Unraveling the complexity of subtropical seagrass nitrogen cycling: a stable isotope approach

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UNRAVELING THE COMPLEXITY OF SUBTROPICAL SEAGRASS NITROGEN CYCLING: A STABLE ISOTOPE APPROACH

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Declaration

I, Natasha L Carlson-Perret, certify that the work presented in this thesis is, to the best of my knowledge and belief, original, except as acknowledged in the text, and that the material has not been submitted, either in whole or in part, for a degree at this or any other university.

I acknowledge that I have read and understood the University’s rules, requirements, procedures and policy relating to my higher degree research award and to my thesis. I certify that I have complied with the rules, requirements, procedures and policy of the University (as they may be from time to time).

[Signature]

Natasha L Carlson-Perret

Date 6/12/18
Abstract

Seagrass habitats are iconic communities with significant economic and cultural value, yet our understanding of nitrogen (N) cycling in subtropical seagrass environments is limited. Nitrogen supply regulates seagrass productivity and also exerts significant control on seagrass community structure and health. Hence understanding the cycling of N in subtropical seagrasses is crucial for future management of the resource.

Constraining seagrass N cycling via N input (dinitrogen fixation) and loss (denitrification and anammox) pathways has been problematic largely due to assumptions inherent in previous methods, and a lack of studies in which more contemporary methods have been employed. The plants themselves add another level of complexity to an already complex set of sediment biogeochemical processes, but to date sediment and plant associated processes have been largely studied in isolation. Furthermore there are few studies in which N cycling processes have been compared between different species. The overarching objective of this research is to use contemporary stable isotope methods to quantify N cycling within and between seagrasses.

The first component of the research investigated techniques for capturing dinitrogen (N₂) fixation in the sediments of a common subtropical seagrass community (Zostera muelleri). A sediment perfusion technique, based on the addition of ¹⁵N labelled N₂ to porewater was developed and subsequently implemented. Comparison of N₂ fixation activity in the above and below ground loci of a Z. muelleri meadow using the ¹⁵N-N₂ perfusion method showed the seagrass leaves were the dominant locus of N₂ fixation. Next, differences in N₂ fixation rates within and between subtropical seagrass species (Z. muelleri and Halophila ovalis) were
measured. Leaves were again the locus of highest $\text{N}_2$ fixation however, differences between the rates of $\text{N}_2$ fixation observed in the two species were determined to be due to species-specific morphological traits such as leaf surface area and density.

Investigations into N loss processes using the newest iteration of the isotope pairing technique (R-IPT-DNRA) in these two seagrass meadows revealed N loss processes varied between the species and showed differences in the fate of fixed N between the seagrass communities. While denitrification rates were low in both species, the $H$. ovalis community was dominated by N loss via anammox. In the $Z$. muelleri system dissimilatory nitrate reduction to ammonium (DNRA) dominated. These differences in N loss was once again attributed to individual species morphology and the impact this can have on the geochemical characteristics of the seagrass rhizosphere. Below ground biomass and the exudation of photosynthates such as oxygen and organic carbon are controlling factors in redox gradients and integral N cycling organisms like sulphate reducing bacteria in the rhizosphere.

Combining the input and loss pathways yielded an N budget for the two seagrass systems that served to confirm the differences in N cycling between the communities. The $H$. ovalis community was found to be a net exporter of N while the $Z$. muelleri community showed net N input.

The application of N isotopic techniques has clarified our understanding of the factors that regulate the input and output of N from seagrass systems. Morphological characteristics and sediment geochemistry play a key role in regulating the net N balance of seagrass communities and must be accounted for when making assessments about the impact of seagrasses on coastal N cycling.
Acknowledgements

Thanks firstly, to my Mum. I dedicate this thesis to you. You have always been my biggest champion even if you weren’t always sure what I was doing or why. Your encouragement has been invaluable and knowing that you are always there for me to laugh, cry, rant at/to is a level of support that not everyone is privileged to have. I am the independent and capable woman that I am today because that is how you taught me to be, by your example.

Thank you to my supervisors Dirk and Brad, your guidance and knowledge helped me navigate a field I was relatively new to when I began. Thanks also to Philip (honorary 3rd supervisor) and Jessica Riekenberg for being fantastic friends with always a helpful, sympathetic and good-humoured ear, you both taught me not to sweat the small stuff even when the small stuff feels huge. Thankyou to the entirety of my family who have had to listen to me bang-on about seagrass at some point or another. Thanks to all those that helped in the field, Jamie David, Jian-Jhih (Kenji) Chen, Philip Riekneberg and Paul Kelly, with analysis Iain Alexander, Mattheus Carvalho and Mustefa Rashid and with kind and helpful advice - far too many to mention. Thanks also goes to the reviewers that have reviewed my published (and submitted) papers, all have helped me make my work better with constructive criticisms. Research cannot be done in a vacuum, it takes a village.

Additional thanks goes to 90s and 00s hip hop and RnB which helped me populate my ‘Auditory Confidence’ playlist with music that is infinitely more effective at boosting serotonin and confidence than any amount of ‘power posing’ and has definitely aided in the betterment of my public and private (meetings) speaking.
Although I am loathe to use the cliché trope of ‘the journey’ to describe my time as a PhD candidate combinations of research problems and personal struggle have meant that the last 4 years have been cumulatively the hardest for me to date. These struggles have strengthened a resilience that I always knew I possessed and helped build confidence I was lacking. I am by far the first or last to experience these hurdles but every set back has made each, even minute, piece of progress feel infinitely sweeter. “I get knocked down but I get up again” – Chumbawumba.
Preface

This thesis is comprised of six chapters, four of which are in various stages of being prepared for, submitted or accepted for publication in peer-reviewed journals.

Chapter 1 presents an introduction to the nitrogen (N) cycle and the specific pathways investigated in this research. Additionally a literature review of how N cycling has been measured in seagrass ecosystems and advances in these techniques that are to be used in this research.

Chapter 2 is largely a method development chapter with some additional background on past techniques that have been used to characterise N cycling in sediments and the seagrass rhizosphere, termed perfusion. This chapter outlines the process used to develop a perfusion technique appropriate for characterising seagrass below ground N\textsubscript{2} fixation that was subsequently used in Chapters 3 and 4. Chapter 2 is being prepared as a review/method development article for publication.

Chapter 3 utilises the method developed in Chapter 2 in concert with a \textsuperscript{15}N-N\textsubscript{2} dissolution method to characterise both above and below ground seagrass N\textsubscript{2} fixation. Individual loci within the seagrass community, i.e. leaves + epiphytes, root/rhizome material and sediment was analysed for \textsuperscript{15}N to determine regions of N\textsubscript{2}-fixing activity. Possible transport pathways of fixed N throughout the seagrass system are discussed. This chapter has been published and the statement of contribution signed by co-authors can be found in Appendix 2:

Carlson-Perret, N. L., Erler, D.V., Eyre, B.D. 2018. Dinitrogen (N\textsubscript{2}) fixation rates in a subtropical seagrass meadow measured with a direct \textsuperscript{15}N-N\textsubscript{2} tracer method, Marine Ecology Progress Series, 605: 87-101

Chapter 4 builds on the work in Chapters 2 and 3 by measuring N\textsubscript{2} fixation, with above and below ground \textsuperscript{15}N-N\textsubscript{2} additions, in two morphologically diverse subtropical
seagrass species (Z. muelleri and H. ovalis) over an artificial diurnal cycle. This chapter addresses the effect species-specific morphological characteristics can have on N\textsubscript{2} fixation and zones of activity within the seagrass community over light and dark. As in Chapter 3 leaf + epiphyte fixation dominated N\textsubscript{2} fixation rates in all but the dark Z. muelleri incubation. Differences in rates between the species due to morphology as well as active N\textsubscript{2} fixing zones are discussed. This chapter has been published, the statement of contribution signed by co-authors can be found in Appendix 3.


**Chapter 5** attempts to close the N cycle in the two seagrass communities of Chapter 4 by measuring N loss and recycling processes, denitrification, anammox and DNRA. In this chapter N loss processes are measured using a new iteration of the isotope pairing technique and the N\textsubscript{2}:Ar technique. Morphological impacts on N loss and recycling are discussed as in N inputs in Chapter 4. This chapter is being prepared for publication.

**Chapter 6** provides N budgets for both seagrass species using data from Chapters 4 and 5 as well as a synthesis, summary and conclusions of the findings from Chapters 2 to 5.

In addition I contributed to the following manuscript during my candidature which was used to calculate rates from the IPT in Chapter 5. It is included in Appendix 1.


sciencedirect.com/science/article/pii/S0016703717302879
Statement of Contribution

The publications,


and

Carlson-Perret, N. L., Erler, D.V., Eyre, B.D. Comparison of dinitrogen fixation in two subtropical seagrass communities. For Marine Chemistry. Submitted

are largely my work. I, the candidate, participated during all stages of these papers and provided an overall contribution greater than that of any co-author. I designed the individual experiments, collected the data, ran analyses and wrote the original manuscripts which were revised with feedback from co-authors.

This also applies to any future chapters to be published from this thesis.

Natasha Carlson-Perret

Dr. Dirk. V. Erler (principle supervisor)
Contents

Chapter 1: Introduction ............................................................................................................. 1

1.1 Nitrogen cycling in seagrass systems ................................................................................. 2

1.1.1 Dinitrogen (N₂) fixation ................................................................................................. 3

1.1.2 Denitrification ............................................................................................................... 5

1.1.3 Anaerobic ammonium oxidation (Anammox) ............................................................... 7

1.1.4 Dissimilatory nitrate reduction to ammonium (DNRA) ............................................... 14

1.2 Current limitations to our understanding of N cycling in seagrass systems .................... 17

1.2.1 Nitrogen fixation in seagrass systems .......................................................................... 20

1.2.2 ¹⁵N-N₂ dissolution method .......................................................................................... 21

1.2.3 Measurement of denitrification, anammox and DNRA in seagrass systems ............... 24

1.2.4 Perfusion technique ..................................................................................................... 28

1.3 Aims and significance of research .................................................................................... 30

1.4 Goals of the research ....................................................................................................... 31

Chapter 2: Perfusion method development. ........................................................................... 33

2.1 Brief introduction to perfusion ......................................................................................... 34

2.1.1 History of perfusion ..................................................................................................... 34

2.2 Development of a suitable perfusion technique ............................................................... 40

Chapter 3: Dinitrogen fixation rates in a subtropical seagrass meadow measured with a direct ¹⁵N-N₂ tracer method .................................................................................... 52

Abstract .................................................................................................................................. 53

3.1 Introduction ....................................................................................................................... 54

3.2 Method .............................................................................................................................. 65

3.2.1 ¹⁵N₂ labelled gas ............................................................................................................. 65

3.2.2 Core collection and maintenance ................................................................................ 66

3.2.3 Core incubations ........................................................................................................... 67

3.2.4 N₂ fixation assay - surface core incubation ..................................................................... 68

3.2.5 N₂ fixation assay - perfusion core incubations .............................................................. 69

3.2.6 Sample analysis ............................................................................................................ 70

3.2.7 N₂ fixation rate calculation .......................................................................................... 71

3.3 Results ............................................................................................................................... 72

3.4 Discussion ......................................................................................................................... 74

3.4.1 N₂ fixation within the seagrass meadow: aboveground .................................................. 74

3.4.2 N₂ fixation within the seagrass meadow: below ground ............................................... 78

3.4.3 Comparison with previous N₂ fixation rates ................................................................. 81

3.5 Conclusions ....................................................................................................................... 84
5.4.3 Conclusions ................................................................................................. 130

Chapter 6: Summary, synthesis and conclusions .................................................. 132

6.1 Moreton Bay seagrass N budget .................................................................. 133

6.2 Summary and synthesis ................................................................................ 134

Chapter 2: Perfusion method development .......................................................... 134

Chapter 3: Dinitrogen fixation rates in a subtropical seagrass meadow measured with a direct $^{15}$N-$N_2$ tracer method ......................................................... 135

Chapter 4: Comparison of dinitrogen fixation rates in two subtropical seagrass communities .......................................................... 137

Chapter 5: Nitrogen loss processes in two subtropical species ......................... 138

6.3 Future directions .......................................................................................... 139

References ........................................................................................................ 142

Appendices ........................................................................................................ 158

Appendix 1 ......................................................................................................... 159

Appendix 2 ......................................................................................................... 174

Appendix 3 ......................................................................................................... 175

Appendice 4 ....................................................................................................... 176

Supplementary materials – Chapter 3 .............................................................. 176

Appendice 5 ....................................................................................................... 180

Supplementary materials – Chapter 4 .............................................................. 180
Figures

**Figure 1.1** Above and below ground N cycling processes (from Thamdrup et al. 2012)

**Figure 2.1** Schematic diagrams of the range of perfusion systems that have been used in previous research. Top row (left to right), Aoki & McGlathery (2017), Penton et al. (2013), Hardison et al. (2011), Koop-Jakobsen & Giblin (2009), de Beer et al. (2005). Middle row (left to right), Sheibley et al. (2003), Ottosen et al. (1999), McGlathery et al. (1998), Risgaard-Petersen et al. (1998). Bottom row (left to right), Risgaard-Petersen & Jensen (1997), Stepanauskas et al. (1996), Risgaard-Petersen et al. (1994), O’Donohue et al. (1991b).

**Figure 2.2** The perfusion disc added to the bottom of sediment cores an 80 mm diameter PVC pipe end cap with ~5 mm holes drilled into the surface.

**Figure 2.3** Perfusion core set-up whereby pore water would be pumped out of the sediment by a peristaltic pump attached to one of the outlet ports (circled in red). This is also the same configuration used for pulling perfusion solution over the sediment into the rhizosphere. The perfusion disc is clearly visible as is some fine sediment which has migrated past the disc. The blue arrow shows the direction of water movement.

**Figure 2.4** The extent of core splitting was still visible even after being left to re-settle for ~24 hours and with pore water drained.

**Figure 2.5** Perfusion core set up for the ‘bottom-up’ iteration of the method where pore water collected separately could be labelled and pumped into the seagrass rhizosphere (water movement indicated by blue arrow) with a peristaltic pump attached to ports in the base of the core (not visible). This iteration commonly caused major core splitting (circled), migration of fine sediment past the perfusion disc is also visible.

**Figure 2.6** A Macro Rhizon sampler used to perfuse the seagrass rhizosphere.
Figure 2.7 Macro Rhizons introduced perfusion solution to the seagrass rhizosphere. A complimentary fitting connected to a peristaltic pump (not shown) was connected to each Macro Rhizon in turn.

Figure 2.8 Sediment was separated into centre and outer edge fractions for lithium concentration analysis to determine if the Macro Rhizon perfusion method had successfully uniformly labelled the rhizosphere.

Figure 2.9 Seagrass cores being injected with perfusion solution into the rhizosphere.

Figure 3.1 Total rates of dinitrogen fixation (mean ± SD, n=3) across all seagrass loci (leaves and epiphytes, sediment and root/rhizome material) in surface and perfusion cores over 5 and 10 hour incubation periods.

Figure 3.2 Dinitrogen fixation rates measured in discrete loci (as mean ± SD µmol N$_2$ m$^{-2}$ h$^{-1}$) when $^{15}$N-N$_2$ labelled site water added to either the water column (a.) or perfused directly into the sediment (b.). Arrows illustrate suggested direction of movement of labelled products around the system. Where S$_{s}$, S$_{e}$ and S$_{r}$ represent surface core fixation in the leaves (including epiphytes), sediment and root/rhizome, and P$_{s}$, P$_{e}$ and P$_{r}$ the N$_2$ fixation in these loci in the perfusion cores. Seagrass vector image from Diana Kleine ian.ucmes.edu/imagelibrary.

Figure 3.3 Dinitrogen fixation (mean ± SD, n=3) over sediment depths in both surface and perfusion cores.

Figure 4.1 Map of the study area located within the Broadwater of southern Moreton Bay, Australia (modified from Eyre et al. 2011a). Sample sites of Zostera muelleri represented by square and Halophila ovalis by diamond.

Figure 4.2 Rates of seagrass N$_2$ fixation (as µmol N$_2$ m$^{-2}$ h$^{-1}$) in various loci within Zostera muelleri (top) and Halophila ovalis (bottom) when $^{15}$N-N$_2$ added above and below ground in both dark (left) and light (right) conditions. Arrows represent potential direction of transport of fixed N and blue circles represent oxygen excretion. Sediment and root/rhizome N$_2$ fixation is represented by P$_{s}$ and P$_{r}$ respectively, while S$_{l}$ represents leaf (including epiphyte) N$_2$.
fixation. Seagrass vector images from Diana Kleine (Z. muelleri) and Catherine Collier (H. ovalis) ian.ucmes.edu/imagelibrary. .......................................................... Error! Bookmark not defined.

**Figure 4.3** Total N\textsubscript{2} fixation rates (i.e. leaf + epiphyte community, root/rhizome material and sediment inclusive) in *Halophila ovalis* and *Zostera muelleri* cores under light and dark conditions and total diel rates. (Mean ± SD, n = 3). ........................................................................................................... 103

**Figure 5.1**: Rates of denitrification (a.), anammox (b.) and DNRA (c.) in seagrasses *Halophila ovalis* and *Zostera muelleri* under both light and dark conditions (mean ± SD, n=3). .................................................................................................................. 120

**Figure 5.2**: Fluxes of N\textsubscript{2} from cores containing seagrasses *Halophila ovalis* and *Zostera muelleri* under light and dark conditions (mean ± SD, n=5). ...... Error! Bookmark not defined.

**Figure 5.3**: Fluxes of ammonium (a.) and NOx (nitrate + nitrite) (b.) from cores containing seagrass plants (*Halophila ovalis* and *Zostera muelleri*) under both light and dark conditions (mean ± SD, n=3) ............................................................................................................................. 125

**Figure 6.1**: Conceptual diagram illustrating the main findings of this research, the differences in N cycling between *Halophila ovalis* and *Zostera muelleri* communities in Moreton Bay. Arrow size indicates the amount of N transported by that process. Where SRB; sulphate reducing bacteria, OM; organic matter, NOx; nitrate + nitrite and DNRA; dissimilatory reduction of nitrate to ammonium.............................................................................................................. 141
Tables

Table 1.1 Rates of denitrification measured in (sub)tropical and temperate seagrass systems, where IPT denotes the use of the isotope pairing technique, $N_2$:Ar is the measurement of fluxes of $N_2$ as compared with the concentration of Ar and AB is the acetylene block technique.

Table 1.2 Rates of dissimilatory nitrate reduction to ammonia (DNRA) in (sub)tropical and temperate seagrass systems.

Table 3.1: Rates of dinitrogen fixation measured in (sub)tropical and temperate seagrass systems, where ARA denotes the acetylene reduction assay and $^{15}$N-$N_2$ as addition of labelled site water. L+E: rates measured on seagrass leaf material with epiphytes; L+E*: epiphytes note removed, reference stated not many were present; L-E: epiphytes removed from leaves prior to incubation; S-RR: sediment minus root/rhizome material; S+RR: sediment with root/rhizome material; R: seagrass root material; Rh: rates measured on seagrass rhizome material; R+R: seagrass root and rhizome material; S: seagrass vegetated sediments; R+Rana: seagrass roots and rhizomes in an anaerobic environment; R+Raero: seagrass roots and rhizome in an aerobic environment; L+Sh: seagrass leaves and shoots in an aerobic environment; (L): light conditions; (D): dark conditions. Note all rates reported as $\mu$mol. N m$^{-2}$ h$^{-1}$.

Table 4.1: Phisical and chemical characteristics of the $H$. ovalis and $Z$. muelleri communities in Moreton Bay.

Table 4.2: Results of two-tailed t-tests comparing $N_2$ fixation rates of various loci within Halophila ovalis and Zostera muelleri seagrass communities. Where results in bold are significant at $p < 0.05$.

Table 5.1: Results of t-tests where results in bold are significant at $p < 0.05$.

Table 5.2: Nitrogen content of above and below ground seagrass biomass measured as % of dry weight.
Table 6.1: The average hourly rates of N either into (input) or out of (export) the *Halophila ovalis* and *Zostera muelleri* seagrass systems of southern Moreton Bay.
Chapter 1: Introduction

Unraveling the complexity of subtropical seagrass nitrogen cycling: a stable isotope approach
1.1 Nitrogen cycling in seagrass systems

Seagrass meadows are dynamic systems that fulfil a multitude of important ecological functions. They are a source of food and provide habitat for marine fauna, they are effective filters of pathogens protecting both humans and corals (Lamb et al. 2017) and they provide stability to sediment limiting erosion and degradation of estuarine environments (Christianen et al. 2013). Arguably one of their most important functions is as efficient nutrient cyclers (Hemminga et al. 1991, Welsh 2001). The cycling of nitrogen (N) in seagrass systems is critical to the health of not only the plants themselves but the ecosystem as a whole. Nitrogen input (dinitrogen fixation) provides valuable bioavailable sources of N not only to the plant but the micro- and megafauna which graze it, and output pathways (denitrification and anammox) control excess nutrients that can lead to eutrophication. Measurements of the import, export and recycling pathways (such as, dissimilatory nitrate reduction to ammonium) of N in seagrass communities have been widely studied (Boon et al. 1986, Welsh et al. 2001, Aoki & McGlathery 2018- Tables 1.1, 1.2) but uncertainty characterising these processes in seagrass still exists. This section serves to highlight and describe the pathways of the N cycle that are relevant in subtropical seagrass habitats and within the scope of this research.

The largest global N reservoir is atmospheric N$_2$ and the largest terrestrial source is igneous rock deposits (Canfield et al. 2005). Terrestrial N is present largely as ammonium (NH$_4^+$) in igneous rocks, weathering of which liberates the biologically available NH$_4^+$ into the local environment however this is quite a slow process. Smaller pools originate from organic N present in

Unraveling the complexity of subtropical seagrass nitrogen cycling: a stable isotope approach

1 - Introduction
terrestrial and aquatic organic detritus and living biomass. Oceanic N inputs are primarily from fixation of atmospheric $\text{N}_2$ by free living cyanobacteria and are much smaller than terrestrial sources of N (Galloway et al. 2004). Coastal and estuarine environments are particularly susceptible to N loads input from anthropogenic sources i.e. N fertiliser runoff from agriculture and industrial processes.

The N cycle in seagrass systems is extremely complex and includes numerous linked processes which ultimately cycle atmospheric $\text{N}_2$ gas to biologically available forms such as $\text{NH}_4^+$ and nitrate ($\text{NO}_3^-$) and back again. The complexity and interconnected-ness of the N cycle is clearly illustrated by Thamdrup (2012) (Figure 1.1). The process affecting the input of N within seagrass environments is $\text{N}_2$ fixation, with denitrification and anammox regulating the export of N from the system (Figure 1.1). Dissimilatory nitrate reduction to ammonium (DNRA) is an N recycling pathway that keeps N within the system creating $\text{NH}_3$ and ultimately $\text{NH}_4^+$, the starting compound for many N processes including nitrification and anammox (Fig. 1.1).

1.1.1 Dinitrogen ($\text{N}_2$) fixation

Dinitrogen fixation is a process largely controlled by bacteria which transform $\text{N}_2$ in the atmosphere into the biologically available $\text{NH}_3$ [1].

$$\text{N}_2 + 8\text{H}^+ + 8\text{e}^- \rightarrow 2\text{NH}_3 + \text{H}_2 \quad [1]$$

These $\text{N}_2$ fixing bacteria are a specialised group of prokaryotes that include aerobic, anaerobic, phototrophic, and chemotrophic organisms. They may be free living or found in symbiotic relationships with plants (Canfield et al. 2005),

Unraveling the complexity of subtropical seagrass nitrogen cycling: a stable isotope approach
Figure 1.1 Above and below ground N cycling processes (from Thamdrup et al. 2012)
in seagrass systems epiphytes commonly found on seagrass leaves and shoots are known to mediate N₂ fixation (Agawin et al. 2016). Chemically mediated dinitrogen fixation may also occur in small amounts due to lightning strikes and may be stimulated by iron deposition from sediments blown into water catchments via wind (Jickells et al., 2005) or present in estuarine sediments. Since the 1990s industrial processes (such as the Haber-Bosch process) are contributing more to the global N budget than naturally occurring N₂ fixation (Gruber and Galloway, 2008).

Dinitrogen fixation is an energetically taxing process. The breaking of the strong triple bond which binds the two N atoms of the N₂ molecule requires a large quantity of energy (bond energy = 945 kJ/mol) (van Loon and Duffy, 2005). Dinitrogen-fixing prokaryotes employ the enzyme nitrogenase which reduces the N₂ molecule to NH₄⁺ at normal environmental temperatures and pressure. This is not an N-specific reaction however, as nitrogenase also reduces any triple bonded molecule present; such as acetylene (C₂H₂) and hydrogen cyanide (Canfield et al. 2005). The low specificity of nitrogenase has been used in methods of measuring N₂ fixation, such as the acetylene reduction assay (ARA) in which reduction of C₂H₂ to ethylene is used as a proxy for N₂ fixation (Stewart et al. 1967, Capone 1982).

1.1.2 Denitrification

Denitrification is an incredibly important process for regulating nutrients especially in estuarine environments. The process reduces bioavailable forms of N (NO₃⁻ and NO₂⁻, and via nitrification NH₄⁺) to gaseous N₂ and released

Unraveling the complexity of subtropical seagrass nitrogen cycling: a stable isotope approach
Introduction

Unraveling the complexity of subtropical seagrass nitrogen cycling: a stable isotope approach

back into the atmosphere. This transformation helps to mitigate biological over proliferation from excess nutrient loading which can lead to serious environmental issues like eutrophication.

Denitrification occurs via a series of stepwise chemical reactions [5] and [6];

\[
\begin{align*}
\text{NO}_3^- & \rightarrow \text{NO}_2^- \rightarrow \text{NO} + \text{N}_2\text{O} \rightarrow \text{N}_2 & \text{[5]} \\
2\text{NO}_3^- + 10e^- + 12\text{H}^+ & \rightarrow \text{N}_2 + 6\text{H}_2\text{O} & \text{[6]}
\end{align*}
\]

Where equation [5] details the steps of the reduction of \( \text{NO}_3^- \) to \( \text{N}_2 \) and equation [6] is the full redox reaction. Gases considered environmentally noxious, nitrous oxide (\( \text{N}_2\text{O} \)) and nitric oxide (\( \text{NO} \)) are produced as intermediates during the denitrification process; with tropospheric \( \text{O}_3 \) depletion being aided by \( \text{N}_2\text{O} \) (Seitzinger et al. 2006).

Denitrification is a largely anaerobic process carried out by a diverse group of prokaryotes. Eukaryotes are also capable of denitrification and many organisms can reduce \( \text{NO}_3^- \) to \( \text{N}_2 \) chemoautotrophically (using iron, sulphur and methane as electron donors) (Risgaard-Petersen et al. 2006).

Denitrification occurs in aquatic sediments, as well as anoxic zones in lakes and oceans. In seagrass systems denitrification often occurs deeper within the anoxic zone of the rhizosphere (i.e. the seagrass root and rhizome and surrounding sediment), with rates usually spiking at night due to the lower \( \text{O}_2 \) status of the sediment (Table 1.1). Oceanic sources of denitrification account for approximately 26% of global values while terrestrial sources are approximately 35-40% (Seitzinger et al. 2006). Due to its export of N from marine and estuarine systems denitrification has been described as the major

Unraveling the complexity of subtropical seagrass nitrogen cycling: a stable isotope approach
factor controlling the size of the non-anthropogenic oceanic fixed N pool. In seagrass systems heterotrophic denitrification is fuelled by the abundant organic carbon released from seagrass roots and from decaying vegetation (Shieh and Yang, 1997; Risgaard-Petersen et al. 1998). Seagrass sediments can therefore be major sites for the denitrification of anthropogenic N due the high availability of carbon.

1.1.3 Anaerobic ammonium oxidation (Anammox)

Anammox is a chemoautotrophic N loss process in which NH$_4^+$ and NO$_2^-$ combine directly to produce N$_2$. Anammox is a relatively new addition to the N cycle being first characterised in waste water treatment bioreactors (Mulder et al. 1995). Anammox has since been detected in an array of locations including;

- oceans (in zones of low oxygen concentration) (Dalsgaard et al. 2003),
- mangrove forests (Meyer et al. 2005),
- wetlands (Erler et al. 2008),
- fresh water lakes (Schubert et al. 2006), and
- marine sediments (Thamdrup and Dalsgaard 2002),

Anammox is carried out by prokaryotes belonging to the Planctomycetes phylum (Mulder et al. 1995, Jetten et al. 2003, Dalsgaard et al. 2005). These bacteria oxidise NH$_4^+$ using NO$_2^-$ as the electron acceptor to produce N$_2$ gas as in equation [7].

\[
\text{NH}_4^+ + \text{NO}_2^- \rightarrow \text{N}_2 + 2\text{H}_2\text{O} \quad \text{[7]}
\]

Unraveling the complexity of subtropical seagrass nitrogen cycling: a stable isotope approach
Table 1.1 Rates of denitrification measured in (sub)tropical and temperate seagrass systems, where IPT denotes the use of the isotope pairing technique, $N_2$:Ar is the measurement of fluxes of $N_2$ as compared with the concentration of Ar and AB is the acetylene-block technique.

