We have carefully considered the reviewers comments and we thank him/her for pushing us on the issue of considering what the slope means. Based on their recommendation we have now focus on this and removed the t-test which they suggest is not appropriate, and we now agree that this hides important information.

In essence, we believe the slope of <1 for the seagrass tissue vs porewater ammonium reflects nitrogen fixation and the slope of < 1 for seagrass for sediment $\delta^{15}N$ versus porewater ammonium reflects isotopically enriched algal material entering the sediment.

We have now modified the discussion, abstract and conclusion to add in this finding, and these changes are visible in track changes.

Regarding the ANOVAs, we believe the statistical significance of temporal and spatial variation in the variables considered here is not important to the focus of the manuscript (and we do not have any central hypothesis in this regard). We now state some of the key general patterns of relevance to the manuscript, most notably the enriched $\delta^{15}N$ values and high porewater NH_4^+ concentrations in the vicinity of the sewage treatment plant.

Negligible isotopic fractionation of nitrogen within temperate *Zostera* spp. meadows

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Abstract

Seagrass meadows form an ecologically important ecosystem in the coastal zone. The $^{15}N/^{14}N$ ratio of seagrass is commonly used to assess extent to which sewage derived nitrogen may be influencing seagrass beds. There have however, been few studies comparing the $^{15}N/^{14}N$ ratios of seagrass beds, their associated sediments and of critical importance, the porewater NH_4^+ pool, which is most bioavailable. Here, we undertook a study of the $^{15}N/^{14}N$ ratios of seagrass tissue, sediment porewater NH_4^+ pool and the bulk sediment to elucidate the extent of any fractionating processes taking place during organic matter mineralisation and nitrogen assimilation. The study was undertaken within two coastal embayments known to receive nitrogen from a range of sources including marine, urban and sewage sources. There was close agreement between the bulk sediment $\delta^{15}N$ and seagrass $\delta^{15}N$ (r^2 of 0.92 and mean offset of 0.9‰); illustrating a close coupling between the plant and sediment pools. The $\delta^{15}N$ of porewater NH_4^+ was strongly correlated with the $\delta^{15}N$ of both the sediment and seagrass tissue. For both of these relationships, however, the intercept of the line was not significantly different from 0 and the slopes were not 1:1, reflecting an enrichment of the porewater NH_4^+ $\delta^{15}N$ pool relative to seagrass tissue and bulk sediment $\delta^{15}N$ at high $\delta^{15}N$ values. We suggest that nitrogen fixation is the most likely explanation for the observation that the $\delta^{15}N$ of seagrass tissue is lower than porewater NH_4^+. Conversely, we suggest that the most likely explanation for the enrichment of porewater NH_4^+ above bulk sediment was through the preferential mineralisation of isotopically enriched algal material (nitrogen derived from sewage sources) within the sediment as $\delta^{15}N$ increased in the vicinity of a sewage treatment plant.

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1 Introduction

Seagrass meadows are widely recognised for their high ecological value, providing a habitat for juvenile fish, stabilising sediment and sequestration of nutrients (Larkum et al., 2006; Nielsen et al., 2004). These ecosystems have been in great decline, due in part to increased nutrient run off and eutrophication (Waycott et al., 2009). Because of the importance of nitrogen in controlling the productivity and eutrophication of coastal environments and seagrass beds, there is great interest in identifying sources of nitrogen to coastal areas.

The ratio of $^{15}N/^{14}N$ (hereafter referred to as $\delta^{15}N$) in seagrass tissue has been widely used to trace nitrogen derived from anthropogenic sources, in particular sewage into seagrass beds (McClelland and Valiela, 1998). The $\delta^{15}N$ of seagrass leaves have also been used as a proxy for nitrogen fixation by seagrass (Hirst et al., 2016; Papadimitriou et al., 2005). A recent global meta-analysis of seagrass $\delta^{15}N$ values found that latitude exerted an overwhelming influence on seagrass $\delta^{15}N$ values, with lighter values being observed in the tropics compared to temperate regions (Christiaen et al., 2014). Possible explanations for this trend included increased nitrogen fixation in tropical waters and an increased predominance of treated sewage as a source of nitrogen in temperate regions. This study highlights the fact that we still have a poor understanding of the factors that control nitrogen isotope ratios in seagrass beds.

