

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

Response to reviewers' comments

We thank the reviewers and the associate editor for their constructive comments. We have addressed the comments by both reviewers (as detailed below) and have revised the manuscript accordingly. Please note that the page and line numbers refer to that in the revised manuscript.

### REVIEWER #1:

It would be good to see some consideration of seagrass physiology and physiognomy in the Introduction. This will help understand why results mostly focused on roots, and also set the context for some of the interpretations and inferences that are considered in the Discussion. This could include what we know about nitrogen species that seagrasses use, and how they get them.

We have briefly mentioned on how seagrasses acquire nutrients in the introduction section of the revised manuscript.

Page 2 Line 19: 'A major pool of nitrogen available to seagrasses is ammonium ( $\text{NH}_4^+$ ) 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.'

Why was the design of the temporal aspect different between the two bays?

The different design of the temporal aspect between the different bays was a reflection of the logistical difficulties encountered accessing and sampling at the different sites. The highly tidal nature of the sites selected meant that access to some of the sites proved problematic and were only able to be accessed sporadically. A more detailed description of the sampling design has been provided in the revised manuscript.

Page 4 Line 22: '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.'

Think more deeply about your hypotheses, and whether the statistical methods used are appropriate. Take the ANOVA: the method is largely inappropriate here, because a two-way fixed factor analysis was used, meaning that the results cannot be broadened beyond the sites and dates surveyed, and the p is largely uninteresting (being overly influenced by sample size). If an ANOVA model is appropriate, I suggest it would be better to use a random-effects model (so that the sites and dates surveyed are considered only a selection of the possible sites and dates that could have been surveyed), and use variance components to examine the important of spatial and temporal variation. Don't forget the interaction term, which is largely ignored here. Also, give the MS in your tables, or the reader doesn't have the information needed to fully examine the results if they wish.

We have thought carefully about these comments. The purpose of the analysis undertaken here was to determine whether there was a statistically significant difference between the sites and times sampled as opposed to whether the difference observed here applied more generally across the systems studied. This is consistent with our discussion of the results solely in terms of whether they are different or not. We do not make any wider regional inferences based on the data. Additionally, the outputs from the statistical analyses requested by the reviewer can be found in the supporting information (Tables S2, S4 – S6).

Also, think about the regression and the paired t-tests. I think the regressions are good, but I also think you can get deeper insights by looking at slope and intercept values, not just  $r^2$ . From the figures, it seems that confidence intervals around the slope do not overlap 1 – so there isn't a 1:1 relationship, which is very interesting (and probably invalidates the use of paired t-tests).

We have now discussed the slope and intercept values of the figures presented in the revised version of the manuscript.

Page 12 Line 4: '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  $\text{NH}_4^+$  (Figure 3a), with the y-intercept of this graph at  $\sim 0.087\%$ .'

Page 14 Line 5: 'We conclude that although there is a slight offset between seagrass tissue and porewater  $\text{NH}_4^+$   $\delta^{15}\text{N}$  values, the  $\delta^{15}\text{N}$  of seagrass is closely coupled to that of bulk sediment  $\delta^{15}\text{N}$  values.'

Also, log-transformation on data  $< 1$  will yield negative results, which probably isn't what you want, did you check that the transformed data make sense?

Whilst this is true, none of the original  $\delta^{15}\text{N}$  signals were  $< 1$ , hence log-transforming the data didn't yield negative results. This has been specifically mentioned in the revised manuscript.

Page 7 Line 9: '(all  $\delta^{15}\text{N}$  values were  $> 1$  ‰, and hence no negative values were obtained with this transformation)'

Describe what you mean by "sediment solid phase".

We were referring to the sediment upon being dried, we agree that this terminology may be slightly confusing and as a result we have substituted "sediment solid phase" with "sediment" in the revised manuscript.

In the methods, more information is needed about the sample collections and preservation, and the seagrass analysis  $\hat{A}^T$  give the information needed to allow others to repeat the methods, much like you have done for the  $\text{NH}_4$  analyses. For example, give the sample size (n) for seagrass, describe in more detail how porewater was separated from the water column, how epiphytes were dealt with and how the seagrass was cleaned, what standards were used for the stable isotope analysis.

As suggested by the reviewer, a more detailed description of the collection, preservation and analysis techniques used relating to the treatment of the seagrass samples has been added to the revised manuscript.

Page 5 Line 5: 'The overlying water column from the intact cores were 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.’

Page 5 Line 14: ‘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.’

Page 5 Line 21: ‘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).’

Page 6 Line 5: ‘Analysis of ERA-certified reference materials (Lot number P2473-505) indicated the accuracy of the spectrophotometric analysis was within 2% relative error.’

Lastly, I would find Figure 2 more useful as a table – think about it.

We still think that Figure 2 is a better way of presenting our data in order to give the readers an idea on the variabilities of the measured parameters across different sampling sites and seasons. We have however changed the colour scheme of the Figure to make it more presentable and easier to understand.

#### REVIEWER #2:

There are several key publications in the area that the authors did not mention in the introduction. Therefore, it is not very convincing and is not giving an overall view to the readers. For example, Papadimitriou et al. (2006) have already reported  $\delta^{15}\text{N}$  in *Zostera noltii* meadows and  $\delta^{15}\text{N}$  in porewater ammonium with a conclusion that reflected each other. So the “no studies” at line 9, page 1 does not appear justified. Also, the “previous studiES” at line 13, page 1 showing a fractionation of 2‰ of N fractionation during OM mineralization could not be only related to the SINGLE study on sapropels. The authors are invited to consult Lehmann et al. (2002); Rooze and Meile (2016) where a full description of the N fractionation process during OM mineralization was provided in either marine/lacustrine environments. Therefore, the “uncertainty” mentioned at line 14 is also not justified. These two examples justified the main problems of this manuscript which are the lack of literature documentation leading to the excessive confirmation of confidence (i.e., “no studies” at line 9 page 1, line 17 page 2). The authors are therefore invited to revise the introduction and provide further details on how and why N isotopes are fractionated by geochemical but also biological processes. This lack of a good literature review is also imputable to the quality of the discussion which is not novel and convincing.

We have now rewritten the introduction and discussion to provide a more comprehensive literature review, with specific references to the publications that the reviewer has suggested. We agree that we missed out on one reference that has described nitrogen fractionation processes in seagrass meadows (Papadimitriou et al. 2006). However, as none of the other papers mentioned here deal with nitrogen fractionation specifically in seagrass meadows means that a far greater body of work is required here to understand these fractionation processes. We feel that whilst this study isn’t the first to tackle this problem, the fact that there has only one previous study at one site on the topic, means this study is nonetheless an important contribution to the ‘limited’ body of work that currently exists, and therefore our use of “uncertainty” in this context is justified.