<table>
<thead>
<tr>
<th>Rate of denitrification ($\mu$mol N m$^{-2}$ h$^{-1}$)</th>
<th>Method</th>
<th>Species, location &amp; season</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Sub)Tropical</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>~0.04s (D)</td>
<td>IPT$^{b,c}$</td>
<td>Zostera muelleri, Shaws Bay, Australia (Winter)</td>
<td>Salk et al. 2017</td>
</tr>
<tr>
<td>~0.16s (D)</td>
<td>N$_2$:Ar</td>
<td>Zostera Capricorni, Hastings River, Australia (Summer)</td>
<td>Eyre et al. 2013</td>
</tr>
<tr>
<td>~445.2 (D)</td>
<td>N$_2$:Ar</td>
<td>Zostera Capricorni, Hastings River, Australia (Summer)</td>
<td>Eyre et al. 2013</td>
</tr>
<tr>
<td>~247.5 (D)</td>
<td>N$_2$:Ar</td>
<td>Zostera Capricorni, Hastings River, Australia (Autumn)</td>
<td>Eyre et al. 2013</td>
</tr>
<tr>
<td>~279.9 (D)</td>
<td>N$_2$:Ar</td>
<td>Zostera Capricorni, Hastings River, Australia (Spring)</td>
<td>Eyre et al. 2013</td>
</tr>
<tr>
<td>65.3 (D)</td>
<td>N$_2$:Ar</td>
<td>Halophila australis, Hastings River, Australia (Summer)</td>
<td>Eyre et al. 2013</td>
</tr>
<tr>
<td>13.1 (L)</td>
<td>N$_2$:Ar</td>
<td>Halophila australis, Hastings River, Australia (Summer)</td>
<td>Eyre et al. 2013</td>
</tr>
<tr>
<td>19.1 (D)</td>
<td>N$_2$:Ar</td>
<td>Zostera Capricorni, Camden Haven, Australia (Summer)</td>
<td>Eyre et al. 2013</td>
</tr>
<tr>
<td>75.8 (L)</td>
<td>N$_2$:Ar</td>
<td>Zostera Capricorni, Camden Haven, Australia (Summer)</td>
<td>Eyre et al. 2013</td>
</tr>
<tr>
<td>102.7 (D)</td>
<td>N$_2$:Ar</td>
<td>Zostera Capricorni, Camden Haven, Australia (Autumn)</td>
<td>Eyre et al. 2013</td>
</tr>
<tr>
<td>118.1 (L)</td>
<td>N$_2$:Ar</td>
<td>Zostera Capricorni, Camden Haven, Australia (Spring)</td>
<td>Eyre et al. 2013</td>
</tr>
<tr>
<td>135.5 (D)</td>
<td>N$_2$:Ar</td>
<td>Zostera Capricorni, Camden Haven, Australia (Spring)</td>
<td>Eyre et al. 2013</td>
</tr>
<tr>
<td>12.3 (L)</td>
<td>N$_2$:Ar</td>
<td>Halophila australis, Camden Haven, Australia (Summer)</td>
<td>Eyre et al. 2013</td>
</tr>
<tr>
<td>L</td>
<td>D</td>
<td>Species, Location, Year</td>
<td></td>
</tr>
<tr>
<td>---</td>
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<td>-------------------------</td>
<td></td>
</tr>
<tr>
<td>21.4</td>
<td>N₂:Ar&lt;sup&gt;B&lt;/sup&gt;</td>
<td><em>Halophila australis</em>, Camden Haven, Australia (Autumn)</td>
<td>Eyre et al. 2013</td>
</tr>
<tr>
<td>29.2</td>
<td>N₂:Ar&lt;sup&gt;B&lt;/sup&gt;</td>
<td><em>Ruppia megacarpa</em>, Camden Haven, Australia (Summer)</td>
<td>Eyre et al. 2013</td>
</tr>
<tr>
<td>206.1</td>
<td>N₂:Ar&lt;sup&gt;B&lt;/sup&gt;</td>
<td><em>Ruppia megacarpa</em>, Camden Haven, Australia (Summer)</td>
<td>Eyre et al. 2013</td>
</tr>
<tr>
<td>147.8</td>
<td>N₂:Ar&lt;sup&gt;B&lt;/sup&gt;</td>
<td><em>Zostera Capricorni</em>, Wallis Lake, Australia (Summer)</td>
<td>Eyre et al. 2013</td>
</tr>
<tr>
<td>86.6</td>
<td>N₂:Ar&lt;sup&gt;B&lt;/sup&gt;</td>
<td><em>Zostera Capricorni</em>, Wallis Lake, Australia (Summer)</td>
<td>Eyre et al. 2013</td>
</tr>
<tr>
<td>150.9</td>
<td>N₂:Ar&lt;sup&gt;B&lt;/sup&gt;</td>
<td><em>Zostera Capricorni</em>, Wallis Lake, Australia (Summer)</td>
<td>Eyre et al. 2013</td>
</tr>
<tr>
<td>78.4</td>
<td>N₂:Ar&lt;sup&gt;B&lt;/sup&gt;</td>
<td><em>Zostera Capricorni</em>, Wallis Lake, Australia (Summer)</td>
<td>Eyre et al. 2013</td>
</tr>
<tr>
<td>203.6</td>
<td>N₂:Ar&lt;sup&gt;B&lt;/sup&gt;</td>
<td><em>Zostera Capricorni</em>, Wallis Lake, Australia (Summer)</td>
<td>Eyre et al. 2013</td>
</tr>
<tr>
<td>75.1</td>
<td>N₂:Ar&lt;sup&gt;B&lt;/sup&gt;</td>
<td><em>Halophila australis</em>, Wallis Lake, Australia (Summer)</td>
<td>Eyre et al. 2013</td>
</tr>
<tr>
<td>121.8</td>
<td>N₂:Ar&lt;sup&gt;B&lt;/sup&gt;</td>
<td><em>Halophila australis</em>, Wallis Lake, Australia (Summer)</td>
<td>Eyre et al. 2013</td>
</tr>
<tr>
<td>119.2</td>
<td>N₂:Ar&lt;sup&gt;B&lt;/sup&gt;</td>
<td><em>Halophila australis</em>, Wallis Lake, Australia (Summer)</td>
<td>Eyre et al. 2013</td>
</tr>
<tr>
<td>62</td>
<td>N₂:Ar&lt;sup&gt;B&lt;/sup&gt;</td>
<td><em>Halophila australis</em>, Wallis Lake, Australia (Spring)</td>
<td>Eyre et al. 2013</td>
</tr>
<tr>
<td>68.1</td>
<td>N₂:Ar&lt;sup&gt;B&lt;/sup&gt;</td>
<td><em>Ruppia megacarpa</em>, Wallis Lake, Australia (Spring)</td>
<td>Eyre et al. 2013</td>
</tr>
<tr>
<td>244.7</td>
<td>N₂:Ar&lt;sup&gt;B&lt;/sup&gt;</td>
<td><em>Ruppia megacarpa</em>, Wallis Lake, Australia (Spring)</td>
<td>Eyre et al. 2013</td>
</tr>
<tr>
<td>281.6</td>
<td>N₂:Ar&lt;sup&gt;B&lt;/sup&gt;</td>
<td><em>Ruppia megacarpa</em>, Wallis Lake, Australia (Spring)</td>
<td>Eyre et al. 2013</td>
</tr>
<tr>
<td>132.1</td>
<td>N₂:Ar&lt;sup&gt;B&lt;/sup&gt;</td>
<td><em>Ruppia megacarpa</em>, Wallis Lake, Australia (Spring)</td>
<td>Eyre et al. 2013</td>
</tr>
<tr>
<td>50.6</td>
<td>N₂:Ar&lt;sup&gt;B&lt;/sup&gt;</td>
<td><em>Posidonia australis</em>, Wallis Lake, Australia (Summer)</td>
<td>Eyre et al. 2013</td>
</tr>
<tr>
<td>33</td>
<td>N₂:Ar&lt;sup&gt;B&lt;/sup&gt;</td>
<td><em>Posidonia australis</em>, Wallis Lake, Australia (Spring)</td>
<td>Eyre et al. 2013</td>
</tr>
<tr>
<td>188.3</td>
<td>N₂:Ar&lt;sup&gt;B&lt;/sup&gt;</td>
<td><em>Posidonia australis</em>, Wallis Lake, Australia (Spring)</td>
<td>Eyre et al. 2013</td>
</tr>
<tr>
<td>93.8</td>
<td>N₂:Ar&lt;sup&gt;B&lt;/sup&gt;</td>
<td><em>Posidonia australis</em>, Wallis Lake, Australia (Spring)</td>
<td>Eyre et al. 2013</td>
</tr>
<tr>
<td>55.1</td>
<td>N₂:Ar&lt;sup&gt;B&lt;/sup&gt;</td>
<td><em>Posidonia australis</em>, Wallis Lake, Australia (Spring)</td>
<td>Eyre et al. 2013</td>
</tr>
<tr>
<td>78</td>
<td>N₂:Ar&lt;sup&gt;B&lt;/sup&gt;</td>
<td><em>Posidonia australis</em>, Wallis Lake, Australia (Winter)</td>
<td>Eyre et al. 2013</td>
</tr>
<tr>
<td>~11.4</td>
<td>N₂:Ar&lt;sup&gt;D&lt;/sup&gt;</td>
<td><em>Halodule wrightii</em> /Zostera marina*, Bogue Sound, USA (Summer)</td>
<td>Smyth et al. 2013</td>
</tr>
<tr>
<td>Net Uptake (D)</td>
<td>N₂:Ar&lt;sup&gt;D&lt;/sup&gt;</td>
<td>Species, Location, Season</td>
<td>Reference</td>
</tr>
<tr>
<td>----------------</td>
<td>-------------------</td>
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<td>-----------</td>
</tr>
<tr>
<td>~200 (D)</td>
<td>N₂:Ar&lt;sup&gt;D&lt;/sup&gt;</td>
<td><em>Halodule wrightii</em> / <em>Zostera marina</em>, Bogue Sound, USA (Autumn, Spring and Winter)</td>
<td>Smyth et al. 2013</td>
</tr>
<tr>
<td>~20 (L)</td>
<td>N₂:Ar&lt;sup&gt;L&lt;/sup&gt;</td>
<td><em>Zostera capricorni</em>, Moreton Bay, Australia (Summer)</td>
<td>Eyre et al. 2011a</td>
</tr>
<tr>
<td>~425 (D)</td>
<td>N₂:Ar&lt;sup&gt;D&lt;/sup&gt;</td>
<td><em>Zostera capricorni</em>, Moreton Bay, Australia (Winter)</td>
<td>Eyre et al. 2011a</td>
</tr>
<tr>
<td>~60 (D)</td>
<td>N₂:Ar&lt;sup&gt;D&lt;/sup&gt;</td>
<td><em>Halophila ovalis</em> / <em>spinulosa</em>, Moreton Bay, Australia (Summer)</td>
<td>Eyre et al. 2011a</td>
</tr>
<tr>
<td>~100 (L)</td>
<td>N₂:Ar&lt;sup&gt;D&lt;/sup&gt;</td>
<td><em>Halophila ovalis</em> / <em>spinulosa</em>, Moreton Bay, Australia (Winter)</td>
<td>Eyre et al. 2011a</td>
</tr>
<tr>
<td>~70 (D)</td>
<td>N₂:Ar&lt;sup&gt;D&lt;/sup&gt;</td>
<td><em>Halophila ovalis</em> / <em>spinulosa</em>, Moreton Bay, Australia (Winter)</td>
<td>Eyre et al. 2011a</td>
</tr>
<tr>
<td>~83-167&lt;sub&gt;L&lt;/sub&gt;</td>
<td>AB</td>
<td><em>Halodule beaudetti</em>, Falmouth Harbour, Jamaica (Winter)</td>
<td>Blackburn et al. 1994</td>
</tr>
</tbody>
</table>

**Temperate**

<table>
<thead>
<tr>
<th>Net Uptake (L)</th>
<th>N₂:Ar&lt;sup&gt;L&lt;/sup&gt;</th>
<th>Species, Location, Season</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>~209 – 351&lt;sub&gt;S,I&lt;/sub&gt; (L)</td>
<td>IPT&lt;sup&gt;L,P&lt;/sup&gt;</td>
<td><em>Zostera marina</em>, South Bay, USA (Summer)</td>
<td>Aoki &amp; McGlathery, 2018</td>
</tr>
<tr>
<td>~53 – 109&lt;sub&gt;S,I&lt;/sub&gt; (L)</td>
<td>IPT&lt;sup&gt;L,P&lt;/sup&gt;</td>
<td><em>Zostera marina</em>, South Bay, USA (Autumn)</td>
<td>Aoki &amp; McGlathery, 2018</td>
</tr>
<tr>
<td>~80 – 81&lt;sub&gt;S,I&lt;/sub&gt; (L)</td>
<td>IPT&lt;sup&gt;L,P&lt;/sup&gt;</td>
<td><em>Zostera marina</em>, South Bay, USA (Spring)</td>
<td>Aoki &amp; McGlathery, 2018</td>
</tr>
<tr>
<td>~17.5&lt;sub&gt;S,I&lt;/sub&gt; (L)</td>
<td>N₂:Ar&lt;sup&gt;L,P&lt;/sup&gt;</td>
<td><em>Zostera marina</em>, South Bay, USA (Summer)</td>
<td>Aoki &amp; McGlathery, 2017</td>
</tr>
<tr>
<td>~109 – 232&lt;sub&gt;S,P&lt;/sub&gt; (D)</td>
<td>N₂:Ar&lt;sup&gt;L,D&lt;/sup&gt;</td>
<td><em>Zostera marina</em>, Shinecock Bay, USA (Summer)</td>
<td>Zarnoch et al. 2017</td>
</tr>
<tr>
<td>~60&lt;sub&gt;P,E&lt;/sub&gt; (L)</td>
<td>N₂:Ar&lt;sup&gt;D&lt;/sup&gt;</td>
<td><em>Zostera capricorni</em>, Lake Macquarie, Australia (Summer)</td>
<td>Eyre and Ferguson 2002</td>
</tr>
<tr>
<td>~70&lt;sub&gt;P,E&lt;/sub&gt; (L)</td>
<td>N₂:Ar&lt;sup&gt;D&lt;/sup&gt;</td>
<td><em>Zostera capricorni</em>, Lake Macquarie, Australia (Summer)</td>
<td>Eyre and Ferguson 2002</td>
</tr>
<tr>
<td>~20&lt;sub&gt;P,E&lt;/sub&gt; (D)</td>
<td>N₂:Ar&lt;sup&gt;D&lt;/sup&gt;</td>
<td><em>Zostera capricorni</em>, Lake Macquarie, Australia (Summer)</td>
<td>Eyre and Ferguson 2002</td>
</tr>
<tr>
<td>~100&lt;sub&gt;P,E&lt;/sub&gt; (D)</td>
<td>N₂:Ar&lt;sup&gt;D&lt;/sup&gt;</td>
<td><em>Zostera capricorni</em>, Lake Macquarie, Australia (Summer)</td>
<td>Eyre and Ferguson 2002</td>
</tr>
</tbody>
</table>
~15.D (L)
~20.D-L (L)
~25.D (D)
~25.D-L (D)
~3.25 (L)
~4 (D)
~2.5 (L)
~5.5 (D)
~3 (L)
~4 (D)
~14.6 – 24*
~4 – 29*
~3 – 35*
~10-12.5*
~4 – 6*
~4 – 5*
~2 – 5*
~6*
~4.17w (L)
~2.08w (D)
~5.8w (L)
~19.2w (D)
| ~5.83s (L) | IPT\textsuperscript{D,N} | *Zostera noltii*, Bassin d’Arcachon, France (Summer) | Rysgaard et al. 1996 |
| ~0.042 (L) | IPT\textsuperscript{D,N} | *Zostera noltii*, Bassin d’Arcachon, France (Summer) | Rysgaard et al. 1996 |
| ~0.042 (D) | IPT\textsuperscript{D,N} | *Zostera noltii*, Bassin d’Arcachon, France (Summer) | Rysgaard et al. 1996 |
| ~0.375 (D) | IPT\textsuperscript{D,N} | *Zostera noltii*, Bassin d’Arcachon, France (Autumn) | Rysgaard et al. 1996 |
| ~3.42 (L) | IPT\textsuperscript{D,N} | *Zostera noltii*, Bassin d’Arcachon, France (Winter) | Rysgaard et al. 1996 |
| ~11.5 (D) | IPT\textsuperscript{D,N} | *Zostera noltii*, Bassin d’Arcachon, France (Winter) | Rysgaard et al. 1996 |
b – R-IPT-DNRA calculation developed by Salk et al. 2017

c – Intact sediment cores without seagrass plants taken from within seagrass bed

D – Intact sediment cores containing seagrass plants

D-L – Intact sediment cores containing defoliated seagrass plants

L – $^{15}$NO$_3^-$ label additions made & measured via MIMS

P – Label added to rhizosphere via push-pull piezometer


B – Incubations in benthic chambers or cores

S – Label added to sediment / rhizosphere

W – Measurements from water column

X – perfusion with $^{15}$NH$_4^+$

I – measurements taken in situ

S+D – Sediment + seagrass detritus

S+P – Sediment + whole seagrass plant

P+E – seagrass plants + epiphytes

P-E – seagrass plants without epiphytes

* - averaged over light and dark rates

(L) – Rates measured under light conditions

(D) – Rates measured under dark conditions
1 - Introduction

Thamdrup and Dalsgaard (2002) were early investigators of anammox in marine sediments; prior to this it had only been described in wastewater treatment processes. They found that anammox was present in marine sediments and that rates were higher in coastal sediments than deep water sediments (Thamdrup and Dalsgaard, 2002), more recent measurements show that N loss by anammox from shallow marine sediments is highly variable and can range from no activity to the dominant N₂ loss pathway (Engström et al. 2005, Rich et al. 2008, Salk et al. 2017). The seagrass rhizosphere is an ideal environment for anammox and has been found to be the dominant N loss process from a seagrass community (Salk et al. 2017). Because anammox and denitrification occur in similar anaerobic environments and yield the same end product (N₂) past denitrification measurements may have unknowingly included anammox and subsequently been overestimated.

1.1.4 Dissimilatory nitrate reduction to ammonium (DNRA)

DNRA reduces NO₃⁻ to ammonium which, unlike anammox and denitrification, retains bioavailable N rather than liberating it as gaseous N₂.

\[ \text{NO}_3^- \rightarrow \text{NH}_4^+ \]  \[8\]

Like denitrification and anammox, DNRA largely occurs under anoxic conditions and in coastal systems it can represent a significant proportion of NO₃⁻ reduction processes (Burgin and Hamilton, 2007; Dong et al. 2011; Giblin et al. 2013) and may even dominate sediment N processes in high carbon, low NO₃⁻ conditions (Algar and Vallino, 2014; Kraft et al. 2014; Brin et al, 2015; Hardison et al. 2015; van den Berg et al. 2015) which means N is retained in the system rather than released via denitrification. This makes it particularly suited to seagrass ecosystems which

Unraveling the complexity of subtropical seagrass nitrogen cycling: a stable isotope approach
Table 1.2 Rates of dissimilatory nitrate reduction to ammonia (DNRA) in (sub)tropical and temperate seagrass systems

<table>
<thead>
<tr>
<th>DNRA rate (μmol N m⁻² h⁻¹)</th>
<th>Method</th>
<th>Species, location &amp; season</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>(Sub)Tropical</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>~ 0.42βs (D)</td>
<td>Intact core + $^{15}$NO₃⁻ additionsₓᵛ</td>
<td>Zostera muelleri, Shaws Bay, Australia (Winter)</td>
<td>Salk et al. 2017</td>
</tr>
<tr>
<td>~1.03s+D (D)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Temperate</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>~10-15α (L)</td>
<td>In situ piezometer + $^{15}$NO₃⁻ additionsᵐ</td>
<td>Zostera marina, South Bay, USA (Summer)</td>
<td>Aoki and McGlathery 2018</td>
</tr>
<tr>
<td>~144-191₁ (L)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>~11α (L)</td>
<td>In situ piezometer + $^{15}$NO₃⁻ additionsᵐ</td>
<td>Zostera marina, South Bay, USA (Autumn)</td>
<td>Aoki and McGlathery 2018</td>
</tr>
<tr>
<td>~60-116₁ (L)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>~7α (L)</td>
<td>In situ piezometer + $^{15}$NO₃⁻ additionsᵐ</td>
<td>Zostera marina, South Bay, USA (Spring)</td>
<td>Aoki and McGlathery 2018</td>
</tr>
<tr>
<td>~48-63₁ (L)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>~14.7₁ (L)</td>
<td>In situ piezometer + $^{15}$NO₃⁻ additionsᵐ</td>
<td>Zostera marina, South Bay, USA (Summer)</td>
<td>Aoki and McGlathery 2017</td>
</tr>
<tr>
<td>~2.9s+p (L)</td>
<td>Intact core + $^{15}$NO₃⁻ additionsᵇ</td>
<td>Zostera noltii, Bassin d’Arcachon, France (Autumn)</td>
<td>Rysgaard et al. 1996</td>
</tr>
<tr>
<td>~5.4s+p (D)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
BS – Rates based in un-vegetated bare sediments without seagrass plants
S+D – Rates based on sediment amended with seagrass detritus
A – Ambient rates without label additions
I – Rates based on $^{15}$NO$_3^-$ additions
S+P – Rates based on sediment cores including seagrass plants
Z – $^{15}$NH$_4^+$ production measured by IRMS (Zhang et al. 2007)
X – Rates calculated using R-IPT-DNRA (Salk et al. 2017)
M – $^{15}$NH$_4^+$ and $^{14}$NH$_4^+$ production measured via MIMS
B – $^{15}$NH$_4^+$ production measured using micro diffusion technique (Blackburn 1993)
(L) – Rates measured under light conditions
(D) – Rates measured under dark conditions
have high concentrations of available organic C from leaf detritus as well as exudates from seagrass roots. DNRA has been characterised in only a few seagrass systems previously (Table 1.2). It is an important process to quantify as it may explain deficits in the N budgets of seagrass systems.

1.2 Current limitations to our understanding of N cycling in seagrass systems

There has been a large volume of research conducted on N cycling in seagrass species over previous decades (McRoy et al. 1973, Boon et al. 1986, Hemminga et al. 1991, Blackburn et al. 1994, Welsh et al. 1996, Risgaard-Petersen et al. 1998, Welsh 2000, Eyre and Ferguson 2002, Eyre et al. 2011a). However, large variability in rates of N inputs, outputs and recycling processes have meant our understanding of N cycling in seagrass communities is still not well constrained. One of the major reasons for this is the use of various methods that contain many assumptions and that more contemporary techniques have not been employed. This section provides a summary of previous research with an emphasis on the methods used in seagrass communities to measure N inputs (N$_2$ fixation), outputs (denitrification and anammox) and recycling processes (DNRA).

Measuring N cycling in seagrass systems is important as rates can vary greatly as compared with bare sediment ecosystems (Caffrey & Kemp 1990, Isaksen & Finster 1996, Zarnoch et al. 2017). The major difference between N cycling in seagrasses compared to sediment dominated systems is the presence of the seagrass themselves. Seagrasses are marine angiosperms meaning they are flowering plants which have roots similar to their terrestrial counterparts, the presence of these submerged root systems can influence N cycling in shallow water sediments (Risgaard-Petersen and Jensen, 1997). The roots of these plants can supply numerous metabolites including O$_2$. Unraveling the complexity of subtropical seagrass nitrogen cycling: a stable isotope approach
organic C and inorganic N to the surrounding sediment. Oxygen released from the roots of seagrasses can drive coupled nitrification-denitrification (Risgaard-Petersen and Jensen 1997, Risgaard-Petersen et al. 1998) and can protect the roots of seagrass plants from toxic sulphides by re-oxidising it to sulphate (Brodersen et al. 2015). This impact on the sulphur species of the sediment can have great repercussions on N₂ fixation and anammox (Welsh 2000, Jensen et al. 2008). Due to this dynamic environment the rhizosphere is thought to be one of the most important regions of N cycling in seagrass systems (McGlathery et al. 1998, Seymour et al. 2018).

Dinitrogen fixation, denitrification, anammox and DNRA have all been observed in the seagrass rhizosphere (Salk et al. 2017, Seymour et al. 2018). However, some regions within the seagrass community have been overlooked in favour of characterising rhizosphere processes. The leaves of seagrasses provide a platform for epiphytes to colonise, which may be of particular importance in seagrass N₂ fixation. It is well know that many seagrass leaf-associated epiphytes are photosynthetically active N₂-fixers (Agawin et al. 2016). Leaf N₂ fixation rates have only been measured in a few studies (Table 3.1), and due to the high above ground biomass of some seagrass species has the potential to be an active site of N₂ fixation, which may be even more important that the rhizosphere.

There has not been much work investigating the impact species-specific morphology may have on seagrass N input and output processes. From what we know, and have discussed above, physical characteristics such as the surface area and density of above ground and below ground biomass
have the potential to impact rates of N\textsubscript{2} fixation, denitrification, anammox and DNRA. Seagrass species vary enormously from small ephemeral species of leaf size ~ 1-4 cm like *Halophila ovalis* (Lanyon 1986) to large dense patches of long (up to 30 cm) leaves like *Zostera muelleri* (Moore & Short 2007), both of which also differ in their below ground morphology (Roberts 1993). These are two subtropical seagrass species commonly found along the east coast of Australia and the differences between these two species alone has the potential to impact on their ability to import and export N and may have implications for local and potentially global N budgets.

One of the reasons seagrass morphology, including active sites of N input and loss processes, has not been investigated is due to limitations by commonly used methods. Dinitrogen fixation in tropical seagrass communities has been measured largely using the ARA (O’Donohue et al. 1991b, Iizumi, 1992, Blackburn et al. 1994, Eyre et al. 2011a, Table 3.1). Other triple bonded molecules may be reduced by nitrogenase and therefore the reduction of acetylene to ethylene has been used as a proxy for N\textsubscript{2} fixation. Estimates of N\textsubscript{2} fixation in seagrass systems have been variable but have always thought to be low (Table 3.1) and there has been very little work in comparing N\textsubscript{2} fixation rates in species based on their morphology. Direct stable isotope techniques may be able to provide greater resolution and insight into the active regions of N\textsubscript{2} fixation within the seagrass community as well as discern differences in these active regions between species. Methodology has also been an issue in the measurement of N loss processes, commonly used methods such as the isotope pairing technique (IPT) and net N\textsubscript{2} flux.
1 - Introduction

measurements (hereon referred to as the N₂:Ar technique) have often provided vastly different estimates (Table 1.1). Due to the IPT being used more in temperate locations and the N₂:Ar technique more commonly employed in subtropical regions, it is not clear if differences are location specific or methodological. The emergence of other N cycling pathways in marine and estuarine sediments, such as anammox and DNRA means that older estimates of denitrification in seagrass systems may have been overestimated.

1.2.1 Nitrogen fixation in seagrass systems

N₂ fixation measurements have been dominated by the ARA which uses the reduction of acetylene to ethylene as a proxy for N₂ reduction to NH₄⁺. The ARA is popular due to its low cost, ease of use in the field and measurement making it the most common method used to measure N₂ fixation in seagrass systems (Stewart et al. 1967, Seitzinger and Garber 1987, Currin et al. 1996, Montoya et al. 1996, Welsh et al. 2000; Table 3.1). To convert the formation of ethylene via reduction of acetylene to rates of N₂ fixation a stoichiometric calculation; often a 3:1 ratio (i.e. 3 moles of acetylene to 1 mole of N₂ fixed) is used as an equivalent for the 6e⁻ transfer involved in the reduction of one mole of N₂ to NH₃ (Hardy et al. 1968, Seitzinger and Garber 1987). This ratio is a major assumption and many different variations on the 3:1 stoichiometry have been used (Table 3.1). Stewart et al. (1967) did suggest that it was advisable to carry out a parallel sample via a direct ¹⁵N₂ method as a ‘calibration’ for the results obtained by acetylene reduction so as to confirm the theoretical 3:1 ratio for individual analyses. Due to the indirect
nature of the ARA and the reagents and products used and formed during analysis there have been suggestions that it may either over- or underestimate rates of N\textsubscript{2} fixation (Seitzinger and Garber 1987, Welsh, 2000). Furthermore, analysis of acetylene reduction samples usually entails the creation of a slurry. This destroys the structure of the sediment profile as all layers are homogenised and the exact location of N\textsubscript{2} fixation within the profile cannot be ascertained. Thus for intact core experiments which are attempting to determine active regions of N\textsubscript{2} fixation within the seagrass system as a whole the ARA is not suitable. The limitations of the ARA with regard to the characterisation of specific loci of N\textsubscript{2} fixation within a seagrass community are discussed in detail in Chapters 3 and 4.

Direct methods that utilise the stable isotope of N (\textsuperscript{15}N) are also available and are arguably more appropriate for measuring rates in the above (i.e. leaves) and below (i.e. rhizosphere) ground loci of seagrass systems, as they have much limited assumptions than proxy methods. The use of stable isotopic measurements in N\textsubscript{2} fixation rate estimates has not been widespread due to the increased cost and technical equipment required as compared with the ARA. To date there has only been one study in which N\textsubscript{2} fixation has been measured in seagrasses using a direct \textsuperscript{15}N-N\textsubscript{2} technique (O’Donohue et al. 1991a).

1.2.2 \textsuperscript{15}N-N\textsubscript{2} dissolution method

A recent improvement in \textsuperscript{15}N-N\textsubscript{2} techniques has been the \textsuperscript{15}N-N\textsubscript{2} dissolution method which involves adding \textsuperscript{15}N-N\textsubscript{2} gas to site water and after an equilibration period adding the enriched solution to a system and tracing the fate of the added \textsuperscript{15}N (Mohr et al. 2010, Grosskopf et al. 2012). The original
\[ ^{15}\text{N}_2 \] technique involved adding \( ^{15}\text{N}-\text{N}_2 \) gas as a bubble directly to the system being studied. This technique required the vigorous shaking to ensure effective dissolution of the \( ^{15}\text{N}-\text{N}_2 \) into the water. One of the major issues with adding \( ^{15}\text{N}-\text{N}_2 \) as a bubble was that post shaking equilibration between the \( ^{15}\text{N}-\text{N}_2 \) gas and the \( \text{N}_2 \) in the water phase was assumed. The addition of a \( ^{15}\text{N}-\text{N}_2 \) bubble and the \( ^{15}\text{N}-\text{N}_2 \) dissolution methods were compared in parallel incubation experiments on artificial seawater and cyanobacterial cultures (Mohr et al. 2010, Grosskopf et al. 2012). It was found the original method of adding \( ^{15}\text{N}_2 \) tracer as a gas bubble significantly underestimated \( \text{N}_2 \) fixation rates (Mohr et al. 2010, Grosskopf et al. 2012). Grosskopf et al. (2012) found poor correlation between the two methods with water column \( \text{N}_2 \) fixation rates in areas of the Atlantic Ocean underestimated by an average of 62% when measured with the \( ^{15}\text{N}-\text{N}_2 \) bubble method. Due to this modification an area previously thought to be unimportant for \( \text{N}_2 \) fixation was actually found to be significant to the global \( \text{N} \) cycle (Grosskopf et al. 2012).

The \( ^{15}\text{N}_2 \) dissolution method not only maintains sedimentary gradients and microbial communities by removing the shaking step of the \( ^{15}\text{N}-\text{N}_2 \) bubble technique (Mohr et al. 2010) it also doesn’t rely on the problematic stoichiometric relationship of the ARA. For intact sediment cores and cores containing whole seagrass plants, the \( ^{15}\text{N}_2 \) dilution method offers the least disruption to the system meaning that micro-zones of \( \text{N}_2 \) fixation are preserved. Additionally, unlike the ARA, the \( ^{15}\text{N}_2 \) method does not interfere with other processes or \( \text{N}_2 \)-fixing microbes which can potentially artificially
inflates or inhibits N$_2$ fixation rates (Welsh 2000), discussed in greater depth in Chapter 3.

Despite the benefits, there are issues associated with the $^{15}$N-N$_2$ dissolution method. The main hindrance being that commercial $^{15}$N-N$_2$ gas may be contaminated with various forms of $^{15}$N, including $^{15}$NH$_4^+$, which can cause overestimation of N$_2$ fixation rates. The contamination of a variety of commercially available $^{15}$N$_2$ gases was investigated by Dabundo et al. (2014). They found varying rates of contamination across the three different brands of gas they analysed (explained in greater detail in Chapter 3). Thus prior to use, gas must be analysed and the level of contamination with $^{15}$N products ascertained. The $^{15}$N$_2$ dissolution method is also a more costly method compared with the ARA. However, the instrumentation required for measurement (isotope ratio mass spectrometry, IRMS) are now widely available in many labs.

As mentioned previously only one study has used a stable isotope technique to determine N$_2$ fixation in seagrasses (O’Donohue et al. 1991a). The $^{15}$N-N$_2$ dissolution technique hasn’t been used to determine regions of N$_2$ fixation in seagrass systems and below ground processes weren’t well constrained by O’Donohue et al. (1991a). Due to the suitability of the $^{15}$N-N$_2$ dissolution method for intact core incubations, where pore water gradients and plant tissue can be maintained, it also makes it ideal for studying the effect morphological characteristics may have on rates of N$_2$ fixation (Chapter 4). The application of this method is critical for the improved understanding of seagrass systems as it has the capacity to
measure rates in a direct fashion and could cause revision to the N\textsubscript{2} fixation capacity of these habitats.

1.2.3 Measurement of denitrification, anammox and DNRA in seagrass systems

The IPT is the most common method used for measuring N loss from seagrass systems (Table 1.2) and is based on measuring the production of single labelled (\textsuperscript{14}N\textsuperscript{15}N) or double labelled (\textsuperscript{15}N\textsuperscript{15}N) N\textsubscript{2} following the addition of \textsuperscript{15}N-NO\textsubscript{3}\textsuperscript{-} to a system commonly, intact cores or chambers (Nielsen, 1992). Since the development of the IPT by Nielsen (1992) (subsequently referred to as IPT\textsubscript{classic}) many researchers have sought to amend and modify the technique to make it more relevant; bringing it in line with current knowledge. Thamdrup and Dalsgaard (2002) were the first to observe the effect of anammox in marine sediments and the potential impact this could have on the measurement of denitrification rates. They carried out IPT incubations with different variations of isotopically labelled and unlabelled NO\textsubscript{3}\textsuperscript{-} and NH\textsubscript{4}\textsuperscript{+} and found that N\textsubscript{2} was not just formed from denitrification but also from a reaction between NO\textsubscript{3}\textsuperscript{-} and NH\textsubscript{4}\textsuperscript{+} i.e. anammox. Various studies in the years following (Risgaard-Petersen et al. 2003, Risgaard-Petersen et al. 2004, Trimmer et al. 2006) attempted to modify the calculations of the IPT\textsubscript{classic} in order to account for N\textsubscript{2} production by anammox. Hsu and Kao (2013) adapted Nielsen’s (1992) IPT\textsubscript{classic} to include anammox and N\textsubscript{2}O production. N\textsubscript{2}O is a by-product of denitrification, and its production had not been not considered in previous IPT iterations as its production was thought to be negligible (Trimmer at al 2006). The premise behind incorporating N\textsubscript{2}O production into IPT calculations is that some \textsuperscript{15}N added to the system may be incorporated into the formation of N\textsubscript{2}O.
Therefore not including N₂O production could lead to an underestimation of denitrification. N₂O fluxes have rarely been reported for seagrass habitats, which makes their inclusion in N loss measurements in seagrass systems even more imperative (Murray et al. 2015).

Another version of the IPT proposed by Master et al. (2005) explored the release of both N₂ and N₂O, however they neglected to account for anammox. The inclusion of anammox in denitrification rate calculations measured by IPT is imperative as the formation of ²⁹N₂ can occur due to¹⁵NO₃⁻ reacting with ¹⁴NH₄⁺ in the anoxic zone. Anammox has been found to be a significant N loss process in shallow estuarine environments (Engström et al. 2005, Crowe et al. 2012), including seagrass systems (Salk et al. 2017). Therefore, if anammox concentrations are significant it could lead to the overestimation of denitrification in these systems. Further adjustments have been made to the IPT calculation by Song et al. (2016) to include DNRA however this iteration of the technique neglected to include N₂O production. Most recently Salk et al. (2017) developed the R-IPT-DNRA which accounts for denitrification, anammox, N₂O production and DNRA, aiming to provide more accurate N loss estimates. This is the version of the IPT calculation used in this research (Chapter 5).

