“Phosphorus availability and microbial driven decomposition in oligotrophic seagrass sediments”

Matthew William Fraser
B.Sc. (Hons.) Marine Science (UWA)

This thesis is presented for the degree of Doctor of Philosophy at the School of Plant Biology, University of Western Australia

2016
Phosphorus cycling in seagrass sediments

Abstract

Phosphorus (P) availability has long been considered to be limiting in many seagrass communities. However, seagrass meadows are often highly productive in oligotrophic waters despite extremely low nutrient concentrations. Seagrass sediments, in contrast, are often enriched in organic matter (OM) that could potentially be a major source of nutrients, particularly of P, following microbial degradation. The main aim of this thesis was to investigate how seagrasses productivity was supported in Shark Bay – an embayment with extremely low soluble reactive phosphorus (SRP) in the water column but with abundant seagrass meadows. I address this key question by focusing on the importance of OM decomposition for P cycling and plant uptake in oligotrophic seagrass sediments.

The research focused on Shark Bay, a World Heritage Site in Western Australia dominated by seagrasses that form the foundation of this important ecosystem. I first document a major pulse of OM inputs to Shark Bay sediments following widespread defoliation and dieback of the dominant seagrass *Amphibolis antarctica* in Shark Bay driven by a marine heatwave and flood event. Given the frequency and magnitude of extreme events are predicted to increase, such events will likely influence biogeochemical cycling in Shark Bay and similar seagrass ecosystems in the near future. I then investigated the effects of seagrass-derived OM on sediment biogeochemistry and microbial communities, and seagrass seedling health in controlled tank systems. Seagrass-derived OM changed the sediment microbial community and significantly increased hydrolytic enzyme expression, particularly phosphatase and glucosidase enzymes. OM enrichment also correlated with decreased N and P content of living seagrass leaves, likely caused by increased microbial competition for nutrients decreasing plant uptake or higher aboveground biomass diluting nutrient impacts. Seagrass derived OM has previously been considered a refractory pool of nutrients, yet here I show its deposition into sediments significantly alters belowground conditions.

I next focused on the specific effects of OM enrichment on P cycling across Shark Bay sediments. I characterised the different fractions of sedimentary phosphorus across the salinity gradient in Shark Bay sediments, including examining the organic P pool to the
compound level using \(^{31}\text{P}\)-NMR (nuclear magnetic resonance). I show that organic P represents a substantial (16-40%) proportion of total P in Shark Bay sediments, and that this pool is strongly correlated with OM content in sediments. The only identifiable organic P compounds detected using \(^{31}\text{P}\)-NMR were phosphate monoesters. The percent organic P was highest at sites with low water SRP, representing a potential source of P at these sites.

I then examined how sediment microbial community composition and functioning relates to patterns in biogeochemical processes and seagrass nutrient status in Shark Bay. I thus characterised the taxonomic and functional diversity of microbial communities within seagrass sediments along a gradient of decreasing water SRP using metagenomic sequencing to better understand P dynamics in the sediments. Genes encoding for phosphorus metabolism increased at low water SRP sites and also correlated to an increase in phosphatase activity. The increase in both the presence of P metabolism gene abundance and the activity of phosphatases suggests that microbial recycling helps support seagrass P demand in Shark Bay through mineralization or phosphate monoesters. Notably, seagrass microbial communities were high in gene abundance for sulfur and phosphorus metabolism when compared with other aquatic and terrestrial ecosystems.

I also sought to investigate the extent to which microbial turnover of organic P plays in seagrass uptake of P across the salinity and P availability gradient, using stable oxygen isotope composition of phosphates (\(\delta^{18}\text{O}_{\text{PO}_4}\)) as a tracer for biological cycling of P. However, my attempts to measure \(\delta^{18}\text{O}_{\text{PO}_4}\) in seagrass and sediment samples were unsuccessful, despite successfully processing and obtaining correct \(\delta^{18}\text{O}_{\text{PO}_4}\) for international isotope standards. Extremely low phosphate concentrations and high salinity (>50‰) prevented successful extraction of pure silver phosphates and led to incorrect \(\delta^{18}\text{O}_{\text{PO}_4}\) for Shark Bay seagrass and sediment samples.

Overall, this thesis shows that seagrass derived OM in sediments cannot be viewed as a passive pool of nutrients in seagrass sediments, and the turnover of OM in seagrass meadows is a potential source of P in oligotrophic seagrass ecosystems. The turnover of OM in strongly oligotrophic seagrass sites is dependent on highly specialised and adapted microbial communities that have a key role in driving mineralization processes, showing
Phosphorus cycling in seagrass sediments

that interactions between seagrasses and sediment microbial communities are a critical knowledge gap within seagrass ecology, and must be further characterised given their impacts on nutrient cycling and primary productivity.
Table of Contents

Abstract...........................................................................................................................................i

Table of Contents..............................................................................................................................iv

Acknowledgements..........................................................................................................................vii

Statement of candidate contribution and list of publications ...........................................................x

Thesis Structure .............................................................................................................................xii

Chapter 1: General introduction.......................................................................................................1
  The productivity of seagrass ecosystems .........................................................................................2
  Marine sediments and the phosphorus cycle ....................................................................................3
  Organic matter and nutrient cycling in near coastal ecosystems ....................................................5
  Phosphorus availability and organic matter turnover .......................................................................5
  Microbial ecology – engines that drive biogeochemical cycles .....................................................7
  Shark Bay: a model oligotrophic ecosystem ....................................................................................9
  Thesis aims and structure ................................................................................................................10
  References .......................................................................................................................................12

Chapter 2: Extreme climate events lower resilience of foundation seagrass at edge of biogeographical range ..................................................................................................................20

Summary .........................................................................................................................................21
  Introduction .....................................................................................................................................22
  Methods .........................................................................................................................................25
    Study site .......................................................................................................................................25
    Spatial extent of Amphibolis defoliation .......................................................................................25
    Data analysis .................................................................................................................................26
  Results ............................................................................................................................................28
    Extreme temperature and rainfall .................................................................................................28
    Response of seagrasses to flooding and temperature events ......................................................29
    Recovery of seagrass meadows .....................................................................................................31
  Discussion ........................................................................................................................................32
  Acknowledgments ..........................................................................................................................38
Chapter 3: Seagrass derived organic matter influences biogeochemistry, microbial communities, and seedling biomass partitioning in seagrass sediments

Abstract

Introduction

Methods

Seedling collection
Experimental design
Post-harvest measurements
Sediment biogeochemistry
Microbial molecular community identification
Statistical analysis

Results

Sediment biogeochemistry
Microbial community structure
Seagrass biomass
Seagrass nutrient concentrations

Discussion

Acknowledgements

References

Chapter 4: Organic phosphorus accumulates in calcareous seagrass sediments with increasing salinity

Abstract

Introduction

Methods

Site description & experimental design
Sediment cores
Sediment chemical and physical characterization
Sequential P extraction of sediments
Solution 31P-NMR spectroscopy
Data analysis

Results

General sediment variation along salinity gradient
Sediment P fractions
Acknowledgements

I must begin by thanking my ever patient supervisors – Gary Kendrick, Pauline Grierson, and Grzegorz Skrzypek. Gary, you have always been extremely generous to me, have looked out for my best interests and allowed me the freedom to explore my own interests without wandering too far. You have given me opportunities beyond my PhD work that most postgraduate students would only dream off, and I am deeply grateful for your belief in my ability. Pauline, as a fellow phosphorus biogeochemistry and coffee tragic you have provided me with wisdom as I often questioned my own research direction. No matter how much I felt I knew coming into a meeting with you, you always managed to give me a fresh perspective and run off to read more papers or run more analyses. Greg, even though our foray into δ18O-PO4 analysis didn’t quite work out as we had hoped, I have learned much from you during my PhD, and you have always been more than generous with your time. Above all, I will be forever grateful with the respect that all my supervisors treated me with – not merely a student-supervisor relationship, but as genuine collaborations built upon trust and respect. I aspire to take the lessons you have taught with me should I ever get the opportunity to be the supervisor instead of the student.

There have been many fantastic collaborators that I have worked with on various aspects of this thesis, which have undoubtedly improved its breadth and content. Their knowledge and expertise inspired me to strive to improve my own research. Bonnie and Deirdre – thanks for introducing me to the small but mighty world of microbes! I loved delving into this work with you, and I’m sure that there will be many further microbial projects in the future. Di – without the countless hours you have put into seagrass research at UWA then I would not have had this opportunity. I am deeply grateful for this, and for you introducing me to the wonderful place that is Shark Bay. And a special thanks to Renae and John – you are both lifelong friends – working together is just a bonus! I likely would be doing something very different if not for you two, you prevented me from heading over to the dark side and sticking with benthic research. There have also been many technical staff that have assisted me on my journey, with a special thanks to Andrea Zavala-Perez, Kate Bowler, Doug Ford and Lindsey Byrne.

I was supported by an Australian Postgraduate Award with a UWA Safety-Net Top-Up
from the University of Western Australia. Funding for the project was primarily provided by a NHT-II Caring for Country Grant. Additional funding was provided by the School of Plant Biology (UWA), the Ray Hart Memorial Scholarship, and the Australian Geographic Society. I thank the Department of Parks and Wildlife for providing permits that allowed me to complete the research presented here. I’d also like to acknowledge the School of Plant Biology – the research culture in the School is fantastic and a credit to all the staff involved that make the PhD experience so rewarding for postgrads like me.

I’ve been lucky enough to be part of two fantastic research groups during my PhD at UWA. The Seagrass Research Group is full of like-minded people that love nothing more than being on the water (and finishing fieldwork days watching the sun go down with a brew!). I couldn’t have completed the fieldwork without you guys, and the many meetings and writing sessions we’ve had have helped shape the way I think about research. Uncle John, Auntie Nae, Andrea, Leo, Liz, Anne, Marion, Ylva, Luke, Napo, Belinda, Dan, Sam, Sahira (thanks for making my first supervisory role so easy!) – you’ve all been great. And thanks for putting up with all of us Gary! To ERGo: you became my home away from home while I was nearing the end of the thesis, which just so happened to be the perfect time to find a group that was always interested in stopping for a coffee break. Pauline, Greg, Kate, Sara, Doug, Alex, Ali, Andre, Rachel, Tegan, Jordan, and Caroline – thank-you all for putting up with me (and all the baby talk) while I was nearing the end of my thesis.

Finally, I wish to thank my family. You are the most important thing in the world to me, and your love and care remind me not to get bogged down in work. Mum and Dad, thank you for providing a loving and stable environment for me to grow up in, sparking off my interest and science and catching me whenever I fell. Scott and CJ – I couldn’t ask for better siblings. Scott and Annie, thanks for convincing us all to come to Australia in the first place, it is without a doubt the best move we all made. And Tara – no words can express how grateful I am for your love. No matter what bumps I encountered along the way, you were always there to remind me to believe in myself and support me through thick and thin. You are the love of my life and I wouldn’t choose to be with any other person on our new adventure. And to my two newborn sons, Harper and Cooper, you provided me with the motivation to finish this thesis. Having identical twins within a
month of your PhD submission may not seem like a good idea, but I wouldn’t have changed it for the world! Like your mum, you both mean the world to me. This thesis is dedicated to my family, I love you all
Statement of candidate contribution and list of publications

The following chapters have been published as scientific manuscripts:

Chapter 2

Chapter 3

In addition, two other chapters have been prepared for submission to scientific journals:

Chapter 4

Chapter 5

I conceived all research questions (in consultation with supervisors), designed the research program, was responsible for the lab work, data analysis and interpretation, as well as the preparation of each manuscript. The thesis is my own work, except where explicitly stated otherwise. The contribution of co-authors to each chapter is described.
Phosphorus cycling in seagrass sediments

in the preamble to each chapter. All co-authors consent to this work being included in this thesis. Co-authors primarily provided advice on initial research direction, specific advice on experimental methods and provided feedback on manuscript drafts.

Signed:

Matthew Fraser (Candidate)

Gary Kendrick (Coordinating Supervisor)

September 2016
Thesis Structure

This thesis has been prepared as a ‘series of papers’ as per Regulation 1.3.1.33 (1) of the University of Western Australia’s General Provisions for Research Higher Degrees (by Thesis). Five experimental chapters (two published, two to be submitted, one a method commentary on oxygen isotope composition of phosphates) are presented, preceded by a General Introduction to place the research in context. A final General Discussion synthesises the overall findings and discusses the implications of the research. The five experimental chapters are written as “stand-alone” manuscripts. Thus, there is some repetition (particularly in materials and methods sections), although every effort has been made to keep this repetition to a minimum. Each experimental chapter is reproduced verbatim, including individual Chapter Abstracts, with the following exceptions:

- Author addresses have been omitted
- Figure and table numbering has been slightly altered to include chapter numbers
- Minor changes have been made to the formatting of each chapter to ensure consistency across the thesis.

References are included at the end of each chapter, in the style of the journal where the research is published/to be submitted to.
Chapter 1: General introduction

The main aim of this thesis is to investigate how seagrasses productivity is supported in oligotrophic environments, specifically focused on the UNESCO World Heritage listed Shark Bay region of Western Australia. In particular, I examined the importance of decomposition and mineralization of organic matter for phosphorus (P) cycling in oligotrophic seagrass sediments. This thesis also emphasizes the key roles that specialist microbial communities in the sediments play in determining organic matter turnover.

How highly productive and frequently diverse ecological communities thrive in low nutrient (oligotrophic) ecosystems has been a central ecological question since the voyages of Darwin (Darwin 1842). In marine ecosystems, this apparent paradox has been most often investigated for coral reefs, which boast high primary productivity and species richness despite existing in some of the most nutrient-poor waters in the world (Smith 1984a; Hatcher 1990; Wyatt et al. 2012). Recently, new methods such as stable isotope tracing have shed light on some of the mechanisms that contribute to higher productivity that might be predicted on the basis of climate and nutrient availability in the water column, including highly efficient nutrient recycling pathways existing in coral reef ecosystems (de Goeij et al. 2013). In comparison, mechanisms driving high productivity in other coastal marine ecosystems are understudied. Seagrass meadows are also highly productive in oligotrophic waters, but we do not fully understand how or why (Romero et al. 2006). Given that seagrasses can take up nutrients from sediments through their roots, the cycling of sediment nutrient pools may also maintain seagrass productivity in oligotrophic ecosystems. The main aim of this thesis is to investigate how seagrasses productivity is supported in oligotrophic environments. I address this key question by looking specifically at the importance of decomposition of organic matter for phosphorus (P) cycling in oligotrophic seagrass sediments.

In this introductory chapter, I provide an overview of drivers of seagrass productivity in oligotrophic environments. I then summarise the importance of phosphorus cycling in seagrass meadows. I also provide an overview of the current understanding of organic matter dynamics in coastal environments, before discussing if the turnover of organic matter in seagrass sediments driven by sediment microorganisms may be important in
supplementing P availability to seagrasses. This background thus provides context for the experimental rationale that is developed in Chapters 2-5 of this thesis.

The productivity of seagrass ecosystems

Seagrasses are marine angiosperms that are the dominant primary producers in many coastal ecosystems, often with high production rates comparable to coral reefs and tropical forests (Duarte & Chiscano 1999). However, some of the most productive seagrass meadows exist in waters with extremely low available nutrient concentrations. For example, Posidonia oceanica (L.) growing in the in Mediterranean Sea, Thalassia testudinum Banks ex König in the Caribbean, and Amphibolis antarctica (Labill.) Sonder et Aschers in Shark Bay all form productive seagrass meadows in nutrient depleted waters (Walker, Kendrick & McComb 1988; Marbà, Duarte & Cebrian 1996; Hemminga, Marbà & Stapel 1999). Indeed, seagrass cover often declines when nutrient availability increases due to increased competition from algae that results in light reduction (Ferdie & Fourqurean 2004). Clearly, seagrasses can thrive in nutrient poor environments such as Shark Bay, which suggests functional adaptation at both the plant and ecosystem level.

Seagrasses possess traits that allow them to efficiently use nutrients. For example, seagrasses can translocate nutrients from old leaves prior to senescence (Hemminga et al. 1999). Some seagrasses also release organic anions that increase nutrient availability, particularly of phosphates, in surrounding calcareous sediments through dissolution (Long et al. 2008). However, such adaptations are unlikely to fully explain high seagrass productivity in oligotrophic environments given their high nutrient requirements (Hemminga et al. 1999). As such, mechanisms other than individual plant traits must contribute to the high primary productivity in oligotrophic seagrass ecosystems.

Seagrasses, like terrestrial angiosperms, interact with sediments through their well-developed root systems. These root systems place seagrasses in a unique position for marine primary producers in that they are able to directly access sediment nutrients through root uptake, as well as take up nutrients from the water column through leaves (Stapel et al. 1996; Nielsen, Koch & Madden 2007). Given seagrasses appear to take up adequate nutrients even when water column concentrations are low suggests that belowground processes may play a key but as yet poorly defined role in maintaining the productivity of seagrass meadows. Furthering our understanding of below-ground
processes in seagrass sediments will therefore improve our understanding of nutrient cycling and maintenance of primary productivity in these ecosystems.

Given seagrass meadows are recognized as important marine carbon sinks it is critical to understand the major constraints on their carbon sequestration (Kennedy et al. 2010; Mcleod et al. 2011; Fourquarean et al. 2012a; Laliberte, Zemunik & Turner 2014), including the limitation or otherwise of particular nutrients. Seagrasses are declining globally, in large part attributable to direct and indirect impacts of nutrient pollution from adjacent coastal development (Orth et al. 2006). Seagrasses growing in oligotrophic environments are particularly susceptible to changes in labile P concentrations. For example, rock phosphate additions of 0.12 g P m$^{-2}$ d$^{-1}$ to nearshore seagrass beds in Florida Bay resulted in increases in the relative abundance of macroalgae and epiphytes, thereby reducing seagrass growth rates (Darwin 1842; Ferdie & Fourquarean 2004). Recovery from the effects of nutrient enrichment can be slow in oligotrophic embayments, with seagrass community structure still altered two decades after enrichment events in Florida Bay (Smith 1984a; Hatcher 1990; Herbert & Fourquarean 2008; Wyatt et al. 2012). Increases in P inputs are thus considered the biggest threat to seagrass communities in sub-tropical embayments around the world, including Shark Bay in Western Australia and Florida Bay in the southern United States (Kendrick et al. 2012; de Goeij et al. 2013). It is thus increasingly evident that a comprehensive understanding of the mechanisms and controls on P cycling is required if we are to fully understand primary productivity and benthic ecology in seagrass ecosystems, and is also fundamental to successful management of seagrass meadows and coastal zones more broadly.

**Marine sediments and the phosphorus cycle**

Marine sediments are a major sink and source of P in coastal ecosystems, and are therefore an important component of the overall P cycle. Phosphorus is present in many different forms within marine sediments, and the availability of P for biological uptake from sediments depends on the distribution of sediment P within these fractions. For example, apatite-P and aluminium–bound P are sinks of P and not readily available for biological uptake (Paytan & McLaughlin 2007; Sinkko et al. 2011). Conversely, loosely adsorbed P is readily available for uptake, while iron-bound P can become available when Fe-compounds are reduced, and organic P can become available for uptake after
mineralization (Sinkko et al. 2011). It is essential to measure more than just total P in sediments, as different P fractions (with varying availabilities) can vary between different sediments (Sinkko et al. 2011). However, in seagrass systems only a few studies have examined P fractionation in sediments (Koch, Benz & Rudnick 2001; Holmer, Carta & Andersen 2006), with the majority inferring availability from porewater P or total P in sediments (Romero et al. 2006).

As described above, the extent of initial adsorption of inorganic P into the sedimentary pool will largely depend on the mineralogy and clay content of the sediments. For example, phosphate ions in solution strongly adsorbs to the surface of calcareous sedimentary particles, limiting the availability of P for primary producers (Short 1987; Duarte & Chiscano 1999). Phosphate can also be adsorbed onto Al and Fe oxide sediment particles, but is more readily desorbed following reduction (Jordan et al. 2008). As such, benthic producers growing in coastal systems dominated by calcareous sediments are often considered to be P-limited (Short 1987; Walker et al. 1988; Marbà et al. 1996; Hemminga et al. 1999). However, this generalization does not always hold true (Erftemeijer et al. 1994; Ferdie & Fourqurean 2004; Fraser et al. 2012), suggesting that other key processes, including the turnover of organic matter, may play a role in maintaining P supply in some seagrass ecosystems.

Coastal sediments are not just a static reservoir of P, but are subject to biological and geochemical processes that redistribute P in ecosystems (Benitez-Nelson 2000). The recycling of P from sediments through plant uptake and then return of OM to the sediment surface can be a significant input in the P budget of coastal ecosystems, often larger than that contributed by allochthonous inputs from mangroves, rivers, or estuaries (Hemminga et al. 1999; Ahlgren et al. 2006; 2011). Despite this, the majority of studies examining marine P cycling have focused on open water systems and thus cycling in the water column, with benthic cycling in sediments only recently receiving limited attention (Stapel et al. 1996; Nielsen et al. 2007; Jilbert et al. 2011; Goldhammer et al. 2011). However, benthic recycling can supply a substantial proportion of nutrients for primary producers in coastal ecosystems, particularly in oligotrophic systems, and is likely disproportionately important for nutrient cycling within these environments compared to systems with higher phosphate concentrations (Fisher, Carlson & Barber 1982; Denis et
Organic matter and nutrient cycling in near coastal ecosystems

Highly productive seagrass meadows in oligotrophic conditions often grow in sediments enriched with OM (Marbà et al. 1996; Kendrick et al. 2012). Seagrasses increase OM concentrations in underlying sediments, primarily through deposition of dead plant material as litter and increased sedimentation rates of plankton (Marbà et al. 2006). OM contents in some seagrass sediments are as high as 100% (Fourquean et al. 2012a). This OM is often assumed to be a refractory pool (Kennedy et al. 2010), but the potential for this OM to sustain productivity in seagrass ecosystems through mineralization has not been examined in great detail. Nutrient release from the breakdown of OM is one of the dominant sources of production, and is particularly important in sustaining productivity in terrestrial oligotrophic ecosystems (Cleveland, Houlton & Smith 2013). For example, the decomposition of OM supports primary productivity of tropical forests growing on low nutrient soils (Reed et al. 2011), in freshwater lakes, streams and rivers (Gächter & Meyer 1993), and in marine ecosystems (Leote et al. 2015). Globally, recycling of OM accounts for 89% of the annual N demand and 98% of the annual P demand (Cleveland et al. 2013). Nevertheless, understanding of the ecological impacts of OM in seagrass sediments is a recognized knowledge gap (Romero et al. 2006). In oligotrophic ecosystems, it is likely that the degradation of OM in seagrass sediments will be particularly important from an ecological perspective by supplementing nutrient availability.

Phosphorus availability and organic matter turnover

There have been remarkably few studies that have characterized sedimentary organic P in seagrass sediments or the processes contributing to P turnover. The recycling of organic compounds containing P is an important and complex component of the P cycle (Grierson, Comerford & Jokela 1998; 1999; Grierson & Adams 2000; Turner, Frossard & Baldwin 2005b). In marine sediments, organic P can comprise a large proportion of the total P in sediments, and is a highly important component of the biogeochemical cycling of P more generally (Gächter & Meyer 1993; Hayes, Richardson & Simpson 2000; Turner et al. 2005a; Giesler et al. 2012). Traditionally, the organic P pool in both soils and
sediments was considered to be refractory (Ahlgren et al. 2011). However, organic P is comprised of a complex mix of known and unknown compounds (Turner, Newman & Newman 2006; Reitzel et al. 2006; Turner 2008; Vincent, Turner & Tanner 2010; Turner & Engelbrecht 2011). These compounds likely differ significantly in their availability for plant uptake (Tarafdar & Claassen 1988; Gerke 2015). For example, phosphate monoesters are of limited availability to plants as they are strongly associated with soil minerals, while phosphate diesters or P lipids are more available for plant uptake due to higher lability (Reitzel et al. 2006, Turner et al. 2011). Changes in the composition of the soil organic P pool may lead to changes in productivity and species composition of primary producers in terrestrial ecosystems (Turner 2008). We might expect that seagrass communities would be similarly influenced by shifts in the amount of OM and its interaction with sediment P fractions. Thus, information on both the amount and chemical composition of sedimentary organic P is fundamental for understanding of P dynamics in aquatic ecosystems (Turner et al. 2006; Zhang et al. 2009). Given low soluble reactive phosphorus (SRP) concentrations are common in marine waters overlying embayments dominated by calcareous sediments, the recycling of P from the organic P pool may represent the primary source of P to seagrass communities.

The biological mineralization of OM by microorganisms is the dominant pathway for organic P to be broken down to inorganic phosphate available for plant uptake (Bünemann et al. 2007; Achat, Bakker & Morel 2009). Biological mineralization is responsible for up to 90% of plant P acquisition in terrestrial environments, and is generally more significant in oligotrophic environments with low inputs of nutrients (van der Heijden, Bardgett & van Straalen 2008). For example, tropical forests are systems with low P inputs and highly weathered soils, where the dominant source of P for uptake by the large pools of aboveground biomass comes from microbially-driven breakdown of OM (Reed et al. 2011). Similarly, the jarrah forests of southwest Australia have been shown to be highly dependent on microbial mineralization of organic P (Grierson & Adams 2000). However, the importance of microbial mineralization for P supply in seagrass ecosystems, including those of coastal Western Australia, is largely unknown. Microorganisms may also compete with seagrass for P through the uptake of phosphate and immobilisation into microbial biomass; this process can accentuate P limitation of plants (Reed et al. 2011; Marschner, Crowley & Rengel 2011). Therefore,
Microorganisms play a complex role within the P cycle as they have the potential to both increase and decrease availabilities of P to higher plants. Competition between plants and microbes for P is particularly strong in nutrient limited systems (van der Heijden et al. 2008). The balance between whether microbial activity is dominated by immobilization or mineralization is dependent on environmental conditions like the relative availability of other nutrients – particularly N – and the “quality” of OM (in its simplest sense characterised by the C:N or C:N:P ratio, (Hobbie 1996; van der Heijden et al. 2008). Consequently, we might expect sediment microbes may act as a sink or source of P in seagrass ecosystems depending on environmental conditions.

Microbial ecology – engines that drive biogeochemical cycles

Microorganisms have been described as a ‘black box’ in ecosystem ecology in recognition of their central importance in regulation biogeochemical processes, yet there is a profound lack of data regarding their quantitative impacts on ecosystem function (Tiedje, Asuming-Brempong & Nüsslein 1999). This is particularly true in seagrass ecosystems. There have only been a limited number of studies examining sediment microbial communities in seagrass ecosystems. Seagrass meadows harbor very high bacterial diversity in sediments (Garcia-Martínez et al. 2009), containing microorganisms that have the capacity to influence ecosystem processes yet are not found in other ecosystems. For example, novel diazotrophs are present in some seagrass sediments that would influence N cycling (Bagwell et al. 2002; Hamisi et al. 2013). Microbial communities adjacent to seagrass roots differ from bulk sediment communities in that *Epsilon*- and *Gammaproteobacteria* increase and *Deltaproteobacteria* decrease (Jensen, Kühl & Preimé 2007). Native bacterial communities are also critical for seagrass transplant success, reducing transplant mortality (Milbrandt, Greenawalt-Boswell & Sokoloff 2008), suggesting the interactions between microbes and seagrasses are ecologically important. However, microbial communities in seagrass communities have primarily been identified from a taxonomic perspective; and the functional roles of these microbial communities have not been investigated. This gap in knowledge limits our understanding of the significance of microbial communities on plant productivity, diversity, and overall ecosystem function in seagrass meadows.