A major pool of nitrogen available to seagrasses is ammonium (NH_4^+) derived from within the sediment, particularly when water column nitrogen concentrations are low (McGlathery et al., 2001). Seagrasses are able to exploit this pool of bioavailable nitrogen through their ability to assimilate nutrients not just through their leaves, but also their roots. Generally, this pool of NH_4^+ within the sediment is present in high concentrations, and as a consequence it is possible that ^{14}N will be preferentially assimilated leading to significant isotopic fractionation (Cook et al., 2015). Conversely, it is possible that the distribution of the NH_4^+ pool is highly heterogeneous, with low concentrations in the vicinity of roots which would lead to minimal fractionation during assimilation. Previous work by Papadimitriou et al. (2006) in *Zostera noltii* meadows found that the isotopic signature of the seagrass tissue reflected the isotopic signature of porewater NH_4^+. This suggests that the benthic pool of nitrogen made a sizeable contribution to the nitrogen requirements of the seagrass (Papadimitriou et al. 2006). These findings are also consistent with studies in other marine sediments colonised by vegetation such as mangroves. McKee et al. (2002) observed that the fractionation of the residual nitrogen pool in the sediment was dependent on the nitrogen (and nutrient) availability, with limited nitrogen fractionation being observed under nitrogen-limiting conditions.

In addition to the assimilation of nitrogen leading to potential isotopic fractionation of the nitrogen pool within the sediment, the breakdown (mineralisation) of organic material is another potential source of nitrogen isotope fractionation. Previous studies have found that due to the metabolic discrimination of ^{14}N over ^{15}N (e.g. Saino and Hattori, 1987; Altabet and Francois, 1994; Sachs and Repeta, 1999), the residual organic material can end up being isotopically enriched. Previous work by Mobius (2013), Lehmann et al. (2002) and Rooze and Meile (2016) found that in a range of marine environments isotopic fractionation was generally between 2 and 4 ‰.

Nitrogen fixation is also known to be a significant process within seagrass sediments (Welsh, 2000). Previous studies conducted in both Western Port and Port Phillip Bay (Cook et al. 2015; Russell et al. 2016) found that nitrogen fixation was responsible for the production of appreciable amounts of bioavailable nitrogen which was utilised by seagrass to meet a portion of its nitrogen requirements. It has been commonly reported that nitrogen fixation results in little isotopic fractionation (Owens 1988), therefore in situations where nitrogen fixation is a significant process (such as in seagrass meadows), this can result in an isotopically lighter nitrogen pool. Consequently, one might expect this to lead to a lower $\delta^{15}N$ of NH_4^+ within the porewater compared to the sediment.

Given this previous research, it is clear that there are several processes that result in potentially significant fractionation of isotopes within seagrass meadows, however few studies have explicitly investigated its occurrence or importance. Given the widespread use of $\delta^{15}N$ as a proxy for nitrogen sources and processes within seagrass, it is critical that we understand the extent of nitrogen fractionation within seagrass colonised sediments. To address this, we collected porewater samples for $\delta^{15}N$ analysis of NH_4^+, bulk sediment and seagrass tissues to compare the $\delta^{15}N$ values from a range of seagrass beds influenced by different sources of nitrogen.

2 Materials and methods

2.1 Study area

A total of 13 sites containing *Zostera muelleri* (except at St. Leonards which contained *Zostera nigricaulis*) were selected for this study, with 10 sites located in Port Philip Bay and 3 sites located in Western Port (Figure 1). Both bays are located in Victoria, Australia and are temperate, intertidal marine embayments. Port Philip Bay is the largest bay in Victoria and has a surface area of ~1930 km^2, and Western Port located roughly 55 km south-east of Melbourne and has a surface area of ~650 km^2. The sites that were selected from Port Phillip Bay have previously been described in Cook et al. (2015) and exhibit a strong gradient in $\delta^{15}N$ from south to north. Whereas, the sites selected from Western Port have been previously described in Russell et al. (2016) and exhibited a range of nutrient and sediment inputs, as well as differences in areal seagrass coverage. Major sources of nitrogen to Port Phillip Bay include the rivers and drains which contribute ~1000 tonnes per year (TN; total nitrogen) and the Western Treatment plant which contributes ~1000-1500 tonnes TN per year (Harris et al., 1996; Hirst et al., 2016). For Western Port, terrestrial sources of nitrogen from the rivers contribute ~650 tonnes TN per year (Russell et al., 2016).

2.2 Sample collection and preservation

Field sampling was carried out in Western Port at intervals of ~2 months between February and November 2016, and sampling in Port Phillip Bay was carried out during August and December in 2016. Differences in the temporal aspect of the sampling regime between Western Port and Port Phillip Bay were a reflection of the logistical difficulties encountered accessing and sampling at the different field sites. The highly tidal nature of the sites selected meant that access to some proved problematic and were only able to be accessed sporadically. Three intact cores containing *Zostera* spp. (65 mm ID × 300 mm long) were obtained from each site to a sediment depth of ~20 cm. Additionally, intact samples (n=2 – 4) of *Zostera* spp. were obtained from each sample site for elemental (N) and stable isotope analysis ($\delta^{15}N$). All samples were returned to Monash University within 4 hours of sampling.