A persistent issue with the IPT and described by Nielsen (1992) as the fundamental limitation of the technique, is the demand for uniform mixing of labelled ¹⁵NO₃⁻ with ¹⁴NO₃⁻ already present within the deeper sediment profile or rhizosphere of seagrasses. It was observed that adding the ¹⁵NO₃⁻ to the water column meant that any coupled nitrification-denitrification that occurs...
around the roots of aquatic macrophytes will not be measured as the label
only penetrates the top few centimetres of the sediment (Nielsen, 1992). This
limitation is recognised by a variety of authors (Nielsen 1992, Cornwell et al.
(2008) and Welsh et al. (2000) found that IPT can underestimate denitrification
rates in sediments covered with seagrass as it only allows measurement of
denitrification in the surface sediments, not deeper in the rhizosphere where
coupled nitrification-denitrification occurs. Thus only labelling the overlying
water in core incubations may not give an accurate estimation of N processes
occurring in the seagrass rhizosphere including denitrification, anammox or
DNRA.

The $N_2$:Ar technique is used to measure the net $N_2$ flux of surface
processes in a closed system (Kana et al. 1994) by measuring the change in
the ratio of the dissolved gases $N_2$ and argon (Ar) in water samples. This
method has been most popular in subtropical seagrass studies and has not
been as widely published as the IPT (Table 1.2). The $N_2$:Ar technique does
not measure discrete denitrification rates in sediments specifically, rather it
provides a measure of the net $N_2$ production of a system, including $N_2$
produced from denitrification and anammox minus $N_2$ fixation. Due to this,
specific regions of denitrification, anammox or $N_2$ loss, are not able to be
identified.

The $N_2$:Ar technique can yield highly precise data, rapid sample
throughput and only requires small sample size (Kana et al. 1994, Cornwell et
al. 1999). Additionally, there is no requirement for expensive $^{15}$N reagents and
potential problematic isotopic mixing issues that can occur with the IPT. The N$_2$:Ar technique does not rely on the assumptions of the IPT, such as uniform mixing of the tracer or penetration of the label beyond superficial sediments and therefore may provide more accurate rates than the IPT.

One of the assumptions inherent in the N$_2$:Ar methodology is that the concentration of Ar remains constant as even a small change in Ar concentration can impact the ratio and alter the calculated rate of N$_2$ loss. It is very easy to compromise the N$_2$:Ar ratio via formation of air or O$_2$ bubbles in the sample collection process which can alter the Ar concentration. The N$_2$:Ar technique is particularly sensitive to the O$_2$ concentration of the sample due to the fact that O$_2$ combines with the N$_2$ produced from denitrification to form NO$^+$ (Jensen et al. 1996). Samples vials must be filled to overflowing before being sealed, incubation apparatus need to be kept submerged and once sampled vials must be kept submerged until analysis. Highly productive sediments are also problematic and samples can only be collected before the core or chamber reaches O$_2$ saturation. As such, N$_2$:Ar measurements can only be collected during the earlier part of the day before O$_2$ saturation becomes too high. As such, light enhanced N$_2$ fixation may be underestimated (Eyre et al. 2013).

Eyre et al. (2002) compared the IPT and N$_2$:Ar on the same estuarine sediments in Denmark and found that the rates of denitrification measured via the IPT were less than those measured by N$_2$:Ar, however they were much more stable and consistent than the N$_2$:Ar results. It is common to obtain higher N$_2$ loss rate estimates with the N$_2$:Ar method as over the course of the
incubation O$_2$ is depleted even with the use of bubbling/airstones (Eyre et al. 2002, Ferguson and Eyre 2007, Gihring et al. 2010, Newell et al., 2016).

The N$_2$:Ar technique only provides the net flux of N$_2$ out of the whole system and cannot measure N cycling processes in discrete loci, especially those within the rhizosphere. In order to accurately characterise seagrass rhizosphere N cycling processes another technique is required.

1.2.4 Perfusion technique
Quantifying rates of N cycling in the seagrass rhizosphere is a difficult but important component to consider when calculating N$_2$ fixation or denitrification rate estimates for these systems. Dinitrogen-fixing bacteria are known to live at depths within the sediment (for example, SRB) and due to the excretion of photosynthetic exudates like O$_2$ and organic C other N cycling processes such as, coupled nitrification-denitrification and anammox may be stimulated (Risgaard-Petersen and Jensen 1997, Risgaard-Petersen et al. 1998). The occurrence of these processes within the seagrass rhizosphere are well-known but poorly quantified. Therefore ignoring rhizosphere N cycling processes may mean that a significant portion of seagrass community N cycling is not included in input and output estimates.

Whilst measuring below ground N cycling processes is important sediment profile studies are difficult to perform without disturbing the naturally occurring redox gradients within the profile and the addition of seagrass plants adds another layer of complexity. In an attempt to determine rhizosphere biogeochemical cycling a number of sediment perfusion techniques have been attempted (Blackburn et al. 1994, Risgaard-Petersen et al. 1994, Risgaard-
Introduction

Petersen and Jensen 1997, Davidsson et al. 1997, Sheibley et al. 2003). Most of these techniques are detailed in only one study each suggesting that their use may be difficult and somewhat ineffective.

Perfusion techniques have largely been undertaken on intact core experiments containing only sediment (Stepanauskas et al. 1996, Davidsson et al. 1997, Sheibley et al. 2003) however, a few studies have used perfusion in order to quantify seagrass rhizosphere processes (Blackburn et al. 1994, Welsh et al. 1996a, McGlathery et al. 1998). Perfusion techniques are often carried out in intact sediment cores as the rhizosphere is easy to see and access. The premise behind most perfusion core techniques is the addition of a reagent (usually a stable isotope tracer) to sediment pore water so as to directly quantify the processes occurring in the rhizosphere. Numerous authors have commented that the IPT only serves to label the superficial sediments and doesn’t penetrate to the rhizosphere (Nielsen 1992, Cornwell et al. 1999, Welsh et al. 2000, Eyre et al. 2002, Ferguson & Eyre 2007, Bartoli et al. 2008) and the N₂:Ar technique doesn’t allow for a measurement of discrete sections of a core just a net flux. Similarly in measurements of N₂ fixation if either isotopic label or acetylene (as in the ARA) is added to the water column it may not penetrate the rhizosphere.

Denitrification and to a lesser extent N₂ fixation in sediments has been measured via a variety of perfusion core studies (Blackburn et al. 1994, Risgaard-Petersen & Jensen 1997, Canion et al. 2014). In N₂ fixation perfusion experiments all except one (O’Donohue et al. 1991a) have used the
ARA (Blackburn et al. 1994, McGlathery et al. 1998, Sheibley et al. 2003, Table 3.1). The addition of acetylene (and its subsequent reduced product ethylene) may stimulate or inhibit N₂ fixers within the rhizosphere (Welsh 2000), therefore it is important that a direct stable isotope technique is used to accurately measure below ground N₂ fixation.

The use of a perfusion method may be the most effective way to ensure uniform and adequate labelling of the rhizosphere however, development of a technique that successfully uniformly labels the rhizosphere whilst mitigating destruction of sedimentary gradients was required for this research. The development of this technique is detailed in Chapter 2. Furthermore there are very few (O’Donohue et al. 1991a,b, Blackburn et al. 1994, McGlathery et al. 1998, Table 3.1) applications of perfusion core techniques in seagrass systems and no work has been done on combining the direct ^15N₂ dissolution method with the perfusion technique. Thus N₂ fixation within the seagrass rhizosphere is not well constrained.

1.3 Aims and significance of research

This research will provide important information on N cycling (N input, output and recycling pathways) in multiple subtropical seagrass species using the most contemporary methods available.

Using the direct ^15N₂ dissolution technique in concert with the perfusion core technique (which has not been used for measuring N₂ fixation in seagrasses) Chapter 3 will present more accurate rates in a subtropical seagrass. It will illustrate N₂ fixation occurring in various loci within the seagrass community i.e. sediment, root/rhizome and leaves + epiphytes. Additionally pathways of N transport through the community will be clarified. In
1 - Introduction

Chapter 4 the N₂ fixation rates of two morphologically diverse seagrass are compared, once again via the ¹⁵N₂ dissolution method and perfusion. By investigating two species of seagrass morphological effects on N₂ fixation activity will be elucidated.

The use of the N₂:Ar technique and IPT in parallel incubations on two subtropical seagrass species will be detailed in Chapter 5. This will aid in bridging the gap between the two commonly used methods and additionally will aid in determining if seagrass morphology plays a role in seagrass system N loss and recycling pathways.

This project is significant because it uses new techniques to characterise N cycling in morphologically diverse subtropical seagrass communities. Rates of N₂ fixation, denitrification, anammox and DNRA measured in this project have the potential to cause a substantial re-evaluation of how these processes are quantified in seagrass systems.

1.4 Goals of the research

This enhanced understanding of N cycling in subtropical seagrass communities will in turn allow a revision of the N budget for Moreton Bay (Chapter 6). In addition to greater understanding of how these dynamic systems cycle N and the pathways and mechanisms used to do so. This research could lead to improved management of iconic seagrass habitats and the catchments they inhabit.

The objectives of the project are:

1. Develop a perfusion technique adapted to measure N cycling in intact sediment cores containing seagrass
1 - Introduction

plants that mitigates disturbance to the seagrass plant and natural sedimentary gradients. Detailed in Chapter 2.

2. Measure surficial and sediment rates of $N_2$ fixation in a subtropical seagrass system using the direct $^{15}N_2$ dissolution method with perfusion to clarify active sites of fixation and transport pathways through the system. Detailed in Chapter 3.

3. Additionally to determine if seagrass species-specific morphology has any impact on $N_2$ fixation and loci of activity over a diurnal cycle - Chapter 4.

4. Characterise rates of N loss via denitrification, anammox and DNRA in two morphologically distinct subtropical seagrass communities using the most recent iteration of the IPT in addition to net fluxes of $N_2$ measured via $N_2$:Ar. To determine if various physical characteristics impact the preference for N loss pathways - Chapter 5.

5. Create a revised N budget for two seagrass species of Moreton Bay based on the results obtained in Chapters 4 and 5 as well as a synthesis and summary of the findings of the research in Chapter 6.
Chapter 2: Perfusion method development.
2 – Perfusion method development

2.1 Brief introduction to perfusion

Nitrogen (N) cycling occurring around the roots of seagrass meadows has been notoriously difficult to quantify. To remedy this, various perfusion techniques have been employed. The basis of the perfusion technique is the addition of a reagent (stable isotopic tracer solutions in this research) to the area of sediment around the roots of seagrass, also referred to as the rhizosphere. Perfusion is largely undertaken in closed ex situ experiments i.e. in sediments incubated in bottles, containers or in this case intact cores containing sediment and whole seagrass plants.

The concept of inundating the rhizosphere of seagrass meadows with a reagent in order to measure the processes occurring there is not new (Moriarty & O’Donohue 1993, Blackburn et al. 1994, Welsh et al. 1996b). The majority of past experiments have used differing techniques to characterise sediment and rhizosphere processes and as such there is no ‘standard’ perfusion method. This chapter will discuss the development of the perfusion technique that was subsequently used to determine below ground seagrass N\textsubscript{2} fixation in Chapters 3 and 4.

2.1.1 History of perfusion

The use of perfusion systems to measure sediment N\textsubscript{2} fixation (Capone & Carpenter 1982, O’Donohue et al. 1991a) and denitrification (Ottosen et al. 1999, Gao et al. 2010) has seen a variety of techniques used due to differences in substrate, reagents and methodology (Fig 2.1).

Whilst many perfusion experiments have been carried out on marine and estuarine sediments, including seagrass vegetated sediments (Jørgensen 1977, Capone and Carpenter 1982, Blackburn and Henriksen 1983), the first perfusion
2 – Perfusion method development

Figure 2.1 Schematic diagrams of the range of perfusion systems that have been used in previous research. Top row (left to right), a.) Aoki & McGlathery (2017), b.) Penton et al. (2013), c.) Hardison et al. (2011), d.) Koop-Jakobsen & Giblin (2009), e.) de Beer et al. (2005). Middle row (left to right), f.) Sheibley et al. (2003), g.) Ottosen et al. (1999), h.) McGlathery et al. (1998), i.) Risgaard-Petersen et al. (1998). Bottom row (left to right), j.) Risgaard-Petersen & Jensen (1997), k.) Stepanauskas et al. (1996), l.) Risgaard-Petersen et al. (1994), m.) O'Donohue et al. (1991b).
Perfusion method development

Unraveling the complexity of subtropical seagrass nitrogen cycling: a stable isotope approach

Experiment to include whole seagrass plants wasn’t until 1991 (O’Donohue et al. 1991a, Fig. 2.1m). The addition of a seagrass plant to an intact core perfusion experiment adds another layer of complexity as care needs to be taken to preserve various micro-zones around the roots.

In formulating the perfusion approach to be used in this research a thorough analysis of past perfusion experiments was undertaken. Due to the number of different perfusion techniques used previously (Fig. 2.1) multiple methods were investigated in order to ascertain the procedure appropriate for characterising N\textsubscript{2} fixation in the seagrass rhizosphere. The aim of the perfusion component of the research was to characterise N\textsubscript{2} fixation in the seagrass rhizosphere whilst mitigating disturbance to natural sedimentary gradients and root interactions.

The earliest techniques used in marine environments involved sediment cores with ports drilled along their length which were injected with an analyte or perfusion solution to measure sulphur-reducing activity (Jørgensen and Fenchel 1974, Jørgensen 1977). This method has been used as a basis for subsequent perfusion experiments (Blackburn and Henriksen 1983) including in seagrass ecosystems (Blackburn et al. 1994, Welsh et al. 1996a,b). The term perfusion wasn’t used until 1982 when Capone and Carpenter (1982) described their method of adding acetylene-treated water to Zostera marina sediments. Their technique involved ‘pulling’ the perfusion solution overlying the core down into the sediment by using a peristaltic pump attached to the bottom of the core to measure N\textsubscript{2} fixation (Capone & Carpenter 1982). Since then the term has become synonymous with inundating sediment in marine and freshwater ecosystems to analyse the processes occurring there. This method of forcing original un-amended pore water out of sediment cores and replacing it with overlying treated water via a peristaltic pump attached to the base of a core has been used in a number of studies (de Beer et al. 2005, Gao et al.)
2010, Gihring et al. 2010b, Fig. 2.1e) however, care must be taken not to clog the pump or lose fine sediment. To combat this some techniques have involved the use of sediment cores with a perforated disc (henceforth referred to as a ‘perfusion disc’) located at the base (McGlathery et al. 1998, Risgaard-Petersen et al. 1998, Ottosen et al. 1999, Fig. 2.1h,i,g). A perfusion disc is often a hard plastic circle with pre-drilled holes in its surface that can fit into the base of a sediment core or other vessel (Fig. 2.2). The holes in the perfusion disc help to keep the majority of sediment in place whilst pore water can still travel through it. Often perfusion discs are covered with fine mesh or some type of robust ‘fabric’ (such as nylon mesh or drain matting) which is water permeable (Ottosen et al. 1999, Fig 2.1g) to mitigate the migration of fine sediment past the disc and into the pump. This system can also be used for pumping water back up into the core, as in McGlathery et al. (1998, Fig. 2.1h). McGlathery et al. (1998) pumped the pore water out of the base of the core but instead of pulling acetylene-treated water overlying the sediment into the rhizosphere at the same time the pore water was transferred into a reservoir where acetylene was added. This treated pore water was then pumped back through the perfusion disc and into the...
Perfusion method development

*Zostera marina* rhizosphere (McGlathery et al. 1998). This method of treating the original pore water is an attempt at keeping rhizosphere chemistry similar to in situ conditions, rather than replacing pore water with oxygenated site water which may have an impact on the redox geochemistry of the rhizosphere. Sheibley et al. (2003) used a similar technique with the addition of bromine as a conservative tracer to their freshwater sediment cores and sampled ports along the length over time (Fig. 2.1f). They found bromine concentrations plateaued after 6 hours suggesting their perfusion solution had taken 6 hours to saturate the sediment (Sheibley et al. 2003). While this gives a good indicator about how long a method like this can take to perfuse a sediment core (and potentially a seagrass rhizosphere) it doesn’t give any clarity on how the perfusion solution is distributed spatially within the core. For example, perfusion solution pumped up from the base of the core will likely take the path of least resistance, i.e. travel up the sides of cores or through any animal burrows or splitting of the sediment that may occur as a product of the perfusion technique used. Also, intact core experiments measuring N cycling including denitrification or N₂ fixation are commonly short (6-12 hours) as the cores are sealed and care must be taken that O₂ concentrations do not drop too low resulting in anoxia, significant changes to sediment redox gradients and core ‘death’.

Other more ‘passive’ perfusion iterations in seagrass ecosystems have included using the plants own internal gas transport system (lacunae). This approach was used by O’Donohue et al. (1991a, b, Fig 2.1m) as well as Moriarty and O’Donohue (1993). In all cases sediment cores containing seagrass plants had overlying water containing either ^15^N₂ (O’Donohue et al. 1991a) or acetylene (O’Donohue et al. 1991b, Moriarty and O’Donohue 1993) added to the surface of the core allowing the plant to transfer the analyte to the rhizosphere via lacunal transport. Fluorescein was used as a conservative tracer to ascertain the uniformity of perfusion within the

Unraveling the complexity of subtropical seagrass nitrogen cycling: a stable isotope approach
rhizosphere (O’Donohue et al. 1991b), however it is not clear whether fluorescein flowed through the plants lacunae or perhaps inundated the sediment via diffusion and if transport through the lacunae was achieved whether the gaseous \(^{15}\text{N}_2\) and acetylene would have travelled via the same mechanism.

One perfusion technique that has been confirmed as very effective is that known as the ‘perfusionator’ developed by Hardison et al. (2011, Fig. 2.1c). Unfortunately the perfusionator was inappropriate for use in this project as the set up requires for the central apparatus to be buried and for sediment to be placed back into the inner ‘core’ of the device. This destroys all natural sedimentary gradients effectively creating a sediment slurry. The particular aim of this research was to measure \(\text{N}_2\) fixation in the seagrass rhizosphere while minimising disturbance to naturally occurring sedimentation. The perfusionator method is not conducive to measuring seagrass \(\text{N}_2\) fixation as plants would need to be removed and subsequently replanted once the perfusionator was in place and the sediment recovered. The removal and subsequent replanting of seagrass plants may cause a stress response that could cause either, plant death or, the seagrass to act anomalously as compared with in situ plants. This disturbance of natural sedimentary gradients means rates of seagrass \(\text{N}_2\) fixation measured using a technique like the perfusionator may not be indicative of in situ rates. More recently Aoki and McGlathery (2017, Fig. 2.1a) developed an amendment to Koop-Jakobsen and Giblin’s (2009) perfusion technique to measure seagrass rhizosphere N cycling in situ (Fig. 2.1d). It involves deploying small push-pull piezometers into the seagrass rhizosphere attached to a peristaltic pump which could remove pore water to be labelled and pumped back into the rhizosphere. The use of this in situ technique means much of the disturbance to the rhizosphere and plant are mitigated, however the spatial distribution of the label within the rhizosphere was not well constrained. This method was used to measure
denitrification and DNRA rates within a *Zostera marina* meadow, processes which commonly occur in anoxic regions of the seagrass rhizosphere. This method may not be as useful for measuring total seagrass $\text{N}_2$ fixation community rates that include above ground processes as the label cannot be added to the overlying water without a suitable enclosure.

One of the major issues with characterising N processes of the rhizosphere is disturbing natural sedimentary gradients and altering the chemistry of discrete areas within it. This means processes may be over or underestimated or perhaps lost entirely. For example, there is a common thought that around the often very fine roots of seagrasses there may be localised areas of lower $\text{NH}_4^+$ concentration where $\text{N}_2$ fixation may be occurring. Inundating the sediment with a large volume of perfusion solution may cause $\text{NH}_4^+$ present in either the pore water or sorbed onto surrounding sediments to destroy these discrete low $\text{NH}_4^+$ environments (Welsh 2000) (this is discussed further in Chapter 3). If this occurs potential $\text{N}_2$ fixation that may be occurring in situ may not be captured in perfusion experiments. This may also be the case in denitrification experiments where micro-zones of coupled nitrification-denitrification (Risgaard-Petersen et al. 1998, Penton et al. 2013) may be disturbed by large volumes of perfusion solution (whether that be $^{15}\text{NO}_3^-$ or $^{15}\text{NH}_4^+$). Large volumes of perfusion solution may even cause physical displacement of sediment depending on the method used destroying micro-zones and natural sedimentary gradients. Consequently there is a fine line between uniformly labelling the rhizosphere and destroying the natural chemical and physical gradients that occur there.

*2.2 Development of a suitable perfusion technique*

Perfusion techniques have been previously used to characterise $\text{N}_2$ fixation processes in seagrass habitats, but these largely involved bare sediment incubations...
Furthermore these early methods were exclusively based on the ARA. The first perfusion experiment undertaken on sediments containing seagrass plants investigated N\textsubscript{2} fixation on the subtropical *Zostera capricorni* in Moreton Bay, Australia (O'Donohue et al. 1991a), the same location as seagrass N\textsubscript{2} fixation and denitrification experiments in Chapters 4 and 5. The addition of seagrass plants to sediment core experiments makes the perfusion technique more difficult but arguably more comparable to in situ rates. The experiments presented in Chapters 3 and 4 are to date only the second (the first being O'Donohue et al. 1991a) to use a \textsuperscript{15}N\textsubscript{2} technique to measure N\textsubscript{2} fixation in seagrass communities. The vast majority of previous N\textsubscript{2} fixation experiments having been performed using the ARA (Capone and Carpenter 1982, Moriarty and O'Donohue 1993, O'Donohue 1991b). O'Donohue et al. (1991a) perfused the rhizosphere by adding \textsuperscript{15}N\textsubscript{2} labelled site water to the surface of intact cores containing whole *Z. capricorni* plants. They coined this perfusion technique ‘lacunal perfusion’ whereby the rhizosphere would become perfused by the labelled \textsuperscript{15}N-N\textsubscript{2} gas travelling through the plants internal gas transport system, or lacunae. Consequently \textsuperscript{15}N-N\textsubscript{2} would be exuded through the roots and into the surrounding sediment. Once perfusion was deemed complete, after ~6 hours, labelled overlying water was replaced with label-free site water. One of the issues with this method is that it is not possible to know how much of the \textsuperscript{15}N\textsubscript{2} actually reached the rhizosphere without being fixed in other areas, for example by epiphytes inhabiting the leaves. There was no conservative tracer used in this experiment, it was assumed sediments were uniformly labelled as O'Donohue et al. (1991b) had used fluorescein to track the inundation of acetylene-treated water into sediment cores. The perfusion methods trialled for this research were variations based on a number of previous methods described in the previous section (2.1). The main aim of
the perfusion facet of this research was to be able to characterise N₂ fixation in the seagrass rhizosphere using a $^{15}$N-N₂ stable isotope technique whilst mitigating disruption to natural sedimentary gradients.

The first attempt at perfusing seagrass sediment was a ‘bottom-up’ approach (like that of, McGlathery et al. 1998, Risgaard-Petersen et al. 1998, Ottosen et al. 1999, Sheibley et al. 2003, Penton et al. 2013; Fig. 2.1h,i,g,f,b). In this trial sediment cores (~15-20 cm, sediment only) were collected in clear acrylic tubes (internal diameter 9 cm, length 46 cm) from a local estuarine embayment Shaws Bay, Ballina NSW, Australia (see Chapter 3 for more information on Shaws Bay). A perfusion disc (Fig. 2.2) consisting of a pvc pipe end cap (80 mm) with multiple ~5 mm holes drilled into its surface was covered in non-woven polypropylene geotextile (150 g m⁻²) drain matting and placed in the bottom of the core between the sediment and the end cap. This allowed movement of pore water while keeping sediment in place (Fig. 2.3). Cores had identical end caps affixed to either end i.e. both end caps were fitted with inflow and outflow taps. The drain matting placed over the perfusion disc also served to prevent blocking of the taps on the bottom of the core. Cores were transported back to the lab where any remaining overlying water was carefully decanted. A peristaltic pump and tubing were attached to the outlet tap at the base of the core in order to pump out the pore water. The concept was to pump out the pore water present in the sediment through the taps located on the bottom of the core, label it and pump it back into the core (Fig. 2.3). This trial was unsuccessful as there was not enough pore water present in the sediment of Shaws Bay (pore water accounted for 18% of wet sediment). Thus only a very small volume of pore water was present,
additionally fine sediment was pulled down through gaps in the sides of the perfusion disc and blocked the taps (Fig. 2.3).

![Image](image)

**Figure 2.3** Perfusion core set-up whereby pore water would be pumped out of the sediment by a peristaltic pump attached to one of the outlet ports (circled in red). This is also the same configuration used for pulling perfusion solution over the sediment into the rhizosphere. The perfusion disc is clearly visible as is some fine sediment which has migrated past the disc, the blue arrow shows the direction of water movement.

The second iteration of this technique was to collect pore water from an area adjacent to where cores were being collected within the seagrass bed instead of pumping it out of the core directly. This meant pore water could be collected in a greater volume which could be labelled in the lab and be incorporated into the sediment cores. A piezometer attached to a peristaltic pump was used (described further in Chapter 3) to collect the pore water into 2 L Schott bottles pre-purged with helium (He). No isotopic label was added to the purged pore water as this trial was primarily to determine if the addition of the pore water to the core was achievable. Any remaining overlying water was decanted from the core and the labelled pore
2 – Perfusion method development

water pumped up through the core via the inlet and outlet taps in the base. The premise behind this perfusion iteration being that labelled pore water pumped up from the base of the sediment core would displace natural pore water pushing it upward thus uniformly labelling the rhizosphere and leaving a layer of ‘original’ unlabelled pore water sitting on top of the core which could be decanted and replaced with overlying site water. This was a method very similar to that of McGlathery et al. (1998, Fig. 2.1h), in their experiment they mention that there was no evidence of core splitting or perfusion solution travelling up natural burrows. This was not the case in our trial, perfusion solution travelling up burrows and core splitting were major issues (Fig. 2.4, 2.5) and clearly evident. This may be a function of differences in the sediment substrate of McGlathery et al. (1998) as compared with our fine muddy sand. We attempted to mitigate core separation by keeping a small amount (~2-3 cm) of overlying water on top of the sediment in order to exert some downward pressure on the core however, this was not enough to prevent splitting. If

![Image](image_url)

**Figure 2. 4** The extent of core splitting was still visible even after being left to re-settle for ~24 hours and with pore water drained.
core splitting couldn’t be avoided it was thought that allowing the core to settle naturally once pore water solution had been added may be a possible solution however, this was also unsuccessful. In most cases post-splitting sediment didn’t settle back into a ‘near-original’ placement in a timely manner and in some cases remained fractured up to 24 hours after the addition of perfusion solution (Fig. 2.5). Additionally, perfusion solution taking the path of least resistance, i.e. up any borrow as well as the sides of the sediment core was observed meaning even without core separation it was evident the sediment wasn’t being evenly inundated. Fluorescein was added to the pore water pumped into the bottom of the core in an attempt to visualise the ‘path’ taken by the perfusion solution. However, this wasn’t successful as the fluorescein either couldn’t be distinguished from the dark, sulphidic sediment under UV light or it was not present.

Figure 2.5 Perfusion core set up for the ‘bottom-up’ iteration of the method where pore water collected separately could be labelled and pumped into the seagrass rhizosphere (water movement indicated by blue arrow) with a peristaltic pump attached to ports in the base of the core (not visible). This iteration commonly caused major core splitting (circled), migration of fine sediment past the perfusion disc is also visible.
Another attempt at a ‘top-down’ strategy which maintained the perfusion disc, drain matting and inlet and outlet taps on the base of the core set up as in the previous approach was trialled. Once sediment cores were collected, any overlying water was decanted and replaced with pore water collected adjacent to the core site as in the previous iteration. A peristaltic pump was attached to the outlet tap on the base of the core in order to displace original pore water and effectively ‘pull down’ labelled pore water into the sediment as used by de Beer et al. (2005, Fig. 2.1e), Gao et al. (2010) and Ghihring et al. (2010b). This approach was ultimately unsuccessful as the perfusion solution was once again observed travelling through the core and out the pump via burrows and along the edges of the core. Thus it was clear this method was not uniformly labelling the rhizosphere.

A popular version of the perfusion method involves drilling ports along the side of cores in order to inject perfusion solution directly into the sediment (Jørgensen & Fenchel 1974, Welsh et al. 1996a,b, Fig. 2.1). I had resisted trying this method as there is a significant risk of cracking the expensive acrylic cores when drilling the ports. Additionally, there were concerns over ports leaking and what could be used to effectively seal them, be robust enough for field collection and be able to be easily re-sealed in the lab once labelled porewater had been injected. Once the previous perfusion iterations had been exhausted it was decided that the port injection method should be trialled. Seven ~6 mm holes were drilled along the length of the 16 cm sediment core (therefore approximately every 2 cm). Instead of using a syringe as has been used in the past (Jørgensen 1977, Blackburn et al. 1994) to inject the rhizosphere, Macro Rhizon soil pore water samplers were used. The Macro Rhizons are made up of a central permeable glass fibre epoxy rod surrounded by a porous ceramic-like (4.5 mm outer diameter, pore size 0.15 µm) layer with a lever lock end (Fig. 2.6). They are designed to sample pore water from soil and have been used to...
sample sediment pore water after perfusion (Seebery-Elverfeldt et al. 2005, Gihring et al. 2010b, Canion et al. 2014). It was thought that these design attributes would make the samplers applicable to introducing labelled solution into sediment. Due to the length of the sampler being porous labelled solution is able to perfuse out along its length in all directions, compared with a syringe, where labelled perfusion solution can only exit from the terminus. Macro Rhizons are potentially better at labelling the sediment and less time consuming as multiple samplers can be inserted at once (Fig. 2.7). The length of the porous portion of the sampler was ~9 cm, large enough that it would fit the internal diameter of the core and would introduce labelled solution along its length. The lever lock ending meant that it could be securely fastened to the tubing of the small peristaltic pump and there was an area of silicon-like material at the base of the porous shaft that helped to seal the ports and prevent excessive leakage (Fig. 2.6). Ports were sealed with duct tape prior to sampling in the field which was replaced post label addition.

The first trial undertaken with the Macro Rhizon samplers consisted of 3 sediment cores again collected from Shaws Bay. Pore water collected from the site via piezometer had lithium chloride (LiCl) added with the distribution of lithium (Li⁺) within the sediment to be used as a conservative tracer for label distribution within the

![Figure 2.6 A Macro Rhizon sampler used to perfuse the seagrass rhizosphere.](image)
sediment. The perfusion solution was injected into the rhizosphere via the Macro Rhizon samplers placed in each port. Perfusion solution was pumped in at ~20 mL minute\(^{-1}\) to a total volume of ~40 mL per port starting from the lowest port and ending with that closest to the surface of the sediment core. The idea behind adding perfusion solution to the Macro Rhizon located the ‘deepest’ within the sediment core first was that as addition of the perfusion solution continued up the core it would push original unlabelled pore water up through the core and eventually be totally displaced. The 40 mL of label solution added per port was an overestimation of the original porosity of this sediment in order to saturate the rhizosphere. No core splitting, air bubble formation or obvious travelling of label solution up burrows or the edges of the core was evident when injecting labelled solution via the Macro Rhizon.

*Figure 2.7* Macro Rhizons introduced perfusion solution to the seagrass rhizosphere. A complimentary fitting connected to a peristaltic pump (not shown) was connected to each Macro Rhizon in turn.
samplers. Cores then had unlabelled site water placed over the top of the core and were allowed to sit for ~24 hours.