Microbial communities are highly impacted by changes in a range of environmental
conditions. Where environmental shifts influence the functional capacity of the microbial communities, there are likely to be shifts in ecosystem processes (Fierer, Leff & Adams 2012). For example, changes in substrate type (Jeffries et al. 2011a), N availability (Fierer et al. 2011), and salinity (Hollister et al. 2010) drive shifts in microbial community function that would likely influence biogeochemical processes and ecosystem functions. Microbial communities mediate many decomposition processes through the production of key enzymes, such as phosphatase (Burns & Dick 2002; Burns et al. 2013). The production of these enzymes is encoded for by functional genes such as PhoA and PhoX (Sebastian & Ammerman 2009). As such, the presence (or absence) of functional genes within a microbial community has been used to assess their potential role in driving key ecosystem processes (Zak, Blackwood & Waldrop 2006), and the loss of one microbial functional group (e.g. N fixation) could lead to declines in plant productivity (van der Heijden et al. 2008). Where unique functional genes are gained or lost from the microbial community, the effects of these environmental drivers on ecosystem function would be most apparent. In seagrass sediments, microbial communities shift in response to the presence of seagrass and changes in season (James et al 2006). Again, the sediment microbial community was only examined on a taxonomic level; the potential influence of these microbial shifts on the function of the seagrass ecosystems has not been examined, and we know very little of other environmental drivers in seagrass systems that may influence microbial community composition. Salinity is a major factor that influences the structure and functioning of coastal ecosystems. The majority of studies examining the impacts of salinity on sediment microbial communities have examined transitions from freshwater to brine to marine salinities (<5-35‰). For example, shifts in microbial communities taxonomy and function in the Baltic Sea were most strongly associated with salinity, including shifts in genes encoding for respiration that would affect C cycling through the system (Dupont et al. 2014). Similar shifts under higher salinities (e.g. hypersaline ecosystems) have received less attention. In hypersaline coastal wetlands, the genes encoding for halotolerance and photosynthesis increased in sediment microbial communities as salinities increased from 37‰ to >100‰ (Jeffries et al. 2011b). Such shifts would also thus have influence on nutrient cycling and ecology across the entire ecosystem.

In addition to salinity, nutrient availability gradients will also likely exert significant
impacts on microbial community composition in sediments (Fierer et al. 2011). Where nutrients become more limiting, genes that promote efficient nutrient uptake and recycling increase, given the microbial requirement for nutrients (Orchard & Webb 2009), altering nutrient cycling rates. Such changes in microbial communities are likely to have the biggest impact in oligotrophic ecosystems where functional redundancy is low (van der Heijden et al. 2008). For example, increased P limitation in microbes across chronosequence soils shifted the functional capacity of the microbial community, reducing decomposition rates and further intensified nutrient limitation (Wardle et al. 2004). Similar shifts in microbial communities along P availability gradients are likely to influence ecosystem processes in seagrass ecosystems. Clearly, understanding the functional capabilities of microbial communities by examining key genes is critical for identifying microbial controls on biogeochemical cycling of P and relationships with seagrass productivity.

Shark Bay: a model oligotrophic ecosystem

The research in this thesis is focused on Shark Bay, Western Australia (25°55'60″S, 113°32'32″E). Shark Bay is an extreme example of an oligotrophic coastal embayment, with very low (0.02 μM – 0.2 μM) concentrations of SRP in the water column (Atkinson, 1987; Fraser et al., 2012). Shark Bay is also an example of an inverse estuary (salinity increases with distance from the ocean) with hypersaline reaches. The salinity gradient is particularly pronounced across the eastern embayment of Shark Bay, rising from 35‰ in the north to over 65‰ in the southern reaches of the Bay. This salinity gradient is inversely correlated with water SRP concentrations in Shark Bay; P concentrations are below detectable limits in hypersaline areas (Atkinson 1987; Fraser et al. 2012). Despite the low P concentrations in the water column, Shark Bay contains productive seagrass meadows that cover over 4000 km² (~30%) of the embayment. The dominant seagrass species are Amphibolis antarctica and Posidonia australis Hook.f. (Kendrick et al. 2012). Both species are large-bodied temperate seagrasses capable of producing large root systems that can take up nutrients from sediments (Pedersen, Paling & Walker 1997; Evrard et al. 2005; Hovey, Cambridge & Kendrick 2012). These meadows support diverse and commercially important fauna, and provide numerous key ecosystem services in the Bay (Kendrick et al. 2012). Seagrasses are clearly foundation species in Shark Bay.
but the question remains as to how these meadows thrive in such a seemingly inhospitable environment?

Shark Bay has been a focus of biogeochemists and ecosystem ecologists for several decades as a marine ecosystem with unique and extreme biogeochemical conditions. Shark Bay is historically important in marine biogeochemistry more broadly as it has been used as an example of a P limited embayment in key biogeochemical papers (Smith & Atkinson 1983; Smith 1984b; a). This early work proposed that P limitation of benthic communities (including seagrass) increased along the salinity gradient due to the decrease in water SRP and total sedimentary P (Atkinson 1987). This pattern of increasing P limitation seems to be reflected in benthic nutrient content across the entire Bay (~100km), with C:P ratios in seagrasses and other benthos increasing along with the salinity gradient (Atkinson 1987; Burkholder, Fourqurean & Heithaus 2013). However, there is considerable variation in C:N:P ratios across smaller spatial scales (10 km), suggesting that factors other than salinity are driving P availability to seagrasses over local scales in Shark Bay (Fraser et al. 2012). Shark Bay has relatively high concentrations of OM in sediments (mean = 3.03%. Fourqurean et al. 2012a) and has even been named as a carbon sequestration hotspot for coastal ecosystems globally (Fourqurean et al. 2012b; a). Given the ecological importance of OM breakdown in maintaining productivity of other terrestrial and aquatic ecosystems (described above), we would expect that OM turnover is extremely important in supplementing nutrient availability for seagrass meadows in Shark Bay.

**Thesis aims and structure**

The broad aim of this PhD thesis was to characterise the sources and ecological impacts of organic matter inputs into seagrass sediments, with particular emphasis on understanding the significance of OM decomposition and mineralization in determining phosphorus availability in an oligotrophic seagrass ecosystem. In particular, the principle objectives of this research were to investigate (i) if extreme climatic events may increase the deposition of OM in sediments, (ii) the effect of seagrass-derived OM on sediment biogeochemistry, microbial community composition, and seagrass physiology, (iii) if organic P proportionally increased in sediments as salinity increased, and (iv) how microbial utilization of OM changed along a gradient of increasing salinities and decreasing SRP concentrations.
Chapter 2 and 3 examine the prospect and influence of OM deposition in oligotrophic seagrass sediments, specifically increases in the deposition of seagrass detritus in sediments (Figure 1.1). Chapter 2 documents widespread defoliation and dieback of the dominant seagrass *A. antarctica* in Shark Bay following a marine heatwave and flood event. These extreme climatic events lead to a large input of seagrass detritus into Shark Bay sediments, representing an organic pulse that would influence the biogeochemical conditions across this oligotrophic bay.

![Figure 1.1](image)

**Figure 1.1** Conceptual model showing main questions in this thesis. 1) What are the impacts of extreme climatic events on the seagrasses of Shark Bay? 2) How do inputs of seagrass-derived organic matter influence the sediment biogeochemistry, microbial community structure, and seagrass seedling health? 3) How does the fractionation of sedimentary P change along the salinity/phosphate gradient in Shark Bay? What compounds dominate the organic P pool in seagrass sediments? 4) How does microbial community structure and function change along the salinity/P availability gradient in Shark Bay sediments? Are microbial communities adapted for low P concentrations at oligotrophic sites? 5) Does the microbial turnover of organic P subsidise seagrass P requirements at sites with low soluble reactive phosphate concentrations?

Chapter 3 examines the impacts of seagrass detritus in sediments using an experimental tank system to control for other environmental factors. In this experiment, I investigated how the addition of OM (as seagrass detritus), influenced sediment biogeochemistry and microbial communities, and seagrass seedling health.

Chapters 4-6 focus on the links between OM decomposition and phosphorus biogeochemistry in oligotrophic seagrass sediments. I characterised the different fractions of sedimentary phosphorus across the salinity gradient in Shark Bay sediments, including examining the organic P pool to the compound level using $^{31}$P-NMR (Chapter 4).
In Chapter 5, I assessed the shift in microbial community structure and function along the salinity/P availability gradient in Shark Bay sediments, and examined correlations to changes in enzyme expression and the genes encoding for organic P metabolism along the salinity/P availability gradient.

In Chapter 6, I describe attempts to use the stable oxygen isotope composition of phosphates ($\delta^{18}O_{\text{PO}_4}$) as a tracer for biological cycling of P. My aim was to use this technique to investigate the extent to which microbial turnover of organic P plays in seagrass uptake of P across the salinity and P availability gradient. However, my attempts to measure $\delta^{18}O_{\text{PO}_4}$ in seagrass and sediment samples were unsuccessful, despite successfully processing and obtaining correct $\delta^{18}O_{\text{PO}_4}$ for international isotope standards. Extremely low phosphate concentrations and high salinity (>50‰) prevented successful extraction of pure silver phosphates and led to incorrect $\delta^{18}O_{\text{PO}_4}$ for Shark Bay seagrass and sediment samples. As such, Chapter 6 is included here as a brief discussion of the methodological challenges of applying this technique to the seagrasses and sediments of Shark Bay, which provides a basis for future attempts for $\delta^{18}O_{\text{PO}_4}$ analysis in hypersaline environments.

Finally, Chapter 7 provides a general discussion of the outcomes of this research, their relevance to the understanding of organic matter dynamics and P cycling in the sediments of Shark Bay, and possible avenues for further research.

References


Phosphorus cycling in seagrass sediments


Phosphorus cycling in seagrass sediments


Phosphorus cycling in seagrass sediments


Chapter 2: Extreme climate events lower resilience of foundation seagrass at edge of biogeographical range

Matthew W. Fraser, Gary A. Kendrick, John Statton, Renae K. Hovey, Andrea Zavala Perez, Diana I. Walker

Preamble: This paper was published in the Journal of Ecology (Fraser et al. 2014), and is formatted to the requirements of the journal (including having a summary instead of a traditional abstract). The work is primarily my own. GAK and DIW received the funding for this project, and assisted me with experimental design. GAK, JS, RKH and AZP all helped me with sample collection and processing. All co-authors provided feedback during the writing of the manuscript. In this chapter, I document the widespread defoliation and dieback of the dominant seagrass *A. antarctica* in Shark Bay following a marine heatwave and flood event. This led to high inputs of seagrass-derived OM entering sediments, with implications for later chapters.

Summary

1. Extreme climatic events will dictate the response of ecosystems to climate change, yet are understudied in marine ecosystems. The interaction of stressors from such events has the potential to amplify negative impacts and drive ecosystems into alternate states.

2. Here, we show a drastic response of a temperate seagrass species (*Amphibolis antarctica*) in Shark Bay - a World Heritage Site in Western Australia at a temperate-tropical transition zone - to two stressors driven by concurrent extreme climatic events: a marine heatwave (Ningaloo Niña) and the Gascoyne Floods that impacted the west coast of Australia in the austral summer of 2010-2011.

3. Widespread defoliation (leaf loss) of *A. antarctica* was observed in the months following the extreme events, and were highest at sites affected by flooding (Wooramel River Floods). We propose that the negative impact was magnified by the synergistic interactions both stressors had on the carbon balance of the plant. The elevated temperatures increased plant demand for carbon, which could not be met through photosynthesis due to turbid floodwaters reducing light availability, resulting in the plant having a negative carbon balance.

4. Recovery of leaf biomass was evident two years after the extreme events, though still 7-20% of historical averages. In contrast, below-ground biomass decreased by an order of magnitude in the two years following the events. As below-ground reserves underpin the tolerance of large seagrass species like *A. antarctica* to disturbances, the declining trajectory of below-ground biomass will likely manifest as a loss of resilience in *A. antarctica* to future disturbances.

5. *Synthesis.* Given the ecological importance of *A. antarctica* in Shark Bay as a foundation species - accounting for 85% (~ 3700 km²) of the cover of seagrasses in Shark Bay - predicted increases in the frequency and magnitude of similar climatic events could have catastrophic implications for the future of this World Heritage embayment. Where extreme climatic events overlap and cause multiple, synergistic stressors to plant communities, ecological responses are likely to be more extreme, particularly in ecosystems where foundation species exist near upper thermal tolerance limits.
Introduction

Climate change research is typically concerned with gradual climatic trends, yet extreme climatic events will also dictate the response of ecosystems to climate change. Understanding how ecosystems respond to extreme climatic events is therefore necessary to predict how ecosystems and biodiversity will respond to climate change (Jentsch, Kreyling & Beierkuhnlein 2007), especially as extreme events are predicted to become more frequent and intense (Easterling et al. 2000). In particular, understanding the response of foundational plant species to extreme climatic events is important, as this will largely shape the ecological response at an ecosystem scale (Jentsch et al. 2007; Royer et al. 2011). However, ecological responses to extreme climatic events can be variable and hard to predict due to species- and system-specific responses (Beierkuhnlein et al. 2011; Arnone et al. 2011; Smith 2011a). To date, most research has focused on the response of terrestrial ecosystems to extreme climatic events, but marine ecosystems may also exhibit extreme ecological responses to these events.

Temperature is unsurprisingly the main focus of climate change research in marine ecosystems, and the relationship between extreme temperatures and physiological performance is relatively well understood (Harley et al. 2006). Temperature, however, is only one of a suite of stressors likely to impact marine ecosystems as a consequence of extreme climatic events. Shifts in freshwater inputs, wave energies, and sediment inputs are other stressors that could result from extreme climatic events in marine ecosystems (Doney et al. 2012). These stressors are unlikely to act singly in natural ecosystems, especially during extreme climatic events. Instead, multiple stressors can result from one extreme climatic, the overlap of two or more extreme climatic events, or in systems that have pre-existing stressors unrelated to extreme events. When these multiple stressors interact, the result can be synergistic where the combined effect is greater than the sum of individual stressors (Darling & Côté 2008). These combined events have the potential to evoke extreme ecological responses (Smith 2011a), reducing resilience or even push ecosystems into alternate states. Chesapeake Bay in the US provides a classic example of a system being pushed into an alternate state as a result of synergistic stressors (Orth & Moore 1983). Accumulated loss of oyster beds, as well as the loss of other buffers such as seagrass, wetlands and forests weakened the bay’s resilience and left it vulnerable to torrential runoff brought by an extreme climatic event - Tropical Storm Agnes - in 1972.
Phosphorus cycling in seagrass sediments
(Orth & Moore 1983). Multiple stressors are particularly concerning for management as the combined effect are notoriously difficult to predict, yet have the potential to erode resilience and lower the threshold for sudden phase shifts in ecosystems (Harley et al. 2006; Hawkins et al. 2008).

The ability of a system to retain essential functions, structure, identity and feedbacks depends on multiple attributes such as the latitude (the maximum amount a system can change before losing the ability to recover), resistance (the ease or difficulty of changing the system) and precariousness (how close the current state of the system is to a limit or threshold) (Walker et al. 2004). The final determining aspect of resilience is how the above three attributes are influenced by states and dynamics of the system at scales above and below the scale of interest (panarchy). As such, not all organisms or ecosystems are equally susceptible to state shifts driven by climate related stressors. In ecosystems where foundation species are at risk from multiple stressors, strong panarchical connections mean that any associated mortality has the potential for catastrophic ecosystem-level effects. Foundation species, like keystone predators (Paine 1966), have a disproportionately large impact on the ecosystems they grow in, with significant cross-scale interactions creating locally stable conditions that support other species and ecosystem processes (Ellison et al. 2005). Loss of foundation species can lead to bottom-up impacts that permeate through the trophic structure of the ecosystem. For example, loss of foundational tree species in North American forests has decreased biodiversity and altered ecosystem processes such as decomposition and nutrient cycling (Ellison et al. 2005). Ecosystems where foundation species exist close to physiological thresholds (e.g. the edge of their biogeographical range) increase the precariousness of the current state of the system, making them particularly vulnerable to multiple stress events, and therefore escalate the potential for catastrophic loss of ecosystem function and services.

Shark Bay is a World Heritage listed marine embayment with a foundational plant species at the edge of its biogeographic range. The temperate seagrass *Amphibolis antarctica* is the dominant primary producer in Shark Bay, covering approximately 3700 km² (~85% of seagrass covered area) of Shark Bay (Walker, Kendrick & McComb 1988), undoubtedly making it the foundation species. One of the key environmental values that this foundation species supports, and that the World Heritage status aims to preserve, is
high marine biodiversity, including significant populations of dugongs, turtles, and tiger sharks (Kendrick et al. 2012). Fish and dugongs forage on *A. antarctica* (Wirsing, Heithaus & Dill 2007; Burkholder et al. 2013) and further evidence suggests that it is indirectly responsible for the characteristic salinity gradient in the Bay, reducing water flow into the upper reaches of the embayment allowing for the development of the world’s most abundant population of stromatolites – mushroom shaped mounds made from layers of cyanobacteria and sediment that form reefs and that are modern analogues for some of Earth’s earliest life - in the hypersaline areas of Shark Bay (Logan, Cebulski & Logan 1970; Jahnert & Collins 2011). These seagrass meadows also sequester some of the highest below-ground carbon stores seen in seagrass meadows worldwide (Fourquarean et al. 2012a). *Amphibolis antarctica* in Shark Bay are near the upper limit of the species thermal tolerance. As such, *A. antarctica* growing in Shark Bay is in a more precarious position when exposed to above-average temperatures or other stressors (Walker & Cambridge 1995). Given the panarchial connections of foundation species, any loss of *A. antarctica* in Shark Bay may have severe consequences for the entire ecosystem and drive it to an alternate state.

Here, we show a potential decrease of resilience in *Amphibolis antarctica* meadows in Shark Bay, with short term (0-6 months) leaf loss and long term (2 years) loss of below-ground biomass. We link this loss of resilience to the synergistic stressors of an extreme temperature event (a marine heatwave) and extreme rainfall events (the Gascoyne Floods) that impacted the west Australian coast in the austral summer of 2010-2011. We then discuss the potential future trajectories of *A. antarctica* in Shark Bay given projected increases in extreme climatic events, and discuss potential ecosystem level implications for the Shark Bay World Heritage Area.
Phosphorus cycling in seagrass sediments

Methods

Study site

The study was conducted at the eastern embayment of Shark Bay, Western Australia (25°55' 60 S, 113° 32' 32 E), a 13000 km² marine embayment (Fig. 2.1a,b). The climate of Shark Bay is hot and dry, with annual evaporation (2000mm) being ten times annual precipitation (200mm). There is a strong salinity gradient in the eastern embayment (36 to > 65‰), driven by the high evaporation rates and restricted circulation with Indian Ocean water (Kendrick et al. 2012). Water temperatures in Shark Bay range from 17°C in winter to 26°C in summer (Walker et al. 1988). Shark Bay is oligotrophic, with phosphorus concentrations below detectable limits in the water column (Fraser et al. 2012). Shark Bay contains some of the most extensive seagrass meadows in the world, and contains up to 12 different species of seagrass (Walker et al. 1988), making it a global hotspot of seagrass diversity. Shark Bay is dominated by monospecific meadows of A. antarctica that cover 3700 km² of the Bay. Posidonia australis and Halodule uninervis (Forsk.) Aschers. also occur in large monospecific meadows in the Bay (Walker et al. 1988). The Wooramel River is the only river that discharges directly into Shark Bay, though only flows during periods of cyclonic events and winter storms. Data on daily sea surface temperatures were downloaded from NOAA’s ¼ degree gridded Advanced Very High-Resolution Radiometer Optimally Interpolated Sea Surface Temperature (Reynolds et al. 2007). Mean monthly sea surface temperature was then calculated from this data. Data on monthly discharge from Wooramel River were downloaded from the Department of Water, Western Australia (http://kumina.water.wa.gov.au/waterinformation/wir/reports/publish/703002/g02.htm).

Spatial extent of Amphibolis defoliation

Twenty-three sites across the eastern embayment were visited in March 2011 and 11 sites were visited in September 2011, incorporating a range of distances to the Wooramel River (6.5 km to 29.9 km; Fig. 2.1c). Water depth was similar across all sites (1-3 m deep). Five sites within 15 km of the River were visited once in March 2011, once in September 2011, and once in March 2013, to investigate longer-term recovery in sites where greatest defoliation was recorded. Amphibolis antarctica was collected at each site with a 25 cm diameter core (18 cm deep)
from the centre of each of five 0.25 m² plots that allowed for both above and below-ground biomass to be sampled in entirety. Plots were randomly chosen by dropping a quadrat from the boat, and flipping the quadrat three times for each individual plot. The entire contents of the core (including below-ground seagrass biomass and sediment) were extracted intact, washed free of sediment and sorted into species. All samples were stored on ice in the field, and then frozen immediately for later processing. Seagrass samples were thawed and epiphytes and sediments removed from seagrass leaves by gentle scraping with a scalpel under flowing deionised water. *Amphibolis antarctica* samples were separated into leaves, shoots, rhizomes, and roots. All dead seagrass biomass (necrotic) was removed prior to weighing. Seagrass was oven dried at 60°C until constant weight, and weighed to estimate biomass.

**Data analysis**

Leaf biomass was regressed against distance from the Wooramel River. Differences in leaf and below-ground biomass were determined using ANOVA. A generalized additive model was used to determine the relationship between seagrass biomass and distance from Wooramel River. Data were transformed where assumptions of heterogeneity were not met. R (version 2.13.1) was used for all statistical analysis.
Fig. 2.1 Location of the study area. (a) Location of Shark Bay. (b) Location of the study site in the eastern embayment of Shark Bay. (c) Location of individual sites within the study area. White and black boxes show sites that were visited in March and September 2011 to determine the effect of proximity to the Wooramel River on seagrass biomass. Black boxes show sites that were visited in March 2011, September 2011, and March 2013 to determine seagrass biomass and potential recovery after the extreme climatic events. Sampling protocols were consistent across all sites and study periods.
Results

Extreme temperature and rainfall

In 2011 an anomalous rise in sea surface temperatures (SST) was observed off the west coast of Australia, with peak SST 5°C warmer than normal (Feng et al. 2013). This marine heatwave, dubbed ‘Ningaloo Niña’, was driven by intense Leeuwin current flows, an extraordinary La Niña, and multi-decadal trends in the Pacific Ocean (Feng et al. 2013). The combination of these events was unprecedented, and can therefore be considered an extreme climatic event (Smith 2011b).

In Shark Bay, the mean monthly SST reached a maximum of 29°C in February 2011, and was above 26°C in January, February, March, and April 2011 (Fig. 2.2a). Mean monthly SST was above 2006-2010 averages in January (2.8°C), February (3.8°C), March (2.0°C), and April 2011 (1.8°C).

A large rainfall event over the Gascoyne region of Western Australia occurred on the 15th December 2010. This rainfall resulted in high river flow of the Wooramel River from December 2010 – February 2011 (Fig. 2.2b), reaching a maximum of 300 Gl discharged in February 2011. This prolonged flood event was extreme for the Wooramel River; with river discharge higher in December 2010 (270 Gl) and February 2011 (300 Gl) than any other month in the previous decade (Fig. 2.2b). The floodwaters brought significant volumes of suspended terrestrial sediments, with flood plumes apparent 10-15 km from the River mouth (Figs 2.3a,b). In sites within 10 km from the river mouth, up to 10 cm of sediment was deposited on seagrass meadows (M.W. Fraser, unpublished data).
Phosphorus cycling in seagrass sediments

**Fig. 2.2** (a) Mean monthly sea surface temperature (°C) in Shark Bay, Western Australia between January 2004 and January 2012. Black outline denotes 2010/2011 ‘marine heatwave’. Data were downloaded from NASA’s MODIS Aqua 4km dataset. (b) Monthly discharge (Gigalitres) from the Wooramel River, between January 2004 and January 2012. Data were downloaded from the Western Australian Department of Water’s Wooramel River station.

*Response of seagrasses to flooding and temperature events*

*Amphibolis antarctica* defoliation (leaf loss) was apparent in meadows in March 2011 (Figs 2.3c,d). Mean *A. antarctica* leaf biomass ranged from 9.2 g m$^{-2}$ to 342.3 g m$^{-2}$ in
March 2011. Leaf biomass was significantly higher as distance from the Wooramel Delta increased in March ($R^2 = 0.694, p < 0.0001, n=23$). Leaf biomass only increased above 100 g m$^{-2}$ at sites further than 14 km from the Wooramel River mouth in March 2011 (Fig. 2.4a), and was always greater at sites further than 14 km from the River mouth. In September 2011, six months after the flood and heatwave events, this pattern in leaf biomass persisted (Fig. 2.4b). Leaf biomass was significantly higher as distance from the Wooramel River increased up to 15 km, after which leaf biomass stayed steady at around 180 g m$^{-2}$ ($R^2 = 0.694, p < 0.0001, n=23$). In the only other seagrass species encountered, *Halodule uninervis*, leaf biomass was not significantly affected by distance to the Wooramel River ($R^2 = 0.01, p=0.63, n=22$).

**Fig. 2.3** (a) Satellite imagery showing study area before the 2010/11 Wooramel River floods. (b) Satellite imagery showing study area 1 month after the 2010/11 Wooramel River floods (courtesy of Curtin University: January 08 2011 RSSRG). Note the flood plume extending out from the coastline. (c) Healthy *Amphibolis antarctica* meadow, showing plants with numerous green leaves. (d) Defoliated *Amphibolis antarctica* in Shark Bay, showing dead plants with all leaf material lost. Photo credit: Gary Kendrick.
Recovery of seagrass meadows

Mean leaf biomass at sites close to the river increased from 8.4 g m\(^{-2}\) to 39.9 g m\(^{-2}\) between March and September 2011 (Fig. 2.5a). By March 2013 - two years after the flood and heatwave events - mean leaf biomass at sites closer to the river had increased to an average of 45.4 g m\(^{-2}\). Leaf biomass was higher at sites further from the Wooramel River during all sampling periods, increasing from 52.9 g m\(^{-2}\) to 68.6 g m\(^{-2}\) between March and September 2011. Mean leaf biomass then increased to 116.1 g m\(^{-2}\) two years after the flood and heatwave event, still far lower than the mean leaf biomass measured in a comparable study using similar methodologies carried out in the same general location (e.g. at similar salinities on the Wooramel Delta) in the 1980s (~600 ± 200 g m\(^{-2}\); (Walker 1985)).

Mean below-ground biomass followed the opposite pattern to leaf biomass, decreasing between March 2011 and March 2013 across all sites (Fig. 2.5b). In sites closer to the Wooramel River, below-ground biomass decreased by an order of magnitude between March 2011 (208.4 g m\(^{-2}\)) and March 2013 (20.8 g m\(^{-2}\)). At sites further from the river, mean below-ground biomass decreased across all three sampling periods, falling from 284 g m\(^{-2}\) in March 2011 to 51 g m\(^{-2}\) in March 2013.
Fig. 2.4 Scatterplots comparing Amphibolis antarctica leaf biomass collected during (a) March 2011 and (b) September 2011 with distance from the Wooramel River. The blue lines represent generalised additive models fitted to the data, grey outline shows 95% confidence interval. Note the logarithmic scale on the y axis.