Page 2 Line 26: ‘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  $\text{NH}_4^+$ . 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 relative nitrogen (and nutrient) availability, with limited nitrogen fractionation being observed under relatively nitrogen-limiting conditions.’

We have also reworded the section dealing with the fractionation effect due to mineralisation, however the papers cited here have values between 2.5-4‰ (for Lehmann et al. 2002) and generally 2-3‰ (for Rooze and Meile 2016). Therefore, in the revised manuscript we have revised this statement about mineralisation fractionation to ~3‰ and have added in the references previously mentioned.

Page 3 Line 7: ‘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  $^{14}\text{N}$  over  $^{15}\text{N}$  (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 ‰.’

Section 2.2, 2.3 and elsewhere, the authors are invited to mention the number of samples/replicate collected and number of observation each time a statistical test has been done.

We have now added the number of samples/replicates that we used for the statistical test.

Page 4 Line 26: ‘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 ( $\delta^{15}\text{N}$ ).’

Which reference materials were used in sections 2.3 to 2.5?

We have now mentioned all the reference materials used for our instrumental analysis in the revised manuscript.

Page 5 Line 21: ‘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).’

Page 6 Line 5: ‘Analysis of ERA-certified reference materials (Lot number P2473-505) indicated the accuracy of the spectrophotometric analysis was within 2% relative error.’

Page 6 line 11: Again, the authors are invited to revise the “no studies” as it is not quite true.

In the revised version of this manuscript, this statement has been revised.

The section 4.1 is very hard to follow. The aim of this section is, so far as I understand, is to attribute a reason for the 1.6 ‰ shift in average between seagrass root and porewater. With the approach used and the way the data are shown by the authors, the difficulty is obvious to find a single reason explaining this shift. In fact, the literature shows clearly the vertical gradient of ammonia in porewater, and the bio-irrigation amplifies the heterogeneity of diagenetic reactions. Therefore, a single sample of a broad sediment depth (20 cm) could not be explanatory for the change in N and N isotope for each seagrass root. The correlation in Fig 4 may be enhanced if the authors correct porewater ammonia concentration by the sediment porosity which may give a better idea of the whole N pool, accessible to the plants. However, the section 5.2.2. in Papadimitriou et al. (2006) has very well discussed the N isotope composition in *Z. noltii* leaves and porewater ammonia. The authors are therefore recommended to shorten and clarify the current section 4.1.

The reviewer has correctly understood the broad aim of this this section. We agree that there are a number of complex factors affecting the possible fractionation of nitrogen and we have broken this down into leaf assimilation from different sources, fractionation during diagenesis and nitrogen fixation. The reviewer has suggested that by sampling the entire sediment pool (rather than profiles) we may have overlooked some important variability of  $\delta^{15}\text{N}$  within the sediment and have therefore overlooked some potentially important diagenetic processes. The literature does indeed show that ammonium concentrations are highly variable with depth, but this does not necessarily apply to the isotope values, and the limited literature on this has not shown a large variation of  $\delta^{15}\text{N}$  with depth (Freudenthal et al 2001). We have now added in a new sentence to note we have overlooked depth variability, but we expect this not to be large based on previous measurements.

Page 5 Line 9: ‘This approach ignores possible depth variation in the porewater  $\delta^{15}\text{N}$  values, however, previous work has shown this has minimal variation with depth (Freudenthal et al. 2001; Prokopenko et al. 2006).’

The reviewer also suggests we shorten this section given that Papadimitriou has also discussed these processes previously. We have carefully read this previous work and we believe the current discussion is warranted because the present study extends to multiple sites, with a much greater variability in the source signature of  $\delta^{15}\text{N}$ . Our discussion also includes a consideration of nitrogen fixation which was not discussed previously.

Finally, the reviewer helpfully suggests we correct the sediment for porosity to tighten up the relationship in Figure 4. Unfortunately, we do not have the data to do this, but in any case, this is only likely to change the data by ~20%. We also do not believe such a correction is warranted because the seagrass are assimilating  $\text{NH}_4^+$  from the porewater, and therefore it seems most logical to report this concentration rather than per unit of bulk sediment, which the reviewer implies here.

Similarly, the section 4.2. shows that key publications in the area are missed. Contradictory to what mentioned, there are several work and models on C, N, and their isotopes during mineralization, e.g., (Lehmann et al., 2002; Bouillon et al., 2012; Rooze and Meile, 2016). - Finally, what is interesting in this study is the correlation between seagrass root, sediment N, and porewater ammonia. Correlations in Fig 3a and 3b show a very similar slope (0.786 vs. 0.773), that means plotting seagrass roots vs. sediment may give a slope of 1. That may lead to a more straightforward conclusion that seagrass roots take the same N isotope signature than sediments rather than trying to explain roots vs. porewater and sediment vs. porewater.

We agree that we could be clearer about how our study is different to the publications that were listed by the reviewer; in essence very few studies have used an experimental approach to look at the differences in  $\delta^{15}\text{N}$  between the sediment and porewater  $\text{NH}_4^+$  pools. Section 4.2 has been revised to include a more thorough discussion of the isotopic fractionation effects of mineralisation, with specific references made to the manuscripts that the reviewer suggested. In the revised manuscript we have also included and discussed the relationship between  $\delta^{15}\text{N}$  of sediment and  $\delta^{15}\text{N}$  of seagrass.

Page 10 Line 2: ‘Overall, our study showed very close agreement between the bulk sediment  $\delta^{15}\text{N}$  and the seagrass tissue  $\delta^{15}\text{N}$  (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  $\text{NH}_4^+$  and subsequent assimilation by seagrass roots is a critical link coupling the nitrogen  $\delta^{15}\text{N}$  values in these two pools. To date, there has only been one study on the  $^{15}\text{N}/^{14}\text{N}$  ratios of porewater  $\text{NH}_4^+$  and its relationship with  $^{15}\text{N}/^{14}\text{N}$  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.’

Technical comments: -Page 5, line 32: The subtraction sign is not a good idea to use in the text as it is confusing with a simple hyphen or a minus, one alternative is using a big delta and having “porewater-seagrass” in subscript.

The equation has been revised to reflect on this suggestion.