Post incubation overlying water was decanted off cores carefully so as to not resuspend or lose any of the fine top layer of sediment. Sediment was then manually extruded while laying horizontally so as to not drain the labelled pore water. The sediment core was gently pushed up and out through the top of the acrylic tube and sectioned into 9 sections (1 cm, 2 cm, 3 cm, 4 cm, 5 cm, 6 cm, 7-9 cm, 10-13 cm and 14-15cm). Slices were then separated into centre and outer edge fractions by using a smaller sub-corer (4.5 cm diameter) (Fig. 2.8). The sub-core was used to separate sediment and pore water from the centre of the core to that closest to the edge of the core to ascertain whether perfusion solution had indeed been distributed uniformly or if there had been any ‘edge effects’, whereby label had primarily travelled up the sides of the cores. These sections were then homogenised, sub-sampled in duplicate, and centrifuged at ~3000 rpm for 10 minutes. The supernatant was collected and filtered through a 0.45 μm syringe filter (Sartorius – cellulose acetate) and water samples collected for Li⁺ concentration measured via inductively coupled plasma mass spectrometry (ICP-MS; as per Chapter 3). No significant difference,

Figure 2.8 Sediment was separated into centre and outer edge fractions for lithium concentration analysis to determine if the Macro Rhizon perfusion method had successfully uniformly labelled the rhizosphere.
via analysis of variance at p <0.05, between Li\(^+\) concentration of the centre or the edge fractions were observed (F\(_{5,47}=1.07\), p>0.05). Additionally, there was no significant change in Li\(^+\) concentration vertically within the sediment profile of either fraction (centre, F\(_{2,23}=0.7\), p>0.05 and edge fraction F\(_{2,23}=0.9\) p>0.05). Consequently it was deemed that the perfusion solution had been distributed uniformly within the sediment via this method. Once this method had been validated it was used to undertake the N\(_2\) fixation experiment in Chapter 3. Lithium chloride was once again used as a conservative tracer and the uniformity of the label solution was again determined (see Chapter 3).

Despite the success of the Macro Rhizon injection method, once the samplers had been through a trial and a full experiment they were largely destroyed. Many had been broken beyond repair and some minor leakage from around the port and the sampler had been observed during the experiment, in most samplers the silicon-like fitting that helped create the seal had been misshapen and damaged. Macro Rhizon samplers are expensive (€255 per set of 10) and can only be ordered from the manufacturer in Europe. Therefore with the cost in addition to the leak issue it was decided to try another slight variation of this perfusion method for future experiments. The more widespread method of injecting the perfusion solution via syringe and large bore needle (Blackburn & Henriksen 1983, Welsh et al. 1996b, de Beer et al. 2005) was adopted for subsequent experiments (Chapter 4).

Using the syringe injection method meant the need for the peristaltic pump was removed and controlling the volume and speed of the perfusion solution to the rhizosphere was much easier (Fig. 2.9). As mentioned in Chapter 1 and further described in Chapter 3, adding too high a volume of perfusion solution may destroy micro-zones of N\(_2\) fixation. Due to the size difference between the sampler and the needle sedimentary gradients would also be less disturbed. Ports were able to be
filled prior to collection with a thin layer of polymer sealant (Soudal Fix-All Flexi) which allowed the needles to pierce the material for injection and still retain a seal once removed. The injection method was as successful as the Macro Rhizon perfusion method with comparable N₂ fixation estimates obtained between them (as per Chapters 3 and 4).

The injection method not only allows greater control of perfusion solution volume and less physical disturbance due to the size of the needle it is also less costly and more easily accessible for researchers than the Macro Rhizon approach. The syringe injection method whilst working well in intact core experiments can still cause inundation of rhizosphere micro-zones, and work developing a more sensitive method that mitigates destruction of sedimentary gradients further is required.

Figure 2.9 Seagrass cores being injected with perfusion solution into the rhizosphere.
Chapter 3: Dinitrogen fixation rates in a subtropical seagrass meadow measured with a direct $^{15}$N-$N_2$ tracer method

3 - Dinitrogen fixation rates in a subtropical seagrass meadow measured with a direct $^{15}$N-$N_2$
tracer method

Abstract

This study used a direct $^{15}$N stable isotope labelling technique to measure
rates of dinitrogen ($N_2$) fixation within above and below ground loci of a subtropical
seagrass meadow (*Zostera muelleri*). The total rate of $N_2$ fixation (i.e. sum of the
above and below ground rates) was $\sim$38 μmol $N_2$ m$^{-2}$ h$^{-1}$, similar to other rates
measured in subtropical systems. Rates of $N_2$ fixation were higher when the $^{15}$N-$N_2$
label was added to the surface water compared to when it was added to the
sediments. Furthermore, the lowest rates of $N_2$ fixation were observed in the
root/rhizome material regardless of whether $^{15}$N-$N_2$ label was added directly to the
rhizosphere (0.12 μmol $N_2$ m$^{-2}$ h$^{-1}$) or the overlying water column (7.3 μmol $N_2$ m$^{-2}$ h$^{-1}$).
This suggests that there was active transport of fixed N from the leaves to the
roots of the seagrass plants, in contrast to other studies in which $N_2$ fixation was
more active in the rhizosphere. This study demonstrates the utility of the direct $^{15}$N-$N_2$
tracer approach for quantifying the spatial heterogeneity of $N_2$ fixation in complex
seagrass environments.
3.1 Introduction

Seagrass meadows play an important role in the nitrogen (N) budgets of coastal systems by enhancing denitrification (N loss) (Eyre & McKee 2002, Eyre et al. 2011, 2016a) and dinitrogen fixation (N uptake) (Welsh 2000, Pereg-Gerk et al. 2002, Cook et al. 2015) compared to unvegetated sediments (Isaksen & Finster, 1996, Risgaard-Petersen et al. 1998). With subtropical and tropical seagrass communities fixing more N than temperate seagrasses (Herbert 1999; Welsh 2000). The range of methods used in measuring dinitrogen (N\textsubscript{2}) fixation in seagrass systems has served to make characterisation of “true” N\textsubscript{2} fixation rates difficult (Table 3.1). While the ARA has dominated seagrass N\textsubscript{2} fixation measurements (Table 3.1) the use of \textsuperscript{15}N-N\textsubscript{2} tracers for determining N\textsubscript{2} fixation rates in other ecosystems has increased substantially (e.g. Bombar et al. 2015, Knapp et al. 2016, Newell et al. 2016). The \textsuperscript{15}N-N\textsubscript{2} tracer method, referred to as the dissolution method, involves completely dissolving gaseous \textsuperscript{15}N-N\textsubscript{2} into site water and quantifying the uptake of \textsuperscript{15}N into biological tissue or sediment (Mohr et al. 2010, Grosskopf et al. 2012). This is an improvement on the original \textsuperscript{15}N-N\textsubscript{2} tracer technique known as the bubble-addition method, where a known volume of \textsuperscript{15}N\textsubscript{2} gas was added directly to a water sample containing the biological organism or sediment. The latter technique assumes equilibration of the \textsuperscript{15}N-N\textsubscript{2} in the bubble with the liquid phase following vigorous shaking (Montoya et al. 1996). Use of the dissolution method eliminates the need for the vigorous shaking required by the bubble-addition method, thus making it suitable for use in intact core incubations as natural sediment gradients are preserved and seagrass plants remain relatively undisturbed.
Table 3.1: Rates of dinitrogen fixation measured in (sub)tropical and temperate seagrass systems, where ARA denotes the acetylene reduction assay and $^{15}$N-$\text{N}_2$ as addition of labelled site water. L+E: rates measured on seagrass leaf material with epiphytes; L+E*: epiphytes note removed, reference stated not many were present; L-E: epiphytes removed from leaves prior to incubation; S-RR: sediment minus root/rhizome material; S+RR: sediment with root/rhizome material; R: seagrass root material; Rh: rates measured on seagrass rhizome material; R+R: seagrass root and rhizome material; S: seagrass vegetated sediments; R+Rana: seagrass roots and rhizomes in an anaerobic environment; R+Raero: seagrass roots and rhizome in an aerobic environment; L+Sh: seagrass leaves and shoots in an aerobic environment; (L): light conditions; (D): dark conditions. Note all rates reported as $\mu\text{mol. N m}^{-2}\text{ h}^{-1}$.

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<th>Rate $\text{N}_2$ Fix ((\mu\text{mol N m}^{-2}\text{ h}^{-1}))</th>
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<th>Species, location &amp; season</th>
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<td>0.08</td>
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<td>0.24</td>
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<tr>
<td>L±R</td>
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<td>6.6</td>
<td>ARA&quot;</td>
<td><em>Halodule beaudetti</em></td>
<td>Falmouth Harbour, Jamaica (Winter)</td>
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<tr>
<td>42-167</td>
<td></td>
<td><em>Syringodium isoetifolium &amp; Cymodecea serrulata</em></td>
<td>Gulf of Carpentaria, Australia (Summer)</td>
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<tr>
<td>~122</td>
<td>ARA'</td>
<td><em>Thalassia hemprichii &amp; Cymodecea rotundata</em></td>
<td>Gulf of Carpentaria, Australia (Summer)</td>
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<td>~45</td>
<td>ARA'</td>
<td><em>Zostera capricorni</em></td>
<td>North Stradbroke Island, Australia (Summer)</td>
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<td>61</td>
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<td>8.5</td>
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<td>0.9-35</td>
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<td>Slurry incubations</td>
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<td>~ 760 – 910</td>
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<td>~ 108-225</td>
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<td>22.3</td>
<td>ARA'</td>
<td><em>Thalassia testudinum</em></td>
<td>Biscayne Bay, USA (Summer)</td>
</tr>
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<td>Reference</td>
<td>Location</td>
<td>Species</td>
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<tr>
<td>Capone et al. 1979</td>
<td>Bimini Harbour, Bahamas (Summer)</td>
<td>Thalassia testudinum</td>
<td>15</td>
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<td>McRoy et al. 1973</td>
<td>Florida, USA (Winter)</td>
<td>Thalassia testudinum</td>
<td>16</td>
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<tr>
<td>Patriquin &amp; Knowles, 1972</td>
<td>St Lawrence, Barbados (Autumn)</td>
<td>Thalassia testudinum</td>
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<tr>
<td>Cardini et al. 2018</td>
<td>Gulf of Aqaba, Jordan (Winter – daily average)</td>
<td>Halophila stipulacea</td>
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<td>Cardini et al. 2018</td>
<td>Gulf of Aqaba, Jordan (Spring – daily average)</td>
<td>Halophila stipulacea</td>
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<td>Cardini et al. 2018</td>
<td>Gulf of Aqaba, Jordan (Summer – daily average)</td>
<td>Halophila stipulacea</td>
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<td>Cardini et al. 2018</td>
<td>Gulf of Aqaba, Jordan (Autumn – daily average)</td>
<td>Halophila stipulacea</td>
<td>21</td>
</tr>
</tbody>
</table>
Net: 45 (L)(D)_{L+Sh} 

**ARA** Posidonia oceanica – Mallorca, Spain (Summer & Spring - average) Agawin et al. 2016

Plants:  
**ARA** Halodule uninervis, Cymodocea rotundata, Thalassia hemprichii, Thalassodendron ciliatum – Dar es Salaam coast, Tanzania, (averaged over one year) Hamisi et al. 2009

*H. uninervis:* ~0.150-0.280  
*C. rotundata:* ~ 0.2-0.4  
*T. hemprichii:* ~0.150-0.5  
*T. ciliatum:* 0.1-0.450

All; *§§

**ARA** Enhalus acoroides, Thalassia hemprichii, Halodule uninervis, Syringodium isoetifolium – Papua New Guinea (Spring) Iizumi, 1992

*E. acoroides:* 4 (L)_{L+E}  
*H. uninervis:* 6.9 (L)_{L+E}  
*T. hemprichii:* 3.4 (L)_{L+E}  
*S. isoetifolium:* 4 (L)_{L+E}

900 *§§*  

**ARA** Thalassia testudinum – Redfish Bay, USA, (Summer) Goering and Parker, 1972

Goering and Parker, 1972
<table>
<thead>
<tr>
<th></th>
<th>Core incubations</th>
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<tr>
<td>~18 (L)&lt;sub&gt;S&lt;/sub&gt;</td>
<td>ARA’ Zostera noltii, Bassin d’Arcachon, France (Spring)</td>
<td>Welsh et al. 2000</td>
<td>26</td>
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<tr>
<td>~8 (D)&lt;sub&gt;S&lt;/sub&gt;</td>
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<tr>
<td>~12 (L)&lt;sub&gt;S&lt;/sub&gt;</td>
<td>ARA’ Zostera noltii, Bassin d’Arcachon, France (Autumn)</td>
<td>Welsh et al. 2000</td>
<td>27</td>
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<tr>
<td>~9 (D)&lt;sub&gt;S&lt;/sub&gt;</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>~7 (L)&lt;sub&gt;S&lt;/sub&gt;</td>
<td>ARA’ Zostera noltii, Bassin d’Arcachon, France (Winter)</td>
<td>Welsh et al. 2000</td>
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<tr>
<td>~5 (D)&lt;sub&gt;S&lt;/sub&gt;</td>
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<tr>
<td>~13(L)&lt;sub&gt;S&lt;/sub&gt;</td>
<td>ARA’ Zostera noltii – Bassin d’Arcachon, France (Summer)</td>
<td>Welsh et al. 1996a</td>
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<tr>
<td>~3 (D)&lt;sub&gt;S&lt;/sub&gt;</td>
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<td>~1.7 (L)&lt;sub&gt;S&lt;/sub&gt;</td>
<td>ARA’ Zostera noltii – Bassin d’Arcachon, France (Winter)</td>
<td>Welsh et al. 1996a</td>
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<td>~0.7 (D)&lt;sub&gt;S&lt;/sub&gt;</td>
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Perfusion

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<tr>
<td>15-20 (L)&lt;sub&gt;S&lt;/sub&gt;</td>
<td>ARA’ Zostera marina, Limfjord, Denmark (Summer)</td>
<td>McGlathery et al. 1998</td>
<td>31</td>
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<tr>
<td>7.5-12.5 (D)&lt;sub&gt;S&lt;/sub&gt;</td>
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</tbody>
</table>
5 -12.5 (L)s  | ARA' | *Zostera marina*, Limfjord, Denmark (Winter) | McGlathery et al. 1998 | 32
4 -13 (D)s
8.75 (L)s  | ARA' | *Zostera marina* – Limfjord, Denmark (Summer) | Risgaard-Petersen et al. 1998 | 33
6.25 (D)s
21 (L)s  | ARA' | *Zostera marina* – Limfjord, Denmark (Spring) | Risgaard-Petersen et al. 1998 | 34
12.5 (D)s

<table>
<thead>
<tr>
<th>Slurry incubations</th>
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</table>
| ~20 (L)s  | ARA' | *Zostera muelleri*, Western Port, Australia (Summer) | Russell et al. 2016 | 35
| ~12 (D)s
| ~6 (L)s  | ARA' | *Zostera muelleri*, Western Port, Australia (Winter) | Russell et al. 2016 | 36
| ~7 (D)s
| ~25 (L)s  | ARA' | *Zostera muelleri*, Western Port, Australia (Spring) | Russell et al. 2016 | 37
| ~18 (D)s
| 5-95 (L)s  | ARA' | *Zostera muelleri / nigricaulis* – Port Philip Bay, Australia (Spring) | Cook et al. 2015 | 38
<table>
<thead>
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<th>Range</th>
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<tr>
<td>5-50 (L)</td>
<td>ARA′</td>
<td>Zostera muelleri / nigricaulis – Port Philip Bay, Australia (Summer)</td>
<td>Cook et al. 2015</td>
<td>39</td>
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<tr>
<td>~9 S+RR</td>
<td>ARA ‡‡</td>
<td>Zostera marina – Great South Bay, USA (Summer)</td>
<td>Capone 1982</td>
<td>40</td>
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<tr>
<td>~9 S+RR</td>
<td>ARA ‡‡</td>
<td>Zostera marina – Vaucluse Shores, USA (Summer)</td>
<td>Capone 1982</td>
<td>41</td>
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<td>6-25 (D) L+E</td>
<td>ARA′</td>
<td>Zostera marina – Virginia Coastal Bays, USA</td>
<td>Cole &amp; McGlathery 2012</td>
<td>42</td>
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<td>0.9-1.5 (D)</td>
<td>ARA ‡</td>
<td>Zostera marina – (Summer)</td>
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<td>0.08 (D)</td>
<td>ARA ‡‡</td>
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<td>1.17(D)S-RR</td>
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<td>Zostera marina – Bassin d'Arcachon, France (Summer)</td>
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<td>8.76 (D)</td>
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<td>Spartina maritima – Bassin d'Arcachon, France</td>
<td>Nielsen et al. 2001</td>
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<td>0.37 (D)</td>
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<td>(Summer)</td>
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<tr>
<td>0.87(D)S-RR</td>
<td>ARA ‡‡</td>
<td>(Summer)</td>
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</table>
- No light/dark information given

* – Theoretical ratio 3:1 used to convert rates of acetylene reduction to N₂ fixation

** – Theoretical ratio of conversion to N₂ fixation not specified

§ – Theoretical ratio of 3:1.9 used to convert rates of acetylene reduction to N₂ fixation

¶ – Theoretical ratio of 1.5:1 used to convert rates of acetylene reduction to N₂ fixation

¶¶ – Theoretical ratio of 4:1 used to convert rates of acetylene reduction to N₂ fixation

§§ – Theoretical ratio of 2.6:1 used to convert rates of acetylene reduction to N₂ fixation

∞ – Rates measured in vials/flasks separately

β – Rates measured in small cores

β – Whole plants incubated in an outdoor aquarium sans sediment
The premise of the ARA is that the reduction of acetylene to ethylene by nitrogenase can be used as a proxy for the reduction of N\textsubscript{2} to ammonium (NH\textsubscript{4}\textsuperscript{+}) (i.e. N\textsubscript{2} fixation) (Stewart et al. 1967, Hardy et al. 1968). While the ARA is cheap and simple to perform, the indirect nature of the technique means it suffers from a range of methodological issues. Predominantly the use of a theoretical ratio, typically calculated as 3 moles of acetylene reduced per mole of N\textsubscript{2} fixed, which is used to calculate N\textsubscript{2} fixation from the reduction of acetylene to ethylene (Stewart et al. 1967, Hardy et al. 1968). The reliability of the 3:1 ratio is the major source of error in the ARA. Previous research in seagrass ecosystems have used direct methods of N\textsubscript{2} fixation, i.e. based on $^{15}$N-N\textsubscript{2} incorporation into the biomass to validate the 3:1 ratio (Patriquin & Knowles 1972, Capone & Budin 1982, O’Donohue et al. 1991a,). However other studies in marine sediments have found large variability in this ratio (0.5 -15.4:1; Welsh 2000, 10-100:1; Seitzinger & Garber 1987). In addition to the variability in the ratio used to calculate N\textsubscript{2} fixation, acetylene can inhibit some N\textsubscript{2} fixing bacteria (Payne 1984, Welsh 2000) and ethylene may be consumed or produced by others (Wynn-Williams & Rhodes 1974, David & Fay 1977). The only non-ARA measurement of N\textsubscript{2} fixation in a seagrass core incubations used a lacunal $^{15}$N-N\textsubscript{2} technique (i.e. O’Donohue et al.1991a; Table 3.1). The use of the direct $^{15}$N-N\textsubscript{2} dissolution method removes many of the uncertainties inherent in the calculation of N\textsubscript{2} fixation rates measured by the ARA.

Methodological issues aside, due to the spatial heterogeneity of seagrass environments, only a few studies have investigated rates of N\textsubscript{2} fixation in discrete loci including leaves, roots/rhizomes and sediments (Table 1; 5,6,7,12 and 13). O’Donohue et al. (1991a) and Iizumi (1992) found the highest N\textsubscript{2} fixation rates associated with the leaves with the lowest rates in the root/rhizome, whereas Moriarty and O’Donohue (1993) found a mixture of rates over their two sites and
Dinitrogen fixation rates in a subtropical seagrass meadow measured with a direct $^{15}$N-$N_2$ tracer method multiple seagrass species. The rhizosphere has long been identified as one of the active $N_2$ fixing zones of seagrasses (Capone & Budin 1982, Welsh et al. 1996a, b, McGlathery et al. 1998) however, it has been particularly difficult to study rhizosphere $N_2$ fixation without disturbing surrounding sediments. In an effort to overcome this, various perfusion core techniques have been developed (Risgaard-Petersen & Jensen 1997, Sheibley et al. 2003, Hardison et al. 2011). Sediment perfusion typically involves collecting intact sediment cores containing seagrass plants with the reagent (commonly acetylene) added to the sediment around the root zone, often via ports located down the length of the core (Moriarty & O'Donohue 1993, Blackburn et al. 1994, Risgaard-Petersen et al. 1998). One study combined the use of $^{15}$N-$N_2$ saturated seawater and a ‘lacunal diffusion’ perfusion technique whereby labelled solution was added to intact sediment seagrass cores and transported into the sediment via the plants own lacunae (O'Donohue et al. 1991a). To date no studies have combined the $^{15}$N-$N_2$ dissolution method and sediment perfusion, where $^{15}$N-$N_2$ saturated seawater is added to the rhizosphere via ports located down the length of the core. Consequently, no study has applied the $^{15}$N-$N_2$ dissolution method to quantify $N_2$ fixation in both the surface material and the rhizosphere.

In this study we apply the $^{15}$N-$N_2$ dissolution method to measure $N_2$ fixation rates within intact cores collected from a subtropical seagrass meadow ($Zostera muelleri$). Dinitrogen fixation was measured in discrete loci by adding $^{15}$N-$N_2$ labelled site water to both the sediment and the water column.

3.2 Method

3.2.1 $^{15}N_2$ labelled gas

A previous study identified major $^{15}$N contamination of commercially available $^{15}$N-$N_2$ gas with $^{15}$N labelled ammonia ($^{15}$N-$NH_3$, measured as $^{15}$N-$NH_4^+$), uptake of
3 - Dinitrogen fixation rates in a subtropical seagrass meadow measured with a direct $^{15}\text{N}-\text{N}_2$ tracer method which can artificially enhance calculated $\text{N}_2$ fixation rates (Dabundo et al 2014). To eliminate the possibility of contamination a preliminary trial was undertaken to check for $^{15}\text{N}-\text{NH}_4^+$ contamination of the gaseous $^{15}\text{N}$-$\text{N}_2$. Labelled gas was purchased from Cambridge Isotope Laboratories (>98% lot no. I-19168A). Dabundo et al. (2014) found this to be the least contaminated commercial $^{15}\text{N}$-$\text{N}_2$ available (~0.014-0.052 μmol $^{15}\text{N}$-$\text{NH}_4^+$). To confirm the gas was free from contamination, aliquots of gas were equilibrated with water samples containing $^{15}\text{N}$-$\text{NH}_4^+$ with a known $\delta^{15}\text{N}$ signature (ammonium sulphate salt IAEA N-1 $\delta^{15}\text{N} +0.43 \%\text{airN}_2 \pm 0.2$ SD). Assays were undertaken in triplicate based on the method outlined in Dabundo et al. (2014). Briefly 60 μL of 1000 μM IAEA N-1 stock standard was added to 12 mL of high purity water (to give a final concentration of 5 μM) and placed in 20 mL crimp top vials. Two mL of $^{15}\text{N}$-$\text{N}_2$ gas was added to each sealed vial and shaken overnight. Once equilibrated three 2.5 mL aliquots of equilibrated solution from each vial were analysed for $^{15}\text{N}$-$\text{NH}_4^+$ (see below). Control samples, which contained IAEA N-1 standard without labelled gas, were also analysed for $^{15}\text{N}$-$\text{NH}_4^+$. Additionally, another batch of $^{15}\text{N}$-$\text{N}_2$ gas (Sigma lot no. MBBB0968V, of the same lot measured by Dabundo et al. 2014) was measured as per the Cambridge $^{15}\text{N}$-$\text{N}_2$ gas.

3.2.2 Core collection and maintenance

Seagrass cores were collected at low tide from a *Zostera muelleri* seagrass stand on the western edge of Shaws Bay, Ballina, located on the north coast of NSW Australia (28° 51' 55.09" S, 153° 34' 58.09" E). Shaws Bay is an artificial estuarine embayment located ~700 m from the mouth of the Richmond River Estuary (Ballina Shire Council, 2015) (for details on the Richmond River Estuary and Catchment see Eyre 1997, McKee & Eyre 2000). Tidal exchange with the greater Richmond River Estuary occurs through the porous wall along the southern edge of the bay, with the depth of the bay ranging from ~1.4 - 7.0 m relative to mean sea level (Ballina Shire
Dinitrogen fixation rates in a subtropical seagrass meadow measured with a direct $^{15}$N-$N_2$ tracer method (Council, 2015). Sediment in the bay ranges from marine sands (of ~ 0.35 mm grain size) along the margins of the bay decreasing in size to silt/mud toward the centre (Ballina Shire Council, 2000). Shaws Bay sediments had a C:N molar ratio of 20.1 (0.15% C; 0.008%N) (Salk et al. 2017).

Cores (18) were collected at low tide during summer, November 2015 via acrylic tubes (46 cm long, 9 cm internal diameter). Cores were inserted into the sediment to a depth of ~15 cm to include the whole seagrass community (i.e. a single seagrass plant, epiphytes, sediment, overlying water and benthic microalgae). Cores were removed via gentle vertical extraction and capped with overlying water for transport. Pore water was collected via a push-pull piezometer and peristaltic pump from 15 cm below the sediment surface and pumped into helium (He) pre-purged sealed 2 L Schott bottles to avoid oxygenation. Temperature and dissolved oxygen (DO) were measured via Hach HQ40D DO meter at the site. Site water was collected (~250 L) in order to fill incubation chambers in the laboratory.

3.2.3 Core incubations

Cores were brought back to the laboratory at Southern Cross University, placed uncapped in incubation chambers and covered with site water. Nine cores which had pre-drilled ports at ~2 cm intervals down the length of the sediment column were selected for rhizosphere label additions (henceforth referred to as perfusion cores). The remaining 9 cores without ports were used as surface cores (where label solution was added to the water column only). Chillers maintained the incubation water at in situ temperature (25° C) and aquarium airstones ensured adequate oxygenation. Magnetic stirrers were fitted to cores at ~10 cm above the sediment surface. Cores were pre-incubated in the dark overnight (Ferguson et al. 2003, 2004).
3 - Dinitrogen fixation rates in a subtropical seagrass meadow measured with a direct $^{15}$N-$\text{N}_2$ tracer method

To generate the $^{15}$N-$\text{N}_2$ labelled solution aliquots of surface and pore water were transferred into 500 mL serum bottles, sealed and degassed under vacuum for 60 minutes. Fifty mL of $^{15}$N-$\text{N}_2$ (Cambridge Isotope Laboratories > 98%) was injected into each serum bottle (over pressurised) and allowed to equilibrate overnight on a shaker table (Klawonn et al. 2015).

3.2.4 $\text{N}_2$ fixation assay - surface core incubation

Following the pre-incubation period high pressure sodium lamps were turned on ~4 hours prior to label addition (~34 PAR $\mu$mol m$^{-2}$ s$^{-1}$) (i.e. only light rates were measured). Cores were sealed and initial samples collected through valves in the lids for DO (which was again measured upon sacrifice), $\delta^{15}$N-$\text{N}_2$ and the $\delta^{15}$N of total dissolved nitrogen (TDN) in the overlying water. For $\delta^{15}$N-$\text{N}_2$ determination, triplicate water samples were collected in 12 mL Exetainers treated with 20 $\mu$L of saturated mercuric chloride and capped without headspace. Samples for $\delta^{15}$N-TDN (~10 mL) were filtered through 0.45 $\mu$m syringe filters (Sartorius) into plastic vials and frozen. Overlying water from cores removed for sampling was immediately replaced with syringes of site water attached to inflow taps in the lid of the cores as samples were removed from outflow taps.

Prior to label addition, three cores were sacrificed in order to determine the background $\delta^{15}$N and N content of organic material and sediment. Water was carefully decanted from cores and samples were collected and stored for analysis of bulk $\delta^{15}$N; the top 5 cm of sediment was collected via duplicate sediment sub-cores taken in modified 50 mL plastic syringes. Additional sediment depths were collected to be used for calculation of perfusion sediment $\text{N}_2$ fixation detailed below. Sediment collected via sub-cores was separated into 1 cm fractions and frozen prior to being freeze-dried and analysed as described below. Seagrass leaves were cut at the sediment surface and root/rhizome material was isolated from the surrounding...
Dinitrogen fixation rates in a subtropical seagrass meadow measured with a direct $^{15}$N-$N_2$ tracer method

sediment by removal and rinsing of any remaining attached sediment with ultra-pure water. Care was taken when separating out the root/rhizome matter, identifying the material collected as being associated with that of the main seagrass plant and not that of neighbouring plants incorporated during the coring process. Once initial cores were sacrificed, 100 mL of the equilibrated $^{15}$N-$N_2$ labelled site water was injected into each of the remaining six cores. After a 30 minute mixing time cores were again sampled for $\delta^{15}$N-$N_2$ as described above. The incubation was carried out over a 10 hour time period with cores sacrificed in triplicate at 5 and 10 hours after tracer addition.

3.2.5 $N_2$ fixation assay - perfusion core incubations

Pre-incubation of the perfusion cores was as described for surface cores. For tracer addition to the sediments 1 L of the labelled pore water solution was added to 1 L of unlabelled pore water that had been purged with He to remove oxygen. Lithium chloride (LiCl at $\sim$2.05 mg L$^{-1}$) was added to the mixture to act as a conservative tracer. Cores were perfused with the $^{15}$N-$N_2$ + Li$^+$ labelled pore water via 9 cm long x 4.5 mm diameter Macro Rhizon samplers that were inserted into pre-drilled holes at 2 cm intervals along the sediment section of the core (Erler et al. 2014). Beginning with the lowest Macro Rhizon a peristaltic pump was used to introduce 40 mL of tracer solution ($\sim$20 mL minute$^{-1}$) into the sediments. Tracer was sequentially added to each of the remaining Macro Rhizons. The volume of labelled pore water solution added was calculated based on previously measured sediment porosity and was intentionally overestimated in order to saturate the rhizosphere.

Cores were capped, incubated in the light (as above) and sacrificed in triplicate after 5 and 10 hours. Samples of the overlying core water and leaves were collected as for surface cores. Additional samples for Li$^+$ concentration were taken from the overlying water of the perfusion cores in order to ascertain if any labelled...
Dinitrogen fixation rates in a subtropical seagrass meadow measured with a direct $^{15}$N-$\text{N}_2$ tracer method solution had diffused from the sediment into the water column. All $\text{Li}^+$ samples were filtered (0.45 µm Sartorius syringe filter) and frozen for later acidification with ~10% nitric acid. Sediment was sectioned into eight horizontal depth fractions (0-1cm, 1-2cm, 2-3cm, 3-4cm, 4-5cm, 5-6cm, 6-9cm and 9-15cm), added to 50-100 mL of 2 M potassium chloride (KCl) to desorb NH$_4^+$ (which could otherwise distort the bulk sediment N estimate), agitated and left overnight. Sediment + KCl were centrifuged at 1509 g for 5 minutes. The supernatant was filtered (0.45 µm Sartorius syringe filter) and collected for TDN concentration, $\delta^{15}$N-TDN and $\text{Li}^+$ concentration analysis. Sediments remaining after KCl extraction were rinsed with ultra-pure water and centrifuged to remove excess salts.

3.2.6 Sample analysis

Sediment, leaf and root/rhizome material were freeze-dried and all sediment fractions were sieved through a 500 µm wire mesh in order to remove any leaf, root/rhizome, or large organic material. All samples were ground in a ring mill prior to being weighed into tin capsules (triplicate samples of ~80 mg sediment, ~8 mg leaves, ~5 mg root/rhizomes) for $\delta^{15}$N analysis. Analysis of the $\delta^{15}$N content of sediment and seagrass material was carried out via elemental analysis (Thermo Finnigan Flash EA 1112) in concert with an IRMS (Thermo Conflo III and a Thermo Delta V Plus) (precision ± 0.3 ‰) (Eyre et al. 2016b).

$\delta^{15}$N-$\text{NH}_4^+$ from the initial tests for gas contamination was measured via oxidation to NO$_2^-$ and subsequent reduction to N$_2$O as per Zhang et al. (2007). The N$_2$O in these samples was purified in liquid N$_2$ with a custom-built purge and trap system. The N isotopic signature of the N$_2$O was determined via a Thermo Delta V Plus IRMS coupled to a continuous flow purge and trap system via a Thermo Fisher GasBench II (analytical precision for this method ± 0.5 ‰).
3 - Dinitrogen fixation rates in a subtropical seagrass meadow measured with a direct $^{15}$N-$\text{N}_2$ tracer method

Samples requiring $\delta^{15}$N-$\text{N}_2$ analysis were headspaced with 2 mL He. Headspace samples (10 μL) were analysed for $\delta^{15}$N-$\text{N}_2$ on a Thermo Trace gas chromatograph (GC) Ultra with a 25 m x 0.32 mm PoraPLOT Q column interfaced to a Thermo Delta V Plus IRMS (precision ± 0.15 ‰).

