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Factors controlling nitrogen fixation in temperate seagrass beds

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ABSTRACT: Nitrogen fixation is an ecologically significant process in marine systems because nitrogen is typically the key limiting nutrient controlling productivity. Seagrass beds are often hot spots for nitrogen fixation owing to the mutualistic relationship between seagrass and nitrogen-fixing sulphate-reducing bacteria. The objectives of this study were to: (1) investigate the factors that controlled nitrogen fixation within seagrass beds (*Zostera muelleri* and *Z. nigricaulis*) on a system scale in a temperate Australian embayment (Port Phillip Bay), and (2) investigate differences in nitrogen isotope ratios ($^{15}N/^{14}N$, $\delta^{15}N$) in seagrass and *Ulva* spp. tissue as a proxy for nitrogen fixation. Nitrogen fixation rates ranged between 3 and 90 µmol m^{-2} h^{-1} and were related to plant biomass during both summer and spring, except at a highly nitrogen-enriched site adjacent to a sewage treatment plant outlet. During spring, biomass-specific nitrogen fixation rates were strongly positively related to leaf C:N ratio, suggesting that nitrogen fixation rates increased with nitrogen limitation during the growing season. Leaves of *Zostera* spp. had $\delta^{15}N$ values that were consistently depleted by 2.4 ± 1.5‰ relative to *Ulva* spp. collected from the same site. We postulate that this isotopic difference arises as a consequence of *Zostera* spp. mainly assimilating N from newly mineralized nitrogen within the sediment (vs. a negligible fraction from N fixation), which is isotopically depleted owing to fractionation during nitrogen mineralization.

KEY WORDS: Seagrass · Nitrogen fixation · Nitrogen isotope · ^{15}N · Nitrogen loading

INTRODUCTION

Seagrass beds are recognized as important habitats in shallow coastal areas globally (Larkum et al. 2006). Seagrasses are able to maintain high rates of productivity in oligotrophic waters, and one of the key reasons for this is their ability to source newly fixed nitrogen from root-associated diazotrophic bacteria (Welsh 2000). Indeed, some of the highest nitrogen fixation rates measured in sediments are associated with seagrass, making these habitats a potentially important source of nitrogen to nitrogen-limited coastal ecosystems. The fraction of seagrass nitrogen demand met by nitrogen fixation is variable; in temperate ecosystems it is typically ~10%, and up to 50% in tropical waters (Welsh 2000). It should be noted, however, that the temperate seagrass beds included in the review by Welsh (2000) were all relatively eutrophic systems in Europe and North America; therefore, the observed difference between tropical and temperate systems may represent a difference in the systems studied as opposed to the climate zone.

Nitrogen fixation within seagrass beds is mostly undertaken by sulphate-reducing bacteria, which have a close mutualistic relationship with seagrass (Nielsen et al. 2001). Various studies have shown a rapid transfer of carbon from seagrass to bacteria and newly fixed nitrogen to seagrass (Welsh 2000). Additionally, rates of nitrogen fixation have been clearly shown to increase in the light, when carbon exudation by seagrass roots is highest (Welsh et al. 1997). Given that nitrogen fixation is an energetically intensive process, one would expect down-regulation of

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the process to occur in the presence of bioavailable nitrogen. A number of previous studies have investigated the role of available nitrogen in controlling nitrogen fixation rates both in laboratory experiments and using field observations; however, the results of experiments to date are equivocal. Additions of NH_4^+ to sediment slurries have been shown to lead to an immediate partial inhibition in the rate of nitrogen fixation (Capone 1982, Welsh et al. 1997), although this effect is not universal (McGlathery et al. 1998). In a field setting, a study in the Bassin d'Arcachon, France, showed that rates of nitrogen fixation were consistently lower in an inner estuary *Zostera noltii* bed exposed to high nutrient loads compared to rates in a *Z. noltii* bed occupying the more oligotrophic outer estuary (de Wit et al. 2001). Based on these previous studies, one would expect nitrogen fixation rates to be positively related to seagrass biomass and negatively related to nitrogen availability. To date, no studies have investigated whether these controlling factors apply across seagrass beds at a system scale (e.g. 10–100 km).

Because of the ecological importance of nitrogen fixation, there is a great deal of interest in quantifying this rate (Welsh 2000). The key methodological obstacle to quantifying nitrogen fixation in the field is the accurate measurement of small changes in nitrogen concentration relative to the high background pool of porewater nitrogen. The most common approach to quantifying nitrogen fixation is the use of the acetylene reduction assay, whereby the reduction rate of acetylene to ethylene is used as a proxy for the nitrogen fixation rate after taking into account a factor of 3–4 to correct for differences in electron transfer between the 2 processes (Capone & Montoya 2001). This reliance on a theoretical conversion ratio between acetylene reduced and nitrogen fixation has raised questions regarding its accuracy (e.g. Seitzinger & Garber 1987). Despite these concerns, most studies that have calibrated this ratio using $^{15}N_2$ have shown the theoretical ratio to be robust (Welsh 2000). Additional problems include sources of ethylene in the sediment other than acetylene reduction, and exposure to acetylene leading to changed nitrogenase activity (Oremland & Capone 1988). Irrespective of the methodological considerations, quantifying nitrogen fixation (as with most process measurements) is very time consuming because intact cores need to be taken and sampled over time. The rates obtained by this process are only representative of that place and time, leading to difficulties with scaling rate estimates beyond the patch scale.