The overlying water column from the intact cores was removed using a syringe filter leaving only saturated sediment. This sediment was subsequently homogenised and the porewater extracted using of a combination of centrifugation and vacuum filtration. The extracted porewater was subsequently filtered through 0.45 µm and 0.20 µm Sartorius Minisart syringe filters, and frozen until analysis, along with the samples of seagrass. This approach ignores possible depth variation in the porewater $\delta^{15}N$ values, however, previous work has shown this has minimal variation with depth (Freudenthal et al. 2001; Prokopenko et al. 2006).

2.3 Seagrass nitrogen isotope ratios

Seagrass samples were collected from each site, washed by hand with deionised water to ensure that all detrital and epiphytic material was removed and then dried to a constant weight at 60 °C for 48 hours. The seagrass samples were separated into leaves and roots/rhizomes before being pulverized using a Retsch MM400 ball mill. All analyses were carried out at Monash University on an ANCA GSL2 elemental analyzer interfaced to a Hydra 20-22 continuous-flow isotope ratio mass-spectrometer (IRMS; Sercon Ltd., UK). The stable isotope data was reported in the delta notation ($\delta^{15}N$) and relative to the isotopic ratio of atmospheric N_2 (R_{Air}= 0.0036765). The precision of the nitrogen analysis was ±0.2‰ (SD; n=5), and ±0.5 µg (SD; n=5). To ensure the accuracy of the isotopic results, the following internal standards (ammonium sulphate, sucrose, gelatine and bream) were run concurrently with the seagrass samples. These internal standards have been calibrated against internationally-recognised reference materials (i.e. USGS 40, USGS 41, IAEA N1, USGS 25, USGS 26 and IAEA C-6).

2.4 Nutrient analysis (NH_4^+, FRP and NO_X)

The concentration of NH_4^+, filterable reactive phosphorous (FRP) and combined NO_3^- and NO_2^- (hereafter NO_X) in the porewater at each site was determined colourimetrically (APHA, 2005) in the National Association of Testing Authorities (NATA) certified laboratory of Monash University (Water Studies Centre). Analysis of ERA-certified reference materials (Lot number P2473-505) indicated the accuracy of the spectrophotometric analysis was within 2% relative error.

2.5 Isotopic analysis of porewater NH_4^+ ($\delta^{15}N$-NH_4^+)

To determine the isotopic signature ($\delta^{15}N$) of the NH_4^+ in the porewater, a slightly modified version of the ammonium diffusion method described by Brooks et al. (1989) was used. Incubations were performed in 250 mL Schott laboratory bottles (Schott AG, Mainz, Germany), with target concentrations of ~17.9 to ~28.6 µM N-NH_4^+ in a final volume 100 mL. Any required dilutions were carried out using NaCl amended ultra-pure water (~35 ppt.) in order to approximate in situ salinities, and prevent swelling of the membranes housing the acid-traps (Holmes et al., 1998). A subsample of 1 mL was removed from each diffusion bottle prior to the addition of the acid trap in order to determine the actual concentration of N-NH_4^+ present in each sample. These samples were filtered through 0.45 µm and 0.20 µm Sartorius Minisart syringe filters and frozen until they were analysed using the indophenol blue method (APHA, 2005). Acid traps were constructed using 4 × 8 mm slices of pre-ashed GF/F paper (Whatman, Buckinghamshire, UK) and acidified with 20 µL of 2.5 M $KHSO_4$, the acidified filter paper was then housed in PTFE membranes (47 mm diameter, 10 µm pore size, Merck Millipore) and crimped shut. These acid traps were added to each diffusion bottle along with ~0.6 g MgO to raise the pH of the solution to ~10. A series of standards were run concurrently using USGS25, USGS26 and IAEA-N1 to ensure that no mass-dependent fractionation effects were encountered. Incubations were carried out at room temperature for 3 weeks on shaker tables at ~135 rpm and the acid traps were then dried in a desiccator in the presence of concentrated HCl for 3 weeks. Afterwards, the dried filter paper was removed from the PTFE membranes and encapsulated in 12 × 8 mm tin capsules (Sercon Ltd., UK). Samples were analysed for their isotopic signature as well as the total mass of nitrogen using the

IRMS described previously. The average recovery obtained for the standards and porewater samples in this study was 100 ± 5%.

2.5 Statistical analysis

Linear regression analysis was carried out using GraphPad Prism 7 to investigate the relationships between variables. For all analyses, the level of significance required for the rejection of the null hypothesis was set at $p<0.05$.

3 Results

3.1 Nutrient concentrations and isotopic signatures ($\delta^{15}N$) of seagrass and porewater NH_4^+

Porewater concentrations of both FRP and NO_X were consistently low throughout the year in Western Port, with FRP ≤2 µM and NO_X ≤27 µM (Table A1). In contrast, the porewater concentrations of NH_4^+ were 1 – 2 orders of magnitude higher (Figure 2a), with generally higher concentrations at both Corinella and Rhyll than compared to Coronet Bay. Similarly, in Port Phillip Bay, little FRP and NO_X were detected in the porewater, with the concentrations at all times ≤44 µM and ≤27 µM respectively (Table A2), whereas the concentration of NH_4^+ was up to an order of magnitude higher (Figure 2b). A high spatial variation in NH_4^+ concentrations were also evident, with the sites in close proximity to terrestrial nitrogen inputs (i.e. Kirk Point and St. Kilda) consistently having the highest porewater NH_4^+ concentrations.