Discussion

In Shark Bay, Amphibolis antarctica is the most abundant seagrass species, occupying 3700 km² (~85% of seagrass covered area) of the shallow subtidal (1-5m depth, Walker et al. 1988), and creates locally stable conditions that support faunal communities and ecological processes (Wirsing et al. 2007; Heithaus et al. 2007), so any negative impacts of global climate change on A. antarctica would have far-reaching effects across the Shark Bay ecosystem. We have found that A. antarctica underwent defoliation after an
extreme climatic event (the marine heatwave). Where the marine heatwave overlapped with the turbid floodwaters from the Wooramel River (the result of another extreme event), there was a more extreme defoliation response (Figs 2.4 and 2.5). We propose that this was a consequence of the interaction between the elevated temperatures and higher turbidity that reduced light availability. Two years after the extreme events, there has been an increase in leaf biomass, suggesting *A. antarctica* is recovering, but below-ground biomass has declined. As below-ground reserves underpin the tolerance of seagrasses to perturbations (Alcoverro *et al.* 1999), the declining trajectory of below-ground biomass will likely manifest as a loss of resilience in *A. antarctica*. Thus, the reduced capacity of the systems foundation species to withstand future perturbations could potentially drive the Shark Bay ecosystem to an alternate state. More generally, ecological responses to extreme climatic events can be variable and hard to predict due to species- and system-specific responses (Smith 2011a). Ecosystems with foundation species growing near upper thermal tolerance limits (like *A. antarctica* in Shark Bay) will be more likely to show extreme ecological responses after extreme climatic events.
Fig. 2.5 Mean *Amphibolis antarctica* (a) leaf biomass and (b) below-ground biomass (rhizome and root biomass) collected in Shark Bay in March 2011 (0 months), September 2011 (6 months), and March 2013 (2 years after the heatwave/flood event). Dark grey bars denote sites within 12 km of the Wooramel River, and light grey bars denote sites further than 12 km from the mouth of the Wooramel River. Error bars indicate ± 1 s.e. Note that mean leaf biomass measured in the 1980s (600 g m\(^{-2}\); Walker 1985) would far exceed the scale of leaf biomass measured in this study.

While *A. antarctica* near the Wooramel River suffered extensive loss of biomass after exposure to both increased temperatures and highly turbid floodwaters, biomass was sustained away from the influence of the turbid waters where sufficient light was able to reach the benthos. This was common across Shark Bay, with meadows in turbid sites off Monkey Mia experiencing highest mortality of seagrasses, while sites characterised by clearer waters were less affected (J.A. Thomson, unpublished data). Large seagrass
Phosphorus cycling in seagrass sediments

species like *A. antarctica* have high light requirements because of their need to balance photosynthetic carbon production with the large respiratory demand from non-photosynthetic below-ground tissues (Hemminga, Marbà & Stapel 1999). Increases in water temperature cause greater respiratory demand for carbon in seagrasses (Ralph *et al.* 2007), and can also inhibit photochemical efficiency and damage photosynthetic pathways (Campbell, McKenzie & Kerville 2006). The interplay between these stressors has also been noted for *A. antarctica*’s congeneric *Amphibolis griffithii* in South-Western Australia (Lavery *et al.* 2009). The significance of catastrophic loss in biomass presented here is that these episodic climatic events are predicted to occur more frequently, eroding the resilience of a foundation species that is already close to its thermal threshold. As mentioned previously, diminished resilience of the foundation species leading to significant loss will likely have a cascading effect on the system, with potential for shifts to alternate states via ecological extinction of entire trophic levels (Ellison *et al.* 2005; Dobson *et al.* 2006). For a system like Shark Bay where the current ecosystem services such as high biodiversity exist due to the presence of *A. antarctica*, any loss of resilience in this species poses a threat to its ecological value and its ability to meet World Heritage status criterion.

Below-ground biomass declined for two years, suggesting that the flood and heatwave events may have a longer-lasting, legacy impact on *A. antarctica* stands, or that this reallocation of biomass may be a strategy for survival within this species. Seagrass use below-ground tissues as stores to initiate recovery or persist through unfavourable conditions (Alcoverro, Manzanera & Romero 2001). Reallocation of biomass from belowground compartments to leaves is a common plant response that contributes to adaptive capacity to changing environmental conditions (McConnaughay & Coleman 1999; Weiner 2004). Despite *A. antarctica* displaying this plasticity, the lack of elasticity (slow recovery time) may change the dimensions of basins of attraction within the resilience landscape, reducing the latitude and resistance to future perturbations. Recovery of leaf biomass at sites to levels measured in the 1980s (Walker 1985) would take 37-38 years at sites close to the river, and 17-18 years at sites further from the river, assuming a linear relationship derived from our data (Fig. 2.5a; \( y = 2.63x + 52.83 \) for sites within 12 km of the Wooramel River and \( y = 1.26x - 18.71 \) for sites further than 12 km from the river). However, climatic models predict an increase in intensity and
frequency of extreme events in Shark Bay (Alexander & Arblaster 2009). Even if decreased belowground biomass aids survival of *A. antarctica* by reducing respiratory C demand, should the frequency of extreme events exceeds recovery times of *A. antarctica*, then the resilience of *A. antarctica* to disturbances is likely to be eroded. Existing *A. antarctica* stands may persist in the short term, but in a condition where they are at more risk to future stressors such as increased temperatures, reduced water quality, disease and physical disturbance.

The flood and heatwave events have led to two responses in *A. antarctica* – a short term, highly visible reduction in leaf biomass; and a long term, less visible reduction in belowground biomass. This presents a clear message to marine conservationists and managers in Shark Bay and elsewhere; not to rely solely upon traditional above-ground metrics to capture ecosystem health and functioning. Management strategies that mitigate local stressors can promote resilience of benthic communities to inevitable disturbances such as increased temperatures (Russell *et al.* 2009). For example, recovery of coral reefs after bleaching events is quicker at sites without chronic human pressures (Carilli *et al.* 2009; Gilmour *et al.* 2013), leading to recommendations that local pressures such as overfishing and pollution should be reduced to aid resilience of corals to climate change (Thompson & Dolman 2010; Ateweberhan, Feary & Keshavmurthy 2013). The Wooramel catchment is dominated by pastoral farms with high rates of ungulate grazing that have led to widespread sediment erosion (Curry *et al.* 1994), which contribute to the loss of terrestrial vegetation and increase of sediment inputs during flood events (McKergow *et al.* 2005). The reduction of sediment inputs through catchment management would help increase long term survival prospects of *A. antarctica* growing on the Wooramel Delta.

As *A. antarctica* is a foundation species in Shark Bay, any loss would be expected to have bottom up influences across the ecosystem if it is not replaced by a functionally equivalent species. *Halodule uninervis* - another seagrass species – did not appear to suffer similar dieback to *A. antarctica* following the heatwave and flood events, though was slightly less likely to be observed in 2012 compared to 2007-2009 (Thomson *et al.* 2014). *Halodule uninervis* is a tropical seagrass species with a life history strategy well suited to disturbance regimes that include low light availabilities and high temperatures (Carruthers *et al.* 2002). *Halodule uninervis* relies on a sedimentary seed bank to persist
Phosphorus cycling in seagrass sediments

through periods of light deprivation, unlike *A. antarctica* that persists through underground biomass stores. As such, *H. uninervis* may replace *A. antarctica* at sites that are marginal for *A. antarctica* growth, such as the Wooramel Delta. However, these two species are not functionally equivalent, and loss of *A. antarctica* could lead to significant ecosystem-scale effects due to the ecological extinction of processes including those that maintain niche habitats for species of high conservation priority. Indeed, even small changes in the abundance of foundation species can potentially lead to feedbacks that cause ecosystem wide effects (Byers *et al.* 2006). Loss of *A. antarctica* could directly lead to loss of forage habitat for species of conservation concern including turtles and the world’s largest population of dugongs (Wirsing *et al.* 2007; Heithaus *et al.* 2007). The flow of important ecosystem services such as sediment trapping or carbon burial (Jahnert & Collins 2011; Fourquearan *et al.* 2012b) will also be significantly impacted, leading to significant ecological, social, and economical costs. Dead seagrass biomass would also enter detrital pathways (Walker, Kendrick & McComb 2006), altering biogeochemical cycling across this oligotrophic embayment. On a regional scale, loss of *A. antarctica* may also lead to a biogeographical range contraction for this endemic seagrass. Though care must be taken when interpreting changes resulting from a single extreme event due to the implicit lack of replication, the documented loss of a foundation species will likely have ecosystem scale consequences in Shark Bay.

State shifts can occur rapidly when drivers interact to alter competition between species (Suding & Hobbs 2009). These abrupt changes in the structure of the system tend to be maintained by feedback processes, making it difficult to return to the previous state (Mayer & Rieterk 2004). Shifts in marine communities from domination by temperate to tropical species has been noted in other marine plants and animals along the Western Australian coastline as a result of the 2010/11 marine heatwave (Wernberg *et al.* 2012), and loss of seagrass following marine heatwaves has been noted in the Mediterranean (Marbà & Duarte 2009). Poleward movement of *A. antarctica* to escape future warming trends would be severely restricted in Shark Bay given its geomorphology, and may lead to a reduction in connectivity of this species along the West Australian coastline (Opdam & Wascher 2004). Loss of *A. antarctica* from Shark Bay would result in dramatic changes to the food web and structure of the shallow sills and banks that are characteristic of the Shark Bay ecosystem, potentially leading to catastrophic collapse of the seagrass
dominated ecosystem. Large scale seagrass loss (>1000 km\(^2\)) in other seagrass dominated systems such as Chesapeake Bay and Cockburn Sound have resulted in significant impacts on benthic metabolism due to large quantities of organic matter entering detrital pathways, and subsequent changes in the physical environment can preclude future recolonization (Kendrick et al. 2002; Kemp et al. 2005). Clearly, multiple stressors resulting from extreme events must be considered alongside gradual warming trends to accurately predict, and mitigate against, the effects of climate change in marine ecosystems.

Acknowledgments

A NHT-II Caring for Country Project grant coordinated by Western Australian Marine Science Institute, and awarded to D.I.W. and G.A.K., funded this research. Additional funding for M.W.F. was received through the Ray Hart Memorial Scholarship, an Australian Geographic Bayerboost Scholarship, and Ph.D. funding from the School of Plant Biology, UWA. Logistical support was provided by the Shark Bay Ecosystem Research Project under the direction of Michael Heithaus, funded by NSF (OCE-0745606). We thank Yasha Hetzel for assistance in obtaining downloading SST data from NOAA, and two anonymous reviewers for comments that contributed greatly to the manuscript.

References


Phosphorus cycling in seagrass sediments


Kendrick, G.A., Fourqurean, J.W., Fraser, M.W., Heithaus, M.R., Jackson, G., Friedman,


Chapter 3: Seagrass derived organic matter influences biogeochemistry, microbial communities, and seedling biomass partitioning in seagrass sediments

M.W Fraser, J. Statton, R.K. Hovey, B. Laverock, G.A. Kendrick

Preamble: This paper was published in Plant & Soil (Fraser et al. 2015), and is formatted to the requirements of the journal. The work is primarily my own. GAK and JS received funding for the project. MWF, JS, and RKH contributed to upkeep and maintenance of tank system. BL and MWF led microbial analysis, MWF and JS led seagrass analysis, MWF carried out biogeochemical analysis. All co-authors contributed to experimental design and provided feedback during the writing of this manuscript. In this chapter, I examine the impacts of seagrass-derived OM on belowground biogeochemistry, microbial communities, and seagrass seedling physiology in a controlled tank environment. This provides context for the potential belowground effects of the dieback of seagrass discussed in the previous chapter, as well as leading into the further investigation of OM as a phosphorus source examined in subsequent chapters.

Citation: Fraser MW, Statton J, Hovey RK, Laverock B, & Kendrick GA. (2015) “Seagrass derived organic matter influences biogeochemistry, microbial communities, and seedling biomass partitioning in seagrass sediments” Plant and Soil. doi:10.1007/s11104-015-2721-0
Abstract

Aims Seedling establishment is a crucial life history stage in seagrasses, yet factors that affect seedling health are poorly characterized. We investigated if organic matter (OM) additions to sediments provided nutritional benefits for seagrass seedlings through microbial degradation.

Methods We tested the effects of sedimentary OM additions on *Posidonia australis* seedlings growing in tank cultures. We focused on sediment biogeochemical processes and microbial communities that may impact seedling growth and physiology.

Results Enrichment of sediments with OM changed microbial community composition (DNA-ARISA) and a significant increase in hydrolytic enzyme expression. Total seedling biomass did not differ between OM treatments, but above:belowground biomass increased with OM enrichment. Nitrogen and phosphorus concentration of seagrass leaves was lower with increasing OM.

Conclusions Seagrass derived OM has been considered a refractory store of carbon, yet here we show its deposition into sediments significantly alters belowground conditions. Remineralization of the OM changes both physical and chemical nature of sediments that leads to greater biochemical activity, change in microbial communities and greater investment into above ground photosynthetic biomass. The presence of OM may assist seagrass seedling survival during early development by enhancing root branching and stability in sediments, but is unlikely to provide nutritional benefits.

Keywords: bacteria, decomposition, enzymes, *Posidonia australis*, roots, seeds
Introduction

Inorganic nutrient concentrations are often below concentrations required to maintain primary productivity in marine sandy habitats where seagrasses dominate (McGlathery et al. 2001). The microbial breakdown of organic matter (OM) supplements primary productivity, and is especially important in low nutrient ecosystems such as tropical forests (Reed et al. 2011). Similarly, OM buried in marine sediments may represent an important source of nutrients to seagrasses (López et al. 1995; Evrard et al. 2005; Kilminister et al. 2006). The role of microorganisms in OM breakdown may be particularly important in seagrass sediments as these sediments contain high concentrations of seagrass derived OM (Kennedy et al. 2010). While this belowground pool has been widely investigated for its role in carbon (C) sequestration (Fourqurean et al. 2012), the ecological implications of these sediments have not been examined in great detail. Seagrass derived OM consists of a range of organic molecules that differ significantly in their biological availability, but most forms of OM are refractory and not readily available for direct uptake by seagrasses (Danovaro 1996). Seagrass therefore rely on the metabolic capabilities of their sedimentary microbes to convert complex molecules to more labile forms of dissolved OM and nutrients that are then available for uptake by seagrasses. Conversely, microbes may also reduce nutrient availability through immobilisation of available nutrients into microbial biomass.

Microbes primarily liberate nutrients from OM through the release of extracellular enzymes. Extracellular enzymes play a crucial role in all biogeochemical cycles by being proximate agents in crucial processes such as decomposition (Burns and Dick 2002; Burns et al. 2013). Enzymes play a complex role in the degradation of plant litter, with numerous enzymes needed to hydrolyse OM at different stages. Plant litter comprises a high proportion of recalcitrant C rich macromolecules such as cellulose, lignin, or hemicellulose. These macromolecules are broken down through the combined enzyme activities of a diverse range of microorganisms (Burns and Dick 2002; Burns et al. 2013). For example, β-glucosidase breaks down cellulose, while α-glucosidase hydrolyses starch (Dick 2011). Consequently, the release of nutrients via microbial enzyme production is partly dependent on the type and quantity of plant litter (Burns et al. 2013). For instance, expression of β-glucosidase and acid phosphatase (associated with C and phosphorus (P))
release from OM) in soils was higher in soils amended with grass litter relative to soils without litter addition (Dornbush 2007). Distinct shifts in the microbial community composition are also apparent during decomposition (Sinsabaugh and Follstad Shah 2012). These shifts can be directly (C availability, chemical composition of litter) or indirectly (redox, pH) related to the presence of OM. The addition of OM to sediments should therefore result in distinct belowground shifts in microbial communities and enzyme production.

The successful establishment of seedlings is a crucial life history stage in seagrasses. Recolonization through seedling establishment may be particularly important in large degraded areas with high rates of seagrass mortality that limits recovery through asexual propagation. Successful recovery would therefore likely depend on new recruits entering from adjacent populations; with several biotic and abiotic bottlenecks to successful settlement and growth of seedlings (Orth et al. 2005; Rivers et al. 2011). However, ecological factors influencing seagrass seedling survival post settlement have received limited attention, despite their importance for maintaining populations (Kendrick et al. 2012). In particular, the nutrient requirements of recently settled seedlings are poorly understood. Additions of inorganic nutrients (N and P) did not increase seedling growth or productivity (Statton et al. 2014). Instead, the release of nutrients from decomposing OM may be an important source of nutrients to *P. australis* seedlings. *Posidonia australis* seedlings grown in tanks with additions of OM showed increased root growth and productivity compared to those grown in control sediments (Statton et al. 2013). However, the belowground processes that increase this root growth are not known.

Here, we tested the effects of OM additions on *P. australis* seedling growth in tank cultures. We also investigated sediment biogeochemical processes and microbial communities that could explain seagrass responses to OM additions. We hypothesised that (i) enzymes involved in C and P cycling would have higher concentrations at higher OM concentrations, (ii) microbial communities would be significantly different in pots with OM compared to pots without OM, and (iii) seagrass seedlings would display higher leaf, root and rhizome biomass and nutrient (N and P) concentrations in sediments with higher concentrations of OM.
Methods

Seedling collection

*Posidonia australis* fruit were collected from Woodman Point (32°08’10”S, 115°44’31”E) during November 2012. Fruit were transported in seawater back to the Fisheries WA Marine Research Facility at Hillarys, Western Australia. Fruit were left to dehisce naturally over 3 days in a holding tank containing constant flow-filtered seawater. Dehiscence of fruit resulted in the negatively buoyant *P. australis* seeds (with emerging radicle and prophyll) collecting in numbers on the bottom of the tank. We assessed seeds for viability based on the presence of an intact seed, developing prophyll, and radicle.

Experimental design

The effect of OM addition on seagrass seedling growth and sediment biogeochemistry was investigated using three tanks, each containing eight replicates of six organic matter treatments. Tanks received a 12h/12h light regime supplied by artificial metal halide lamps positioned above each tank (250 μmol photons m⁻² s⁻¹ or 10.8 mols photons d⁻¹), with constantly replaced seawater filtered to 0.2μm. Replicate seedlings were planted into square pots (70 x 70 mm, 300 mm height) containing one of the six OM treatments (0, 0.5, 1, 1.5, 2, 2.5% sediment dry weight). Given the quantitative nature of our treatments, we used a ‘replicated regression’ design to increase the power and flexibility of our experiment (Cottingham et al. 2005). These OM concentrations represented levels common in natural seagrass sediments, and are comparable to previous *P. australis* aquaculture experiments (Statton et al. 2013). Sediment was collected from Woodman Point beach. This sediment was primarily calcareous, and contained negligible quantities of OM (<0.1% dry wt.) Sediment was dried at 60°C for 72 hours prior to addition to pots. Organic matter was sourced from Woodman Point beach wrack and consisted primarily of *Posidonia sp.* leaves, roots and fibrous leaf sheaths. Organic matter was dried at 60°C for 3 days and ground to <2mm using a coffee grinder. Sediments were homogenously mixed to a known mass of organic matter according to treatment (Table 1). A total of 144 replicate seedlings were each randomly planted in individual pots with a randomly assigned treatment. Seedlings were cleaned by hand every 2-3 days to ensure no algal overgrowth that could limit light availability.
Post-harvest measurements

All seedlings were harvested after six months. Prior to harvesting (7 days), five replicate shoots from each treatment within each tank were marked by punching a hole with a pin through all leaves at the top of each shoot sheath (Short and Duarte 2001). After harvesting, leaves were removed then separated into old and young leaves with ages based on position on shoot (oldest leaf with the lowest vertical position on a shoot, and successively younger leaves positioned in ascending order along the shoot). New leaf growth was defined as the distance between the initial marking scar and base of the leaf (young and old leaves), plus any new unmarked leaves (young) formed during the seven day period. New leaf growth was dried (see above) and weighed for leaf productivity estimates. Seagrass biomass was determined from five replicates in each tank. Seedlings were cleaned of epiphytes and separated in component parts (individual leaves, roots, and rhizomes). These components were then dried at 60°C for 72 hours and weighed to obtain biomass. Due to low biomass, leaves from each treatment in each tank were pooled before nutrient and stable isotope analyses. Leaves were ground to a fine powder using a steel ball-mill. Carbon (C) and N concentrations were determined using an Automated C/N Analyser-Mass Spectrometer consisting of a 20/22 mass spectrometer connected to an ANCA-S1 preparation system (Sercon, Crewe, UK) at the Western Australian Biogeochemistry Centre at the University of Western Australia. All samples were standardized using multi-point normalization against a secondary reference of Radish collegiate (3.167% N, 41.51% C.), which was in turn standardized against primary analytical standards (International Atomic Energy Agency, Vienna) (Paul et al. 2007). The external error of analyses (one standard deviation) was no more than 0.1 for C:N ratio. Phosphorus contents of seagrass and sediment samples were determined using a dry-oxidation and acid hydrolysis technique, followed by a colourimetric analysis of the phosphate concentration of the extract (Fraser et al. 2012). Phosphate concentrations were compared with Australian Soil and Plant Analysis Council Standards 102 (Pinus radiata) and 105 (Eucalyptus sp.), which were treated accordingly. We found this technique to yield 94-101% of the reported P content of ASPAC Standards 102 and 105. Elemental concentrations of seagrass and sediment samples were calculated as a percentage of dry weight, and converted to elemental ratios.
Phosphorus cycling in seagrass sediments

**Sediment biogeochemistry**

The activities of four hydrolytic enzymes were measured using standard colourimetric methods (Dick 2011). The enzymes and substrates used were (i) α-glucosidase assayed with 4-Nitrophenyl α-D-glucopyranoside, (ii) β-glucosidase assayed with 4-Nitrophenyl β-glucopyranoside, (iii) acid phosphatase and (iv) alkaline phosphatase both assayed with 4-nitrophenyl phosphate disodium hexahydrate. Briefly, 1.00 g of sediment was added to a 50 ml Erlenmeyer flasks with 4 mL of modified universal buffer (pH=6.00 for α-glucosidase and β-glucosidase, pH=6.50 for acid phosphatase and pH=11.00 for alkaline phosphatase) and 1 mL substrate, and were incubated at 37 ºC for one hour. Following incubation, 1 mL of 0.5M CaCl₂ and 4 ml of 0.5M NaOH (for phosphatases) or 4 ml 10mM tris(hydroxymethyl) aminomethane (pH=12, for glucosidases) were added to stop the reaction. The final nitrophenol concentrations were determined photometrically at 400 nm. Resin extractable P was measured using anion exchange membranes that are a good representation of P that is available for uptake by plants (Cooperband and Logan 1994; Cheesman et al. 2010; Bünemann et al. 2013). Membranes were cut into 4 cm x 1 cm strips, and four were inserted into sediments in each pot. The membranes were left for four weeks to reach equilibrium with porewaters, before being removed, rinsed in DI water, and placed in 30 ml 0.5M HCl. Resin extractable P was then measured from HCl extracts using the Murphy Riley method for colourimetric determination of P concentrations (Murphy and Riley 1962). Sediment redox potential was measured six months after sediments were installed using a platinum electrode connected to a pH electrode and measured as millivolts. Platinum electrodes were left in sediments for 48 hours to allow biogeochemical profiles to be re-established after initial disturbance. No data was collected for redox potential in 2.5% OM treatments due to logistical difficulties. Sediment penetration resistance was measured using a Pocket Penetrometer (Humboldt Mfg. Co., Illinois, US).

**Microbial molecular community identification**

Surface sediment samples (2 ml, 0-2 cm depth) for DNA extraction and genetic analysis were collected from pots using a 3 ml syringe. Samples were transferred to a 2 ml cryovial, and immediately frozen in liquid nitrogen (N) before storage at -80ºC. Sediment samples were then homogenized using a sterile metal spatula, and 0.5 g was added to a 2 ml Eppendorf tube. DNA was extracted from sediments using the MP Bio Fast DNA
SPIN kits for soil (MP Bio, California, United States of America) with some modifications (Abell et al. 2011). DNA was eluted in nuclease-free water and DNA extraction was confirmed by agarose gel electrophoresis.

DNA was amplified using universal bacterial primers 16S-1392F (5'-GYACACACCGCCCGT-3’) and a 5'HEX labelled version of the reverse primer 23S-125R (5'-GGGTTBCCCCATCRG-3’) (Hewson and Fuhrman 2004). 50 μL reactions containing 1 μL DNA, 25 μL of GoTaq Green Master Mix (Promega, Australia), 2 μL of Bovine Serum Albumin (Promega), 1 nM of each primer were amplified as follows: initial denaturation at 94°C for 3 minutes, followed by 35 cycles of 94°C for 1 min, 55°C for 1 min 30 sec and 72°C for 1 min 30 sec, and a final extension step of 72°C for 10 min. Successful amplification was confirmed by gel electrophoresis, and PCR products were purified using AMPure magnetic beads (Agencourt, Beckman Coulter Life Science, USA) according to the manufacturer’s instructions, resuspended in 40 μL of TE buffer and quantified using a Nanodrop 8000 spectrophotometer (ThermoScientific, Wilmington, USA).

Microbial community DNA was analysed by Automated Ribosomal Intergenic Spacer Analysis (ARISA). Approximately 25-40 ng of purified amplified PCR reaction was mixed with 0.4 μL of internal size standard (GS1200-Liz, Applied Biosystems) in deionised formamide and denatured at 94°C for 2 minutes. Denaturing capillary electrophoresis (on a 50cm capillary array) was carried out using an ABI 3130XL genetic analyzer (Applied Biosystems). ARISA profiles were analysed using GeneMapper v3.7 (Applied Biosystems), with peak height as a measure of community presence, and a peak threshold of 50 relative fluorescence units greater than the baseline.

While the drying of sediments likely altered the initial microbial community composition associated with the sediments, this was necessary to ensure the quantitative accuracy of each OM treatment. All OM treatments were received the same protocol, removing any potential treatment effect. Any introduced sampling artefacts would be minimized by collecting samples for microbial community identification at the end of the experiment (i.e. six months after being submerged), giving microbial communities time to restablish.

Statistical analysis

Relationships between seagrass biomass, productivity, nutrient content, sediment enzyme
expression and organic matter content were analysed using linear regression analysis. Prior to analysis, data were checked for normality and homogeneity of variances. The relationship between the sediment microbial community and measured environmental variables was investigated using a distance-based multivariate multiple regression model (distance linear based model [DistLM]) marginal tests with a Bray-Curtis distance matrix based on 9,999 permutations. Seagrass and sediment biogeochemistry data were analysed using R version 3.1.1 (R Core Team 2014) using the base program and the ‘ggplot2’ package for graphics (Wickham 2009). Variation in microbial community structure between samples was analysed in PRIMER v6.0 (Clarke and Gorley 2006) with the PERMANOVA+ Add-on (Anderson et al. 2008).

Results

Sediment biogeochemistry

Redox potential in sediments strongly decreased with the addition of organic matter (Fig. 3.1a, $R^2 = 0.86$, $p<0.0001$, $n=15$), indicating more reducing conditions in sediments. Redox potential ranged from a maximum of -92.9 mV ($\pm$ 9.9 mV) in sediments with 0% OM to -375.4 mV ($\pm$14.2 mV) in sediments with 2.0% OM. Penetration resistance in sediments was significantly greater with the addition of organic matter (Fig. 3.1b, $R^2 = 0.93$, $p < 0.0001$, $n=18$), indicating a higher force needed for roots to penetrate into sediments. Resin extractable P tended to decrease in sediments as the concentration of OM increased, although this was not statistically significant at $p = 0.05$ ($R^2 = 0.15$, $p = 0.059$, $n=24$, data not shown).