#### REFERENCES CITED:

- Altabet, M. A., and Francois, R.: Sedimentary nitrogen isotopic ratio as a recorder for surface ocean nitrate utilization. *Global Biogeochem. Cy.*, 8, 103-116, doi: 10.1029/93GB03396, 1994.
- Barrón, C., Middelburg, J. J., and Duarte, C. M.: Phytoplankton trapped within seagrass (*Posidonia oceanica*) sediments are a nitrogen source: An in situ isotope labeling experiment, *Limnol. Oceanogr.*, 51, 1648-1653, 2006.
- Freudenthal, T., Wagner, T., Wenzhöfer, F., Zabel, M., and Wefer, G.: Early diagenesis of organic matter from sediments of the Eastern subtropical Atlantic: Evidence from stable nitrogen and carbon isotopes, *Geochim. Cosmochim. Acta*, 65, 1795-1808, 10.1016/S0016-7037(01)00554-3, 2001.
- Kennedy, H., Beggins, J., Duarte, C. M., Fourqurean, J. W., Holmer, M., Marbà, N., and Middelburg, J. J.: Seagrass sediments as a global carbon sink: Isotopic constraints, *Global Biogeochem. Cy.*, 24, 10.1029/2010GB003848, 2010.
- Lehmann, M. F., Bernasconi, S. M., Barbieri, A., and McKenzie, J.A.: Preservation of organic matter and alteration of its carbon and nitrogen isotope composition during simulated and in situ early sedimentary diagenesis. *Geochim. Cosmochim. Acta*, 66, 3573-3584, doi: 10.1016/S0016-7037(02)00968-7, 2002.

- McGlathery, K. J., Berg, P., and Marino, R.: Using porewater profiles to assess nutrient availability in seagrass-vegetated carbonate sediments, *Biogeochemistry*, 56, 239-263, doi: 10.1023/A:1013129811827, 2001.
- McKee, K. L., Feller, I.C., Popp, M., and Wanek, W.: Mangrove isotopic ( $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$ ) fractionation across a nitrogen vs. phosphorous limitation gradient. *Ecology*, 83, 1065-1075, doi: 10.1890/0012-9658(2002)083[1065:MINACF]2.0.CO;2, 2002.
- Mobius, J.: Isotope fractionation during nitrogen remineralization (ammonification): Implications for nitrogen isotope biogeochemistry, *Geochim. Cosmochim. Acta*, 105, 422-432, doi: 10.1016/j.gca.2012.11.048, 2013.
- Papadimitriou, S., Kennedy, H., Rodrigues, R.M.N.V., Kennedy, D.P., and Heaton, T.H.E.: Using variation in the chemical and stable isotopic composition of *Zostera noltii* to assess nutrient dynamics in a temperate seagrass meadow, *Org. Geochem.*, 37, 1343-1358, doi: 10.1016/j.orggeochem.2006.01.007, 2006.
- Prokopenko, M.G., Hammond, D.E., Berelson, W.M., Bernhard, J.M., Stott, L., and Douglas, R.: Nitrogen cycling in the sediments of Santa Barbara basin and Eastern Subtropical North Pacific: nitrogen isotopes, diagenesis and possible chemosymbiosis between two lithotrophs (*Thioploca* and Anammox) – “riding on a glider”, *Earth Planet. Sc. Lett.*, 242, 186-204, doi:10.1016/j.epsl.2005.11.044, 2006.
- Rooze, J., and Meile, C.: The effect of redox conditions and bioirrigation on nitrogen isotope fractionation in marine sediments. *Geochim. Cosmochim. Acta*, 184, 227-239, doi: 10.1016/j.gca.2016.04.040, 2016.
- Sachs, J. P., and Repeta, D. J.: Oligotrophy and nitrogen fixation during eastern Mediterranean sapropel events. *Science*, 286, 2485-2488, doi: 10.1126/science.286.5449.2485, 1999.
- Saino, T., and Hattori, A.: Geographical variation of the water column distribution of suspended particulate organic nitrogen and its  $^{15}\text{N}$  natural abundance in the Pacific and its marginal seas. *Deep Sea Res. Part A*, 34, 807-827, doi: [10.1016/0198-0149\(87\)90038-0](https://doi.org/10.1016/0198-0149(87)90038-0), 1987.



# 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. Excessive nitrogen inputs to the coastal zone pose a key threat to seagrass through eutrophication and associated algal overgrowth. The <sup>15</sup>N/<sup>14</sup>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 <sup>15</sup>N/<sup>14</sup>N ratios of seagrass beds, their associated sediments and of critical importance, the porewater NH<sub>4</sub><sup>+</sup> pool, which is most bioavailable. Here, we undertook a study of the <sup>15</sup>N/<sup>14</sup>N ratios of seagrass tissue, sediment porewater NH<sub>4</sub><sup>+</sup> pool and the 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 extremely close agreement between the bulk sediment δ<sup>15</sup>N and seagrass δ<sup>15</sup>N (r<sup>2</sup> of 0.92 and mean offset of 0.9‰); illustrating a close coupling between the plant and sediment pools. The δ<sup>15</sup>N of porewater ammonium was strongly correlated with the δ<sup>15</sup>N of both the sediment and seagrass tissue (r<sup>2</sup> of 0.89 and 0.85) respectively. The δ<sup>15</sup>N porewater NH<sub>4</sub><sup>+</sup> minus the δ<sup>15</sup>N seagrass tissue ranged between -1.4 and 7‰ with an average 1.6‰. We suggest the most likely explanation for this was fractionation during assimilation as a consequence of diffusion limitation, although the magnitude of this change was relatively small. Nitrogen fixation may have also contributed a small amount to the observed isotopic depletion of the plants relative to the sediment porewater NH<sub>4</sub><sup>+</sup> pool. A consideration of the nitrogen isotope values of the seagrass bed nitrogen pools compared to external sources suggest the dominant source of nitrogen to seagrass is recycling from within the bed, with a relatively small contribution from water column assimilation, particulate trapping and nitrogen fixation.

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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.

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The ratio of  $^{15}\text{N}/^{14}\text{N}$  (hereafter referred to as  $\delta^{15}\text{N}$ ) 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  $\delta^{15}\text{N}$  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  $\delta^{15}\text{N}$  values found that latitude exerted an overwhelming influence on seagrass  $\delta^{15}\text{N}$  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.

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A major pool of nitrogen available to seagrasses is ammonium ( $\text{NH}_4^+$ ) 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  $\text{NH}_4^+$  within the sediment is present in high concentrations, and as a consequence it is possible that  $^{14}\text{N}$  will be preferentially assimilated leading to significant isotopic fractionation (Cook et al., 2015). Conversely, it is possible that the distribution of the  $\text{NH}_4^+$  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  $\text{NH}_4^+$ . This suggests that the benthic pool of nitrogen made a sizeable contribution to the

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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.

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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  $^{14}\text{N}$  over  $^{15}\text{N}$  (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 ‰.

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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  $\delta^{15}\text{N}$  of  $\text{NH}_4^+$  within the porewater compared to the sediment.

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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  $\delta^{15}\text{N}$  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  $\delta^{15}\text{N}$  analysis of  $\text{NH}_4^+$ , bulk

sediment and seagrass tissues to compare the  $\delta^{15}\text{N}$  values from a range of seagrass beds influenced by different sources of nitrogen.

