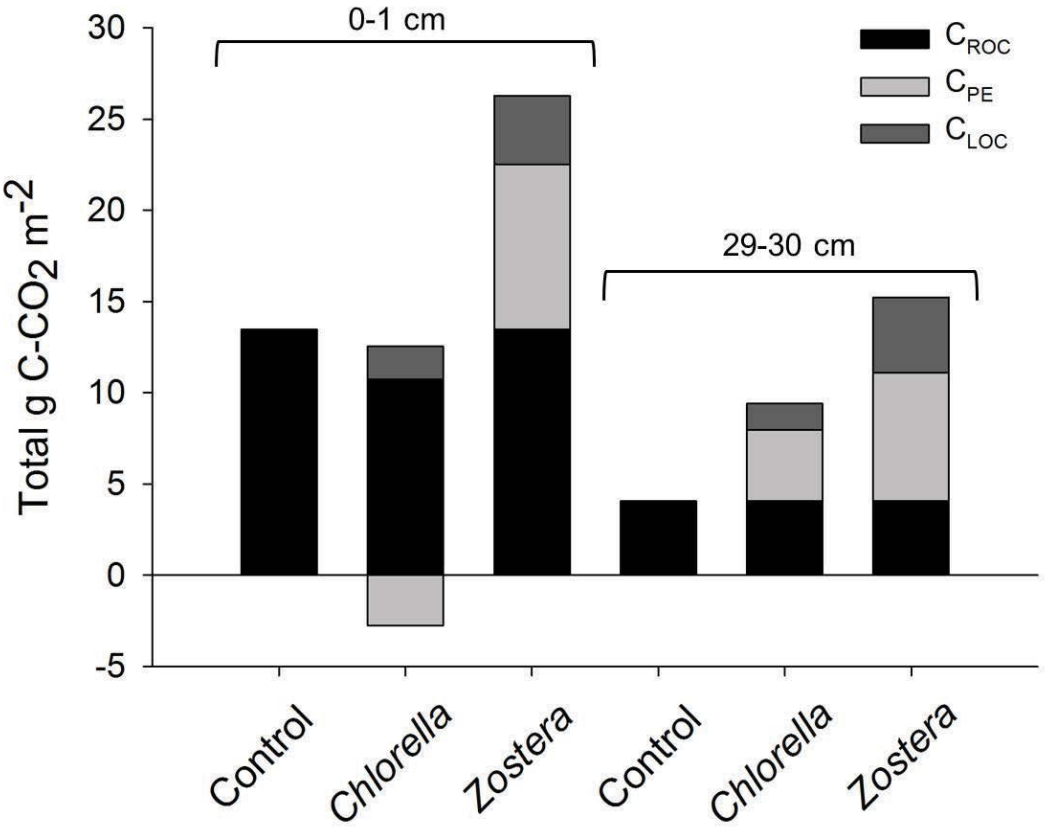


Figure 4.3: Estimated contributions of labile organic carbon (LOC), refractory organic carbon (ROC) and priming effect (PE) sources to respiration. Each bar stack represents a fraction of the total C-CO_2 respired, with the fractions of ROC and LOC calculated from stable isotopes signatures using a mixing model (See Methods: Calculations and Statistical Analyses). Values represent means.

The deep sediments had significantly lower respiration rates ($P = 0.002$) and amount of CO_2 respired ($P = 0.024$) than surface sediments (Figs. 4.1c, d & 4.2). The decreased activity could possibly be due to a combination of reasons. First, bacteria from deeper sediments have been shown to have lower enzymatic activities, which would affect the rate and the amount of OC being remineralised (Costa et al. 2007). Second, the deeper sediments had less OC and N available for utilisation compared to surface sediments (1% vs 4% OC, 0.35% vs 0.07% N). Greater nutrient limitations of microbes have shown to promote enhanced ROC remineralisation with the addition of fresh biomass, e.g., real priming effect (Blagodatskaya and Kuzyakov 2008). Measuring N remineralisation in addition to OC when adding fresh OM sources has been suggested for future priming experiments (Blagodatskaya and Kuzyakov 2008). Lastly, assessment of the organic matter (OM) quality using thermogravimetric analysis showed there was a higher proportion of recalcitrant/refractory OM in the deep sediments than surface sediments (62% vs 52% of total OM, respectively; Fig. 4.4). Therefore, in addition to having less ROC available for remineralisation, the deep ROC would have likely already been heavily processed by microbes since burial (Burdige 2007), leaving less easily available OC to be primed. Regardless, addition of LOC to the deeper sediments still elicited 2-2.7-times more CO_2 released as a result of priming compared to the deep control sediments (Fig. 4.3). Interestingly, the priming effect was still occurring for both deep, LOC-amended sediments at the end of the experiment. This may indicate that, while remineralisation of LOC and ROC was slower than the surface sediments, the microbial populations in the deep sediments can sustain priming for a longer period of time (Figs. 4.1c, d)

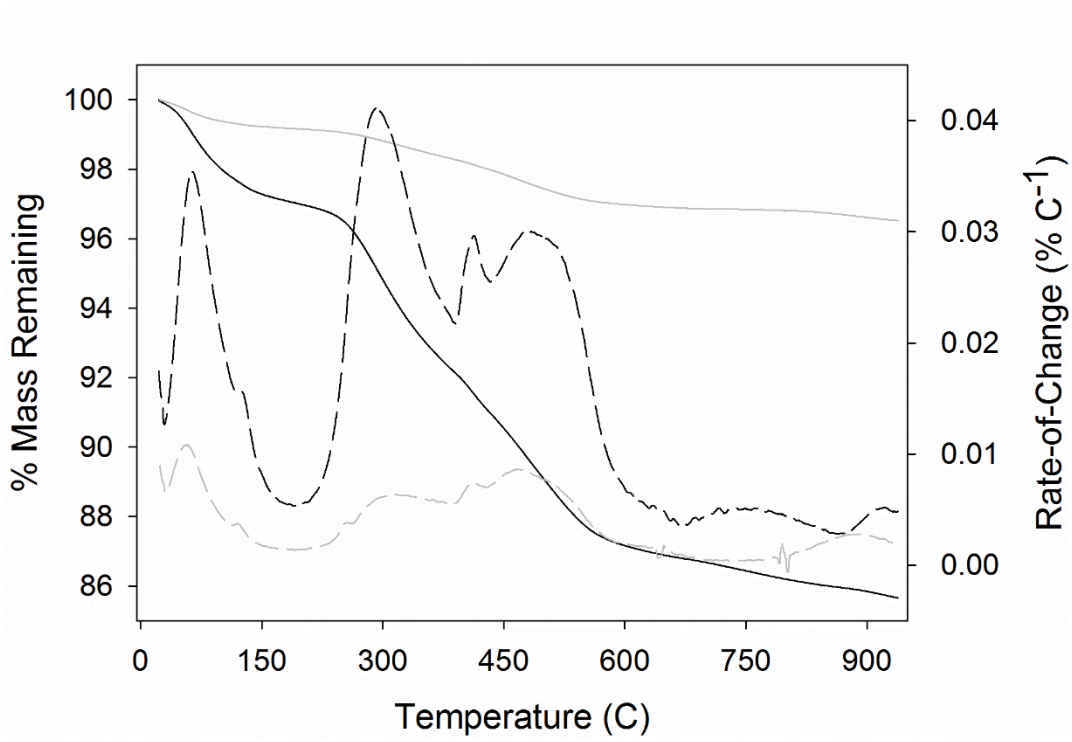


Figure 4.4: Thermogravimetric analysis of 0-1 cm (black) and 29-30 cm (grey) sediments. Solid lines represent % mass remaining, and dashed lines represent the rate-of-change (% mass loss $°C^{-1}$). Labile (200-400°C) and recalcitrant/refractory (400-600°C) organic matter (OM) was normalised to total OM (200-650°C). Mass loss above 650°C represents inorganics, and mass loss below 200°C represents dehydration.

The present results provide evidence that the microbial priming effect can exist for seagrass sediments. The magnitude of priming in coastal sediments seemed to be highly dependent on the quality and structure of LOC, with + *Zostera* sediments producing the greatest priming response. This enhanced response could potentially be attributed to the high diversity of organic substrates provided by the detritus, which could likewise support greater diversity of microbial groups and, therefore, enzymes to remineralise the detritus (Guenet et al. 2012). These difference in priming response could also be due do the differing physical structure between the two LOC sources. Future experiments on more similarly structured LOC (e.g., macroalgae vs. seagrass), will allow us to better define the roles of diversity and lability of substrates on the priming effect. The presence of a negative and apparent priming effect also highlighted the importance of environmental conditions and nutrient limitations of the microbial

populations, particularly in systems that receive inputs from land and the time frame of the experimental incubation (Kuzyakov et al. 2000, Blagodatskaya and Kuzyakov 2008, Guenet et al. 2010).

Since this is the first study to quantify marine microbial priming, it undoubtedly opens a wealth of additional questions and hypotheses. For example, it is important to consider that the inputs of LOC from microalgae blooms or seagrass detrital production to surface sediments in coastal ecosystems are a natural, continual process. The results in this study, i.e., negative and apparent priming from *Chlorella* additions and potential preservation of remaining *Zostera* detritus, suggest that such LOC additions to surface sediments may have a small impact on long-term ROC losses. On the contrary, scenarios, such as dredging and boating activities, that lead to the reintroduction of deep sediment (and nutrient-limited microbes) to the water column or sediment surface, followed by the interaction with LOC, can produce a priming effect that will increase the vulnerability of ROC to remineralisation. Consequently, priming may pose a major, global threat to the carbon sink capacity of blue carbon systems undergoing physical disturbances. We encourage more research to explore how other variables such as the changes in microbial biomass, community and functions, temperature or repeated physical disturbances can influence the priming effect as a mechanism in weakening ROC storage in blue carbon habitats.

Chapter 5:
Small-scale seagrass habitat loss affects quantity and quality of sedimentary carbon

Abstract

The degradation and loss of seagrass habitats have been repeatedly shown to reduce ecosystem function, including the ability of seagrass meadows to sequester carbon. While there have been global efforts to mitigate the occurrence of large-scale anthropogenic disturbances like dredging, the smaller local disturbances like propeller and anchoring scars can be more difficult to quantify, and thus to manage and mitigate. Furthermore, it is unclear how small or patchy habitat loss affects carbon sequestration in seagrass habitats. In this study, we simulated a small-scale, patchy die-off of two subtropical seagrass species, *Halodule wrightii* and *Thalassia testudinum*, by shading 2.89 m^2 plots for six months. The die-off 'kill' plots were compared to control (non-shaded) and bare sediments before treatment, immediately after treatment and after 11 months of recovery. We analysed the quantity and quality of sediment organic matter (OM) and organic carbon (C_{org}) using thermogravimetric, stable isotope and elemental analyses. The small-scale die-off led to a loss of 50-65% OM in the top 8 cm of *H. wrightii* kill plots, but no losses in C_{org} were detected. In contrast, *T. testudinum* lost significant portions of both OM (50%) and C_{org} (21-47%), but this was limited to the top 1 cm of sediment. TGA and stable isotope analyses showed a reduction of labile OM and depletion of $\delta^{13}C$ and $\delta^{15}N$ signatures after the kill treatment indicating microbial remineralisation of isotopically heavier and biochemically more labile carbohydrates and proteins. This study provides evidence that small-scale seagrass die-offs can lead to ecologically significant reductions in seagrass C-sequestration capacity, i.e., we estimate that 0.08-2.02 Mt C_{org} are being lost from seagrass meadows globally due to small-scale anthropogenic habitat loss. This study highlights the importance in accounting for these losses and creating efforts to monitor and mitigate future small-scale habitat loss.

Introduction

Seagrass meadows provide important services to coastal ecosystems, such as acting as a nursery habitat for fish, providing primary production for food webs, and preserving water quality by trapping suspended particles and nutrients (Orth et al. 2006). More recently, it has been discovered that seagrasses play a globally-significant role in capturing and storing carbon dioxide (CO_2) (Fourqurean et al. 2012), which has attracted attention from research and management entities seeking to mitigate climate change using the natural process of 'biosequestration' (Laffoley and Grimsditch 2009, Macreadie et al. 2014a, Sutton-Grier et al. 2014). Seagrass habitats bury organic carbon (C_{org}) on average 40-times faster than terrestrial habitats and can store large C_{org} pools within sediments for millennia (Laffoley and Grimsditch 2009, McLeod et al. 2011). These qualities make seagrass habitats hot spots for C_{org} sequestration and greenhouse gas mitigation.

Within the last century, 29% of seagrass habitats have disappeared globally, with rates of loss approximately 110 km^2 yr^{-1} since 1980 (Waycott et al. 2009). While some of these losses are attributed to natural events like storms, most are caused by anthropogenic factors, including eutrophication, dredging, trawling, and boating activities, which negatively affect ecosystem functions (Orth et al. 2006, Waycott et al. 2009, Marbà et al. 2014). Several studies have addressed the loss of C_{org} storage and the potential for the amplified release of CO_2 back into the atmosphere following seagrass habitat disappearance, but with varying outcomes. Global estimates have suggested that the top metre of C_{org} could be remineralised back into CO_2, representing a flux of 63-297 Tg C yr^{-1} as a result of seagrass loss (Fourqurean et al. 2012), in addition to losses associated with biomass and reduction of the sediment-capturing ability of a meadow (Fourqurean et al. 2012, Marbà et al. 2015). The loss of some species like *Posidonia oceanica* in the Mediterranean had a 11-25% reduction in C_{org} sequestration after habitat loss (Marbà et al. 2014); however, habitat loss of other species like *Zostera nigricaulis* had no effect on C_{org} stocks (Macreadie et al. 2014b). These reports suggest that the effect of habitat loss on C_{org} sequestration and storage is more variable than previously assumed in models (Fourqurean et al. 2012, Pendleton et al. 2012).

Anthropogenic physical disturbances can cause direct habitat destruction as well as the associated decrease in water quality (Erftemeijer and Lewis 2006). Dredging alone has caused nearly 24,000 ha of seagrass loss from 1876 to 2005 (Erftemeijer and

Lewis 2006). Based on average global C stock estimates (Fourqurean et al. 2012), dredging has led to approximately 60.5 Gg C_{org} lost as living biomass and 7.9 Tg C_{org} lost from the top metre of sediments. Smaller-scale disturbances like boating damage, propeller scars and human trampling can also have significant damaging effects on seagrass habitats and functions. For example, boat moorings may produce relatively large circular scours within meadows (3-300 m^2) (Walker et al. 1989), while propeller scars of ~3-12 cm depth can reduce seagrass cover and macrofaunal abundances (Uhrin and Holmquist 2003). Anchoring causes breakage and uprooting of seagrass shoots in the order of 50-840 shoots m^{-2} (Francour et al. 1999, Milazzo et al. 2004). In addition to acute losses, small-scale disturbances can also weaken the stability of the remaining meadow and lead to increased fragmentation and decline in ecological function (Fonseca and Bell 1998, Whitfield et al. 2002, Tewfik et al. 2007). Unfortunately, unless there are monitoring programs in place like those for boating scars in Florida, USA (Sargent et al. 1995), small-scale damage may remain unreported, and the subsequent effects on C_{org} stocks and sequestration will remain unquantified.

The aim of this study was to study the effects of shading-induced, small-scale habitat loss of two morphologically different seagrass species on the quality and quantity of C_{org} stocks over the short-term (< 1 yr). While this approach will not simulate the physical loss of seagrass and sediments from experimental plot, we can track the response of carbon stocks after above- and below-ground seagrass loss, e.g, low light conditions, salinity or temperature stress, etc.). We examined beds containing either *Halodule wrightii* or *Thalassia testudinum* in Big Lagoon (Florida, USA). We hypothesised that the morphologically smaller *H. wrightii* will rapidly lose C_{org} following die-off, but would recolonise the die-off area more quickly. In contrast, we hypothesised that the slower-growing, more robust seagrass species, *T. testudinum*, will lose C_{org} more slowly following die-off, but revegetation will also be slower.

Methods

Site description and sample collection

The study took place at Johnson's Beach in Big Lagoon, Florida, USA (30.3036 N, 87.4134 W) from May 2013 to December 2014 in a seagrass meadow containing *Halodule wrightii* Ascherson and *Thalassia testudinum* Banks *ex* König. There was

clear zonation between the two species, with *H. wrightii* closer to the shoreline and *T. testudinum* appearing in deeper regions (Stutes et al. 2007). The meadow resides in the Johnson's Beach National Seashore Federal Park and has relatively minimal anthropogenic impact (Stutes et al. 2007). It is surrounded by saltmarsh (*Spartina alterniflora*) with no residential development. The area is tidally influenced and salinities typically remain ~23 with lows near 10 and maximums of 32 (recorded at the nearby State Park site; Stutes et al. 2007, Cebrián et al. 2009, Christiaen et al. in press). Annual water temperatures typically range from 11-33°C (recorded at State Park; Christiaen et al. in press). The area has clear water and relatively low water-column nutrient and chlorophyll concentrations (Stutes et al. 2007, Cebrián et al. 2009, Christiaen et al. in press). Recreational activities by park visitors primarily include kayaking, paddle-boarding, fishing, manual shellfish collection and small boat activity.

The experiment was performed in a block design with each of the 3 blocks containing 5 treatment plots (1.7 x 1.7 m): bare sand, *H. wrightii* control, *H. wrightii* kill, *T. testudinum* control and *T. testudinum* kill (Fig. S5.1). The 'kill' treatment was performed by shading (Hemminga 1998, Longstaff and Dennison 1999), which involved a cage (1.7 x 1.7 x 0.25 m) made of PVC tubing and fiberglass screen that block > 90% of incident light. The rhizomes were severed along the plot edge to prevent resource translocation. Cages were deployed in June 2013 and remained until January 2014 to ensure complete die-off (i.e., we confirmed that all above-ground biomass was lost and below-ground structures were dead at that time). Seagrass control plots received no shading treatment and rhizomes remained intact, and bare plots were void of vegetation.

Samples were collected three times during the experiment. Pre-kill (or 'Pre') sampling occurred in June 2013 prior to applying the kill treatment. Time 0 (T_0) was sampled upon removal of the cages in January 2014, and Time 1 (T_1) was sampled at approximately ~11 months later (November 2014). At each sampling time, one sediment core per plot was taken for geochemical analyses. Cores were taken with 10 cm diameter PVC piping to a depth of 12 cm for *H. wrightii* and 15 cm for *T. testudinum* in order to capture the entire rhizosphere. Sediment cores were sliced in 1 cm intervals, and live infauna and seagrass were removed before drying at 60°C. Dry bulk density (g cm^{-3}) was subsequently calculated (Howard et al. 2014). Subsamples

were taken for loss-on-ignition, thermogravimetric analysis (TGA), and elemental and stable isotope analyses.

Geochemical Analysis

Total organic matter content (% OM) content was quantified by loss-on-ignition (LOI). Approximately 8 g of dried sediments from selected core depths (0-1, 2-3, 4-5, 7-8, 9-10, 11-12, 14-15 cm) at each time point were placed in pre-combusted aluminium tins and heated in a muffled furnace for 4 hours at 550°C (Fourqurean et al. 2012). Organic matter content was calculated as the percent mass loss during ignition.

Surface (0-1 cm), subsurface (2-3 cm) and deep (11-12 cm) sediment samples were analysed for elemental and stable isotope content from each time point (Stable Isotope Geosciences Facility, Texas A&M University). Sediment samples were ground to a fine size fraction (< 64 μm) using mortar and pestle, and then subsequently treated to remove the calcium carbonate fraction via fumigation with concentrated hydrochloric acid (Harris et al. 2001). Samples were analysed using an elemental analyser (Carlo Erba NA 1500 Series 2) attached to an isotope ratio mass spectrometer (IRMS; Thermo Finnigan Conflo III and a Thermo Finnigan Delta Plus XP; Thermo Electron, GmbH, Bremen, Germany). The L-glutamic acid standards utilised were USGS 40 ($\delta^{15}N$ = -4.52‰ Air, $\delta^{13}C$ = -26.39‰ VPDB) and USGS 41 ($\delta^{15}N$ = 47.57‰ Air, $\delta^{13}C$ = 37.63‰ VPDB).

To determine the macromolecular content and organic matter quality of the sediments, thermogravimetric analysis (TGA) analyses were performed on samples from block three at 0-1 cm, 2-3 cm and 11-12 cm depths from pre-kill and T_1 samplings. TGA was performed at the University of Technology Sydney, Australia. Data from this analysis provided information of the recalcitrance of the sediment OM, determined by the temperature at which OM was combusted. All samples were analysed using a thermogravimetric analyser (SDT Q600, TA Instruments, New Castle, DE, USA) with a 0.1 μg balance sensitivity. An aliquot of ground sample (~60 mg) was place in a platinum cup and heated under air (gas flow 50 mL min^{-1}) at 20°C min^{-1} to 900°C (Capel et al. 2006). Universal Analysis software (TA Instruments, New Castle, DE, USA) was used to aid in the identification and quantification of mass loss within specific exotherms (Exo). Delineation of thermal intervals was based on the rate-of-change derivative (% mass loss $°C^{-1}$), which indicated distinct temperature mass loss.