Enzymes involved in C cycling showed variable responses to OM enrichment (Fig. 3.2). $\alpha$-glucosidase expression was not significantly related to concentration of OM in sediments (Fig. 3.2a, $R^2=0.08$, $p=0.13$, $n=18$). $\beta$-glucosidase expression significantly increased as the concentration of OM in sediments increased, indicating the potential for increased cellulose degradation (Fig. 3.2b, $R^2 = 0.79$, $p<0.0001$, $n=24$).
Fig. 3.1 Relationship between sediment organic matter % and (a) sediment redox potential (n=3) and (b) sediment penetration resistance (n=3). Black dots represent data points, dashed lines represent linear models fitted to the data, and the grey outline shows 95% confidence intervals. Note that no data was collected for redox potential in 2.5% OM treatments.

Enzymes involved in releasing phosphate from OM had higher expression in sediments with OM added, in comparison to the 0% OM treatment. Alkaline phosphatase expression also significantly increased as OM content in sediments increased (Fig. 3.2c, \( R^2=0.45, \ p=0.0009, \ n=24 \)). Acid phosphatase expression significantly increased as sediment OM concentration increased (Fig. 2d, \( R^2=0.53, \ p<0.0001, \ n=24 \)).
Fig. 3.2 Expression of the enzymes α-glucosidase (a, n=3), β-glucosidase (b, n=4), alkaline phosphatase (c, n=4), and acid phosphatase (d, n=3) in sediments with *Posidonia australis* seedlings ranging from 0% to 2.5% OM (d.w.). Enzyme activities are in μmol substrate consumed hr⁻¹ g⁻¹ dry sediment. Black dots represent data points, dashed lines represent linear models (where significant) fitted to the data, and the grey outline shows 95% confidence intervals.

**Microbial community structure**

The sediment microbial community composition was significantly altered upon enrichment with OM (PERMANOVA p<0.001). Pairwise differences between treatments tended towards significance where differences in OM content were largest. For example, there were no significant pairwise differences between the 0%, 0.5%, 1%, and 1.5% OM treatments, whereas the 2.5% OM treatment was significantly different to all treatments.
except 1.5% OM, which showed overlap with communities from enriched and non-enriched treatments.

The remaining environmental data measured in the experiment (hydrolytic enzymes, sediment redox potential, and resin P) only accounted for 16% of the total variation in the microbial community (Fig. 3.3). Changes in microbial communities were most strongly correlated to changes in sedimentary organic matter content (accounting for 12.2% of the variation), β-glucosidase expression (10.8% of total variation), and sediment redox potential (10.6% of total variation). All other variables accounted for <2% of the variation in the microbial community (i.e. over 50% of the microbial community variability is unexplained by our measured variables).

**Seagrass biomass**

Total seagrass seedling biomass did not significantly change with OM amendments (Fig. 3.4a, $R^2 = 0.003$, $p = 0.092$, n=90). Similarly, seedling leaf biomass did not change significantly with OM addition (Fig. 4b, $R^2 = 0.01$, $p = 0.302$, n=90). Organic matter
enrichment did not change total leaf productivity (Fig. 3.4c, $R^2 = 0.01$, $p = 0.47$, n=90) or productivity in young leaves ($R^2 < 0.01$, $p = 0.9$, n=90, n=19). There were strong relationships in the biomass allocation between different leaves among OM treatments. Biomass was significantly greater in older leaves at lower OM concentrations (Fig. 3.4d, $R^2 = 0.32$, $p < 0.0001$, n=90), while biomass was significantly greater in newer leaves at higher OM concentrations ($R^2 = 0.13$, $p = 0.016$, n=90).

**Fig. 3.4** Total seedling biomass (a), total leaf biomass (b), leaf productivity (c), and leaf specific biomass (d) for *Posidonia australis* seedlings growing in sediments with organic matter concentrations ranging from 0% to 2.5% OM (d.w.). Values are means from 15 replicates, with bars showing ± 1 s.e.

Rhizome biomass decreased significantly with sediment OM enrichment, although this relationship was weak (Fig. 3.5a, $R^2 = 0.059$, $p = 0.012$, n=90) and non-linear. Root biomass also decreased significantly with OM enrichment; again this relationship was
weak and non-linear (Fig. 3.5b, $R^2 = 0.047$, $p = 0.023$, n=90). Total belowground biomass decreased significantly upon OM addition (Fig. 3.5c, $R^2 = 0.078$, $p = 0.008$, n=90). The decrease of root and rhizome biomass at high OM led to a significant increase in above:belowground biomass with OM enrichment (Fig. 3.5d, $R^2 = 0.046$, $p = 0.024$, n=90).

![Graphs showing biomass changes with OM concentration](image)

**Fig. 3.5** Total rhizome biomass (a), total root biomass (b), total belowground biomass (c), and aboveground:belowground biomass ratios (d) for *Posidonia australis* seedlings growing in sediments with organic matter concentrations ranging from 0% to 2.5% OM (d.w.). Values are means from 15 replicates, with bars showing ± 1 s.e.

**Seagrass nutrient concentrations**

The nutrient concentration of seagrass leaves was variable with OM enrichment (Table
Carbon (R^2 = 0.05, p = 0.78, n=18), nitrogen (R^2 = 0.15, p = 0.06, n=18), and phosphorus (R^2 = 0.13, p = 0.08, n=18) concentrations in seagrass leaves did not significantly change with OM addition. However, the carbon:nitrogen (C:N) ratio of seagrass leaves significantly increased with increasing OM concentrations (R^2 = 0.41, p = 0.0042, n=18). Carbon:phosphorus (C:P) ratios of seagrass leaves tended to increase with elevated OM concentrations, though this relationship was weaker and not significant at the 0.05 level (R^2 = 0.20, p = 0.062, n=18). Nitrogen:phosphorus (N:P) ratios of seagrass leaves showed no significant relationship to OM concentrations (R^2 = 0.02, p = 0.2768, n=18). No significant relationships were detected between sediment OM and total seagrass nutrient content after correction for seedling biomass (i.e. total leaf biomass x C/N/P (%d.w.)).

Table 3.1 Carbon (C), nitrogen (N), and phosphorus content (% d.w), and carbon:nitrogen (C:N), carbon:phosphorus (C:P), and nitrogen:phosphorus (N:P) ratios of seagrass leaves growing in sediments with different concentrations of organic matter. Values are means ± standard error (in parentheses).

<table>
<thead>
<tr>
<th>Sedimentary organic matter concentration (% dry weight)</th>
<th>0%</th>
<th>0.5%</th>
<th>1%</th>
<th>1.5%</th>
<th>2%</th>
<th>2.5%</th>
</tr>
</thead>
<tbody>
<tr>
<td>C (% d.w.)</td>
<td>37.3 (0.3)</td>
<td>37.5 (0.1)</td>
<td>37.1 (0.3)</td>
<td>37.0 (0.2)</td>
<td>37.3 (0.5)</td>
<td>37.3 (0.2)</td>
</tr>
<tr>
<td>N (% d.w.)</td>
<td>2.0 (0.1)</td>
<td>2.0 (0)</td>
<td>1.9 (0.1)</td>
<td>1.9 (0.1)</td>
<td>1.9 (0.1)</td>
<td>1.9 (0.1)</td>
</tr>
<tr>
<td>P (% d.w.)</td>
<td>0.15 (0.01)</td>
<td>0.16 (0.01)</td>
<td>0.14 (0.01)</td>
<td>0.15 (0.002)</td>
<td>0.14 (0.004)</td>
<td>0.13 (0.02)</td>
</tr>
<tr>
<td>C:N</td>
<td>18.6 (0.3)</td>
<td>18.6 (0.2)</td>
<td>19.4 (0.7)</td>
<td>19.4 (0.5)</td>
<td>19.8 (0.1)</td>
<td>20.0 (0.4)</td>
</tr>
<tr>
<td>C:P</td>
<td>248 (17)</td>
<td>226 (16)</td>
<td>269 (12)</td>
<td>239 (4)</td>
<td>268 (7)</td>
<td>287 (33)</td>
</tr>
<tr>
<td>N:P</td>
<td>13.4 (1.1)</td>
<td>12.1 (0.9)</td>
<td>13.9 (0.1)</td>
<td>12.4 (0.4)</td>
<td>13.6 (0.30)</td>
<td>14.3 (1.4)</td>
</tr>
</tbody>
</table>

Discussion

Seagrass detritus is a ubiquitous component of OM in seagrass sediments. Consequently, established seagrass meadows have been considered as hotspots for C burial over long temporal and spatial scales (Fourqurean et al. 2012), but the ecological importance of this OM over smaller temporal and spatial scales is largely unknown. Here, we show the significant impacts of seagrass-derived OM on the biogeochemistry and microbial communities within sediments, and on biomass partitioning in *P. australis* seedlings.
Elevated seagrass OM concentrations in sediments strongly increased the expression of hydrolysing enzymes in sediments, confirming our first hypothesis. The enzymes examined were those involved with C and P release from complex organic molecules (Fig. 3.2). We found that the addition of OM led to distinct changes in biogeochemical conditions in sediments between treatments, which could alter sediment nutrient availability. Resin P tended to decrease with OM enrichment, and increased production of phosphatase indicated a likely alteration in the physiological demands of the microbial community. For example, the addition of OM could have released the microbial community from C limitation, shifting demands onto other nutrients (e.g. P) (Haugwitz and Michelsen 2011). The immobilization of P and N into microbial biomass could then reduce the concentrations in porewaters, resulting in increased plant-microbial competition for nutrients. An increase in plant-microbe competition for nutrients after OM enrichment has been well established in agricultural systems (Lehmann et al. 2011; Quilliam et al. 2012). Alternatively, the greater aboveground biomass in OM enriched treatments may have led to a dilution of potential nutrient impacts, though the necessary pooling of samples for nutrient analysis prevent us from exploring in further detail. Regardless, the ecological role of seagrass detritus on sediments is poorly known, and has largely been considered as a refractory pool of C due to low turnover rates (Mcleod et al. 2011). We suggest that in natural meadows, the deposition of detritus may increase competition for nutrients between seagrasses and microbes as meadows age and deposit more OM into sediments, shifting nutrient limitations as meadows develop. However, the extent of nutrient limitation would depend on other nutrient inputs, such as sedimentation of suspended materials, which are also enhanced by seagrass cover (Risgaard-Petersen and Dalsgaard 1998; Garcia and Duarte 2001). Clearly, the ecological role that seagrass detritus plays in coastal sediments must be considered when examining the biogeochemistry of seagrass meadows.

There were significant shifts in the sedimentary microbial community as OM concentrations increased, confirming our second hypothesis. Increased seagrass OM concentrations may have benefitted microbial groups that were actively able to utilise the substrate by releasing enzymes (Coolen et al. 2011; Khodadad et al. 2011), or by altering environmental conditions (Allison and Martiny 2008). We observed here that seagrass
OM addition resulted in more reduced sediments (Fig. 3.1a), which would favour microbes that require little or no oxygen, such as sulphate reducers (Rosselló-Mora et al. 1999). These bacterial taxa play an important role in OM remineralization in marine sediments, and within the plant rhizosphere may even form mutualistic relationships with seagrasses, due to the capacity of many sulphate reducers to fix N, thereby making it available for uptake by the plant (Welsh 2000). It appears that 1.5% OM in sediments may represent a threshold concentration, after which a significant shift in the microbial community composition occurs. It is unknown whether such a threshold would also hold true in situ; especially given the capacity of large seagrass root systems to oxygenate sediments (Greve et al. 2003; Pedersen et al. 2004; Borum et al. 2005), therefore buffering environmental conditions and, presumably, microbial metabolic processes. Given that sulphide intrusion from sediments has been suggested as a contributor of seagrass decline (Borum et al. 2013; Holmer and Hasler-Sheetal 2014), the role of sulphate reducers in seagrass sediments warrants further investigation.

There were modest decreases in root and rhizome biomass in seedlings growing in enriched OM treatments (Fig. 3.5). *Posidonia australis* exhibits strong architectural and morphological plasticity in root growth under changing environmental conditions. For example, *P. australis* growing in summer at Oyster Harbour, Australia had more lateral roots than in winter (Hovey et al. 2011; Hovey et al. 2012). Drivers of root plasticity are well known in many terrestrial plants, but factors that influence root growth and morphology in seagrasses are poorly characterised, despite the importance of seagrass roots for nutrient uptake and anchorage (Alcoverro et al. 2000; Belzunce et al. 2008). Our study shows that *P. australis* seedlings demonstrate plasticity in root growth even at an early developmental stage (<6 months after settling) when exposed to variable OM concentrations, even if the mechanisms for this are still unknown. Signalling compounds such as hormones may play a role in seedling root development, though have shown variable results in previous experiments with *Posidonia spp.* (Balestri and Bertini 2003; Glasby et al. 2015).

Seagrass OM addition led to limited changes in aboveground seedling biomass and productivity (Fig. 3.4, Table 3.1). The use of nutritional reserves in seedlings or uptake of nutrients from seawater may have homogenised responses to increased OM, even
where some treatments may confer an ecological advantage over a longer timescale. Though productivity did not significantly change between treatments, seedlings allocated more biomass to old leaves when growing in low OM sediments, and to new leaves when growing in high OM sediments. This suggests changes in leaf senescence rates. Leaf senescence is a plastic trait that is highly dependent on environmental conditions such as nutrient availability (Guo and Gan 2005). By signalling a high availability of C relative to N in old leaves, sugar accumulation triggers leaf senescence, remobilizing N into new leaves and preventing loss of N from the plant (Wingler 2005). Nitrogen accumulation was lower in high OM treatments (Table 3.1), suggesting that a decrease in N availability driven by OM additions may alter leaf senescence rates.

We did not find significant evidence supporting the previous hypotheses that seagrass derived OM assists *P. australis* seedlings through sediment microbial activity (Statton et al. 2013), but there are other ecological advantages that higher seagrass OM concentrations in sediments may provide to seedlings. Though sediment penetration resistance was greatest in sediments with higher OM concentrations (Fig. 3.1b), the level of physical force required to remove seedlings from sediments was also greater in sediments with higher OM concentrations (J. Statton, pers. comm). Sediment OM enrichment increases secondary and tertiary branching of *P. australis* roots (Statton et al. 2013), which increase stability in sediments (Grossnickle 2005). As a result, the presence of OM may assist seedlings in early survival by limiting hydraulic removal; a major bottleneck to seedling survival (Kirkman 1999; Statton et al. 2012). An increase in anchorage potential in organic rich sediments would have implications for seagrass recovery following dieback events that can input large volumes of seagrass-derived OM into sediments (Walker et al. 2006; Fraser et al. 2014). This may also have restoration implications, as hydrodynamic scouring is a major problem in seagrass restoration projects (Statton et al. 2012). Choosing sites with higher seagrass OM in sediment or preconditioning sediments with seagrass OM prior to transplanting may provide means of increasing restoration success.

The addition of seagrass OM to sediments also changed other physical conditions that could have influenced seedling health. Sediments with higher OM concentrations had significantly more negative redox potentials (Fig. 3.1a), indicative of lower oxygen
concentrations resulting from increased respiration rates. Increased OM concentrations in seagrass sediments also increase sulphide in porewater, which can contribute to seagrass dieback through sulphide intrusion (Borum et al. 2005). Seagrasses are particularly susceptible to sulphide intrusion during times of low oxygen concentrations. However, we found no decrease in seagrass health or survival in high OM treatments in this experiment. The contribution of sedimentary organic matter to toxic sulphide intrusion may be overestimated due to the nature and concentrations of OM added in previous experiments. For example, experimental additions of OM are often in unique forms that would not be found in significant concentrations in natural seagrass sediments such as sugar (Pérez et al. 2007) or sodium acetate (Ruiz-Halpern et al. 2008), and therefore would not be ecologically representative of in situ conditions. Similarly, conclusions drawn from natural experiments have often been conducted in highly degraded systems such as fish farms with anthropogenically derived OM additions that again may not represent conditions that are typically found in healthy seagrass meadows (Delgado et al. 1999). The dominant source of OM in most natural seagrass meadows would be seagrass detritus, especially in meadows dominated by large, long-lived species such as *P. australis* (Fourqurean et al. 2012). Seagrass detritus is notoriously refractory in nature. The high difference in lability between the OM additions in this and other experiments would result in very different below ground impacts - with differences in decomposition rates, and microbial and environmental shifts resulting from the additions. This is evidenced by the lack of negative impacts on seedlings here and in similar experiments (Statton et al. 2013), and the often-abundant growth of seagrasses upon sediments naturally enriched with seagrass derived OM. For example, seagrasses form highly productive meadows in blue carbon hotspots like the Mediterranean Sea and Shark Bay, Western Australia, even where sedimentary sulphide concentrations are high (Cambridge et al. 2012). The ecological consequences of OM enrichment of seagrass sediments will be highly dependent on the types and quantities of OM added. Future experiments should focus on adding OM types and concentrations that are representative of natural conditions if ecologically relevant conclusions are desired.
Acknowledgements

We thank the support staff at the Department of Fisheries, WA for logistical support throughout the experiment. We also thank the CSIRO Marine and Atmospheric Research (CMAR) Environmental Genomics team in Hobart, Tasmania, for their help with microbial community analyses; in particular Dr Guy Abell. This project was funded through Australian Research Council Linkage Grants to GAK and JS (LP100200429 & LP130100155). BL was funded by an Indian Ocean Marine Research Centre (IOMRC) Postdoctoral Fellowship, supported by AIMS, CSIRO and the University of Western Australia (UWA). MF supported by Ph.D. funding from the School of Plant Biology, UWA. We thank 3 anonymous reviewers who provided thoughtful comments that greatly improved the manuscript.

References


Phosphorus cycling in seagrass sediments


66


Chapter 4: Organic phosphorus accumulates in calcareous seagrass sediments with increasing salinity

Matthew W. Fraser, Gary A. Kendrick, Grzegorz D. Skrzypek, Lindsay T. Byrne, Pauline F. Grierson

Preamble: This manuscript will be submitted to Limnology and Oceanography, and is formatted to the requirements of the journal. The work is primarily my own. GAK received funding for the project. GAK and PFG assisted with experimental design. LB provided help with NMR analysis and interpretation. GAK, GS, and PFG all provided feedback on the manuscript. This chapter examines the different fractions of sedimentary phosphorus across the salinity gradient in Shark Bay sediments, including examining the organic P pool to the compound level using $^{31}$P-NMR, providing a unique insight into the organic P pool in seagrass ecosystems.
Phosphorus cycling in seagrass sediments

Abstract

Seagrasses growing on calcareous sediments in oligotrophic waters are often assumed to be phosphorus (P) limited given the high P adsorption capacity of these sediments. However, little is known of P speciation in seagrass sediments and if seagrasses are able to utilize P from other fractions such as calcareous bound-P or reducible P complexes in sediments. In particular, mineralization of organic P from seagrass litter could play an important role in reducing P limitation in oligotrophic seagrass meadows. Here, we sought to quantify the amounts and proportion of inorganic and organic fractions in calcareous sediments of Shark Bay, a subtropical embayment characterised by large seagrass meadows and a strong salinity gradient. We used a sequential extraction method to examine P fractions in sediments across a range of salinities (39-47‰). We also used \(^{31}\)P-nuclear magnetic resonance (NMR) to characterise the sediment organic P fraction to the compound level - the first time this has been done is seagrass sediments. We found that the most available P fractions (magnesium extractable P and reducible P) were not significantly related to salinity, but tended to be greater at low salinity sites, while the proportion of organic P in sediments significantly increased with salinity. The proportion of organic P was highly correlated with sedimentary organic matter content (R\(^2\) = 0.50, P < 0.0001). The only organic P compounds detected by NMR were phosphate monoesters, which are a readily mineralisable form of P and thus a potential source of P to seagrass. We propose that cycling of seagrass detritus is of increasing importance in maintaining P requirements of seagrasses as salinity increases and soluble reactive phosphorus in the water column decreases.

Keywords: \(^{31}\)P-nuclear magnetic resonance, nutrient availability, phosphate monoesters, sediment organic phosphorus, SEDEX, Shark Bay
Introduction

Seagrasses growing in oligotrophic coastal habitats with calcareous sediments and low phosphorus (P) inputs are often considered P limited because of low soluble reactive P (SRP) concentrations in overlying waters (Short 1987). However, root uptake of P from sediments can supply a substantial proportion (up to 100%) of the P requirements of seagrasses, particularly when growing in ecosystems with low concentrations of SRP in the water column (Jensen et al. 1998; Gras et al. 2003). Phosphates in seagrass sediments are present as a diverse range of compounds that differ in their availability for biological uptake (Koch et al. 2001; Holmer et al. 2006), yet for the most part are undescribed. The organic P fraction in particular is poorly understood in marine sediments, despite growing recognition of its ecological importance and complexity in terrestrial soils and freshwater sediments (Turner et al. 2005; Baldwin 2013). In seagrass sediments of Florida Bay, organic P can comprise almost half of the total sediment P (Koch et al. 2001). However, the chemical composition of organic P and therefore its potential mineralization and availability for uptake by plants is unknown. Further, the environmental drivers influencing the spatial distribution of different P fractions across marine ecosystems, including coastal embayments, are not well known.

Previous studies have shown that sediment P in coastal ecosystems can be strongly influenced by salinity of the overlying water column. Shifts in P fractions of seagrass sediments have been examined along freshwater-marine transitions to salinities from 5-37‰ at Florida Bay (Koch et al. 2001). This study found that porewater P did not increase with salinity, given the high adsorption capacity of the predominately calcareous sediments. Elsewhere, in an analysis of a temperate estuary in South Carolina, porewater P increased as salinity increased, but sediment sorbed P (measured as bicarbonate-extractable P) decreased (Sundareshwar and Morris 1999). However, this study did not assess concurrent change in organic P. Other studies have found that under high salinity, organic P compounds such as phosphate monoesters may become more mineralizable due to increased desorption from sediment surfaces, and thus available for root uptake (Monbet et al. 2009). However, it remains unclear if and how the distribution of P forms in calcareous sediments is affected under hypersaline (>40‰) conditions and how this may influence benthic production, limiting our understanding of P cycling and nutrient limitation in these ecosystems.
Different fractions of sedimentary P can be operationally grouped based on sequential extractions, which provide a more comprehensive assessment of overall P availability than simply measuring single extracts of inorganic P or total P. One such extraction technique is SEDEX (Ruttenberg 1992), which has been developed and modified for specific use with marine sediments. The SEDEX technique splits total sedimentary P into four operationally defined pools that differ in their bioavailability: loosely sorbed P, reducible P, calcium-bound P, and residual organic P. While sequential extraction methods necessarily define P pools based on the types of extractant used, the results can provide ecologically relevant information. For example, loosely sorbed P primarily consists of inorganic phosphate that is readily available for uptake by plants, whereas calcium-bound P is relatively unavailable for direct uptake by plants (Short 1987). Reducible P comprises P adsorbed onto reducible metal oxyhydroxides, and is strongly affected by the concentrations of metals in sediments such as Fe, Mg and Al, as well as sulfur that can sequester Fe and potentially release P (Koch et al. 2001). Splitting sedimentary P into such fractions provides detailed information about the potential availability of sedimentary P for biological uptake, therefore allowing more accurate predictions for biological responses to external P inputs.

Shark Bay is a large sub-tropical marine embayment with extensive seagrass communities that form some of the largest continuous meadows in the world (Kendrick et al. 2012). Salinity across Shark Bay ranges from 35‰ in the north to ~70‰ in the south (Kendrick et al. 2012). While the relationship between seagrass physiology and growth is reasonably well understood for Shark Bay (Walker 1985; Kendrick et al. 2012), remarkably little is understood of the interactions between seagrass distributions and sediment characteristics. However, sediments are known to be predominantly calcareous (mean = 71.3% CaCO₃) and sediment organic matter content (OM) also increases with salinity (Fourqurean et al. 2012). SRP concentrations in the water column are low throughout Shark Bay and frequently below detectable limits (<0.02 μM) in its southern, hypersaline reaches (Atkinson 1987; Fraser et al. 2012). As such, seagrasses in Shark Bay are considered to be P limited, especially in hypersaline areas (Burkholder et al. 2013). However, sediments may also represent a source of P to seagrasses given the dominant species (*Amphibolis antarctica* and *Posidonia australis*) can absorb nutrients via their root systems (Pedersen et al. 1997; Alcoverro et al. 2000). The highly variable P content
of seagrass leaves observed over small spatial scales in Shark Bay (~10km; Fraser et al. 2012) suggests that (i) root uptake from sediments at least partially meets P demand of these plants and (ii) that the distribution of labile P fractions in the sediments is heterogeneous.

In this study, our objective was to determine how the total and proportional concentrations of different phosphorus fractions varied in sediments along the salinity gradient in Shark Bay, and evaluate the potential of sediment as a P source for seagrasses. We used sequential extraction and $^{31}\text{P}$-NMR to determine the concentrations of different sediment P fractions and to identify dominant organic P compounds. Specifically, we hypothesized that: (i) calcareous-bound P will be the dominant fraction throughout the majority of sediments in Shark Bay, given that calcareous sediments dominate; (ii) concentrations of labile P (magnesium-extractable P and reducible P) would be lower and comprise proportionally less of the total sediment P at hypersaline sites, given the lower overlying SRP in the water column; (iii) concentrations of organic P fractions would be highest and comprise a greater proportion of total sediment P at high salinity sites, given that sediment OM increases with salinity (Fourqurean et al. 2012). We also identified organic P compounds across the salinity gradient in Shark Bay using $^{31}\text{P}$-NMR.

Methods

Site description & experimental design

Eight sites were selected across the eastern embayment of Shark Bay (Fig. 4.1). All sites ~1.5 km apart and had overlying water of 1-2m deep. Three sites (sites 6-8) were adjacent to the mouth of the ephemeral Wooramel River, the only river that flows directly into Shark Bay. The benthic community at all sites was dominated by *Amphibolis antarctica* with *Halodule uninervis* growing as an understory species. Salinity of the water column was determined at each site with a TPS 90FL-T analyser (TPS Pty Ltd, Springwood, Queensland, Australia). Salinity ranged from 37‰ at site 1 to a maximum of 46.7‰ at site 7 (Table 1).
Phosphorus cycling in seagrass sediments

Figure 4.1: Location of the study site at Shark Bay, Western Australia. Black circles denote sites. Haloclines showing salinity at time of sampling are shown in dashed lines, with corresponding salinity ranges measured in this study in italics. Inset shows location of Shark Bay.

Sediment cores

At each site, six replicate sediment cores (50 mm diameter x 500 mm length) were collected in March 2013. PVC Cores were driven into the sediment using a hammer, then carefully removed from the sediment, capped at both ends, and placed in a cooler with ice. Cores were frozen prior to transport back to the laboratory in Perth (800 km away) to prevent multiple thawing and freezing of samples that can influence P concentrations (Vaz et al. 1994). We acknowledge that this treatment may result in overestimation of inorganic phosphate and underestimation of pyrophosphates (Turner et al. 2007), but was unavoidable given the remoteness of the site. Each core was divided into six different depth sections: 0-2 cm, 2-5 cm, 5-10 cm, 10-20 cm, 20-30 cm, and 30-40 cm; each size section was then homogenized by mixing the sample in a sealed bag. Large seagrass
fragments and shells (>5mm) were removed. A 1 g sub-sample was taken from each bag for sequential P extraction, while the remainder was used for chemical and physical characterisation of sediments.