The $\delta^{15}N$ of seagrass in the context of this study refers to the $\delta^{15}N$ of the roots. There was, however; no significant difference between the $\delta^{15}N$ of the seagrass roots and that of the leaves (Figure A1). The heaviest values were consistently found at both Corinella and Rhyll, which were in the closest proximity to human activities and catchments inputs. In contrast, the site at Coronet Bay was the furthest from these inputs and showed a correspondingly low isotopic signature. In contrast, there was appreciable variation in the isotopic signature of the seagrass in Port Phillip Bay (Figure 2d), with

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values varying from ~2.2‰ to in excess of 16‰ over the course of 2016. The highest isotopic signatures were consistently found in the northerly sites (Kirk Point, Altona and St. Kilda), and south-eastern sites (Blairgowrie and Rosebud), with all isotopic signatures ≥6.9‰. In contrast, the seagrass meadows located in the south-west of Port Phillip Bay (Swan Bay – North Corio) consistently exhibited the lowest isotopic signatures of between 2.2‰ and 6.4‰.

The isotopic signature of porewater NH_4^+ in Western Port was found to exhibit relatively little variation, with values ranging from ~3.9 to 7‰ throughout the course of this study (Figure 2e). Whilst there was no evidence of significant temporal variability in the isotopic signature at each site, there was, however, an apparent north-south gradient in isotopic signatures. In general, the least isotopically enriched porewater NH_4^+ was found in the northern sites (Corinella and Coronet Bay), whilst the highest was found at Rhyll.

Unlike Western Port, appreciable spatial variation in the isotopic signature of porewater NH_4^+ was observed throughout Port Phillip Bay. The highest values of between 11.4 and 19.4‰ were consistently observed in the northern sections of the bay from Kirk Point to St. Kilda, whilst sites such as Portarlington and North Corio consistently displayed the lowest values of between 4.2 and 6.4‰ (Figure 2f). The isotopic signature was found to remain reasonably constant throughout the year with the exception of St. Leonards, which displayed an appreciable decrease from winter to summer.

3.2 Potential isotopic effects associated with vegetative assimilation and mineralisation

An extremely strong positive and statistically significant linear correlation was observed between the isotopic signatures of seagrass and porewater NH_4^+ for all sites throughout this study (Figure 3a; $r^2 = 0.86$, $p<0.001$), with a gradient of 0.79 (95% CI 0.67-0.90) and a y-intercept of 0.09 (95% CI -0.97-1.1). This showed that there was no consistent offset in the nitrogen isotope signatures for the porewater NH_4^+ and seagrass tissue (the y-intercept was not significantly different from 0), but that there was an increasing difference between the seagrass tissue and the sediment porewater NH_4^+ pool at higher $\delta^{15}N$

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values, with seagrass roots being ~5‰ less than the porewater NH_4^+ pool at the highest $\delta^{15}N$ values. A strong relationship was also observed between the isotopic signatures of sedimentary nitrogen pool and porewater NH_4^+ (Figure 3b; $r^2 = 0.89$, $p<0.001$), with a gradient of 0.77 (95% CI 0.67 – 0.88), and a y-intercept of 0.72 (95% CI -0.27 – 1.7). This showed that there was no consistent offset in the nitrogen isotope signatures of the porewater NH_4^+ and the sediment $\delta^{15}N$ (the y-intercept was not significantly different from 0), but there was an increasing difference between the sediment $\delta^{15}N$ and the sediment porewater NH_4^+ pool at higher $\delta^{15}N$ values, with the sediment $\delta^{15}N$ being ~5‰ less than the porewater NH_4^+ pool at the highest $\delta^{15}N$ values. Bulk sediment and seagrass $\delta^{15}N$ values were also tightly correlated with an r^2 of 0.92 (Figure 4), with a gradient of 0.944 (95% CI 0.84-1.05) and a y-intercept of 0.9 (95% CI 0.092-1.71). This showed that there was a small consistent offset of ~1‰ between the $\delta^{15}N$ of the seagrass and the sediment. There was no relationship between $\Delta(\delta^{15}N_{\text{porewater } NH_4^+ \text{–seagrass}})$ and porewater NH_4^+ concentration, nor $\Delta(\delta^{15}N_{\text{porewater } NH_4^+ \text{–sediment}})$ and porewater NH_4^+ concentration (Figure 5).

