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

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ABSTRACT: This study used a direct ^{15}N stable isotope labelling technique to measure rates of dinitrogen (N_2) fixation within above- and belowground loci of a subtropical seagrass meadow (*Zostera muelleri*). The total rate of N_2 fixation (i.e. sum of the above- and belowground rates) was ~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 the ^{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}). These results suggest 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. Our 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.

KEY WORDS: Nitrogen fixation · Stable isotope tracer · *Zostera muelleri*

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). However, the range of methods used in measuring dinitrogen (N_2) fixation in seagrass systems has served to make characterisation of 'true' N_2 fixation rates difficult (Table 1).

While the acetylene reduction assay (ARA) has dominated seagrass N_2 fixation measurements (Table 1), the use of ^{15}N-N_2 tracers for determining N_2 fixation rates in other ecosystems has increased substantially (e.g. Bombar et al. 2015, Knapp et al. 2016, Newell et al. 2016). The ^{15}N-N_2 tracer method, referred to as the dissolution method, involves completely dissolving gaseous ^{15}N-N_2 into site water and quantifying the uptake of ^{15}N into biological tissue or sediment (Mohr et al. 2010, Großkopf et al. 2012). This is an improvement on the original ^{15}N-N_2 tracer technique known as the bubble-addition method, where a known volume of $^{15}N_2$ gas was added directly to a water sample containing the biological organism or sediment. The latter technique assumes equilibration of the ^{15}N-N_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.

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Table 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 indicates addition of labelled site water. L+E: rates measured on seagrass leaf material with epiphytes; L+E*: epiphytes not removed, reference stated that 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 rhizomes in an aerobic environment; L+Sh: seagrass leaves and shoots in an aerobic environment; (L): light conditions; (D): dark conditions

N_2 fixation (µmol N m^{-2} h^{-1})	Method	Species, location & season	Reference	Expt
(Sub)Tropical				
Core incubations				
35.6 $(L)_{L+E}$ 19.8 $(L)_{S-RR}$ 14.6 $(L)_{R+R}$	^{15}N-N_2	*Zostera muelleri* – Shaws Bay, Australia (Summer)	This study	1
2.3 $(L)_S$ ~1 (D)	ARA[b]	*Zostera capricorni* – Moreton Bay, Australia (Summer)	Eyre et al. (2011)	2
2.8 $(L)_S$ ~0.25 $(D)_S$	ARA[b]	*Halophila ovalis / spinulosa* – Moreton Bay, Australia (Summer)	Eyre et al. (2011)	3
42–167 $(L)_S$	ARA[c]	*Halodule beaudetti* – Falmouth Harbour, Jamaica (Winter)	Blackburn et al. (1994)	4
6.5 $(L)_{L+E}$ 7.7 $(L)_R$ 48–140 $(L)_S$	ARA[b]	*Syringodium isoetifolium* & *Cymodecea serrulata* – Gulf of Carpentaria, Australia (Summer)	Moriarty & O'Donohue (1993)	5
0.89 $(L)_{L+E}$ 4.8 $(L)_R$ 38–57 $(L)_S$	ARA[b]	*Thalassia hemprichii* & *Cymodecea rotundata* – Gulf of Carpentaria, Australia (Summer)	Moriarty & O'Donohue (1993)	6
12.5 $(L)_{L+E}$ 0.89 $(L)_R$ 0.30 $(L)_{Rh}$ 74 $(L)_S$	ARA[b]	*Enhalus acoroides* – Gulf of Carpentaria, Australia (Summer)	Moriarty & O'Donohue (1993)	7
Perfusion				
0.08 $(L)_{L+E}$ 0.24 $(L)_{R+R}$ 6.6 $(L)_{S-R}$	^{15}N-N_2	*Zostera muelleri* – Shaws Bay, Australia (Summer)	This study	8
42–167 $(L)_S$	ARA[c]	*Halodule beaudetti* – Falmouth Harbour, Jamaica (Winter)	Blackburn et al. (1994)	9
~122 $(L)_S$	ARA[b]	*Syringodium isoetifolium* & *Cymodecea serrulata* – Gulf of Carpentaria, Australia (Summer)	Moriarty & O'Donohue (1993)	10
~45 $(L)_S$	ARA[b]	*Thalassia hemprichii* & *Cymodecea rotundata* – Gulf of Carpentaria, Australia (Summer)	Moriarty & O'Donohue (1993)	11
61 $(L)_{L+E*}$ 8.5 $(L)_{R+R}$ 0.9–35 $(L)_S$	^{15}N-N_2	*Zostera capricorni* – North Stradbroke Island, Australia (Summer)	O'Donohue et al. (1991a)	12
Slurry incubations				
~324–390 $(L)_{R+Rana}$ ~ 760–910 $(L)_{R+Raero}$ ~108–225 $(L)_{S-R}$	ARA[d]	*Zostera capricorni* – Moreton Bay, Australia (Autumn)	Hansen et al. (2000)	13