## 2 Materials and methods

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### 5 2.1 Study area

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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 Phillip Bay and 3 sites located in Western Port (Figure 1). Both bays are located in Victoria, Australia and are temperate, intertidal marine embayments. Port Phillip Bay is the largest bay in Victoria and has a surface area of  $\sim 1930 \text{ km}^2$ , and Western Port located roughly 55 km south-east of Melbourne and has a surface area of  $\sim 650 \text{ km}^2$ . The sites that were selected from Port Phillip Bay have previously been described in Cook et al. (2015) and exhibit a strong gradient in  $\delta^{15}\text{N}$  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  $\sim 1000$  tonnes per year (TN; total nitrogen) and the Western Treatment plant which contributes  $\sim 1000$ - $1500$  tonnes TN per year (Harris et al., 1996; Hirst et al., 2016). For Western Port, terrestrial sources of nitrogen from the rivers contribute  $\sim 650$  tonnes TN per year (Russell et al., 2016).

### 20 2.2 Sample collection and preservation

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Field sampling was carried out in Western Port at intervals of  $\sim 2$  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  $\times$  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 ( $\delta^{15}\text{N}$ ). All samples were returned to Monash University within 4 hours of sampling.

5 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 a combination of centrifugation and vacuum filtration. The extracted porewater was subsequently filtered through 0.45  $\mu\text{m}$  and 0.20  $\mu\text{m}$  Sartorius Minisart syringe filters, and frozen until analysis, along with the samples of seagrass. This approach ignores possible depth variation in the porewater  $\delta^{15}\text{N}$  values, however, previous work has shown this has minimal variation with depth (Freudenthal et al. 2001; Prokopenko et al. 2006).

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### 2.3 Seagrass nitrogen isotope ratios

Seagrass samples were collected from each site, washed by hand with deionised water to ensure that all  
15 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 ( $\delta^{15}\text{N}$ ) and relative to the  
20 isotopic ratio of atmospheric  $\text{N}_2$  ( $R_{\text{Air}}= 0.0036765$ ). The precision of the nitrogen analysis was  $\pm 0.2\%$  (SD;  $n=5$ ), and  $\pm 0.5 \mu\text{g}$  (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).

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## 2.4 Nutrient analysis (NH<sub>4</sub><sup>+</sup>, FRP and NO<sub>x</sub>)

The concentration of NH<sub>4</sub><sup>+</sup>, filterable reactive phosphorous (FRP) and combined NO<sub>3</sub><sup>-</sup> and NO<sub>2</sub><sup>-</sup> (hereafter NO<sub>x</sub>) in the porewater at each site was determined colorimetrically (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.](#)

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## 2.5 Isotopic analysis of porewater NH<sub>4</sub><sup>+</sup> (δ<sup>15</sup>N-NH<sub>4</sub><sup>+</sup>)

To determine the isotopic signature (δ<sup>15</sup>N) of the NH<sub>4</sub><sup>+</sup> 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<sub>4</sub><sup>+</sup> 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<sub>4</sub><sup>+</sup> 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<sub>4</sub>, 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

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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  $100 \pm 5\%$ .

## 2.5 Statistical analysis

5 Two-factor analysis of variance (ANOVA) was undertaken to compare the spatial and temporal differences in porewater nutrient concentrations, as well as seagrass tissue nitrogen isotopic signature. Plots of residuals and boxplots were used to test assumptions of homogeneity and normality of variance (Quinn and Keough, 2002). Where data failed these tests,  $\ln(x)$  transformation of the data was carried out and then reassessed for homogeneity and normality of variance (all  $\delta^{15}\text{N}$  values were  $>1\%$ , and hence  
10 no negative values were obtained with this transformation). In the case of significant responses, post-hoc comparisons were carried out using the TukeyHSD post-hoc test. These analyses were done using R 3.3.0 (R Core Team, 2015). 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  $p < 0.05$ .

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## 3 Results

### 3.1 Nutrient concentrations and isotopic signatures ( $\delta^{15}\text{N}$ ) of seagrass and porewater $\text{NH}_4^+$

Porewater concentrations of both FRP and  $\text{NO}_x$  were consistently low throughout the year in Western Port, with FRP  $\leq 2 \mu\text{M}$  and  $\text{NO}_x \leq 27 \mu\text{M}$  (Table S1). In contrast, the porewater concentrations of  $\text{NH}_4^+$   
20 were 1 – 2 orders of magnitude higher (Figure 2a), with significantly higher concentrations at both Corinella and Rhyll than compared to Coronet Bay ( $p < 0.05$ ; Table S2). Furthermore, significant temporal variation in concentrations were also found in Western Port ( $p < 0.05$ ; Table S2), with concentrations reaching a maximum during late-autumn/mid-winter. Similarly, in Port Phillip Bay, little FRP and  $\text{NO}_x$  were detected in the porewater, with the concentrations at all times  $\leq 44 \mu\text{M}$  and  $\leq 27 \mu\text{M}$  respectively  
25 (Table S3), whereas the concentration of  $\text{NH}_4^+$  was up to an order of magnitude higher (Figure 2b). Furthermore, significant spatial variation in  $\text{NH}_4^+$  concentrations were also evident ( $p < 0.001$ ; Table S4)

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with the sites in close proximity to terrestrial nitrogen inputs (i.e. Kirk Point and St. Kilda) consistently having the highest porewater  $\text{NH}_4^+$  concentrations. Significant temporal variation in the porewater concentration of  $\text{NH}_4^+$  was also evident in Port Phillip Bay ( $p=0.037$ ; Table [S4](#)), with the highest concentrations generally found during the winter sampling period.

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The  $\delta^{15}\text{N}$  of seagrass in the context of this study refers to the  $\delta^{15}\text{N}$  of the roots. There was, however, no significant difference between the  $\delta^{15}\text{N}$  of the seagrass roots and that of the leaves (Figure [S1](#)). Despite

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the fact that the isotopic signature of nitrogen in seagrass was only found to vary between 2 and 5‰ in Western Port (Figure 2c), significant differences in the isotopic signature were found between the sites

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( $p<0.001$ ; Table [S5](#)). 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. Statistically significant differences in the isotopic signature of the seagrass were also evident throughout the year ( $p<0.001$ ; Table [S5](#)), with the samples obtained during late autumn consistently lower throughout the bay

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than at any other time of the year. In contrast, there was appreciable variation in the isotopic signature of the seagrass in Port Phillip Bay (Figure 2d), with values varying from  $\sim 2.2\text{‰}$  to in excess of  $16\text{‰}$  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  $\geq 6.9\text{‰}$ . In contrast, the seagrass meadows located in the south-west of Port Phillip Bay (Swan

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Bay – North Corio) consistently exhibited the lowest isotopic signatures of between  $2.2\text{‰}$  and  $6.4\text{‰}$ . Whilst this resulted in statistically significant differences in isotopic values between sites ( $p<0.001$ ; Table [S6](#)), no statistically significant changes in isotopic signatures were observed from winter to summer ( $p=0.350$ ; Table [S6](#)).