Another, but much less common, approach that has been used to quantify nitrogen fixation rates uses natural abundance $^{15}N/^{14}N$ ratios. The approach relies on the fact that newly fixed nitrogen typically has a $\delta^{15}N$ of −2 to 0‰ owing to minimal fractionation of atmospheric N_2 (which by definition has a $\delta^{15}N$ = 0‰) during nitrogen fixation (Fry 2006). In environments where the fixed nitrogen pool has a substantially different $\delta^{15}N$ to the background nitrogen pool, this difference in isotope ratios can be used to estimate new inputs. In the case of the marine environment, the isotopic signature of nitrogen in nitrate is ~6–8‰, which means that the contribution of diazotrophic nitrogen to the requirements of organisms with high rates of nitrogen fixation such as cyanobacteria can be detected (Woodland & Cook 2014). To date, there have been no reports of $^{15}N/^{14}N$ ratios being used to quantify nitrogen fixation in seagrass beds. For this approach to be feasible, it requires a readily observable discrepancy in the $\delta^{15}N$ of seagrass compared to bioavailable sources and a means of quantifying the fixed nitrogen endmember.

The aims of this study were to: (1) Quantify nitrogen fixation rates within shallow *Zostera* spp. beds in a temperate coastal embayment to identify controlling factors at the regional scale. We hypothesized that rates of nitrogen fixation would be positively mediated by biomass and negatively mediated by nitrogen availability. (2) Investigate the potential to use nitrogen isotope ratios of seagrass as a proxy for nitrogen fixation. We hypothesized that a macro-alga such as the ubiquitous *Ulva* spp. could serve as a proxy for the local ambient nitrogen endmember and that the presence of an offset between *Ulva.* spp. and seagrass $\delta^{15}N$ values at the same site would reflect the influence of nitrogen fixation.

MATERIALS AND METHODS

Study site

Samples were collected from 10 shallow sites (water depth ~0.5 m) within Port Phillip Bay, Australia (Fig. 1), a temperate marine embayment with an area of ~1900 km^2. The dominant species of seagrass in the shallower, intertidal areas of the bay are *Zostera nigricaulis* and *Z. muelleri*. The dominant source of nitrogen to the bay (~50% of annual input, ~2000 Mg yr^{-1}) is the Western Treatment Plant in the northwest (Fig. 1) which results in seagrass having a strong gradient in $\delta^{15}N$ ranging from ~18‰ in the north of the bay, through 6–8‰ in the open, southern

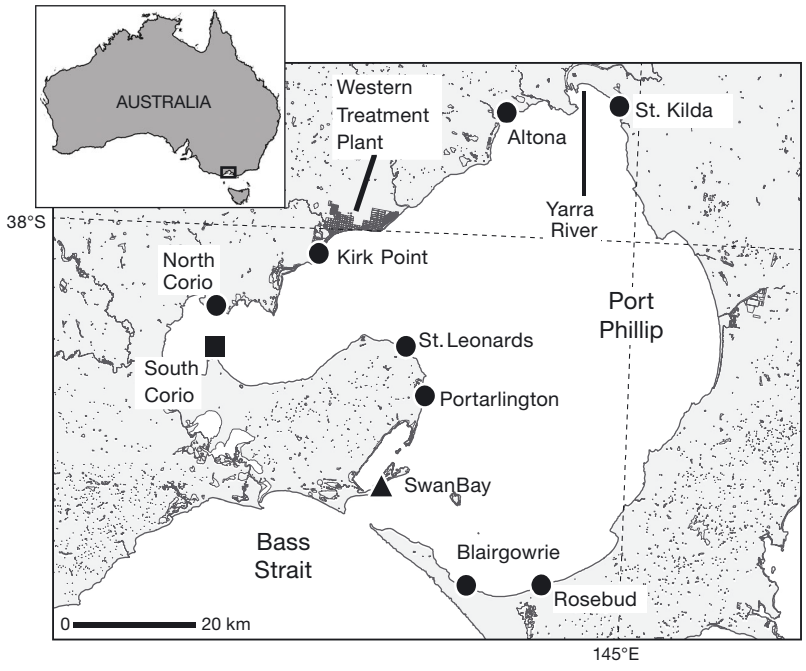

Fig. 1. Port Phillip Bay, Australia, showing the study sites as well as the Western Treatment Plant and the mouth of the Yarra River, which are the 2 major sources of nitrogen to the bay. Sites marked with circles were sampled in both seasons; South Corio (square) and Swan Bay (triangle) were sampled only in summer and spring, respectively

section of the bay, and as low as 0‰ in isolated inlets such as Swan Bay. The Yarra River discharges into the far northern end of the bay, delivering ~25% of the total nutrient load to the bay. The balance of the nitrogen load to the bay comes from diffuse sources including smaller rivers and urban drains (Harris et al. 1996). The sampling sites (Fig. 1) were selected to cover this isotope gradient, and the average distance between sites was 17 km. Dissolved inorganic nitrogen is typically very low throughout the bay (<1 µM), except within the Yarra River plume and within the immediate vicinity of outlets from the Western Treatment Plant.

Sampling

At each sample site, 4 intact cores (tube dimension: 67 mm ID × 300 mm length) were collected at each field site (water depth ~0.5 m), with an average sediment height of 15 cm core^{-1}. Tissue samples of *Zostera* spp. and *Ulva* spp. were collected at each field site for later isotope analysis. Samples were collected in January 2013 (summer) and late October to early November 2013 (spring). Upon return to the laboratory (within 4 h of collection), the cores were placed in a tank filled with aerated seawater that was collected at one of the field sites and maintained at *in situ* temperature. A magnetic stirrer bar was suspended in each core and driven by an external magnet rotating at ~60 rpm. The water level in the tank was adjusted to just above the core height. All cores were stored without lids, and exchange between the water column in the core and tank water was allowed to occur.