*Sediment chemical and physical characterization*

Sediment grain size fractions were determined using a dry sieving technique using 1180, 850, 500, 355, 250, 106 and 63 μm sieves (Endecotts Ltd.). The OM content of sediments was estimated from loss on ignition (LOI), which was determined by the difference in sediment weight after combustion of 1g sediment samples at 550 °C for 8 hours (Fourqurean et al. 2012). Elemental analyses of sediment NaOH/EDTA extracts (Al, Ca, Fe, Mg, Mn, P, S, and As) were performed on a PerkinElmer Optima 5300DV inductively couple plasma optical emission spectrometer (ICP-OES).

*Sequential P extraction of sediments*

We used a sequential extraction method for marine sediments (Ruttenberg 1992), with modifications for carbonate-dominated sediments developed by Jensen et al. (1998) and Koch et al. (2001). Magnesium extractable P, which is considered the most biologically available fraction, was extracted using 49 mL of 1 M MgCl₂ (pH = 8.00). 1 mL of 2 M sulfuric acid was added to the sample extracts for storage prior to P measurement. 50 mL of bicarbonate-dithionite solution (0.11 M NaHCO₃ and 0.11 M Na₂S₂O₄, pH = 7.00) was then added to the residues from the previous procedure to extract reducible P adsorbed by crystalline and amorphous iron and manganese compounds (Reducible P) (Jensen and Thamdrup 1993). Prior to preservation, the supernatant was bubbled with nitrogen gas (N₂) for 20 minutes and then the supernatant preserved with the addition of 4 mL of 1 N sulfuric acid, and 21 mL of deionised water. Preservation resulted in the precipitation of elemental sulfur, necessary to exclude sulfides and limit interference during analysis (Koch et al. 2001). Sediment residues from the bicarbonate-dithionite extraction were then extracted with 30 mL of 1 N HCl to measure calcareous-bound P (calcareous P). Sediments were shaken several times and purged to release pressure prior to extraction, until the release of CO₂ stopped. 20 ml of deionised water was then added to the samples. Residual organic phosphorus was then extracted from the remaining sediment using a dry-oxidation acid hydrolysis technique (Residual organic P) (Solórzano and Sharp 1980). Residual organic P comprises the majority of sediment organic P, although small
amounts of labile organic P compounds are likely to be lost in the preceding bicarbonate-dithionite extraction (Wang et al. 2013). After each extraction, inorganic phosphate concentrations were measured spectrophotometrically using a modified ascorbic acid method (Murphy and Riley 1962; Kuo 1996).

Solution $^{31}$P-NMR spectroscopy
Surficial sediments (0-10 cm) were extracted with a standard NaOH/EDTA reagent and analysed by solution $^{31}$P-NMR spectroscopy following the approach of (Cade-Menun and Liu 2014). Phosphorus was extracted by adding 20 g of sediment to a 50 mL centrifuge tube with 40 mL of 0.25M NaOH + 0.05M EDTA solution. The high ratio of sediment to extractant was necessary to obtain adequate NMR signals. Samples were then shaken for 16 hours, centrifuged (2000 rpm for 30 minutes) and then filtered through a Whatman #42 filter, with the supernatant being retained. Samples of the supernatant were then lyophilized and 1 g of freeze-dried extract was dissolved in 3.6 ml of deionised water, centrifuged (1400 rpm for 20 minutes), and then added to a 10 mm NMR tube with 0.4mL of deuterated water ($D_2O$).

$^{31}$P-NMR spectra were determined on each sample using a Bruker AV500 (Bruker, Germany) spectrometer at 298ºK. Data were acquired with a 90º pulse of 41.0 µs, relaxation delay of 10 s and an acquisition time of 1 s. Typically 5440 scans were acquired over 16 h 43 min. Chemical shifts were referenced to an orthophosphoric acid standard. The spectra presented have a line broadening of 5 Hz, and are $^1$H decoupled.

Data analysis
All data analyses were performed in R version 3.1.1 (R Core Team 2014) using the base program and ‘ggplot2’ package (Wickham 2009). Relationships between sediment characteristics, salinity, and sediment P fractions were determined using linear regression analysis. Relationships between sediment depth, salinity, and P fractions were determined using two-way ANOVA. Data were log transformed where normality and/or heterogeneity assumptions were not met. Peak assignment in $^{31}$P-NMR was based on previous reported procedure (Turner et al. 2003; Cade-Menun et al. 2005; Cade-Menun and Liu 2014). Changes in the organic P pool at the compound level were analysed.
visually based on $^{31}$P-NMR spectra, as quantitative analysis was not possible given the low signal:noise ratio encountered in these samples.

Results

General sediment variation along salinity gradient

Shark Bay sediments were predominately composed of particles in the size class 250-500 µm; however finer particles (106-250 µm) were more dominant at sites 1 and 7 (Table 4.1). Organic matter content ranged from 1% in surface sediments at site 1 (mean = 2.3%) to over 16% at 2-5cm depth at site 6 (mean = 11.0%). Organic matter concentration in sediments significantly increased with salinity ($R^2 = 0.63$, $P < 0.0001$, $n = 144$).

Calcium concentrations of sediments ranged between 2193 µg g$^{-1}$ at site 3 to 3137 µg g$^{-1}$ at site 1 (Table 4.2). Concentrations of iron and aluminium in sediments were also higher at higher salinity sites. Magnesium concentrations at site 8 were almost six-fold higher than any other site. Manganese was only detected at high salinity sites (6-8); sulfur concentrations were also highest at these sites. Arsenic was highest at sites 7 and 8, and lowest at site 2. Sediment sulfur concentrations were positively correlated with salinity ($R^2 = 0.8$, $P < 0.002$, $n = 8$).

Sediment P fractions

The concentrations and relative proportions of different sediment P fractions changed significantly across the salinity gradient in Shark Bay (Table 4.2). Both residual organic P concentration and proportion of total sediment P significantly increased with salinity. In contrast the concentration and proportion of calcareous P (notionally the least labile fraction) significantly decreased from >65% (60 µg P g$^{-1}$) to <40% (23 µg P g$^{-1}$) along the salinity gradient. Reducible P concentrations and proportions were weakly positively correlated to salinity. However, the proportion of magnesium-extractable P – the most available for biological uptake – did not change in concentration or as a proportion of total P along the salinity gradient.

Magnesium-extractable P was the smallest P pool in Shark Bay sediments, contributing an average of 7.4% to the total P in Shark Bay sediments. Magnesium-extractable P concentration was not significantly related to salinity ($R^2 = 0.02$, $P = 0.13$, $n = 144$).
Phosphorus cycling in seagrass sediments

Similarly, the proportion of total P present as magnesium-extractable P did not differ across the salinity gradient ($R^2 = 0.04$, $P = 0.2$, $n = 48$). Magnesium-extractable P was highest in sediments at intermediate salinities, with a mean of 8.9 μg P g$^{-1}$ (9.2% of total P) at site 4 and 7.7 μg (9.4%) at site 5. While not related to salinity overall, the highest salinity sites (sites 6, 7 and 8) consistently had the lowest concentrations of magnesium-extractable P (Fig. 4.2). Site 8 had the lowest mean concentration of magnesium-extractable P (2.2 μg P g$^{-1}$; 4.4%), while site 6 had the lowest concentrations at 20-30 cm (1.2 μg P g$^{-1}$; 3.8%) and >30 cm depths (1.2 μg P g$^{-1}$; 2.4%). Overall, magnesium-extractable P significantly decreased with sediment depth ($F_{5,138} = 47.0$, $P < 0.0001$, Fig. 4.2), but there was no significant interaction between magnesium-extractable P with salinity and sediment depth.
**Table 4.1:** Summary data for water and sediment chemical and physical characterization at each site. Sediment % fines is the percentage of sediments with a grain size under 106μm. Sediment aluminium (Al), calcium (Ca), iron (Fe), magnesium (Mg), manganese (Mn), sulfur (S), and arsenic (As) concentrations are all in µg g⁻¹.

<table>
<thead>
<tr>
<th>Site</th>
<th>Salinity (%)</th>
<th>Sediment OM (%)</th>
<th>Sediment fraction &lt;106 μm (%)</th>
<th>Al</th>
<th>Ca</th>
<th>Fe</th>
<th>Mg</th>
<th>Mn</th>
<th>S</th>
<th>As</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>39.5</td>
<td>2.3</td>
<td>8.3</td>
<td>0.02</td>
<td>3137</td>
<td>0.1</td>
<td>0</td>
<td>0.0</td>
<td>156</td>
<td>0.7</td>
</tr>
<tr>
<td>2</td>
<td>39.2</td>
<td>3.2</td>
<td>12.3</td>
<td>0.42</td>
<td>2402</td>
<td>0.3</td>
<td>1</td>
<td>0.0</td>
<td>125</td>
<td>0.2</td>
</tr>
<tr>
<td>3</td>
<td>42.4</td>
<td>2.6</td>
<td>5.2</td>
<td>0.34</td>
<td>2193</td>
<td>0.3</td>
<td>5</td>
<td>0.0</td>
<td>164</td>
<td>1.2</td>
</tr>
<tr>
<td>4</td>
<td>43.8</td>
<td>7.2</td>
<td>22.9</td>
<td>0.38</td>
<td>2975</td>
<td>0.2</td>
<td>8</td>
<td>0.0</td>
<td>497</td>
<td>1.0</td>
</tr>
<tr>
<td>5</td>
<td>43.9</td>
<td>5.0</td>
<td>12.4</td>
<td>0.14</td>
<td>3001</td>
<td>0.2</td>
<td>2</td>
<td>0.0</td>
<td>375</td>
<td>1.5</td>
</tr>
<tr>
<td>6</td>
<td>44.1</td>
<td>11.0</td>
<td>20.4</td>
<td>3.55</td>
<td>2798</td>
<td>1.1</td>
<td>59</td>
<td>0.9</td>
<td>862</td>
<td>0.9</td>
</tr>
<tr>
<td>7</td>
<td>46.1</td>
<td>11.1</td>
<td>23.0</td>
<td>9.79</td>
<td>2929</td>
<td>2.8</td>
<td>10</td>
<td>0.7</td>
<td>933</td>
<td>2.2</td>
</tr>
<tr>
<td>8</td>
<td>45.7</td>
<td>8.5</td>
<td>16.1</td>
<td>2.65</td>
<td>3050</td>
<td>0.9</td>
<td>1</td>
<td>0.1</td>
<td>705</td>
<td>2.8</td>
</tr>
</tbody>
</table>
Table 4.2: Mean sediment P fractions and proportions of total sediment P across Shark Bay, averaged across all sediment depths. PMgCl = Magnesium extractable P. All concentrations are in μg P g DW⁻¹, with standard errors in brackets (N=18).

<table>
<thead>
<tr>
<th>Site</th>
<th>PMgCl (μg g⁻¹)</th>
<th>Reducible P (μg g⁻¹)</th>
<th>Calcereous P (μg g⁻¹)</th>
<th>Residual organic P (μg g⁻¹)</th>
<th>PMgCl (% total P)</th>
<th>Reducible P (% total P)</th>
<th>Calcereous P (% total P)</th>
<th>Residual organic P (% total P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5.23 (±1.15)</td>
<td>5.70 (±2.01)</td>
<td>37.53 (±8.01)</td>
<td>8.36 (±0.90)</td>
<td>9.3</td>
<td>9.8</td>
<td>65.9</td>
<td>16.1</td>
</tr>
<tr>
<td>2</td>
<td>5.75 (±0.77)</td>
<td>6.69 (±1.05)</td>
<td>60.52 (±9.22)</td>
<td>13.74 (±1.09)</td>
<td>6.6</td>
<td>7.6</td>
<td>69.5</td>
<td>16.2</td>
</tr>
<tr>
<td>3</td>
<td>3.46 (±2.11)</td>
<td>4.84 (±0.90)</td>
<td>25.26 (±2.78)</td>
<td>7.23 (±1.77)</td>
<td>7.9</td>
<td>11.6</td>
<td>63.1</td>
<td>17.4</td>
</tr>
<tr>
<td>4</td>
<td>8.92 (±1.05)</td>
<td>10.51 (±2.24)</td>
<td>47.92 (±6.6)</td>
<td>25.47 (±2.59)</td>
<td>9.2</td>
<td>10.7</td>
<td>52.0</td>
<td>28.1</td>
</tr>
<tr>
<td>5</td>
<td>7.68 (±0.91)</td>
<td>10.04 (±1.79)</td>
<td>45.65 (±7.62)</td>
<td>15.81 (±3.24)</td>
<td>9.5</td>
<td>12.4</td>
<td>57.9</td>
<td>20.2</td>
</tr>
<tr>
<td>6</td>
<td>3.37 (±0.40)</td>
<td>8.39 (±1.10)</td>
<td>22.55 (±4.51)</td>
<td>20.67 (±2.61)</td>
<td>5.9</td>
<td>14.9</td>
<td>41.2</td>
<td>38.0</td>
</tr>
<tr>
<td>7</td>
<td>3.62 (±0.30)</td>
<td>9.21 (±1.41)</td>
<td>23.52 (±4.43)</td>
<td>23.28 (±1.78)</td>
<td>6.4</td>
<td>14.6</td>
<td>39.4</td>
<td>39.5</td>
</tr>
<tr>
<td>8</td>
<td>2.20 (±0.58)</td>
<td>5.56 (±0.70)</td>
<td>26.77 (±6.53)</td>
<td>17.77 (±0.96)</td>
<td>4.4</td>
<td>11.1</td>
<td>49.1</td>
<td>35.3</td>
</tr>
</tbody>
</table>
Reducible P pool comprised an average of 11.6% of total P in Shark Bay sediments (Table 4.2, Fig. 4.2). Reducible P ranged from 4.84 μg P g⁻¹ at site 3 to 10.6 μg P g⁻¹ at site 4. There was a weak positive relationship between salinity and reducible P proportions ($R^2 = 0.17$, $P = 0.003$, $n = 48$). Reducible P concentrations were more strongly correlated with depth than salinity, significantly decreasing as sediment depth increased ($F_{5,138} = 20.45$, $P < 0.0001$, Fig. 4.2), from a mean of 11.9 μg P g⁻¹ (16.2%) at 0-2 cm to 4.4 μg P g⁻¹ (7.7%) at depths over 30 cm. There was no significant relationship between sediment reducible P concentrations and Fe ($R^2 = 0.01$, $P = 0.75$, $n = 9$) or Al concentrations ($R^2 = 0.04$, $P = 0.62$, $n = 9$).

Figure 4.2: Mean sediment phosphorus fractions across Shark Bay, separated by site and depth. White bars represent magnesium extractable P, light gray bars represent reducible P, dark grey bars show calcareous bound P, and black bars denote residual organic phosphorus.
Residual organic P was the second largest sediment P fraction at all sites, comprising on average 26.4% of P in sediments. Residual organic P concentrations significantly increased with salinity ($R^2 = 0.29$, $P < 0.0001$, $n = 144$), and the proportion of total P present as residual organic P also increased with salinity ($R^2 = 0.66$, $P < 0.0001$, $n = 48$). Residual organic P concentrations were highest at intermediate salinities sites, with a maximum mean of $25.5 \mu g \text{ P g}^{-1}$ (28%) at site 4 (Table 4.2). However, residual organic P made up the largest proportion of total P in high salinity sites (sites 6-8, 35-40%), owing to the lower total P at these sites. Conversely, residual organic P was proportionally lowest at the low salinity sites (sites 1-3), comprising only 16-17% of total P. There was no strong relationship between sediment depth and residual organic P concentrations ($F_{5,138} = 1.22$, $P = 0.3$), which remained relatively stable across all depths (Figure 2). The concentration of residual organic P in sediments was positively correlated to the OM content of sediments ($R^2 = 0.50$, $P < 0.0001$, $n = 144$, Fig. 4.3a). There was also a strong positive relationship between the proportion of residual organic P in sediments and sulfur concentrations ($R^2 = 0.98$, $P < 0.0001$, $n = 9$, Fig. 4.3b).

Calcareous-bound P was the largest sedimentary P pool in Shark Bay, comprising an average of 54.8% of total P in Shark Bay sediments (Table 4.3). The concentration ($R^2 = 0.2$, $P < 0.0001$, $n = 144$) and proportion ($R^2 = 0.66$, $P < 0.0001$, $n = 48$) of calcareous bound P in sediments significantly decreased with increasing salinity. Low salinity sites had the highest concentrations and proportions of calcareous P, with a maximum of $60.5 \mu g \text{ P g}^{-1}$ (69.6%) at site 2 (Table 4.2). High salinity sites had the lowest concentrations and proportions of calcareous P in sediments. There was no relationship between calcareous P concentrations and sediment depth ($F_{5,138} = 0.25$, $P = 0.94$), with mean concentrations remaining relatively constant in the upper 40 cm of sediment ($34.3 \mu g \text{ P g}^{-1}$ at 20-30 cm to $37.6 \mu g \text{ P g}^{-1}$ at 5-10cm; Fig. 4.2).
Fraser (2016)

Figure 4.3: Scatterplot showing relationship between (a) sediment residual organic P concentrations and sediment organic matter concentrations ($R^2 = 0.50, P < 0.0001, n = 144$) and (b) total sedimentary sulfur and proportion of residual organic P in sediments ($R^2 = 0.98, P < 0.0001, n = 9$). Dashed line represents a linear model added to each dataset; grey outline shows 95% confidence interval.

Solution $^{31}$P-NMR spectroscopy

Inorganic orthophosphate was the dominant P compound extracted from sediments by NaOH-EDTA, and gave an $^{31}$P-NMR signal of $\delta=6.5$ppm; however this signal shifted to $\delta=4.5$ppm at site 6, likely due to this extract containing higher concentrations of the paramagnetic magnesium than other samples (Table 4.1). Inorganic phosphate extracted by EDTA was higher at low and intermediate salinity sites, and decreased at higher salinities. Phosphate monoesters were the only forms of organic P detected, which gave
Phosphorus cycling in seagrass sediments

Signals between $\delta=4.5\text{ppm}$ and $5.0\text{ppm}$ (Fig. 4.4, inset). Phosphate monoester peaks were greatest at intermediate salinities (sites 4 and 5), but could not be detected at sites 6 and 7 (the sites of highest salinity). Site 8 had relatively high monoester peaks, even though the inorganic phosphate peak at this site was lower relative to lower salinity sites.

![Figure 4.4: Solution $^{31}\text{P-NMR}$ spectra from Shark Bay sediments by NaOH-EDTA extract. Figure inset shows area of spectrum from site 5, where organic P in samples was detected, most likely as phosphate monoesters. A line broadening of 5 Hz was used in the spectra.](image)

**Discussion**

The concentrations and relative proportions of sediment P fractions changed substantially across the salinity gradient in Shark Bay, although not exactly as we had expected. For example, more labile fractions like magnesium-extractable P and reducible P did not decrease in a predictable manner with increased salinity. However, we did find complex shifts in the overall composition of sediment P that has significant implications for primary productivity of seagrass meadows. At low salinity sites (e.g. sites 1-3, <40‰), the bulk (>60%) of sedimentary P is calcareous bound P (Table 4.2, Fig. 4.2). This is considered the least available P fraction in marine environments given that the pH changes required for P release are unlikely to yield much P in buffered marine waters.
These low salinity sites also have the highest SRP (~0.2µM; Atkinson 1987) in the overlying water column, and is likely the dominant source of P for seagrasses at these sites. At intermediate salinities (sites 4 and 5, 40-44‰), SRP concentrations in the overlying water column are lower but labile P fractions like magnesium-extractable P and reducible P fractions increase in the sediment. At these sites, root uptake is likely an effective and more important strategy for P acquisition. The likely increasing importance of sediment P for plant growth at sites where SRP is low is backed up by the strong decline in magnesium extractable P with increasing sediment depth at these sites. At high salinity sites (sites 6-8, >44‰), total sediment P and water SRP are at a minimum, yet the proportion of organic P is highest at these sites. Therefore, recycling of sedimentary organic P (primarily from seagrass detritus) and efficient uptake strategies would be required at these high salinity sites. Given that salinity, sediment OM and SRP in the water column all differ along the environmental gradient studied in this experiment, it is not possible to identify if one variable in particular drives the fractionation of P in sediments. We hypothesise that the combination of all factors combine to significantly alter biogeochemistry along the gradient. Nevertheless, as salinity increases and water SRP decreases in oligotrophic seagrass meadows, strategies to access sedimentary organic P fractions become increasingly important to sustain primary productivity, with the organic P fractions quantitatively increasing given higher OM in sediments.

The only organic P compounds detectable with $^{31}$P-NMR analysis were phosphate monoesters (Fig. 4.4). This is the first study that has sought to characterise the organic P fraction in seagrass sediments and, the findings share some similarities but also have key differences with studies of other terrestrial and aquatic ecosystems. Though phosphate monoesters typically dominate the organic P pool in natural soils and both freshwater and marine sediments, other organic P compounds are usually detected (Doolette and Smernik 2011; Baldwin 2013; Kraal et al. 2015; McLaren et al. 2015). For example, while phosphate monoesters were the dominant organic P pool in Baltic Sea sediments, DNA-P, techoic acid P and pyrophosphates were also detected (Ahlgren et al. 2006; Kraal et al. 2015). Similarly, phosphate monoesters dominate the organic P pool in mangrove sediments, but phospholipids and phosphate diesters were also found (El-Rifai et al. 2008), with similar patterns seen in terrestrial (Doolette and Smernik 2011; Turner and Engelbrecht 2011; Celi et al. 2013; McLaren et al. 2015) and wetland ecosystems (Turner...
Phosphorus cycling in seagrass sediments

et al. 2007; Cheesman et al. 2012). Here the total organic P fraction comprised only 7-25 μg P g⁻¹, and this low concentration accounts for the weak NMR signals. Regardless, the lack of detection of readily degraded organic P compounds such as phosphate diesters also indicate that if other organic compounds are present they are in very low concentrations in Shark Bay sediments. Phosphate monoesters are typically the by-product of plant tissues (Turner et al. 2002), and can be quite stable and thus persist longer after deposition, requiring phosphatase enzymes to be released for hydrolysis to occur before P was released (Ladd and Butler 1972; Burns 1982 Nannipieri 1998). Given that OM in Shark Bay sediments is composed primarily of seagrass detritus (Cawley et al. 2012) and low SRP concentrations limit planktonic productivity (Kimmerer et al. 1985), P compounds from seagrass litter likely form the bulk of the organic P pool in Shark Bay sediments. We suspect that phosphate monoesters also dominate the sedimentary organic P pool at other sites where the main sources of OM are seagrass detritus; further sampling across a range of sites is needed to confirm this hypothesis.

Phosphate monoesters are essential signaling molecules present in all plants, commonly released in response to stresses (Gillaspy 2011). For example, inositol phosphates have been implicated in tolerance to increases in salinity in rice and tobacco (Majee et al. 2004; Das-Chatterjee et al. 2006). A similar role in seagrasses may represent an adaptation that allows the survival of these vascular plants in the saline marine environment, and may partly explain the higher peaks in the NMR spectra from some of the higher salinity sites (Fig. 4.4). However, concentrations of inositol compounds did not increase in Cymodocea nodosa at sites with elevated salinities from the Mediterranean (Drew 1978), suggesting that this stress response may be species-specific. The role of inositol phosphates for salinity tolerance in seagrasses is largely unknown, yet may be a key physiological response to salinity stress. Further ³¹P-NMR analysis in different seagrass sediments would show if the dominance of phosphate monoesters in the organic P pool of Shark Bay sediments were a result of the increased salinities, the low concentrations of phosphate available for plant uptake, or is a common feature across all seagrass sediments. Such information would assist in determining the general mobility and ecological importance of organic P in seagrass ecosystems.

The dominance of phosphate monoesters and lack of more readily degraded organic P
compounds suggests that internal recycling of phosphorus is important in Shark Bay sediments, which is typical of low P environments (Reed et al. 2011). To utilize the organic P pool in Shark Bay, organisms must either produce enzymes that can hydrolyse phosphate monoesters, or rapidly recycle and take up more labile organic P compounds present at very low concentrations. Seagrasses and attached epiphytes release alkaline phosphatase on leaf surfaces when P limited, alleviating P limitation by hydrolysing phosphate monoesters (Pérez and Romero 1993; Martínez-Crego et al. 2006; Koch et al. 2009). However, the capacity for seagrass roots to release phosphatases into the rhizosphere is largely unknown, despite the large proportion of P in organic forms in sediments. Microbiologically derived phosphatases are also important in phosphate monoester cycling in terrestrial soils (Richardson and Simpson 2011), and can be more efficient than plant derived phosphatases (Tarafdar et al. 2001), but their ecological importance in seagrass sediments is unknown. Activity of microbial and seagrass derived phosphatases in sediments would likely be important in ecosystems like Shark Bay that are characterised by low available P concentrations yet contain large organic P pools dominated by phosphate monoesters that require hydrolysis before seagrass uptake.

Table 4.3: Summary table comparing P fractions in Shark Bay sediments to other seagrass sediments. Mean sediment pools for magnesium extractable P (PMgCl), reducible P, calcareous P, and residual organic P are provided, as well as dominant seagrass species and sediment OM concentrations. All P fractions are in μg g⁻¹. The proportion of each P fraction as total sedimentary P are also given below concentrations in brackets. * indicates data was presented as calcareous + residual organic P combined.