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The isotopic signature of porewater  $\text{NH}_4^+$  in Western Port was found to exhibit relatively little variation, with values ranging from  $\sim 3.9$  to  $7\text{‰}$  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  $\text{NH}_4^+$  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  $\text{NH}_4^+$  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  $\text{NH}_4^+$  for all sites throughout this study (Figure 3a;  $r^2 = 0.86$ ,  $p < 0.001$ ). The difference between  $\delta^{15}\text{N}$  porewater  $\text{NH}_4^+$  and  $\delta^{15}\text{N}$  seagrass ( $\Delta \delta^{15}\text{N}_{\text{NH}_4^+ \text{ porewater-seagrass}}$ ) was statistically significant (paired t-test,  $p < 0.005$ ) and ranged between -1.4 to 7‰, with a mean value of 1.6‰. A strong relationship was also observed between the isotopic signatures of sedimentary nitrogen pool and porewater  $\text{NH}_4^+$  (Figure 3b;  $r^2 = 0.89$ ,  $p < 0.001$ ). The difference between  $\delta^{15}\text{N}$  porewater  $\text{NH}_4^+$  and  $\delta^{15}\text{N}$  sediment was statistically significant (paired t-test  $p < 0.005$ ) and ranged between -3.6 and 5.9‰, with a mean of 0.9‰. Bulk sediment and seagrass  $\delta^{15}\text{N}$  values were also tightly correlated with an  $r^2$  of 0.92 (Figure 4). There was a statistically significant difference between seagrass  $\delta^{15}\text{N}$  and sediment  $\delta^{15}\text{N}$  (paired t-test  $p < 0.05$ ) and this ranged between -3.6 to 3.7‰ with a mean value of -0.5‰. There was no relationship between  $\Delta(\delta^{15}\text{N}_{\text{porewater NH}_4^+ \text{-seagrass}})$  and porewater  $\text{NH}_4^+$  concentration, nor  $\Delta(\delta^{15}\text{N}_{\text{porewater NH}_4^+ \text{-sediment}})$  and porewater  $\text{NH}_4^+$  concentration (Figure 5).

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## 4 Discussion

Overall, our study showed very close agreement between the bulk sediment  $\delta^{15}\text{N}$  and the seagrass tissue  $\delta^{15}\text{N}$  (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  $\text{NH}_4^+$  and subsequent assimilation by seagrass roots is a critical link coupling the nitrogen  $\delta^{15}\text{N}$  values in these two pools. To date, there has only been one study on the  $^{15}\text{N}/^{14}\text{N}$  ratios of porewater  $\text{NH}_4^+$  and its relationship with  $^{15}\text{N}/^{14}\text{N}$  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 $\text{NH}_4^+$ pool

Our results showed that on average the seagrass had a  $\delta^{15}\text{N}$  of 1.6 ‰ less than the  $\text{NH}_4^+$  in the porewater, with a range of -1.4 to 7‰. Given that there were no significant temporal changes in isotopic signature ( $p > 0.05$ ), we assume that the offsets observed here are not an artefact of lags associated with changing isotope pools over time. Three possible explanations for these offsets are considered as follows:

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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  $\text{NH}_4^+$  and  $\text{NO}_3^-$  concentrations in the water column are typically  $< 1\mu\text{M}$  at the study sites, and there is therefore unlikely to be significant fractionation taking place.

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

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at these sites. The east coast of Port Phillip Bay including sites St Kilda, Rosebud and Blairgowrie are likely to be influenced by the Yarra River plume ( $\delta^{15}\text{N} = \sim 9.7\text{‰}$ ), Western Treatment Plant and marine sources of nitrogen (Hirst et al., 2016). At all of these sites, the  $\delta^{15}\text{N}$  of seagrass was  $< 9\text{‰}$  and therefore assimilation of water column nitrogen is an unlikely explanation for the lower  $\delta^{15}\text{N}$  values observed in

5 seagrass compared to porewater at these sites. The sites in the west of Port Phillip Bay including North and South Corio, Portarlington and Swan Bay are likely to be dominated by marine sources of nitrogen from the water column (Hirst et al., 2016). At all these sites, the seagrass  $\delta^{15}\text{N}$  was  $< 6.5\text{‰}$  and therefore assimilation of marine nitrogen from the water column is unlikely to explain the generally lower  $\delta^{15}\text{N}$  in seagrass compared to porewater at these sites. Within Western Port, there was considerable variation in

10 the offset of the seagrass and porewater  $\delta^{15}\text{N}$  values, with  $\sim 1/3$  of the samples showing a  $\delta^{15}\text{N}$  enrichment in the seagrass compared to the porewater  $\text{NH}_4^+$ . The  $\delta^{15}\text{N}$  values of seagrass within Western Port were always  $< 5\text{‰}$  and therefore the enriched values of  $\delta^{15}\text{N}$  in seagrass relative to the porewater could be explained by the assimilation of a small amount of terrestrial or marine derived nitrogen from the water column. For the sites where the seagrass was depleted in  $\delta^{15}\text{N}$  relative to the porewater  $\text{NH}_4^+$ , it is unlikely

15 that significant nitrogen assimilation was taking place from the water column. Taken together these results, suggest there is very limited direct assimilation of nitrogen from the water column, which is consistent with the low concentrations of inorganic nitrogen in the water column in this region (Russell et al., 2016), and is in agreement with a previous study of seagrass nitrogen sources at an open coastal site (McGlathery et al., 2001).

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## 2. Fractionation of nitrogen during assimilation from the porewater $\text{NH}_4^+$ pool:

Handley and Raven (1992) reported that the isotopic fractionation associated with the vegetative assimilation of  $\text{NH}_4^+$  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  $\text{NH}_4^+$  in the sediment. One possible explanation for this is that  $\text{NH}_4^+$  concentrations are highly heterogeneous in the sediment resulting in very low concentrations directly within the vicinity of roots where active assimilation is

occurring (Welsh et al., 1997). As such, the assimilation of  $\text{NH}_4^+$  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  $\Delta(\delta^{15}\text{N}_{\text{porewater NH}_4^+ - \text{seagrass}})$  and the bulk porewater  $\text{NH}_4^+$  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  $\text{NH}_4^+$  (Figure 3a), with the y-intercept of this graph at  $\sim 0.087\%$ . 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  $\delta^{15}\text{N}$  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  $\delta^{15}\text{N}$  of  $\sim 0\%$ ) 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  $\text{NH}_4^+$  pool. To test the plausibility of this, we can undertake some simple calculations with a linear mixing model (Fry, 2006) to estimate the fraction of newly fixed nitrogen assimilated by seagrass assuming a nitrogen fixation end member of  $0\%$  and using the measured porewater  $\delta^{15}\text{N}$  values. Using this approach, it was found that nitrogen fixation contributed between 0 and 68% with a mean of 20% to the nitrogen being assimilated by the seagrass in Port Phillip Bay. Direct measurements of nitrogen fixation in Port Phillip Bay have previously suggested nitrogen fixation contributes a maximum of  $\sim 15\%$  to nitrogen demand, with a mean of  $\sim 5\%$  (Cook et al., 2015). Within Western Port, this mass balance is complicated by the fact that seagrass was sometimes enriched in  $\delta^{15}\text{N}$  compared to the sediment. In the instances where  $\delta^{15}\text{N}$  was depleted in the seagrass compared to the porewater, this mass balance yielded a mean contribution of 30%, which is well above previously estimated mean contributions of  $\sim 15\%$  by (Russell et al., 2016). We therefore suggest it is unlikely that nitrogen fixation can account for all of the isotopic depletion generally observed within the seagrass relative to the porewater, although we cannot rule out a small contribution.