4 Discussion

Overall, our study showed very close agreement between the bulk sediment $\delta^{15}N$ and the seagrass tissue $\delta^{15}N$ (Figure 4). This finding is not surprising and consistent with the paradigm that seagrasses rely on sediment derived nitrogen (Barrón et al., 2006), and that a significant fraction of organic matter within seagrass sediments is derived from seagrass itself (Kennedy et al., 2010). The mineralisation of organic matter to NH_4^+ and subsequent assimilation by seagrass roots is a critical link coupling the nitrogen $\delta^{15}N$ values in these two pools. To date, there has only been one study on the $^{15}N/^{14}N$ ratios of porewater NH_4^+ and its relationship with $^{15}N/^{14}N$ ratios in bulk sediment and vegetation in coastal sediments (Papadimitriou et al., 2006). That study focused on one location in Wales over a seasonal cycle and the present study greatly extends the geographical spread of simultaneous isotope measurements of seagrass tissue, sediment and porewater.

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4.1 Isotopic signatures of the seagrass pool relative to the porewater NH_4^+ pool

Our results showed that at low $\delta^{15}N$ values, there was typically a close agreement between the porewater NH_4^+ and seagrass isotopic signatures, but this increased to ~5‰ at the highest $\delta^{15}N$ values. The highest $\delta^{15}N$ values were observed in the north of Port Phillip Bay which is influenced by the Western Treatment Plant (WTP). Given that there were generally negligible temporal changes in isotopic signature at these sites (Figure 2f), we assume that the offsets observed here were not artefacts of lags associated with changing isotope pools over time. Three possible explanations for these offsets are considered as follows:

1. Seagrass are assimilating another source of nitrogen through their leaves:

In the following discussion, we assume negligible fractionation of nitrogen during leaf assimilation from the water column. We justify this on the basis that NH_4^+ and NO_3^- concentrations in the water column are typically < 1µM at the study sites, and therefore unlikely to be significant fractionation taking place.

If we compare the likely $\delta^{15}N$ values of nitrogen source to the seagrass at each of the sites (Table 1), they are typically higher than the porewater values. At the sites closest to the WTP (Kirk Point and Altona), the $\delta^{15}N$ of the seagrass is ~15‰ which is ~ 8‰ lighter than sewage derived DIN (22.5‰) and therefore direct assimilation of sewage derived by the seagrass leaves seems unlikely at these sites.

2. Fractionation of nitrogen during assimilation from the porewater NH_4^+ pool:

Handley and Raven (1992) reported that the isotopic fractionation associated with the vegetative assimilation of NH_4^+ in a range of environments can vary from 9 to 18‰. Within soils, there is typically a fractionation of only 1 – 2‰ in association with plant assimilation owing to diffusion limitation (Kendall and McDonnell, 1998; Michener and Lajtha, 2007). At first glance, the notion of diffusion limitation seems at odds with the observation that there were often high concentrations of NH_4^+ in the sediment. One possible explanation for this is that NH_4^+ concentrations are highly heterogeneous in the sediment resulting in very low concentrations directly within the vicinity of roots where active

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assimilation is occurring (Welsh et al., 1997). As such, the assimilation of NH_4^+ from the porewater is effectively diffusion limited, leading to minimal isotope fractionation. Evidence to support this comes from the observation that there was no relationship between the Δ ($\delta^{15}N_{porewater\ NH_4^+ - seagrass}$) and the bulk porewater NH_4^+ concentration (Figure 5). Such a conclusion is also supported by the lack of an offset between the isotopic signature of the seagrass roots relative to that of the porewater NH_4^+ (Figure 3a), with the y-intercept of this graph at ~0.09‰. This finding is in agreement with Papadimitriou et al. (2006) who also concluded diffusion limitation was a likely explanation for the small offset observed between porewater and seagrass $\delta^{15}N$ values.

3. Nitrogen fixation within the rhizosphere:

Nitrogen fixation within the rhizosphere of seagrass is well documented and it is thought to be mediated by sulfate reducing bacteria, tightly coupled to the exudation of organic carbon from seagrass roots (Welsh, 2000). As such it is possible that newly fixed nitrogen (which has a $\delta^{15}N$ of ~ 0‰) is rapidly assimilated by seagrass rather than entering the bulk sediment pool. Under this scenario, seagrass nitrogen would become isotopically depleted compared to the porewater NH_4^+ pool. This explanation is also consistent with the increasing difference (i.e. gradient of 0.79) between the $\delta^{15}N$ of porewater NH_4^+ and seagrass roots at high $\delta^{15}N$ value because there is a larger difference in $\delta^{15}N$ between the nitrogen fixation and porewater NH_4^+ endmembers. This means that for a given amount of nitrogen fixation, there will be a much larger offset between the $\delta^{15}N$ of seagrass tissue and porewater NH_4^+. Indeed if we use a simple linear mixing model (Fry, 2006) assuming a $\delta^{15}N$ for nitrogen fixation of 0‰ and no fractionation during assimilation, then the slope in Figure 3a represents the proportion of nitrogen derived from the porewater, and the remainder is derived from nitrogen fixation, which is ~20% in this instance. Direct measurements of nitrogen fixation in Port Phillip Bay have previously suggested nitrogen fixation contributes a maximum of ~15% to nitrogen demand, with a mean of ~5% (Cook et al., 2015), however, there are large uncertainties inherent in scaling instantaneous rate measurements of nitrogen fixation over the entire growth cycle of seagrass. More broadly, an estimate of 20% contribution of nitrogen fixation to seagrass nitrogen requirements is consistent with estimates of up to 30% in other systems (Cole and McGlathery, 2012; Russell et al., 2016). It is therefore plausible that