Samples for Li$^+$ concentration were analysed via inductively coupled plasma – mass spectrometry (precision ± 0.009 mg L$^{-1}$) (Erler et al. 2010). A Lachat QuickChem 8000 four channel Flow Injection Analyser was used to measure NH$_4^+$ concentration from sediments desorbed with KCl and TDN concentration (following persulphate oxidation) (Eyre 2000). Determination of $\delta^{15}$N-TDN followed the protocol detailed in Knapp et al. (2005). Briefly, persulphate oxidation of TDN to NO$_3^-$ was followed by conversion of NO$_3^-$ to N$_2$O via the denitrifier method (precision ± 0.2 ‰) (Sigman et al. 2001) and measured via GC-IRMS as described above.

3.2.7 $\text{N}_2$ fixation rate calculation

Rates of $\text{N}_2$ fixation (in units of μmol $\text{N}_2$ m$^{-2}$ h$^{-1}$) were calculated using the following equation (based on Wilson et al. 2012);

$$V(t^{-1}) = \frac{[(\text{AP}_{\text{PN-Final}}) - \text{AP}_{\text{PN-Initial}}]}{(\text{AP}_{\text{N}_2} - \text{AP}_{\text{PN-Initial}}))} \times \frac{1}{\Delta t} \times \frac{\text{PN}_{\text{Final}}}{2} \quad [1]$$

Where $\text{AP}_{\text{PN-Final}}$ is the $^{15}$N atom% of the organic N pools (i.e. bulk sediment, leaves and roots) following incubation; $\text{AP}_{\text{PN-Initial}}$ denotes the $^{15}$N atom% of the background N pool in control samples prior to addition of $^{15}$N-$\text{N}_2$. The $\text{AP}_{\text{N}_2}$ term represents the $^{15}$N atom% of the enriched $\text{N}_2$ pool half an hour post addition of $^{15}$N-$\text{N}_2$ labelled site water. The $^{15}$N content of each locus, post incubation, in μmol is designated by the $\text{PN}_{\text{Final}}$ term; $\Delta t$ is the incubation time. Finally, the division by two is to convert the subsequent value from gram- atoms of N to moles of $\text{N}_2$ as detailed in Montoya et al. (1996). The accumulation of $^{15}$N in any particular pool is deemed to be equivalent to the rate of $\text{N}_2$ fixation in that pool. Alternatively $^{15}$N-NH$_4^+$ produced from $\text{N}_2$ fixation could be incorporated into the organic N pool. We would argue that in the

Unraveling the complexity of subtropical seagrass nitrogen cycling: a stable isotope approach
3 - Dinitrogen fixation rates in a subtropical seagrass meadow measured with a direct $^{15}$N-$\text{N}_2$ tracer method short incubation time, $^{15}$N appearing in a locus is most likely to have been fixed there. This was supported by the analysis of $^{15}$N-TDN, which showed minimal amounts of $^{15}$N appearing in the TDN pool (see results below). We would have expected this to be higher if fixed N was being metabolised and transported through the system.

Total N$_2$ fixation was calculated as the sum of the fixed N accumulated in the different loci across both core types as in:

$$\text{Total N fixed} = P_s + P_r + P_l + S_s + S_r + S_l$$

Where $P_s$, $P_r$ and $P_l$ represent N$_2$ fixation rates (in μmol N$_2$ m$^{-2}$ h$^{-1}$) calculated in the perfusion core sediment, root/rhizome and leaf (including epiphytes) fractions. The surface sediment, root/rhizome and leaf (including epiphytes) associated N$_2$ fixation rate (in μmol N$_2$ m$^{-2}$ h$^{-1}$) are represented by $S_s$, $S_r$ and $S_l$ respectively. All rates are reported as mean ± SD. Analysis of variance was used to determine if sediment N$_2$ fixation rates varied significantly over depth in both surface and perfusion cores (p < 0.05).

3.3 Results

The pure $^{15}$N-N$_2$ gas was found to be free from contamination with $^{15}$N-NH$_4^+$ (Table S.1, Appendix 4). As such, the rates of N$_2$ fixation presented in this study are based on true N$_2$ fixation. Concentration of $^{15}$N contaminants within the $^{15}$N-N$_2$ gas were not measured as per Dabundo et al. (2014). Instead, $\delta^{15}$N ‰ values and peak area were compared between the Cambridge and Sigma $^{15}$N-N$_2$ gas batches and the IAEA N-1 standard. Results of this contamination assessment are given in the supplementary material (Table S.1, Appendix 4).

The Li$^+$ tracer used in the perfusion cores was distributed homogenously throughout the sectioned rhizosphere. A one-way analysis of variance (ANOVA) of Li$^+$ concentrations between sediment sections showed they were not significantly different ($F_{7,44} = 2.15, p < 0.05$). Concentrations of Li$^+$ measured in each sediment
Dinitrogen fixation rates in a subtropical seagrass meadow measured with a direct $^{15}$N-$\text{N}_2$ tracer method fraction are presented in the supplementary material (Table S.2, Appendix 4). The amount of $^{15}$N recovered in the TDN pool of the water column of the surface cores was minor ($\sim0.0088 \pm 0.0015 \text{ μmol N}_2 \text{ m}^{-2} \text{ h}^{-1}$) representing $\sim0.003$ % of the total recovered $^{15}$N in the surface cores. The Li$^+$ concentration in the overlying water increased from $\sim0.16 \pm 0.0032 \text{ mg L}^{-1}$ prior to perfusion to $\sim0.18 \pm 0.011 \text{ mg L}^{-1}$ in the 10 hour incubated perfusion cores. This change in concentration represents only 1.5% of the volume of the water column overlying the perfused sediment and increased the background $\delta^{15}$N-$\text{N}_2$ to 75‰ (assuming 2.1 L of overlying water which was the average across all cores).

Background $\delta^{15}$N or atom percent measurements of all loci, including the water column are presented in the supplementary materials (Tables S.3 and S.4, Appendix 4). The total rate of $\text{N}_2$ fixation of the *Zostera muelleri* community was $\sim38 \pm 28 \text{ μmol N}_2 \text{ m}^{-2} \text{ h}^{-1}$ calculated as the sum of averages across all loci in both core types (i.e. equation [2]). Surface core and perfusion $\text{N}_2$ fixation were

![Figure 3.1](image)

**Figure 3.1** Total rates of dinitrogen fixation (mean ± SD, n=3) across all seagrass loci (leaves and epiphytes, sediment and root/rhizome material) in surface and perfusion cores over 5 and 10 hour incubation periods.
Dinitrogen fixation rates in a subtropical seagrass meadow measured with a direct $^{15}$N-$N_2$ tracer method

$34 \pm 18 \ \mu$mol N$_2$ m$^{-2}$ h$^{-1}$ and $4.8 \pm 5.7 \ \mu$mol N$_2$ m$^{-2}$ h$^{-1}$ respectively for the 5 hour incubation and $36 \pm 7.2 \ \mu$mol N$_2$ m$^{-2}$ h$^{-1}$ and $2.5 \pm 4.7 \ \mu$mol N$_2$ m$^{-2}$ h$^{-1}$ for the 10 hour incubation (Fig. 3.1).

Dinitrogen fixation rates observed across all loci were greater in the surface incubations as compared with the perfusion incubations (Fig. 3.2). The leaf material, which includes epiphytes, exhibited the highest N$_2$ fixation rates in the surface incubations ($18 \pm 25 \ \mu$mol N$_2$ m$^{-2}$ h$^{-1}$) (Fig. 3.2). While in perfusion cores the sediment was the area of highest activity ($3.3 \pm 2.1 \ \mu$mol N$_2$ m$^{-2}$ h$^{-1}$) (Fig. 3.2). There was a much higher accumulation of fixed N in the root/rhizome fraction of the surface cores ($7.3 \pm 8.3 \ \mu$mol N$_2$ m$^{-2}$ h$^{-1}$) than in the root/rhizome fraction of the perfusion cores ($0.12 \pm 0.16 \ \mu$mol N$_2$ m$^{-2}$ h$^{-1}$) (Fig. 3.2).

The rate of N$_2$ fixed in the top 5 cm of sediments was higher when label was added to the water column compared with the equivalent depth perfusion sediments (Fig. 3.3). In fact, on average, the N$_2$ fixation rates of the top five fractions (1 cm each) of the surface cores (corresponding to a depth of 1-5 cm of sediment) were higher ($\sim 9.9 \pm 6.6 \ \mu$mol N$_2$ m$^{-2}$ h$^{-1}$) than the average of the total perfusion profile ($\sim 3.3 \pm 2.1 \ \mu$mol N$_2$ m$^{-2}$ h$^{-1}$). A decrease in the rate of fixation with core depth was observed over the top 5cm of the surface cores ($F_{(4,55)}=6.3$, $p > 0.05$), this was not observed in the perfusion sediment ($F_{(7,42)} = 1.01$, $p < 0.05$; Fig. 3.3).

Background $\delta^{15}$N or atom percent measurements of all loci, including the water column are presented in Tables S.3 and S.4 in Appendix 4.

3.4 Discussion

3.4.1 N$_2$ fixation within the seagrass meadow: aboveground

By analysing N$_2$ fixation rates in discrete locations of a seagrass community we have been able to quantify the most important zones of N$_2$ fixing activity. Such
Dinitrogen fixation rates in a subtropical seagrass meadow measured with a direct \(^{15}\)N-N\(_2\) tracer method quantification has been undertaken previously largely using the ARA, with \(N_2\) fixation estimates ranging from <1 to 61 \(\mu\)mol N m\(^{-2}\) h\(^{-1}\) for leaf material, and <1 to 8.5 \(\mu\)mol N m\(^{-2}\) h\(^{-1}\) in root/rhizomes (Table 3.1; 5, 6, 7, 12, 27), which bracket the rates we have presented here (Table 3.1; 1, 8). More recently, sections of leaves and roots have been incubated in isotopically labelled solutions (Lehnen et al. 2016), with the highest rates measured in the root material (2.9 \(\mu\)mol N g\(^{-1}\) d\(^{-1}\) – dry weight).

In both surface and perfusion cores, the highest \(N_2\) fixation rates were observed in the loci initially in direct contact with the labelled solution (i.e. leaf material in the surface incubations and sediment in the perfusion incubations) (Fig. 3.2). Root/rhizome material displayed the lowest \(N_2\) fixation rates across both core types (Fig. 3.2).

In the surface addition cores, \(N_2\) fixation was likely driven by epiphytes colonising the leaves. As we did not compare leaves scraped of epiphytes with un-scraped samples it is unclear if N released by epiphytes was incorporated into the leaf tissue or if what we measured was fixed N incorporated into the epiphytes themselves. In studies that have compared leaves cleaned of epiphytes no \(N_2\) fixing activity was observed (Goering & Parker, 1972). If epiphytes were excreting fixed N to the leaves we would expect to see some evidence of this \(^{15}\)N (in the form of TDN) lost to the water column. Given that \(\delta^{15}\)N-TDN in the water column remained unchanged during the incubation (Fig. 3.2), coupled with the short time frame of the experiment, it seems unlikely that epiphytes were secreting \(^{15}\)N for assimilation by the seagrass leaves. Thus, \(^{15}\)N measured in surface incubation leaf material was likely that incorporated within epiphytes.

Leaf material \(N_2\) fixation was much lower in the perfusion cores (Fig. 3.2). This is not surprising as no \(^{15}\)N-N\(_2\) was added to the water column of the perfusion cores. There was a small increase in the concentration of Li\(^+\) tracer in the water column of
Dinitrogen fixation rates in a subtropical seagrass meadow measured with a direct $^{15}$N-$N_2$ tracer method

the perfusion cores which suggests there was some leakage of labelled pore water to the overlying water. The low rates of leaf fixation in the perfusion cores is likely due to a small amount of $^{15}$N-$N_2$ in the labelled pore water solution diffusing into the overlying water and being fixed by leaf-associated epiphytes. Alternatively, labelled products from $^{15}$N-$N_2$ fixed in the rhizosphere may have been transported to the leaves. O'Donohue et al. (1991a) suggested that the Zostera capricorni meadow in their study fixed N largely in the perfused rhizosphere which was subsequently transported to the leaves over a 6 hour incubation. Fixed N may have been directed toward more metabolically active regions of the plant (i.e. younger leaves) rather than the established root/rhizome material. O'Donohue et al. (1991a) reported the highest $N_2$ fixation rate in the leaves of their perfused cores (Table 3.1). Due to the lacunal diffusion method (whereby labelled solution is added to the intact core and transported into the sediment via the plants own lacunae) used by O'Donohue et al. (1991a) the leaves of the seagrass were in contact with $^{15}$N-$N_2$ labelled solution (i.e. more similar to our surface incubations). Therefore epiphytic $N_2$ fixation may have contributed to the estimates reported by O'Donohue et al (1991a), even though minimal epiphytic colonisation of their seagrass leaves was observed.

Although we did not quantify epiphyte density, colonies were clearly present on leaves. The dominance of epiphytic $N_2$ fixation in the surface incubations may explain the reduced total $N_2$ fixation observed in the perfusion cores (Fig. 3.2).

As leaves in the perfusion incubations were not exposed directly to the $^{15}$N-$N_2$ labelled solution there was much reduced leaf epiphytic incorporation of fixed $^{15}$N and thus lower detection of the tracer in other areas of the plant (such as the root/rhizome) which may suggest leaf epiphytic fixation is the dominant source of
**Figure 3.2** Dinitrogen fixation rates measured in discrete loci (as mean ± SD μmol N₂ m⁻² h⁻¹) when ¹⁵N-N₂ labelled site water added to either the water column (a.) or perfused directly into the sediment (b.). Arrows illustrate suggested direction of movement of labelled products around the system. Where S₁, S₂ and S₃ represent surface core fixation in the leaves (including epiphytes), sediment and root/rhizome, and P₁, P₂ and P₃ the N₂ fixation in these loci in the perfusion cores. Seagrass vector image from Diana Kleine ian.ucmes.edu/imagelibrary.
3 - Dinitrogen fixation rates in a subtropical seagrass meadow measured with a direct $^{15}$N-$N_2$ tracer method

Importantly, we have found that the rates of $N_2$ fixation were much higher above the sediments, when $^{15}$N-$N_2$ was added to the overlying water, than below (Fig. 3.2). This contrasts with past studies that show a large proportion of $N_2$ fixation occurs in the rhizosphere (Welsh et al. 1996a, Hansen et al. 2000, Nielsen et al. 2001) followed by mobilisation of fixed N to the leaves to aid in photosynthesis (O’Donohue et al. 1991a). Relatively low recovery of $^{15}$N in root/rhizome material was therefore unexpected in the perfusion cores where $^{15}$N-$N_2$ was added directly to the rhizosphere.

### 3.4.2 $N_2$ fixation within the seagrass meadow: below ground

The Li$^+$ tracer showed that $^{15}$N-$N_2$ label was distributed evenly throughout the sediment layers of the perfusion cores. The large $NH_4^+$ pool already present in the pore water and sorbed onto sediments ($\sim 65 – 1100$ μmol L$^{-1}$ Table S.5) may have limited $N_2$ fixation. Welsh et al. (1997) observed 40-60% inhibition of nitrogenase in a

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**Figure 3.3** Dinitrogen fixation (mean ± SD, n=3) over sediment depths in both surface and perfusion cores

Unraveling the complexity of subtropical seagrass nitrogen cycling: a stable isotope approach
Dinitrogen fixation rates in a subtropical seagrass meadow measured with a direct $^{15}$N-N$_2$ tracer method

*Zostera noltii* meadow at NH$_4^+$ concentrations of between 5-10 μM, but that even after additions of up to 1 mM 30% activity persisted. McGlathery et al. (1998) also found that even concentrations of up to 650 μM pore water NH$_4^+$ did not have a negative effect on N$_2$ fixation rates. Any potential inhibition of nitrogenase activity may be further exacerbated by the large volume of labelled pore water solution added to perfusion cores. This could disrupt natural gradients and cause transport of NH$_4^+$ into localised areas of low NH$_4^+$ concentration surrounding the roots (Welsh, 2000). This inundation of NH$_4^+$ into discrete N$_2$ fixing zones around the roots of the seagrass may have inhibited the action of N$_2$ fixing bacteria, by either reversible inhibition of nitrogenase (Hartmann et al. 1986, Fu & Burris 1989), or by reducing the flow of electrons to nitrogenase and acting as a decoupler (Laane et al. 1980).

Disturbance to natural sedimentary gradients was absent in the surface cores, which may be why rates in those sediments were higher than the perfusion sediments (Fig. 3.2). Thus, reduced N$_2$ fixation rates in the sediment and root/rhizome material of perfusion cores is likely due to a combination of destruction of micro-zones of N$_2$ fixation and high NH$_4^+$ concentration.

This potential destruction of micro-zones of low NH$_4^+$ concentration may also explain the lack of N$_2$ fixation in the root/rhizomes of the perfusion cores as compared with that in the surface cores (Fig. 3.2). A lack of N$_2$ fixation in the sediment may mean that labelled products are not supplied to the root/rhizome. Alternatively, the addition of $^{15}$N-N$_2$ to the water column in the surface cores rather than the rhizosphere could suggest that $^{15}$N observed in surface core root/rhizomes is a result of $^{15}$N-N$_2$ being transported through the internal lacunae from the overlying water to the roots. The lack of additional $^{15}$N-N$_2$ in the water column of the perfusion...
Dinitrogen fixation rates in a subtropical seagrass meadow measured with a direct $^{15}$N-$N_2$ tracer method

cores would mean this pathway would be much reduced, resulting in the lower rates we observed (Fig. 3.2).

Sediment $N_2$ fixation rates in the 1-5 cm samples of the surface cores were found to be elevated, relative to the perfusion cores, and decreased with depth (Fig. 3.3). Unexpectedly these rates were still higher than the total sediment fractions of the perfusion cores (Fig. 3.3), even though any $^{15}$N would have had to come from labelled solution added to the water column rather than that perfused directly into the sediment. Superficial sediments may have picked up some of this labelled solution via diffusion allowing $^{15}$N to be fixed by various N$_2$-fixing bacteria, while at greater depths uptake is likely due to the transport of gases through the plant itself (Fig. 3.2). The decrease in N$_2$ fixation seen in the surface core 1-5 cm fractions, seems to show diffusion from the labelled overlying water (Fig. 3.3). A system of gas-filled lacunae transport gases such as carbon dioxide and oxygen throughout the plant which are subsequently released at the roots into the surrounding sediment (O'Donohue et al. 1991b). Thus, labelled and unlabelled N$_2$ not fixed by epiphytes may be transported through the plant and out into the sediment where it is fixed by sulphate reducing bacteria (SRB). Nielsen et al. (2001) found increased N$_2$ fixation rates on the root/rhizome material of the seagrasses *Z. noltii* and *Spartina maritima* compared to the surrounding unvegetated sediment, which was positively related to the amount of SRB in these locations. Dinitrogen fixation appears to be minimal at the root/rhizome of this *Z. muelleri* bed compared to leaf and sediment rates (Fig. 3.2). This may be due to a lack of SRB populating these structures or the large pool of NH$_4^+$ present in the sediment.

We propose that the disturbance of natural sedimentary gradients coupled with the high pore water NH$_4^+$ concentration could explain why N$_2$ fixation rates in the
rhizosphere were less than sediments in surface cores (Fig. 3.2). There are clearly some technical constraints in the application of perfusion techniques to core incubations containing seagrass plants whether it be injecting label solution into the sediment via ports or using a ‘lacunal diffusion’ approach as in O’Donohue et al (1991a). Quantifying the N₂ fixation processes accurately in the rhizosphere while maintaining natural gradients remains challenging.

3.4.3 Comparison with previous N₂ fixation rates

The N₂ fixation rates presented here via the addition and recovery of ¹⁵N-N₂, are within the range of previously reported rates in seagrass communities (Table 3.1). Although we did not directly compare the ¹⁵N-N₂ dissolution method and the ARA in this Z. muelleri meadow, methodological differences between the techniques have potential to effect rate estimates. The function of N₂ fixing bacteria (such as SRB) can be inhibited by acetylene (Payne 1984, Welsh 2000, Fulweiler et al. 2015). Inhibition of SRB can have a significant impact on N₂ fixation rates in the seagrass rhizosphere (Capone 1982, Isaksen & Finster 1996, Welsh et al. 1996b); SRB have been found to constitute up to 78% of total N₂ fixation in a seagrass system (Nielsen et al. 2001). Thus, any inhibition of SRB could cause major underestimation of N₂ fixation. Furthermore, the product of the ARA, ethylene, may be either consumed or produced by various microbial communities (Welsh, 2000) which may lead to artificially heightened or diminished rates. Additionally, the variability in the ratio used to calculate N₂ fixation from acetylene reduction (Seitzinger & Garber 1987, Welsh 2000) may have an effect on the difference between N₂ fixation rates measured via ¹⁵N-N₂ dissolution and the ARA.

Another possible reason for variability in previous N₂ fixation measurements may stem from the practice of incubating individual components of the seagrass
Dinitrogen fixation rates in a subtropical seagrass meadow measured with a direct $^{15}$N-$\text{N}_2$ tracer method

system (i.e. roots, leaves, sediment) separately (Table 3.1; 25, 42, 43 and 44). Often this is accompanied by severe disturbance of natural sediment redox gradients through sediment slurrification which modifies the uptake of isotope tracers (Riekenberg et al. 2017). Incubating these individual components of the seagrass ecosystem separately may not provide a realistic ‘whole system’ view of $\text{N}_2$ fixation in seagrass habitats. During isolated incubations discrete $\text{N}_2$ fixing zones around the root/rhizome may become inundated by nearby regions of high NH$_4^+$ concentration (Welsh, 2000), naturally occurring $\text{N}_2$ fixing bacterial community distribution may be effected (Langezaal et al. 2003, Boer et al. 2009), and links between structures that affect $\text{N}_2$ fixation may be destroyed. For example, when leaves are incubated separately to roots and sediments $\text{N}_2$ fixation occurring from lacunal transport is not observed.

The $^{15}$N-$\text{N}_2$ dissolution method can be performed on intact cores with minimal disturbance to natural sediment biogeochemical gradients and whole seagrass plants. Thus, the $^{15}$N-$\text{N}_2$ dissolution method may provide $\text{N}_2$ fixation rates much more indicative of in situ rates. O’Donohue et al. (1991a) measured similar rates of fixation relative to this study, 120 $\mu$mol N m$^{-2}$ h$^{-1}$, using a $^{15}$N-$\text{N}_2$ dissolution method in a subtropical $Z$. capricorni meadow. However, if the lacunal diffusion method of perfusion used by O’Donohue et al. (1991a) was not successful in uniformly labelling the rhizosphere then possible sites of $\text{N}_2$ fixation may have not been captured in the measurement.

The Li$^+$ data in our perfusion cores showed uniform labelling of the sediment and minimal contribution of labelled pore water to the overlying water column. Therefore, we are confident that $^{15}$N-$\text{N}_2$ was available to all sediment layers and that oxygenated water did not infiltrate into the sediments during perfusion. It is possible
that there is some overlap between N₂ fixation measured in the rhizosphere of the surface and perfusion cores in the calculation of the total N₂ fixation rate (equation [2]). However, perfusion core sediment fixation represents 14.6% of the total rate estimate, while perfusion root/rhizome fixation only 0.3% (Fig. 2). We do not think this is enough to warrant deleting either from the total N₂ fixation rate calculation.

Accurate measurement of N₂ fixation in the rhizosphere is complex due to natural sediment heterogeneity. The rates in our experiment may have been negatively affected by the relatively short time frame of the incubations (up to 10 hours). However, our results seem to suggest that the majority of fixation occurred in the first 5 hours (Fig. 1). This would explain the reduced perfusion rates at the 10 hour time-point as rates averaged over a longer time frame (10 hours) appear lower (Fig. 1). This tapering off of rates may be due to changing redox conditions in the sediment over the incubation period.

Although the ¹⁵N-N₂ dissolution method does provide a valuable direct measure of N₂ fixation in seagrass systems it is more costly than previous methods in that access to specialised analytical instruments is required and labelled gas is expensive. Additionally, the potential for ¹⁵N-N₂ gas to be contaminated with ¹⁵N-NH₄⁺ can artificially inflate experimental findings, making the gas and results of any experiment potentially unusable. We would recommend confirming any contamination between different batch numbers and brands of gas (see supplementary material, Table S.1) prior to any experimental procedure although this does increase the cost incurred. The ¹⁵N-N₂ dissolution method also does not lend itself as easily to in situ incubations as the ARA as the ¹⁵N-N₂ site water solution needs to be equilibrated while shaking over many hours.
3 - Dinitrogen fixation rates in a subtropical seagrass meadow measured with a direct $^{15}$N-$N_2$
tracer method

The $N_2$ fixation rates we have measured are a similar order of magnitude to rates measured in previous studies by the ARA (Table 3.1). Despite this, and although we did not directly compare the $^{15}$N-$N_2$ dissolution method and the ARA on this seagrass bed, we suggest the $^{15}$N$_2$ dissolution method is the preferred method for calculating $N_2$ fixation in seagrass communities as it removes many of the existing uncertainties in the ARA methodology. Our whole system approach to measuring $N_2$ fixation in discrete zones in core incubations further serves to clarify $N_2$ fixing pathways in seagrass systems and zones of highest $N_2$ fixing activity where the majority of past studies have focused on sediments.

3.5 Conclusions

To our knowledge this is the first study that applies the $^{15}$N-$N_2$ dissolution method to intact seagrass cores with both surface and rhizosphere additions. Leaf epiphytic activity appears to have dominated $N_2$ fixation in the surface addition cores. While sediment $N_2$ rates were highest in perfusion cores, previously mentioned issues with perfusion methodology may have negatively affected these estimates. As our measurements were only conducted in summer and under light conditions, they can be thought of as maximum rates. Rates over winter and in dark conditions would be expected to be lower (Goering & Parker 1972). It may be possible for leaf-associated epiphyte $N_2$ fixation rates, and sediment surface $N_2$ fixing bacteria to be stimulated further by higher intensity light conditions closer to ambient rates rather than the sodium lamps we used. The location of Shaws Bay, with its high concentration of $NH_4^+$, may have also had a negative impact on $N_2$ fixation in the $Z. muelleri$ meadow. Further research using the $^{15}$N-$N_2$ dissolution method for measuring $N_2$ fixation in other subtropical seagrass species is needed to clarify if morphological and/or location effects have an impact on $N_2$ fixation rates.
Chapter 4: Comparison of dinitrogen fixation rates in two subtropical seagrass communities

Comparison of dinitrogen fixation rates in two subtropical seagrass communities

Abstract

Seagrass morphology varies greatly between species and can impact their nitrogen (N) cycling capacity, including dinitrogen (N₂) fixation. We used a ^15N-N₂ stable isotope technique to measure N₂ fixation rates in different zones of two morphologically diverse seagrass communities, *Zostera muelleri* and *Halophila ovalis* in Moreton Bay, Australia. Isotope label additions were made to both the surface and rhizosphere of sediment cores containing whole plants over an artificial diurnal cycle. In both species the highest rates of N₂ fixation were found in the leaves (including epiphytes) in the light with areal leaf related N₂ fixation rates higher in the *Z. muelleri* (46 ± 26 µmol N₂ m⁻² h⁻¹) community compared to *H. ovalis* (11 ± 7.2 µmol N₂ m⁻² h⁻¹). There was a switch in the location of N₂ fixation from the leaves to the sediments in the dark in the *Z. muelleri* community which was not observed in *H. ovalis*. The change in the active site of N₂ fixation in the *Z. muelleri* community is likely related to the reduction in oxygen supply and associated increase in sulphate reducing bacterial activity at night. Overall the *Z. muelleri* community fixed N₂ at ~3 times (~75 µmol N m⁻² h⁻¹) the rate of *H. ovalis* (~25 µmol N m⁻² h⁻¹). The results of this study indicate that plant morphology, and its associated influence on sediment biogeochemistry, may have a substantial impact on regulating N₂ fixation rates in seagrass communities.
4.1 Introduction

Seagrass communities are effective nutrient cyclers and facilitate much of the dinitrogen (N\textsubscript{2}) fixation in the coastal zone (Welsh 2000, Garcias-Bonet et al. 2016, Cardini et al. 2017). However little is known about how species-specific morphological traits may impact the plants N\textsubscript{2} fixation capacity. Physical attributes such as leaf and root/rhizome surface area play an important role not just as surfaces for the colonisation of N\textsubscript{2}-fixing diazotrophs, but may also impact the geochemical characteristics of the rhizosphere, via exudation of oxygen (O\textsubscript{2}) and organic carbon (C) from the root system. As such, the species composition of seagrass meadows can greatly impact their capacity to regulate nitrogen (N) biogeochemistry in coastal systems (Eyre at al 2011a, 2016a). Additionally, our understanding about how different seagrass species affect N cycling processes and in particular N\textsubscript{2} fixation rates, has not been updated to include measurements using contemporary stable isotope techniques. The major objective of this study, therefore, is to apply the \textsuperscript{15}N-N\textsubscript{2} dissolution technique to quantify N\textsubscript{2} fixation in various locations within the seagrass community of two morphologically different subtropical species. This method has not been used to characterise and compare above and below ground N\textsubscript{2} fixation rates of different seagrass species over a diurnal cycle.

Dinitrogen fixation rates above the sediment surface are influenced by the surface area available for N\textsubscript{2} fixing epiphytes to colonise (Cole and McGalthery 2012, Cook et al. 2015), and therefore differences in leaf surface area and density can have a significant impact on N\textsubscript{2} fixation rates between seagrass species. Below ground sediment processes are much more complex and are controlled by the availability of O\textsubscript{2}, organic C, and inorganic N (Knapp 2012) all of which may be influenced by species-specific morphological characteristics. For instance,
Comparison of dinitrogen fixation rates in two subtropical seagrass communities

Subtropical sediment N₂ fixation rates have been reported up to >100 μmol N m⁻² h⁻¹ in *Halodule beaudetti* (Blackburn et al. 1994) and as low as ~3 μmol N m⁻² h⁻¹ in *Halophila ovalis/spinulosa* communities (Eyre et al. 2011a).

Some seagrass species may have an indirect impact on coastal N₂ fixation through the effect of grazing preferences of marine herbivores. *Halophila ovalis* is frequently grazed by large marine fauna such as, green sea turtles and dugongs (Preen 1995; Brand-Gardner et al. 1999). Regular grazing of *H. ovalis* in comparison to other species such as *Z. muelleri* may exacerbate differences in N₂ fixation by decreasing leaf surface area for epiphyte colonisation. Dinitrogen-fixing epiphytes on seagrass leaves have been identified as a major source of bioavailable N to the plant and may be the main driver of N₂ fixation during the day (Carlson-Perret et al. 2018), with removal of seagrass leaves representing a drop in N₂ fixation rates of up to 89% (Agawin et al. 2017). Conversely frequent grazing may cause N₂ fixation to be stimulated in order to cater for new growth with plant N content increasing 15-30% in simulated grazing experiments of *H. ovalis* (Aragones et al. 2006). Seagrass leaves are not the only area of the plant where physical variations between species may impact N₂ fixing activity.

The seagrass rhizosphere (i.e. the root, rhizome and surrounding sediment) is a complex system with many biological, chemical and physical aspects working in concert. Seagrass roots release metabolites such as O₂, organic C, and inorganic N which can impact rhizosphere N₂ fixation. The presence of these compounds is in turn regulated by the physical structure of the roots (i.e. their surface area and vertical penetration of the sediment) which can vary significantly between species. For example, the root zone of *Z. muelleri* is characterised by numerous rhizomes with many long (~ 6-10 cm) fine roots whilst *H. ovalis* rhizomes tend to have one (~ 5-8 cm) root per shoot covered in tiny root hairs which serve to increase the root surface area.
Comparison of dinitrogen fixation rates in two subtropical seagrass communities area (Roberts 1993). The penetration of metabolites released from seagrass roots into the surrounding sediments, coupled with sediment organic matter content, can regulate the activity of sulphate reducing bacteria (SRB) in the rhizosphere. Sulphate reducing bacteria are one of the key N2-fixers in seagrass communities and have been found to have a mutualistic relationship with the surfaces of plant root and rhizomes (Welsh 2000). Release of O2 from seagrass roots largely during photosynthesis may affect N2-fixers by acting as either a stimulant to aerobic N2-fixing diazotrophs, or an inhibitor to anaerobic fixers like SRB (Pedersen et al. 1998, Koren et al. 2015). Whilst organic C, either from roots or seagrass detritus, acts as a source of energy for N2 fixing bacteria and has an overall positive effect on rhizosphere N2 fixation (Holmer et al. 2004, Cole and McGlathery 2012). The concentration of inorganic N species (i.e. specifically NH4+) in the rhizosphere or water column has been found to impact N2 fixation by inhibiting nitrogenase activity at high concentrations in some cases (Postgate and Kent 1984, Welsh et al. 1997, Knapp 2012), whilst inhibition in other was minimal (McGlathery et al. 1998).