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#### 4.2 Isotopic signatures of the sediment pool relative to the porewater $\text{NH}_4^+$ pool

In general, the porewater  $\text{NH}_4^+$  was isotopically enriched compared to the sediment pool by 0.9‰ (Figures 3b and 5b), although this was observed to be highly variable in certain circumstances. 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  $\text{NH}_4^+$  isotope pool, then the  $\delta^{15}\text{N}$  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  $\delta^{15}\text{N}$  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  $\text{NH}_4^+$  by a range of organisms such macroalgae and benthic 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  $^{15}\text{N}\text{-NH}_4^+$  tracer injected into the sediment (Russell et al., 2016). Based on the previous discussion in section 4.1 then, it is most likely that the isotopic enrichment of the porewater compared to the sediment is the result of isotopic fractionation during assimilation by plant roots.

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#### 5 Conclusions

The strong relationship between the  $\delta^{15}\text{N}$  values of the seagrass roots, porewater  $\text{NH}_4^+$  and sediment support the current paradigm that nitrogen is tightly recycled within seagrass beds. On average, nitrogen

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within seagrass roots had a  $\delta^{15}\text{N}$  of 1.6 ‰ lower than the porewater, which are most likely explained by isotope fractionation during assimilation of nitrogen from the porewater. This relatively low apparent fractionation factor suggests that seagrass roots are exposed to low sediment  $\text{NH}_4^+$  concentrations despite high bulk concentrations in the porewater. This apparent discrepancy suggests a high degree of heterogeneity of  $\text{NH}_4^+$  within the sediment caused by diffusion limitation of nitrogen assimilation. We conclude that although there is a slight offset between seagrass tissue and porewater  $\text{NH}_4^+$   $\delta^{15}\text{N}$  values, the  $\delta^{15}\text{N}$  of seagrass is closely coupled to that of bulk sediment  $\delta^{15}\text{N}$  values.

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### References

APHA: Standard methods for the examination of water and wastewater, American Public Health Association, American Water Works Association, and Water Environment Federation., Washington, DC, 2005.

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Altabet, M. A., and Francois, R.: Sedimentary nitrogen isotopic ratio as a recorder for surface ocean nitrate utilization. *Global Biogeochem. Cy.*, 8, 103-116, doi: 10.1029/93GB03396, 1994.

Barrón, C., Middelburg, J. J., and Duarte, C. M.: Phytoplankton trapped within seagrass (*Posidonia oceanica*) sediments are a nitrogen source: An in situ isotope labeling experiment, *Limnol. Oceanogr.*, 51, 1648-1653, 2006.

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Brodersen, K. E., Nielsen, D. A., Ralph, P. J., and Kühl, M.: Oxic microshield and local pH enhancement protects *Zostera muelleri* from sediment derived hydrogen sulphide, *New Phytol.*, 205, 1264-1276, doi: 10.1111/nph.13124, 2015.

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Brooks, P. D., Stark, J. M., McInteer, B. B., and Preston, T.: Diffusion Method To Prepare Soil Extracts For Automated Nitrogen-15 Analysis, *Soil Sci. Soc. Am. J.*, 53, 1707-1711, doi: 10.2136/sssaj1989.03615995005300060016x, 1989.

Christiaen, B., Bernard, R. J., Mortazavi, B., Cebrian, J., and Ortmann, A. C.: The degree of urbanization across the globe is not reflected in the delta N-15 of seagrass leaves, *Mar. Pollut. Bull.*, 83, 440-445, doi: 10.1016/j.marpolbul.2013.06.024, 2014.

Cook, P. L. M., Evrard, V., and Woodland, R. J.: Factors controlling nitrogen fixation in temperate seagrass beds *Mar. Ecol. Prog. Ser.*, 525, 41-51, doi: 10.3354/meps11247, 2015.

Freudenthal, T., Wagner, T., Wenzhöfer, F., Zabel, M., and Wefer, G.: Early diagenesis of organic matter from sediments of the Eastern subtropical Atlantic: Evidence from stable nitrogen and carbon isotopes, *Geochim. Cosmochim. Acta*, 65, 1795-1808, doi: 10.1016/S0016-7037(01)00554-3, 2001.

Frederiksen, M. S. and Glud, R. N.: Oxygen dynamics in the rhizosphere of *Zostera marina*: A two-dimensional planar optode study, *Limnol. and Oceanogr.*, 51, 1072-1083, doi: 10.4319/lo.2006.51.2.1072, 2006.

Fry, B.: *Stable Isotope Ecology*, Springer, New York, 2006.

Handley, L. L. and Raven, J. A.: The use of natural abundance of nitrogen isotopes in plant physiology and ecology, *Plant, Cell & Environment*, 15, 965-985, doi: 10.1111/j.1365-3040.1992.tb01650.x, 1992.

Harris, G., Batley, G., Fox, D., Hall, D., Jernakoff, P., Molloy, R., Murray, A., Newell, B., Parslow, J., and Skyring, G.: *Port Phillip Bay Environmental Study Final Report*, CSIRO, Canberra, Australia, 1996.

Hirst, A. J., Longmore, A. R., Ball, D., Cook, P. L. M., and Jenkins, G. P.: Linking nitrogen sources utilised by seagrass in a temperate marine embayment to patterns of seagrass change during drought, *Mar. Ecol. Prog. Ser.*, 549, 79-88, doi: 10.3354/meps11708, 2016.

Holmes, R. M., McClelland, J. W., Sigman, D. M., Fry, B., and Peterson, B. J.: Measuring 15N-NH4+ in marine, estuarine and fresh waters: An adaptation of the ammonia diffusion method for samples with low ammonium concentrations, *Mar. Chem.*, 60, 235-243, doi: 10.1016/S0304-4203(97)00099-6, 1998.

Kendall, C. and McDonnell, J. J. (Eds.): *Isotope Tracers in Catchment Hydrology*, Elsevier, Amsterdam, 1998.