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nitrogen fixation accounts for the observed difference between the δ^{15}N of porewater NH_4^+ and seagrass tissue, in addition to a small but variable contribution of isotope fractionation during assimilation.

4.2 Isotopic signatures of the sediment pool relative to the porewater NH_4^+ pool

In general, the porewater NH_4^+ was isotopically enriched compared to the sediment pool (Figures 3b and 5b), and this enrichment was greatest at highest δ^{15}N values (sites influenced by the WTP as discussed previously) as indicated by the slope of 0.77 in Figure 3b. Once again this is generally consistent with previous studies of soils (Kendall and McDonnell, 1998; Michener and Lajtha, 2007) and sediment vegetated with seagrass (Papadimitriou et al 2006). Previous work investigating the isotopic fractionation of nitrogen during mineralisation in marine sediments has found that this fractionation is generally in the range of 2 – 4.5‰ (Lehmann et al., 2002; Möbius, 2013 and Rooze and Meile, 2016). This suggests that if mineralisation was having a dominant effect on the NH_4^+ isotope pool, then the δ^{15}N of this pool should be lower than the sediment, which was not the case in this study. Nitrification is another possible process that could lead to an enrichment of the porewater δ^{15}N pool, however, we believe it is unlikely to explain the fractionation observed here. Nitrification is an obligate aerobic process and it is generally confined to the top few millimetres of sediment owing to the limited penetration of oxygen (Rysgaard et al., 1996). Research has also found that ammonia oxidising bacteria (AOB) are generally outcompeted for available NH_4^+ by a range of organisms such macroalgae and bethnic macrophytes (Risgaard-Petersen et al., 2004; Rysgaard et al., 1996). Whilst benthic primary producers such as seagrass can create micro-oxic zones deeper within the sediment (Brodersen et al., 2015; Frederiksen and Glud, 2006), these same seagrasses will also be actively competing with the nitrifiers for bioavailable nitrogen (Vonk et al., 2008). Consistent with this, we have measured negligible rates of nitrification coupled to denitrification in intact cores with $^{15}N\text{-}NH_4^+$ tracer injected into the sediment (Russell et al., 2016).

As discussed previously, fractionation during assimilation could possibly lead to a small enrichment of the δ^{15}N of porewater NH_4^+, however this does not explain the increasing discrepancies between the

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δ^{15}N of porewater NH$_4^+$ and bulk sediment observed at high δ^{15}N values (as indicated by the slope of 0.77 in Figure 3b) within the vicinity of the WTP. Another possibility is that the NH$_4^+$ pool within the sediment is derived from an isotopically enriched sub pool of organic matter with a high turnover and hence release of NH$_4^+$. We speculate that isotopically-enriched phytoplankton and macroalgae which have assimilated nitrogen from the WTP enter the sediment pool and are rapidly turned over. This leads to an enrichment of the NH$_4^+$ pool compared to the bulk nitrogen pool which is probably associated with more recalcitrant and isotopically-depleted seagrass organic matter. The plausibility of this hypothesis is supported by the δ^{15}N signatures of macroalgae previously reported in Port Phillip Bay, which were ~ 2.5‰ higher than adjacent seagrass (Cook et al., 2015). Furthermore, the bulk sediment δ^{13}C values of ~ -17‰ from the sites within the vicinity of the WTP, suggest the organic matter within the sediment is a ~50% mix of algal material (δ^{13}C ~ -23 ‰) and seagrass (δ^{13}C ~ -11‰, Cook et al., 2015). This is also consistent with the slight enrichment of the bulk sediment above seagrass in Port Phillip Bay by ~1‰ reflecting the 50:50 mix between seagrass derived organic matter and algal material enriched by a δ^{15}N of 2.5‰. If this algal material were to undergo degradation at a rate much higher than seagrass material, then it is quite conceivable that the δ^{15}N of the porewater will become enriched towards the algal δ^{15}N which can be as high as 20‰ in the vicinity of the WTP (Cook et al., 2015).