<table>
<thead>
<tr>
<th>Location</th>
<th>PMgCl (μg g⁻¹)</th>
<th>Reducible P (μg g⁻¹)</th>
<th>Calcareous P (μg g⁻¹)</th>
<th>Residual organic P (μg g⁻¹)</th>
<th>Seagrass species</th>
<th>Climate</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shark Bay</td>
<td>2.2 – 8.9 (4.4 - 9.5%)</td>
<td>5.6 – 10.5 (7.6 - 14.9%)</td>
<td>22.6 – 60.5 (39.4 - 69.5%)</td>
<td>7.2 – 25.5 (16.1 - 39.5%)</td>
<td>Amphibolis antarctica</td>
<td>Subtropical</td>
<td>This study</td>
</tr>
<tr>
<td>Florida Bay</td>
<td>0.1 – 19.0 (1.9 - 5.1%)</td>
<td>&lt;1.0 – 68 (&lt;0.6 - 18.4%)</td>
<td>74 – 151 (40.9 - 86.0%)</td>
<td>28 – 70 (18.1 - 56.5%)</td>
<td>Thalassia testudinum/ Halodule wrightii</td>
<td>Subtropical</td>
<td>Koch et al. (2001)</td>
</tr>
<tr>
<td>Bermuda</td>
<td>2.2 – 6.2 (2.7 - 3.7%)</td>
<td>14 – 28 (16.7 - 23.2%)</td>
<td>58 – 129 (70.7 - 76.8%)</td>
<td>2.2 – 4.6 (2.6 - 2.7%)</td>
<td>Thalassia testudinum/ Syringodium filiforme</td>
<td>Tropical</td>
<td>Jensen et al. (1998)</td>
</tr>
<tr>
<td>Denmark</td>
<td>5.3 – 10.4 (1.6 - 3.8%)</td>
<td>4.3 – 13 (1.6 - 4.6%)</td>
<td>259.0 – 320.0 (92.2 - 95.2%)</td>
<td></td>
<td>Zostera marina</td>
<td>Temperate</td>
<td>Holmer et al. (2006)</td>
</tr>
</tbody>
</table>
The ephemeral Wooramel River has previously been hypothesised as a source of P for seagrass uptake in Shark Bay after significant flow events, with inputs likely to be in the reducible P fraction given the iron-dominated hinterland (Fourqurean et al. 2012; Fraser et al. 2012). However, the Wooramel River had not flowed for over two years at the time of sampling and we found no evidence of higher reducible P concentrations at the sites adjacent to the Wooramel River (sites 6-8), even though iron and aluminium concentrations were highest in these sediments (Table 4.1). While flood events in this arid region are quite rare (once or twice a decade), the Wooramel River potentially can introduce significant amounts of sediment to Shark Bay. Even though P concentrations in Western Australian soils are low due to the highly-weathered nature of the continent, rivers in semi-arid Australia still have the potential to bring large pulses of P after rewetting following large rainfall events (Mitchell et al. 1997; McIntyre et al. 2009). Concentrations of reducible P are significantly affected by changes in abiotic variables such as pH and redox potential (Wang et al. 2013). Under anoxic conditions and oceanic pH, P is likely to be desorbed from sediments quite quickly following large rainfall events and released to the overlying water column, promoting release of P and removing it from the sedimentary pool (Jordan et al. 2008). The high salinities at the sites would also promote release of P from reducible sediments (Sundareshwar and Morris 1999). Total P in sediments and seagrasses adjacent to the Wooramel River were elevated following the 2010/11 Wooramel River floods (Fraser et al. 2012), but this appears to be short-lived as all labile fractions were low by the time of sampling for this study 24 months later. However, given the low bioavailable P concentrations at the Wooramel Delta, such pulse events may be important for seagrasses to periodically meet P demands, especially if the P taken up by seagrasses can then be recycled into the organic P pool, preventing export from the immediate area.

Overall, this study has revealed that Shark Bay may not be as oligotrophic and P-limited as previously thought (Atkinson 1987; Burkholder et al. 2013). Shark Bay does indeed have lower total sediment P concentrations in comparison to other similar embayments (Table 4.3). However, we found that only 40-60% of total P is bound to calcareous P, which is much less than other tropical seagrass ecosystems such as Bermuda, where up to 77% is present in calcareous bound fractions. Proportions of labile P (magnesium-extractable P) are also relatively high in Shark Bay compared to similar embayments.
However, Shark Bay has similar proportions of calcareous bound P (~50%) and organic fractions to sediments in Florida Bay (16-40% in Shark Bay, ~40% in Florida Bay). The relatively high organic P is likely almost solely derived from seagrass detritus in both ecosystems (Koch et al. 2001; Cawley et al. 2012). Thus, the recycling of autochthonous seagrass OM likely plays a key role in P cycling in sub-tropical oligotrophic embayments, and becomes increasingly important as SRP decreases and salinity increases.

Acknowledgments

An NHT-II Caring for our Country project grant coordinated by Western Australian Marine Science Institute awarded to G.A.K. funded this research. Additional funding for M.W.F was received through PhD funding from the School of Plant Biology, UWA. Logistical support was provided by the Shark Bay Ecosystem Research Project under the direction of Michael Heithaus, funded by NSF (OCE-0745606). We thank Oscar Serrano-Gras, John Statton and Amy Griffiths for help collecting samples, and Kate Bowler for assistance in sequential extractions. The authors acknowledge the facilities, and the scientific and technical assistance of the Australian Microscopy & Microanalysis Research Facility at the Centre for Microscopy, Characterisation & Analysis, The University of Western Australia, a facility funded by the University, State and Commonwealth Governments.

References


Phosphorus cycling in seagrass sediments


Fraser (2016)


Phosphorus cycling in seagrass sediments


Vaz, M. R., A. C. Edwards, C. A. Shand, and M. S. Cresser. 1994. Changes in the chemistry of soil solution and acetic-acid extractable P following different types of
Chapter 5: Taxonomic and functional structure of microbial communities in seagrass sediments across salinity and phosphorus gradients

Matthew W. Fraser, Deirdre B. Gleeson, Pauline F. Grierson, Bonnie Laverock, Gary A. Kendrick

Preamble: This manuscript is to be submitted to Nature Microbiology, and is formatted to the requirements of the journal. The requirements of Nature Microbiology include having an introductory paragraph with results and implications instead of having an abstract, methods being presented after the main body of text, and no distinct results or discussions sections. The work is primarily my own. DBG and BL provided advice regarding microbial analysis. GAK and PFG provided advice regarding experimental design. All co-authors provided feedback on the manuscript. This chapter examines the shift in microbial community structure and function along the salinity/P availability gradient in Shark Bay sediments described in previous chapters, and examines correlations to changes in sediment biogeochemistry. I also compare the metagenomic structure of microbial communities in seagrass sediments to other ecosystems to provide context for the results.
Sediment microorganisms influence primary producer composition and ecosystem functions given their critical role in regulating biogeochemical processes. Sediment microorganisms are particularly importantly in oligotrophic ecosystems where small changes in the availability of macronutrients such as phosphorus (P) have the greatest impacts. Seagrass meadows are marine ecosystems that present a unique ecological niche to sediment microbial communities as seagrasses possess true root systems that release oxygen and organic matter. Rhizosphere microorganisms mediate nutrient cycling and organic matter turnover in seagrass sediments\(^1\), but the identity of the microbes and how they may respond to chemical change in their aquatic environment is unknown. Here, we used metagenomic sequencing in seagrass sediments in order to investigate the structure and functioning of the microbial community across a salinity and P availability gradient in Shark Bay – an oligotrophic, hypersaline, and arid ecosystem that boasts abundant seagrass meadows that directly contribute to its World Heritage Status\(^2\). We show that microbial communities change appreciably across salinity and P availability gradients, with increases in phosphonate metabolism genes showing adaptations toward increasingly P-limited conditions. Given that sediment organic P concentrations are highest where P concentrations in the water column are low, the microbial processing of organic P may support P requirements of seagrasses at particularly oligotrophic sites.

Microorganisms have an integral role in maintaining productivity in oligotrophic ecosystems by possessing genes that encode for novel enzymes that enable the uptake of organic phosphorus pools\(^3\). Where nutrients become more limiting, genes that promote efficient nutrient uptake and recycling increase in microbial communities, leading to increases in the release of extracellular enzymes capable of degrading organic matter and altering nutrient cycling rates\(^4,5\). For example, in the ultra-low P Sargasso Sea, novel organic phosphorus compounds such as polyphosphate are used as the main P source, driven by microorganisms that have an increased capacity for storing and degrading these compounds\(^6,7\). Similarly, soil microorganisms often form close associations with plants in terrestrial oligotrophic ecosystems, and are important in maintaining ecosystem productivity given their ability to degrade refractory organic P forms, with biological mineralization of organic P responsible for up to 90% of plant P uptake\(^8\). Seagrass
meadows present unique/novel habitats for sediment microorganisms in a marine setting as the only group of marine angiosperms. As angiosperms, seagrasses possess true root systems and rhizospheres, oxygenating surrounding rhizosphere sediments and creating unique conditions that support high levels of bacterial diversity. Microbial communities within the seagrass rhizosphere may also increase nutrient availability, especially in oligotrophic ecosystems. However, studies of microbial communities in seagrass rhizosphere sediments have to date primarily focused on their taxonomic structure, with limited information on their functional capacity to influence biogeochemical processes and ecosystem function in seagrass meadows. This information is an important knowledge gap given the numerous ecosystem services seagrass meadows provide, including provision of habitat for other marine organisms including important fisheries species, improvement of water quality through nutrient uptake, and enrichment of the organic matter content of sediments, contributing greatly to the global carbon cycle as a key blue carbon habitat.

Environmental gradients alter microbial community function and thus enzyme expression and biogeochemical processing. Salinity and nutrient availability are two key environmental drivers in seagrass ecosystems, influencing productivity and trophic structure. Shark Bay has one of the largest seagrass meadows in the world, and contains a natural salinity and phosphorus (P) availability gradient. Salinity rises from normal marine salinity (~35‰) in the north of the Bay to over 65‰ in the southern, hypersaline reaches of the Bay. Soluble reactive phosphorus (SRP) – the most available source of P for biological uptake – decreases along this same gradient and is extremely low (<0.02µM) in high salinity areas. Seagrasses are extremely important in maintaining the status of Shark Bay as a World Heritage Site, underpinning a diverse ecosystem including turtle and dugong populations, and contributing to the salinity gradient (by restricting hydrodynamic circulation) that supports Earth’s most extensive stromatolite populations. In addition, the seagrasses of Shark Bay sequester 243 Mg organic carbon ha⁻¹ in sediments, making it a global hotspot for coastal organic carbon burial and comparable with organic C stored in terrestrial forests. However, seagrass-derived organic matter also influences biogeochemical processes and microbial community structure in sediments, and may be an important source of nutrients for seagrasses in oligotrophic areas. It is unknown how the current environmental conditions...
within Shark Bay impact upon seagrass-associated microbial communities and the processes they mediate, and how future environmental changes (e.g. increases in temperature, increased salinities, changes in nutrient availability) may also influence these microbial communities.

Here, we used metagenomic sequencing to determine taxonomic and functional changes in the rhizosphere sediment microbial communities of 6 seagrass meadows in Shark Bay (Fig. 5.1) encompassing a range of salinities (39.2‰ – 53.2‰, Appendix 1) and water SRP concentrations from 0.1µM to below detectable concentrations (<0.02µM). We hypothesised that: (i) rhizosphere microbial communities would significantly change in taxonomy and function along the gradient; (ii) microbial communities at high salinity/low SRP sites would possess functions that are advantageous for P uptake in such environments (i.e. higher abundance of phosphorus metabolism genes); and (iii) rhizosphere microbial function would be correlated to enzyme activities. We also compared the microbial metagenomes of seagrass rhizospheres from this study with other aquatic and terrestrial ecosystems to ascertain if there are consistent patterns in microbial community structure and key biogeochemical parameters that would in turn indicate potential “universality” in the adaptations of microbial communities to particular conditions.
Across the salinity/P availability gradient, there were distinct shifts in the taxonomic and functional structure of seagrass-associated microbial communities. Sites above 46‰ salinity showed separation in taxonomic profiles compared with profiles generated from communities sampled at lower salinities (Fig. 5.2a). Proteobacteria were underrepresented in the high salinity sites (7 and 9, Fig. 5.2a). Gene abundances for Betaproteobacteria ($\eta^2=0.64$, $P=0.015$), Deltaproteobacteria ($\eta^2=0.57$, $P=0.029$), Gammaproteobacteria ($\eta^2=0.78$, $P=0.0023$), and Zetaproteobacteria ($\eta^2=0.88$, $P=0.0002$) all significantly decreased along the salinity gradient. In contrast, Alphaproteobacteria tended to increase with increasing salinity ($\eta^2=0.53$, $P=0.038$), as did Planctomycetes ($\eta^2=0.79$, $P=0.005$) and Verrucomicrobia ($\eta^2=0.85$, $P=0.001$), with highest abundances in sites 7 and 9 (Fig. 5.2b). The class Cytophagia (phylum =
Bacteriodes) also increased significantly with salinity ($\eta^2 = 0.73$, $P=0.0059$).

Functional gene abundances within the microbial community also shifted along the salinity gradient and matched patterns in taxonomic composition, with sites above 46‰ differentiated from lower salinity sites (Fig. 5.3a, Appendix 2). Genes related to microbial photosynthesis ($P=0.008$), DNA metabolism ($P=0.01$), and P metabolism ($P=0.015$) were all significantly more abundant in high salinity sites, while nitrogen metabolism ($P=0.015$), and cell signaling ($P=0.044$) were significantly lower. Clearly, changes in environmental conditions (in this case, salinity and P availability) can alter both the taxonomic and functional capacity of seagrass-associated microbial communities, and these changes have the potential to fundamentally alter biogeochemical cycling of P, N, and C (photosynthesis) in sediments.

The gradient in P availability also contributed to significant changes in the capacity of microbial communities to utilize organic phosphorus compounds – in particular, phosphonates. Phosphonates are a significant component of the dissolved organic P pool in oceans$^{24}$, and become an increasingly important P source to relieve P limitation in for diazotrophs in oligotrophic ocean ecosystems$^{25}$. Genes related to phosphonate degradation, transport, and metabolism were all significantly more abundant at high salinity sites where SRP concentrations decreased below detectable concentrations (Fig. 5.3b). Genes for phosphoacetaldehyde hydrolyse, important for phosphate release from phosphonates, also showed greater abundance at higher salinities (Fig. 5.3b). The increased abundance of phosphonate metabolism genes suggests an increased reliance of microbes on organic P within the high salinity sites. Given that sediment organic P increases in both concentration and as a proportion of total P as salinity increases in Shark Bay, microorganisms with the functional traits that allow them to use this resource would be at a competitive advantage. Phosphonates may therefore be an important source of biological P uptake in oligotrophic coastal marine ecosystems as they are in open ocean ecosystems.
Figure 5.2 Taxonomic profiles from metagenomic sequencing of microbial communities in Shark Bay seagrass sediments. (a) Principal component analysis of taxonomic profiles between samples at the class level. Sites gradually increase in salinity from green (lowest salinity, site 1) to dark blue (highest salinity, site 9). (b) Differences in relative abundance of classes between high salinity sites (>46‰, sites 7 and 9, teal) and lower salinity sites (<46‰, grey). Only significantly different classes are shown, with corrected $P$-values calculated using Storey’s false discovery rate approach ($P<0.05$).
Figure 5.3 Functional profiles from metagenomic sequencing of microbial communities in Shark Bay seagrass sediments. (a) Principal component analysis of functional profiles between samples at the class level. Sites gradually increase in salinity from green (lowest salinity, site 1) to dark blue (highest salinity, site 9). (b) Differences in relative abundance of P metabolism functions annotated using the SEED Subsystems database between high salinity sites (>46‰, sites 7 and 9, teal) and lower salinity sites (<46‰, grey). Functions related to phosphonate metabolism are in bold. Only P-metabolism functions that were significantly different between high and lower salinity sites are shown, with corrected P-values calculated using Storey’s false discovery rate approach (P<0.05)
Patterns in gene abundance do not always necessarily dictate actual expression of genes and subsequent biogeochemical shifts\(^2\). In this study, gene abundance for secreted alkaline phosphatase significantly increased with salinity \((R^2=0.98, P<0.0001)\) and were strongly correlated with extracellular activity of the alkaline phosphatase enzyme measured in sediments along the gradient \((R^2=0.88, P<0.0001)\). This suggests that the alkaline phosphatase in the sediments is likely of microbial origin. In agreement to previous studies\(^20\), there was, however, no corresponding increase in P content of seagrass leaves (Appendix 1). Assuming enough substrate, the release of phosphatase enzymes produced by bacteria would increase the availability of organic P to both microorganisms and seagrasses. As such, the activities of microorganisms in sediments may assist seagrasses in meeting P requirements in extremely oligotrophic areas such as our low-SRP sites; and may constitute an important ecological interaction between seagrasses and rhizosphere microbial communities.

Genes related to P, nitrogen, and sulfur metabolism were more abundant in seagrass-associated microbial communities than in most other ecosystems (Fig. 5.5c). In particular, genes related to organic S and P metabolism were highly abundant in seagrass ecosystems. Given that seagrasses can take up nutrients from the water column through their leaves\(^27\), the remineralization of organic matter in sediments has often been overlooked as a potential nutrient source, especially given that organic matter in seagrass sediments is often refractory. However, seagrasses can receive organic inputs from terrestrial and oceanic sources, and high sedimentation rates ensure much of this material is retained in seagrass sediments. In addition, seagrass sediments are often enriched in seagrass detritus itself. The sediment organic pool is potentially a major source of nutrients in seagrass ecosystems, and may play an important role in maintaining productivity. If so, the functional diversity of seagrass-associated microbial communities will play a central role in nutrient cycling, primary productivity, and the maintenance of ecosystem services that seagrasses provide.
Figure 5.4 Taxonomic profiles from metagenomic sequencing of microbial communities from a range of different ecosystems. (a) Principal component analysis of taxonomic profiles (level=class) between microbial communities from all ecosystems examined. Seagrass communities from this study shown in bright green. (b) Principal component analysis of taxonomic profiles (level=class) from a subset of ecosystems, with freshwater and stromatolite communities removed to show fine-scale patterns. (c) Relative abundance of classes between seagrass microbial communities and microbial communities from other ecosystems. Only significantly different classes are shown, with corrected $P$-values calculated using Storey’s false discovery rate approach ($P<0.05$).
Seagrass-associated microbial communities, somewhat unsurprisingly, showed the strongest similarity to mangrove microbial communities when compared to profiles from a range of different terrestrial and aquatic ecosystems (Fig. 5.5). Mangroves and seagrass habitats share many environmental similarities; both are dominated by rooted macrophytes growing in organic-rich, anoxic sediments submerged in saline water. Microbial communities from other marine benthic environments, such as salt marsh and estuarine sediments, also showed significant similarity to those from seagrass sediments. However, when comparing the functional profiles for the same communities, profiles for seagrass rhizosphere communities showed greater overlap with functional profiles from mangrove, estuarine, and even some terrestrial ecosystems than with salt marshes (Fig. 5.5). This suggests that the environmental factors driving microbial community structure and functional capacity are to some extent independent of each other. Microbial community structure in marine habitats can also be largely driven by communities in adjacent habitats such as the surrounding water or sediments, with subsequent selection based on functional capabilities. Seagrasses have unique microbial communities with a distinct taxonomic and functional structure, likely driven by a combination of environmental and biological factors that are yet to be fully characterised.

In addition to genes regulating the P cycle, there were also significant shifts in taxonomic composition of sediment microbial communities sampled. The bacterial phylum *Planctomycetes* was more abundant within the high salinity sites of Shark Bay (Fig. 5.2b). *Planctomycetes* also appeared more abundant in seagrass sediments compared with other ecosystems (Fig. 5.4). The *Planctomycetes* can have significant impacts on nutrient cycling. For example, some anaerobic *Planctomycetes* can perform anaerobic ammonia oxidation, greatly altering N availability in anaerobic environments, and are responsible for up to 50% of fixed N removal from the ocean. *Planctomycetes* also produce abundant sulfatases, catalysing the hydrolysis of organosulfates and impacting the sulfur cycle. There has been recent focus on their interactions with macroalgae given the high abundance of *Planctomycetes* on macroalgal biofilms in comparison to their negligible abundance in seawater. The interaction between some macroalgal species and *Planctomycetes* is thought to be symbiotic; with the *Planctomycetes* contributing to the remineralization of organic matter and mobilization of nutrients, in return for habitat and substrate provision by the macroalgae. *Planctomycetes* have previously been found in
other seagrass sediments\textsuperscript{9-11,36}, but their specific interactions with seagrasses are unknown. \textit{Planctomycetes} abundances may be high in seagrass sediments because of seagrass-derived organic exudates or litter\textsuperscript{23,37}, providing an ideal niche where they can utilize a range of chemical compounds for energy\textsuperscript{33}. Seagrasses may also benefit from this association by making use of the nutrients released by \textit{Planctomycetes} metabolic processes. If so, \textit{Planctomycetes} may be key organisms mediating the turnover of organic matter in seagrass ecosystems, becoming increasingly important as nutrient availability decreases.
Figure 5.5 Functional profiles from metagenomic sequencing of microbial communities from a range of different ecosystems. (a) Principal component analysis of functional profiles (level 1 functions) between microbial communities from all ecosystems examined. Seagrass communities from this study shown in bright green. (b) Principal component analysis of functional profiles (level 1 functions) from a subset of ecosystems, with coral, freshwater and stromatolite communities removed to show fine-scale patterns. (c) Differences in relative abundance of functions between seagrass microbial communities (green) and microbial communities from other ecosystems (dark grey). Only significantly different classes are shown, with corrected $P$-values calculated using Storey’s false discovery rate approach ($P<0.05$).
Methods

Sampling sites and experimental design
Rhizosphere sediments and plants were collected from nine shallow (1.5 – 2.5 m) subtidal sites across Shark Bay, Western Australia in March 2014. Shark Bay is a World Heritage listed embayment, with the seagrasses in the Bay crucial to this status. The seagrasses of Shark Bay support a diverse food web, are highly productive, and species rich. The seagrass communities at all sites were dominated by the temperate seagrass *Amphibolis antarctica*, with the tropical species *Halodule uninervis* as a sparse understory.

The nine sites were chosen to encompass a range of salinities and SRP concentrations, from site 1 (lowest salinity, Guichenault Point) to site 9 (highest salinity, L’haridon Bight) (Fig. 5.1). All sites were separated by at least 5km. At each site, four sediment samples (0-10 cm deep) were taken using 50 mL plastic syringe core, and transferred to 50 mL centrifuge tubes, for later biogeochemical and enzyme analysis. *Amphibolis antarctica* leaves (n=100) were also hand-collected at each site for P content analysis. Samples were stored on ice in the field for no longer than 8 hours, and subsequently stored at 4°C (sediment samples) or -20°C (seagrass leaves) until processing. In addition, five extra sediment samples (0-5 cm) were collected using an ethanol-sterilized 3ml syringe core, transferred to 3 mL cryovials and snap frozen immediately in liquid nitrogen. Cryovials were then stored at -80°C until DNA extraction. On return to the laboratory, seagrass leaves were thawed and epiphytes were removed from leaves by gentle scraping with a razor blade under deionised water. Leaves were dried at 60°C for 3 days, then ground using a mortar and pestle. P content on a subsample was determined using standard dry oxidation acid hydrolysis techniques. SRP for water samples was measured colorimetrically on filtered (0.45 µm) water samples collected from each site using standard methods.

Enzymes
The activities of three hydrolytic enzymes were measured using standard colorimetric methods. The enzymes and substrates used were (i) β-glucosidase assayed with 4-nitrophenyl β-glucopyranoside, (ii) acid phosphatase and (iii) alkaline phosphatase both assayed with 4-nitrophenyl phosphate disodium hexahydrate. For each assay, 1.00 g of sediment was added to a 50 mL Erlenmeyer flasks with 4 mL of modified universal buffer.
Phosphorus cycling in seagrass sediments

(pH=6.00 for β-glucosidase, pH=6.50 for acid phosphatase and pH=11.00 for alkaline phosphatase) and 1 mL substrate, and were incubated at 37 °C for one hour. Following incubation, 1 mL of 0.5M CaCl₂ and 4 mL of 0.5M NaOH (for phosphatases) or 4 mL 10mM tris(hydroxymethyl) aminomethane (pH=12, for glucosidases) were added to stop the reaction. The final nitrophenol concentrations were determined photometrically at 400 nm against a standard curve. Microbial P was operationally calculated as the difference in resin-extractable P between non-fumigated and hexanol-fumigated sediment samples using standard methodology.

Microbial community identification

Differences in sediment microbial communities were firstly identified across all nine sites using automated ribosomal intergenic spacer analysis (ARISA), before a subset of samples was chosen for further metagenomic analysis. A subset of six sites was selected for metagenomic analysis (sites 1, 3, 5, 6, 7 and 9). DNA extraction from 500 mg of three replicate sediments per site was performed using PowerSoil DNA Isolation Kit (MoBio, California, CA, USA) according to the manufacturer’s instructions. Where extractions yielded less than 200 ng of DNA, additional extractions were performed to ensure final concentrations of neat DNA were above 200 ng. In total, 18 DNA samples were sequenced (Australian Genome Research Facility, Australia) on an Illuma MiSeq sequencer. Two runs were performed to ensure that a target of 2 million sequence reads per sample.

Metagenomic annotation and statistical analysis

Raw, unassembled reads were annotated using the Metagenomics Rapid Annotation (MG-RAST) pipeline version 3.6. Taxonomic profiles were generated using Representative Hit Classification of the M5NR database to allow comparisons of multiple metagenomes, and functional profiles were generated using Hierarchical Classification against the Subsystems database, with a minimum alignment length of 50 bp and E value cutoff of $E < 1 \times 10^{-5}$ (Appendix 3).

To examine statistical differences in taxonomic and functional profiles across Shark Bay sediments, data were analysed using the Statistical Analysis of Metagenomic Profiles (STAMP) package. $P$ values for all tests were calculated using ANOVA for multiple
test comparisons and Tukey-Kramer test for posthoc analysis. Storey’s false discovery rate (FDR) was used for multiple test corrections, given its higher power than other correction methods such as Benjamini-Hochberg FDR\(^{46,47}\). Effect sizes (\(\eta^2\)) are provided with \(P\) values to show magnitude of shifts and give context to the biological significance of significant results\(^{48}\).

To compare microbial communities from seagrass rhizosphere sediments in this study to those from other ecosystems we downloaded metagenomic profiles from a range of terrestrial, aquatic, and marine habitats publicly available on MG-RAST (full details Appendix 4). Where possible, we chose profiles obtained with similar average sequence lengths (~300 bp) and using Ilumina as a sequencing platform, though this was not always possible. Again, taxonomic profiles were generated using Representative Hit Classification of the M5NR database, and functional profiles were generated using Hierarchical Classification against the Subsystems database, with a minimum alignment length of 50 bp and E value cutoff of E<1x10\(^{-5}\). Profiles were combined and analysed using STAMP, with post-hoc tests using the Games-Howell test to account for the unequal sample sizes\(^{45}\). Storey’s FDR was used for multiple test corrections.

Relationships between sediment enzyme activity, microbial P, sediment organic matter content, and selected metagenomic groups were analysed using linear regression analysis against salinity. Prior to analysis, data were checked for normality and homogeneity of variances. Due to assumptions being violated, non-parametric tests (Kruskal Wallis) were used to examine the relationships for seagrass P content. Statistical tests were analysed using R version 3.1.1\(^{48}\) using the base program and the ‘ggplot2’ package for graphics\(^{49}\).
References


Phosphorus cycling in seagrass sediments


Chapter 6: Tracking phosphorus cycling in seagrass meadows using oxygen isotopes: Methodological challenges and future approaches

Matthew W. Fraser, Pauline F. Grierson, Gary A. Kendrick, Grzegorz Skrzypek

Preamble: This chapter describes an ultimately unsuccessful attempt to investigate the extent to which microbial turnover of organic P and subsequent uptake of mineralised P by seagrass using stable oxygen isotope composition of phosphates ($\delta^{18}$O-PO$_4$) as a tracer for biological cycling of P. An analysis of the approach taken and some of the challenges faced in this research is included in the thesis in order to inform future efforts of $\delta^{18}$O-PO$_4$ measurements in seagrass ecosystems.
Introduction

In oligotrophic seagrass meadows, concentrations of soluble reactive phosphate (SRP) in the water column may be too low to sustain productivity, and uptake of P from sediment sources become increasingly important in meeting seagrass P demands (Chapters 4 and 5). While the effect of geochemical processes such as sorption or desorption on P availability in marine sediments are relatively well understood (Paytan & McLaughlin 2007), less is known about the importance of the ecological and biological mechanisms of P regeneration from microbial biomass. In oligotrophic meadows with low inorganic P concentrations, mineralization of sediment organic P compounds including the mineralization of the microbial biomass may be important in sustaining P requirements of seagrasses.