Kennedy, H., Beggins, J., Duarte, C. M., Fourqurean, J. W., Holmer, M., Marbà, N., and Middelburg, J. J.: Seagrass sediments as a global carbon sink: Isotopic constraints, *Global Biogeochem. Cy.*, 24, doi: 10.1029/2010GB003848, 2010.

Larkum, A. W. D., Orth, R. J., and Duarte, C. M. (Eds.): *Seagrasses: biology, ecology and conservation*, Springer, The Netherlands, 2006.

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Lehmann, M. F., Bernasconi, S. M., Barbieri, A., and McKenzie, J.A.: Preservation of organic matter and alteration of its carbon and nitrogen isotope composition during simulated and in situ early sedimentary diagenesis. *Geochim. Cosmochim. Acta*, 66, 3573-3584, doi: 10.1016/S0016-7037(02)00968-7, 2002.

5 McClelland, J. W. and Valiela, I.: Linking nitrogen in estuarine producers to land derived sources, *Limnol. Oceanogr.*, 43, 577-585, doi: 10.4319/lo.1998.43.4.0577, 1998.

McGlathery, K. J., Berg, P., and Marino, R.: Using porewater profiles to assess nutrient availability in seagrass-vegetated carbonate sediments, *Biogeochemistry*, 56, 239-263, doi: 10.1023/A:1013129811827, 2001.

10 McKee, K. L., Feller, I.C., Popp, M., and Wanek, W.: Mangrove isotopic ( $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$ ) fractionation across a nitrogen vs. phosphorous limitation gradient. *Ecology*, 83, 1065-1075, doi: 10.1890/0012-9658(2002)083[1065:MINACF]2.0.CO;2, 2002.

Michener, R. and Lajtha, K. (Eds.): *Stable isotopes in Ecology and Environmental Science*, Blackwell Publishing Malden, 2007.

Mobius, J.: Isotope fractionation during nitrogen remineralization (ammonification): Implications for nitrogen isotope biogeochemistry, *Geochim. Cosmochim. Acta*, 105, 422-432, doi: 10.1016/j.gca.2012.11.048, 2013.

15 Nicholson, G., Longmore, A., and Spooner, D.: Assessment of the impact of effluent from the Western Treatment Plant on trace metals in sentinel organisms and sediment. Fisheries Victoria Internal Report No.32, Victorian Department of Primary Industries, Queenscliff, 2011.

Nielsen, S. L., Banta, G. T., and Pedersen, M. F. (Eds.): *Estuarine nutrient cycling: The influence of primary producers*, Kluwer, Dordrecht, 2004.

20 Owens, N. J. P.: Natural variations in  $15\text{N}$  in the marine environment, *Adv. Mar. Biol.*, 24, 389-451, doi: 10.1016/S0065-2881(08)60077-2, 1988.

Papadimitriou, S., Kennedy, H., Rodrigues, R.M.N.V., Kennedy, D.P., and Heaton, T.H.E.: Using variation in the chemical and stable isotopic composition of *Zostera noltii* to assess nutrient dynamics in a temperate seagrass meadow, *Org. Geochem.*, 37, 1343-1358, doi: 10.1016/j.orggeochem.2006.01.007, 2006.

25 Papadimitriou, S., Kennedy, H., Kennedy, D., Duarte, C. M., and Marbà, N.: Sources of organic matter in seagrass-colonized sediments: A stable isotope study of the silt and clay fraction from *Posidonia oceanica* meadows in the western Mediterranean, *Org. Geochem.*, 36, 949-961, doi: 10.1016/j.orggeochem.2004.12.002, 2005.

Prokopenko, M.G., Hammond, D.E., Berelson, W.M., Bernhard, J.M., Stott, L., and Douglas, R.: Nitrogen cycling in the sediments of Santa Barbara basin and Eastern Subtropical North Pacific: nitrogen isotopes, diagenesis and possible chemosymbiosis between two lithotrophs (*Thioploca* and *Anammox*) – “riding on a glider”. *Earth Planet. Sc. Lett.*, 242, 186-204, doi:10.1016/j.epsl.2005.11.044, 2006.

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Quinn, G. P. and Keough, M. J.: Experimental design and data analysis for biologists, Cambridge University Press, 2002.

R Core Team: R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria, 2015.

5 Risgaard-Petersen, N., Nicolaisen, M. H., Revsbech, N. P., and Lomstein, B. A.: Competition between Ammonia-Oxidizing Bacteria and Benthic Microalgae, *Appl. Environ. Microb.*, 70, 5528-5537, doi: 10.1128/AEM.70.9.5528-5537.2004, 2004.

Rooze, J., and Meile, C.: The effect of redox conditions and bioirrigation on nitrogen isotope fractionation in marine sediments. *Geochim. Cosmochim. Acta*, 184, 227-239, doi: 10.1016/j.gca.2016.04.040, 2016.

10 Russell, D. G., Kessler, A. J., Wong, W. W., and Cook, P. L. M.: The importance of nitrogen fixation to a temperate, intertidal embayment determined using a stable isotope mass balance approach, *Biogeosciences Discuss.*, 2017, 1-28, doi: 10.5194/bg-2017-418, 2017.

Russell, D. G., Warry, F. Y., and Cook, P. L. M.: The balance between nitrogen fixation and denitrification on vegetated and non-vegetated intertidal sediments, *Limnol. Oceanogr.*, 61, 2058-2075, doi: 10.1002/lno.10353, 15 2016.

Rysgaard, S., Risgaard-Petersen, N., and Sloth, N. P.: Nitrification, denitrification, and nitrate ammonification in sediments of two coastal lagoons in Southern France. In: *Coastal Lagoon Eutrophication and Anaerobic Processes (CLE AN.)*, Springer, 1996.

20 Sachs, J. P., and Repeta, D. J.: Oligotrophy and nitrogen fixation during eastern Mediterranean sapropel events. *Science*, 286, 2485-2488, doi: 10.1126/science.286.5449.2485, 1999.

Saino, T., and Hattori, A.: Geographical variation of the water column distribution of suspended particulate organic nitrogen and its <sup>15</sup>N natural abundance in the Pacific and its marginal seas. *Deep Sea Res. Part A*, 34, 807-827, doi: 10.1016/0198-0149(87)90038-0, 1987.

Vonk, J. A., Middelburg, J. J., Stapel, J., and Bouma, T. J.: Dissolved organic nitrogen uptake by seagrasses, *Limnol. Oceanogr.*, 53, 542-548, doi: 10.4319/lno.2008.53.2.0542, 2008.

25 Waycott, M., Duarte, C. M., Carruthers, T. J. B., Orth, R. J., Dennison, W. C., Olyarnik, S., Calladine, A., Fourqurean, J. W., Heck, K. L., and Hughes, A. R.: Accelerating loss of seagrasses across the globe threatens coastal ecosystems, *Proc. Natl. Acad. Sci.*, 106, 12377-12381, doi: 10.1073/pnas.0905620106, 2009.