5 Conclusions

The strong relationships between the δ^{15}N values of the seagrass roots, porewater NH$_4^+$ and sediment support the current paradigm that nitrogen is tightly recycled within seagrass beds. However, the slight offset between δ^{15}N of sediment and porewater NH$_4^+$ particularly in the vicinity of the WTP also suggests that mineralisation of the isotopically-enriched macroalgae could be a potential external source of nitrogen to the porewater NH$_4^+$ pool. On average, nitrogen within seagrass roots had a δ^{15}N of 1.6‰ lower than the porewater NH$_4^+$, which are most likely explained by nitrogen fixation in addition to a small isotope fractionation effect during assimilation of nitrogen from the porewater. The gradient of δ^{15}N of porewater NH$_4^+$ vs seagrass suggests that porewater NH$_4^+$ is likely to contribute up to 80% of

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the nitrogen acquired by seagrasses in both Port Phillip Bay and Western Port while nitrogen fixation is accounted for the remainder 20%. Our study shows that simultaneous investigations of $\delta^{15}N$ of porewater NH_4^+, bulk sediment and seagrass and support 3 key paradigms in seagrass nitrogen acquisition 1. Seagrass obtain the majority of their nitrogen from the sediment; 2. A small, but significant proportion of this demand (~20%) can be met by nitrogen fixation; 3. Algal inputs to the sediment represent a significant source of nitrogen that is ultimately assimilated by seagrass.

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Table 1: Summary of possible nitrogen isotopic end-members in Western Port and Port Phillip Bay

Source	Isotopic End-Member (‰)	Reference
Port Phillip Bay		
Nitrogen fixation	0.0	Owens, 1988
Oceanic	6.9	Russell et al., 2017
Yarra River	9.7	Hirst et al., 2016
Western Treatment Plant (WTP)	22.8	Nicholson et al., 2011
Western Port		
Nitrogen Fixation	0.0	Owens, 1988
Oceanic	6.9	Russell et al., 2017
Riverine	9.2	Russell et al., 2017

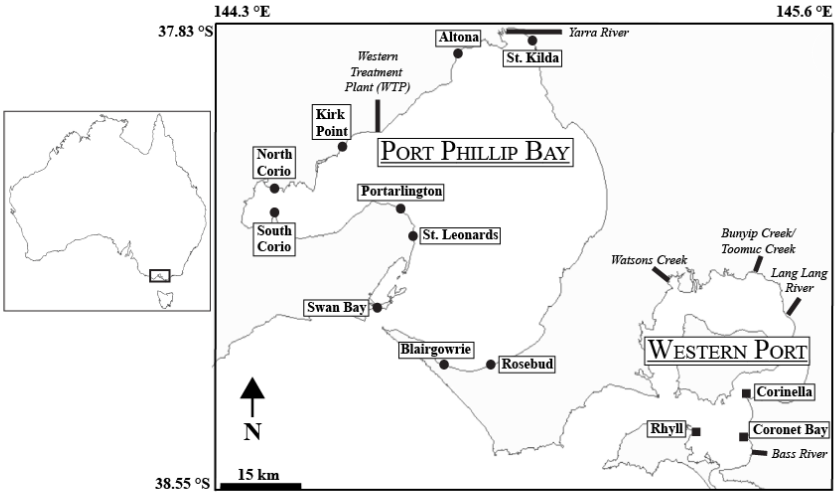

Figure 1: Western Port and Port Phillip Bay, Australia, showing the field sites. The sites marked with circles were sampled during August and December 2016, and the sites marked with squares were sampled approximately bimonthly over the period February – November 2016.