Table continued on next page

Table 1 (continued)

N_2 fixation (μmol N m^{-2} h^{-1})	Method	Species, location & season	Reference	Expt
22.3_S[a]	ARA[b]	*Thalassia testudinum* – Biscayne Bay, USA (Summer)	Capone & Taylor (1980)	14
$14–36_S$[a]	ARA[b]	*Thalassia testudinum* – Bimini Harbour, Bahamas (Summer)	Capone et al. (1979)	15
0.09 $(L)_{S+RR}$	ARA[e]	*Thalassia testudinum* – Florida, USA (Winter)	McRoy et al. (1973)	16
$80–417_{S+RR}$[a]	ARA[b]	*Thalassia testudinum* – St Lawrence, Barbados (Autumn)	Patriquin &Knowles (1972)	17
Other				
~1.7 $(L)(D)$[j]	ARA[b]	*Halophila stipulacea* – Gulf of Aqaba, Jordan (Winter – daily average)	Cardini et al. (2018)	18
~17.5 $(L)(D)$[j]	ARA[b]	*Halophila stipulacea* – Gulf of Aqaba, Jordan (Spring – daily average)	Cardini et al. (2018)	19
~192 $(L)(D)$[j]	ARA[b]	*Halophila stipulacea* – Gulf of Aqaba, Jordan (Summer – daily average)	Cardini et al. (2018)	20
~16.7 $(L)(D)$[j]	ARA[b]	*Halophila stipulacea* – Gulf of Aqaba, Jordan (Autumn – daily average)	Cardini et al. (2018)	21
Net: 45 $(L)(D)_{L+Sh}$[h]	ARA[f]	*Posidonia oceanica* – Mallorca, Spain (Summer & Spring – average)	Agawin et al. (2016)	22
Plants: *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;[a,h]	ARA[f]	*Halodule uninervis, Cymodocea rotundata, Thalassia hemprichii, Thalassodendron ciliatum* – Dar es Salaam coast, Tanzania, (averaged over 1 year)	Hamisi et al. (2009)	23
E. acoroides: 4 $(L)_{L+E}$ nil $(D)_{L+E}$ *T. hemprichii*: 3.4 $(L)_{L+E}$ *H. uninervis*: 6.9 $(L)_{L+E}$ *S. isoetifolium*: 4 $(L)_{L+E}$	ARA[b]	*Enhalus acoroides, Thalassia hemprichii, Halodule uninervis, Syringodium isoetifolium* – Papua New Guinea (Spring)	Iizumi (1992)	24
900_{L+E}[a,h] Nil_{L-E}[a,h]	ARA[e]	*Thalassia testudinum* – Redfish Bay, USA (Summer)	Goering & Parker (1972)	25
Temperate				
Core incubations				
~18 $(L)_S$ ~8 $(D)_S$	ARA[b]	*Zostera noltii*, Bassin d'Arcachon, France (Spring)	Welsh et al. (2000)	26
~12 $(L)_S$ ~9 $(D)_S$	ARA[b]	*Zostera noltii*, Bassin d'Arcachon, France (Autumn)	Welsh et al. (2000)	27
~7 $(L)_S$ ~5 $(D)_S$	ARA[b]	*Zostera noltii*, Bassin d'Arcachon, France (Winter)	Welsh et al. (2000)	28
~13$(L)_S$ ~3 $(D)_S$	ARA[b]	*Zostera noltii* – Bassin d'Arcachon, France (Summer)	Welsh et al. (1996a)	29
~1.7 $(L)_S$ ~0.7 $(D)_S$	ARA[b]	*Zostera noltii* – Bassin d'Arcachon, France (Winter)	Welsh et al. (1996a)	30