Mass loss occurring from the start to 180°C was associated with the loss of moisture. According to Lopez-Capel et al. (2006), the first exotherm interval (Exo 1: labile OM corresponding to aliphatics and carbohydrates) ranged from 200°C to 400°C, followed by Exo 2 (recalcitrant OM primarily as aromatics) from 400°C to 580°C. Exo 3 (refractory OM as polycondensed forms of lipids, aromatic C and organic residues) extended from 580°C to 650°C, and finally inorganics (e.g., carbonates) from 650-900°C. Exos 1–3 were recalculated as a proportion of total organic matter from 200°C to 650°C.

Statistical Analysis

An Analysis of Variance (ANOVA) with repeated measures was used to analyse the change in total organic matter content and C_{org} pools (mg C_{org} cm^{-3}). A preliminary two-way ANOVA testing *time* and *treatment* at each depth show no significant difference between bare, controls and kill treatments. Therefore, a one-way ANOVA was performed to look differences over *time* (pre-kill, T_0, T_1) for each depth for each treatment separately (Bare, *H. wrightii* control, *H. wrightii* kill, *T. testudinum* control, *T. testudinum* kill) (SPSS, IBM, Armonk, NY, USA). The Bonferroni correction was applied for pairwise comparisons. Cohen's *d* was used to calculate effect size for changes in C_{org} through time.

Multivariate Permutational Analysis of Variance (PERMANOVA) was used to statistically analyse the shifts in organics quality (TGA) and C source (stable isotope and C:N ratio) data. For TGA, analyses were time-consuming, so we were limited in using one replicate for the analyses. Therefore, species were pooled in order to perform a two-way PERMANOVA on each control and kill treatments separately to analyse differences across *time* (pre-kill, T_1) and among *depths* (0-1 cm, 2-3 cm, 11-12 cm). Shifts in stable isotopes with C:N constraints were analysed with a one-way PERMANOVA at each depth (0-1 cm, 2-3 cm, 11-12 cm) for each treatment separately over *time* (pre-kill, T_0, T_1). For all PERMANOVAs, Monte Carlo tests (*P(MC)*) were used for tests with less than 100 unique permutations. Similarity percentage (SIMPER) analysis was used to identify exotherm or elemental and isotopic signature contributing to the significant differences. Analyses were based on untransformed data and Euclidean distance resemblance matrices calculated with PRIMER v6 with PERMANOVA+ add on (Clark 1993; PRIMER-E Ltd., Ivybridge, UK).

Mixing model

We further analysed changes in isotope signatures and C:N ratios with IsoSource (Phillips and Gregg 2003) to estimate the contributing OM sources in the sediments. Models were run at 1% increments and the lowest possible tolerance using five sources and two isotopes ($\delta^{13}C$ and $\delta^{15}N$) with C:N ratio constraints. The five reference sources included three fresh OM sources for Johnson's Beach: *Spartina alterniflora*, composite seagrass (*H. wrightii* and *T. testudinum*) and seston/plankton filtered from the water column on a pre-combusted GFF filter. To take into account diagenesis of stable isotopes during decomposition, two additional sources were included in the model. These additional sources were decomposed *S. alterniflora* and decomposed seagrass. The decomposed *S. alterniflora* values were calculated by applying the relative shifts in $\delta^{13}C$, $\delta^{15}N$ and C:N of *S. alterniflora* that decayed for 18 months provided by Benner et al. (1991) to the fresh values measured in this study. There were no available estimates for changes in stable isotope during the decomposition of *H. wrightii*, so the decomposed seagrass values were calculated using the shifts in stable isotope and elemental signatures for only *T. testudinum* after ~12 months of decomposition provided by Fourqurean and Schrlau (2003).

Results

Re-vegetation in the seagrass die-off plots did not occur for either seagrass species approximately one year after removing the kill treatment cages. On 28-29 April 2014, between T_0 and T_1 samplings, there was a large rain event (> 50 cm) that caused lagoon salinities to decline from 19 to 0.5 in 3 days. Normal salinities did not return until 14 days after the rain event. As a result of continued low salinities, there was a thinning of seagrass densities for both species at Johnson's Beach (Fig. S5.1). Despite the shifts in seagrass abundance across all experimental blocks, we did detect significant changes in OM and C_{org} content as well as the isotope/element signatures of the sediments as a result of small-scale seagrass die-off.

Organics Content

OM was highly variable among treatments over time (Fig. S5.2). Bare plots had consistently lower OM than seagrass plots and did not significantly change throughout the experiment (Figs. 5.1a, S5.2). The down-core OM content of the seagrass plots ranged from 0.5-3.0%, and the controls did not significantly vary through time (Fig. 5.1b-e). Kill plots for both seagrasses experienced significant reductions in OM in the upper sediment layers following the die-off (Fig. 5.1b-e). Namely, for *H. wrightii*, there was a 50-65% loss of OM between pre-kill and T_1 in the top 8 cm (Fig. 5.1c), and % OM became increasingly similar to that of the bare plots by T_1 (Figs. 5.1, S5.2c). Pairwise tests with Bonferroni correction indicated significant losses only occurred for *H. wrightii* kill plots at 2-3 cm between pre-kill and T_0 (Table 5.1, Fig 5.1c). *Thalassia testudinum* kill plots experienced a 50% OM reduction in the top 1 cm, which was not significant with a Bonferroni correction between pre-kill and T_1 samplings ($P = 0.087$; Table 5.1, Fig. 5.1e). During the processing of the *T. testudinum* cores, we noticed substantial rhizome decomposition in the kill plots (i.e., hollowed rhizome cylinder; Fig. S5.3).

For sediment C_{org} pools (mg C cm^{-3}) calculated with dry bulk density data (Fig. S5.4), the bare plots had ~2 mg C_{org} cm^{-3} except for the deep samples at T_0 (~4%, $P > 0.05$; Fig. 5.1f). Seagrass plots averaged 2-8 mg C_{org} cm^{-3} and were highly variable both down-core and across time (Fig. 5.1g-j). C_{org} pools of control plots did not change significantly throughout the experiment (Table 5.1, Fig. 5.1g, i). Contrary to the trends in OM, the upper sediment layers did not significantly vary in C_{org} for *H. wrightii*; rather, the C_{org} content in the deep sediments of *H. wrightii* kill plots significantly increased between pre-kill and T_1 (Table 5.1, Fig. 5.1h). The C_{org} pools at the surface *T. testudinum* kill plots did significantly decline during the post-kill period by 21-47% (Table 5.1, Fig. 5.1j). Although, the loss of C_{org} was limited to the top 1 cm for *T. testudinum* kill plots, the effect size was large ($d = 2.1$-3.7).

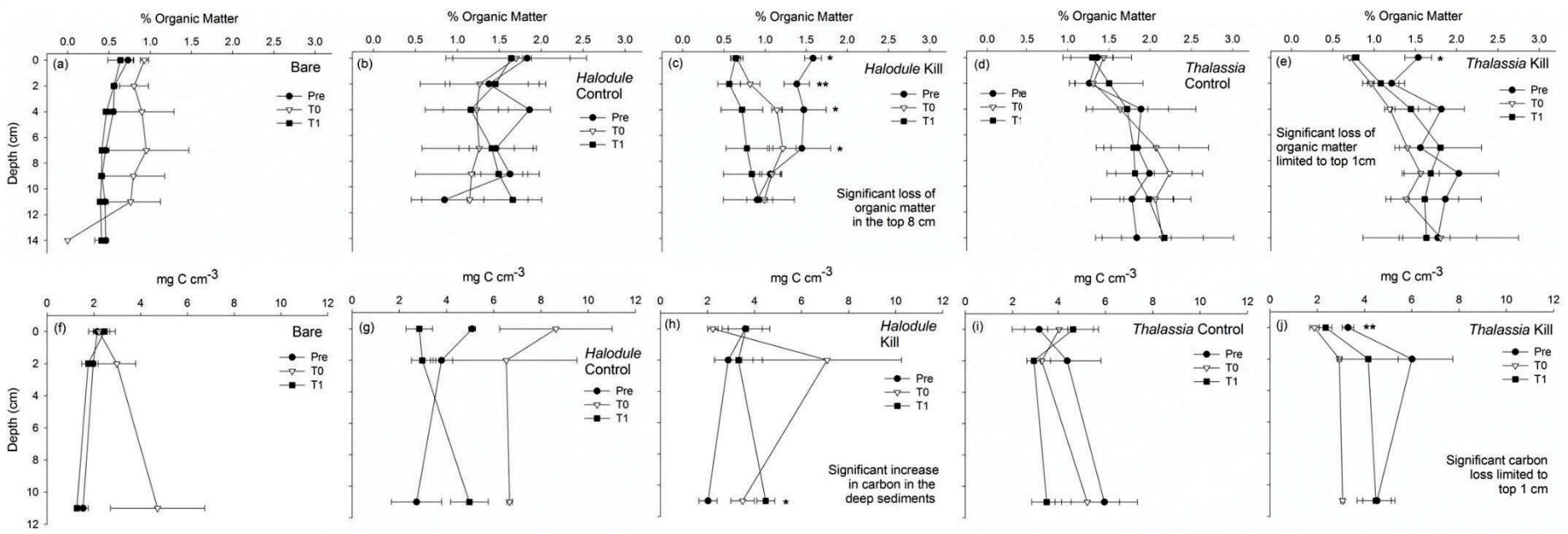


Figure 5.1: Down-core variation in organic matter and organic carbon for each plot through time. Panels a-e represent changes in % OM down-core through time, and panels f-j represent changes in mg C cm^{-3} down-core through time. Significant shifts at each depth are indicated with annotated text, and asterisks represent non-adjusted (*) and Bonferroni adjusted (**) post-hoc analyses. Pre = pre-kill sampling, T_0 = Time 0 sampling, T_1 = Time 1 sampling.

Table 5.1: OM, C_{org}, and stable isotope and elemental statistical analyses over time. One-way repeated measures ANOVA for OM and C_{org} data, and one-way PERMANOVA for isotope and elemental data. Non-adjusted and Bonferroni correction (BC) or Monte Carlo (*P(MC)*) P-values reported for post-hoc analyses. Hw = *Halodule wrightii*, Tt = *Thalassia testudinum*, Ctrl = control, Pre = pre-kill, T_0 = Time 0, T_1 = Time 1.

		% Organic Matter					mg C cm^{-3}					Stable Isotopes and C:N				
Plot	Depth (cm)	df	F	P	Non-adj	BC	df	F	P	Non-adj	BC	df	*Pseudo-F*	*P (perm)*	t	*P(MC)*
Bare	0-1	2	2.338	0.213			2	0.120	0.890			2	0.299	0.972		
	2-3	2	1.675	0.296			2	1.810	0.276			2	0.39151	0.831		
	4-5	2	0.884	0.481												
	7-8	2	0.928	0.467												
	9-10	2	0.920	0.469												
	11-12	1*	0.757	0.476			2	2.634	0.186			2	3.8829	0.074		
Hw, Ctrl	0-1	2	0.060	0.943			2	6.037	0.142			2	0.53289	0.735		
	2-3	2	0.346	0.727			2	0.912	0.472			2	2.1531	**0.007**	-	> 0.05
	4-5	2	0.561	0.641												
	7-8	2	0.071	0.933												
	9-10	2	0.033	0.968												
	11-12	2	0.481	0.675			2	16.449	0.057			2	1.4008	0.300		
Hw, Kill	0-1	2	30.483	**0.004**	0.035†, 0.019‡	0.056‡	2	0.908	0.472			2	4.5067	**0.025**	2.6798†, 2.507‡	0.031†, 0.023‡
	2-3	2	9.737	**0.029**	0.011†	0.032†	2	1.262	0.376			2	1.4345	0.250		
	4-5	2	8.576	**0.036**	0.030‡	0.089‡										
	7-8	2	10.690	**0.025**	0.023‡	0.068‡										
	9-10	2	1.091	0.419												
	11-12	2	0.052	0.950			2	19.901	**0.008**	0.016‡	0.048‡	2	1.5979	0.197		

		% Organic Matter					mg C cm^{-3}					Stable Isotopes and C:N				
Plot	Depth (cm)	df	F	P	Non-adj	BC	df	F	P	Non-adj	BC	df	*Pseudo-F*	*P (perm)*	t	*P(MC)*
Tt, Ctrl	0-1	2	0.088	0.917			2	1.793	0.278			2	1.8445	0.136		
	2-3	2	1.213	0.387			2	0.748	0.530			2	1.753	**0.049**	-	> 0.05
	4-5	2	0.574	0.604												
	7-8	2	0.307	0.751												
	9-10	2	1.126	0.409												
	11-12	2	1.209	0.388			2	1.520	0.323			2	0.44578	0.812		
	14-15	2	0.651	0.569												
Tt, Kill	0-1	2	19.499	**0.009**	0.049†, 0.029‡	0.087‡	2	84.262	**0.001**	0.001†, 0.018‡	0.003†, 0.055‡	2	9.0096	0.005	3.8656‡, 3.1091¶	0.007‡, 0.027¶
	2-3	2	0.702	0.548			2	1.467	0.333			2	2.0302	0.255		
	4-5	2	2.420	0.205												
	7-8	2	0.887	0.530												
	9-10	2	2.247	0.222												
	11-12	2	4.661	0.090			2	3.116	0.153			2	0.82363	0.570		
	14-15	2	0.071	0.933												

*No sphericity; Greenhouse-Geisser Correction

†Pre vs. T_0

‡Pre vs. T_1

¶T0 vs. T_1

Organics Quality

TGA analysis revealed significant changes in OM quality over time for both control (*Psuedo-*$F_{1,6}$ = 6.8065, *P(perm)* = 0.023) and kill (*Psuedo-*$F_{1,6}$ = 15.772, *P(perm)* = 0.008) treatments (Fig. 5.2). Neither depth nor the depth x treatment interaction was significantly different. SIMPER analysis revealed that differences between pre-kill and T_1 samplings for control samples were driven by approximately 4% loss of labile OM (Exo 1; 50.8% contribution to dissimilarity) and a 3% increase in recalcitrant OM (Exo 2; 40.3% contribution). For the kill treatments, shifts in OM quality between pre-kill and T_1 samplings were driven almost equally by a 5.6% decrease in labile OM (47.4% contribution) and a 5.1% increase in recalcitrant OM (48.5% contribution).

Stable isotope and elemental values were obtained from plant reference sources and the sediment samples (Fig. 5.3). The polygon created from the stable isotope signatures of the reference OM encompassed ~70% of the sediment samples (Fig. 5.3a). However, when C:N ratios were included into the scatter plot, the spread of the sediment samples became less inclusive, driven by the high C:N ratio of fresh *Spartina* (Fig. 5.3b). Statistical analyses of stable isotope and elemental signatures indicated a significant change over time for the surface samples of *H. wrightii* kill (*Pseudo-*$F_{2,6}$ = 4.5067, *P(perm)* = 0.025) and *T. testudinum* kill (*Pseudo-*$F_{2,6}$ = 9.0096, *P(perm)* = 0.005) treatments (Table 5.1, Fig. 5.4). *Halodule wrightii* kill 0-1 cm had significantly different signatures between pre-kill and T_0 (*P(MC)* = 0.031) and T_1 (*P(MC)* = 0.023). T_1 for *T. testudinum* kill 0-1 cm was significantly different than pre-kill (*P(MC)* = 0.007) and T_0 (*P(MC)* = 0.027). SIMPER analysis showed that the dissimilarity across the sampling times was driven by a depletion of both isotopes between pre-kill and T_0 (28-56% contribution) followed by an enrichment of $\delta^{13}C$ between T_0 and T_1 (56-73% contribution). Additionally, SIMPER analysis revealed a portion of the dissimilarity through time was also driven by increasing C:N ratios (17-45% contribution; Fig. 5.4).

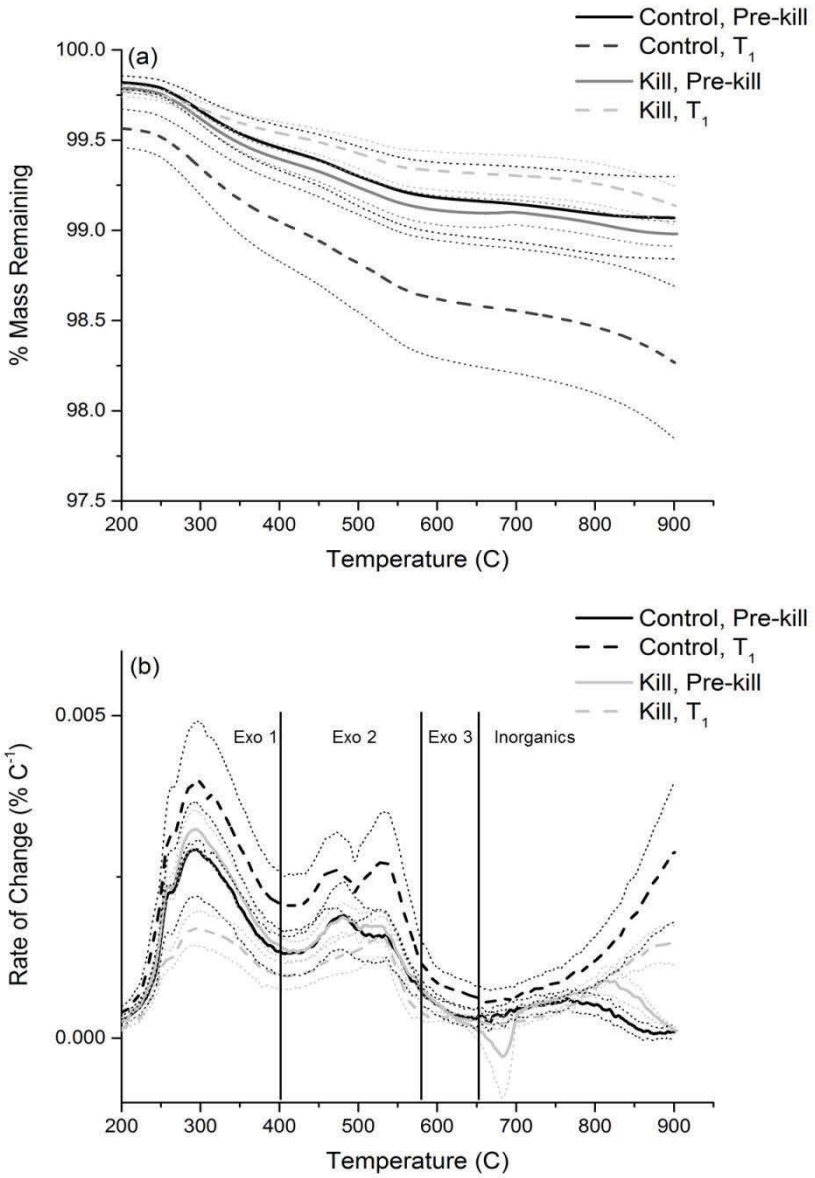


Figure 5.2: Thermograms for the sediment (a) mass loss and (b) rate-of-change with increasing temperatures. Exo 1 (200°C to 400°C) coincides with labile OM (aliphatics and carbohydrates), followed by Exo 2 that defines recalcitrant OM (aromatics) from 400°C to 580°C. Exo 3 (580°C to 650°C) represents refractory OM (polycondensed lipids, aromatic C and organic residues), and finally inorganics (e.g., carbonates) from 650-900°C. Thick lines represent means ± 1 S.E.M. shown by thin lines.