The following day, the tank was covered in a blackout material, the cores were sealed, and the dissolved oxygen concentration was monitored in each core at 10 to 30 min intervals using a probe (see 'Analytical methods') until a time series of 4 concentration points was obtained. A halogen light bank with a constant average light intensity of ~650 µmol photons m^{-2} s^{-1} at the canopy was then placed over the tank. The first dissolved oxygen reading was taken approximately 30 min after the light bank was switched on, after which a similar time series of 4 points was obtained. The lids were then removed and the core left open overnight in the bath. The following day, the light bank was switched on and remained on for the duration of the incubation, and the water level in the tank was lowered below the top of the core liners to prevent water exchange between the cores and the tank. About 160 ml of seawater were removed from the water column above each sediment core. Using a 10 cm needle with a closed end and open slits at 1 cm intervals, 40 ml of acetylene-saturated seawater were injected vertically into the sediment of each core in 4 injections of 10 ml evenly around the circumference of the core. Following this, 120 ml of acetylene-saturated seawater were added to the water column of the cores and topped up to the rim of the core with seawater from the tank. The volume of seawater replaced (~160 ml) with acetylene-saturated seawater was equivalent to a final ~20% acetylene-saturated seawater. The cores were then closed with lids. The 4 cores from each field site were slurried in a time series, with the first core sampled and slurried 30 min after the replacement of seawater with acetylene-saturated water. This approach was used to ensure that ethylene, which will accumulate to much higher concentrations within the sediment than the water column (O'Donohue et al.

1991), was quantitatively recovered and analysed. Nitrogen fixation was only measured in the light, and the rates measured here are therefore likely to be maximum estimates (Welsh 2000). Each subsequent core was slurried approximately 90 min after the previous core, giving a time series of 4 samples. For each core slurry, a 12 ml water sample was collected in a gas-tight 12 ml Exetainer (Labco) and preserved with 250 µl 50% w/v $ZnCl_2$ for analysis of ethylene. A 10 ml water sample was collected, filtered (0.45 µm cartridge filter) and frozen for analysis of ammonium (NH_4), nitrate + nitrite (NOx) and filterable reactive phosphorus (FRP) concentrations.

Seagrass leaves and *Ulva* spp. were sampled independently at each site and prepared for stable isotope analysis: epibionts were removed from leaf samples of *Zostera* spp. and from *Ulva* spp. with a scalpel blade; macrophytes were then rinsed in distilled water and dried at 60°C until a constant weight was reached (~48 h). Replicate samples (n = 3) of dried seagrass leaves and *Ulva* spp. were powdered and homogenised with a ball mill grinder.

Analytical methods and calculations

All samples were analysed at the Water Studies Centre (Monash University). Ethylene was analysed on a Varian 3300 gas chromatograph equipped with an Alltech AT Q capillary column and a flame ionisation detector and quantified against standards diluted from a certified gas mixture (BOC gases). Ethylene (C_2H_4) production rates were calculated from the slope of the linear regression of C_2H_4 concentration versus time (which was typically very linear), and typically had r^2 values >0.7. Nitrogen fixation rates were calculated from C_2H_4 production rates using a 3:1 C_2H_4:N_2 ratio (Welsh 2000) and the standard deviation about the slope of the regression propagated through the calculation. Nutrients were analysed using standard colorimetric methods (APHA 1992) in a National Association of Testing Authorities (NATA)-certified laboratory. Oxygen concentrations were measured using a Hach LDO probe attached to an HQ40d meter. Nitrogen and carbon ($\delta^{13}C$) isotope analysis was carried out on an elemental analyser interfaced to a Sercon 20-22 mass spectrometer. Stable isotope samples were analysed on an ANCA GSL2 elemental analyser interfaced to a Hydra 20-22 continuous-flow isotope ratio mass spectrometer (Sercon). The precision of the stable isotope analysis was ±0.1‰ for ^{13}C and ±0.2‰ for ^{15}N (SD, n = 5). Stable isotope data are expressed in the delta notation ($\delta^{13}C$ and $\delta^{15}N$), relative to the stable isotopic ratio of Vienna Pee Dee Belemnite standard (R_{VPDB}= 0.0111797) for C and atmospheric N_2 (R_{Air} = 0.0036765) for nitrogen.

Hourly gross primary production (GPP) was calculated by subtracting the dark O_2 flux from the light O_2 flux. Net daily community production (NCP) was calculated by multiplying GPP by a 12 h light period plus the daily dark O_2 flux (12 × GPP + 24 × dark O_2 flux). To estimate daily nitrogen demand, GPP was integrated over a 12 h period and then divided by the leaf C:N ratio (mol) measured at each site. Daily nitrogen fixation was calculated by multiplying the light nitrogen fixation rates by 24 h. Rates of nitrogen fixation in the light typically increase by a factor of ~2 in seagrass beds compared to the dark (Welsh 2000), so the contribution of daily nitrogen fixation to total nitrogen demand is most likely overestimated by ~25%.

RESULTS

With the exception of St Leonards, which was vegetated with *Zostera nigricaulis*, all sites were vegetated with *Z. muelleri*. There was a wide range of $\delta^{15}N$ values for *Zostera* spp. collected around the bay, with the most isotopically enriched samples being found at Altona and Kirk Point ($\delta^{15}N$ = 14.5–18.7‰), consistent with their proximity to the Western Treatment Plant and known water circulation patterns within the bay. Water typically flows from south to north in the centre of the bay and splits into northeast and southwest flowing water masses on the northern coastline. The lowest values ($\delta^{15}N$ ~ 1–3‰) were found in Swan Bay and North Corio Bay, which are relatively isolated water masses. *Zostera* spp. leaf $\delta^{13}C$ values fell in the range −9 to −13 ‰ and were generally most isotopically enriched in spring (Table 1). *Zostera* spp. leaf C:N ratio (mol:mol) was consistently highest during summer and ranged between 13.4 (St Kilda, spring) and 34.4 (Rosebud, summer; Table 1). Sediment organic carbon content was only measured during spring and was highly variable, ranging from 0.1% at St Leonards up to 6.5% at North Corio, consistent with the observed dominance of sand and soft dark mud at each of the sites, respectively (Table 2). The sediment $\delta^{13}C$ composition ranged from −22.8‰ at St Leonards, indicating the dominance of algal detritus, up to −13.8‰ at North Corio, indicating the dominance of *Zostera* spp. detritus (Table 2). Integrated pools of porewater