Table continued on next page

Table 1 (continued)

N_2 fixation (μmol N m^{-2} h^{-1})	Method	Species, location & season	Reference	Expt
Perfusion				
15–20 $(L)_S$	ARA[b]	*Zostera marina*, Limfjord, Denmark (Summer)	McGlathery et al. (1998)	31
7.5–12.5 $(D)_S$				
5–12.5 $(L)_S$	ARA[b]	*Zostera marina*, Limfjord, Denmark (Winter)	McGlathery et al. (1998)	32
4–13 $(D)_S$				
8.75 $(L)_S$	ARA[b]	*Zostera marina* – Limfjord, Denmark (Summer)	Risgaard-Petersen et al. (1998)	33
6.25 $(D)_S$				
21 $(L)_S$	ARA[b]	*Zostera marina* – Limfjord, Denmark (Spring)	Risgaard-Petersen et al. (1998)	34
12.5 $(D)_S$				
Slurry incubations				
~20 $(L)_S$	ARA[b]	*Zostera muelleri*, Western Port, Australia (Summer)	Russell et al. (2016)	35
~12 $(D)_S$				
~6 $(L)_S$	ARA[b]	*Zostera muelleri*, Western Port, Australia (Winter)	Russell et al. (2016)	36
~7 $(D)_S$				
~25 $(L)_S$	ARA[b]	*Zostera muelleri*, Western Port, Australia (Spring)	Russell et al. (2016)	37
~18 $(D)_S$				
5–95 $(L)_S$	ARA[b]	*Zostera muelleri / nigricaulis* – Port Philip Bay, Australia (Spring)	Cook et al. (2015)	38
5–50 $(L)_S$	ARA[b]	*Zostera muelleri / nigricaulis* – Port Philip Bay, Australia (Summer)	Cook et al. (2015)	39
~9 $_{S+RR}$[a]	ARA[g]	*Zostera marina* – Great South Bay, USA (Summer)	Capone (1982)	40
~9 $_{S+RR}$[a]	ARA[g]	*Zostera marina* – Vaucluse Shores, USA (Summer)	Capone (1982)	41
Other				
6–25 $(D)_{L+E}$[h]	ARA[b]	*Zostera marina* – Virginia Coastal Bays, USA (Summer)	Cole & McGlathery (2012)	42
0.9–1.5 $(D)_S$[i]				
0.08 (D)[h]	ARA[c]	*Zostera noltii* – Bassin d'Arcachon, France (Summer)	Nielsen et al. (2001)	43
0.46 (D)[h]				
1.17$(D)_{S\text{-}RR}$[h]				
8.76 (D)[h]	ARA[c]	*Spartina maritima* – Bassin d'Arcachon, France (Summer)	Nielsen et al. (2001)	44
0.37 (D)[h]				
0.87$(D)_{S\text{-}RR}$[h]				