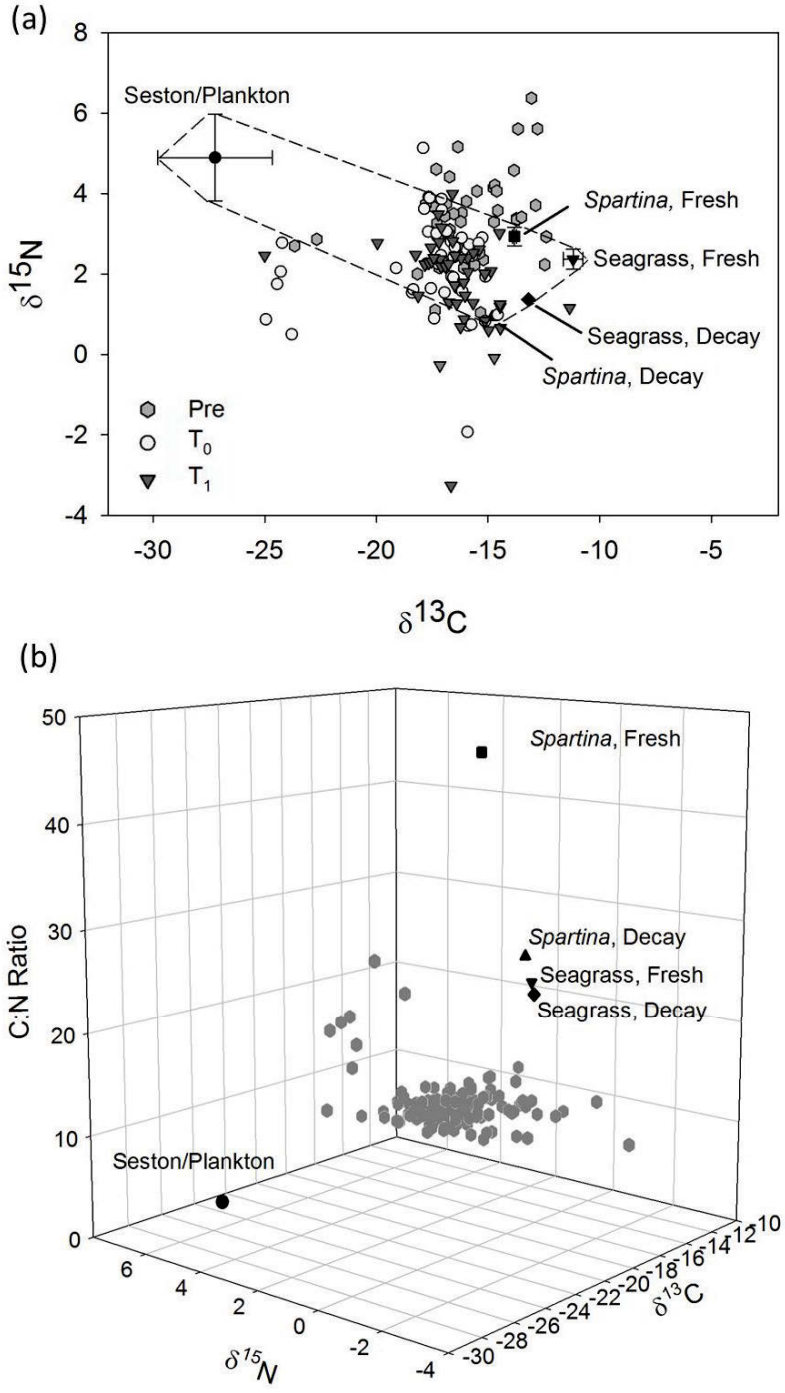


Figure 5.3: Stable isotope signatures of sediments in comparison with reference organic matter: (a) Stable isotope signatures from pre-kill (Pre), Time 0 (T_0) and Time 1 (T_1) samplings in association with the polygon of reference signatures created by fresh and predicted decaying OM sources, (b) Stable isotope signatures including C:N ratios for all sediments (circles) and fresh and predicted decaying OM.

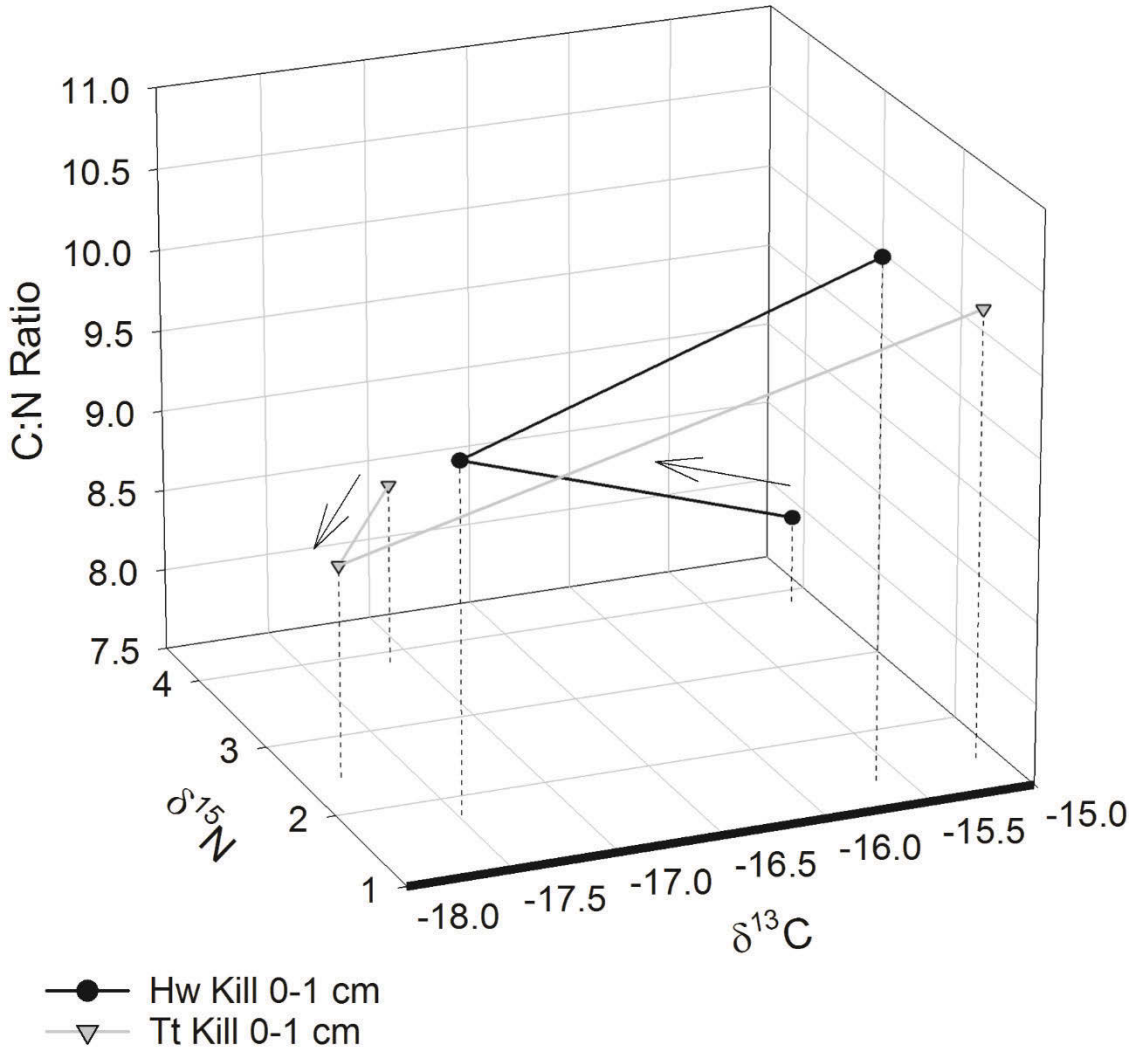


Figure 5.4: Stable isotope and elemental signatures of sediment samples that significantly changed through time. Arrows indicate direction of change through time from pre-kill to Time 0 to Time 1. Hw = *Halodule wrightii*, Tt = *Thalassia testudinum*.

Mixing model analysis of the stable isotope signatures with C:N ratio constraints was used to predict the contributions of the five OM reference sources to the sediment samples (Figs. 5.5, S5.5-S5.9). The possible solutions for most samples typically consisted of 50:50 contributions of seston/plankton and fresh seagrass with decayed seagrass contributing roughly 0-10% (Figs. 5.5, S5.5-S5.9). The two samples that had significant shifts in elemental and stable isotope signatures showed no change in source distribution across sampling times (Fig. 5.5). Similarly, the source distributions of approximately half of the remaining samples varied little throughout the experiment. In

some cases, there was a shift from fresh OM to decayed OM between pre-kill and T_0 (Figs. S5.5, S5.6, S5.7). *Spartina* OM had little to no contributions to the sediments except for *H. wrightii* control at 0-1 cm and *T. testudinum* kill at 2-3 cm, the latter samples having the most diverse span of predicted contributions from all sources (Figs. S5.6, S5.9).

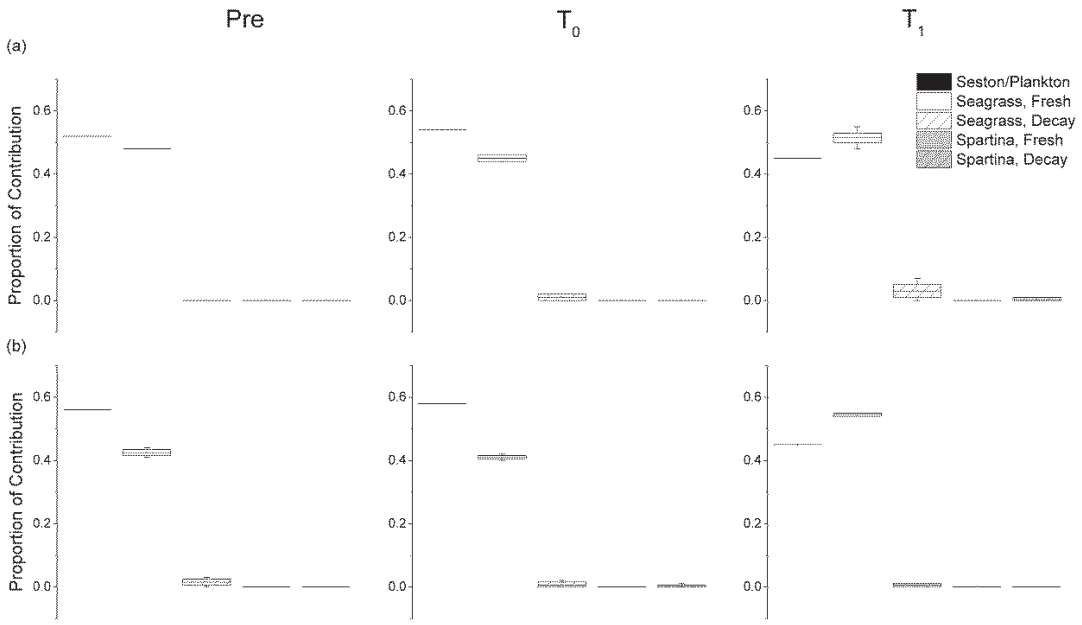


Figure 5.5: Box plots of the contributions of OM calculated by the mixing model for the samples with significant changes in stable isotopes and C:N through time: (a) *H. wrightii*, kill, 0-1 cm and (b) *T. testudinum*, kill, 0-1 cm sediments. See Supplementary Figures for predicted contributions for the remaining treatments and depths.

Discussion

The aim of this study was to quantify the short-term effects of small-scale seagrass loss on sediment organics content and quality. After applying a shading treatment to *H. wrightii* and *T. testudinum* meadows to simulate habitat loss, we recorded significant changes in sediments limited to the top few cm after the kill treatment for both species, which supported our hypothesis. We also hypothesised that *H. wrightii* would be able to recover prior to T_1 as previously shown by Creed and Amando Fliho (1999), while *T. testudinum* would be expected to recover over the next

7-10 years (Dawes et al. 1997). Lack of recovery of seagrass in the die-off plots, particularly for fast-growing *H. wrightii*, was likely related to the natural loss of seagrass abundance and densities during the low salinity event between T_0 and T_1 samplings (Fig. S5.1). While this event affected revegetation progress, the organic content data in the control plots indicate that the sediments were not affected (Fig. 5.1).

The organic matter and C_{org} pools were highly variable among species and treatments within a given sampling time (Figs. 5.1, S5.2). However, when pre-kill and post-kill samples were compared at specific depths within an individual plot, the surface sediments of the kill treatments changed significantly in organic content and quality. The greatest amount of OM loss occurred in the top 8 cm of *H. wrightii* kill plots, although the C_{org} content did not coincide with this OM loss (Fig. 5.1). This disconnect between percent OM and C_{org} is likely related the methodology used to analyse elemental content (e.g., in conjunction with stable isotope anlaysis). Stable isotope analysis uses very small masses of sediment, which could misrepresent the C_{org} content of a bulk sample. We recommend that extreme caution be used when interpreting C_{org} and N data obtained in this manner. For the top 1 cm of *T. testudinum* kill plots, there was a significant loss of both OM and C_{org} after the kill treatment. The loss of C_{org} in *T. testudinum* kill plots (21-47%) was both statistically and ecologically significant (Fig. 5.1). The loss of organics may have been limited to the top 1 cm for *T. testudinum* plots due the greater stability of the remaining below-ground structures, which could limit scouring and diffusion of oxygen into the sediment that would have otherwise led to stimulated microbial remineralisation (Jørgensen and Revsbech 1985). Significant decomposition of below-ground tissues was evident from hollowed rhizome material observed during T_1 sampling (Fig. S5.3). The microbial remineralisation of labile storage compounds inside the rhizome, leaving behind the more refractory structural components, is typical for long-term *T. testudinum* decomposition (Kenworthy and Thayer 1984). The limited loss of C_{org} in the *T. testudinum* kill plots could also be attributed to the addition of this detrital carbon from the newly dead below-ground tissues that could have masked the net loss of C_{org} below 1 cm. Although the deeper sediment in the *H. wrightii* kill plots did not have remnant rhizomes, the shift from live C to detrital C could similarly explain the significant 2.5-times gain in C_{org} in the deepest layer of these *H. wrightii* plots (Fig. 5.1h).

Thermogravimetric, stable isotope and elemental analyses provided some insight into the changes in the quality of sediment organics after the loss of seagrass. TGA analyses indicated that differences between pre- and post-kill OM was due to a relative loss of labile OM for both control and kill treatments (Fig. 5.2). The loss of labile OM coincided with an increase in C:N ratios (Fig. 5.4b), suggesting there may be preferential loss of labile and N-containing compounds by microbial communities (Harrison 1989, Fourqurean and Schrlau 2003, Chapter 2). The relative shifts in stable isotopes also provide evidence of sediment organic OM decomposition between pre- and post-kill samplings. Firstly, depletion of $\delta^{15}N$ was consistent for most samples (Fig. 5.3a), which is typical for mangrove and long-term seagrass and saltmarsh decomposition (Zieman et al. 1984, Benner et al. 1991, Fourqurean and Schrlau 2003). Depletion of $\delta^{15}N$ signatures also suggest microbial degradation of the OM was supplemented by exogenous N sources rather than utilisation of plant N (Benner et al. 1991).

There was also a depletion of $\delta^{13}C$ values between pre-kill and T_0 samplings (Fig. 5.4). This depletion has shown to be due to preferential degradation of isotopically heavier C-rich carbohydrates and proteins during early stages of decomposition, leaving behind lighter and more refractory lignocellulose compounds (Benner et al. 1987, Benner et al. 1991). The depletion of $\delta^{13}C$-rich labile compounds was consistent with the shift in OM contributions between pre-kill and T_0 from fresh OM to decayed OM (Figs. S5.5, S5.6, S5.7). There was a subsequent shift back to fresh seagrass OM predictions between T_0 and T_1, during which many of the sediments had an increase in $\delta^{13}C$ values (Figs. 5.4, S5.5, S5.6, S5.7). Fresh seagrass inputs are likely not to explain this enrichment, especially for the subsurface or deep samples. Rather, enrichment of $\delta^{13}C$ values are commonly seen for well processed, deeper soils due to progressive colonisation of microbial biomass, which is more enriched than bulk soils (Ehleringer et al. 2000). This cause of $\delta^{13}C$ enrichment is not as well established for coastal sediments, although some amino acids associated with enriched microbial biomass have been shown to become more abundant during decomposition of sediments in seagrass meadows (Macko et al. 1994).

Despite shifts in isotope and elemental content occurring during the experiment, the mixing model analysis did not reflect these changes. First, the mixing model showed that fresh *S. alterniflora* was not a contributor to the sediment OM due to a high C:N

ratio compared to bulk sediments. Secondly, the model predictions may also be partly limited by the references used. For example, the highly deplete $\delta^{15}N$ (< 1 per mil) are typically indicative of terrestrial or emergent coastal plant OM diagenesis during decomposition (Zieman et al. 1984, Benner et al. 1991), suggesting the sediment may contain some decayed terrestrial OM not accounted for this in study. Lastly, since seagrasses do not undergo significant diagenesis during decomposition (< 2 per mil shift for $\delta^{15}N$) (Zieman et al. 1984, Fourqurean and Schrlau 2003), the larger shifts seen in this study could be related to increasing contributions of microbial biomass (Lehmann et al. 2002).

Combining OM and C_{org} loss with shifts in TGA and stable isotope signatures suggests that small-scale losses of seagrass do lead to significant losses of organics, particularly labile fractions, due to microbial decomposition. This could be due to (1) lack of additional fresh inputs that would occur by trapping of particles by above-ground biomass or inputs from exudates from live seagrasses (Macreadie et al. 2014a), resulting in (2) microbial remineralisation of sediment OM instead of fresh inputs (Guenet et al. 2010) or (3) erosion and transport of OM to other habitats (Marbà et al. 2015). However, this loss of OM and C_{org} was limited to the top few centimeters, which is much shallower than the predicted losses of up to a metre deep (Fourqurean et al. 2012). Since it has been shown in other sub-tropical and tropical meadows that small-scale disturbances can exacerbate seagrass fragmentation and reduction in recovery and ecological function (Fonseca and Bell 1998, Uhrin et al. 2011), these experimental plots will be continuously monitored in the next decade to assess this possibility for additional losses of biomass, OM and C_{org} in the future. We also suggest using age-dating and hydrodynamics techniques to better inform the dynamics of decomposition, inputs and export of the sediment over time.

Even though these C_{org} losses were much shallower than expected, small-scale loss of seagrass meadows can results in 21-47% reduction in C_{org} pools (Fig. 5.1i). An extensive survey along the Florida coast estimated that 6.4% (700,106 m^2) of total meadow cover was lost due to boating activities (Sargent et al. 1995). Other smaller-scale studies found that such disturbances affect < 2% of the local meadows (Walker et al. 1989, Martin et al. 2008). If we apply a range of 0.5-6.4% loss of habitat attributed to scarring to global estimates of seagrass cover (300,000 – 600,000 km^2; Fourqurean et al. 2012), between 1,500 and 38,400 km^2 could be destroyed from small-scale habitat loss.

Using the mean of C_{org} stocks in the top 1 cm reported for 12 seagrass species from temperate and tropical regions (5.26 mg C cm^{-3}; Lavery et al. 2013a) and the percent C_{org} lost in this study, 0.08 – 2.0 Mt of C_{org} could be released as CO_2. Using global carbon values, US$6-89 per tonne (World_Bank 2015), the loss of C_{org} due to scarring is roughly estimated to be US$5 to $180 million.

With the increased interest in blue carbon management and restoration research, we are only beginning to understand the recovery of C_{org} sequestration with the recovery of seagrass habitat. Smaller seagrass species like *Halodule* are able to colonise within a year after dredging aided by their fast horizontal rhizome elongation rates (Marbà and Duarte 1998, Sheridan 2004), leading to improved sediment and water quality for slower-growing genera, e.g., *Thalassia*. Unfortunately, most recovery times are estimated to be ≥ 2 years for large morphotypes, if revegetation happens at all (Erftemeijer and Lewis 2006). Large-scale restoration of seagrass meadows has shown the recovery of a meadow's C-sequestering function can occur decades after revegetation (Greiner et al. 2013, Marbà et al. 2015). However, there is less information on the restoration of live biomass and C_{org} stocks from small-scale disturbances (Macreadie et al. 2014b, Macreadie et al. 2015). A recent study found that scarring could lead to loss of sediment OM, N and P in Florida Bay seagrass meadows for at least 5 years and up to 25-30 cm deep, but even this loss was quite variable depending on site and time given for recovery (Bourque et al. 2015).

We have shown that during the first year after small-scale seagrass loss there are shallow but ecologically significant losses in organic carbon. These damages can potentially represent significant global ecological and economic losses associated with declined C-sequestration. The long-term effects of small-scale habitat loss still need to be studied further, but with the results shown herein, we suggest small-scale losses of habitat should be rigorously monitored with the intent to move toward better management and public awareness of this type of seagrass habitat destruction globally.

Acknowledgements

I would like to thank the Dauphin Island Sea Lab for hosting me and this experiment, as well as Josh Goff and all the volunteers for their time in the field and laboratory. Australian Quarantine Import Permit IP14016607 was used.