Table 1. Summary of carbon and nitrogen isotope ratios and C:N ratios measured in the leaves of *Zostera* spp. and *Ulva* spp. ($\delta^{15}N$ only), during the spring and summer sampling campaigns (n = 3). –: no data

Site name	Location (DD)		Summer (January 2013)								Spring (October–November 2013)							
			Zostera spp.				*Ulva* spp.				*Zostera* spp.				*Ulva* spp.			
	Latitude	Longitude	$\delta^{13}C$		$\delta^{15}N$		C:N		$\delta^{15}N$		$\delta^{13}C$		$\delta^{15}N$		C:N		$\delta^{15}N$	
			Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Altona	–37.874832	144.816523	–12.0	0.5	18.7	0.2	19.2	1.4	20.6	0.6	–9.4	0.5	14.5	0.8	15.0	1.5	14.0	0.3
Blairgowrie	–38.361829	144.785722	–11.2	0.5	11.3	0.9	28.5	6.8	11.7	0.3	–8.8	0.7	10.7	1.0	20.5	2.0	13.2	0.9
Kirk Point	–38.027393	144.565307	–11.8	0.3	18.5	0.5	16.9	0.6	20.9	0.5	–9.9	0.1	17.6	1.1	13.5	1.9	21.2	0.5
North Corio	–38.085248	144.426548	–11.2	0.5	5.4	0.5	23.0	1.3	8.4	0.7	–9.8	0.5	1.4	0.6	14.0	1.8	10.3	2.7
Portarlington	–38.118992	144.689825	–11.0	0.9	5.7	0.5	23.8	2.6	10.9	0.4	–12.4	0.7	4.4	0.4	20.0	1.9	7.6	1.5
Rosebud	–38.358069	144.889194	–13.4	0.7	7.7	0.6	34.4	9.7	9.1	2.2	–12.6	1.1	8.2	0.1	24.5	5.3	11.3	0.6
St Kilda	–37.863595	144.970614	–12.3	1.0	10.7	0.6	16.4	1.8	12.4	1.4	–8.7	0.7	10.4	0.6	13.4	0.5	11.0	0.5
St Leonards	–38.169311	144.718836	–12.2	0.2	8.5	1.2	29.8	2.7	14.5	NA	–9.7	0.2	6.8	0.5	23.6	3.8	7.4	0.0
South Corio	–38.127594	144.426872	–9.0	0.5	4.6	0.7	22.4	2.8	6.9	1.0	–	–	–	–	–	–	–	–
Swan Bay	–38.263056	144.663416	–	–	–	–	–	–	–	–	–9.4	0.3	3.2	0.9	21.1	3.8	5.8	0.2

NH_4^+ were typically highest during summer, with concentrations ranging from 11.6 µM at Rosebud during spring up to 239 µM during summer at Kirk Point. Porewater FRP showed a similar pattern, with the lowest concentration of 1.3 µM being observed in Swan Bay and the highest concentration of 58.1 µM being observed at Kirk Point (Table 2).

GPP was generally in the range 4.7 (North Corio in spring) to 25 mmol m^{-2} h^{-1} (Blairgowrie, spring, Fig. 2a,b). Dark O_2 consumption was weakly, but significantly ($r^2 = 0.46$, $p < 0.005$) correlated with light O_2 fluxes, and ranged from 5 to 12 mmol m^{-2} h^{-1} over the 12 h dark period. In general, there was a net consumption of oxygen, with 13 out of 18 measurements being net heterotrophic (i.e. more carbon was decomposed than produced) with rates ranging from −72 (Kirk Point, spring) to 38 mmol m^{-2} d^{-1} (St Kilda, summer, Fig. 2c). Rates of nitrogen fixation were typically highest during spring, with rates of up to 90 µmol N m^{-2} h^{-1} being observed. More generally, rates were within the range of 10 to 40 µmol N m^{-2} h^{-1} (Fig. 2d,e). Estimated rates of nitrogen fixation as a proportion of total nitrogen demand ranged from <1% at St Kilda in spring up to 15% at Rosebud in spring (Fig. 2f).

During summer there was a significant relationship between *Zostera* spp. biomass and nitrogen fixation ($r^2 = 0.5$, $p < 0.05$). In spring the positive relationship between *Zostera* spp. biomass and nitrogen fixation was similar ($r^2 = 0.6$, $p < 0.05$), provided that the datum for Kirk Point, which is the site most exposed to the Western Treatment Plant (i.e. receives the most nutrients), was excluded from the non-linear regression (Fig. 3).

Concentrations of NO_x were insignificant compared to NH_4^+ and are not presented. During summer, there was no relationship between nitrogen fixation and integrated porewater NH_4^+ and FRP. During spring, there was a weak and statistically insignificant relationship between porewater NH_4^+ and biomass-normalized nitrogen fixation rate ($r^2 = 0.43$, $p > 0.05$, Fig. 4a). Also during spring, we found a strong significant relationship between leaf C:N ratio and the biomass-normalized nitrogen fixation rate (Fig. 4b).

There was a strong relationship between the $\delta^{15}N$ of *Zostera* spp. leaves and *Ulva* spp. collected from each study site, with *Zostera* spp. leaves showing a positive offset (isotopic enrichment) of ~2.4‰ compared to *Ulva* spp. (Fig. 5a). There was no significant relationship between the offset in $\delta^{15}N$ of *Ulva* spp. and *Zostera* spp. leaves and biomass-normalized nitrogen fixation at each site (Fig. 5b).