[a]No light/dark information given
[b]Theoretical ratio of 3:1 used to convert rates of acetylene reduction to N_2 fixation
[c]Theoretical ratio of conversion to N_2 fixation not specified
[d]Theoretical ratio of 3:1.9 used to convert rates of acetylene reduction to N_2 fixation
[e]Theoretical ratio of 1.5:1 used to convert rates of acetylene reduction to N_2 fixation
[f]Theoretical ratio of 4:1 used to convert rates of acetylene reduction to N_2 fixation
[g]Theoretical ratio of 2.6:1 used to convert rates of acetylene reduction to N_2 fixation
[h]Rates measured in vials/flasks separately
[i]Rates measured in small cores
[j]Whole plants incubated in an outdoor aquarium without 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_2 to ammonium (NH_4^+) (i.e. N_2 fixation) (Stewart et al. 1967, Hardy et al. 1968). While the ARA is inexpensive 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_2 fixed, which is used to calculate N_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_2 fixation, i.e. based on ^{15}N-N_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_2 fixation, acetylene can inhibit some N_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_2 fixation in a seagrass core incubation used a lacunal ^{15}N-N_2 technique (O'Donohue et al. 1991a; Table 1). The use of the direct ^{15}N-N_2 dissolution method removes many of the uncertainties inherent in the calculation of N_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_2 fixation in discrete loci including leaves, roots/rhizomes and sediments (see entries 5, 6, 7, 12 and 13 in Table 1). O'Donohue et al. (1991a) and Iizumi (1992) found the highest N_2 fixation rates associated with the leaves and the lowest rates in the root/rhizome, whereas Moriarty & O'Donohue (1993) found a mixture of rates over their 2 sites and 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 problem, 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 applied the ^{15}N-N_2 dissolution method to measure N_2 fixation rates within intact cores collected from a subtropical seagrass meadow (*Zostera muelleri*). N_2 fixation was measured in discrete loci by adding ^{15}N-N_2 labelled site water to both the sediment and the water column.

MATERIALS AND METHODS

^{15}N-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 which can artificially enhance calculated N_2 fixation rates (Dabundo et al. 2014). To eliminate the possibility of contamination, a preliminary trial was undertaken to check for ^{15}N-NH_4^+ contamination of the gaseous ^{15}N-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}N-N_2 available (~0.014–0.052 µmol ^{15}N-NH_4^+). To confirm that the gas was free from contamination, aliquots of gas were equilibrated with water samples containing ^{15}N-NH_4^+ with a known $\delta^{15}N$ signature (ammonium sulphate salt IAEA N-1 $\delta^{15}N$ $+0.43‰_{airN_2}$ ± 0.2 SD). Assays were undertaken in triplicate based on the method outlined by Dabundo et al. (2014). Briefly, 60 µl of 1000 µM IAEA-N-1 stock standard were 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}N-N_2 gas were added to each sealed vial and shaken overnight. Once equilibrated, 3 aliquots (2.5 ml each) of equilibrated solution from each vial were analysed for ^{15}N-NH_4^+ (see below). Control samples, which contained IAEA N-1 standard without labelled gas, were also analysed for ^{15}N-NH_4^+. Additionally,

another batch of ^{15}N-N_2 gas (Sigma lot no. MBBB09 68V, of the same lot measured by Dabundo et al. 2014) was measured as per the Cambridge ^{15}N-N_2 gas.

Core collection and maintenance

Seagrass cores were collected at low tide from a *Zostera muelleri* stand on the western edge of Shaws Bay, Ballina, located on the north coast of New South Wales, 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 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 (n = 18) were collected at low tide during summer (November) of 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 a Hach HQ40D DO meter at the site. Site water was collected (~250 l) in order to fill incubation chambers in the laboratory.

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).

To generate the ^{15}N-N_2 labelled solution, aliquots of surface and pore water were transferred into 500 ml serum bottles, sealed and degassed under vacuum for 60 min. Fifty ml of ^{15}N-N_2 (Cambridge Isotope Laboratories >98%) were injected into each serum bottle (over pressurised) and allowed to equilibrate overnight on a shaker table (Klawonn et al. 2015).

N_2 fixation assay - surface core incubation

Following the pre-incubation period, high-pressure sodium lamps were turned on ~4 h prior to label addition (~34 µmol m^{-2} s^{-1} PAR; i.e. only light rates were measured). Cores were sealed, and initial samples were collected through valves in the lids for DO (which was again measured upon sacrifice), $\delta^{15}N$-N_2 and the $\delta^{15}N$ of total dissolved nitrogen (TDN) in the overlying water. For $\delta^{15}N$-N_2 determination, triplicate water samples were collected in 12 ml exetainers treated with 20 µl of saturated mercuric chloride and capped without headspace. Samples for $\delta^{15}N$-TDN (~10 ml) were filtered through 0.45 µm 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, 3 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 were 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 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 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 were injected into each of the remaining 6 cores. After a 30 min mixing time, cores were again sampled for $\delta^{15}N$-N_2 as described above. The incubation was carried out over a 10 h time period, with cores sacrificed in triplicate at 5 and 10 h after tracer addition.