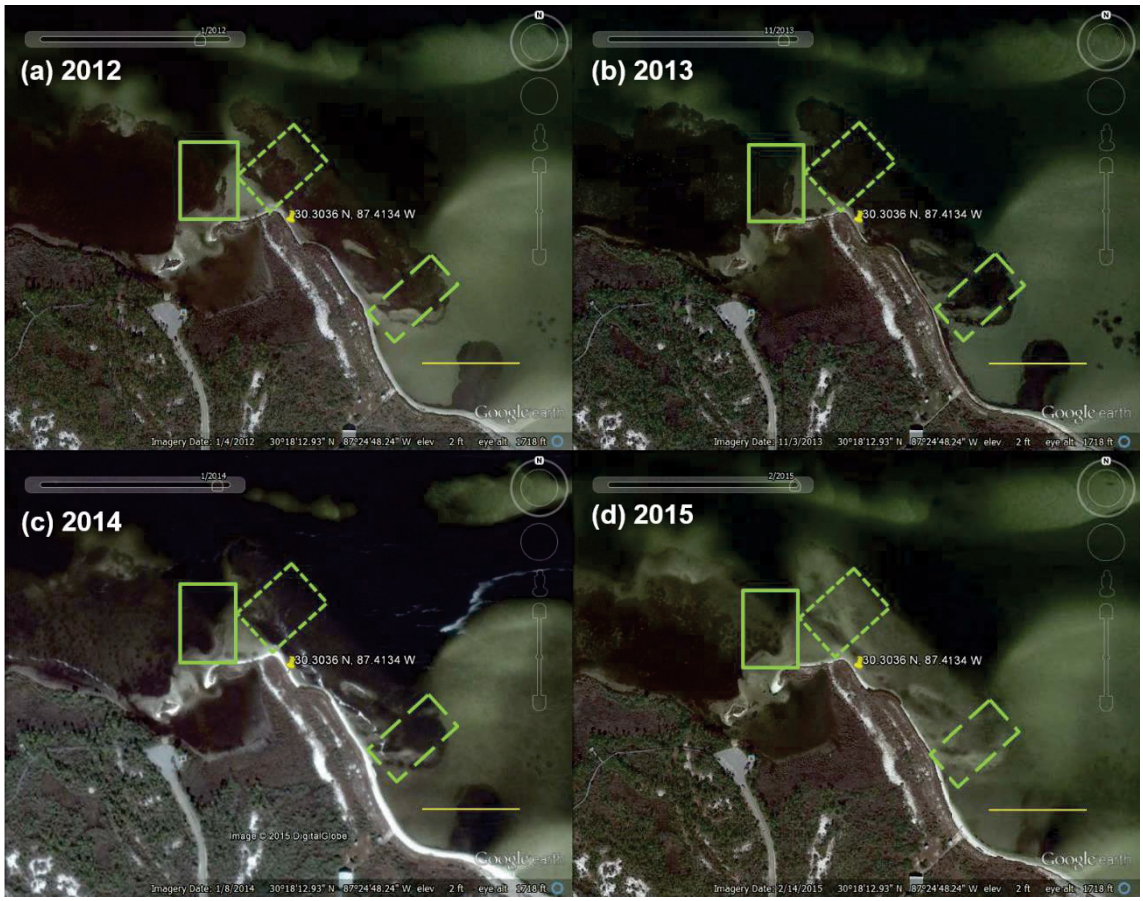


Figure S5.1: Historical time lapse of the seagrass meadows at the Johnson's Beach site from Google Earth®: (a) January 2012, (b) November 2013, (c) January 2014, and (d) February 2015. Yellow line is a 100 m scale bar. Green boxes indicate the location of the three treatment blocks.

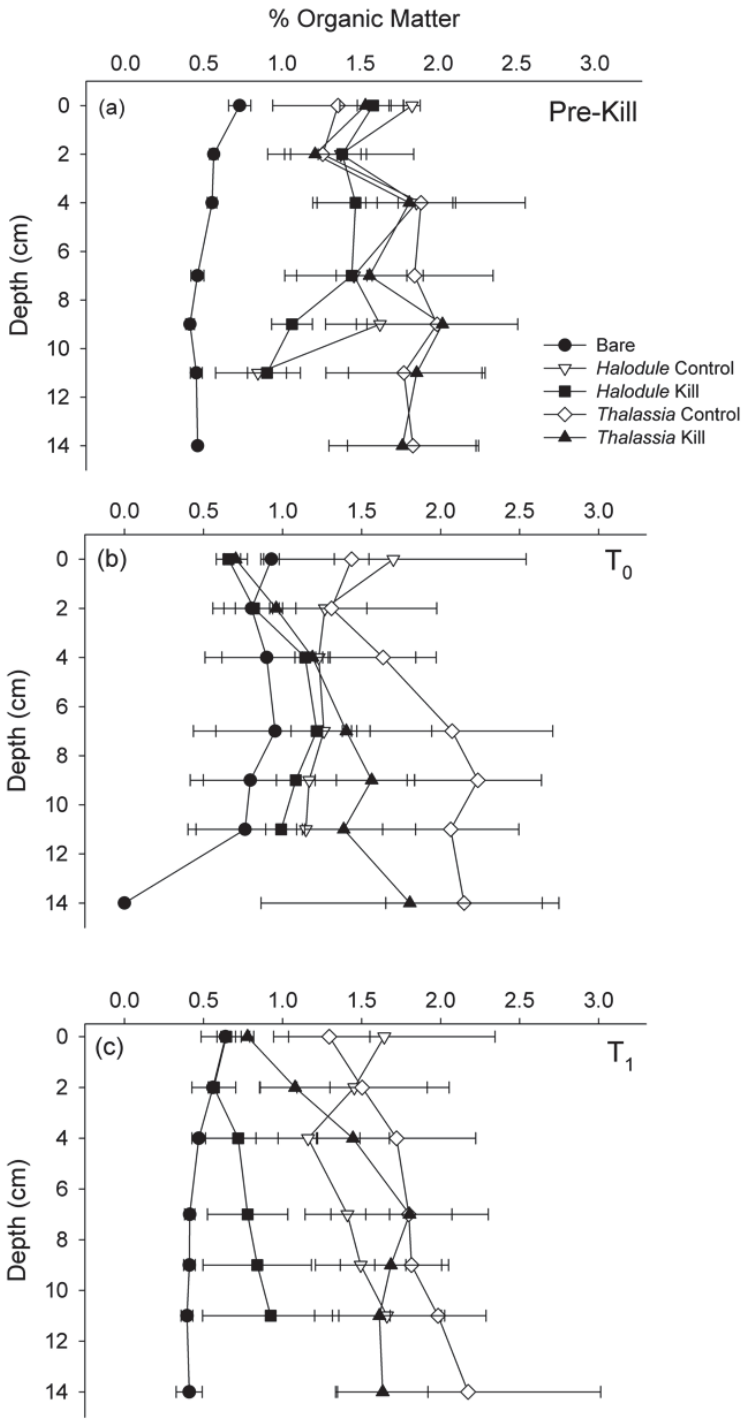


Figure S5.2: Differences in % organic matter down-core for each treatment at (a) pre-kill, (b) Time 0 (T_0) and (c) Time 1 (T_1) samplings. Values represent means ± S.E.M.

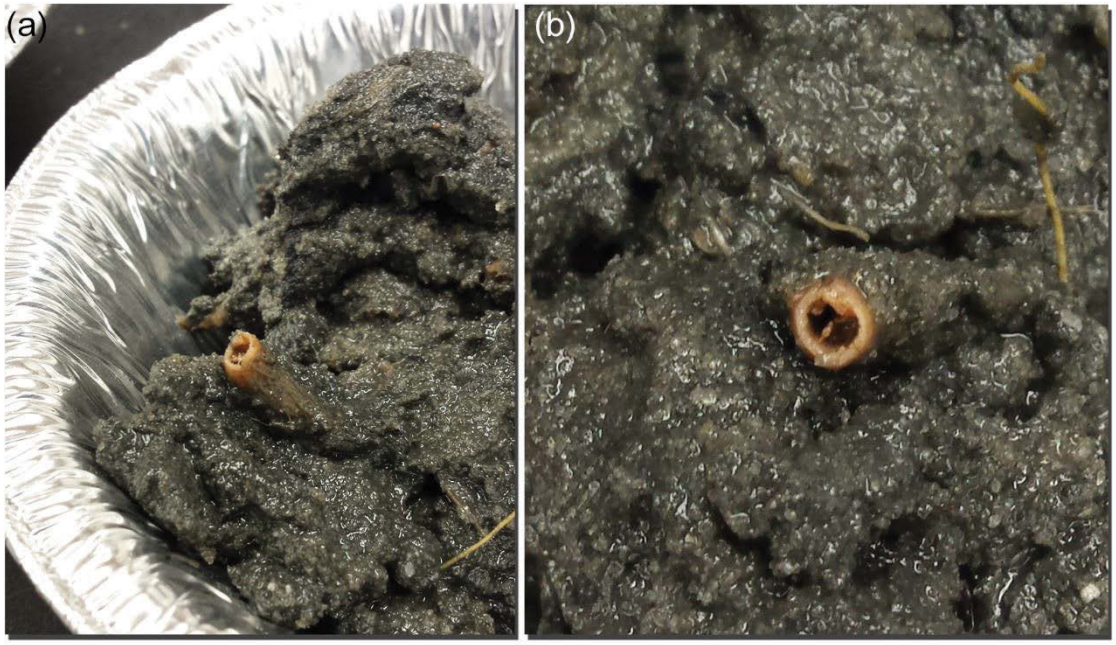


Figure S5.3: Remnants of hollow, decaying rhizome tissue from *Thalassia testudinum* kill plot. Panels show loss of inner biomass likely from microbial decomposition leaving behind structurally rigid and refractory outer walls.

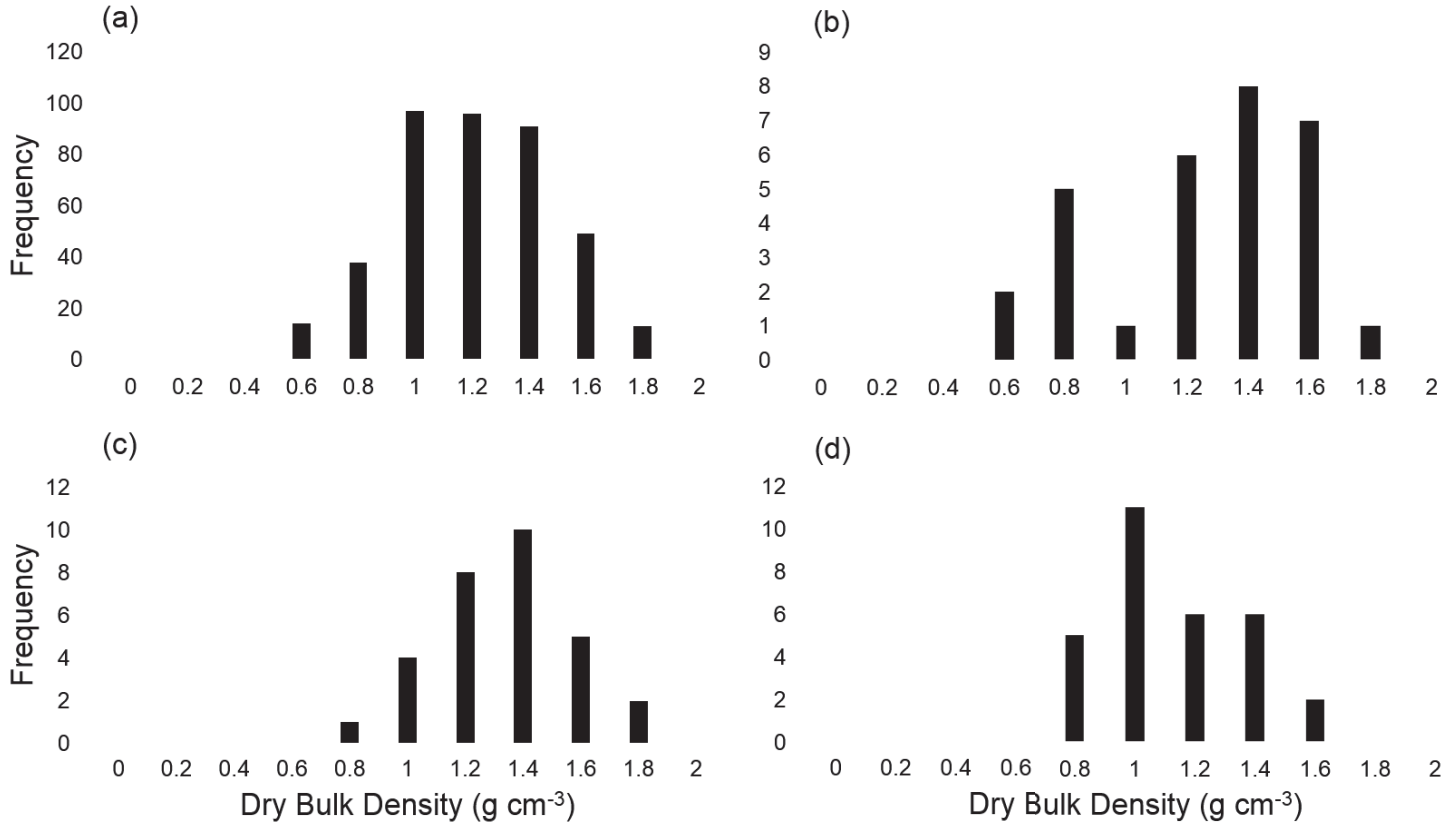


Figure S5.4: Histogram of the dry bulk density values for (a) all sediment depths and the depths analysed of C_{org}: (b) 0-1 cm. (c) 2-3 cm and (d) 11-12 cm.

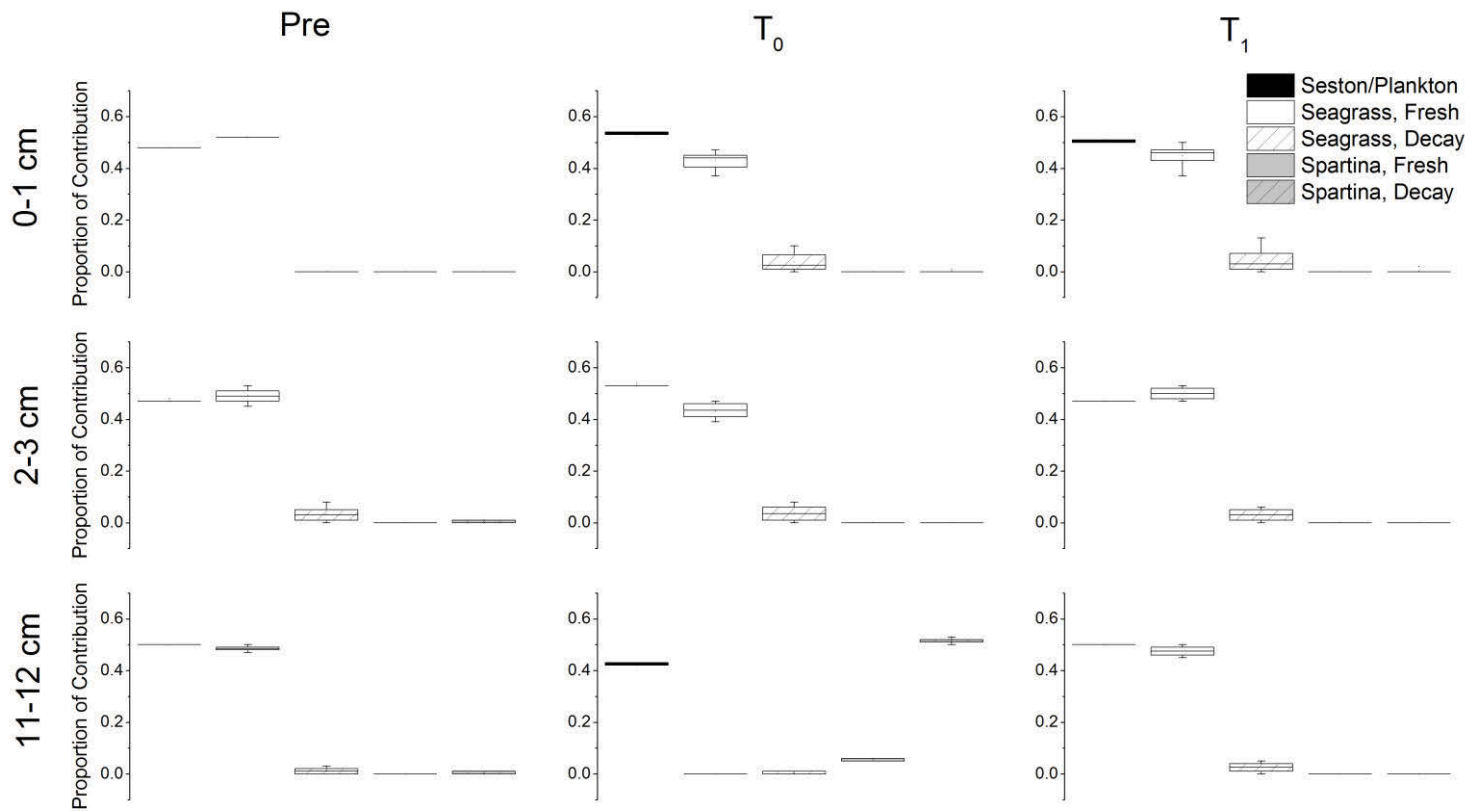


Figure S5.5: Box plots of predicted organic matter sources for sediments from Bare plots. The predictions are based on stable isotope ($\delta^{13}C$, $\delta^{15}N$) and elemental (C:N ratio) signatures analysed with the IsoSource mixing model. Pre = pre-kill sampling, T_0 = Time 0 sampling, T_1 = Time 1 sampling. Values are based on means.

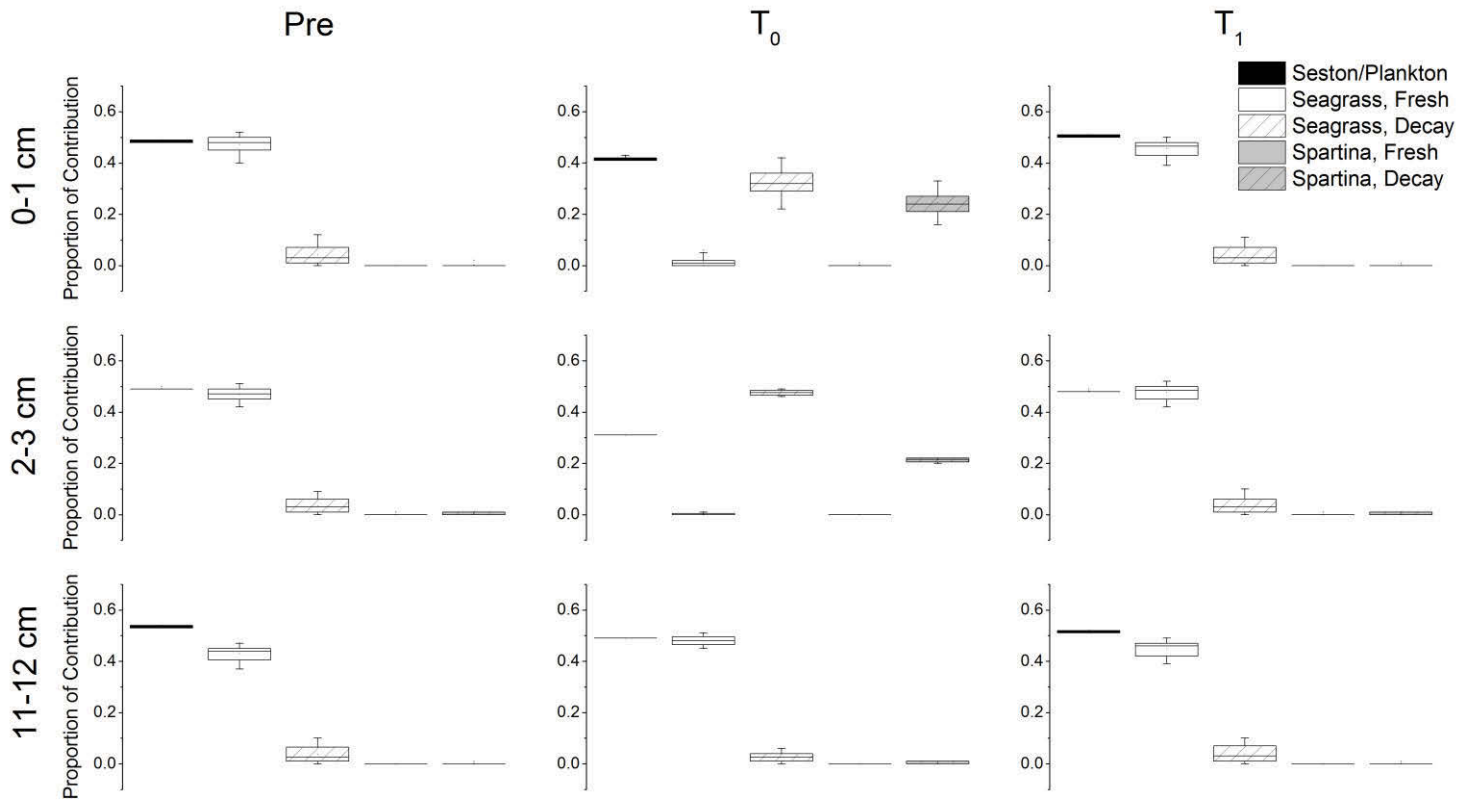


Figure S5.6: Box plots of predicted organic matter sources for sediments from *Halodule wrightii* control plots. The predictions are based on stable isotope ($\delta^{13}C$, $\delta^{15}N$) and elemental (C:N ratio) signatures analysed with the IsoSource mixing model. Pre = pre-kill sampling, T_0 = Time 0 sampling, T_1 = Time 1 sampling. Values are based on means.