Table 2. Summary of integrated porewater NH_4^+, filterable reactive phosphorus (FRP), % sediment organic carbon content (%C) and sediment carbon isotope ratios (top 10 mm) measured at each study site during summer and spring (n = 3, except for %C and carbon isotope ratios, n = 1). –: no data

Site name	Summer (January 2013)				Spring (October – November 2013)					
	NH_4^+ (µM)		FRP (µM)		NH_4^+ (µM)		FRP (µM)		%C	$\delta^{13}C$
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	n = 1	n = 1
Altona	55.5	19.0	9.6	1.8	108.4	103.6	7.3	3.5	0.5	–16.6
Blairgowrie	66.6	37.2	5.0	1.5	58.9	94.1	2.2	2.9	0.2	–17.6
Kirk Point	239.3	169.1	58.1	16.0	78.4	32.8	14.2	3.6	1.1	–17.6
North Corio	28.6	10.2	8.6	2.7	122.0	124.2	7.4	6.1	6.5	–13.8
Portarlington	22.3	18.5	11.1	4.1	23.0	10.4	2.2	0.4	0.3	–14.5
Rosebud	19.9	10.5	4.9	1.4	11.6	3.7	2.3	0.7	0.2	–20.1
St Kilda	200.7	148.1	8.4	3.9	48.4	24.4	2.9	0.9	0.3	–21.6
St Leonards	58.9	47.4	9.7	4.3	49.8	64.5	2.8	2.9	0.1	–22.8
South Corio	198.2	78.5	12.8	4.2	–	–	–	–	–	–
Swan Bay	–	–	–	–	23.2	12.7	1.3	0.2	0.4	–16.0

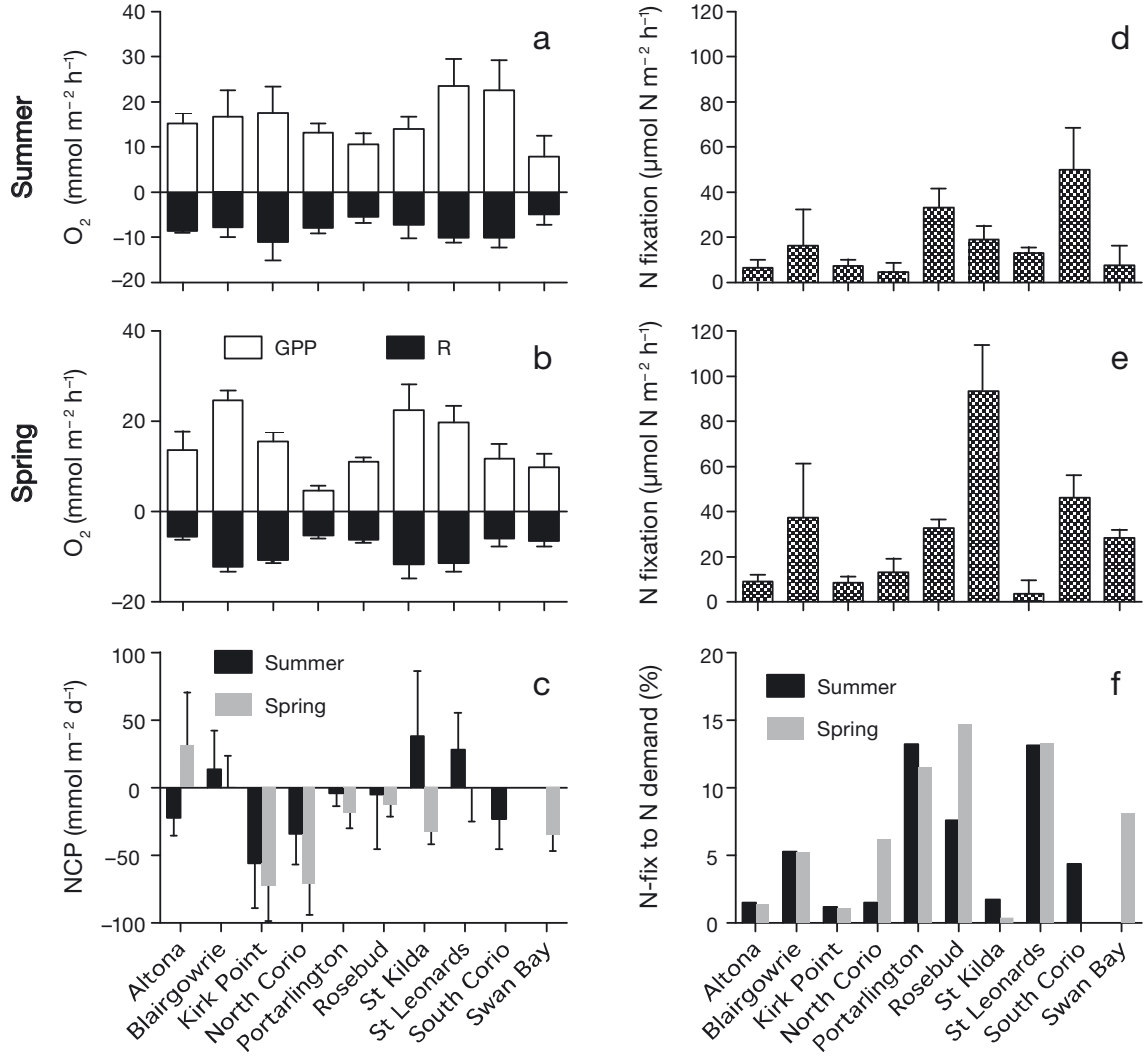

Fig. 2. Rates of gross primary production (GPP) and respiration (R) during (a) summer and (b) spring. (c) Rates of daily net community production (NCP) during both seasons. Nitrogen fixation during (d) summer and (e) spring (mean ± SD, n = 4). (f) Rate of nitrogen fixation as a percent of total estimated nitrogen demand by *Zostera* spp. (see 'Materials and methods' for calculation)