In addition to species-specific morphology, light availability influences N2 fixation rates in seagrass systems. Previous studies on seagrass N2 fixation have reported higher rates in the light compared to those measured under dark conditions (McGlathery et al. 1998, Welsh et al. 2000, Russell et al. 2016). This is largely due to photosynthetically active epiphyte mediated leaf N2 fixation which suggests that regions of N2 fixation under dark conditions may be different. Variations in the release of photosynthates (O2 and organic C) from roots under light and dark conditions may cause a change in the aerobic status of the sediment surrounding the roots meaning that largely anaerobic SRB may become the dominant source of fixed N to the plant at night. Whilst N2 fixation in seagrasses has been investigated extensively, very few of these studies have measured above and below ground rates.
Comparison of dinitrogen fixation rates in two subtropical seagrass communities over a diurnal cycle in different species. The use of stable isotopic tracer methods to measure above and below ground seagrass N\textsubscript{2} fixation has only been undertaken twice (O’Donohue et al. 1991, Carlson-Perret et al. 2018), and has never been applied to compare different species over a diurnal cycle. The direct $^{15}$N-N\textsubscript{2} dissolution method is ideal for teasing apart the complexity of N\textsubscript{2} fixation in seagrass systems as its addition to the system is relatively non-invasive disturbing natural sedimentary gradients less than other techniques such as the indirect acetylene reduction assay.

In this study we measured N\textsubscript{2} fixation rates in two morphologically distinct seagrass species, *H. ovalis* and *Z. muelleri*. In subtropical Australia (Moreton Bay), using the $^{15}$N-N\textsubscript{2} dissolution method. Above and below ground N\textsubscript{2} fixation rates were measured in intact core incubations via $^{15}$N-N\textsubscript{2} additions to the overlying water and perfusion of the rhizosphere over an artificial diurnal cycle i.e. under lights or wrapped in foil ex situ. We hypothesised that *Z. muelleri* would fix N\textsubscript{2} at a greater rate than *H. ovalis* under light and dark conditions as their meadows are often more dense with greater above and below ground biomass (Samper-Villarreal et al. 2017). Under light conditions the larger leaf surface area of *Z. muelleri* allow more photosynthetically active N\textsubscript{2}-fixing epiphytes to colonise. Under dark conditions we also expected greater N\textsubscript{2} fixation rates from *Z. muelleri* as compared with *H. ovalis* as SRB in the rhizosphere become the dominant N\textsubscript{2}-fixers and are known to be associated with the root and rhizome of seagrass plants (Welsh 2000).

4.2 Method

4.2.1 Sample Site and collection

Moreton Bay is located on the subtropical south-eastern coast of Queensland, Australia. It has an area of 3400 km\textsuperscript{2} and stretches from the Gold Coast in the south...
4 – Comparison of dinitrogen fixation rates in two subtropical seagrass communities to Caloundra in the north. Sample sites were located at the southern end of the bay known as the Broadwater (Fig. 4.1). For more detailed information on southern

Moreton Bay see Eyre et al. (2011a,b) and Eyre and McKee (2002). Seagrass species in the southern part of the bay are largely dominated by *Zostera muelleri*, *Halophila ovalis* and *Halophila spinulosa* (Cuttriss et al. 2013), the macro alga *Caulerpa taxifolia* is also present. To test the impact seagrass morphology may have on N₂ fixation rates we chose two species that are dominant in subtropical Australian systems, *Z. muelleri* and *H. ovalis*. These two species are often found in the same coastal environments but vary markedly in terms of morphology. For example, *Z. muelleri* is characterised by long, thin strap-like leaves (~30 cm long and 0.5-5 mm wide) (Moore & Short, 2006) and dense growth (Samper-Villarreal et al. 2017), ideal for leaf epiphyte colonisation. Whereas, the leaves of *H. ovalis* are much shorter (1-4

Figure 4.1 Map of the study area located within the Broadwater of southern Moreton Bay, Australia (modified from Eyre et al. 2011a). Sample sites of *Zostera muelleri* represented by square and *Halophila ovalis* by diamond.
Comparison of dinitrogen fixation rates in two subtropical seagrass communities cm long and 5-20 mm wide) (Lanyon 1986) and grow in less dense ephemeral patches with less surface area for N2-fixing epiphytes to colonise.

Samples were collected in October 2016 (during mid austral spring) using clear acrylic cores (46 cm long with an internal diameter of 9 cm). Cores (n=9 per species) were collected comprising ~15 cm of sediment containing individual H. ovalis and Z. muelleri plants and the associated overlying water. Sediment cores containing H. ovalis and Z. muelleri were collected from southern Moreton Bay (Fig. 4.1, Table 4.1). All cores were as described in Carlson-Perret et al. (2018) as perfusion cores, i.e. ~6 mm ports (sealed with Soudal Fix-All Flexi multi-use polymer sealant) located ~2 cm along the length of the sediment column.

Site water temperature was measured with a Hach HQ40D meter during core collection (± 0.1°C). Additional site water ~250 L was collected to top up cores and fill incubation chambers (see below).

4.2.2 Core incubations

Cores were transported back to the laboratory where they were pre-incubated uncapped, overnight (Ferguson et al. 2003, 2004) in clear acrylic incubation chambers with ~100 L site water. In situ water temperature (23 ± 1°C) was maintained with aquarium temperature regulators. Stir bars, controlled by a central magnet, were placed ~10cm above the sediment surface and spun at just below the sediment resuspension rate (Eyre and Ferguson, 2005). Aquarium airstones in the acrylic incubation chamber kept the cores oxygenated during the pre-incubation period (~14 hours).

Additional samples of site water were placed in four 500 mL serum bottles, sealed and degassed for ~1 hour. Fifty mL of 15N-N2 gas (> 98% Cambridge Isotope laboratories, lot no. I-19168A) was injected into each bottle and agitated on a shaker table overnight. The 15N-N2 gas was tested prior to use for contamination by enriched.
Comparison of dinitrogen fixation rates in two subtropical seagrass communities
forms of ammonium (NH$_4^+$), contamination was found to be minimal (see Appendix 4
for details).

Following the pre-incubation period water samples were collected from three
cores (termed background cores), filtered with a 0.45 μm syringe filter (Sartorius,
cellulose acetate) and stored for dissolved inorganic nitrogen (DIN) including NOx
(nitrate + nitrite) and NH$_4^+$ and total dissolved nitrogen (TDN) determination. Filtered
samples were frozen at -20 °C until analysis. Unfiltered water column samples were
collected in triplicate for isotopic N$_2$ determination (δ$^{15}$N-N$_2$) in 12 mL glass gas-tight
vials (Exetainers) without headspace and were immediately poisoned with 20 μL of
saturated (~8% w/v) mercuric chloride.

Following water sample collection, seagrass leaves and root/rhizome material
were collected from the background cores by removing the entire plant via gentle
vertical extraction. Leaves and root/rhizome were rinsed with ultra-pure water,
separated and immediately frozen at -20 °C. The sediment portion of the cores were
sectioned into nine varying depth fractions (0-1 cm, 1-2 cm, 2-3 cm, 3-4 cm, 4-5 cm,
5-6 cm, 6-9 cm, 9-13 cm, 13-15 cm) and frozen (-20 °C).

The remaining 12 cores were sealed and 100 mL of $^{15}$N-N$_2$ labelled site water
was injected into each through gas-tight ports in the lid. An additional 100 mL of $^{15}$N-
N$_2$ labelled site water was added to the length of the rhizosphere via a 90 mm spinal
needle (Terumo 22G) attached to a 20 mL plastic syringe. The $^{15}$N-N$_2$ solution was
injected equally into each port located equidistantly along the sediment fraction of the
cores (~10 mL per port). As the label solution was injected the needle was slowly
withdrawn from sediment so that the solution would be distributed evenly within the
rhizosphere (Jørgensen 1977, McGlathery et al. 1998).
Three cores of each species were incubated under light conditions (high pressure sodium Agri-Gro lamps at ~34 PAR μmol m$^{-2}$ s$^{-1}$) with the remaining cores wrapped in aluminium foil to simulate dark conditions (Welsh et al. 2000, Russell et al. 2016). All cores were incubated for 6 hours to mitigate cores becoming anoxic. Once incubations were complete cores were sacrificed and sampled following the same procedure as background cores.

### 4.2.3 Sample analysis

Samples collected for DIN and TDN concentration were analysed via a Lachat QuickChem 8000 four channel Flow Injection Analyser. For details of the methods used see Eyre and Pont (2003).

Prior to analysis samples to be analysed for $\delta^{15}$N-$N_2$ were injected with 2 mL helium and left to equilibrate overnight. Headspace samples (10 μL) were analysed via a Thermo Trace gas chromatograph (GC) Ultra with a 25 m x 0.32 mm PoraPLOT Q column interfaced to a Thermo Delta V Plus isotope ratio mass spectrometer (IRMS) (precision ± 0.15‰).

Sediment fractions were homogenised separately with a glass rod and two ~10 g (wet weight) sub-samples were taken from each. Sub-samples were placed in 50 mL falcon tubes with 20 mL 2M potassium chloride solution (KCl) (Morin and Morse, 1999) in order to desorb porewater NH$_4^+$ from sediment surfaces which may distort sediment bulk $\delta^{15}$N. Sediment sub-samples + KCl were agitated and left to desorb for ~24 hours after which they were centrifuged at ~3000 rpm for 5 minutes. Supernatant was decanted and sediment sub-samples were resuspended in 20 mL high purity water and centrifuged at ~3000 rpm for 5 minutes to remove excess remaining salts. This washing procedure was repeated 3 times.

Leaves, root/rhizome material and sediment (i.e. the washed sediment sub-samples) were all freeze-dried. Dried sediments were sieved through a 500 μm mesh.
4 – Comparison of dinitrogen fixation rates in two subtropical seagrass communities
to isolate and exclude any leaf and root/rhizome detritus. Dried leaf, root/rhizome and
sediment samples were ground individually in a ring mill to ensure homogeneity.
Samples were weighed into tin capsules in duplicate (leaves ~8-15 mg, root/rhizomes
~5-20 mg, sediments ~80-160 mg) and analysed for %N and $\delta^{15}$N (with %C
measured in sediments only). Seagrass biological matter and sediments were
analysed via an elemental analyser (Thermo Finnigan Flash EA 1112) coupled to an
IRMS (Thermo Conflo III and a Thermo Delta V Plus) (precision ± 0.3 ‰) (Eyre et al.
2016b).

4.2.4 $N_2$ fixation calculation

Dinitrogen fixation rates in leaves, root/rhizome and sediment was calculated
as per equation [1] (Wilson et al. 2012) and detailed in (Carlson-Perret et al. 2018)
as,

$$V(t^{-1}) = \frac{(AP_{(PN-Final)} - AP_{(PN-Initial)})}{(AP_{N_2} - AP_{(PN-Initial)})} \times \frac{1}{\Delta t} \times \frac{PN_{Final}}{2} \quad [1]$$

Briefly, $PN_{Final}$ was calculated based on $\delta^{15}$N and %N measured in each locus
(i.e. leaves + epiphytes, root/rhizome and sediment) via EA-IRMS. The atom percent
$AP_{(PN-Initial)}$ represent atom percent values of the $^{15}$N in the unlabelled loci of the
background cores and $AP_{(PN-Final)}$ atom percent values of the individual loci measured
at the end of the $^{15}$N-$N_2$ amended incubations. The $AP_{N_2}$ used to calculate surface
(i.e. leaves + epiphytes) $N_2$ fixation rates was determined from water column samples
collected 30 minutes after label addition. Whilst the $AP_{N_2}$ used in rhizosphere
(including root/rhizome material and sediment) rate estimates was determined from
the undiluted perfusion solution. Raw data for initial and final PN and atom% values
for leaf (Table S.6), root/rhizome material (Table S.7) and sediment (Table S.8) is
presented in Appendix 5.

Total $N_2$ fixation per species was calculated by adding the rates from each loci
as per equation [2],

Unraveling the complexity of subtropical seagrass nitrogen cycling: a stable isotope
approach
4 – Comparison of dinitrogen fixation rates in two subtropical seagrass communities

\[
\text{Total N fixed} = S_l + P_s + P_r \tag{2}
\]

Where \(S_l\) represents surface \(N_2\) fixation (i.e. leaf + epiphyte as \(\mu\text{mol} \ N_2 \ m^{-2} \ h^{-1}\)) and \(P_s\) and \(P_r\) denote below ground sediment and root/rhizome \(N_2\) fixation as \(\mu\text{mol} \ N_2 \ m^{-2} \ h^{-1}\) respectively.

Combined daily rates of \(N_2\) fixation were calculated by assuming a 12 hour cycle of light and dark, i.e. light rates of each individual species were combined as in [2], multiplied by 12 and added to dark rates calculated in the same way. The combined total of 12-hourly light and dark rates was then divided by 24 to achieve an average daily hourly rate. Standard deviation of all combined rates was calculated as in equation [3],

\[
\text{Total SD} = \sqrt{(x^2)+(y^2)}\ldots \tag{3}
\]

Where \(x\) and \(y\) represent SD of each measure.

Due to unequal variance across the data sets, largely due to the innate variability of sediment and biological matter analysis as well as each loci having a small number of replicates (\(n=3\)), a multi-factor ANOVA could not be used. Instead two-sample t-tests assuming unequal variance were run on \(N_2\) fixation rates obtained between light levels, species and location to determine if rates were significantly different.

4.3 Results

4.3.1 Seagrass community characteristics

Seagrass community sediment and water column characteristics are detailed in Table 4.1, note that morphological features of *Halophila ovalis* and *Zostera muelleri* communities of Moreton Bay come from Samper-Villarreal et al. (2017). On average *Z. muelleri* has a much higher above ground, below ground biomass, and shoot density than *H. ovalis* (Table 4.1). Water column \(NH_4^+\) concentration was ~4
Comparison of dinitrogen fixation rates in two subtropical seagrass communities

times higher in the Z. muelleri cores in comparison to H. ovalis and the sediment C:N ratio was also higher in the Z. muelleri community (Table 4.1).

4.3.2 N\textsubscript{2} fixation under light conditions

Above ground N\textsubscript{2} fixation rates (i.e. leaves + epiphytes) under light conditions were higher in Z. muelleri than H. ovalis (46.3 ± 25.9 μmol N\textsubscript{2} m\textsuperscript{-2} h\textsuperscript{-1} and 10.7 ± 7.18 μmol N\textsubscript{2} m\textsuperscript{-2} h\textsuperscript{-1} respectively) with the Z. muelleri leaf community fixing N\textsubscript{2} at almost 5 times the rate of the H. ovalis leaf community (Fig. 4.2). In both species, above ground N\textsubscript{2} fixation rates dominated over both the root/rhizome and sediment rates (Fig. 4.2). The Z. muelleri leaf community was by far the highest rate observed under light conditions however, root/rhizome and sediment rates in the light were higher in H. ovalis compared to the same loci in Z. muelleri (Fig. 4.2). In both species light N\textsubscript{2} fixation rates were higher in the sediments than in the root/rhizome material (Fig. 4.2).

4.3.3 N\textsubscript{2} fixation under dark conditions

Whilst above ground N\textsubscript{2} fixation rates dominated across both species under light conditions, this trend was reversed under dark conditions, particularly in Z. muelleri. Sediment rates in Z. muelleri (17 ± 10 μmol N\textsubscript{2} m\textsuperscript{-2} h\textsuperscript{-1}) exceeded those of the Z. muelleri leaf community under dark conditions (10 ± 4.8 μmol N\textsubscript{2} m\textsuperscript{-2} h\textsuperscript{-1}). Zostera muelleri dark sediment N\textsubscript{2} fixation rates were ~14 times greater than that of the sediments under light conditions whilst H. ovalis saw no significant difference between light and dark sediment N\textsubscript{2} fixation (t\textsubscript{(43)} = 1.13, p = 0.27) (Fig. 4.2, Table 4.2). In the dark the Z. muelleri community had higher N\textsubscript{2} fixation rates than H. ovalis in the sediment and leaf community (Fig. 4.2). Root/rhizome N\textsubscript{2} fixation rates were consistently the lowest region of activity across both species and light treatments
4 – Comparison of dinitrogen fixation rates in two subtropical seagrass communities (Fig. 4.2). In both *Z. muelleri* and *H. ovalis* root/rhizome there was no significant difference between light and dark N$_2$ fixation rates (Table 4.2).

### 4.3.4 Total N$_2$ fixation

Total rates of N$_2$ fixation (i.e. leaves + sediment + root/rhizome material) were greater in the light than the dark in both species (Fig. 4.3), with *Z. muelleri* N$_2$ fixation rates consistently higher than *H. ovalis* over both light levels (Fig. 4.3). The total mean diel N$_2$ fixation rate of the *Z. muelleri* community tended to exceed that of *H. ovalis* (36 ± 28 μmol N$_2$ m$^{-2}$ h$^{-1}$ and 12 ± 7.7 μmol N$_2$ m$^{-2}$ h$^{-1}$ respectively) (Fig. 4.3). Statistically significant differences between these rates aren’t clear due to high sample rate variability.
Table 4.1: Physical and chemical characteristics of the *H. ovalis* and *Z. muelleri* communities in Moreton Bay.

<table>
<thead>
<tr>
<th></th>
<th>Halophila ovalis</th>
<th>Zostera muelleri</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sediment C:N ratio</td>
<td>15.6</td>
<td>23.2</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>(0.12% C; 0.0090% N)</td>
<td>(0.73% C; 0.037% N)</td>
<td></td>
</tr>
<tr>
<td>Water column measurements;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NH$_4^+$ (µg L$^{-1}$)</td>
<td>6.1 ± 3.2</td>
<td>24 ± 5.9</td>
<td>This study</td>
</tr>
<tr>
<td>NO$_x$ (µg L$^{-1}$)</td>
<td>&lt; 2</td>
<td>3.5 ± 2.3</td>
<td>This study</td>
</tr>
<tr>
<td>TDN (µg L$^{-1}$)</td>
<td>249 ± 10</td>
<td>287 ± 60</td>
<td>This study</td>
</tr>
<tr>
<td>Morphological characteristics;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Average leaf width (cm)</td>
<td>0.81 ± 0.23</td>
<td>0.25 ± 0.10</td>
<td>Samper-Villarreal et al. 2017</td>
</tr>
<tr>
<td>Average leaf length (cm)</td>
<td>1.9 ± 0.65</td>
<td>5.5 ± 1.8</td>
<td>Samper-Villarreal et al. 2017</td>
</tr>
<tr>
<td>Average shoot density (m$^2$)</td>
<td>1082 ± 929</td>
<td>2701 ± 3430</td>
<td>Samper-Villarreal et al. 2017</td>
</tr>
<tr>
<td>Average above ground biomass (dry weight – g m$^{-2}$)</td>
<td>5.5 ± 7.5</td>
<td>29 ± 29</td>
<td>Samper-Villarreal et al. 2017</td>
</tr>
<tr>
<td>Average below ground biomass (dry weight – g m$^{-2}$)</td>
<td>10 ± 14</td>
<td>218 ± 167</td>
<td>Samper-Villarreal et al. 2017</td>
</tr>
<tr>
<td>Area (km$^2$)</td>
<td>1.7 ± 0.1</td>
<td>3.2 ± 0.2</td>
<td>Eyre et al. 2011a</td>
</tr>
</tbody>
</table>
Figure 4.2 Rates of seagrass N\textsubscript{2} fixation (as µmol N\textsubscript{2} m\textsuperscript{-2} h\textsuperscript{-1}) in various loci within Zostera muelleri (top) and Halophila ovalis (bottom) when \textsuperscript{15}N-N\textsubscript{2} added above and below ground in both dark (left) and light (right) conditions. Arrows represent potential direction of transport of fixed N and blue circles represent oxygen excretion. Sediment and root/rhizome N\textsubscript{2} fixation is represented by P\textsubscript{s} and P\textsubscript{r} respectively, while S\textsubscript{i} represents leaf (including epiphyte) N\textsubscript{2} fixation. Seagrass vector images from Diana Kleine (Z. muelleri) and Catherine Collier (H. ovalis) ian.ucmes.edu/imagelibrary.
4 – Comparison of dinitrogen fixation rates in two subtropical seagrass communities

4.4 Discussion

The $^{15}$N-$\text{N}_2$ dissolution technique has not previously been used to compare $\text{N}_2$ fixation rates between seagrass species over a diurnal cycle, and between the loci of $\text{N}_2$ fixing activity within the seagrass community. The major findings of this study are that; 1) seagrass $\text{N}_2$ fixation rates are highest at the surface under light conditions, 2) there is a switch in $\text{N}_2$ fixation activity dominance from above ground in the light to below ground in the dark, and 3) total rates of $\text{N}_2$ fixation are on average higher in the *Zostera muelleri* community as compared to the *Halophila ovalis* community.

4.4.1 Influence of light on $\text{N}_2$ fixation

Sites of $\text{N}_2$ fixation activity appeared to change with light availability. In the light, rates were higher in the above ground biomass than in the rhizosphere for both *Z. muelleri* and *H. ovalis* (Fig. 4.2) consistent with a previous study employing the same $^{15}$N-$\text{N}_2$ dissolution method (Carlson-Perret et al. 2018). Under dark conditions however, *Z. muelleri* sediment $\text{N}_2$ fixation was higher than leaf + epiphyte rates. The decrease in above ground $\text{N}_2$ fixation associated with dark conditions is not unexpected given that photosynthetically active leaf-associated epiphytes are active $\text{N}_2$-fixers (Agawin et al. 2017). Furthermore, light may increase the $\text{O}_2$ supply to the sediments via seagrass roots (Hemminga 1998, Koren et al. 2015) which may inhibit $\text{N}_2$ fixation in the sediments. In the majority of previous seagrass $\text{N}_2$ fixation studies that compare light and dark conditions, rates are reported as higher in the light for sediments (McGlathery et al. 1998, Risgaard-Petersen et al. 1998, Welsh et al. 2000, Russell et al. 2016) and leaves (Iizumi 1992). The only other study that has compared $\text{N}_2$ fixation rates between these same two seagrass species also undertaken in the Broadwater (Eyre et al. 2011a) found rates in the light were similar in both species with both *H. ovalis* and *Z. muelleri* sediment $\text{N}_2$ fixation rates decreasing in the dark.

Unraveling the complexity of subtropical seagrass nitrogen cycling: a stable isotope approach
Table 4.2: Results of two-tailed t-tests comparing N\(_2\) fixation rates of various loci within *Halophila ovalis* and *Zostera muelleri* seagrass communities. Where results in bold are significant at \( p < 0.05 \).

<table>
<thead>
<tr>
<th></th>
<th>Sediment</th>
<th>Leaves</th>
<th>Root/Rhizome material</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>H. ovalis</em> (^a,b)</td>
<td>( t_{(43)} = 1.13, p = 0.27 )</td>
<td>( t_{(2)} = 1.07, p = 0.40 )</td>
<td>( t_{(2)} = 0.80, p = 0.51 )</td>
</tr>
<tr>
<td><em>Z. muelleri</em> (^a,b)</td>
<td>( t_{(22)} = -5.49, p = 0.0014 )</td>
<td>( t_{(2)} = 2.04, p = 0.18 )</td>
<td>( t_{(2)} = 2.03, p = 0.18 )</td>
</tr>
<tr>
<td><em>H. ovalis</em> (^a) &amp; <em>Z. muelleri</em> (^a)</td>
<td>( t_{(9)} = 2.40, p = 0.04 )</td>
<td>( t_{(2)} = -2.29, p = 0.15 )</td>
<td>( t_{(2)} = 1.83, p = 0.21 )</td>
</tr>
<tr>
<td><em>H. ovalis</em> (^b) &amp; <em>Z. muelleri</em> (^b)</td>
<td>( t_{(8)} = -4.78, p = 0.001 )</td>
<td>( t_{(2)} = -1.46, p = 0.28 )</td>
<td>( t_{(3)} = 7.85, p = 0.004 )</td>
</tr>
</tbody>
</table>

\(^a\) - Light N\(_2\) fixation rates  
\(^b\) - Dark N\(_2\) fixation rates
Comparison of dinitrogen fixation rates in two subtropical seagrass communities

Unraveling the complexity of subtropical seagrass nitrogen cycling: a stable isotope approach

**Figure 4.3** Total N\(_2\) fixation rates (i.e. leaf + epiphyte community, root/rhizome material and sediment inclusive) in *Halophila ovalis* and *Zostera muelleri* cores under light and dark conditions and total diel rates. (Mean ± SD, n = 3).

In these types of ‘whole system’ measurements resolution of particular locations of N\(_2\) fixing activity cannot be discerned. Without our measurement of N\(_2\) fixation rates in different loci within the seagrass community we would also report a decrease in total N\(_2\) fixation rates in the dark as compared to light conditions (Fig. 4.3). Due to these measurements we are able to tease out different process that may be driving the varying rates of N\(_2\)-fixing activity in each loci of *Z. muelleri* and *H. ovalis* in the light and dark.

The spike in sediment rates in *Z. muelleri* at night (Fig. 4.2) suggests that species-specific below ground morphology has a significant impact on N\(_2\) fixation rates, especially in the dark (Table 2). This is likely to do with the extensive root system of *Z. muelleri* and its ability to release O\(_2\) along its entire root and rhizome (Brodersen et al. 2015). This supports the hypothesis that SRB are the dominant N\(_2\)-
Comparison of dinitrogen fixation rates in two subtropical seagrass communities

Fixers in the *Z. muelleri* sediment profile (Carlson-Perret et al. 2018), perhaps due in part to a more sulphidic sediment. Nitrogenase the enzyme that cleaves the triple bonds joining the atoms of N₂, can also be inhibited by O₂ (Canfield et al. 2010). Thus the dark sediment N₂ fixation rates observed in the *Z. muelleri* community in this study are likely due to the drop in O₂ released from the root system at night, allowing a more anoxic sediment environment to form stimulating the activity of the largely anaerobic SRB. Oxygen release from the *Z. muelleri* root system has been described as an additional protective mechanism of the plant against toxic sulphides which may accumulate in the sediment (Brodersen et al. 2015). During the day, as more O₂ is released, sulphides in the rhizosphere are re-oxidised to sulphate, and at night when SRB are active in a lower O₂ environment they reduce sulphate and, as their action is non-specific, also reduce N₂.

An increase in sulphate reduction at night has been observed in the rhizosphere of *Z. muelleri* (Holmer et al. 2017) and *Z. capricorni* (conspecific with *Z. muelleri*) (Pagès et al. 2012) via colourimetric gel (diffuse gradients in thin-flim samplers). There were also other clear geochemical differences in their seagrass community over the diel cycle including increases in iron (II) (Fe²⁺), sulphate (SO₄²⁻) and NH₄⁺ in the dark. Iron (Fe) can stimulate or limit N₂ fixation as diazotrophs such as *Trichodesmium* require large amounts of Fe to survive (Kustka et al. 2003, Jickells et al. 2017). Oxygen released from seagrass roots can oxidise dissolved Fe²⁺ to Fe³⁺ which then precipitates and can oxidise sulphides to elemental S or other sulphur compounds which may in turn help stimulate SRB. An increase in NH₄⁺ would be expected as a product of increased N₂ fixation in the night and *Z. muelleri* water column NH₄⁺ was much higher than *H. ovalis* which may be as a result of fluxes out of the sediment or leaf fixation (Table 4.1). These increases in key chemical drivers...
4 – Comparison of dinitrogen fixation rates in two subtropical seagrass communities

of sediment N₂ fixation in the dark were attributed to root/rhizome O₂ loss to the surrounding sediment during the day (Pagès et al. 2012). The fact that an increase in rhizosphere N₂ fixation in the dark wasn’t observed in H. ovalis suggests that light is not the sole driver of N₂ fixation activity in seagrass communities and that seagrass morphology may have a greater impact on rates than first thought.

4.4.2 Species related differences in N₂ fixation

Across the two species, Z. muelleri leaves (including associated epiphytes) exhibited higher N₂ fixation rates than the H. ovalis leaf community in both light and dark conditions (Fig. 4.2). One reason for the difference in the rates of N₂ fixation between these two species is that Z. muelleri plants are characterised by their long thin strap-like leaves (Lanyon 1986, Moore and Short 2007) as opposed to the small ovoid leaves of H. ovalis (Lanyon 1986). The greater above ground biomass (Table 4.1) and surface area of Z. muelleri leaves creates more space for N₂-fixing epiphytes to colonise relative to H. ovalis. Additionally, H. ovalis is preferentially grazed by the dugong (Dugong dugon) and green sea turtle over Zostera seagrass species, both of which are active in Moreton Bay (Preen 1995, Brand-Gardner et al. 1999). The Z. muelleri community of Moreton Bay does have a higher plant density than H. ovalis (Table 4.1) with plant density being found related to N₂ fixation in previous studies (Cole and McGlathery 2012, Cook et al. 2015). Conversely, frequent grazing may stimulate N₂ fixation and the rapid shunting of bioavailable N to areas of new growth (Howarth & Marino 2006) which has been observed in Thalassia testudinum after simulated green sea turtle grazing experiments (Moran and Bjorndal, 2005). With a similar response observed in terrestrial grasses with defoliated plants increasing the N allocation to new leaves (Jaramillo and Detling, 1988), although that doesn’t appear to be the case in this H. ovalis community.
4 – Comparison of dinitrogen fixation rates in two subtropical seagrass communities

It is likely that morphological characteristics of *H. ovalis* and *Z. muelleri* impact the variation in N$_2$ fixation of not only the leaves of these species but also in the root/rhizome. The root system of *Z. muelleri* is commonly much deeper and denser with many long (up to 10 cm) thin hair like roots emanating off the length of the rhizome (Lanyon 1986). The root structure of *H. ovalis* is less extensive and shallower relative to *Z. muelleri*, made up of one central root per leaf shoot (Lanyon 1986) covered with microscopic hairs (Roberts 1993). The increased surface area from the small root hairs may explain why root/rhizome fixation was higher over both light and dark conditions in *H. ovalis* compared with *Z. muelleri* (Fig. 4.2). *Halophila ovalis* roots have been observed releasing O$_2$ largely from their root tip (Connell et al. 1999) rather than along the length of their root and rhizome as seen in *Z. muelleri* (Brodersen et al. 2015). This may mean O$_2$-sensitive N$_2$-fixing bacteria (like SRB) can be in closer contact with the root system of *H. ovalis* and thus make the transfer of fixed N to the plant easier. Even when O$_2$ release slows at night a ‘microshield’ of aerobic sediment exists around the fine roots of *Z. muelleri* (Brodersen et al. 2015). This may cause SRB that are within this microshield to be less active in turn limiting N$_2$ fixation supplied to the plant from the sediments directly in contact with the root system. The difference in below ground biomass between the species (Table 4.1) may also be a contributing factor to the difference observed in root/rhizome rates. With *H. ovalis* having a reduced below ground biomass with comparison to *Z. muelleri* this also means less storage capacity for fixed N and potentially less opportunity for transfer of fixed N into the root/rhizome by sediment N$_2$-fixers.

As well as root and rhizome morphology and biomass, the amount of organic C exudates released from the seagrass roots may impact root/rhizome N$_2$ fixation rates. One of the greatest factors limiting rates of N$_2$ fixation in marine sediments is
thought to be the amount of available C (Wetzel and Penhale 1979, Hansen et al. 2000). It may be the case that *H. ovalis* is releasing greater amounts of organic C from its roots which could promote N\(_2\) fixation in small pockets around the roots, stimulating N\(_2\)-fixing bacteria there thus generating a source of easily accessible fixed N to the plant. The idea of bacterial interactions with seagrass root/rhizomes in the rhizosphere is well accepted however very little information on the mechanisms of transfer of fixed N to the plant or the rates of this transfer is available (Welsh 2000). The N\(_2\)-fixers dominant in the *H. ovalis* sediment profile may be more sensitive to the amount of organic C available than those in the *Z. muelleri* sediment. Sulfate reducing bacteria account for almost 50% of organic C oxidation in marine sediments (Jørgensen 1982, Thode Andersen and Jørgensen 1989). The C:N ratio of *Z. muelleri* sediments is higher than that of *H. ovalis* which suggests more labile organic C is available in the *Z. muelleri* rhizosphere (Table 4.1) which may be due in part to greater leaf detritus in the *Z. muelleri* community. The role of SRB and organic C in N\(_2\) fixation could also explain why sediment N\(_2\) fixation rates decreased, albeit minimally, in *H. ovalis* under dark conditions, which contrasts to the significant rise in dark sediment N\(_2\) fixation in *Z. muelleri* (Fig. 4.2, Table 4.2), reflecting the more active SRB in the *Z. mueleiri* sediment than in *H. ovalis*.