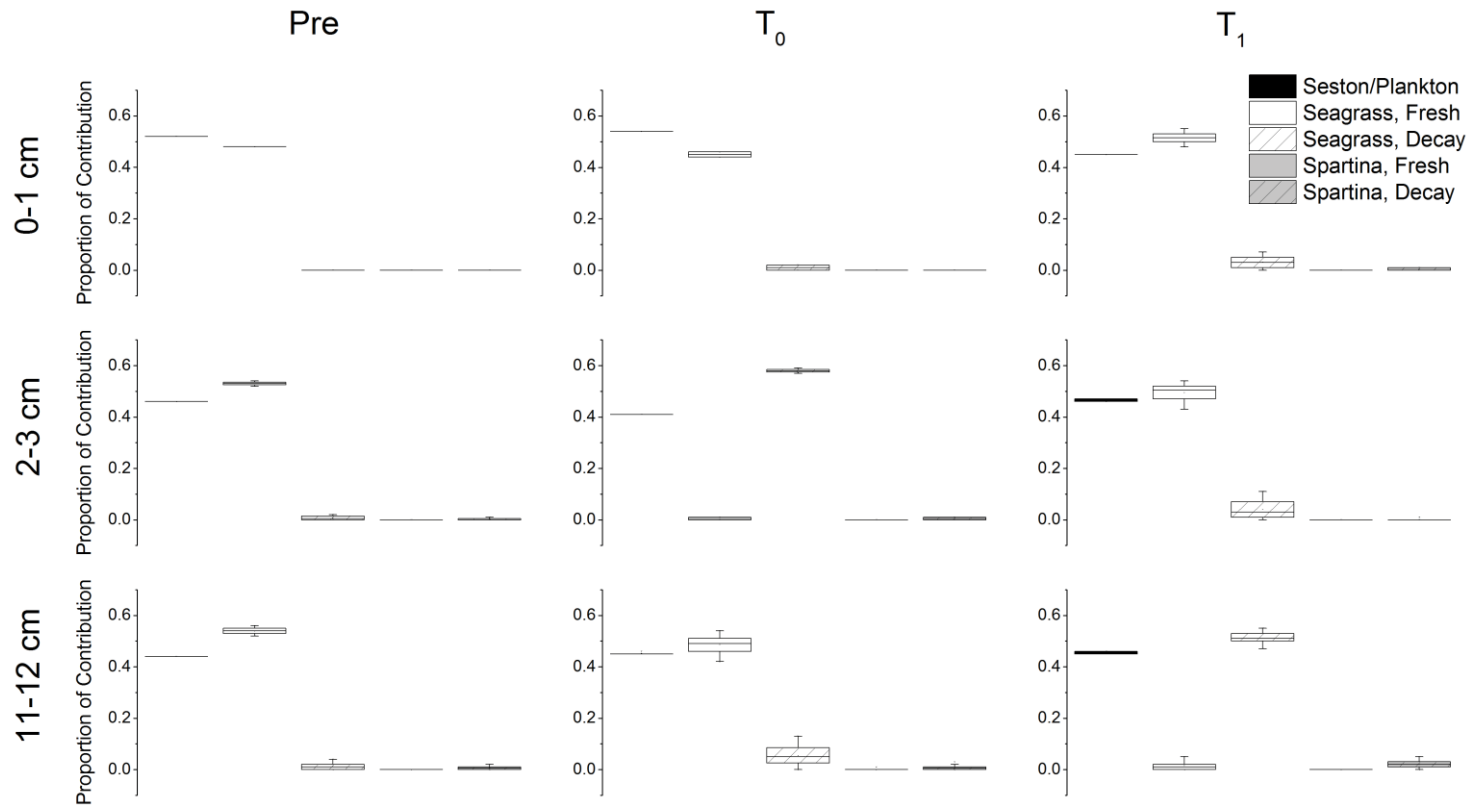


Figure S5.7: Box plots of predicted organic matter sources for sediments from *Halodule wrightii* kill plots. The predictions are based on stable isotope ($\delta^{13}C$, $\delta^{15}N$) and elemental (C:N ratio) signatures analysed with the IsoSource mixing model. Pre = pre-kill sampling, T_0 = Time 0 sampling, T_1 = Time 1 sampling. Values are based on means.

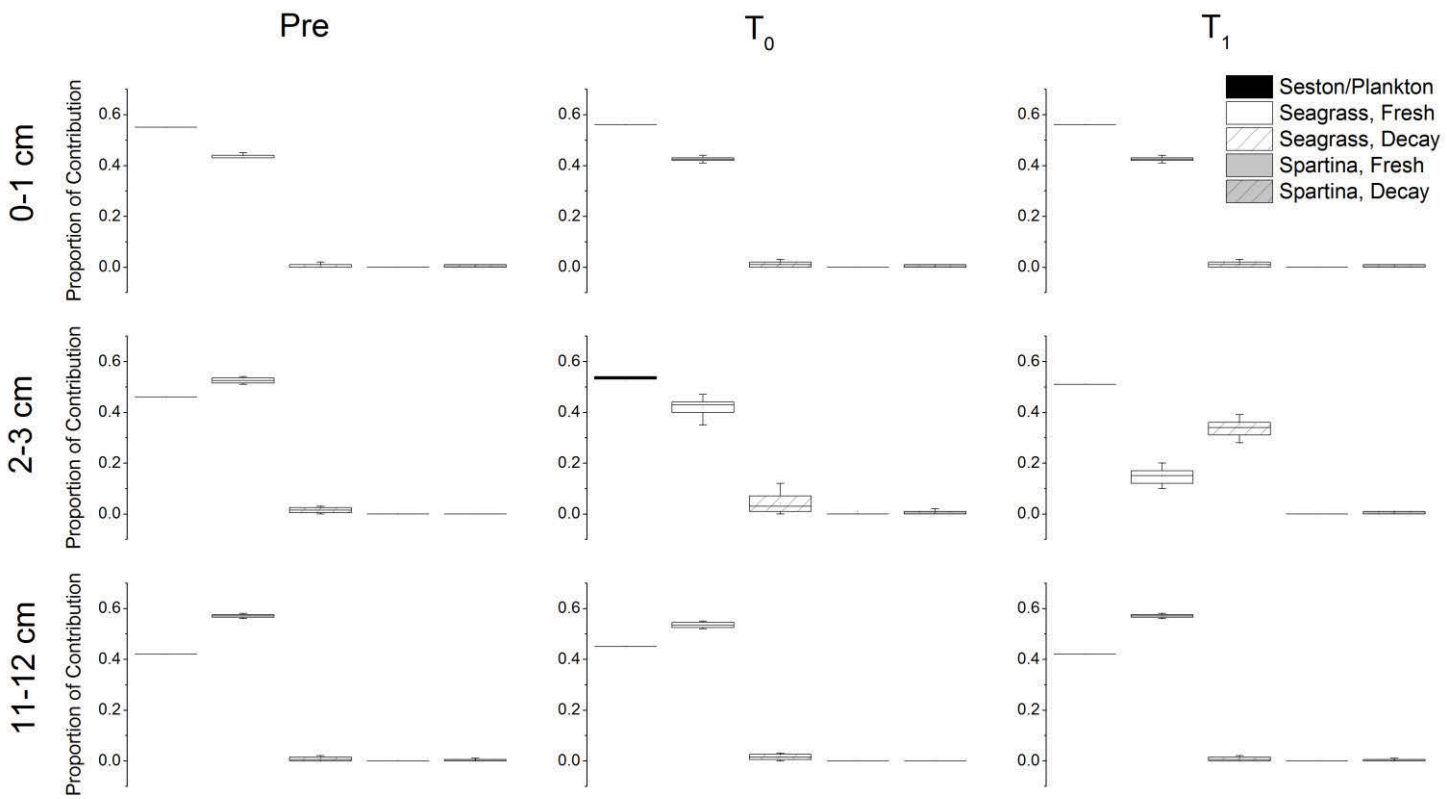


Figure S5.8: Box plots of predicted organic matter sources for sediments from *Thalassia testudinum* control plots. The predictions are based on stable isotope ($\delta^{13}C$, $\delta^{15}N$) and elemental (C:N ratio) signatures analysed with the IsoSource mixing model. Pre = pre-kill sampling, T_0 = Time 0 sampling, T_1 = Time 1 sampling. Values are based on means.

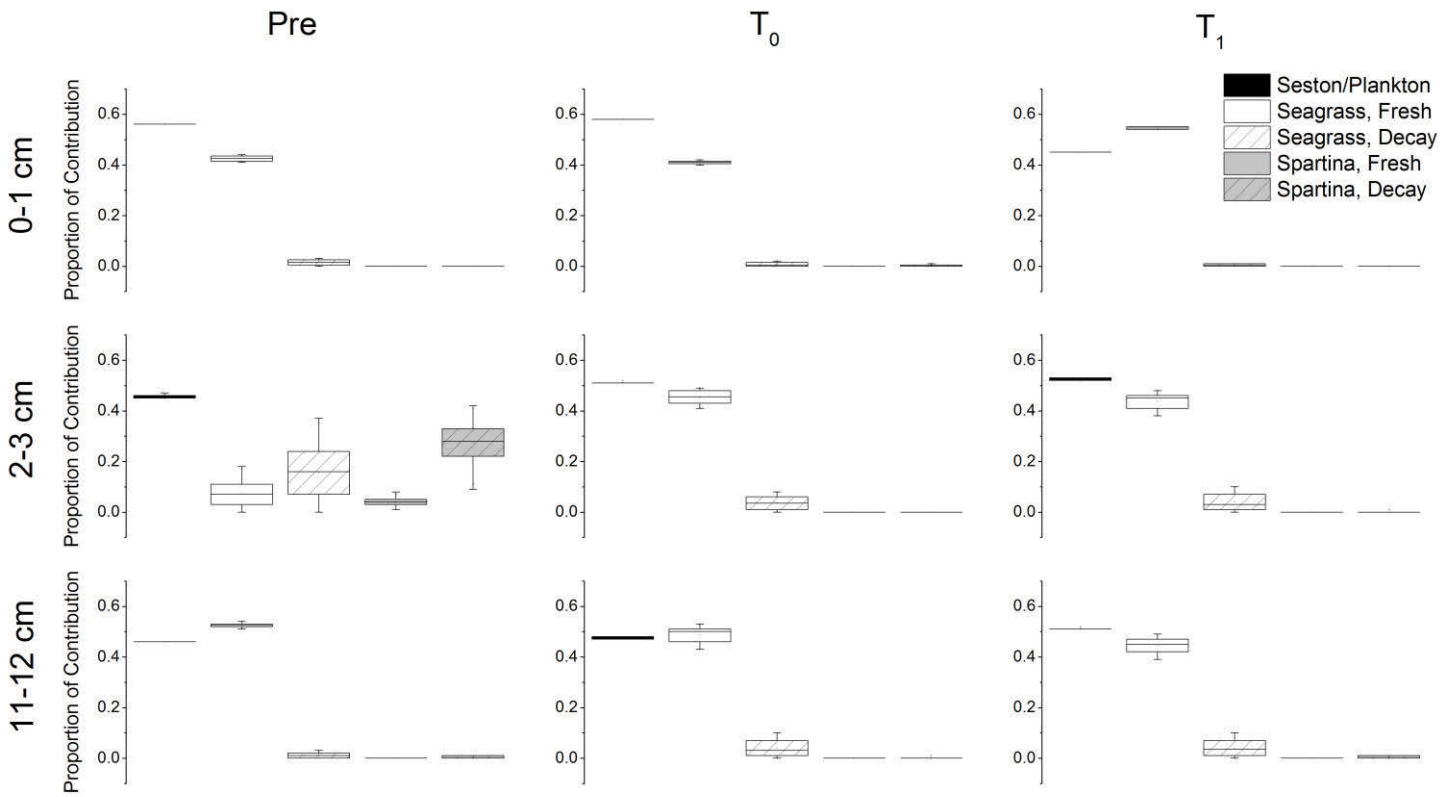


Figure S5.9: Box plots of predicted organic matter sources for sediments from *Thalassia testudinum* kill plots. The predictions are based on stable isotope ($\delta^{13}C$, $\delta^{15}N$) and elemental (C:N ratio) signatures analysed with the IsoSource mixing model. Pre = pre-kill sampling, T_0 = Time 0 sampling, T_1 = Time 1 sampling. Values are based on means.

Synthesis, Conclusion and Outlooks

The refractory characteristics of carbon-containing compounds is one of the main factors that can influence the sequestration and storage of carbon within blue carbon ecosystems. The aim of this thesis was to unravel the processes and factors that influence, and even promote, the preservation of refractory carbon in seagrass meadows, beginning with the refractory carbon in live plants and following it through decomposition to its storage within the sediments. To accomplish this aim, a multi-variable approach was taken, which involved assessing the main and interaction effects of biological, chemical and environmental/physical variables on carbon remineralisation and storage.

In Chapter 1, refractory carbon content of 23 species of seagrass and 4 different tissue types was assessed in a global survey. This study revealed that climatic region, either temperate or tropical, was an important determinant of macromolecular composition and refractory carbon content in seagrass tissues (tropical > temperate). This novel finding was possibly related to the relatively higher labile biomass and production in seagrasses at higher latitudes and increased grazing pressures at lower latitudes. It was also shown that tissue type was a significant driver of refractory carbon content (root > leaf > sheath/stem > rhizome). Interestingly, rhizomes showed less *relative* refractory carbon content due to a high quantity of labile storage products within the tissues despite previous literature showing otherwise. This study also provided new information on the refractory carbon content in sheath/stem tissues. Previously under-reported in the literature, refractory carbon content in sheaths and stems was highly variable across seagrass taxa and was influenced by the size and morphologies of the seagrass, whereby, larger morphologies typically contained higher refractory carbon content.

Chapters 2 and 3 investigated factors that influence the microbial remineralisation of seagrass detritus and refractory carbon in two experiments, one short-term 3-month study and one long-term 2-year study involving *Zostera muelleri*. We discussed how temperature and nutrients affected seagrass remineralisation processes primarily under oxic conditions, but did not affect remineralisation under anoxic conditions (Ainley and Bishop 2015). These results indicate the potential for oxygen availability to constrain the effects of temperature and nutrients to enhance the

remineralisation of seagrass detritus (Ainley and Bishop 2015). Remineralisation of refractory carbon was dependent also on tissue type, environmental conditions and duration of remineralisation. Degradation of refractory carbon was significantly enhanced under (oxygenated) increased temperature. Conversely, labile organic matter was degraded in the rhizome/root detritus over a longer period of time, shown by a significant shift from labile-dominated to refractory-dominated organic matter under anoxic conditions.

Chapters 2 and 3 also captured the distinct succession of seagrass-associated microbial communities on each tissue type throughout the decomposition process. Despite the difference in time-scales and incubation conditions between the two studies, when the two data sets were compared, the bacterial communities colonising the detritus were not significantly different between the stages of decay during the first few months of incubation (Fig. C1). In the 'early' stages of decay (7 days for short-term and 14 days for long-term experiment), the bacterial communities were dominated by *r*-strategists, like Alpha- and Gammaproteobacteria, Bacilli, Clostridia and Bacteroidia, which are typically characterised as fast-colonisers that take advantage of easily-degradable labile compounds (Fig. C2). During the later or 'advanced' stages of decay (84 days for short-term and 168 days for long-term), *K*-strategists became more prevalent, especially facultative or obligate anaerobes (e.g., Deltaproteobacteria, Spirochaetes, Cytophagia, Anaerolineae and Acidimicrobia) due to their ability to degrade more refractory compounds like lignocellulose (Fig. C2).

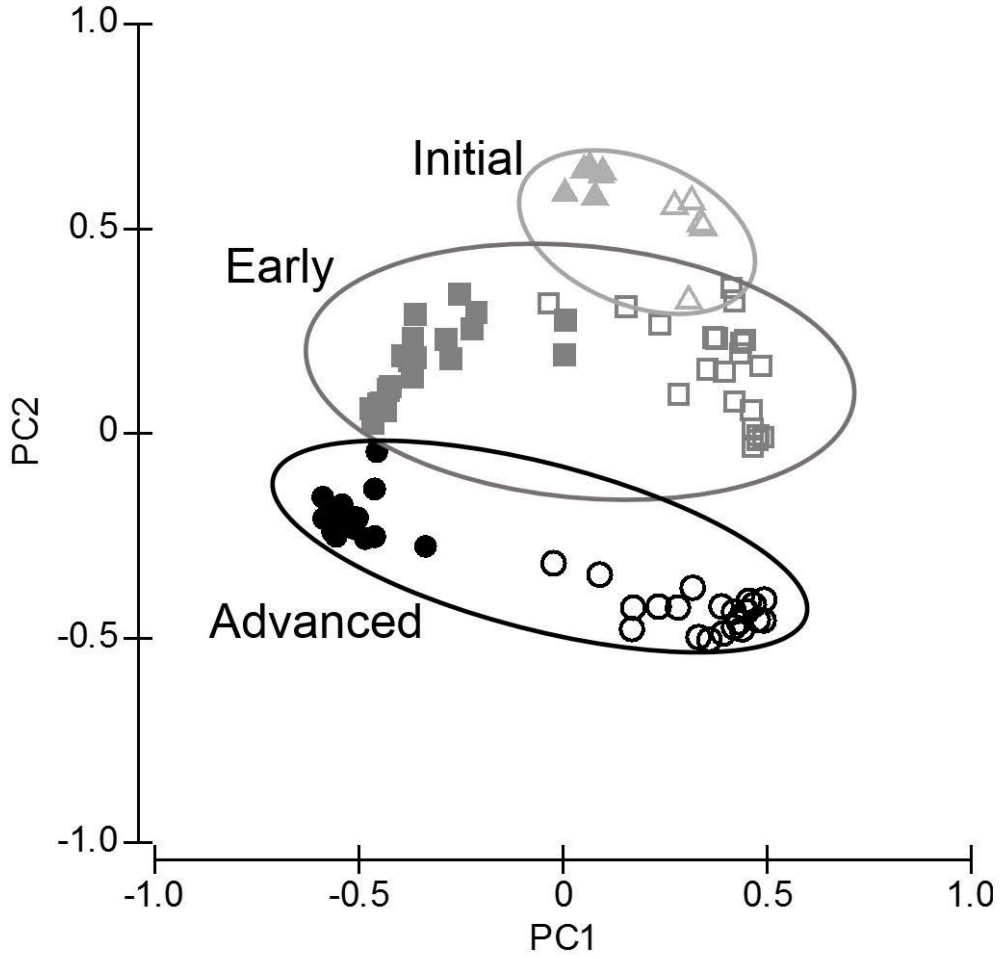


Figure C1: Principal components analysis of the seagrass-associated bacterial communities from the short-term laboratory and long-term field decomposition data sets (Chapters 1 and 2). PC1 = 31.5% of the variation explained by tissue type, and PC2 = 21.3% of the variation explained by stage of decomposition. Leaf-associated communities are represented by the open symbols, and rhizome/root-associated communities are represented by the closed symbols.