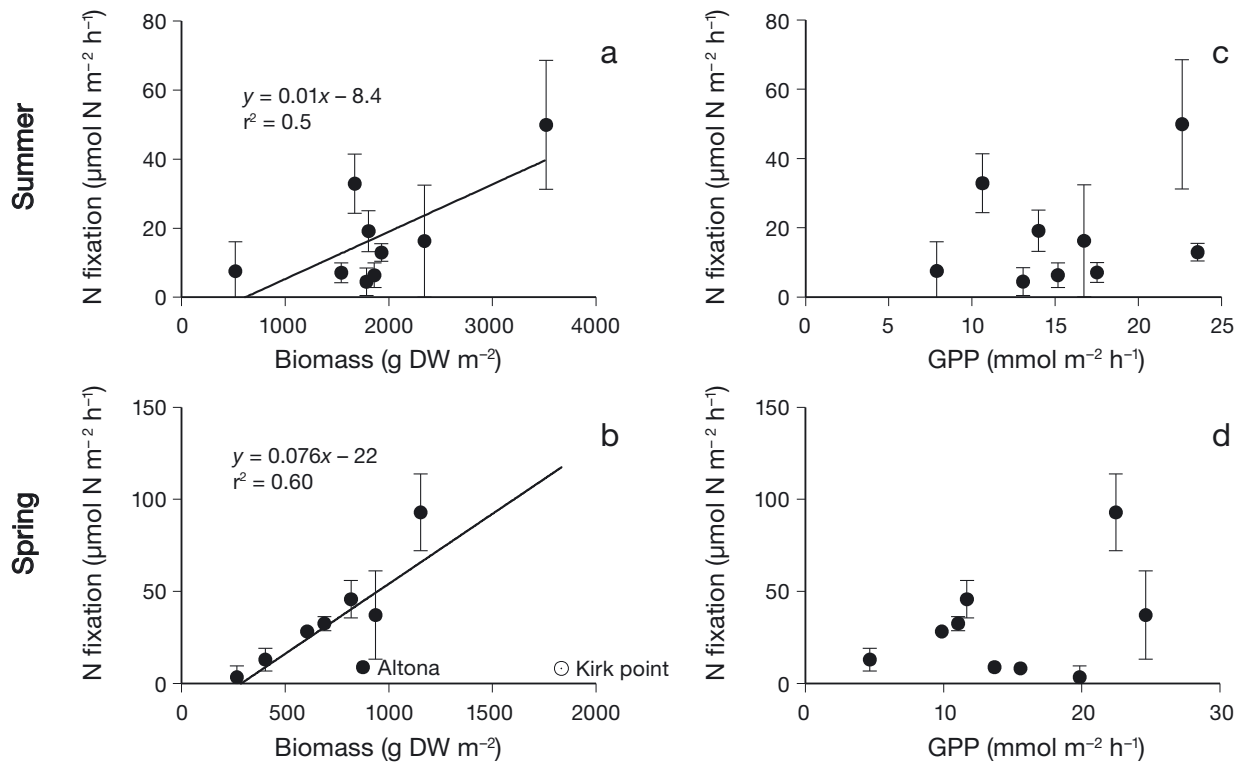

Fig. 3. Areal rates of nitrogen fixation (mean ± SD, n = 4) versus plant biomass in (a) summer and (b) spring, as well as areal rates of nitrogen fixation versus gross primary production (GPP: light O_2 flux minus dark O_2 flux) during (c) summer and (d) spring. Only statistically significant relationships are shown ($p < 0.05$). Note that the Kirk Point datum was excluded from the relationship during spring

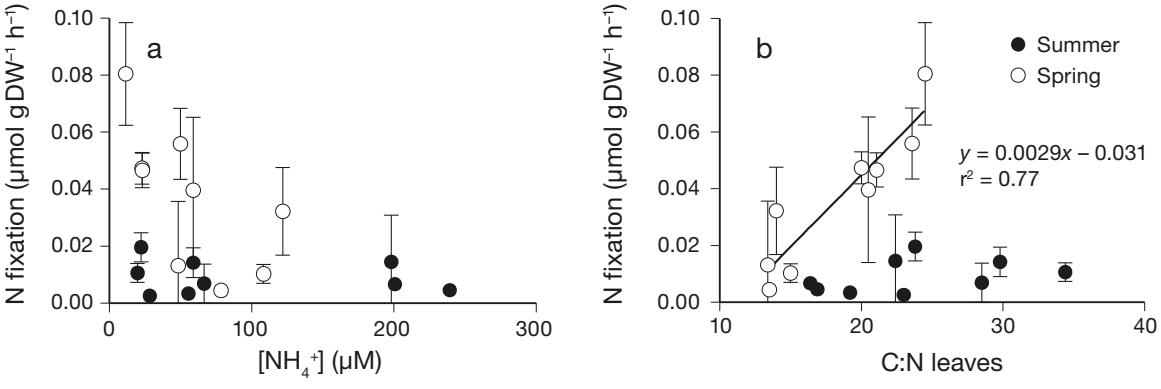

Fig. 4. Plant biomass normalized rates (mean ± SD, n = 4) of nitrogen fixation versus (a) integrated average porewater NH_4^+ concentrations and (b) C:N ratio of *Zostera* spp. leaves. Only statistically significant relationships are shown ($p < 0.05$)