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 ~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 × 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 (~20 ml min^{-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 h. 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 solution had diffused from the sediment into the water column. All Li^+ samples were filtered (0.45 µm Sartorius syringe filter) and frozen for later acidification with ~10% nitric acid. Sediment was sectioned into 8 horizontal depth fractions (0–1, 1–2, 2–3, 3–4, 4–5, 5–6, 6–9 and 9–15 cm), 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 min. The supernatant was filtered (0.45 µm Sartorius syringe filter) and collected for TDN concentration, $\delta^{15}N$-TDN and Li^+ concentration analysis. Sediments remaining after KCl extraction were rinsed with ultra-pure water and centrifuged to remove excess salts.

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 isotope ratio mass spectrometer (IRMS) (Thermo Conflo III and a Thermo Delta V Plus) (precision ±0.3‰) (Eyre et al. 2016b).

$\delta^{15}N$-NH_4^+ from the initial tests for gas contamination was measured via oxidation to NO_2^- and subsequent reduction to N_2O as per Zhang et al. (2007). The N_2O in these samples was purified in liquid N_2 with a custom-built purge and trap system. The N isotopic signature of the N_2O 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 was ±0.5‰).

Samples requiring $\delta^{15}N$-N_2 analysis were headspaced with 2 ml He. Headspace samples (10 µl) were analysed for $\delta^{15}N$-N_2 on a Thermo Trace gas chromatograph (GC) Ultra with a 25 m × 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 4-channel flow injection analyser (Lachat QuickChem 8000) 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 by Knapp et al. (2005). Briefly, persulphate oxidation of TDN to NO_3^- was followed by conversion of NO_3^- to N_2O via the denitrifier method (precision: ±0.2‰) (Sigman et al. 2001) and measured via GC-IRMS as described above.

N_2 fixation rate calculation

Rates of N_2 fixation (in units of µmol N_2 m^{-2} h^{-1}) were calculated using the following equation (based on Wilson et al. 2012):

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

where $AP_{(PN\text{-Final})}$ is the ^{15}N atom% of the organic N pools (i.e. bulk sediment, leaves and roots) following incubation; $AP_{(PN\text{-Initial})}$ denotes the ^{15}N atom% of the background N pool in control samples prior to addition of ^{15}N-N_2. The AP_{N_2} term represents the ^{15}N atom% of the enriched N_2 pool 30 min post addition of ^{15}N-N_2 labelled site water. The ^{15}N content of each locus, post incubation, in µmol is designated by the PN_{Final} term; Δt is the incubation time. Finally, the division by 2 is performed to convert the subsequent value from gram-atoms of N to moles of N_2 as detailed by Montoya et al. (1996). The accumulation of ^{15}N in any particular pool is deemed to be equivalent to the rate of N_2 fixation in that pool. Alternatively ^{15}N-NH_4^+ produced from N_2 fixation could be incorporated into the organic N pool. We would argue that in the 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:

$$\text{Total N fixed} = P_s + P_r + P_l + S_s + S_r + S_l \quad (2)$$

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 ($p < 0.05$).

RESULTS

The pure ^{15}N-N_2 gas was found to be free from contamination with ^{15}N-NH_4^+ (see Table S1 in the Supplement at www.int-res.com/articles/suppl/m605p087_supp.pdf). 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 Table S1.