The isotopic composition of oxygen ($\delta^{18}O$) in soluble reactive phosphorus (SRP) is thought to reveal the balance between phosphate transport and biological turnover rates in marine ecosystems (Colman et al. 2005). Tracking the importance of biological P cycling is more difficult than carbon or nitrogen cycling given that P only has one naturally occurring stable isotope ($^{31}$P). Radioisotopes ($^{32}$P and $^{33}$P) can be used to measure biological P cycling (Van Veen et al. 1987; Benitez-Nelson & Karl 2002; Nielsen et al. 2006), but only in short term studies that are often cost-prohibitive. Recently, isotopes of oxygen on phosphate molecules have been recognized as a potential method for tracking sources and cycling of P in aquatic environments (Paytan & McLaughlin 2011). The P-O bond in phosphate is resistant to inorganic hydrolysis at most temperatures on Earth, and isotope exchange of O can only occur during biological processes, when $\delta^{18}$O-PO$_4$ is equilibrated with water available for the organism, making it an excellent signature for biological activity (Tudge 1960; Blake, Alt & Martini 2001). Two processes control the $\delta^{18}$O-PO$_4$ signature of plants or microorganisms. Direct uptake of surrounding inorganic phosphate results in a $\delta^{18}$O-PO$_4$ signature equal to surrounding water/porewater, and shows a negligible contribution of P from organic sources. Conversely, the extracellular degradation of organic P (and subsequent uptake) results in $\delta^{18}$O-PO$_4$ values lower than the $\delta^{18}$O-PO$_4$ of surrounding water (Blake, O'Neil & Surkov 2005). The most crucial processes in this exchange is typically the hydrolysis of phosphoesters (Blake et al. 2005; Liang & Blake 2009), which often
constitute the majority of organic P compounds. Given the capacity for the $\delta^{18}$O-$\text{PO}_4$ to indicate use of organic P compounds, Tamburini et al. (2012) used $\delta^{18}$O-$\text{PO}_4$ measurements from sediments, vegetation, and microbial pools to determine that P is cycled through soil microorganisms before being released to the available pool. By comparing $\delta^{18}$O-$\text{PO}_4$ signatures from all potential sources to the available P signature, it may be possible to determine key processes leading to P becoming available for plant uptake.

Previously, I have shown that organic P in the sediments of Shark Bay is dominated by phosphate monoesters (Chapter 4). Soluble reactive P in the water column tends to be negatively correlated to organic P in sediments. The expression of extracellular phosphatase enzymes also increases along the SRP gradient, and microorganisms have increased abundance of genes for the metabolism of organic P compounds at low SRP sites (Chapter 5). Viewed collectively, these findings suggest that the microbial-driven mineralization of organic P is an increasingly important P source for seagrasses along the SRP gradient in Shark Bay. Here, we aimed to track biological turnover of phosphates by measuring sediment, microbial, and seagrass $\delta^{18}$O-$\text{PO}_4$ pools across the SRP gradient in Shark Bay. We expected that seagrasses at the most oligotrophic (i.e. low SRP) sites would show a $\delta^{18}$O-$\text{PO}_4$ isotopic signature closest to the microbial P pool, reflecting an increased uptake of P that had previously been recycled through sediment microorganisms.

**Methods**

*Sampling sites and experimental design*

Sediment and seagrass samples were selected at nine shallow sites (1.5 – 2.5m) across Shark Bay, Western Australia in March 2014 as described in Chapter 5 (see Fig. 5.1 for full study map). The nine sites were chosen to encompass a range of salinities and SRP concentrations, from site 1 (lowest salinity, Guichenault Point) to site 9 (highest salinity, L’haridon Bight), with all sites separated by at least 5 km. The seagrass communities at all sites were dominated by the temperate seagrass *Amphibolis antarctica*, with the tropical species *Halodule uninervis* as a sparse understory. At each site, five sediment samples (0-10 cm deep) were taken using a 50 mL plastic syringe core, and transferred to 50 mL centrifuge tubes, for later isotopic analysis of the sediment, microbial, and resin
extractable $\delta^{18}$O-PO$_4$ pools. *Amphibolis antarctica* leaves (n=100) were also hand-collected at each site for P content analysis. Samples were stored on ice in the field for no longer than 8 hours, and subsequently stored at 4°C (sediment samples) or -20°C (seagrass leaves) until processing.

**Phosphorus extraction**

Phosphorus was extracted from plants and sediments using the methodology of Tamburini et al. (2012). Phosphate from three different sediment pools (resin extractable P, microbial P, and calcareous sediment P) were extracted. Resin and microbial P fractions were extracted as described by (Grierson & Adams 2000) in a modification of the method of (Kouno, Tuchiya & Ando 1995). Resin extractable P represents P that is readily desorbed from sediment particles and is considered available for plant uptake. For resin extractable P, 20 g of fresh sediment was shaken with 4 cm$^2$ strips of anion exchange membranes (Part #BDH-551642S from VWR International ®, USA) in a 1:4 sediment:water suspension for 16h. Membranes were saturated with HCO$_3$$^-$ prior to extraction. Membranes were then removed from the sediment:water suspension, and extracted phosphate was eluted from membranes by shaking the membranes in 0.2M HNO$_3$ for 30 minutes. Microbial P was also extracted using 20g of fresh sediment with anion exchange membranes, but with 1 mL of hexanol added. Microbial P is thereafter defined as the amount of extracted by the resin in the presence of hexanol minus resin-extractable P without hexanol. Calcareous sediment P was extracted by adding 40 mL 1M HCl to 10 g of sediment. Extracts were shaken overnight, filtered, and P was extracted from the filtrate. This extraction was chosen as it is considered a good representation of P present in Ca-P minerals (Tamburini, Bernasconi & Angert 2010). Foliar P of seagrasses was extracted from 2 g of seagrass leaf material using a concentrated (10 M) HNO$_3$ extraction (Pfahler et al. 2012). Prior to extraction, seagrass leaves were scraped clean of epiphytes, dried at 30°C for 48 hours, and ground using a mortar and pestle. Phosphorus from phosphate rock standards (Standard NBS120c, National Institute of Standards and Technology, USA) were also extracted using the same procedure as calcareous P, and analysed to check P recovery.

**Sample preparation and isotopic analysis**

Phosphorus extracts from sediment and seagrass as well as reference material of known
phosphate concentrations were purified and converted to silver phosphate in order to remove any other compounds from samples that could contain oxygen that would interfere with the analysis (Tamburini et al. 2010; 2012). Dissolved organic matter was removed from P extracts using DAX-8 Amberlite Resin that had been preconditioned using 100% methanol. Extracts were then precipitated as ammonium phospho-molybdate (APM) by adding 4.2 M NH₄NO₃ and 0.1 M (NH₄)Mo₇O₂₄•4H₂O (ammonium molybdate). APM crystals were left to form overnight, before being dissolved in citric acid-NH₄OH solution (10 g citric acid, 140 ml conc. NH₄OH, 300 ml deionised water). Extracts were then precipitated as magnesium ammonium phosphate (MAP) by adding 25 ml of a magnesia solution (50 g MgCl₂•6H₂O and 100 g NH₄Cl dissolved in 1 L deinoinised water acidified to pH 1 with 12 M HCl), ammonia hydroxide solution (1:1 v:v NH₄OH/H₂O) and leaving overnight. The MAP crystals were then thoroughly rinsed with ammonia hydroxide solution, to remove traces of Cl that would interfere with later silver phosphate precipitation. The MAP crystals were then dissolved in 0.5 M HNO₃. The resulting solution was then shaken overnight with BioRad AG50-X8 cation resin (H⁺ form, 100-200 mesh, preconditioned with HNO₃) to remove cations from solution. Finally, the samples were precipitated as Ag₃PO₄ by adding a Ag-amine solution (10.2 g AgNO₃, 9.6 g NH₄NO₃, 18.5 ml of concentrated NH₄OH, and 81.5 ml deionised water). Crystals were left to form overnight, before being vacuum filtered, rinsed with deionised water, and left to dry at 50°C overnight. Typically, Ag₃PO₄ crystals should be yellow and euhedral (Tamburini et al. 2010).

Extracted Ag₃PO₄ was weighed into silver capsules, and then pyrolysed in the presence of carbon at 1450°C in a helium stream in a TC/EA (Thermal Conversion/ Elemental Analyser). After separation from other gases on a GC column, the yield CO was analysed on a Delta XL Mass Spectrometer in continuous flow mode (Thermo-Fisher Scientific), at the West Australian Biogeochemistry Centre. All δ¹⁸O-PO₄ ratios are reported relative to the VSMOW standard in per mil (‰) (Halas et al. 2011). Normalization to the VSMOW scale was based on two replicated international reference materials provided by the International Atomic Energy Agency (IAEA): IAEA-601, IAEA-602 following multipoint normalization (Skrzypek & Sadler 2011). The analytical combined uncertainty was <0.4‰.
Results

Using standard extraction procedures, we successfully precipitated Ag$_3$PO$_4$ crystals from phosphate rock standards. Ag$_3$PO$_4$ crystals from phosphate rock standards were yellow and euhedral, consistent with previously successful experiments extracting Ag$_3$PO$_4$ from sediments (Tamburini et al. 2010). Precipitate was also formed from calcareous sediment and seagrass extracts, but had a very different appearance to the precipitates prepared from the rock phosphate standards. Crystals from seagrass and calcareous sediment extracts were brown-black, very fine, and flocculent. The appearance of the calcareous sediment and seagrass precipitates was consistent across several extractions. Despite repeated attempts, no precipitate was yielded from microbial or resin-extractable P extracts.

Precipitates from standards, seagrass, and mineral P extractions were then analysed on the isotope ratio mass spectrometer (IRMS). The $\delta^{18}$O-PO$_4$ values from control standard NBS120c were matching the commonly accepted values within typical for TC/EA method precision. (Table 6.1). The CO yields for NBS120c where in the expected range for pure AgPO$_4$.

However, the signals for precipitates from seagrass and calcareous sediment P pools were ten times lower than NBS120c standards and expected values had pure Ag$_3$PO$_4$ had been extracted, and in several cases just above the background level typical for TC/EA. These extremely low yields suggest that samples contained very little Ag$_3$PO$_4$, and likely contained other unexpected low-oxygen chemical compounds that had been co-precipitated. Therefore, these results were discarded as not reliable.

Discussion

Given logistical and time constraints, I was unable to satisfactorily refine the $\delta^{18}$O-PO$_4$ methodology to address the aims of this chapter. Though precipitates were extracted from sediment and seagrass P pools, the low O$_2$ yields prevent any confidence that the extraction process was completed and that the extract contained pure Ag$_3$PO$_4$. The successful precipitation and analysis of the phosphate rock NBS120c standard suggests that the laboratory procedure was not the main cause for the unreliable results, and instead
points towards problems with processing samples through current methods. Below, I outline several potential reasons why the $\delta^{18}$O-PO$_4$ analysis of Shark Bay sediments and seagrasses was not successful and suggest future steps that may help overcome these analytical issues.

Table 6.1: Maximum amplitude of mass 28 signal sampled (i.e. CO$_2$ yield, mV) and $\delta^{18}$O-PO$_4$ signatures of Ag$_3$PO$_4$ precipitates from rock phosphate standards, seagrasses, and sediments. * - Oxygen yield too low on samples, indicating presence of compounds other than Ag$_3$PO$_4$.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Sample mass (g)</th>
<th>Phosphorus concentration (mg g$^{-1}$)</th>
<th>P content of sample (mg)</th>
<th>Mass 28 CO$_2$ yield (mV)</th>
<th>$\delta^{18}$O-PO$_4$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rock phosphate standard</td>
<td>0.0005 -0.01</td>
<td>330</td>
<td>0.17 - 3.3</td>
<td>2321 - 2370</td>
<td>21.4-22.8 (±0.3) (std. accepted value=21.7)</td>
</tr>
<tr>
<td>Seagrass</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Site 1</td>
<td>2</td>
<td>0.63</td>
<td>1.26</td>
<td>282</td>
<td>19.2*</td>
</tr>
<tr>
<td>Site 2</td>
<td>2</td>
<td>0.51</td>
<td>1.02</td>
<td>257</td>
<td>21.7*</td>
</tr>
<tr>
<td>Site 3</td>
<td>2</td>
<td>0.5</td>
<td>1</td>
<td>346</td>
<td>20.2*</td>
</tr>
<tr>
<td>Site 4</td>
<td>2</td>
<td>0.48</td>
<td>0.96</td>
<td>328</td>
<td>20.8*</td>
</tr>
<tr>
<td>Site 5</td>
<td>2</td>
<td>0.52</td>
<td>1.04</td>
<td>228</td>
<td>21.3*</td>
</tr>
<tr>
<td>Site 6</td>
<td>2</td>
<td>0.74</td>
<td>1.48</td>
<td>530</td>
<td>22.1*</td>
</tr>
<tr>
<td>Site 7</td>
<td>2</td>
<td>0.53</td>
<td>1.06</td>
<td>471</td>
<td>23.1*</td>
</tr>
<tr>
<td>Site 8</td>
<td>2</td>
<td>0.57</td>
<td>1.14</td>
<td>408</td>
<td>24.0*</td>
</tr>
<tr>
<td>Site 9</td>
<td>2</td>
<td>0.65</td>
<td>1.3</td>
<td>1102</td>
<td>22.4*</td>
</tr>
<tr>
<td>Sediment</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Site 1</td>
<td>20</td>
<td>0.056</td>
<td>1.12</td>
<td>358</td>
<td>24.6*</td>
</tr>
<tr>
<td>Site 4</td>
<td>20</td>
<td>0.057</td>
<td>1.14</td>
<td>609</td>
<td>14.2*</td>
</tr>
<tr>
<td>Site 5</td>
<td>20</td>
<td>0.046</td>
<td>0.91</td>
<td>376</td>
<td>17.5*</td>
</tr>
<tr>
<td>Site 6</td>
<td>20</td>
<td>0.051</td>
<td>1.02</td>
<td>271</td>
<td>4.4*</td>
</tr>
<tr>
<td>Site 7</td>
<td>20</td>
<td>0.074</td>
<td>1.48</td>
<td>190</td>
<td>11.5*</td>
</tr>
</tbody>
</table>

Compounds within seagrasses that inhibit precipitation processes

The procedures used here have been successfully used to measure $\delta^{18}$O-PO$_4$ in terrestrial plants and surrounding soils (Tamburini et al. 2012; Pfahler et al. 2012) as well as marine sediments (McLaughlin et al. 2006; Goldhammer et al. 2011b). However, to my knowledge this is the first attempt to assess the $\delta^{18}$O-PO$_4$ of seagrasses or their surface sediments. Given the challenges faced here, and the appearance of the precipitates, it may
be that seagrasses contain compounds that cause contamination or interfere with the formation of a pure Ag₃PO₄ precipitate. For example, seagrasses contain abundant sulfated phenolic compounds to assist survival in a saline habitat and to deter herbivores (McMillan, Zapata & Escobar 1980; Vergés et al. 2007). The concentration of these types of compounds is rarely associated with other marine or terrestrial communities where δ¹⁸O-PO₄ has previously been measured. However, several precipitation and rinsing steps are included in the published methodology which are designed to remove ions that could potentially interfere with Ag₃PO₄ precipitation or δ¹⁸O-PO₄ signals (Tamburini et al. 2010). Similarly, samples are initially treated with DAX-8 resin to remove dissolved organic matter. The DAX-8 resin is highly effective at removing aquatic humic OM compounds, but has primarily been used in freshwater environments, with limited use in seagrass ecosystems (Lavery et al. 2013). We might speculate that organic compounds that are more abundant in seagrasses may not be completely removed by this step, and could have interfered with the precipitation of Ag₃PO₄. Further investigation utilizing multiple methods for dissolved organic matter removal, coupled with spectroscopic analysis of extracts, would help to determine how effective the DAX-8 resin is at cleaning the samples of dissolved organic matter and whether an alternative procedure may be required.

Hypersalinity of Shark Bay

Presence of ions such as Na⁺ and Cl⁻ can limit the precipitation of Ag₃PO₄ (Tamburini et al. 2014). As such, the high salinities in Shark Bay could be contributing to the limited precipitation of Ag₃PO₄ in Shark Bay samples by concentrating specific ions that interfere with the methodology. However, the methods used in this experiment have also been used successfully in marine ecosystems (Colman et al. 2005; Goldhammer et al. 2011b; a; Joshi, Kukkadapu & Burdige 2015) where salinities would also have been over 20‰. The various precipitations used prior to the final Ag₃PO₄ precipitation include several rinsing steps, and are designed to eliminate interfering ions. As such, salinity alone is unlikely to have prevented proper Ag₃PO₄ precipitation. However, in hypersaline environments like Shark Bay, it may be required to increase the number of rinses of precipitated crystals at all stages of the method to account for the higher concentrations of anions and cations. Cerium phosphate precipitations have been used in some marine δ¹⁸O-PO₄ experiments as an alternative to the APM precipitation (McLaughlin et al. 2004; 2006; Paytan &
McLaughlin 2007). I also tried cerium phosphate extractions in place of APM precipitations in this experiment, but again failed to purify Ag₃PO₄ from extracts.

*Low phosphate concentrations*
Both the waters of Shark Bay and the underlying sediments are highly oligotrophic. Thus, there may have been insufficient phosphate in the sediment extracts for successful precipitation of Ag₃PO₄. The purification of extracts into Ag₃PO₄ (with several different precipitation and rinsing steps) likely leads to loss of phosphates, exacerbating the already low concentrations. However, low concentrations (0.5 µmol) of porewater P have successfully been extracted using similar methodologies, allowing ~200 µg Ag₃PO₄ that was enough to generate adequate δ¹⁸O-PO₄ signals (Goldhammer et al. 2011b). The microbial and resin P extracts here were close to this lower limit of analysis; we suspect loss of P through experimental handling likely left P concentrations too low. However, calcareous sediment P and phosphates extracted from seagrass leaves were all above 1000 µg (Table 6.1), well in excess of the 200 µg limit successfully measured by Goldhammer et al. (2011b). Overall low phosphate concentrations alone are unlikely to fully explain the lack of Ag₃PO₄ precipitation.

*Resin preparation*
Goldhammer (2011) noted that using cation resin membranes prepared the previous day resulted in reddish discolouration of samples that made proper Ag₃PO₄ impossible. The reason for this discolouration was never fully determined, but was prevented by preparing resin membranes immediately before analysis (Goldhammer, pers. comm.). I prepared our resin immediately before use but despite this caution noticed a reddish discolouration in some samples similar to that described by Goldhammer (2011). This may have contributed to improper Ag₃PO₄ precipitation but at this point I have no clear chemistry-based explanation as to why.

*Final comment*
My original objective for this study was to analyse the δ¹⁸O-PO₄ signatures of different P pools in seagrass ecosystems to elucidate biological P cycling in these environments. However, I was unable to adequately extract purified Ag₃PO₄ from seagrasses or their surrounding sediments. I propose that the poor precipitation of Ag₃PO₄ in this
experiments may be attributable to a combination of unique organic and sulfur rich compounds in seagrass tissues, high salinities, and low P concentrations. Further experiments should focus on determining the compounds in seagrass tissues that may interfere with current analytical procedure, and find a method to remedy this (e.g. removal of compounds in separate step, or new precipitation methodology). A first step would be to spike samples from seagrass ecosystems with rock phosphate and test relative recovery. It would also be useful to perform this experiment in an ecosystem with higher ambient P concentrations and lower salinities, to avoid potential problems with high salinities and low P concentrations. Systematic concentration-precipitation experiments need to be undertaken to identify detection limits across a range of systems. It might also useful to test procedures for concentrating P extracts prior to the precipitation step. New and modified methods that are rigorously tested across a diversity of ecosystems and sample types are thus needed in order to increase confidence in the δ¹⁸O-PO₄ approach to understand biological turnover of phosphates.
References


Phosphorus cycling in seagrass sediments


Chapter 7: General discussion: organic matter cycling and the phosphorus biogeochemistry of Shark Bay

The findings of this thesis expand the knowledge of the different sources and significance of organic matter in seagrass sediments, and the role OM plays in influencing P biogeochemistry and sustaining the P demands of seagrasses in Shark Bay. More generally, this research also provides new understanding of the ways in which seagrass productivity is maintained in oligotrophic environments. This research also contributes to the growing recognition of the significance of microbial communities and their functioning to understanding the biogeochemical processes that sustain life in near coastal ecosystems around the world. In particular, this research emphasizes the importance of understanding the functional capabilities of rhizosphere microbial communities, given the influence this has on key biogeochemical processes and ultimately primary productivity.

The primary aim of this PhD study was to characterise the sources and ecological impacts of organic matter (OM) inputs into seagrass sediments, and then examine the role that microbially-driven breakdown and turnover of this OM plays in supplementing phosphorus (P) availability in an oligotrophic seagrass ecosystem (Figure 7.1). My research demonstrates that OM plays a significant role in the P cycle of seagrass meadows, and may supplement seagrass productivity in oligotrophic areas after microbial mineralization. In this concluding discussion, I first briefly summarise the main findings from my research. I then discuss how my research fits into wider paradigms emerging within marine ecology more broadly, focusing on extreme climatic events, organic matter cycling, and microbial ecology. I then discuss how my research changes our knowledge of the biogeochemistry of Shark Bay, before concluding.
Figure 7.1 Conceptual model showing main results from this thesis. 1) Seagrasses showed short term defoliation and longer-term loss of belowground biomass in response to the 2010/11 marine heatwave, with biomass losses greatest in areas where light availability was reduced as a result of the 2010/11 Wooramel River flood, leading to an input of seagrass-derived OM into sediments. 2) Seagrass-derived OM into sediments led to shifts in nutrient availability, enzyme expression, and microbial community structure in sediments, while seagrass seedlings has shifts in biomass allocation. 3) Sediment P fractionation across the salinity and phosphate gradient in Shark Bay. High salinity sites had higher concentrations and proportions of organic P compounds, with phosphate monoesters dominating this fraction. 4) Microbial communities showed an increase in the abundance of organic P at high salinity/low SRP sites, correlating with an increase in the expression of phosphatases that could hydrolyse phosphate monoesters. 5) The microbial degradation could support the P requirements of seagrasses in the sites with lowest SRP concentrations, but this could not be experimentally confirmed (using δ18O-PO4).
Summary of main findings of this research

I show that extreme climatic events that cause seagrass dieback lead to declines in seagrass belowground biomass up to 2 years after disturbance, with high quantities of seagrass-derived OM entering sediments (Chapter 2). Seagrass-derived OM significantly changes belowground conditions in seagrass sediments; altering nutrient availability, microbial community composition, and hydrolytic enzyme expression (Chapter 3), and could be a major factor dictating sediment biogeochemistry in seagrass sediments during seagrass colonization, meadow growth, and following dieback events. Further, OM in sediments was found to be an important component of the P cycle in Shark Bay. As salinity increased along the salinity gradient and soluble reactive phosphate (SRP) in the water column decreased, the concentration and proportion of P in the organic P fraction increased in sediments, even though total P decreased (Chapter 4). The organic P fraction was dominated by phosphate monoesters, likely resulting from seagrass litter dominating OM inputs into sediments. Microbial community structure and function in sediments also significantly changed along the salinity/SRP gradient possibly in response to increased sedimentary organic P (Chapter 5). Microbial communities had higher abundances of genes related to the metabolism of organic P compounds at sites with high salinity/low SRP sites, and were strongly correlated with an increased expression of phosphatase enzymes that could hydrolyse phosphate monoesters. Though it is likely that the microbial degradation of organic P at least partially supplements seagrass P requirements in sites with low SRP, this could not be experimentally validated using δ18OPO4 as a tracer of P sources due to methodological issues (Chapter 6). Nevertheless, this thesis clearly show that seagrass-derived OM directly influences P biogeochemistry and microbial communities within seagrass ecosystems. This thesis reveals new insights into the significance of seagrass-derived OM, the characteristics of the organic P pool, and the functional characteristics in seagrass sediments.

Sediment organic matter in seagrass meadows

Organic matter in seagrass sediments has recently received attention as a globally important carbon sink, often noted for its refractory nature (Mcleod et al. 2011; Fourquean et al. 2012a). However, this thesis shows that OM cannot be considered as
just a passive C sink in seagrass ecosystems, instead influencing biogeochemical processes, microbial community structure, and seagrass physiology (Chapter 3-5). As such, the gradual enrichment of sediments with OM after seagrass colonization may play an important role in biogeochemical cycling and ecosystem processes in coastal ecosystems. Furthermore, seagrass-derived OM contributes to detrital food webs that are often more important to energy transfer up the food chain than direct grazing on seagrasses, showing the bottom-up impacts this resource can have (Mcleod et al. 2011).

Shark Bay itself has been stated as a global C sequestration hotspot (Fourqurean et al. 2012b), but this sediment OM pool also has a contemporary ecological importance in directly influencing biogeochemistry and productivity within Shark Bay.

The ecological and geochemical impacts of OM in seagrass sediments will largely depend on the types of inputs the meadows receives. Oligotrophic ecosystems with abundant seagrass meadows like Shark Bay have sediment OM pools dominated by seagrass detritus characterised with high C:N and C:P ratios (Gacia, Duarte & Middelburg 2002; Fourqurean et al. 2012b; Fraser et al. 2012). Conversely, settling organic material from planktonic sources would make up a substantial component of total sedimentary OM in mesotrophic or eutrophic ecosystems where nutrient availability and plankton biomass is higher (Marbà et al. 2006; Krause-Jensen, Markager & Dalsgaard 2011). Plankton detritus has lower C:N and C:P ratios than seagrass detritus (Duarte 1990), and this difference in elemental composition could affect how quickly OM is mineralised, the products of mineralization, and the physiological impacts of mineralization (Enriquez, Duarte & Sand-Jensen 1993; Eyre & Ferguson 2002). Similarly, inputs of OM resulting from anthropogenic activities will also have different chemical composition and lability, yet the distinction between types of OM is rarely made. Inputs of OM into seagrass meadows are often expected to cause detrimental effects based on previous experiments that focus on highly degraded ecosystems (Delgado et al. 1999) or from experiments that use unnatural OM forms that would not be found in natural seagrass sediments (Pérez et al. 2007; Ruiz-Halpern, Macko & Fourqurean 2008), but the actual effects of OM enrichment will depend largely on the dominant source of OM inputs. Characterising the origins and chemical composition of OM inputs in seagrass sediments will be important in predicting subsequent ecological effects.
Seagrass ecosystems – a new focus on organic P

In this thesis, I show that the OM in seagrass sediments influences P cycling in seagrass sediments, and that organic P may be an important contributor to the P requirements of seagrasses growing in carbonate sediments that would normally be expected to be P limited.

Models of P cycling and availability in seagrass ecosystems have focused on inorganic or labile P pools in sediments and in the water column, primarily driven by an early focus on the detrimental effects of inorganic nutrient enrichment in seagrass meadows (Short 1987). This focus has led to the paradigm of P limitation of seagrass growth in tropical, carbonate sediments given the low SRP concentrations that are common in these ecosystems, as well as the high adsorption of P onto calcareous sediment particles and N being in excess due to nitrogen fixation (Short 1987; Romero et al. 2006). However, N limitation has been found of seagrasses growing on carbonate sediments (Erftemeijer et al. 1994; Udy, Dennison & Lee Long 1999), while P limitation has been found in seagrass growing on sediments with low carbonate content (Pérez et al. 1991), showing factors other than sediment type influence nutrient limitation.