DISCUSSION

Factors controlling nitrogen fixation

The only variable significantly correlated with nitrogen fixation rates for both seasons was plant biomass (excluding Kirk Point in spring, see below for further discussion). This finding is consistent with previous studies (Cole & McGlathery 2012) and the well accepted paradigm that seagrass have a mutualistic relationship with nitrogen-fixing sulphate-reducing bacteria through the supply of labile organic carbon (Nielsen et al. 2001). During spring, the biomass-specific nitrogen fixation rates were a factor of 2 to 3 higher than summer. Higher rates of nitrogen fixation in spring compared to summer contrasts with previous studies of nitrogen fixation in seagrass beds in temperate systems, where highest

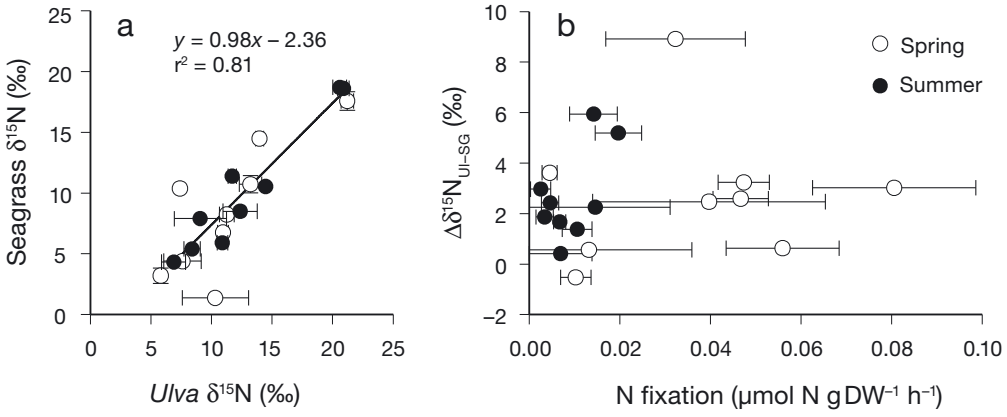

Fig. 5. (a) *Zostera* spp. leaf $\delta^{15}N$ versus *Ulva* spp. $\delta^{15}N$ collected from the same site and (b) the difference between *Ulva* spp. $\delta^{15}N$ and *Zostera* spp. $\delta^{15}N$ versus plant biomass-normalized nitrogen fixation rate. Only statistically significant relationships are shown ($p < 0.05$)

rates are typically observed at the height of summer (Welsh et al. 1996a, McGlathery et al. 1998, de Wit et al. 2001). The fact that these systems are located at slightly higher latitudes than Port Phillip Bay means that they have less light and cooler temperatures, conditions that prevent high nitrogen fixation rates in spring. The most likely explanation for the pattern we observed is that new plant growth had ceased by summer when average site biomass was ~2 times higher than spring, consistent with previous studies (Derosa et al. 1990). The cessation of growth would correspond with a reduction in overall plant nitrogen demand. By contrast, new plant growth was high during spring (possibly also in part due to higher catchment nutrient inputs during this period), which increased nitrogen demand and led to significantly higher rates of nitrogen fixation per unit of plant biomass.

The other key factor that has previously been shown to control nitrogen fixation is the availability of nitrogen. Porewater NH_4^+ concentration had no significant relationship between nitrogen fixation rates in both spring and summer. It should be noted, however, that porewater NH_4^+ concentration may not be the best indicator of nitrogen availability. Although most sediment NH_4^+ concentrations observed were consistent with expected patterns from the study sites (with respect to the proximity or isolation from nitrogen sources), sediment NH_4^+ concentrations are likely to be extremely heterogeneous on the mm–cm scale and there can be regions of very high NH_4^+ in nearby pockets laterally or vertically separated from the root zone (likely correlated to the presence of fauna). Under such a scenario, diffusive transport to the roots could be quite slow and the actual supply rate to the plant very low. In addition, some sites may have a high supply of nitrogen from the water column (for example Kirk Point), further confounding the use of porewater NH_4^+ as a proxy for nitrogen availability to the plant.

Another common proxy for nitrogen availability to seagrass is leaf C:N ratio, with C:N ratios of ~12–15 being observed in relatively nutrient-rich areas and C:N ratios >20 more typical of oligotrophic waters (Fourqurean et al. 1997, Fourqurean & Zieman 2002). Within Port Phillip Bay, we observed a very similar pattern to these previous studies, with the lowest leaf C:N ratios (<15) being observed in the northern section of the bay in the vicinity of the Western Treatment Plant and the Yarra River plume, and the highest C:N ratios typically being observed in the southern section of the bay away from nitrogen inputs. The strong positive relationship between C:N ratio and biomass-normalized nitrogen fixation rate during spring (Fig. 4b) indicates that nitrogen availability exerted a strong influence on nitrogen fixation rates. This contrasts sharply with the absence of a similar relationship during summer. As discussed above, we hypothesize that during summer, when seagrass biomass is highest, assimilative nitrogen demand is relatively low. There are 2 possible mechanisms for such regulation of nitrogen fixation at the community scale. The first is that sulphate reducers down-regulate nitrogen fixation, as has been indicated by previous studies (Capone 1982, Welsh et al. 1997), although this finding is not universal (McGlathery et al. 1998). Another possible, but as yet unexplored mechanism, is that the plants themselves regulate the amount of carbon exuded from their roots to control the activity of sulfphate-reducing bacteria. During times of peak nitrogen demand relative to availability, it is possible that seagrass will exude more carbon to stimulate sulphate-reducing bacteria and nitrogen fixation. Conversely, during the later growing season when nitrogen demand is low, carbon exudation decreases, leading to lower rates of nitrogen fixation. To

further test such a hypothesis requires measurements of both nitrogen fixation and carbon exudation rates on a mm scale, which is technically challenging.