The Li^+ tracer used in the perfusion cores was distributed homogenously throughout the sectioned rhizosphere. A 1-way 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 fraction are presented in Table S2. The amount of ^{15}N recovered in the TDN pool of the water column of the surface cores was minor (~0.0088 ± 0.0015 µmol N_2 m^{-2} h^{-1}), representing ~0.003% of the total recovered ^{15}N in the surface cores. The Li^+ concentration in the overlying water increased from ~0.16 ± 0.0032 mg l^{-1} prior to perfusion to ~0.18 ± 0.011 mg l^{-1} in the 10 h 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 $\delta^{15}N$-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 Tables S3 & S4. The total rate of N_2 fixation of the *Zostera muelleri* community was ~38 ± 28 µmol N_2 m^{-2} h^{-1} calculated as the sum of averages across all loci in both core types (i.e. Eq. 2). Surface core and perfusion N_2 fixation were 34 ± 18 and 4.8 ± 5.7 µmol N_2 m^{-2} h^{-1}, respectively, for the 5 h incubation and 36 ± 7.2 and 2.5 ± 4.7 µmol N_2 m^{-2} h^{-1} for the 10 h incubation (Fig. 1).

Dinitrogen fixation rates observed across all loci were greater in the surface incubations than in the perfusion incubations (Fig. 2). The leaf material, which includes epiphytes, exhibited the highest N_2

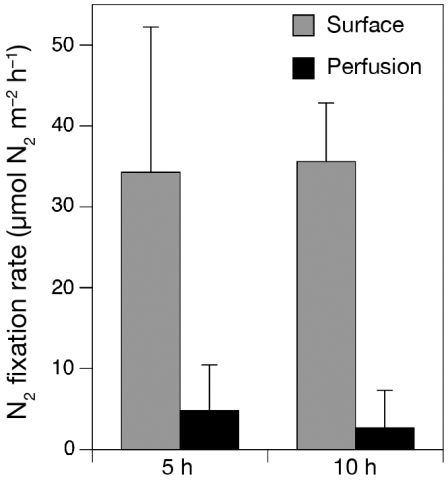

Fig. 1. Total rates of dinitrogen fixation (mean ± SD, n = 3) across all seagrass loci (leaves and epiphytes, sediment and root/rhizome material) in surface and perfusion cores over 5 and 10 h incubation periods

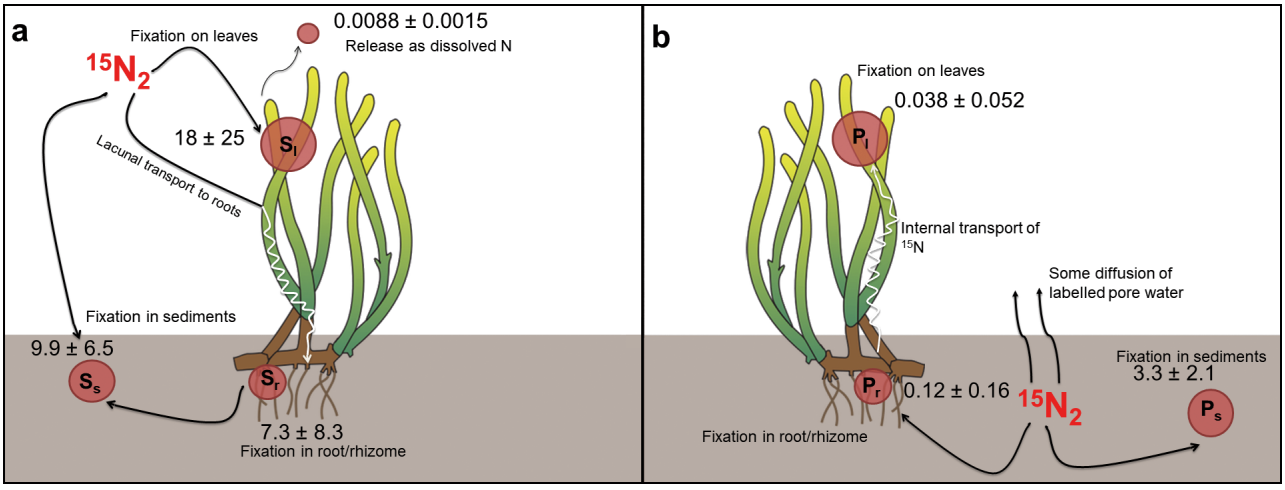

Fig. 2. Dinitrogen fixation rates measured in discrete loci (as mean ± SD µmol N_2 m^{-2} h^{-1}) when ^{15}N-N_2 labelled site water was either (a) added to the water column or (b) perfused directly into the sediment. Arrows illustrate suggested direction of movement of labelled products around the system. S_l, S_s and S_r represent surface core fixation in the leaves (including epiphytes), sediment and root/rhizome, and P_l, P_s and P_r represent the N_2 fixation in these loci in the perfusion cores. Seagrass vector image from Diana Kleine (ian.umces.edu/imagelibrary)