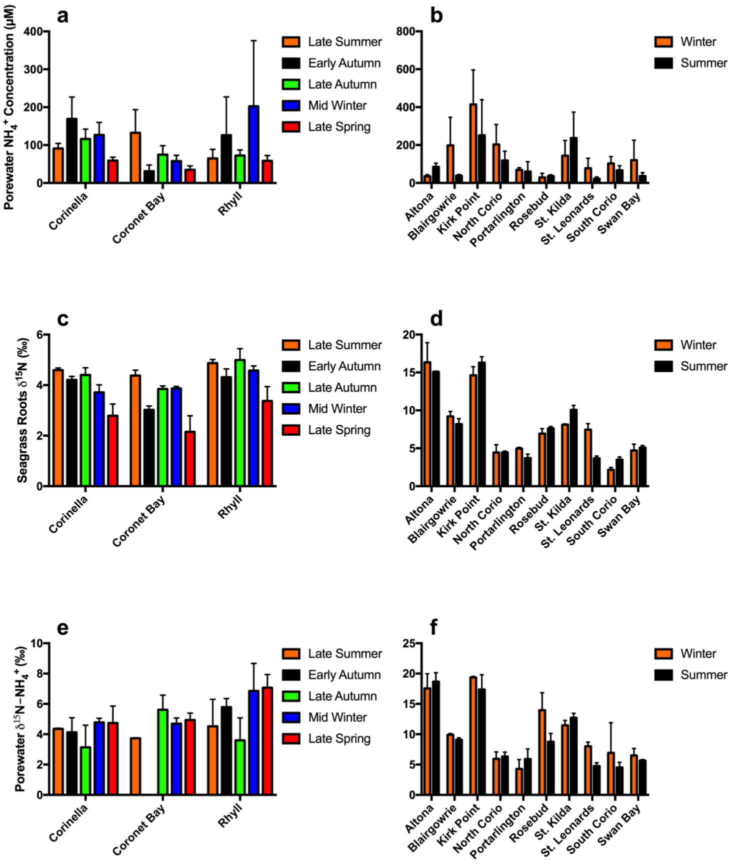

Figure 2: Porewater NH$_4^+$ concentrations for (a) Western Port and (b) Port Phillip Bay, seagrass root isotopic signature (δ^{15}N) for (c) Western Port and (d) Port Phillip Bay, and porewater NH$_4^+$ isotopic signature for (e) Western Port and (f) Port Phillip Bay. ***Note: No results are available for the isotopic signature of porewater NH$_4^+$ in early autumn at Coronet Bay.*** **All values are mean ± S.D.**

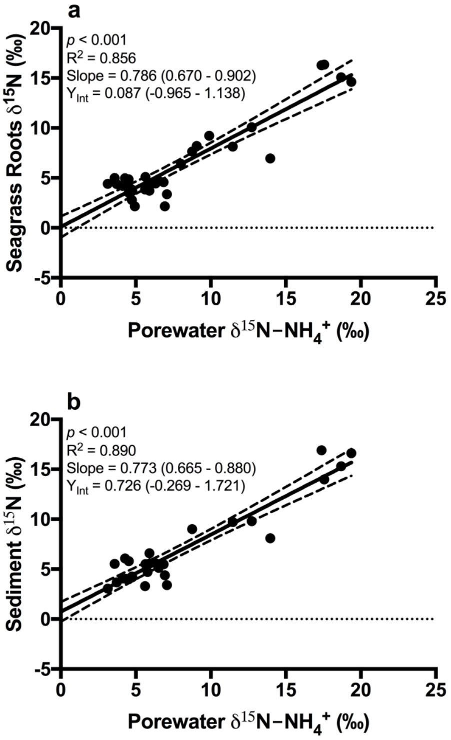

Figure 3: Plot of the porewater $\delta^{15}N\text{-}NH_4^+$ against (a) $\delta^{15}N$ of seagrass roots and (b) $\delta^{15}N$ of the sedimentary nitrogen pool. The 95% confidence intervals of the linear regression are depicted by the dashed lines, and the values in parentheses represent the 95% confidence interval range for the slope and y-intercept.

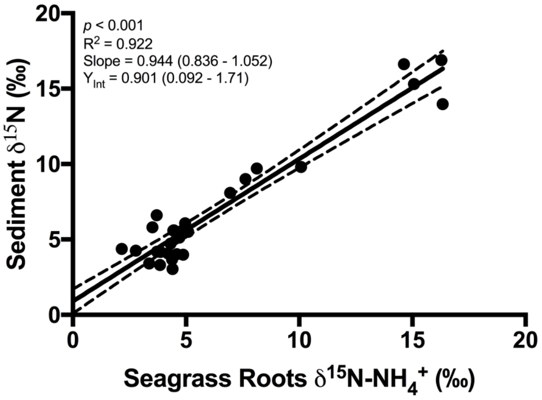

Figure 4: Plot of seagrass δ^{15}N against sediment δ^{15}N.

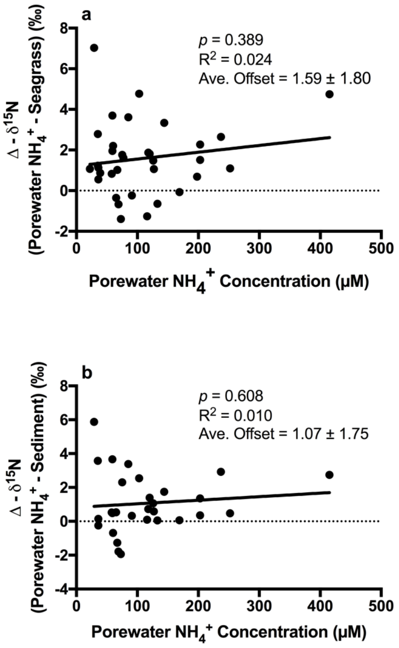

Figure 5: Plot of porewater NH_4^+ concentration against (a) the difference between the porewater $\delta^{15}N$-NH_4^+ and the seagrass $\delta^{15}N$ (b) the difference between the porewater $\delta^{15}N$-NH_4^+ and the bulk sediment $\delta^{15}N$.