The re-oxidation of sulphides leading to increased SRB activity at night may also explain the lower sediment fixation rates in *H. ovalis*, particularly under dark conditions. As O\(_2\) is largely released from the *H. ovalis* root tip there may be more sulphides present around the length of the root as re-oxidation of sulphides to sulphate is occurring largely around the tip. Sulphide concentrations are higher around seagrass roots than in bare sediment due to the release of C exudates from roots (Simpson et al. 2018). Therefore, with higher sulphide concentrations around
the root coupled with the shorter length of *H. ovalis* roots and O\(_2\) being released largely at the tip only, not as much O\(_2\) is penetrating into the surrounding sediment. This means less re-oxidation of sulphide to sulphate which may inhibit SRB activity. A preferential association of SRB with root/rhizome surfaces exists (Isaksen and Finster 1996) and therefore the greater surface area of root material in *Z. muelleri* plants compared with *H. ovalis* may explain the dominant role of SRB as N\(_2\) fixers in the *Z. muelleri* rhizosphere. This indicates sediment fixation is not as critical for *H. ovalis* and perhaps leaf fixation is the main source of fixed N to the plant. The N\(_2\) fixation rates that we have measured in root/rhizome samples of *H. ovalis* may be fixed N transported to the roots from the leaves. It is also possible that any inadvertent root/rhizome severing occurring during the sampling process has altered rates measured there. Another potential source of fixed N in the root and rhizome may be from root-associated endophytes. Root-associated endophytes live within the plant tissue and have been observed in *Posidonia oceanica* via *nifH* gene testing (Garcias-Bonet et al. 2016). These root-associated endophytes may be absent or less prevalent in *Z. muelleri* explaining the lower N\(_2\) fixation rates between the species. Species-specific morphology combined with sediment biogeochemistry can have a significant impact on loci of N\(_2\) fixation activity as well as overall community N\(_2\) fixation rates.

Characterising below ground processes remains challenging and the perfusion method can disrupt micro-zones of N\(_2\) fixation activity. While we tried our best to mitigate destruction of these micro-zones by using a smaller volume of pore water labelling solution we are not able to discern if this was effective or not. Newer methods such as the push-pull piezometer method described by Aoki and McGlathery (2017) may be more successful in characterising rhizosphere processes.
Comparison of dinitrogen fixation rates in two subtropical seagrass communities and can be used in situ. Accurate measurement of below ground processes is important for system biogeochemical measurements and shouldn’t be disregarded by researchers.

Other factors which weren’t included in this study have the potential to impact seagrass $N_2$ fixation, for example the individual microbiomes of the two seagrass stands which could be vastly different between species, the intensity of grazing to each stand and general community composition such as occurrence of macro algae and other fauna which may contribute to greater organic C and N concentrations in either stand. Greater clarity could be discerned on the impact of these factors by comparing independent meadows of seagrass of the same species.

4.4.3 Implications on the system-wide N budget

Using the areas of $Z.\ muelleri$ and $H.\ ovalis$ communities within southern Moreton Bay (Eyre et al. 2011a, Table 4.1) $Z.\ muelleri$ fixes $\sim27$ t of N y$^{-1}$ with $H.\ ovalis$ fixing $\sim5$ t of N y$^{-1}$. Together these two species fix $\sim32$ t of N y$^{-1}$ in the Broadwater area alone. The difference in total areal rates of $N_2$ fixation for each species ($Z.\ muelleri$ $\sim9$ t N km$^{-2}$ yr$^{-1}$ and $H.\ ovalis$ $\sim3$ t N km$^{-2}$ yr$^{-1}$) illustrates how important species morphology can be when scaling up $N_2$ fixation estimates of seagrass. Even though this research was only conducted on incubations during spring we believe that scaling rates up to a yearly estimate illustrates the potential for large differences in N cycling behaviour between seagrass species, the rates we present here are an upper estimate of yearly $N_2$ fixation. Thus considering seagrass species when calculating $N_2$ fixation, and potentially denitrification, for system N budgets that include seagrass communities could lead to significant changes in N estimations.
Chapter 5: Nitrogen loss in two subtropical seagrass communities

This chapter is currently being prepared for journal submission.
Abstract

Seagrasses are important nutrient cyclers in the coastal zone and are especially valuable in regulating bioavailable nitrogen (N) via N loss processes. We investigated N loss and recycling pathways in two morphologically distinct subtropical seagrass species using the isotope pairing technique (IPT) and net fluxes of N\textsubscript{2} measured via membrane inlet mass spectrometry (MIMS). There was a difference in the N cycling pathways between the species, the *Halophila ovalis* community was dominated by anammox (~34 µmol N m\textsuperscript{-2} h\textsuperscript{-1}) and in *Zostera muelleri* by dissimilatory nitrate reduction to ammonium (DNRA) (~56 µmol N m\textsuperscript{-2} h\textsuperscript{-1}). Denitrification rates were low in both systems (~ 5 µmol N m\textsuperscript{-2} h\textsuperscript{-1} and ~3 µmol N m\textsuperscript{-2} h\textsuperscript{-1} for *H. ovalis* and *Z. muelleri* respectively). The high organic content and sulphidic nature of the *Z. muelleri* rhizosphere likely promoted DNRA over anammox and denitrification, whereas the stable nature and low organic content of the *H. ovalis* sediments appear to have promoted anammox at the expense of denitrification. Dinitrogen fluxes measured with MIMS were higher than those measured by IPT but both techniques showed that N loss from the *H. ovalis* system was higher than the *Z. muelleri* community. This research indicates that species-specific seagrass morphology could play a significant role in regulating N processes in seagrass systems.
5.1 Introduction

Seagrass communities are dynamic ecosystems that are susceptible to excess nutrient inputs from ever increasing coastal development. Nitrogen (N) discharge into many of the world’s coastal environments has led to eutrophication of waterways, proliferation of algae and loss of biodiversity (Conley et al. 2009, Canfield et al. 2010). The assimilation and processing of N by seagrass is an important ecosystem service (Costanza et al. 1997), particularly the uptake of ammonium (NH$_4^+$) and nitrate (NO$_3^-$). The primary N loss pathway is often thought to be denitrification which has been studied widely in seagrass communities (Blackburn et al. 1994, Risgaard-Petersen & Ottosen 2000, Zarnoch et al. 2017). However, newer research in seagrass ecosystems has shown there may be other N loss processes operating, such as anammox (Salk et al. 2017) and dissimilatory nitrate reduction to ammonium (DNRA) (Aoki & McGlathery 2018), making N loss difficult to characterise in these systems.

A popular method used to investigate denitrification in seagrass communities is the isotope pairing technique (IPT) (Welsh et al. 2001, Risgaard-Petersen et al. 1998). The IPT uses additions of $^{15}$NO$_3^-$ to quantify the production of $^{15}$N-N$_2$ (Nielsen 1992). Initially the IPT only took into account denitrification, but the discovery of anammox in marine sediments (Thamdrup & Dalsgaard 2002) saw the amendment of the calculation to take into account N$_2$ production from this process (Risgaard-Petersen et al. 2003). Thus previous rates attributed to denitrification may have been overestimated due to the presence of anammox. Since then many researchers have sought to improve the IPT in order to include more competing N process that may affect N loss estimates, including N$_2$O production (Hsu and Kao 2013) and DNRA (Song et al. 2016). The most recent iteration is the R-IPT-DNRA which is the only
5 – Investigating nitrogen loss processes in two subtropical seagrass species
calculation to take into account denitrification, anammox, N₂O production and DNRA
(Salk et al. 2017).

To date no-one has used the R-IPT-DNRA calculation to estimate N loss
between seagrass species. And to our knowledge none have sought to specifically
investigate the impact seagrass morphology may have on rates of N loss over a
diurnal cycle. Seagrass morphology can vary dramatically between species, from
dense meadows of long strap-like leaves (as in *Zostera muelleri*) to small ephemeral
patches of short ovoid leaves (like *Halophila ovalis*) (Lanyon 1986, Moore & Short
2006). Although less obvious, below ground biomass can also differ considerably
between species and arguably this ‘invisible’ component of seagrass morphology has
the greatest impact on rates of N loss.

Denitrification and anammox occur largely in anoxic environments making the
seagrass rhizosphere a likely location for these processes to occur. This can be
attributed to the impact that roots and rhizome of seagrass can have on the
geochemical characteristics of the sediment via release of photosynthetic exudates
such as oxygen (O₂) and organic carbon (C). The release of O₂ from seagrass roots
has been described as an adaptation to protect the plant from toxic sulphides formed
in the sediment (Brodersen et al. 2015, Martin et al. 2018), while organic C can
regulate both anammox and denitrification (Eyre and Ferguson 2002, Chang et al.
2014). In addition, sulphur species are commonly found in shallow marine sediments
with a high organic content and may have an inhibitory effect on anammox (Jensen
et al. 2008).

While denitrification in seagrass systems has been well studied there are very
few studies of anammox and DNRA in seagrass communities (Salk et al. 2017, Aoki
& McGlathery 2017, 2018), and none comparing potential differences between
species. Anammox is well suited to the seagrass rhizosphere especially at night
5 – Investigating nitrogen loss processes in two subtropical seagrass species when a more anoxic environment is created by the diminished release of O₂ exudates from seagrass root and rhizomes. Dissimilatory reduction of nitrate to ammonia is known to be an important estuarine N cycling process (An & Gardner 2002, Dong et al. 2011, Bernard et al. 2015) but has only been measured in a few seagrass systems (Table 1.2) and hasn’t been compared between seagrass species. Sulphate reducing bacteria (SRB) are common dinitrogen fixers in seagrass systems but have also been found to have high DNRA activity, this could lead to competition between denitrifiers and SRB for available NO₃⁻ (Tiedje et al. 1982, Welsh et al. 2001, Erler et al. 2017). Salk et al. (2017) found anammox and DNRA to be dominant in seagrass sediments which may have implications seagrass N budget estimates.

Another commonly used method for measuring N loss is the quantification of N₂ fluxes by membrane inlet mass spectrometry (MIMS). This method uses the change in ratio of dissolved gaseous N₂ to argon (Ar) to calculate a net flux of whole system N₂ loss and uptake (henceforth referred to as the N₂:Ar technique). Denitrification studies in seagrass meadows have been largely split along climatic lines, with most (sub)tropical studies using the N₂:Ar technique (Eyre et al. 2011a, 2013, Eyre & Ferguson 2002). These N₂:Ar estimations often report higher N₂ loss rates than those measured in temperate locations which have overwhelmingly used the IPT (Rysgaard et al. 1996, Welsh et al. 2000). This disparity is often attributed to the idea that the N₂:Ar technique takes into account processes occurring deeper in the rhizosphere which the IPT does not. It is suggested that adding isotopic label only to the surface water of cores only allows superficial penetration of the top few centimetres of the sediment profile (Nielsen 1992). The N₂:Ar technique isn’t without its weaknesses, care must be taken during incubation set up and sample collection as even small O₂ bubble formation can lead to erroneous results (Eyre et al. 2002) making it difficult to use in highly productive communities (Eyre et al. 2013).
In this study we characterised N loss (denitrification and anammox) and N recycling (DNRA) in two subtropical seagrasses (Z. muelleri and H. ovalis) over an artificial diurnal cycle using the R-IPT-DNRA. Additionally we measured N₂ fluxes in both species to ascertain if general N loss trends were consistent with the IPT. We hypothesise that the morphological diversity between the two seagrass species will have an impact on the processes that dominate in each community. Higher sulphate concentration and SRB activity in the Z. muelleri rhizosphere due to greater O₂ release from the roots and rhizome may favour denitrification over anammox, whereas we expect to see an inverse relationship in H. ovalis due to less O₂ release to the rhizosphere.

5.2 Method

5.2.1 Sample collection

Sediment cores (acrylic 46 cm long, internal diameter 9 cm containing ~15 cm of sediment and ~ 36 cm site water) containing whole seagrass plants (Halophila ovalis and Zostera muelleri) were collected from the Broadwater, southern Moreton Bay during spring 2016 (as per Fig 4.1). Moreton Bay is a subtropical embayment located in south-eastern Queensland, Australia. Multiple seagrass species are common in the area which are grazed by marine fauna such as the dugong (Dugong dugong) and green sea turtles. For more information regarding Moreton Bay see (Eyre et al. 2011a, Carlson-Perret et al. 2018). These two seagrass species were chosen for their distinctly different morphology and because they are widespread throughout subtropical regions of the east coast of Australia.

Cores were collected as detailed in Chapter 4, briefly, 9 intact cores were collected per species, 6 designated for N₂ flux measurements and subsequent isotopic label additions to the overlying water (surface cores), with the remaining 3 cores to be sampled for pre-labelling (or background) measurements. Cores were
5 – Investigating nitrogen loss processes in two subtropical seagrass species returned to the lab and pre-incubated for ~24 hours in 100 L chambers filled with site water maintained at in situ temperature (23 ± 1 °C). Oxygenation of water was maintained via aquarium airstones and magnetic stir bars were fitted to approximately 10 cm above the sediment surface and rotated by a central magnet (Ferguson et al. 2003, 2004).

5.2.2 \( \text{N}_2 \) flux measurements

Five surface cores per species were capped in the dark with an additional core per species used to monitor dissolved oxygen (DO). Dark incubations were run prior to light incubations so as to limit the formation of oxygen bubbles (Eyre and Ferguson, 2002). Regular triplicate samples (~every 1.5-2 hours depending on DO in \( \text{O}_2 \) core) were taken for \( \text{N}_2 \) flux measurements in 7 mL gas-tight glass vials which were filled under gravity to overflowing. Samples were immediately poisoned with 20 µL of saturated mercuric chloride solution (\( \text{HgCl}_2 \)) (~8% w/v), capped with glass stoppers without headspace and stored submerged at water regulated to in situ temperature until analysed. Water removed from sealed cores during sampling was immediately replaced via gravity-fed site water from gas-tight bags attached to an inflow port in the lid of each core. Replacement water was kept at the same temperature and under the same conditions (light or dark) as cores being sampled to maintain constant Ar concentration (Eyre and Ferguson 2005, Eyre et al. 2011a).

Samples for dissolved inorganic N (DIN) analysis (\( \text{NO}_x \), as \( \text{NO}_3^- + \text{NO}_2^- \), and \( \text{NH}_4^+ \)) were taken from the water column of sealed cores and filtered (Sartorius 0.45 µm syringe filter, cellulose acetate) into 10 mL polypropylene vials. A small headspace was left to allow for expansion and samples were frozen at -20 °C until analysed. Water removed for nutrient concentrations was replaced by the gas-tight reservoir bags as above.
Oxygen saturation was measured in the dedicated oxygen core at regular intervals (Hach HQ40D meter). When the DO approached ~20% less than its starting concentration lights (sodium Agri-Gro ~ 34 PAR μmol m⁻² s⁻¹) were either turned on (i.e. at the end ending the dark incubation and the commencement of the light incubation) or the cores opened (ending the light incubation). The gravity-fed reservoir bags were disconnected and cores opened and allowed to re-oxygenate for the isotope pairing experiment.

5.2.3 Isotope pairing technique (IPT)

Once DO had increased to ~100% (~2-3 hours) in all 12 cores they were sealed. Isotopic label (10 mL of 10.5 mM K¹⁵N-NO₃⁻ prepared in site water) was added to each core via plastic syringe injection into the inlet port giving a final concentration of the overlying water within the cores of ~50 μM K¹⁵N-NO₃⁻. As label solution was injected into the cores excess overlying water was allowed to flow out of the outlet ports. Six cores (3 per species) were incubated under lights (as per the flux incubations) and an additional 6 cores were double-wrapped in aluminium foil to simulate dark conditions (Welsh et al. 2000, Russell et al. 2016). Dark and light cores were incubated for 6 hours. Once the incubation was complete cores were sacrificed and the water column sampled for DIN and DO. A sub-core (2.3 cm internal diameter, 50 cm long) of the total sediment profile (~15 cm) was taken for DNRA determination. The sub-core was extracted and immediately placed into a 1 L plastic bucket containing ~20g potassium chloride (KCl) in order to form a ~2M solution with the sediment porewater (based on assumed sediment porosity ~18%). The bucket was sealed, agitated and left for ~24 hours. The remainder of the core was slurrified and samples taken for ¹⁵N-N₂ and ¹⁵N-N₂O. Samples for δ¹⁵N-N₂ analysis were taken from the slurrified sediment resuspension into 12 mL glass Exetainers without headspace. Each Exetainer was immediately dosed with 20 μL of saturated (~8%
5 – Investigating nitrogen loss processes in two subtropical seagrass species

\( \text{w/v) HgCl}_2 \) solution as a biocide. A siphon was set up from the core so that slurrified samples could flow directly into 250 mL amber glass bottles without headspace for \( ^{15}\text{N}-\text{N}_2\text{O} \) analysis. Immediately 200 \( \mu \text{L} \) of saturated \( \text{HgCl}_2 \) was added to each bottle to halt microbial activity.

### 5.2.4 Sample analysis

Fluxes of \( \text{N}_2 \) were measured via the change in the ratio of the dissolved gases \( \text{N}_2 \) and \( \text{Ar} \) via MIMS as detailed in Eyre et al. (2002). Concentrations of DIN were analysed via a Lachat QuickChem 8000 four channel Flow Injection Analyser (see Eyre and Pont 2003 for method details, errors and detection limits).

Sub-cores taken for determination of DNRA activity and extracted with 2M KCl were sub-sampled and centrifuged at \( \sim 3000 \text{ rpm for } \sim 10 \text{ minutes} \). The supernatant was collected and filtered through a 0.45 \( \mu \text{m} \) syringe filter (Sartorius – cellulose acetate) and frozen at -20\(^\circ\)C until analysis. Supernatant samples were analysed for \( ^{\delta^{15}}\text{N-}\text{NH}_4^+ \) following the method outlined by Zhang et al. (2007). Briefly, \( \text{NH}_4^+ \) is oxidised to \( \text{NO}_2^- \) and subsequently reduced to \( \text{N}_2\text{O} \). The N isotopic ratio of \( \text{N}_2\text{O} \) is measured by a continuous flow purge and trap system connected to a Thermo Fisher GasBench II in concert with a Thermo Delta V Plus IRMS (analytical precision for this method \( \pm 0.5 \% \)).

Core slurry samples to be analysed for \( ^{30}\text{N}_2 \) and \( ^{29}\text{N}_2 \) were headspaced with 2 mL of helium (He) prior to analysis. Measurement of the \( ^{30}\text{N}_2 \) and \( ^{29}\text{N}_2 \) content of the equilibrated headspace (10 \( \mu \text{L} \) samples) was via a Thermo Trace gas chromatograph Ultra with a 25 m x 0.32 mm PoraPLOT Q column teamed with a Thermo Delta V Plus IRMS (precision \( \pm 0.15 \% \)).

Water samples for \( ^{\delta^{15}}\text{N-N}_2\text{O} \) determination were headspaced with 25 mL of He and analysed via IRMS as per \( ^{\delta^{15}}\text{N-}\text{NH}_4^+ \) analysis minus the chemical reduction steps.
Nitrogen composition (%N) of seagrass leaf and root/rhizome biomass was determined by EA-IRMS on background cores sampled for baseline analysis (precision ± 0.003%) (detailed in Chapter 4).

5.2.5 N\textsubscript{2} flux calculation

Fluxes of N\textsubscript{2} from the seagrass system were calculated by linear regression of the concentration of N\textsubscript{2} and Ar and corrected for the addition of replacement water, as a function of incubation time, core water volume and surface area (Eyre et al. 2011a). Concentration data from the dark part of the incubation was used to calculate dark flux rates while light flux rates were calculated based on data from the light part of the incubation. (Eyre et al. 2011a). Net rates were calculated from the beginning and end of the incubation as a whole i.e. the first flux measurements taken at the beginning of the dark incubation and the last measurement taken in the light.

5.2.6 IPT calculation

Rates of denitrification, anammox and DNRA were calculated using the modified IPT calculation R-IPT-DNRA detailed in Salk et al. (2017) which takes into consideration NO\textsubscript{3}- reduction pathways other than denitrification, including N\textsubscript{2}O production, anammox and DNRA.

5.2.7 Statistical analysis

Due to unequal variance and often small sample size (n=3) ANOVA could not be reliably used to determine the significance between rates of denitrification, anammox and DNRA between both seagrass species. Instead two-tailed t-tests assuming unequal variance were used at p < 0.05 to determine significance.
Figure 5.1: Rates of denitrification (a.), anammox (b.) and DNRA (c.) in seagrasses *Halophila ovalis* and *Zostera muelleri* under both light and dark conditions (mean ± SD, n=3).
5.3 Results

5.3.1 IPT rate calculations

Rates of denitrification in both species were similar, low and didn’t change significantly over either light or dark conditions (Fig. 5.1a, Table 5.1). Anammox dominated N loss in *Halophila ovalis* and was 6 times higher at night than those measured under light conditions, $58.25 \pm 90.11 \, \mu\text{mol} \, \text{N} \, \text{m}^{-2} \, \text{h}^{-1}$ and $9.55 \pm 1.24 \, \mu\text{mol} \, \text{N} \, \text{m}^{-2} \, \text{h}^{-1}$ respectively. Rates of anammox in the *Zostera muelleri* community were lower than denitrification and were similar over light and dark (Fig. 5.1b). The *Z. muelleri* community was dominated by DNRA which was not statistically significantly different during the light and dark (Fig. 5.1c, Table 5.1).

5.3.2 N$_2$ fluxes

There was uptake of N$_2$ in the *Z. muelleri* community under light conditions while *H. ovalis* produced N$_2$ in the light (Fig. 5.2). Both communities produced N$_2$ under dark conditions (Fig. 5.2). Diel fluxes of N$_2$ showed the *H. ovalis* community was a net producer of N$_2$ and *Z. muelleri* a net consumer (Fig. 5.2).

5.3.3 Nutrient fluxes

There was uptake of NH$_4^+$ in the *Z. muelleri* community and small fluxes of NH$_4^+$ in the *H. ovalis* system (Fig. 5.3a). Both species however, were net producers of NOx ($\text{NO}_3^- + \text{NO}_2^-$) (Fig. 5.3b).
Table 5.1 Results of t-tests where results in bold are significant at \( p < 0.05 \).

<table>
<thead>
<tr>
<th></th>
<th>Denitrification</th>
<th>Anammox</th>
<th>DNRA</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>H. ovalis(^{a,b})</strong></td>
<td>( t(2) = -0.13, p = 0.91 )</td>
<td>( t(2) = -0.94, p = 0.45 )</td>
<td>( t(2) = 0.95, p = 0.44 )</td>
</tr>
<tr>
<td><strong>Z. muelleri(^{a,b})</strong></td>
<td>( t(4) = -1.95, p = 0.19 )</td>
<td>( t(4) = 1.73, p = 0.16 )</td>
<td>( t(2) = -1.36, p = 0.31 )</td>
</tr>
<tr>
<td><strong>H. ovalis(^{a}) &amp; Z. muelleri(^{a})</strong></td>
<td>( t(2) = 4.64, p = 0.04 )</td>
<td><strong>( t(2) = 12.3, p = 0.0066 )</strong></td>
<td>( t(2) = 0.67, p = 0.57 )</td>
</tr>
<tr>
<td><strong>H. ovalis(^{b}) &amp; Z. muelleri(^{b})</strong></td>
<td>( t(3) = 0.30, p = 0.78 )</td>
<td>( t(2) = 1.12, p = 0.38 )</td>
<td>( t(3) = -1.83, p = 0.16 )</td>
</tr>
<tr>
<td><strong>H. ovalis(^{c}) &amp; Z. muelleri(^{c})</strong></td>
<td>( t(8) = 1.22, p = 0.26 )</td>
<td>( t(5) = 1.31, p = 0.25 )</td>
<td>( t(7) = -0.22, p = 0.83 )</td>
</tr>
</tbody>
</table>

\(^{a}\) - Light N\(_2\) fixation rates  
\(^{b}\) - Dark N\(_2\) fixation rates  
\(^{c}\) - Total diel N\(_2\) fixation rates
5.3.4 Biomass N composition

Analysis of N composition (%N) of seagrass above and below ground biomass showed the *Z. muelleri* system had a higher %N in the leaves and almost double the %N of *H. ovalis* (Table 5.2).
5.4 Discussion

5.4.1 Results of R-IPT-DNRA

Denitrification rates measured in both *Zostera muelleri* and *Halophila ovalis* were similar across the species and are in the range of rates measured by IPT in previous seagrass experiments (Risgaard-Petersen and Ottosen 2000, Welsh et al. 2001, Table 1.1), and in subtropical sediments (Dunn et al. 2012, Salk et al. 2017). What was surprising was that although the seagrass communities were located relatively close to one another (Fig. 4.1) they were dominated by different N loss pathways.

Nitrogen loss from *H. ovalis* was dominated by anammox with dark rates ~6 times higher than those measured under light conditions (Fig. 5.1b). This spike in anammox activity at night is likely due to lower O\textsubscript{2} concentrations in the rhizosphere than during the day. Seagrass roots excrete photosynthetically derived O\textsubscript{2} thus at night the drop in O\textsubscript{2} release causes a shift in the oxic status of the rhizosphere. Anammox is a largely anaerobic process however, therefore its occurrence in the light in *H. ovalis* suggests anaerobic micro-zones exist in sediments in the day. Conversely, no real discernible anammox activity was observed in the *Z. muelleri* community over either light or dark conditions. The lack of anammox in the *Z. muelleri* community suggests there are different geochemical conditions within the rhizosphere of the two seagrass species. Sulphidic compounds, commonly found in shallow marine sediments are known to inhibit anammox activity (Jensen et al. 2008, Thamrup 2012). It may be that concentrations of sulphide are higher in the *Z. muelleri* rhizosphere as compared with the *H. ovalis* community which could be inhibiting anammox activity in the *Z. muelleri* community.
Figure 5.3 Fluxes of ammonium (a.) and NOx (nitrate + nitrite) (b.) from cores containing seagrass plants (*Halophila ovalis* and *Zostera muelleri*) under both light and dark conditions (mean ± SD, n=3)
This is consistent with Chapter 4 where a spike in N\textsubscript{2} fixation rates at night was observed in the sediment of \textit{Z. muelleri} and not in \textit{H. ovalis}. This difference was suggested to be caused by higher activity of SRB due partly to re-oxidation of sulphides as a result of O\textsubscript{2} release from roots. The availability of sulphide in the rhizosphere is a result of higher availability of organic C in the \textit{Z. muelleri} rhizosphere, shown by the higher sediment %C reported in this system compared with the \textit{H. ovalis} community (Table 4.1). The presence of sulphide in coastal sediments is thought to be the reason why anammox is more common in deeper water sediments (Jensen et al. 2008, Trimmer and Nichols 2009, Thamdrup 2012). However, high anammox rates in shallow marine sediments are being increasingly observed, with between 0-86\% of N\textsubscript{2} loss being attributed to anammox in coastal areas (Engström et al. 2005, Rich et al. 2008, Salk et al. 2017), although rates are highly variable. Anammox rates measured by Salk et al. (2017) on bare sediments colonised by \textit{Z. muelleri} are in a similar range to our rates which is likely due to sulphide concentrations increased by the greater organic matter content present in the generally denser \textit{Z. muelleri} system compared with the ephemeral \textit{H. ovalis}, with increases in organic matter known to stimulate N\textsubscript{2} loss (Babbin et al. 2014). Differences in how \textit{H. ovalis} and \textit{Z. muelleri} release O\textsubscript{2} into the rhizosphere (discussed in Chapter 4) coupled with the sulphidic sediments and higher organic C content of the \textit{Z. muelleri} community may explain the difference in rates of anammox observed between the two species.

This lack of appreciable anammox in \textit{Z. muelleri} during both light and dark incubations is surprising given that rates of denitrification were also minimal, and comparable with that of \textit{H. ovalis}. The rates of N\textsubscript{2} fixation in these same species were compared in Chapter 4 and \textit{Z. muelleri} was found to have fixed \textasciitilde3 times the amount of N\textsubscript{2} as compared with \textit{H. ovalis}. This indicates that there must be other
processes utilising the fixed N in the *Z. muelleri* community as the majority of it is not being released as N\(_2\) via denitrification or anammox.

Rates of DNRA were not significantly different between the two seagrass communities. The significant DNRA rates observed in both species is likely due to the high amount of organic C present in seagrass systems from root exudates as well as plant detritus, which favours DNRA over denitrification in NO\(_3^-\) limited systems (Tiedje et al. 1982, Burgin & Hamilton 2007, Kraft et al. 2014, van den Berg et al. 2015). Increases in DNRA rates, at the expense of denitrification, was observed in *Z. muelleri* sediment amended with seagrass detritus (Salk et al. 2017). The increased sulphide concentration and activity of SRB in the *Z. muelleri* rhizosphere can also stimulate DNRA, as sulphide acts as an electron donor in the transformation of NO\(_3^-\) to NH\(_4^+\) (An & Gardner 2002) and SRB has been shown to have DNRA activity (Tiedje et al. 1982, Welsh et al. 2001). Although colonised by the same species of seagrass and located only ~150 km south of our field site the rates of DNRA obtained from bare sediments by Salk et al. (2017) are ~140 times less than ours. This suggests that including whole seagrass plants in estimations of N processes can make a substantial difference to rate estimates, and that seagrass morphology, especially below ground biomass, can have a significant impact on the biogeochemical gradients affecting N cycling. A potential explanation for the lack of N\(_2\) export seen in our IPT experiment is the fact that we only measured rates based on surface label additions, is advisable to remember that when adding labelled \(^{15}\)N species to the overlying water column in a closed system such as core incubations that only superficial sediment processes will be captured and not those deeper within the rhizosphere (Nielsen 1992). Therefore N\(_2\) producing processes, like anammox and denitrification, may be occurring deeper within the sediment profile but weren’t captured in this experiment. Perfusing the sediment with a \(^{15}\)NH\(_4^+\) tracer would serve
to capture deeper rhizosphere processes without over-stimulating N cycling.

Characterising rhizosphere N loss and recycling rates could impact the estimates observed in surface-only additions.

What is curious about the high DNRA rates in the *Z. muelleri* community is that if no real export of N\(_2\) is occurring from the system (lack of denitrification and anammox) and we know there are high rates of N\(_2\) inputs (~38 ± 28 µmol N m\(^{-2}\) h\(^{-1}\), Chapter 4) then what is happening to this bioavailable N? Fluxes of NH\(_4^+\) and NO\(_x\) also do not account for these large inputs, with only low export of NH\(_4^+\) and NO\(_x\) across the two species (Fig. 5.3). Therefore it is likely that much of this excess N is being re-assimilated by the *Z. muelleri* community and incorporated into its biomass.

When total %N of seagrass biomass was compared between the two species *Z. muelleri* had almost 2 times the %N of *H. ovalis*. It is likely that the need for N of *Z. muelleri* outstrips that of *H. ovalis* due to its larger denser meadows and its greater above ground biomass (Table 4.2). This greater need for N coupled with the greater organic C from larger amounts of year-round detritus as compared and sulphidic content of the rhizosphere means the *Z. muelleri* community is well suited to favour DNRA.