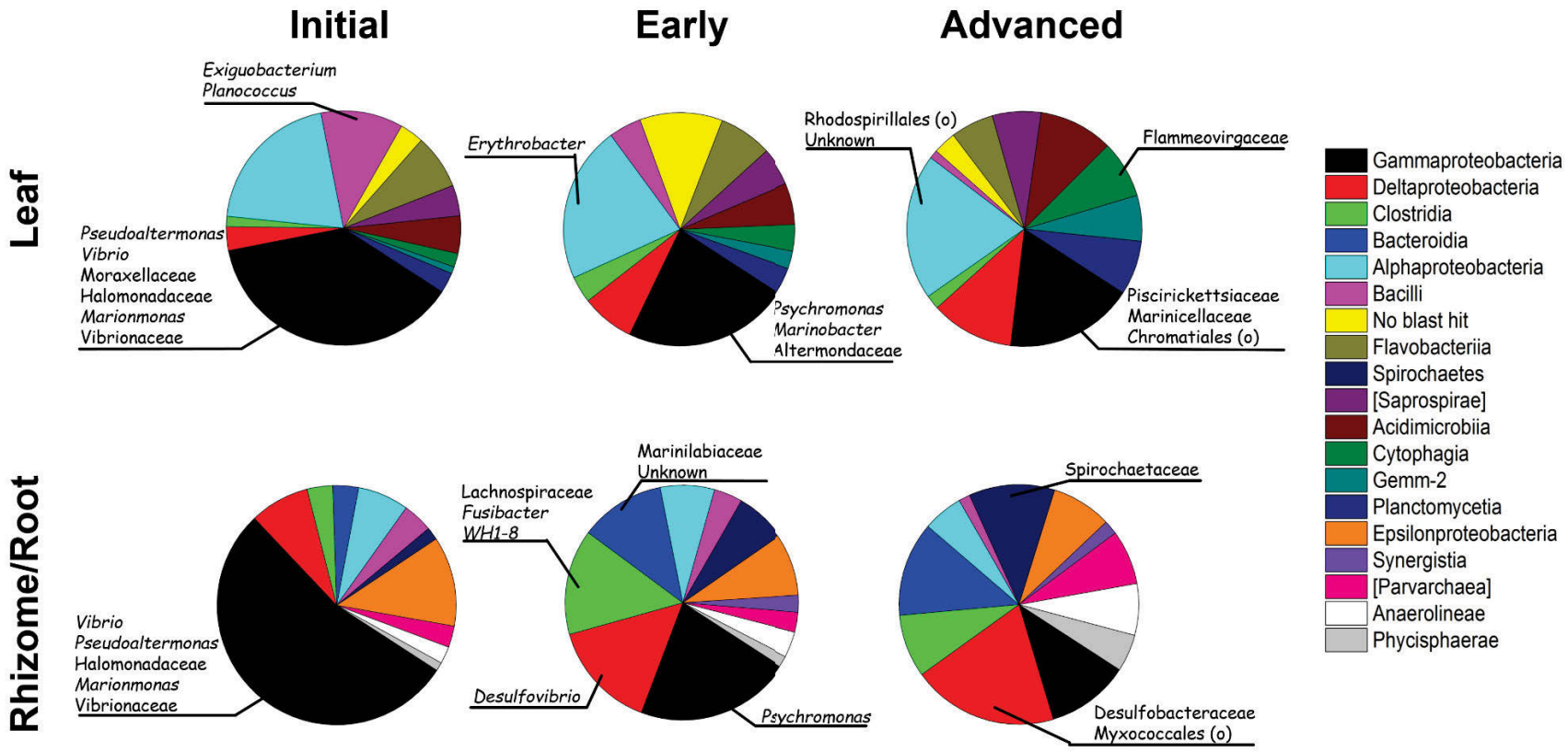


Figure C2: Comparison of the classes driving the differences in bacterial communities during initial, early and late-stage seagrass decomposition for both tissue types. Dominant families and genera in each class are labelled. Both short-term laboratory and long-term field decomposition data sets (Chapters 1 and 2) were combined, and the top 12 contributing classes for each tissue type are shown.

In the last two chapters, the effect of physical disturbance on sediment refractory carbon was investigated. First, it was shown that physical disturbance via simulating the displacement of deep sediments into the water column (e.g., dredging) and the subsequent contact with fresh, labile carbon (e.g., detritus from an algae bloom or senescent seagrass tissue), enhanced microbial remineralisation of millennial refractory carbon. This study was the first to quantify the response known as the 'microbial priming effect' for seagrass sediments, which led to a 2-3 times greater loss in stored carbon than undisturbed sediments. It was also shown that small-scale seagrass habitat loss had a relatively rapid and ecologically significant effect on carbon stocks that varied between meadow type. The smaller seagrass species *Halodule wrightii* experience reductions in organic matter loss in the top 8 cm, while the meadow dominated by the larger *Thalassia testudinum* species lost both organic matter and organic carbon in the top 1 cm. The differential response between the two species was related to their below-ground biomass and structure, which was larger and more stable for *T. testudinum* and thus minimised the loss of sediment carbon. In both cases, the losses were in the form of labile organic matter from the degradation of fresh plant sources.

In the next sections of the Synthesis, Conclusion and Outlooks, the data obtained from the thesis chapters will be critically evaluated and synthesised with the aim to highlight this research's position and novel insights in the field of blue carbon research. Additionally, the limitations of this thesis and the prospects of future research will also be assessed.

Trends in microbial remineralisation of refractory carbon

Climatic region and tissue type were the driving factors that determined the biochemical composition and refractory carbon content of seagrasses globally. Yet, are these factors still important once the process of microbial remineralisation is considered?

Climate or latitude have been shown to play a primary (Irons et al. 1994, Aerts 1997) or secondary (Couteaux et al. 1995, Silver and Miya 2001) role in influencing decay rates for terrestrial detritus. Higher decays rates in lower latitudes were attributed to higher and more consistent temperatures and increased humidity compared to higher

latitudes (Irons et al. 1994, Couteaux et al. 1995). Furthermore, bacterial growth rates typically increase with increasing temperature in coastal environments (White et al. 1991). If these trends found in terrestrial ecosystems parallel the processes occurring in marine ecosystems, we would expect to find that even though tropical seagrasses had high proportions of refractory carbon in their tissues, preservation of this carbon may be reduced as a result of higher microbial remineralisation under higher ambient temperatures. However, when seagrass decay rates from reviewed field decomposition studies were compared across latitudes (Table I2), there was a weak correlation between the two variables and was driven by the high rates for a few data points from latitudes 4-5°S ($R^2 = 0.2375$; Fig. C3). If these four tropical data points were removed, the relationship between latitude and decay rate would be negligible ($R^2 = 0.0043$). The lack of robust observations at low latitudes makes it impossible to predict with certainty whether climatic region is an important factor in understanding microbial remineralisation. There are a few studies have found that elevated temperatures could enhance microbial remineralisation (Godshalk and Wetzel 1978, Pedersen et al. 2011, Ainley and Bishop 2015, Chapter 2) , suggesting future seawater temperature increases caused by global warming will enhance carbon remineralisation. However, these studies are relatively short-term and do not take into account adaptations by microbial communities in response to thermal regimes (Pietikäinen et al. 2005, Hall et al. 2008, Pedersen et al. 2011). Understanding how differences in climate and long-term warming projections will affect refractory carbon remineralisation in blue carbon systems will benefit from incorporating acclimation responses of cold- vs. warm-adapted bacterial community metabolism to changes in predicative models (Hall et al. 2008).

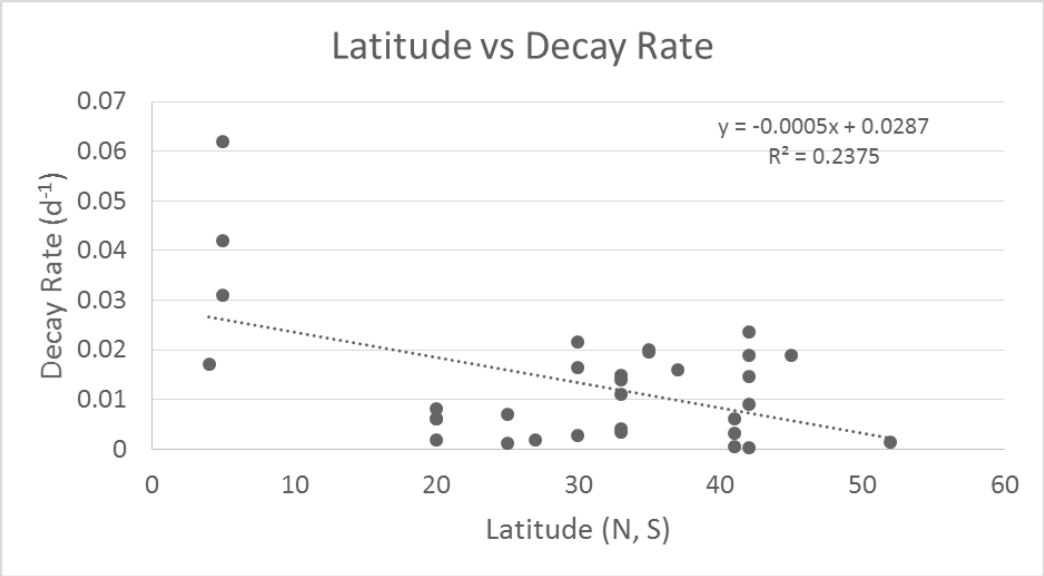


Figure C3: Relationship between latitude and decay rates of seagrass. A linear regression was performed on the decay rates (d^{-1}) from seagrass decomposition studies performed in the field and the latitude of the site rounded to the nearest whole degree.

In addition to temperature differences, temperate and tropical seagrass ecosystems often have different habitat characteristics, such as sediment type, which may influence microbial remineralisation. Experiments that have investigated the effect of sediment type on seagrass decomposition showed no difference between clay/silt or sandy sediments during the *early* stages of anaerobic rhizome and root decay, which were attributed to the low metabolic efficiency of these bacteria (Kenworthy and Thayer 1984). However, there was enhanced decay during the *later* stages of decay for rhizomes and roots after 150 days and 40 days, respectively (Kenworthy and Thayer 1984). The reason for this was not discussed, but could potentially be related to nutrient or OM limitations in the sandy sediment. As bacteria are the primary nutrient cyclers of detritus in tropical carbonate sediments, their metabolism is controlled by fresh detritus inputs, as well as P-availability (Alongi et al. 1993, Erftemeijer and Middelburg 1993, Alongi 1994, McGlathery et al. 1994). It is possible that the late stages of decay in sandy or carbonate sediments can become more nutrient limited and lead to a reduction in microbial remineralisation.

Tissue type was the secondary determinant of refractory carbon content in seagrasses with roots and leaves having the highest refractory carbon content followed

by sheaths and rhizomes. In terrestrial systems, litter quality is one of the primary factors influencing decomposition rates, and refractility is often evaluated as higher C:N and lignin:N ratios (Melillo et al. 1982, Enríquez et al. 1993), and is especially important during the later stages of decay, especially during humus formation (Couteaux et al. 1995, Silver and Miya 2001, Cleveland et al. 2014). A similar index to lignin:N ratios using the thermogravimetric data in Chapter 1 can assess refractility of seagrass tissues. This modified index is based solely on lignocellulose since nitrogen content can only be assessed during pyrolysis in conjunction with mass spectrometry (range of nitrogen pyrolysis = 200-600°C) (López-González et al. 2014). This index can also be used to assess the changes in refractory OM content during decomposition (e.g., Chapter 3; Kristensen 1990). In the proposed R (refractory) index, the proportion of lignocellulose content normalised to the total OM is divided by the proportion of the total OM available in the plant biomass (Table C1; see also Fig. 1.7 for visualisation of proportions). For the fresh tissues in Chapter 1, $R = (TI_3 + TI_4)/ (TI_{total})$, whereby TI_3 is the mass lost between 300-400°C, TI_4 is the mass lost between 400-600°C, TI_{total} is the total OM lost between 180-600°C and TI_3 and TI_4 are represented as proportions of TI_{total}. For decomposed tissues in Chapter 3, there was no distinction between the two labile thermal intervals TI_1 and TI_2, so these intervals were combined as $TI_{\boldsymbol{1}}$. In this case, the refractory lignocellulose components are $TI_{\boldsymbol{2}}$ and $TI_{\boldsymbol{3}}$, so $R = (TI_{\boldsymbol{2}} + TI_{\boldsymbol{3}})/ (TI_{total})$, whereby $TI_{\boldsymbol{2}}$ is the mass lost between 300-400°C, $TI_{\boldsymbol{3}}$ is the mass lost between 400-600°C, TI_{total} is the total OM lost between 180-600°C and TI_2 and $TI_{\boldsymbol{3}}$ are represented as proportions on TI_{total}. R-index calculations for the fresh tissues align with the predictions for refractory carbon content in Chapter 1 (root > leaf > sheath/stem > rhizome), and also quantifies the shifts in refractility of decaying seagrass (Table C1). However, quantification of refractory carbon/OM content is only part of the story. The ultimate preservation of seagrass refractory carbon in seagrass ecosystems will be influenced by their fate after senescence during microbial remineralisation.

Table C1: R (refractory) index calculated from thermogravimetric analysis for fresh tissues from the international study (Chapter 1) and the long-term decomposition study (Chapter 3). R is calculated by dividing the proportion of lignocellulose-associated organic matter by the total OM in a sample (see also Fig. 1.7). Values represent mean ± 1 S.E.M.

Fresh	R	Days of Decay	R, Leaf wrack	R, Rhizome/root
Leaf	1.45 ± 0.07	0	1.45 ± 0	1.60 ± 0
Sheath/Stem	1.34 ± 0.08	14	1.85 ± 0.06	1.36 ± 0.02
Rhizome	1.08 ± 0.05	98	2.00 ± 0.15	1.67 ± 0.07
Root	2.18 ± 0.23	168	1.62 ± 0.10	1.17 ± 0.07
		389	1.77 ± 0.11	1.69 ± 0.08
		519	1.57 ± 0.07	1.58 ± 0.06
		789	1.96 ± 0.04	2.68 ± 0.62

For leaves, herbivory and export of biomass need to be considered when calculating the likelihood of carbon sequestration. Approximately, 6-19%, and at times up to 90%, of the total leaf production can be exported from the meadow due to wave-action (Ochieng and Erftemeijer 1999, Cebrián and Duarte 2001, Mateo et al. 2006), and 5-50% from herbivory (Cebrián et al. 1997). Therefore, much of the carbon that could potentially be sequestered is often removed from the ecosystem prior to burial. Although intertidal leaf decomposition occurs more slowly than their subtidal counterparts (Hemminga and Nieuwenhuize 1991, Machás et al. 2006), wrack collecting on the shoreline is considered a greenhouse gas hotspot due to the lack of photosynthetic production to offset the CO_2 release (Coupland et al. 2007, Lavery et al. 2013b). While there is still hope for a proportion of the wrack to be re-submerged (Oldham et al. 2010), the contributions of reintroduced wrack biomass to blue carbon stocks has not been quantified. Furthermore, the majority of decay studies have shown that leaves decomposing in the meadows under oxic conditions typically lasts less than a year before complete remineralisation (mean = 3.9 yr; median = 1.7 yr; Table I2). In only a handful of studies have leaves persisted for more than 4 years and under conditions that promoted slower decay rates ($k \leq 0.25$), e.g., high refractility, low temperature and low oxygen availability, (Godshalk and Wetzel 1978, Rice and Tenore 1981, Peduzzi and Herndl 1991, Vichkovitten and Holmer 2004, Chapter 3) . However,

these estimates assume constant decay, rather than multiple phases, and thus likely represent an underestimation of the time until full remineralisation.

There is very little information on sheath/stem decomposition dynamics. The exception are the sheaths that contribute ~20% of the carbon in the mattes formed by Mediterranean *Posidonia oceanica* (Pergent et al. 1994). However, we can estimate that sheaths and stems can have the potential to make significant contributions to blue carbon stocks due to (a) their attachment to below-ground biomass that increases their chances of burial (Romero et al. 1992, Pergent et al. 1994, Cebrián et al. 1997), and (b) the high refractory carbon content, especially in morphologically larger species (Chapter 1).

Rhizome and roots have traditionally been viewed as the most important carbon-contributing seagrass tissue types due to their high refractory and predominantly anoxic conditions during decomposition (Mateo et al. 2006). We showed that the refractory carbon content was highest in roots and lowest in rhizomes (Table C1), which was attributed to the high amount of labile carbon stored in rhizome tissues (Chapter 1). However, outside of this thesis, rhizome decay rates have been shown to be consistently slower than root decay rates due to physical structures, e.g., thicker outer epidermal cells, which reduced the amount of labile carbon readily accessible to microbes in the later stages of decomposition (Kenworthy and Thayer 1984, Fourqurean and Schrlau 2003, Vichkovitten and Holmer 2004). Even though rhizomes had the lowest R index, they would likely provide some of the greatest contributions to carbon stocks due to their resistance to microbial remineralisation after the labile compounds are leached or degraded.

Despite some unknowns concerning the effects of climate and sediments on long-term decay dynamics, we can make predictions on how both initial refractory carbon content and decomposition dynamics will affect carbon sequestration in seagrass meadows. In considering C lost to herbivory or export as well as C remaining in the meadow via burial, the contributions of tissue types to carbon sequestration likely follows sheath/stem ~ rhizome > root > leaf. Seagrasses with large morphologies (and refractory carbon) and biomass and production will also likely contribute more to long-term carbon stocks. These conclusions align with a review of the fate of production of four temperate seagrasses, whereby the percent of seagrass production destined for burial (after herbivory, export and one year of oxic decomposition) ranged from 0.15-

7.7% and 0.05-6.7% for sheaths and rhizomes, respectively (Cebrián et al. 1997). Roots and leaves likewise contributed 0.66-2.3% and 0-3.1%, respectively, of their production to burial (Cebrián et al. 1997). In summary, refractory carbon content, low rate of export or herbivory and anoxic conditions promote the sequestration of seagrass carbon. We suggest that the influence of climatic variation and sheath/stem contributions to refractory carbon stocks need better assessments and prioritisation in future research and modelling exercises.

Incorporating microbial responses in seagrass decomposition models

Thus far, microbial responses have played a minor role in assessing seagrass carbon cycling in the context of blue carbon sequestration; however, this thesis has shown that bacterial and archaeal communities, their inferred functions and the factors that affect their metabolism strongly influence seagrass decomposition. In fact, marine microbes have been proposed as the 'gatekeepers' of marine carbon cycling (Arnosti 2011), and likely drive blue carbon sequestration. While the research on microbial dynamics in marine systems is still forthcoming, some of the recent research coming from terrestrial systems in the last 5-10 years may be able to inform the expanding research in blue carbon systems (Six et al. 2006, Schimel and Schaeffer 2012). First, enzymatic functions of the microbes have been considered one of the most important factors influencing carbon remineralisation and biogeochemistry (Zak et al. 2006, Schimel and Schaeffer 2012). For example, enzymatic functions have been shown to be specialised for local litter quality (Freschet et al. 2012) and strongly influence resource acquisition and among-taxa interactions (Allison 2012). A recent review on extracellular enzymes in marine sediments suggested that surface area and functional diversity were important factors for enzyme activity, while temperature and pH were less important due to their relatively small temporal fluctuations (Arnosti et al. 2014). Furthermore, some of the unanswered questions concerning the microbial-carbon sequestration relationships proposed in this thesis (e.g., sediment types, latitudinal variation, etc.) could be answered by further researching the bacterial enzymatic 'toolbox' and their utilisation of these enzymes under a range of conditions (Arnosti 2011, Arnosti et al. 2014).

In addition to enzymatic functions, community composition has been useful for understanding decomposition dynamics. For example, 32% of the variation in respiration rates in soils have been shown to be attributed to bacterial community composition with a positive relationship between species richness and decay (Cleveland et al. 2014), i.e., a more diverse community was able to degrade litter more quickly than less diverse communities. The same study showed that beta diversity (among-community variation) decreased during advanced stages of decay because the taxa became more specialised in the breakdown of cellulose and lignin (Cleveland et al. 2014). We saw a similar convergence of bacterial communities during the long-term decomposition study between tissue types and between detritus and bulk sediment communities (Chapter 3). These shifts in bacterial communities were likely related to increasingly similar R indices (substrate quality; R = 3.7-5.1 for 0-3 cm sediments at Fagans Bay) and environments (anoxic, sulphate-dominated metabolism) between the buried litter and the bulk sediments.

One factor that seemed particularly important for seagrass carbon remineralisation, but often overlooked in terrestrial systems, was oxygen availability and the potential protection anoxic conditions provide from enhanced remineralisation under increased temperature and exogenous nutrient inputs (Chapters 2 and 3). While we did not place the leaves in anoxic conditions in the short-term laboratory experiment, we can infer from previous studies that lack of oxygen seems to be a stronger limiting factor in microbial metabolism than temperature and nutrient availability for both tissue types (Godshalk and Wetzel 1978, Pellikaan 1984, Wilson et al. 1986a, Fourqurean and Schrlau 2003). More research needs to be done to tease apart what aspects of anaerobic remineralisation hinder refractory carbon degradation and aids in its preservation. Is remineralisation simply hindered by reduced energy transfer efficiency (Kenworthy and Thayer 1984)? Or does refractory carbon remineralisation depend on the presence of certain facultative or obligate anaerobic bacteria and their enzymes (Arnosti 2011) or the physical structure and protection provided by lignocellulose (Benner et al. 1984)?