When examining the availability and importance of sediment organic P in seagrass meadows, I found it was important to couple traditional sequential extractions with techniques such as $^{31}$P-NMR to examine the organic P fraction to a compound level. Only a few studies had previously examined P fractionation in seagrass sediments (Jensen et al. 1998; Koch, Benz & Rudnick 2001; Holmer, Carta & Andersen 2006; Reimer & Huerta-Diaz 2010) and, in all of these experiments, the organic P fractions was operationally defined based on extraction techniques. While providing valuable information of P fractionation in sediments in general, this does not provide any information on the potential turnover and availability of the organic P pool itself, given that the variety of compounds that contribute to the organic P pool can vary greatly in their availability and residence times in sediments (Turner, Frossard & Baldwin 2005; Baldwin 2013). I found that the dominant organic P compound in Shark Bay sediments were phosphate monoesters. Phosphate monoesters are not labile, and often accumulate in soils and sediments with high amounts of plant litter (Turner et al. 2002), and their
dominance in seagrass sediments is not surprising given that seagrass litter is often the major component of OM in seagrass sediments (Cawley et al. 2012). Further research in other seagrass ecosystems is required to determine if phosphate monoesters dominate the organic P pool in all seagrass sediments, or if other organic P compounds may also form a substantial proportion of the fraction. Again, the ubiquitous presence of alkaline phosphatase – which could hydrolyse phosphate monoesters - in many different seagrass ecosystems (Pérez & Romero 1993; Martínez-Crego, Romero & Alcoverro 2006; Koch, Kletou & Tursi 2009) suggests that phosphate monoesters make up a substantial proportion of organic P in these environments.

The composition of the organic P fraction in seagrass sediments will depend largely on the types of organic inputs into meadows. Where other OM inputs such as planktonic- or terrestrially-derived OM contribute substantially to the total sediment OM pool, the composition of the sediment organic P fraction is likely to be different. For example, sediment traps from a range of different coastal and open ocean ecosystems showed that settling particulate matter consisted of phosphate monoesters, phosphate diesters, and phosphonates (Paytan et al. 2003), while detrital inputs from adjacent coastal ecosystems such as mangroves or wetlands would also have detectable concentrations of phosphate diesters and phosphonates in the organic P pools (Cheesman, Turner & Ramesh Reddy 2012; Baldwin 2013). Further research examining the drivers of organic P speciation in seagrass sediments would help determine the potential availability of this overlooked nutrient pool.

**Extreme events: an emerging threat to seagrass meadows**

This thesis showed the drastic impacts that short-term, extreme climatic events like the 2010/11 marine heatwave can have on seagrass meadows. Though the effects of increasing temperature on seagrass physiology and ecology have been widely studied (Walker & Cambridge 1995; Campbell, McKenzie & Kerville 2006; Marbà & Duarte 2009), the impacts of increasing temperatures resulting from climate change has generally been considered as a gradual, press disturbance where mean temperatures slowly increase, giving seagrasses time to acclimate and adapt. However, extreme events that lead to short lived periods of temperatures above mean levels are expected to increase in frequency and intensity in the near future (Alexander & Arblaster 2009), and will present
Phosphorus cycling in seagrass sediments

short-term pulse disturbances to seagrass ecosystems over time scales that limit acclimation or adaptation. The capacity for seagrasses to persist through extreme events will depend primarily on their upper thermal tolerance limits. Temperate seagrasses growing near their upper thermal tolerance thresholds, like *Amphibolis antarctica* in Shark Bay, will be most at risk to suffer negative effects following extreme events, and may be replaced by smaller tropical seagrasses better suited for survival through high temperature events. As such, seagrass ecosystems may respond to warming through ‘tropicalization’ similar to fish species, with transition zones from tropical to temperate species gradually move polewards (Vergés et al. 2014). Impacts on seagrasses will be particularly acute when extreme temperatures coincide with additional stressors. In this thesis I showed that areas with reduced light availability had increased rates of dieback, showing the capacity for multiple stressors to create patchy responses within an ecosystem. Effective management of important seagrass ecosystems (especially at species range limit) will require recognition of both pulse and press impacts of climate change, and potentially with additive or synergistic stressors that would exacerbate loss. This may require a shift of management strategies: away from direct strategies solely preventing local loss driven by coastal development to strategies that also mitigate loss driven by regional-global scale changes in climatology that acknowledge multiple stressors.

Shark Bay’s status as a World Heritage listed ecosystem is intrinsically linked to the health and functioning of seagrass meadows within the Bay that could be affected by future environmental changes (Kendrick et al. 2012). Clearly, extreme events like the 2010/11 heatwave have the potential to cause direct impacts to seagrasses, and therefore key World Heritage values, within Shark Bay. Future extreme events could drive further diebacks of temperate, meadow-forming seagrasses such as *A. antarctica*, which may be replaced by smaller tropical seagrasses such as *Halodule uninervis* at sites that are marginal for *A. antarctica* growth (Fraser et al. 2014). However, small tropical seagrasses like *H. uninervis* do not offer the same ecosystem functions as *A. antarctica*, which is a foundation species that is a niche habitat for species of high conservation priority in Shark Bay (Fraser et al. 2014; Thomson et al. 2014). For example, the health of green turtles – that use the seagrass meadows for foraging – decreased following the seagrass dieback of Shark Bay (Thomson et al. 2014). Other species are also likely to have been impacted following seagrass dieback in Shark Bay, but the full ecosystem effects are difficult to
identify without long-term data sets to compare population changes before and after the heatwave within Shark Bay. In addition, a shift towards a benthic community dominated by tropical seagrasses would impact other ecosystem services such as sedimentation, carbon sequestration, and nutrient cycling in Shark Bay (Walker, Kendrick & McComb 2006; Jahnert & Collins 2011; Fourqurean et al. 2012b). Clearly, any further loss of seagrass in the Shark Bay World Heritage Area would have significant bottom-up impacts on the ecologically and socially important functions that seagrasses contribute in Shark Bay.

**Microbial ecology of seagrass sediments**

Microbial ecology and biogeochemistry are intimately linked in seagrass sediments, with the functional composition of the microbial community largely determining the availability of the organic P fraction and the decomposition of OM in sediments. In this thesis I showed that microorganisms in seagrass sediments respond strongly to environmental conditions (salinity and P availability), and shifts in microbial community structure subsequently alter biogeochemistry and P availability in seagrass sediments. However, microorganisms have a myriad of roles in plant rhizospheres that can influence plant productivity; altering nutrient cycles, causing or increasing resistance to disease, and regulating responses to potential stressors (Miki et al. 2010; Mangan et al. 2010; Van Der Putten et al. 2013). The strength of such interactions would also have important implications for species diversity within seagrass meadows; with strong positive feedbacks likely to promote monospecific meadows and negative feedbacks promoting meadows with higher species diversity (van der Heijden, Bardgett & van Straalen 2008; Mangan et al. 2010; Van Der Putten et al. 2013). In seagrass sediments specifically, microorganisms also mediate the availability of oxygen (Borum et al. 2005) and potential toxins such as hydrogen sulfides in sediments that can influence seagrass productivity and health (Pedersen, Binzer & Borum 2004). The interactions between seagrasses and microorganisms underpin the functioning of seagrass meadows, yet these interactions remain relatively understudied in seagrass ecosystems. As such, key questions regarding microbial ecology in seagrass sediments remain unanswered. How do seagrasses interact with microorganisms in sediments? What are the major drivers of microbial community structure and function in seagrass rhizospheres? Are seagrass-associated microbial communities species-specific, or do they share similarities among seagrass species? Are
there key microbial groups that are critical to healthy seagrasses? I believe microbial ecology is one of the most pressing areas of research in seagrass ecology, and emerging techniques will allow us to address some of these key questions.

A rapid increase in our understanding of microbial ecology in seagrass ecosystems will be facilitated by recent advances in molecular methods, which are increasing in power and efficiency while decreasing in cost (Simon & Daniel 2011). In this thesis, I used metagenomic sequencing to examine the taxonomic and functional structure of the entire microbial community in seagrass sediments. Microbial community taxonomy and function do not always change concurrently in marine ecosystems (Frias-Lopez et al. 2008). The functional diversity ultimately determines the microbial contribution to ecosystem processes such as decomposition and nutrient cycling (Zak, Blackwood & Waldrop 2006), yet most studies examining microorganisms associated with seagrasses have focused on taxonomic measurements (Jensen, Kühl & Priemé 2007; Ikenaga et al. 2010; Green-García & Engel 2012). In the future, it will be more appropriate to examine functional genes along with taxonomic structure in seagrass-associated microbial communities when addressing the effects of microorganisms on ecosystem processes or seagrass function. Complementary techniques such as metatranscriptomics and metaproteomics can be used to examine changes in gene and protein expression respectively (Wilmes & Bond 2006; Gilbert et al. 2008), and used alongside molecular methods like metagenomics to show real time microbial community responses to changes in physical conditions. In situ hybridisation methods such as fluorescent in-situ hybridization (FISH) and high resolution secondary ion mass spectrometry (nano-SIMS) could identify specific microbial groups and their locations within seagrass tissues and - in the case of nano-SIMS - allow us to determine their importance for nutrient transfer into seagrasses using stable isotope labelling (Amann, Fuchs & Behrens 2001; Behrens, Kappler & Obst 2012). These techniques could be combined with manipulative experiments under controlled conditions to explicitly investigate the importance of microorganisms to seagrass physiology. Plant-soil feedback experiments have become increasingly common in terrestrial plant ecology, and involve experimentally altering soil microbial communities (e.g. sterilizing sediments or preconditioning with another species prior to planting) and testing the effects on plant health (Van Der Putten et al. 2013). These techniques will allow us to move beyond simply cataloguing what microbial
species are present in seagrass meadows, and instead focus on the ecological functions they perform.

**A new assessment of the biogeochemistry of Shark Bay**

This thesis has revealed new insights into the dependence of biogeochemical processes and microbial functioning of Shark Bay that demonstrate a previously unappreciated role of seagrass OM in maintaining P availability. Previously, Shark Bay has been widely considered a notable example of an oligotrophic marine embayment, where primary productivity is strongly P limited, especially at high salinity sites (Smith & Atkinson 1983; Smith 1984a; b). This thesis shows a clear shift in the predominance of different sedi\-mentary P fractions (Chapter 4) and microbial community function (Chapter 5) along the salinity gradient of Shark Bay that changes the availability of sedimentary P, and may explain why seagrasses do not always show signs of increasing P limitation across the salinity gradient (Fraser et al. 2012). Patterns in sediment P speciation, microbial P metabolism gene abundance, and enzyme expression suggest microbial communities were increasingly targeting the organic P fraction in high salinity areas.

This thesis also further shows the capacity of seagrasses to act as ecosystem engineers by modifying their surrounding environment. Seagrasses are generally considered ecosystem engineers through their role modifying the physical structure of the environment (e.g. Bos et al. 2007), as they do in Shark Bay by creating shallow banks through increased sedimentation (Kendrick et al. 2012). However, this thesis demonstrates that the seagrasses of Shark Bay also contribute to biogeochemical gradients in sediment OM and P availability, as well as salinity, creating a unique environment in southern, hypersaline reaches where they are able to dominate as primary producers in spite of extreme conditions of oligotrophy and hypersalinity. Sediment microorganisms are likely an important part of seagrasses creating and surviving within such extreme environments; containing novel genes and potentially forming close associations with seagrasses that enable nutrient demands to be met from organic sources.

If recycling of nutrients from seagrass-derived OM is important in supporting primary productivity in Shark Bay, further loss of seagrasses would impact biogeochemical cycling in Shark Bay. Seagrasses provide microorganisms with a unique environment by
Phosphorus cycling in seagrass sediments

creating a gradient in oxygen around roots and through enriching sediments with seagrass trapped and derived OM (Welsh 2000; Garcia-Martínez et al. 2009). Interactions between plants and microorganisms also become increasingly important for plant nutrition under oligotrophic conditions, while functional redundancy is decreased for important and often highly specialized functions (van der Heijden et al. 2008). Consequently, Shark Bay may be highly sensitive to reductions in microbial diversity arising from shifts in environmental conditions. Predicted increases in the magnitude and frequency of extreme events like the 2010/11 heatwave and floods in Shark Bay would directly influence microbial communities by changing environmental conditions, and resultant significant seagrass dieback would also indirectly impact seagrass-associated microbial communities through a loss of habitat (Jensen et al. 2007; Williams et al. 2009). Extreme climatic events leading to increased sea temperatures and flooding will increase in Shark Bay (Alexander & Arblaster 2009; Ummenhofer et al. 2015), while global ocean temperatures are expected to increase 0.2°C per decade under most projections (IPCC 2007). In addition, associated changes to ocean circulation (Hobday & Lough 2011) and hydrology (Ummenhofer et al. 2015) could influence salinity or nutrient availability that would also likely impact seagrass and microbial communities. The subsequent effects on biogeochemical processes, especially of phosphorus and carbon in Shark Bay are hard to predict. For example, increased temperatures could increase seagrass-derived OM in sediments by causing seagrass dieback, but would also increase mineralization rates of existing sediment OM.

Shark Bay has limited pressures from surrounding anthropogenic activities, which has contributed to it remaining a relatively pristine ecosystem (Kendrick et al. 2012). However, Shark Bay would be particularly vulnerable to future nutrient inputs. Currently, Shark Bay has been largely protected from eutrophication issues faced by many other seagrass communities around the world owing to its extreme isolation (Burkholder, Tomasko & Touchette 2007). However, even small nutrient additions can lead to changes in benthic community structure away from temperate, structurally-important seagrasses to tropical seagrasses, macroalgae, or even unvegetated sediments (Armitage et al. 2005; Armitage, Frankovich & Fourquarean 2011). Increases in nutrients in readily available inorganic forms like nitrates or phosphates could rapidly alter benthic biogeochemistry and ecology in surrounding meadows, especially since biogeochemical cycling within
Shark Bay seems dominated by internal recycling processes mediated by microbial activity. As such, any future development that could increase nutrient inputs (e.g. population growth, increased tourism) could have significant negative impacts on the seagrass meadows of Shark Bay, and must be managed accordingly. Nutrient addition experiments would show how benthic communities would respond to potential changes in nutrient inputs, allowing predictions of ecosystem responses that would inform the future management of Shark Bay.

**Conclusion**

Nutrient cycling in seagrass sediments is a major driver of seagrass ecology, yet the contribution of nutrients from organic pools have largely been ignored, in spite of seagrass sediments often being enriched in OM. This thesis showed that OM in seagrass sediments has significant effects on biogeochemical processes and microbial ecology in the oligotrophic seagrass meadows of Shark Bay, and has the potential to drive changes in seagrass productivity. Even seagrass-derived OM – previously considered as refractory in seagrass sediments – greatly alters belowground processes, and will likely become more prevalent in key seagrass ecosystems like Shark Bay with increases in extreme climatic events. Interactions between sediment OM, microbial communities, and seagrasses should be further elucidated to better understand the belowground drivers of seagrass ecology which have been often overlooked in favour of more visible changes in water quality. Given that belowground interactions have the potential to alter nutrient budgets, primary productivity, and even seagrass diversity, this should be a research priority. Plant ecology, sediment biogeochemistry, and microbial ecology are intrinsically linked to one another by a series of complex interactions that together determine key ecosystem functions like primary productivity, and must be examined concurrently to understand belowground drivers of coastal ecosystem ecology. Quantifying the link between microbially derived recycled organic P and uptake of P by seagrasses remains the missing link in this thesis given the methodological difficulties with extracting $\delta^{18}$O$_{PO_4}$ from seagrasses and sediments, but deserves immediate further investigation.

**References**

Phosphorus cycling in seagrass sediments


Behrens, S., Kappler, A. & Obst, M. (2012) Linking environmental processes to the in situ functioning of microorganisms by high-resolution secondary ion mass spectrometry (NanoSIMS) and scanning transmission X-ray microscopy (STXM). Environmental Microbiology, 14, 2851–2869.


Phosphorus cycling in seagrass sediments


Short, F. (1987) Effects of sediment nutrients on seagrasses: literature review and
Phosphorus cycling in seagrass sediments


Appendices
Appendix 1: Summary of physical and biogeochemical data at each site. Depth, temperature, and salinity all averages over 5 CTD casts. Sediment organic matter, enzyme expressions, microbial P biomass, and seagrass P content are averages (n=3 per site). SRP = Soluble reactive phosphorus. BD = Below detection.

<table>
<thead>
<tr>
<th>Site</th>
<th>Depth (m)</th>
<th>Temp (°C)</th>
<th>Water SRP (µM)</th>
<th>Salinity (%)</th>
<th>Organic matter (% d.w)</th>
<th>Alkaline phosphatase (µmol l⁻¹ hr⁻¹)</th>
<th>Acid phosphatase (µmol l⁻¹ hr⁻¹)</th>
<th>Beta glucosidase (µmol l⁻¹ hr⁻¹)</th>
<th>Microbial P biomass (µg g⁻¹)</th>
<th>Seagrass P (% d.w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.8</td>
<td>25.7</td>
<td>0.1</td>
<td>39.2</td>
<td>1.7</td>
<td>123.9</td>
<td>40.4</td>
<td>4.7</td>
<td>35.0</td>
<td>0.06</td>
</tr>
<tr>
<td>2</td>
<td>1.6</td>
<td>26.3</td>
<td>0.1</td>
<td>39.7</td>
<td>2.7</td>
<td>31.0</td>
<td>23.9</td>
<td>4.9</td>
<td>16.0</td>
<td>0.05</td>
</tr>
<tr>
<td>3</td>
<td>1.9</td>
<td>26.4</td>
<td>0.06</td>
<td>40.6</td>
<td>1.5</td>
<td>26.5</td>
<td>17.1</td>
<td>3.4</td>
<td>8.1</td>
<td>0.05</td>
</tr>
<tr>
<td>4</td>
<td>1.3</td>
<td>25.0</td>
<td>0.08</td>
<td>42.3</td>
<td>4.3</td>
<td>102.2</td>
<td>43.3</td>
<td>8.5</td>
<td>7.3</td>
<td>0.05</td>
</tr>
<tr>
<td>5</td>
<td>1.5</td>
<td>24.4</td>
<td>0.06</td>
<td>43.6</td>
<td>2.0</td>
<td>66.8</td>
<td>39.3</td>
<td>4.7</td>
<td>15.5</td>
<td>0.06</td>
</tr>
<tr>
<td>6</td>
<td>1.6</td>
<td>26.0</td>
<td>0.05</td>
<td>42.7</td>
<td>4.1</td>
<td>131.0</td>
<td>71.0</td>
<td>17.4</td>
<td>52.3</td>
<td>0.07</td>
</tr>
<tr>
<td>7</td>
<td>1.5</td>
<td>26.1</td>
<td>BD</td>
<td>46.8</td>
<td>4.3</td>
<td>493.3</td>
<td>202.9</td>
<td>16.7</td>
<td>66.4</td>
<td>0.05</td>
</tr>
<tr>
<td>8</td>
<td>2.3</td>
<td>25.2</td>
<td>BD</td>
<td>51.2</td>
<td>2.1</td>
<td>239.0</td>
<td>79.3</td>
<td>3.1</td>
<td>54.7</td>
<td>0.06</td>
</tr>
<tr>
<td>9</td>
<td>1.8</td>
<td>25.3</td>
<td>BD</td>
<td>53.2</td>
<td>2.8</td>
<td>702.3</td>
<td>230.0</td>
<td>11.2</td>
<td>30.5</td>
<td>0.07</td>
</tr>
</tbody>
</table>
Appendix 2: Post-hoc analysis (Games-Howell) of differences in abundance of level 1 functions annotated using the SEED database between high salinity sites (>46‰, white) and lower salinity sites (<46‰, dark grey). Level 1 functions that were significantly different between high and lower salinity sites are denoted by *, with corrected $P$-values using Storey’s false discovery rate approach ($P<0.05$, see Chapter 5 methods for full description).
Appendix 3: Summary of metagenomic sequencing results for Shark Bay sediments. Number of base pairs, sequencing reads, annotated proteins and % predicted using the SEED subsystem database after quality control on the MG-RAST pipeline.

<table>
<thead>
<tr>
<th>MG-RAST ID</th>
<th>Site</th>
<th>Base pair count</th>
<th>Sequence count</th>
<th>Average sequence length</th>
<th>Annotated protein (%)</th>
<th>Number of subsystem proteins</th>
<th>Predicted Subsystems proteins (%)</th>
<th>α-diversity (species)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4661592.3</td>
<td>1</td>
<td>1,466,828,783</td>
<td>4,493,000</td>
<td>326</td>
<td>38.2</td>
<td>1,664,043</td>
<td>47</td>
<td>671</td>
</tr>
<tr>
<td>4661595.3</td>
<td>1</td>
<td>1,214,340,697</td>
<td>3,776,363</td>
<td>321</td>
<td>36</td>
<td>1,333,736</td>
<td>43</td>
<td>718</td>
</tr>
<tr>
<td>4661600.3</td>
<td>1</td>
<td>1,233,436,148</td>
<td>3,836,447</td>
<td>321</td>
<td>32.6</td>
<td>1,281,710</td>
<td>44.2</td>
<td>696</td>
</tr>
<tr>
<td>4661594.3</td>
<td>3</td>
<td>1,167,798,847</td>
<td>3,528,382</td>
<td>330</td>
<td>17.7</td>
<td>301,420</td>
<td>12.3</td>
<td>758</td>
</tr>
<tr>
<td>4661590.3</td>
<td>3</td>
<td>1,125,452,740</td>
<td>3,495,614</td>
<td>321</td>
<td>25.5</td>
<td>845,917</td>
<td>33</td>
<td>757</td>
</tr>
<tr>
<td>4661591.3</td>
<td>3</td>
<td>1,197,070,339</td>
<td>3,753,866</td>
<td>318</td>
<td>30.5</td>
<td>1,006,349</td>
<td>35</td>
<td>706</td>
</tr>
<tr>
<td>4661604.3</td>
<td>5</td>
<td>1,160,096,662</td>
<td>3,634,852</td>
<td>319</td>
<td>25.5</td>
<td>853,589</td>
<td>31</td>
<td>701</td>
</tr>
<tr>
<td>4661593.3</td>
<td>5</td>
<td>1,192,194,650</td>
<td>3,714,494</td>
<td>320</td>
<td>31.7</td>
<td>1,149,229</td>
<td>38</td>
<td>748</td>
</tr>
<tr>
<td>4661603.3</td>
<td>5</td>
<td>1,214,421,809</td>
<td>3,754,913</td>
<td>323</td>
<td>33.7</td>
<td>1,207,849</td>
<td>38</td>
<td>725</td>
</tr>
<tr>
<td>4661596.3</td>
<td>6</td>
<td>1,184,808,271</td>
<td>3,672,117</td>
<td>322</td>
<td>30.1</td>
<td>1,029,926</td>
<td>33</td>
<td>753</td>
</tr>
<tr>
<td>4661597.3</td>
<td>6</td>
<td>1,224,919,206</td>
<td>3,854,469</td>
<td>317</td>
<td>30.5</td>
<td>1,040,510</td>
<td>33</td>
<td>741</td>
</tr>
<tr>
<td>4661601.3</td>
<td>6</td>
<td>1,202,976,906</td>
<td>3,755,410</td>
<td>320</td>
<td>33</td>
<td>1,101,648</td>
<td>34</td>
<td>743</td>
</tr>
<tr>
<td>4661602.3</td>
<td>7</td>
<td>1,206,253,769</td>
<td>3,777,840</td>
<td>319</td>
<td>32.2</td>
<td>1,124,935</td>
<td>36</td>
<td>719</td>
</tr>
<tr>
<td>4661599.3</td>
<td>7</td>
<td>1,186,351,414</td>
<td>3,678,190</td>
<td>322</td>
<td>31</td>
<td>1,084,656</td>
<td>35</td>
<td>740</td>
</tr>
<tr>
<td>4661598.3</td>
<td>7</td>
<td>1,209,795,764</td>
<td>3,786,067</td>
<td>319</td>
<td>31</td>
<td>1,028,746</td>
<td>33</td>
<td>724</td>
</tr>
<tr>
<td>4661587.3</td>
<td>9</td>
<td>1,167,185,047</td>
<td>3,599,493</td>
<td>324</td>
<td>30</td>
<td>1,013,308</td>
<td>34</td>
<td>768</td>
</tr>
<tr>
<td>4661588.3</td>
<td>9</td>
<td>1,197,907,978</td>
<td>3,757,584</td>
<td>318</td>
<td>32.6</td>
<td>1,175,768</td>
<td>39</td>
<td>743</td>
</tr>
<tr>
<td>4661589.3</td>
<td>9</td>
<td>1,207,337,003</td>
<td>3,841,773</td>
<td>314</td>
<td>33.4</td>
<td>1,144,768</td>
<td>37</td>
<td>727</td>
</tr>
</tbody>
</table>
Appendix 4: Summary of metagenomes used to compare microbial communities in seagrass rhizosphere sediments to other ecosystems. All metagenomes are publicly available on the MG-RAST server.

<table>
<thead>
<tr>
<th>Biome Type</th>
<th>MG-RAST IDs</th>
<th>Location</th>
<th>Sequencing method</th>
<th>Samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Seagrass</td>
<td>4661587.3 – 4661604.3</td>
<td>Shark Bay, Western Australia</td>
<td>Illumina</td>
<td>18</td>
</tr>
<tr>
<td>Coral</td>
<td>4445755.3 – 4445756.3</td>
<td>Magnetic Island, Australia</td>
<td>454</td>
<td>2</td>
</tr>
<tr>
<td>Deep Sea</td>
<td>4487294.3, 4487295.3</td>
<td>Offshore sediment, South China Sea</td>
<td>Illumina</td>
<td>2</td>
</tr>
<tr>
<td>Desert</td>
<td>(i) 4477805.3, (ii) 4477872.3–4477873.3</td>
<td>(i) Mojave Desert, California</td>
<td>454</td>
<td>3</td>
</tr>
<tr>
<td>Estuary</td>
<td>4440948.3, 4441020.3 - 4441022.3</td>
<td>Coorong Lagoon, South Australia</td>
<td>Ilumina</td>
<td>3</td>
</tr>
<tr>
<td>Freshwater</td>
<td>4465820.3, 4465821.3, 4465822.3</td>
<td>Colorado River, USA</td>
<td>454</td>
<td>4</td>
</tr>
<tr>
<td>Grassland</td>
<td>4539064.3, 4541651.3</td>
<td>Cedar Creek, USA</td>
<td>454</td>
<td>3</td>
</tr>
<tr>
<td>Mangroves</td>
<td>4523017.3 – 4523020.3</td>
<td>Red Sea, Saudi Arabia</td>
<td>Ilumina</td>
<td>2</td>
</tr>
<tr>
<td>Rainforest soil</td>
<td>4497403.3, 4497404.3</td>
<td>Amazon Forest, Brazil</td>
<td>454</td>
<td>4</td>
</tr>
<tr>
<td>Salt marsh</td>
<td>4520021.3, 4520022.3, 4520023.3</td>
<td>Plum Island, USA</td>
<td>Ilumina</td>
<td>2</td>
</tr>
<tr>
<td>Sponge</td>
<td>4530252.3, 4530290.3, 4530370.3</td>
<td>Botany Bay, Australia</td>
<td>Ilumina</td>
<td>3</td>
</tr>
<tr>
<td>Stromatolite</td>
<td>4604137.3, 4604139.3, 4604141.3</td>
<td>Shark Bay, Western Australia</td>
<td>Ilumina</td>
<td>3</td>
</tr>
</tbody>
</table>