fixation rates in the surface incubations (18 ± 25 µmol N_2 m^{-2} h^{-1}; Fig. 2), whereas in perfusion cores the sediment was the area of highest activity (3.3 ± 2.1 µmol N_2 m^{-2} h^{-1}; Fig. 2). There was a much higher accumulation of fixed N in the root/rhizome fraction of the surface cores (7.3 ± 8.3 µmol N_2 m^{-2} h^{-1}) than in the root/rhizome fraction of the perfusion cores (0.12 ± 0.16 µmol N_2 m^{-2} h^{-1}; Fig. 2).

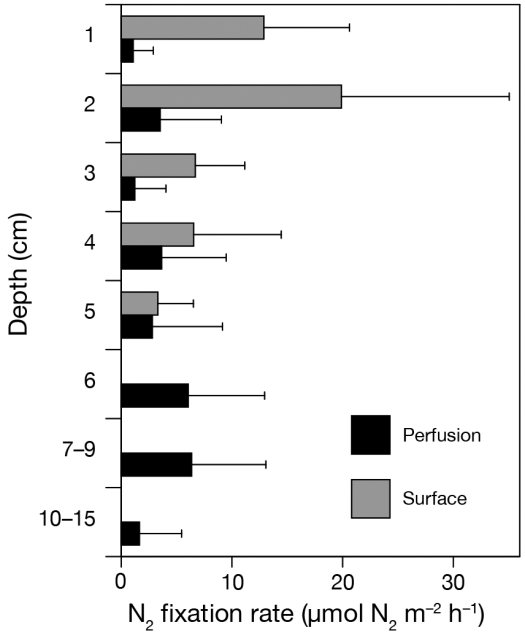

Fig. 3. Dinitrogen fixation (mean ± SD, n = 3) over sediment depths in both surface and perfusion cores

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). In fact, on average, the N_2 fixation rates of the top 5 fractions (1 cm each) of the surface cores (corresponding to a depth of 1–5 cm of sediment) were higher (~9.9 ± 6.6 µmol N_2 m^{-2} h^{-1}) than the average of the total perfusion profile (~3.3 ± 2.1 µmol N_2 m^{-2} h^{-1}). A decrease in the rate of fixation with core depth was observed over the top 5 cm 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).

Background $\delta^{15}N$ or atom percent measurements of all loci, including the water column, are presented in Tables S3 & S4.

DISCUSSION

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 quantification has been undertaken previously largely using the ARA, with N_2 fixation estimates ranging from <1 to 61 µmol N m^{-2} h^{-1} for leaf material, and <1 to 8.5 µmol N m^{-2} h^{-1} in roots/rhizomes (Table 1, entries 5, 6, 7, 12 and 27), which bracket the

rates we have presented here (Table 1, entries 1 and 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 µmol 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. 2). Root/rhizome material displayed the lowest N_2 fixation rates across both core types (Fig. 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 unscraped 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. 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. 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 the perfusion cores, which suggests 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 h 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 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. 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 that leaf epiphytic fixation is the dominant source of root/rhizome fixed N. 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. 2). This contrasts with past studies which showed that 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.

N_2 fixation within the seagrass meadow: belowground

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 (~65–1100 µmol l^{-1}; Table S5) may have limited N_2 fixation. Welsh et al. (1997) observed 40–60% inhibition of nitrogenase in a *Z. noltii* meadow at NH_4^+ concentrations of between 5 and 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. 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 roots/rhizomes of the perfusion cores as compared with that in the surface cores (Fig. 2). A lack of N_2 fixation in the sediment may mean that labelled products are not supplied to the roots/rhizomes. 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 roots/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 cores would mean this pathway would be much reduced, resulting in the lower rates we observed (Fig. 2).