Refractory compounds that contain aromatic components (lignin, polyphenolics, amino acids, etc.) and eventually accumulate in seagrass ecosystems can come from several sources other than seagrass, such as mangrove, saltmarsh and terrestrial detritus and dissolve organic matter (Bianchi 2011, Nebbioso and Piccolo 2013). Do the microbes and their enzymes that typically breakdown this allochthonous refractory

carbon (e.g., terrestrial microbes) also perform the same functions in marine systems (i.e., facultative microorganisms)? It is known that terrestrial pathogens can be transferred to marine ecosystems via runoff, pollution and sedimentation (Harvell et al. 1999), but do refractory carbon-degrading microorganisms also persist in the transition between terrestrial or freshwater environments to marine environments (Richards et al. 2012)? And, more importantly, can they function in anoxic sediments? There is evidence that anaerobic bacteria have the capacity to degrade aromatics, but most of these studies have been performed in the context of the remediation of aromatic pollutants via several mechanisms, such as methanogenic fermentation, benzoyl-CoA reductase and decarboxylases (Balba and Evans 1977, Heider and Fuchs 1997, Fuchs et al. 2011). We were limited in this thesis by describing aromatic-degrading function of bacteria as a proxy of taxa present (*r*- v *K*-strategists) or predicted metabolic KEGG pathways of the bacteria in Chapter 2. While we could only infer function with these analyses, the aerobic and anaerobic aromatic degradation pathways were present in the KEGG xenobiotic metabolism pathways of taxa present under these conditions. As future research on this topic is pursued, it will be important to explicitly quantify the metabolic pathways of plant aromatic degradation and then to extrapolate what it implies for blue carbon sequestration.

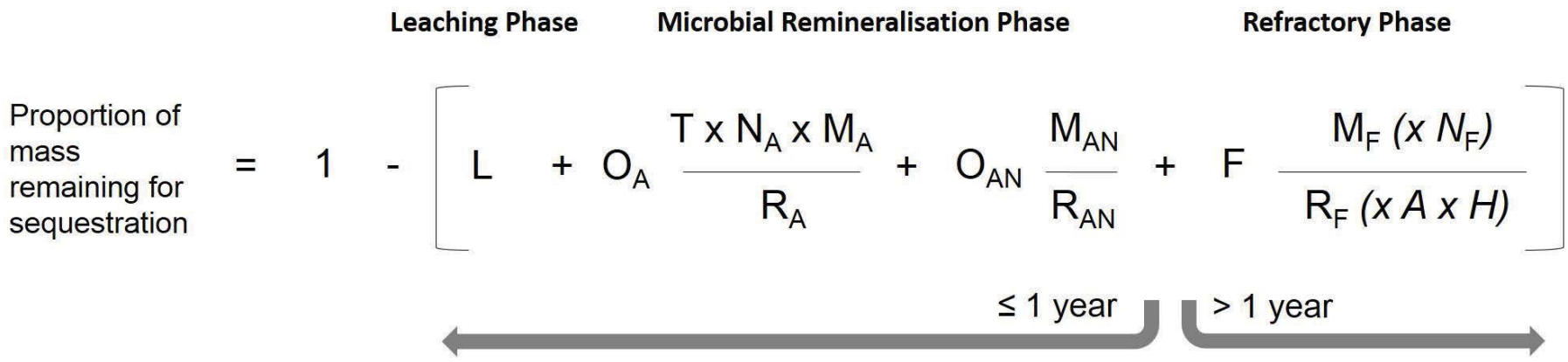
To summarise the findings of Chapter 1-3, we propose a modified conceptual decomposition model for seagrasses (Fig. C4). This model combines the traditional 3-phase decomposition model (leaching, microbial remineralisation and refractory phases) with the 5-factor seagrass decomposition model (temperature, nutrients, oxygen, particle size, refractility; Godshalk and Wetzel 1978). Like the Godshalk and Wetzel (1978) model, the parameters in this model are indices and proportions to allow for multiple response variables to be used to predict the proportion of mass remaining for burial and sequestration, and thus, the values for now represent an overall effect (increase or decrease) on seagrass decomposition. However, the new model does include several modifications. First, the microbial remineralisation phase is separated into oxic and anoxic components to reflect the effects of temperature (T) and nutrients (N) in enhancing remineralisation in the oxic phase, as well as the different microbial communities/functions and refractory carbon content under these two conditions. T and N are indices calculated from the treatment effect of an appropriate response variable relative to the control (Response of Treatment)/(Response of Control), whereby larger

values indicate enhanced mass loss. R is represented by the refractory index for the decomposing detritus explained above. The factor M is also included in both remineralisation and refractory phases to account for the microbial functional groups present that can degrade lignocellulose. Since M represents functional groups, this index could be calculated from enzymatic degradation of lignocellulose (e.g., Michaelis–Menten kinetics, V_{max}, K_m; Allison 2012), and will likely have a maximum value so that no more than 100% of the mass is able to be degraded. The M index may also be represented from data produced from metagenomics or metatranscriptomics analyses. However, as these datasets for lignocellulose degradation are rare, more research needs to be done to parameterise M in the model. Lastly, this model excludes the particle size (S) factor in the Godshalk and Wetzel (1978) model since this factor was not assessed in this thesis.

There are several assumptions and constraints of this conceptual model:

1) Leachate is assumed to be completely consumed by bacteria (Vichkovitten and Holmer 2004), so the leaching phase (L) is simply the proportion of mass lost for that phase,
2) The parameters O_A, O_{AN} and F are the proportions of time spent in oxic remineralisation, anoxic remineralisation and refractory phases, respectively. The sum of these is equal to 1,
3) The M parameter has a maximum value (to be determined),
4) The leaching and the beginning of the microbial phases occur in approximately a year or less (i.e., 50% of mass decaying aerobically typically occurs in less than a year, mean = 0.59 y, Table I2),
5) The refractory phase, if present, begins after at least two years of decay (i.e., 50% of mass decaying anaerobically lasts for ~2.5 years: mean = 2.4 y, Table I2),
6) The refractory phase, F, occurs only in anoxic conditions and assumes no physical disturbance,
7) The refractory parameter, R, increases as decay progresses, i.e., the more labile compounds are being consumed over time,

8) The microbial degradation of lignocellulose, M, decreases when shifting from oxic to anoxic conditions and from the remineralisation phase to refractory phase, and
9) The ranges of mass loss for each phase have been estimated from the following sources: Romero et al. 1992, Vichkovitten and Holmer 2004, Chapter 2, Chapter 3.



L = proportion of mass leached; assumes complete utilisation by microbes

O_A = proportion of time spent under microbial remineralisation under oxic conditions

O_{AN} = proportion of time spent under microbial remineralisation under anoxic conditions

F = proportion of time spent under microbial remineralisation under the refractory phase; assumes 100% anoxic conditions

M = lignocellulose-degrading microbes (functional group)

A = adsorption to minerals

H = humification/geopolymerisation

Indices:

T = temperature index = (T_{trmt} / T_{ctrl})

N = nutrient index = (N_{trmt} / N_{ctrl})

T, N > 1 is enhancement of remineralisation

T, N < 1 is reduction of remineralisation

R = refractory index (TGA)

M = TBD index ~ (M_{trmt} / M_{ctrl})

A & H = Indices TBD

Constraints:

$O_A + O_{AN} + F = 1$

T, N > 0

$R_A \leq R_{AN} \leq R_F$

$M_A \geq M_{AN} \geq M_F$

Ranges of mass loss:

Leaching = 0 - 0.5

Oxic Remin. = 0.2 – 0.6

Anoxic Remin. = 0.2 – 0.6

Refractory = 0 - 0.5

Figure C4: Modified conceptual model for seagrass decomposition based on the new insights derived from this thesis.

There are several other factors that may be considered for future modifications of this model. Sediment type may be influential to seagrass decomposition (Kenworthy and Thayer 1984, Kristensen and Hansen 1995, Neubauer et al. 2004), due to differences in oxygen diffusion and nutrient cycling conditions among sediment types (Revsbech et al. 1980, Alongi 1994, McGlathery et al. 1994, Rasheed et al. 2004). Additionally, the inclusion of a refractory phase is new for seagrass decomposition, so the factors influencing this stage still needs additional assessment. By the time seagrass material reaches this advanced stage of decay, it is possible that the factors affecting sediment carbon-cycling like mineral adsorption (A) and humification (H) would also promote the preservation of seagrass carbon (Burdige 2007). Exogenous nitrogen (N_F) availability has been hypothesised to enhance terrestrial litter decay after several years (Melillo et al. 1989). In Chapter 3, we also showed that N inputs can degrade in sediments over time-scales that likely represent decades to centuries along with organic carbon. However, the marine sediment refractory carbon-nitrogen dynamics (A, H, N_F) still need to be assessed for this model.

Perspectives on refractory carbon loss after disturbance

Our aims in Chapters 4 and 5 were to inform the sparse but growing field of research concerning the cause and effect relationship between disturbance and sediment carbon preservation in blue carbon ecosystems. The response to a small-scale seagrass die-off in Chapter 5 (50-65% loss of OM in the top 8 cm for *H. wrightii* and 20-50% loss of C_{org} in the top 1 cm of sediments for *T. testudinum*) was much smaller than the predicted responses of 100% C_{org} loss in the top 1 m of sediments (Fourqurean et al. 2012, Pendleton et al. 2012). These disparate results are a part of a growing set of data that not only indicate that the previous predictions may be overestimating the effects of carbon remineralisation after seagrass loss, but are also providing insight into the factors that may be driving these variations. Based on the results of this thesis and recent literature (Macreadie et al. 2013, Macreadie et al. 2014b, Marbà et al. 2014, Macreadie et al. 2015, Marbà et al. 2015, Apichanangkool et al. in review), we consider the following variables important to the magnitude of carbon loss after seagrass loss or disturbance and deserve further investigation: (1) Size and type of disturbance, (2) Sediment and hydrology dynamics and (3) Seagrass morphology, structure and

decomposition. First, much of the conclusions in the literature thus far suggest that the effect of disturbance size and type on C_{org} losses is still quite unclear and highly variable. Macreadie et al. (2014b) showed that small-scale losses simulated by several types of disturbances (above-ground loss and whole plants physically removed) led to no change in C_{org} stocks over the course of two years. Conversely, we measured a relative loss in C_{org} in *Thalassia testudinum* and *Halodule wrightii* meadows comparable to long-term large-scale of *P. oceanica* and *P. australis* (Marbà et al. 2014, Macreadie et al. 2015). The variation in the response to small-scale losses may be due to greater susceptibility of small plots to other inputs of carbon, such as the 'leakage' of carbon from adjacent sediments or allochthonous inputs like phytoplankton, seston or benthic microalgae (Macreadie et al. 2014b).

This brings us to the second factor concerning the effects of sedimentation and hydrology. As sediment inputs and hydrodynamics have been hypothesised to influence C_{org} burial in blue carbon systems (Duarte et al. 2013b, Lavery et al. 2013a). For example, extremely impacted sites, like Fagans Bay studied in this thesis, high sedimentation would mask the differences typically expected in C_{org} stocks between seagrass meadows (higher) and unvegetated areas (lower) due to enhanced particle trapping by the meadow. In these cases, seagrass habitat loss would likely not show a loss in C_{org} stocks. It would be interesting to investigate whether or not low-organics, high carbonate sediments respond differently to seagrass loss than those with high terrigenous inputs. Additionally, the quality of C_{org} lost will be important when assessing the effect of disturbance on C_{org}. We found that the OM lost in Chapter 5 was mostly labile OM, while the refractory OM was maintained or relatively increased. To this end, we need to ask: to what degree is refractory C_{org} protected from remineralisation? Does adsorption or humification provide protection of C_{org} regardless of disturbance?

Lastly, we found that seagrass morphology, structure and decomposition affected the response of C_{org} to seagrass loss, whereby larger species with large below-ground structures seemed to buffer C_{org} losses, possible due to increased sediment stabilisation (Chapter 5). Furthermore, we hypothesised that the decomposition of freshly produced detritus as result of seagrass die-off counteracted some of the C_{org} loss. How long will this fresh carbon input last and will it become a component of refractory C_{org} in time? Furthermore, this input of fresh labile carbon could potentially produce a

localised, subsurface priming effect via enhanced microbial remineralisation and respiration of the fresh detrital carbon. Yet, this response may have gone undetected since we used a long-term sampling scheme (months vs. 1 yr; Chapters 4 and 5).

The small-scale, short-term study in Chapter 4 (microbial priming experiment) served as a complement to the larger-scale, long-term study in Chapter 5. The results therein suggested that disturbance in an environment that has high inputs of labile organic carbon (LOC) may stimulate the loss of centuries-old C_{org} over a period of a few months. However, the fact that the LOC treatments that induced a microbial priming response was short-lived and that the majority of the LOC (seagrass) biomass remained at the end of the experiment may mean that in the long-term, the priming effect may be a minor source of refractory C_{org} loss and is potentially only limited to the leaching phase of detrital decay. However, much more research on the priming effect needs to be done to resolve the spatial and temporal impacts of priming in blue carbon habitats. For example, does the microbial priming effect occur seasonally coinciding with micro- or macroalgae blooms (White et al. 1991, Hansen and Blackburn 1992, Lignell et al. 1993, Danovaro et al. 1994, Hardison et al. 2010) or annual seagrass leaf senescence events (Kenworthy and Thayer 1984)? Will the microbial priming effect responses recorded in Chapter 4 be similar in areas where there are multiple or continuous disturbances occur, such as large dredging projects or meadows at common boat anchoring or boat ramp sites? In other words, does priming continuously occur if disturbance rates are constant, or is there a maximum priming response for sediment refractory organic carbon (ROC)? Additionally, we only showed the priming effect in response to LOC additions of two very different sources, structurally and nutritionally. Will macroalgae have a similar priming effect to seagrass since it is physically large like seagrasses or will the higher nutrients in macroalgae give a larger priming response? Lastly, will priming occur with the addition of inorganic nutrients to sediments like predicted in previous reviews (Kuzyakov et al. 2000, Guenet et al. 2010). Some evidence suggests that fertilisation will enhance microbial remineralisation of sediment C_{org} (López et al. 1998), and may have a particularly large effect in nitrogen- or phosphorus-limited areas (Sundareshwar et al. 2003).

Concluding remarks and research outlooks

The holistic approach taken in this thesis provided us some insight into the processes and factors that affect refractory carbon in seagrass meadows, from its production in seagrass tissues to its persistence (or remineralisation) during decomposition to its preservation in sediments and the mechanisms that provoke further remineralisation (Fig. C5). We showed that while refractory carbon content in seagrass tissues will support carbon sequestration, the ultimate fate during and after decomposition is a stronger influence on the refractory carbon preservation. In summary, anoxic conditions and structural complexity of the tissues promote refractory carbon preservation and are likely strongly dependent on the functions of the microbial communities present. Warming and the addition of labile organic carbon and nutrients only enhanced decay in the short-term and under oxic conditions, while physical disturbance and habitat loss may cause losses of sediment refractory carbon over the course of months to years depending on the type of disturbance. In light of these results, we now have more questions and avenues of research to pursue in the future.

As we have mentioned above, there is much more to understand about the functional microbial groups and their enzymatic remineralisation of refractory carbon in blue carbon ecosystems, and in particular the possibility of anaerobic remineralisation of aromatics by bacteria. Methodologies such an enzymatic assays and genomics (metagenomics and metatranscriptomics), would advance our understanding by providing direct quantification of the enzymatic pathways bacteria use to degrade aromatic refractory carbon. We also need to tease apart the factors that affect carbon remineralisation during the refractory phase of decomposition (e.g., nutrient availability, adsorption, humification), and discern if these factors hinder further remineralisation during priming or disturbance events.

Our knowledge on the roles of bacteria in marine carbon cycling and blue carbon sequestration is still growing. Yet, while eukaryotic microbes like fungi and protists have been known to provide important organic cycling functions in marine and coastal systems (Hyde et al. 1998, Raghukumar 2004, Richards et al. 2012), our empirical knowledge on their diversity, communities and functions in these systems is comparatively much less than what is known for prokaryote microorganisms. In terrestrial systems, fungi are the primary decomposers of aromatics and lignin. There is evidence that fungi are abundant and diverse on seagrass tissues and have the capability

to degrade aromatic chemicals *in vitro* (Panno et al. 2013), but this has yet to be shown to happen with plant-based lignocellulose. In addition to fungi, saprobic protists like thraustochytrids and labyrinthulids are also important degraders of seagrass detritus (Sathe and Raghukumar 1991). Thraustrochytrids can produce a variety of enzymes to degrade lipids, starch and proteins (Bongiorni et al. 2005, Taoka et al. 2009), though carbohydrate and cellulose degradation can be poor or highly varied across species and conditions (Taoka et al. 2009, Nagano et al. 2011). Labyrinthulid degradation of cellulose or other molecules has only been assessed qualitatively (Muehlstein 1992, Bremer 1995), but not directly quantified. Since eukaryotic microorganisms may be able to degrade a diversity of compounds including lignocellulose, blue carbon research would benefit from understanding their role in decomposition under various conditions (oxygen availability, temperature) and seagrass substrates, in addition to the potential for fungal competition or co-metabolism interactions with bacteria.

The final avenue of future research we believe will be important to the future of blue carbon research is the idea of carbon 'up-cycling'. Bacterial degradation of organic matter in itself has been shown to promote refractory organic matter accumulation in marine environments. During the degradation of labile organic matter (carbon-, nitrogen- and phosphorus-containing compounds), bacteria convert or 'up-cycle' the molecules into more refractory and highly diverse metabolites (Rice and Hanson 1984, Gächter and Meyer 1993, Ogawa et al. 2001, Lechtenfeld et al. 2015). This up-cycling of carbon has previously been shown for bacterial utilisation of seagrass exudates (Kaldy et al. 2006), and potentially detected in Chapter 5 with the increased microbial biomass in seagrass sediments coinciding with increasing detrital stable isotope signatures. Not only can the transformed refractory organic matter persist for more than a year (Ogawa et al. 2001), but microbial biomass can account for a quarter of the carbon and half of the nitrogen (Kaiser and Benner 2008) making them a significant part of organic matter accumulation in the ocean and likewise have large implications on carbon sequestration models. In fact, they may be the key to refractory carbon accumulation in ocean (Lechtenfeld et al. 2015) and provide the link between seagrass refractory carbon accumulation during decomposition (years to decades) and the storage and preservation of refractory carbon in sediments (centuries to millennia).

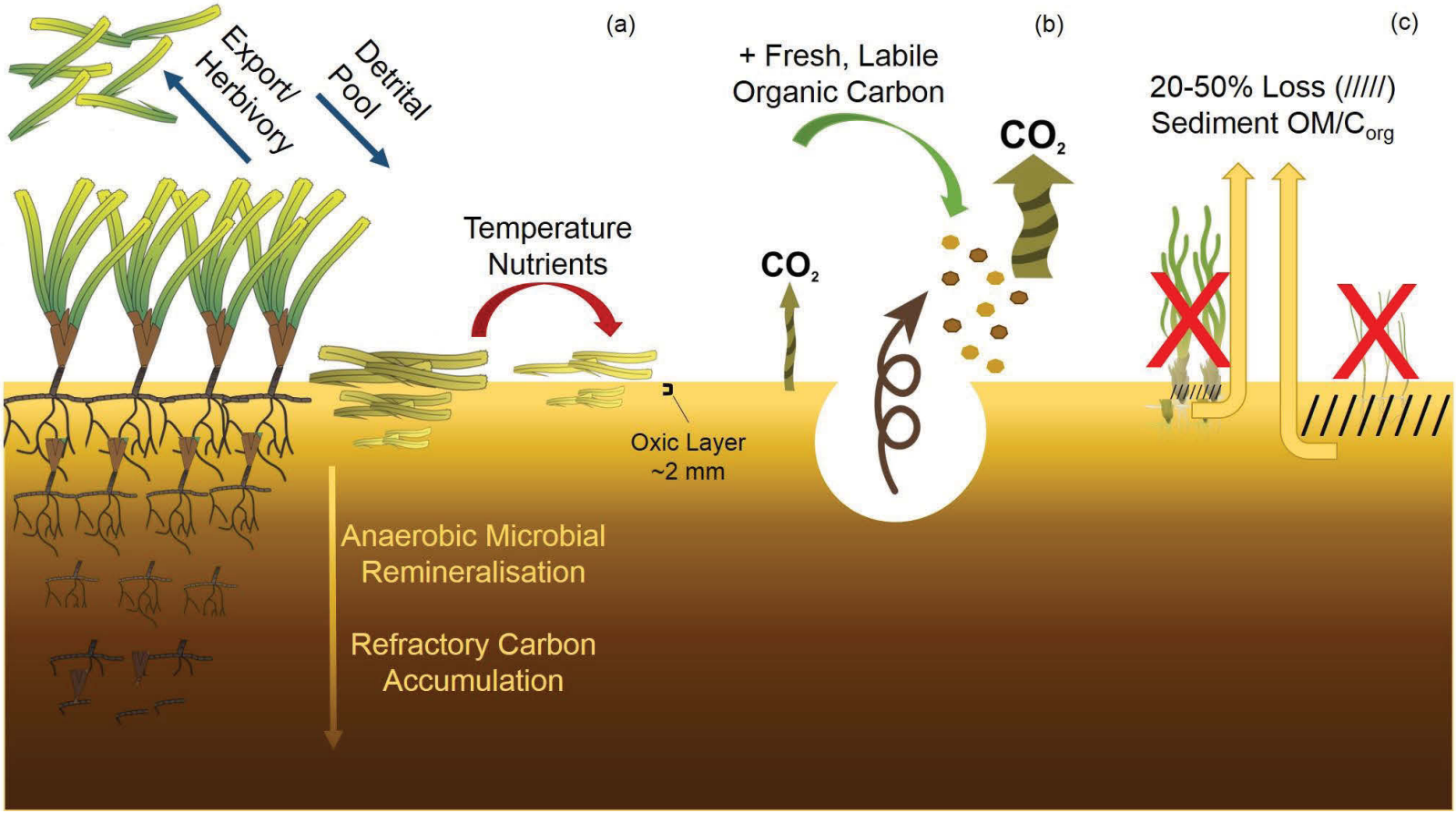


Figure C5: Conceptual design illustrating the processes affecting refractory carbon (increasingly darker colour) accumulation and remineralisation with (a) decomposition, (b) the microbial priming effect and (c) habitat loss. Symbols used here are courtesy of the Integration and Application Network, University of Maryland Center for Environmental Science (ian.umces.edu/symbols/).