Previous studies of nitrogen fixation by *Zostera* spp. in temperate systems have measured rates falling in the range of 5 to 20 µmol m^{-2} h^{-1} in the light (Welsh et al. 1996b, 2000, McGlathery et al. 1998, Risgaard-Petersen et al. 1998, de Wit et al. 2001). Although broadly within the same order of magnitude, the rates measured in our study (3–90 µmol m^{-2} h^{-1}) are generally higher than in these previous studies. We suggest the key reason for the relatively high rates of nitrogen fixation observed at some of the study sites within Port Phillip Bay is the availability of nitrogen. Port Phillip Bay receives a total nitrogen load of ~200 mmol m^{-2} yr^{-1} (Harris et al. 1996) compared to 460 mmol m^{-2} yr^{-1} in the Basin d'Arcachon (de Wit et al. 2001) and 2700 mmol m^{-2} yr^{-1} in Limfjord (Møhlenberg 1999), where these previous studies took place. Conclusions about geographic influences on nitrogen fixation should therefore be tempered against regional nitrogen loading rates.

It has previously been shown that nitrogen fixation in temperate seagrass beds comprises a relatively small fraction of nitrogen demand (Risgaard-Petersen et al. 1998, Welsh 2000). In this study, we estimated that nitrogen fixation comprises a variable fraction of nitrogen demand, with a maximum of 15% (more likely to be ~10% given that all of our nitrogen fixation rates were measured in the light), which is broadly consistent with previous studies (Welsh 2000). The balance of nitrogen assimilated was most likely nitrogen derived from the water column as well as nitrogen remineralized within the sediment. Indeed, if net daily metabolism is considered, there was generally a close balance between production and remineralization of organic material with a slight tendency towards heterotrophy, indicating that nutrient supplies from mineralization matched demand for production. This observation is consistent with the emerging paradigm that seagrass beds trap sediment and organic material within the bed, leading to a net import of nutrients in a particulate form that are subsequently supplied to the seagrass within the bed (Evrard et al. 2005, Barrín et al. 2006). Further support for this hypothesis comes from the sediment stable isotope data, which had $\delta^{13}C$ signatures of −13.8 to −22.8‰. Given that the average $\delta^{13}C$ for *Zostera* spp. leaves was −10.7‰ and assuming an algal (including phytoplankton, macroalgae and microphytobenthos) endmember of −23‰, then this equates to ~25 to 100% organic matter derived from algal sources.

Can stable isotopes of nitrogen be used as a proxy for nitrogen fixation?

The second aim of this study was to investigate whether there is any evidence that the stable isotope ratio of *Zostera* spp. leaves could be used as a proxy for nitrogen fixation. Given the strong isotope gradient caused by the Western Treatment Plant in Port Phillip Bay, we estimated the water $\delta^{15}N$ endmember using the $\delta^{15}N$ of *Ulva* spp., which has not been reported to be associated with nitrogen-fixing organisms. Consistent with our initial hypothesis, *Ulva* spp.was on average ~2.4‰ heavier than *Zostera* spp. collected from the same site. There was, however, no relationship between the difference in *Zostera* spp. $\delta^{15}N$ and *Ulva.* spp. $\delta^{15}N$ and the biomass normalized nitrogen fixation rate, suggesting that nitrogen fixation did not play a role in the observed offset. This finding is also consistent with the finding that nitrogen fixation contributed <15% of nitrogen demand in the sites studied here. In tropical systems, where nitrogen fixation can comprise a much larger fraction of nitrogen requirements (Welsh 2000), we speculate that the $\delta^{15}N$ of seagrass tissue compared to external sources may provide a proxy for nitrogen fixation.

The cause of the consistent offset in the $\delta^{15}N$ of *Zostera* spp. and *Ulva* spp. remains to be explained. One hypothesis is that nitrogen fractionation during decomposition leads to the accumulation of isotopically light bioavailable nitrogen within the porewater, which is subsequently assimilated by *Zostera* spp. This hypothesis is consistent with a recent study which reported that the fractionation of nitrogen during anoxic decomposition is −1.4 to −2.3‰, a value similar to our observed offset (Möbius 2013). Another hypothesis to explain this offset is that *Zostera* spp. are able to fractionate nitrogen in favour of ^{14}N during assimilation of the typically high concentrations of NH_4^+ observed in the sediment porewater. In contrast, *Ulva* spp. are only able to assimilate nitrogen from the water column, where less fractionation occurs due to lower nitrogen concentrations. If this was the case, we would expect to see an increase in fractionation with higher NH_4^+ in the porewaters or with lower leaf C:N ratio, which, as discussed previously, appears to act as a good proxy for nitrogen availability in spring. However, we observed no significant relationship between these variables. We therefore believe that isotope fractionation during nitrogen decomposition to NH_4^+ and subsequent assimilation from the porewater is the most likely explanation for the consistent offset observed between *Zostera* spp. and *Ulva* spp.

Conclusion

In conclusion, beds of *Zostera* spp. in the temperate embayment studied derived the majority of their nitrogen from the sediment porewater, which is ultimately derived from the decay of phytoplankton trapped within the bed. This is consistent with: (1) measures of net metabolism, which show that the sites had a tendency towards net heterotrophy (i.e. net import of organic matter); (2) stable isotopes of carbon which show that algal material contributes 25 to 100% of organic material to the sediment; and (3) a consistent offset of ~2.4‰ $\delta^{15}N$ between *Zostera* spp. and locally collected *Ulva.* spp. During periods of nitrogen limitation and peak nitrogen demand in spring, nitrogen fixation rates are highest, supplying up to 15% of total nitrogen demand. By contrast, nitrogen-replete sites had relatively low rates of nitrogen fixation during the spring growing season. This suggests that nitrogen fixation is regulated based on supply and demand at the community scale. The exact mechanisms regulating nitrogen fixation are yet to be fully elucidated and require further research.

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