Sediment N_2 fixation rates in the 1–5 cm samples of the surface cores were elevated, relative to the perfusion cores, and decreased with depth (Fig. 3). Unexpectedly these rates were still higher than the total sediment fractions of the perfusion cores (Fig. 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 gasses through the plant itself (Fig. 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). A system of gas-filled lacunae transports gasses 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. 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 lower than in sediments in surface cores (Fig. 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 N_2 fixation processes accurately in the rhizosphere while maintaining natural gradients remains challenging.

Comparison with previously reported N_2 fixation rates

The N_2 fixation rates presented here via the addition and recovery of ^{15}N-N_2 are within the range of previously reported rates in seagrass communities (Table 1). Although we did not directly compare the ^{15}N-N_2 dissolution method and the ARA in this *Z. muelleri* meadow, methodological differences between the techniques have the potential to affect rate estimates. The function of N_2 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 N_2 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 N_2 fixation in a seagrass system (Nielsen et al. 2001). Thus, any inhibition of SRB could cause major underestimation of N_2 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 N_2 fixation from acetylene reduction (Seitzinger & Garber 1987, Welsh 2000) may have an effect

on the difference between N_2 fixation rates measured via ^{15}N-N_2 dissolution and the ARA.

Another possible reason for variability in previous N_2 fixation measurements may stem from the practice of incubating individual components of the seagrass system (i.e. roots, leaves, sediment) separately (Table 1, entries 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 N_2 fixation in seagrass habitats. During isolated incubations, discrete N_2 fixing zones around the root/rhizome may become inundated by nearby regions of high NH_4^+ concentration (Welsh 2000), naturally occurring N_2 fixing bacterial community distribution may be affected (Langezaal et al. 2003, Böer et al. 2009), and links between structures that affect N_2 fixation may be destroyed. For example, when leaves are incubated separately from roots and sediments, N_2 fixation occurring from lacunal transport is not observed.

The ^{15}N-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-N_2 dissolution method may result in N_2 fixation rates that are much more indicative of *in situ* rates. O'Donohue et al. (1991a) measured similar rates of fixation relative to this study, 120 µmol N m^{-2} h^{-1}, using a ^{15}N-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 N_2 fixation may not have 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-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 N_2 fixation measured in the rhizosphere of the surface and perfusion cores in the calculation of the total N_2 fixation rate (Eq. 2). However, perfusion core sediment fixation represents 14.6% of the total rate estimate, while perfusion root/rhizome fixation represents only 0.3% (Fig. 2). We do not think this is enough to warrant deleting either from the total N_2 fixation rate calculation. Accurate measurement of N_2 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 h). However, our results suggest that the majority of fixation occurred in the first 5 h (Fig. 1). This would explain the reduced perfusion rates at the 10 h timepoint as rates averaged over a longer time frame (10 h) 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 ^{15}N-N_2 dissolution method does provide a valuable direct measure of N_2 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 ^{15}N-N_2 gas to be contaminated with ^{15}N-NH_4^+ can artificially inflate experimental findings, making the gas and results of any experiment potentially unusable. We recommend confirming any contamination between different batch numbers and brands of gas (Table S1) prior to any experimental procedure, although this does increase the cost incurred. The ^{15}N-N_2 dissolution method also does not lend itself as easily to *in situ* incubations as the ARA, as the ^{15}N-N_2 site water solution needs to be equilibrated while shaking over many hours.

The N_2 fixation rates we have measured are of a similar order of magnitude to rates measured in previous studies by the ARA (Table 1). Despite this, and although we did not directly compare the ^{15}N-N_2 dissolution method and the ARA in 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 focussed on sediments.

CONCLUSIONS

To our knowledge, this is the first study to apply 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 the 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 whether morphological and/or location effects have an impact on N_2 fixation rates.

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