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Appendix

Appendix Table 1: Review of literature that reports refractory organic matter as a structural carbohydrates and fibre content for above-ground and below-ground seagrass tissues. All data are reported as % dry weight. NDF = neutral detergent fibre (hemicellulose + cellulose + lignin), ADF = acid detergent fibre (cellulose + lignin). Data were compiled for Chapter 1.

				Structural Carbohydrates			Fibre		
Tissue	Temperate/ Tropical	Family	Species	Cellulose	Hemi-cellulose	Lignin	NDF	ADF	Reference
Leaf	Temperate	Cymodoceaceae	*Amphibolis antarctica*				43.50		1
	Temperate	Cymodoceaceae	*Amphibolis griffithii*				47.80		1
	Temperate	Cymodoceaceae	*Cymodocea nodosa*				51.00		2[e]
	Temperate	Cymodoceaceae	*Cymodocea nodosa*				50.00		2[e]
	Temperate	Cymodoceaceae	*Cymodocea nodosa*				46.00		2[e]
	Temperate	Cymodoceaceae	*Cymodocea nodosa*				51.00		2[e]
	Temperate	Cymodoceaceae	*Syringodium isoetifolium*				38.10		1
	Temperate	Cymodoceaceae	*Thalassodendron pachyrhizum*				49.40		1
	Temperate	Hydrocharitaceae	*Halophila ovalis*				43.90		1
	Temperate	Posidoniaceae	*Posidonia australis*	20.20	11.70	14.90[a]	46.80	35.10	3
	Temperate	Posidoniaceae	*Posidonia australis*				57.20		1
	Temperate	Posidoniaceae	*Posidonia coriacea*				58.40		1
	Temperate	Posidoniaceae	*Posidonia oceanica*	31.40	25.70	24.70			4
	Temperate	Posidoniaceae	*Posidonia sinuosa*				59.50		1
	Temperate	Zosteraceae	*Zostera marina*	19.93					5[e]
	Temperate	Zosteraceae	*Zostera marina*				52.60		6

Tissue	Temperate/ Tropical	Family	Species	Structural Carbohydrates			Fibre		Reference
				Cellulose	Hemi-cellulose	Lignin	NDF	ADF	
	Temperate	Zosteraceae	*Zostera marina*	12.20	13.80	4.30[a]			7
	Temperate	Zosteraceae	*Zostera muelleri*				44.00		8[e]
	Temperate	Zosteraceae	*Zostera nigricaulis*				55.20		1
	Temperate	Zosteraceae	*Zostera tasmanica*			4.90			9
Temperate Avg ± 1 S.E.M				*20.93 ± 3.95*	*17.07 ± 4.36*	*12.20 ± 4.82*	*49.65 ± 1.49*	*35.10 ± 0*	
	Tropical	Cymodoceaceae	*Cymodocea rotundata*	13.90[b]	17.80[c]	19.00[a]	50.70	32.90	10
	Tropical	Cymodoceaceae	*Cymodocea rotundata*				25.20		11[f]
	Tropical	Cymodoceaceae	*Cymodocea serrulata*	13.90[b]	17.00[c]	15.30[a]	46.20	29.20	10
	Tropical	Cymodoceaceae	*Cymodocea serrulata*				23.93		11[f]
	Tropical	Cymodoceaceae	*Cymodocea serrulata*				2.07		12[ef]
	Tropical	Cymodoceaceae	*Halodule pinifolia*				16.50		11[f]
	Tropical	Cymodoceaceae	*Halodule pinifolia*				4.17		12[ef]
	Tropical	Cymodoceaceae	*Halodule uninervis*	13.70[b]	15.60[c]	18.90[a]	48.20	32.60	10
	Tropical	Cymodoceaceae	*Halodule uninervis*			20.16	52.50		13
	Tropical	Cymodoceaceae	*Halodule wrightii*	16.15[b]	9.50[c]	1.85[ad]	27.50d	18.00[d]	14
	Tropical	Cymodoceaceae	*Syringodium filiforme*	13.95[b]	8.00[c]	2.05[ad]	24.00d	16.00[d]	14
	Tropical	Cymodoceaceae	*Syringodium isoetifolium*	16.50[b]	10.30[c]	10.20[a]	37.00	26.70	10
	Tropical	Cymodoceaceae	*Syringodium isoetifolium*				21.40		11[f]
	Tropical	Cymodoceaceae	*Syringodium isoetifolium*				4.55		12[ef]
	Tropical	Hydrocharitaceae	*Enhalus acoroides*				20.30		11[f]

Tissue	Temperate/ Tropical	Family	Species	Structural Carbohydrates			Fibre		Reference
				Cellulose	Hemi-cellulose	Lignin	NDF	ADF	
	Tropical	Hydrocharitaceae	*Halophila engelmanni*	12.73		1.00			15e
	Tropical	Hydrocharitaceae	*Halophila minor*	9.20[b]	11.20[c]	11.50[a]	31.90	20.70	10
	Tropical	Hydrocharitaceae	*Halophila ovalis*	11.10[b]	10.40[c]	10.80[a]	32.30	21.90	10
	Tropical	Hydrocharitaceae	*Halophila ovalis*			16.53	51.14		13
	Tropical	Hydrocharitaceae	*Halophila ovalis*				4.42		12[ef]
	Tropical	Hydrocharitaceae	*Halophila spinulosa*	15.80[b]	8.10[c]	11.20[a]	35.10	27.00	10
	Tropical	Hydrocharitaceae	*Halophila spinulosa*			13.99	52.81		13
	Tropical	Hydrocharitaceae	*Thalassia hemprichii*				16.76		11[f]
	Tropical	Hydrocharitaceae	*Thalassia testudinum*	26.53[b]	5.22[c]	5.92[a]	37.67	32.46	16
	Tropical	Hydrocharitaceae	*Thalassia testudinum*	24.50[b]	8.10[c]	2.30[a]	34.90	26.80	17
	Tropical	Hydrocharitaceae	*Thalassia testudinum*	18.15[b]	10.00[c]	1.85[ad]	30.00[d]	20.00[d]	14
	Tropical	Hydrocharitaceae	*Thalassia testudinum*	17.50	19.10	9.10[a]			18
	Tropical	Zosteraceae	*Zostera muelleri*	12.60[b]	14.30[c]	15.40[a]	42.30	28.00	10
	Tropical	Zosteraceae	*Zostera muelleri*			18.37	48.43		13
Tropical Avg ± 1 S.E.M				*15.75 ± 1.20*	*11.76 ± 1.14*	*10.81 ± 1.52*	*30.44 ± 3.05*	*25.56 ± 1.58*	
Total Leaf Avg ± 1 S.E.M				*16.84 ± 1.29*	*12.70 ± 1.24*	*11.05 ± 1.46*	*37.59 ± 2.44*	*26.24 ± 1.62*	
Leaf + Stem	Tropical	Cymodoceaceae	*Cymodocea serrulata*	27.89[b]	21.60[c]	6.99[a]	56.48	34.88	19
	Tropical	Cymodoceaceae	*Syringodium isoetifolium*				40.07	42.19	19

Tissue	Temperate/ Tropical	Family	Species	Structural Carbohydrates			Fibre		Reference
				Cellulose	Hemi-cellulose	Lignin	NDF	ADF	
	Tropical	Hydrocharitaceae	*Halophila ovalis*	20.42[b]	19.04[c]	4.13[a]	43.59	24.55	19
	Tropical	Hydrocharitaceae	*Halophila spinulosa*	28.70[b]	13.89[c]	4.48[a]	47.07	33.18	19
Total Leaf + Stem Avg ± 1 S.E.M				*25.67 ± 2.64*	*18.18 ± 2.27*	*5.20 ± 0.90*	*46.80 ± 3.53*	*33.70 ± 3.62*	
Non-photosynthetic AG	Temperate	Posidoniaceae	*Posidonia oceanica*	38.00	21.00	27.00			20
	Temperate	Zosteraceae	*Zostera tasmanica*			2.10			9
	Temperate	Zosteraceae	*Zostera tasmanica*			5.40			9
	Tropical	Hydrocharitaceae	*Halophila engelmanni*	23.63		0.70			15[e]
Total Non-photosynthetic Avg ± 1 S.E.M				*30.82 ± 7.18*	*21.00 ± 0*	*8.80 ± 6.15*	*N/A*	*N/A*	
Total AG Avg ± 1 S.E.M				*19.12 ± 1.49*	*13.87 ± 1.17*	*10.17 ± 1.37*	*38.37 ± 2.28*	*27.90 ± 1.62*	
Rhizome	Temperate	Zosteraceae	*Zostera marina*	18.30	28.90	3.50[a]			7
	Temperate	Zosteraceae	*Zostera tasmanica*			5.40			9
Temperate Avg ± 1 S.E.M				*18.30 ± 0*	*28.90 ± 0*	*4.45 ± 0.95*	*N/A*	*N/A*	

Tissue	Temperate/ Tropical	Family	Species	Structural Carbohydrates			Fibre		Reference
				Cellulose	Hemi-cellulose	Lignin	NDF	ADF	
	Tropical	Cymodoceaceae	*Cymodocea serrulata*				3.22		12[ef]
	Tropical	Cymodoceaceae	*Halodule pinifolia*				3.92		12[ef]
	Tropical	Cymodoceaceae	*Halodule wrightii*	12.70[b]	11.00[c]	1.80[ad]	25.50[d]	14.50[d]	14
	Tropical	Cymodoceaceae	*Syringodium filiforme*	17.85[b]	3.50[c]	1.65[ad]	23.00[d]	19.50[d]	14
	Tropical	Cymodoceaceae	*Syringodium isoetifolium*				3.93		12[ef]
	Tropical	Hydrocharitaceae	*Halophila engelmanni*	22.67		0.47			15[e]
	Tropical	Hydrocharitaceae	*Halophila ovalis*				4.97		12[ef]
	Tropical	Hydrocharitaceae	*Thalassia testudinum*	19.30[b]	0.00[c]	3.50[a]	22.80	22.80	17
	Tropical	Hydrocharitaceae	*Thalassia testudinum*	13.35[b]	6.00[c]	1.15[ad]	20.50[d]	14.50[d]	14
	Tropical	Hydrocharitaceae	*Thalassia testudinum*	20.20	21.10	6.80[a]			7
Tropical Avg ± 1 S.E.M				*17.68 ± 1.61*	*8.32 ± 3.66*	*2.56 ± 0.94*	*13.48 ± 3.61*	*17.83 ± 2.03*	
Total Rhizome Avg ± 1 S.E.M				*17.77 ± 1.36*	*11.75 ± 4.55*	*3.03 ± 0.78*	*13.48 ± 3.61*	*17.83 ± 2.03*	
Root	Temperate	Zosteraceae	*Zostera marina*	21.30	40.90	5.70[a]			7
	Temperate	Zosteraceae	*Zostera tasmanica*			6.50			9
Temperate Avg ± 1 S.E.M				*21.30 ± 0*	*40.90 ± 0*	*6.10 ± 0.40*	*N/A*	*N/A*	

Tissue	Temperate/ Tropical	Family	Species	Structural Carbohydrates			Fibre		Reference
				Cellulose	Hemi-cellulose	Lignin	NDF	ADF	
	Tropical	Cymodoceaceae	*Cymodocea serrulata*				3.28		12[ef]
	Tropical	Cymodoceaceae	*Halodule pinifolia*				2.58		12[ef]
	Tropical	Cymodoceaceae	*Syringodium isoetifolium*				2.97		12[ef]
	Tropical	Hydrocharitaceae	*Halophila engelmanni*	13.23		2.07			15[e]
	Tropical	Hydrocharitaceae	*Halophila ovalis*				3.52		12[ef]
	Tropical	Hydrocharitaceae	*Thalassia testudinum*	23.50	28.70	5.70[a]			7
Tropical Avg ± 1 S.E.M				*18.37 ± 5.13*	*28.70 ± 0*	*3.88 ± 1.82*	*3.09 ± 0.20*	*N/A*	
Total Root Avg ± 1 S.E.M				*19.34 ± 3.12*	*34.80 ± 6.10*	*4.99 ± 0.99*	*3.09 ± 0.20*	*N/A*	
Rhizome + Root	Temperate	Zosteraceae	*Zostera marina*	16.67					5[e]
	Tropical	Cymodoceaceae	*Cymodocea rotundata*	12.60[b]	9.70[c]	19.90[a]	42.20	32.50	10
	Tropical	Cymodoceaceae	*Cymodocea serrulata*	25.70[b]	4.68[c]	7.46[a]	37.84	33.16	19
	Tropical	Cymodoceaceae	*Cymodocea serrulata*	14.40[b]	9.30[c]	15.80[a]	39.50	30.20	10
	Tropical	Cymodoceaceae	*Halodule uninervis*	11.60[b]	8.30[c]	8.40[a]	28.30	20.00	10
	Tropical	Cymodoceaceae	*Halodule uninervis*			18.85	51.41		13
	Tropical	Cymodoceaceae	*Syringodium isoetifolium*			6.48[a]	31.09	33.55	19
	Tropical	Cymodoceaceae	*Syringodium isoetifolium*	15.20[b]	8.00[c]	11.10[a]	34.30	26.30	10
	Tropical	Hydrocharitaceae	*Halophila minor*	11.00[b]	4.50[c]	8.80[a]	24.30	19.80	10

Tissue	Temperate/ Tropical	Family	Species	Structural Carbohydrates			Fibre		Reference
				Cellulose	Hemi-cellulose	Lignin	NDF	ADF	
	Tropical	Hydrocharitaceae	*Halophila ovalis*	26.25[b]	7.43[c]	4.50[a]	38.18	30.75	19
	Tropical	Hydrocharitaceae	*Halophila ovalis*	12.40[b]	5.10[c]	9.00[a]	26.50	21.40	10
	Tropical	Hydrocharitaceae	*Halophila ovalis*			15.08	46.53		13
	Tropical	Hydrocharitaceae	*Halophila spinulosa*	30.79[b]	13.41[c]	4.86[a]	49.06	35.65	19
	Tropical	Hydrocharitaceae	*Halophila spinulosa*	17.90[b]	9.70[c]	8.80[a]	36.40	26.70	10
	Tropical	Hydrocharitaceae	*Halophila spinulosa*			13.92	50.09		13
	Tropical	Zosteraceae	*Zostera muelleri*	11.10[b]	8.10[c]	15.10[a]	34.30	26.20	10
	Tropical	Zosteraceae	*Zostera muelleri*			13.19	43.03		13
Total Rhizome + Root Avg ± 1 S.E.M				*17.13 ± 2.14*	*8.02 ± 0.79*	*11.33 ± 1.19*	*38.31 ± 2.10*	*28.02 ± 1.58*	
Total BG Avg ± 1 S.E.M				*17.64 ± 1.21*	*12.02 ± 2.41*	*8.05 ± 1.03*	*26.19 ± 3.16*	*25.47 ± 1.70*	
Whole plant	Tropical	Cymodoceaceae	*Cymodocea serrulata*	26.97[b]	14.57[c]	7.16[a]	48.70	34.13	19
	Tropical	Cymodoceaceae	*Halodule uninervis*			19.17	51.60		13
	Tropical	Cymodoceaceae	*Syringodium isoetifolium*				35.05	38.21	19
	Tropical	Hydrocharitaceae	*Halophila decipiens*	15.50[b]	7.10[c]	4.90[a]	27.50	20.40	10
	Tropical	Hydrocharitaceae	*Halophila ovalis*	23.05[b]	14.16[c]	4.10[a]	41.31	27.15	19
	Tropical	Hydrocharitaceae	*Halophila ovalis*			15.73	48.86		13
	Tropical	Hydrocharitaceae	*Halophila spinulosa*	29.44[b]	14.17[c]	4.56[a]	48.17	34.00	19

Tissue	Temperate/ Tropical	Family	Species	Structural Carbohydrates			Fibre		Reference
				Cellulose	Hemi-cellulose	Lignin	NDF	ADF	
	Tropical	Hydrocharitaceae	*Halophila spinulosa*			13.54	50.83		13
	Tropical	Hydrocharitaceae	*Halophila trichosata*	16.60[b]	8.40[c]	8.10[a]	33.10	24.70	10
	Tropical	Zosteraceae	*Zostera muelleri*			14.32	44.04		13
Total Whole Plant Avg ± 1 S.E.M				*22.31 ± 2.78*	*11.68 ± 1.62*	*10.18 ± 1.86*	*42.92 ± 2.65*	*29.77 ± 2.78*	

[a] Lignin reported as acid detergent lignin (ADL).
[b] Cellulose calculated from ADF – ADL.
[c] Hemicellulose calculated from NDF – ADF.
[d] Reference used values of a range, so the median was reported in table.
[e] Value represents a mean reported over > 1 season.
[f] NDF was described as TDF.

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Note

Detachment and flow cytometric quantification of seagrass-associated bacteria ☆

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ABSTRACT

A new protocol was developed to detach bacteria from seagrass tissue and subsequently enumerate cells using flow cytometry (FCM). A method involving addition of the surfactant Tween 80 and vortexing resulted in maximum detachment efficiency of seagrass attached bacteria, providing a robust protocol for precisely enumerating seagrass-associated bacteria with FCM. Using this approach we detected cell concentrations between 2.0×10^5 and 8.0×10^6 cells mg^{-1} DW tissue.

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Comparison of marine macrophytes for their contributions to blue carbon sequestration

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Abstract. Many marine ecosystems have the capacity for long-term storage of organic carbon (C) in what are termed "blue carbon" systems. While blue carbon systems (saltmarsh, mangrove, and seagrass) are efficient at long-term sequestration of organic carbon (C), much of their sequestered C may originate from other (allochthonous) habitats. Macroalgae, due to their high rates of production, fragmentation, and ability to be transported, would also appear to be able to make a significant contribution as C donors to blue C habitats. In order to assess the stability of macroalgal tissues and their likely contribution to long-term pools of C, we applied thermogravimetric analysis (TGA) to 14 taxa of marine macroalgae and coastal vascular plants. We assessed the structural complexity of multiple lineages of plant and tissue types with differing cell wall structures and found that decomposition dynamics varied significantly according to differences in cell wall structure and composition among taxonomic groups and tissue function (photosynthetic vs. attachment). Vascular plant tissues generally exhibited greater stability with a greater proportion of mass loss at temperatures >300°C (peak mass loss ~320°C) than macroalgae (peak mass loss between 175–300°C), consistent with the lignocellulose matrix of vascular plants. Greater variation in thermogravimetric signatures within and among macroalgal taxa, relative to vascular plants, was also consistent with the diversity of cell wall structure and composition among groups. Significant degradation above 600°C for some macroalgae, as well as some belowground seagrass tissues, is likely due to the presence of taxon-specific compounds. The results of this study highlight the importance of the lignocellulose matrix to the stability of vascular plant sources and the potentially significant role of refractory, taxon-specific compounds (carbonates, long-chain lipids, alginates, xylans, and sulfated polysaccharides) from macroalgae and seagrasses for their long-term sedimentary C storage.

This study shows that marine macroalgae do contain refractory compounds and thus may be more valuable to long-term carbon sequestration than we previously have considered.

Key words: blue carbon; carbon sequestration; macroalgae; mangrove; plant cell wall; pyrolysis; saltmarsh; seagrass; thermogravimetry.

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