5.4.2 N\(_2\) fluxes

Fluxes of N\(_2\) show a similar relationship to those measured by the IPT in the *H. ovalis* community, with higher rates (Fig. 5.2). Under both light and dark conditions there was a net efflux of N\(_2\) from the *H. ovalis* system, which also agrees with the *H. ovalis* N\(_2\) fixation rates (Chapter 4) where low N\(_2\) fixation in was observed in this community. The *H. ovalis* community fixed less N\(_2\) than *Z. muelleri* and N\(_2\) uptake shows that in the light the *Z. muelleri* system is consuming N\(_2\). What is surprising however is that dark N\(_2\) fluxes were higher in *Z. muelleri* than *H. ovalis*. Higher net N\(_2\) fluxes were also observed in N\(_2\):Ar measurements by Eyre et al. (2011a) between *H. ovalis* and *Z. muelleri*.
5 – Investigating nitrogen loss processes in two subtropical seagrass species *H. ovalis/spinulosa* and *Z. capricorni* (analogous with *Z. muelleri*). This is not observed in the IPT with N loss via denitrification being minimal and on par with *H. ovalis* and anammox being almost non-existent in the *Z. muelleri* system. The differences seen in the N₂ loss estimates between the two methods is likely due to the fact that the N₂:Ar method takes into consideration the whole closed system i.e. fluxes from the whole core including the sediment and plant. The IPT however only characterises N process in the top few centimetres of sediment when label is added to the surface water of the core. Thus it is possible that the higher efflux of N₂ in *Z. muelleri* during the night is from denitrification and/or anammox occurring deeper within the sediment profile that was not captured via the IPT. As well as differences in the patterns of N₂ production, the fluxes measured via the N₂:Ar technique were higher across both species than rates we measured with the IPT, again this may be related to the superficial nature of surface label additions. The N₂ flux data presented here suggests that *Z. muelleri* is a net consumer of N₂ fixation which fits with the high N₂ fixation rates we measured (which also included the rhizosphere) previously, coupled with the rates of DNRA and minimal N₂ loss processes we measured via the IPT. Conversely *H. ovalis* seems to be a net producer of N₂ and N₂ fixation rates measured in the *H. ovalis* community were much lower (~3 times) than in the *Z. muelleri* community (Chapter 4). As discussed in Chapter 4 much of the difference in N₂ fixation rates between the two seagrasses could be attributed to their morphological differences and the effect these physical characteristics had on the biogeochemical processes in the rhizosphere. It follows that N loss is also effected by species-specific morphology which does seem to be evident in this instance with both the IPT and N₂:Ar methods showing marked differences in N loss between the species.
In terms of future research into seagrass N loss processes it would be pertinent to undertake the IPT (using the amended R-IPT-DNRA) with both surface and below ground additions of label in conjunction with the N₂:Ar method to see if the divide between rates obtained via each technique can be breached. As mentioned previously perfusing the sediment with $^{15}$NH$_4^+$ would serve to characterise N cycling processes deeper within the rhizosphere that additions to the surface water do not capture. Aoki and McGlathery (2018) measured much higher rates of DNRA and denitrification in a Zostera marina community (Table 1.1, 1.2) with their push-pull perfusion technique, where the seagrass rhizosphere was labelled with $^{15}$NH$_4^+$ in situ. In fact their measurements of these processes without label additions were closer to those we present here (Aoki and McGlathery 2018), and illustrates the impact rhizosphere processes can have on total N loss estimates. Additionally, factors outside the morphology and geochemistry of the particular seagrass stands investigated may have an impact on the rates measured here. For example, it may be pertinent to investigate the microbiome of seagrasses studied in future experiments to determine the distribution of microorganisms known to facilitate N loss processes between different stands of the same seagrass species. This may be particularly useful in determining why there was such a marked increase in anammox rates between the species and whether it was a function of the individual seagrass microbiome and not related to changes in geochemistry related to differing seagrass morphology. In that way the true impact of morphology, local geochemistry and differences in individual microbiomes could be further clarified.

5.4.3 Conclusions

This research clearly illustrates the difference in N loss and recycling processes between seagrass species from the same location can be significant. Morphological features such as root/rhizome density can have a significant impact on
Investigating nitrogen loss processes in two subtropical seagrass species

the geochemical gradients in the rhizosphere via their exudates, especially O₂ and sulphur concentrations, influencing the dominance of one N loss pathway over another. We found that anammox dominates in the *H. ovalis* community because of lower sulphide and organic C concentrations. While the *Z. muelleri* community is dominated by DNRA because of its highly sulfidic rhizosphere and activity of SRB which outcompete denitrifiers for NO₃⁻. The higher organic C load in the *Z. muelleri* community combined with its increased N requirement compared with *H. ovalis* due to its larger biomass density explains its role as a net importer of N.
Chapter 6: Summary, synthesis and conclusions.
6 – Summary, synthesis and conclusions.

This Chapter provides a summary of the work of the previous chapters, the objectives accomplished and conclusions reached. An N budget for Morton Bay utilising the data of Chapters 4 and 5 and a conceptual diagram is also presented.

6.1 Moreton Bay seagrass N budget

Constructing separate N budgets for each of the two seagrass communities (Zostera muelleri and Halophila ovalis) from data presented in Chapters 4 and 5 revealed distinct differences between them. The H. ovalis system was found to be a net exporter of N (~ 20 µmol N m\(^{-2}\) h\(^{-1}\)) while Z. muelleri was found to be an N sink (~73 µmol N m\(^{-2}\) h\(^{-1}\)) (Table 6.1). This suggests that conclusions made in Chapters 4 and 5 regarding morphological characteristics of seagrass potentially having a significant impact on the way N is cycled, are indeed correct. The individual seagrass N budgets were calculated by adding the N loss (anammox and denitrification) rates obtained via IPT in Chapter 5 and subtracting them from the N input (N\(_2\) fixation) rates reported in Chapter 4. This was then compared to the net N\(_2\) fluxes presented in Chapter 5 and all presented in Table 6.1. The N deficit in each community cannot be made up by net N\(_2\) fluxes in Z. muelleri and is actually higher in H. ovalis (Table 6.1) and there would be some overlap between rates measured via IPT and N\(_2\):Ar. In the Z. muelleri system extra N may be used to support the greater biomass of this community as discussed in Chapter 5. Fluxes of DIN (Fig. 5.3) and measurements of DNRA (Fig. 5.1c) do not explain the deficits in each system. It is difficult to know whether these fluxes are from N cycling processes like N\(_2\) fixation or decomposition of organic matter in the sediment. These measurements were taken only in spring and N cycling behaviour changes due to seasonality (Table 1.1, 3.1) may alter these estimations.

Even when combining the budgets of the two systems, there was still an
overall N input of ~ 54 µmol N m$^{-2}$ h$^{-1}$. Spring is a common time of growth and reproduction which may explain why *Z. muelleri* has such a high N requirement, it's higher above and below ground biomass may also be why the *Z. muelleri* community requires more N than *H. ovalis*.

Due to the research presented in this project being focused on the microbiologically and geochemically mediated short-term processes of N loss and import in seagrass communities unfortunately other potentially important factors were not investigated. For example N loss through burial, seagrass death and detrital matter, and the impact of macro faunal grazing were not explored. Nitrogen burial measured in warm temperate Australian *Z. capricorni* and *H. ovalis* communities ranged from 22 - 65 µmol N m$^{-2}$ h$^{-1}$ and 11 - 29 µmol N m$^{-2}$ h$^{-1}$ (Eyre et al 2016a). Growth rates of *Z. capricorni* measured over spring-summer in Moreton Bay possibly contributing ~13 µmol N m$^{-2}$ h$^{-1}$ from leaves and 12 µmol N m$^{-2}$ h$^{-1}$ from root/rhizome growth (Udy and Dennison 1997), with rates expected to slow over the autumn/winter period. Consequently these processes may contribute significantly to N loss and import measured in these seagrass systems and aid in closing the deficits observed in each species budget.

6.2 Summary and synthesis

*Chapter 2: Perfusion method development.*

In this chapter a brief overview of the types of perfusion methods was presented along with details of the iterations of those trialled for this research. The method of using either Macro Rhizon pore water samplers or long large bore needles to inject $^{15}$N-N$_2$ labelled solution through ports into the rhizosphere was found to be successful in uniformly labelling the seagrass rhizosphere as determined by distribution of the conservative tracer LiCl.
Although this method may not completely mitigate the destruction of micro-zones of N\textsubscript{2} fixation activity in the rhizosphere (via inundation of areas of low NH\textsubscript{4}\textsuperscript{+} with solution of higher NH\textsubscript{4}\textsuperscript{+} from the surrounding sediment). It is the first intact core incubation technique to use the \textsuperscript{15}N-N\textsubscript{2} dissolution technique to characterise seagrass rhizosphere N\textsubscript{2} fixation.

Chapter 3: Dinitrogen fixation rates in a subtropical seagrass meadow measured with a direct \textsuperscript{15}N-N\textsubscript{2} tracer method.

In Chapter 3 the perfusion technique detailed in Chapter 2 was used in addition to surface \textsuperscript{15}N-N\textsubscript{2} label additions to characterise above and below ground N\textsubscript{2} fixation in a Z. muelleri community. The use of the \textsuperscript{15}N-N\textsubscript{2} dissolution method in concert with the Macro Rhizon-delivered perfusion method meant active regions of N\textsubscript{2} fixation within the seagrass community could be elucidated. The leaves of Z. muelleri and their associated epiphytes were found to dominate N\textsubscript{2} fixation rate estimates (Fig. 6.1) in contrast with many studies that show the rhizosphere as the dominant region for N\textsubscript{2} fixation activity in seagrasses (Table 3.1). In fact, sediment N\textsubscript{2} fixation rates were higher than those of the root/rhizome material even when \textsuperscript{15}N-N\textsubscript{2} was added directly into the rhizosphere. This suggests that under light conditions bioavailable N from N\textsubscript{2} fixation occurs largely in the leaves and is distributed around the plant. The fact that this experiment was conducted only in light conditions, in one location and on one seagrass species meant that these findings could not be applied to other subtropical species existing in other locations, or activity at night. The results of this experiment were integral in forming the basis of those that followed in Chapters 4 and 5.

This experiment did illustrate the useful application of the \textsuperscript{15}N-N\textsubscript{2} dissolution method for measuring N\textsubscript{2} fixation estimates in seagrass systems, most previous
Table 6.1: The average hourly rates of N either into (input) or out of (export) the *Halophila ovalis* and *Zostera muelleri* seagrass systems of southern Moreton Bay.

<table>
<thead>
<tr>
<th></th>
<th>Input (µmol N m(^{-2}) h(^{-1}))</th>
<th>Export (µmol N m(^{-2}) h(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Halophila ovalis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(N_2) fixation</td>
<td>16.275</td>
<td>Denitrification</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Anammox</td>
</tr>
<tr>
<td>Total</td>
<td>16.275</td>
<td>Total</td>
</tr>
<tr>
<td>Deficit</td>
<td>19.93</td>
<td></td>
</tr>
<tr>
<td>Net (N_2) Flux</td>
<td>+88.374</td>
<td></td>
</tr>
<tr>
<td><strong>Net N Export</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Zostera muelleri</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(N_2) fixation</td>
<td>74.93</td>
<td>Denitrification</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Anammox</td>
</tr>
<tr>
<td>Total</td>
<td>74.93</td>
<td>Total</td>
</tr>
<tr>
<td>Deficit</td>
<td>73.144</td>
<td></td>
</tr>
<tr>
<td>Net (N_2) Flux</td>
<td>-36.34</td>
<td></td>
</tr>
<tr>
<td><strong>Net N Input</strong></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Unraveling the complexity of subtropical seagrass nitrogen cycling: a stable isotope approach

Studies have used the ARA (Table 3.1). The direct nature of this method removes the issues associated with the indirect ARA and may explain some of the variability seen across previous measurements.

Chapter 4: Comparison of dinitrogen fixation rates in two subtropical seagrass communities.

This chapter builds on the work of the preceding two chapters in that Chapter 4 investigates the N₂ fixation of two subtropical seagrass species, *H. ovalis* and *Z. muelleri*. Intact sediment cores containing whole seagrass plants were collected from Moreton Bay located ~150 km north of Shaws Bay (the location of the *Z. muelleri* meadow sampled in Chapter 3). The major premise behind this chapter was to investigate if different morphological characteristics impacted seagrass N₂ fixation activity over dark and light conditions. *Halophila ovalis* and *Z. muelleri* differ greatly in their above and below ground biomass, especially leaf surface area which, from the results of Chapter 3, could significantly impact their rates of N₂ fixation. The inclusion of *Z. muelleri* in this experiment was to determine if there had been any significant location effects associated with Shaws Bay that may have influenced N₂ fixation activity in that particular stand however, rates between the two locations were comparable.

The use of the ¹⁵N-N₂ dissolution method via label additions to the surface and rhizosphere meant that a clearer picture of N₂ fixation and zones of activity could be ascertained. Species-specific morphology had a significant impact on loci of N₂-fixing activity, while leaf fixation still dominated in both species it was significantly higher in *Z. muelleri* which forms a much denser meadow with greater leaf surface area for epiphyte colonisation than the ephemeral *H. ovalis*. The inclusion of rates under dark conditions revealed that below ground dark activity is different between the two...
species likely due to their differing root/rhizome morphology and how O$_2$ is released into the respective rhizospheres. The spike in $Z. \text{muelleri}$ sediment dark rates was unexpected and served to illustrate that not all seagrass N$_2$ fixation activity occurs in the same loci. This chapter illustrated how different N$_2$ fixation rates can be between species and could impact how local estuarine and marine N budgets are calculated.

**Chapter 5: Nitrogen loss processes in two subtropical species**

Chapter 5 aims to ‘close the loop’ of N cycling in seagrasses by characterising N loss processes in the two seagrass species from Chapter 4, *H. ovalis* and *Z. muelleri*. The measurement of N loss via the most recent amendment of the IPT (R-IPT-DNRA) has not been used on intact cores containing seagrass plants and allows the calculation of N loss rates accounting for denitrification, anammox, N$_2$O production and N recycling by DNRA. Again significant differences in these rates were seen between the species suggesting morphological characteristics impacted the preference for one N loss process over another. Anammox was the dominant N loss process in *H. ovalis* which was basically non-existant in the *Z. muelleri* community which was dominated by DNRA. The difference in root/rhizome characteristics is thought to be one of the major factors affecting the presence of one process over another. In Chapter 4 the dominant N$_2$-fixers in the *Z. muelleri* rhizosphere was suggested as being SRB, the higher sulphate concentrations in the *Z. muelleri* community could be suppressing anammox activity. The higher N content of the *Z. muelleri* system and its higher N demand due to its dense above and below ground biomass is suggested as the reason that rates of N loss are much lower in this system.

The use of the N$_2$:Ar method to confirm relationships of N loss between the species illustrated that *Z. muelleri* was indeed a net consumer of N while *H. ovalis*...
was a net producer. The difference in rates between the IPT and N\textsubscript{2}:Ar are due to
the fact that the N\textsubscript{2} flux rates are based on net whole system measurements which
may take into account processes occurring deeper within the rhizosphere and the
IPT possibly not infiltrating micro-zones of N loss activity deeper within the
rhizosphere. The N\textsubscript{2}:Ar technique however doesn’t distinguish between the individual
N\textsubscript{2} production processes, or where other processes like DNRA may be present.

6.3 Future directions

Temperate seagrass systems are often limited by different nutrients than their
subtropical and tropical counterparts and may cycle N differently as a result. Thus
using the techniques employed in this research do determine N\textsubscript{2} fixation (\textsuperscript{15}N-N\textsubscript{2}
dissolution and perfusion) and N loss (R-IPT-DNRA as well as net N\textsubscript{2} fluxes) could
elucidate potentially different active zones in temperate seagrass communities and N
cycling pathways than those investigated here. Generalised changes to seagrass N
cycling and N budgets cannot be made until temperate seagrasses are also
investigated.

Including perfusion with \textsuperscript{15}NH\textsubscript{4}\textsuperscript{+} in the measurement of N loss via the R-IPT-
DNRA would be valuable in ascertaining if the gaps between the IPT and N\textsubscript{2}:Ar N
loss rates can be met. Ideally if these measurements could be undertaken in situ it
would likely remove many of the potential issues of disturbances of micro-zones
within the sediment as well as possibly providing rates closer to that of actual insitu
processes. Some work on in situ seagrass N cycling is being done with \textsuperscript{15}N isotopic
techniques (Aoki & McGlathery 2017, 2018) so far however, these have largely
investigated N loss processes (denitrification and DNRA). Aoki and McGlathery’s
(2017,2018) use of mini piezometers may not be suited to N\textsubscript{2} fixation estimates as
leaves are not able to be enclosed and thus leaf + epiphyte mediated N$_2$ fixation is not captured.

Additionally it would be interesting to explore further the interplay between sulphides, SRB activity, O$_2$ and organic C on N loss processes in the seagrass rhizosphere particularly anammox and DNRA. To more clearly understand the role of these chemical and biological factors and how they drive or inhibit particular N pathways. As mentioned in Chapter 5, the local microbiology of individual seagrass stands has the potential to impact rates of N loss and N import. Deeper investigation into the true influence of the seagrass microbiome on these processes may be achieved by investigating individual seagrass communities of the same species in different locations. This would aid in clarifying the differences in rates attributed to differences in morphology and local geochemistry. In this way comparing seagrass communities in different locations may also aid in clarifying the impact of local macrofaunal grazing.
Figure 6.1: Conceptual diagram illustrating the main findings of this research, the differences in N cycling between *Halophila ovalis* and *Zostera muelleri* communities in Moreton Bay. Arrow size indicates the amount of N transported by that process. Where SRB; sulphate reducing bacteria, OM; organic matter, NOx; nitrate + nitrite and DNRA; dissimilatory nitrate reduction to ammonium.
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Erler DV, Eyre BD, Davison L (2008) The contribution of anammox and denitrification to sediment N\textsubscript{2} production in a surface flow constructed wetland. Environmental science & technology 42:9144-9150


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Unraveling the complexity of subtropical seagrass nitrogen cycling: a stable isotope approach
References

Koop-Jakobsen K, Giblin AEJL, Methods O (2009) New approach for measuring denitrification in the rhizosphere of vegetated marsh sediments. 7:626-637


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References


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Appendices

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Appendices

Appendix 1

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Appendices

Appendix 2

The following people and institutions contributed to the publication of work undertaken as part of this thesis:

<table>
<thead>
<tr>
<th>Author 1 (Candidate)</th>
<th>Netasha L. Carlson-Perret, Southern Cross University</th>
</tr>
</thead>
<tbody>
<tr>
<td>Author 2 (Primary supervisor)</td>
<td>Dr. Dirk V. Eriker, Southern Cross University</td>
</tr>
<tr>
<td>Author 3 (Supervisor)</td>
<td>Prof. Bradley D. Eyre, Southern Cross University</td>
</tr>
</tbody>
</table>

Authorship details;


Located in Chapter 3.

Candidate contribution: The candidate was the primary author and with author 2 and 3 contributed to the conception and design of the research project. The candidate collected the data, was primarily responsible for sample and data analysis, with guidance from authors 2 and 3 and prepared the first draft of the manuscript. Overall the candidate contributed ~60% to the planning, execution and preparation of the work for the paper.

Author 2: Contributed to the project planning, fieldwork and guidance in data analysis and edited the manuscript.

Author 3: Contributed to the project funding and initial design and development and edited the manuscript.

We the undersigned agree with the above stated ‘proportion of work undertaken’ for the above published peer-reviewed manuscripts contributing to this thesis:

Signed Author 1 (Candidate):

Signed Author 2:

Signed Author 3:
Appendices

Appendix 3

The following people and institutions contributed to the publication of work undertaken as part of this thesis:

<table>
<thead>
<tr>
<th>Author 1 (Candidate)</th>
<th>Natasha L. Carlson-Perret, Southern Cross University</th>
</tr>
</thead>
<tbody>
<tr>
<td>Author 2 (Primary supervisor)</td>
<td>Dr. Dirk V. Erler, Southern Cross University</td>
</tr>
<tr>
<td>Author 3 (Supervisor)</td>
<td>Prof. Bradley D. Eyre, Southern Cross University</td>
</tr>
</tbody>
</table>

Authorship details:

Carlson-Perret NL, Erler DV, Eyre BD Comparison of dinitrogen fixation rates in two subtropical seagrass communities, submitted/reviewed to Marine Chemistry

Located in Chapter 4.

Candidate contribution: The candidate was the primary author and with author 2 and 3 contributed to the conception and design of the research project. The candidate collected the data, was primarily responsible for sample and data analysis, with guidance from authors 2 and 3 and prepared the first draft of the manuscript. Overall the candidate contributed ~60% to the planning, execution and preparation of the work for the paper.

Author 2: Contributed to the project planning, fieldwork and guidance in data analysis and edited the manuscript.

Author 3: Contributed to the project funding and initial design and development and edited the manuscript.

We the undersigned agree with the above stated ‘proportion of work undertaken’ for the above submitted and peer-reviewed manuscripts contributing to this thesis:

Signed Author 1 (Candidate)

Signed Author 2:

Signed Author 3
Appendix 4

Supplementary materials – Chapter 3

Table S.1: Average $\delta^{15}$N ‰ of the Cambridge isotope $^{15}$N-$^2$N gas (n=6) used in this study, the IAEA N1 (n=3) standard and a Sigma gas (n=6) not used in this project.

<table>
<thead>
<tr>
<th></th>
<th>Average $\delta^{15}$N ‰</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cambridge $^{15}$N-$^2$N (lot no. I-19168A)</td>
<td>-5.13</td>
<td>0.80</td>
</tr>
<tr>
<td>N-1 standard (IAEA +0.43 ‰air$^2$N ± 0.2 SD)</td>
<td>0.43</td>
<td>2.12</td>
</tr>
<tr>
<td>Sigma $^{15}$N-$^2$N (lot no. MBBB0968V)</td>
<td>70678.13</td>
<td>20350.012</td>
</tr>
</tbody>
</table>

Table S.2: Concentration of lithium (Li$^+$) in each labelled perfusion core sediment fraction. Fractions 9-13 and 13-15 cm were combined as one fraction when calculating $^2$N fixation rates. Where sample ID is as follows e.g. P.T1.1 corresponds to perfusion time 1. Core1. Dashes indicate samples that were lost and not measured.

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Li$^+$ concentration mg L$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0-1 cm</td>
</tr>
<tr>
<td>P.T1.1</td>
<td></td>
</tr>
<tr>
<td>P.T1.2</td>
<td>0.284</td>
</tr>
<tr>
<td>P.T1.3</td>
<td>0.244</td>
</tr>
<tr>
<td>P.T2.1</td>
<td>0.279</td>
</tr>
<tr>
<td>P.T2.2</td>
<td>0.245</td>
</tr>
<tr>
<td>P.T2.3</td>
<td>0.209</td>
</tr>
</tbody>
</table>
Table S.3: Background atom percent and $\delta^{15}\text{N}$ ‰ values for each locus used to calculate $\text{N}_2$ fixation. All values are averages (n=3).

<table>
<thead>
<tr>
<th>Loci</th>
<th>Average Atom %</th>
<th>SD</th>
<th>Average $\delta^{15}\text{N}$ ‰</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Surface leaves</td>
<td>0.36886</td>
<td>0.00021</td>
<td>7.00</td>
<td>0.59</td>
</tr>
<tr>
<td>Surface root/rhizome</td>
<td>0.36884</td>
<td>0.00033</td>
<td>6.94</td>
<td>0.90</td>
</tr>
<tr>
<td>Surface sediment (0-1 cm)</td>
<td>0.36812</td>
<td>0.09203</td>
<td>4.99</td>
<td>0.50</td>
</tr>
<tr>
<td>Surface sediment (1-2 cm)</td>
<td>0.36809</td>
<td>0.00013</td>
<td>4.90</td>
<td>0.35</td>
</tr>
<tr>
<td>Surface sediment (2-3 cm)</td>
<td>0.36766</td>
<td>5.11399E-05</td>
<td>3.71</td>
<td>0.14</td>
</tr>
<tr>
<td>Surface sediment (3-4 cm)</td>
<td>0.36768</td>
<td>0.00023</td>
<td>3.76</td>
<td>0.63</td>
</tr>
<tr>
<td>Surface sediment (4-5 cm)</td>
<td>0.36768</td>
<td>0.00018</td>
<td>3.77</td>
<td>0.49</td>
</tr>
<tr>
<td>Perfusion leaves</td>
<td>0.36890</td>
<td>0.00089</td>
<td>7.11</td>
<td>2.44</td>
</tr>
<tr>
<td>Perfusion root/rhizome</td>
<td>0.36791</td>
<td>0.00035</td>
<td>4.41</td>
<td>0.97</td>
</tr>
<tr>
<td>Perfusion sediment (0-1 cm)</td>
<td>0.36801</td>
<td>0.00020</td>
<td>4.67</td>
<td>0.54</td>
</tr>
<tr>
<td>Perfusion sediment (1-2 cm)</td>
<td>0.36798</td>
<td>0.00026</td>
<td>4.59</td>
<td>0.71</td>
</tr>
<tr>
<td>Perfusion sediment (2-3 cm)</td>
<td>0.36794</td>
<td>0.00016</td>
<td>4.48</td>
<td>0.45</td>
</tr>
<tr>
<td>Perfusion sediment (3-4 cm)</td>
<td>0.36796</td>
<td>0.00011</td>
<td>4.53</td>
<td>0.31</td>
</tr>
<tr>
<td>Perfusion sediment (4-5 cm)</td>
<td>0.36793</td>
<td>0.00013</td>
<td>4.44</td>
<td>0.36</td>
</tr>
<tr>
<td>Perfusion sediment (5-6 cm)</td>
<td>0.36787</td>
<td>0.00013</td>
<td>4.30</td>
<td>0.37</td>
</tr>
<tr>
<td>Perfusion sediment (6-9 cm)</td>
<td>0.36805</td>
<td>0.00033</td>
<td>4.78</td>
<td>0.91</td>
</tr>
<tr>
<td>Perfusion sediment (9-15 cm)</td>
<td>0.36837</td>
<td>0.00032</td>
<td>5.65</td>
<td>0.89</td>
</tr>
</tbody>
</table>
Table S.4: Average atom % of overlying water background, labelled overlying water and perfusion solution.

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Average Atom %</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water column, post core sealing, no label</td>
<td>0.36590</td>
<td>0.00009</td>
</tr>
<tr>
<td>water column (surface cores) 0.5 hours post label addition</td>
<td>1.3</td>
<td>0.039</td>
</tr>
<tr>
<td>Perfusion core solution (pore water + $^{15}$N$_2$ + Li$^+$)</td>
<td>6</td>
<td>0.180</td>
</tr>
</tbody>
</table>
Table S.5: Concentration of NH$_4^+$ in perfusion background core pore water and sorbed onto sediment over varying depths. Where sample ID is as follows e.g. P.T1.1 corresponds to Perfusion. Time 1. Core 1.

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>NH$_4^+$ µg L$^{-1}$</th>
<th>NH$_4^+$ µmol. L$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>P.T.O.1  0-1 cm</td>
<td>10110</td>
<td>562</td>
</tr>
<tr>
<td>1-2 cm</td>
<td>15890</td>
<td>883</td>
</tr>
<tr>
<td>2-3 cm</td>
<td>11190</td>
<td>622</td>
</tr>
<tr>
<td>3-4 cm</td>
<td>8931</td>
<td>496</td>
</tr>
<tr>
<td>4-5 cm</td>
<td>2601</td>
<td>145</td>
</tr>
<tr>
<td>5-6 cm</td>
<td>6955</td>
<td>386</td>
</tr>
<tr>
<td>6-9 cm</td>
<td>13700</td>
<td>761</td>
</tr>
<tr>
<td>9-15 cm</td>
<td>19540</td>
<td>1086</td>
</tr>
<tr>
<td>P.T.0.2  0-1 cm</td>
<td>10760</td>
<td>598</td>
</tr>
<tr>
<td>1-2 cm</td>
<td>7300.0</td>
<td>406</td>
</tr>
<tr>
<td>2-3 cm</td>
<td>13400</td>
<td>744</td>
</tr>
<tr>
<td>3-4 cm</td>
<td>18420</td>
<td>1023</td>
</tr>
<tr>
<td>4-5 cm</td>
<td>6006</td>
<td>334</td>
</tr>
<tr>
<td>5-6 cm</td>
<td>4976</td>
<td>276</td>
</tr>
<tr>
<td>6-9 cm</td>
<td>13140</td>
<td>730</td>
</tr>
<tr>
<td>9-15 cm</td>
<td>3527</td>
<td>196</td>
</tr>
<tr>
<td>P.T.0.3  0-1 cm</td>
<td>11280</td>
<td>627</td>
</tr>
<tr>
<td>1-2 cm</td>
<td>3459</td>
<td>192</td>
</tr>
<tr>
<td>2-3 cm</td>
<td>14190</td>
<td>788</td>
</tr>
<tr>
<td>3-4 cm</td>
<td>7016</td>
<td>390</td>
</tr>
<tr>
<td>4-5 cm</td>
<td>4682</td>
<td>260</td>
</tr>
<tr>
<td>5-6 cm</td>
<td>2106</td>
<td>117</td>
</tr>
<tr>
<td>6-9 cm</td>
<td>1165</td>
<td>65</td>
</tr>
<tr>
<td>9-15 cm</td>
<td>1185</td>
<td>66</td>
</tr>
</tbody>
</table>
Appendix 5
Supplementary materials – Chapter 4

Table S6: Values of particulate nitrogen (PN) and concentration of 15N (atom %) in seagrass leaf material including epiphytes, where the number 1, 2 or 3 denotes the core number.

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>PN (µmol)</th>
<th>(^{15}\text{N} (\text{A} %) )</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>H. ovalis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Background</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 Leaves</td>
<td>93</td>
<td>0.368302383</td>
</tr>
<tr>
<td>2 Leaves</td>
<td>138</td>
<td>0.36811699</td>
</tr>
<tr>
<td>3 Leaves</td>
<td>57</td>
<td>0.368031714</td>
</tr>
<tr>
<td>Light</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 Leaves</td>
<td>94</td>
<td>0.370166801</td>
</tr>
<tr>
<td>2 Leaves</td>
<td>121</td>
<td>0.373645769</td>
</tr>
<tr>
<td>3 Leaves</td>
<td>146</td>
<td>0.375173615</td>
</tr>
<tr>
<td>Dark</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 Leaves</td>
<td>135</td>
<td>0.371108502</td>
</tr>
<tr>
<td>3 Leaves</td>
<td>182</td>
<td>0.369987071</td>
</tr>
<tr>
<td><strong>Z. muelleri</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Background</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 Leaves</td>
<td>1096</td>
<td>0.368214918</td>
</tr>
<tr>
<td>2 Leaves</td>
<td>1229</td>
<td>0.368304695</td>
</tr>
<tr>
<td>3 Leaves</td>
<td>1044</td>
<td>0.368152025</td>
</tr>
<tr>
<td>Light</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 Leaves</td>
<td>1628</td>
<td>0.368859289</td>
</tr>
<tr>
<td>2 Leaves</td>
<td>1087</td>
<td>0.369801319</td>
</tr>
<tr>
<td>3 Leaves</td>
<td>381</td>
<td>0.369550364</td>
</tr>
<tr>
<td>Dark</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 Leaves</td>
<td>1012</td>
<td>0.368446537</td>
</tr>
<tr>
<td>2 Leaves</td>
<td>1649</td>
<td>0.368309926</td>
</tr>
<tr>
<td>3 Leaves</td>
<td>1153</td>
<td>0.368538503</td>
</tr>
</tbody>
</table>
Appendices

**Table S.7:** Values of particulate nitrogen (PN) and concentration of 15N (atom %) in seagrass root/rhizome material, where the number 1, 2 or 3 denotes the core number.

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>PN (µmol)</th>
<th>15N (A%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>H. ovalis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Background</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 Roots</td>
<td>60</td>
<td>0.367719073</td>
</tr>
<tr>
<td>2 Roots</td>
<td>21</td>
<td>0.368187181</td>
</tr>
<tr>
<td>3 Roots</td>
<td>16</td>
<td>0.367509652</td>
</tr>
<tr>
<td>Light</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 Roots</td>
<td>173</td>
<td>0.371190002</td>
</tr>
<tr>
<td>2 Roots</td>
<td>41</td>
<td>0.373801585</td>
</tr>
<tr>
<td>3 Roots</td>
<td>104</td>
<td>0.382724576</td>
</tr>
<tr>
<td>Dark</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 Roots</td>
<td>108</td>
<td>0.372942466</td>
</tr>
<tr>
<td>3 Roots</td>
<td>95</td>
<td>0.37302591</td>
</tr>
<tr>
<td><strong>Z. muelleri</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Background</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 Roots</td>
<td>192</td>
<td>0.368541908</td>
</tr>
<tr>
<td>2 Roots</td>
<td>330</td>
<td>0.367753743</td>
</tr>
<tr>
<td>3 Roots</td>
<td>57</td>
<td>0.36757662</td>
</tr>
<tr>
<td>Light</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 Roots</td>
<td>247</td>
<td>0.368504806</td>
</tr>
<tr>
<td>2 Roots</td>
<td>83</td>
<td>0.368449334</td>
</tr>
<tr>
<td>3 Roots</td>
<td>92</td>
<td>0.368656624</td>
</tr>
<tr>
<td>Dark</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 Roots</td>
<td>154</td>
<td>0.368824863</td>
</tr>
<tr>
<td>2 Roots</td>
<td>232</td>
<td>0.367707638</td>
</tr>
<tr>
<td>3 Roots</td>
<td>96</td>
<td>0.367798754</td>
</tr>
</tbody>
</table>
Table S.8: Average values (n=3) of particulate nitrogen (PN) and concentration of 15N (atom %) per sediment fraction (mean ± SD).

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>PN (µmol)</th>
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<td>HB1 13-15cm</td>
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### Appendices

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<tr>
<th>Depth Range</th>
<th>N (mg) ± Error</th>
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<td>2496 ± 1252</td>
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<td>13-15cm</td>
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</table>

Unraveling the complexity of subtropical seagrass nitrogen cycling: a stable isotope approach