Welsh, D. T.: Nitrogen fixation in seagrass meadows: Regulation, plant-bacteria interactions and significance to primary productivity, *Ecol. Lett.*, 3, 58-71, doi: 10.1046/j.1461-0248.2000.00111.x, 2000.

30

**Deleted:** Revsbech, N. P., Sorensen, J., Blackburn, T. H., and Lomholt, J. P.: Distribution of oxygen in marine sediments measured with microelectrodes, *Limnol. Oceanogr.*, 25, 403-411, doi: 10.4319/lno.1980.25.3.0403, 1980. .

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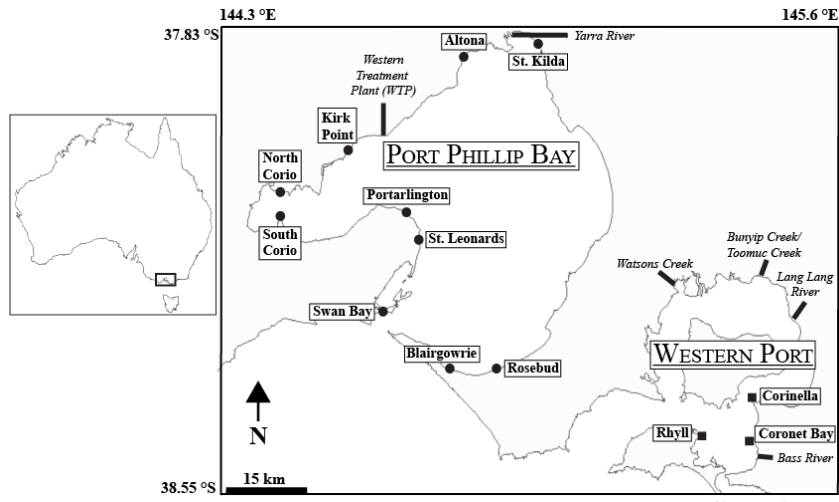
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**Table 1:** Summary of possible nitrogen isotopic end-members in Western Port and Port Phillip Bay

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Source	Isotopic End-Member (‰)	Reference
<b>Port Phillip Bay</b>		
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
<b>Western Port</b>		
Nitrogen Fixation	0.0	Owens, 1988
Oceanic	6.9	Russell et al., 2017
Riverine	9.2	Russell et al., 2017

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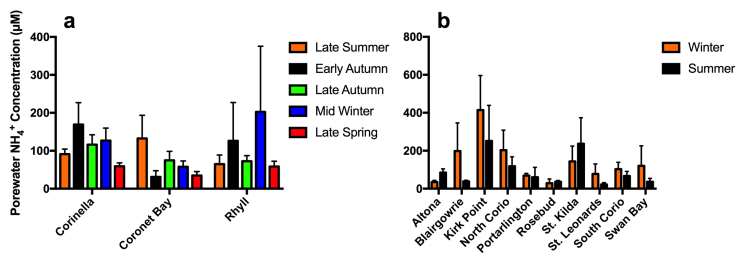


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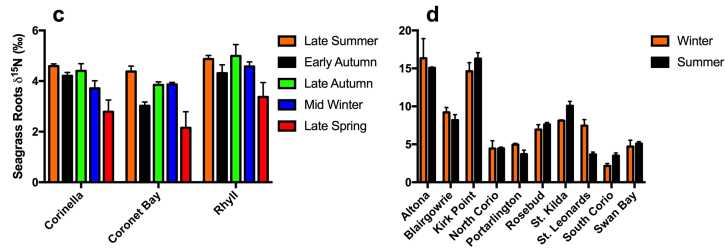
Figure 1: Western Port and Port Phillip Bay, Australia, showing the field sites. The sites marked with circles were sampled during August and December 2016, and the sites marked with squares were sampled approximately bimonthly over the period February – November 2016.

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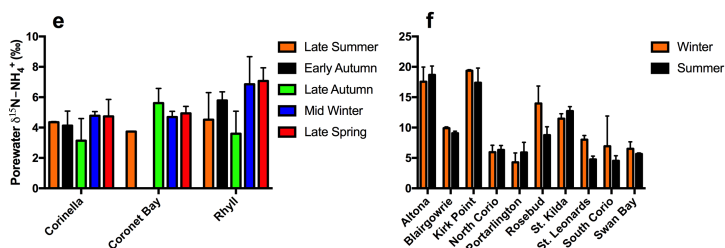
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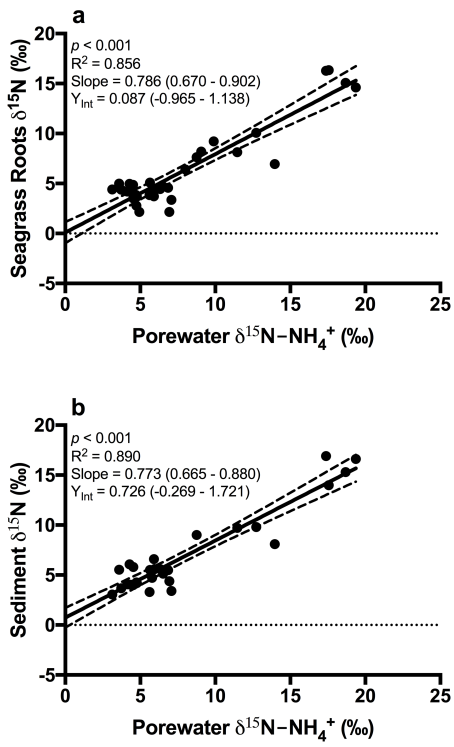
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Figure 2: Porewater  $\text{NH}_4^+$  concentrations for (a) Western Port and (b) Port Phillip Bay, seagrass root isotopic signature ( $\delta^{15}\text{N}$ ) for (c) Western Port and (d) Port Phillip Bay, and porewater  $\text{NH}_4^+$  isotopic signature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

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Figure 3: Plot of the porewater  $\delta^{15}N-NH_4^+$  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.

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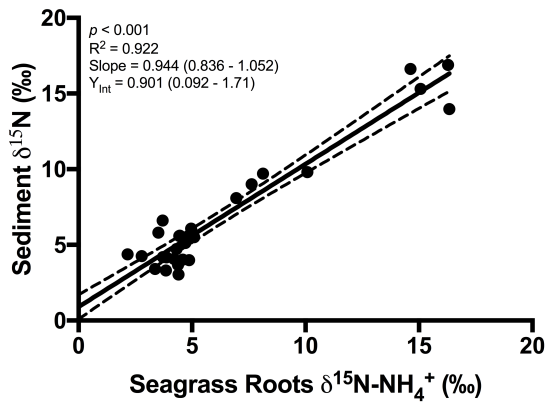
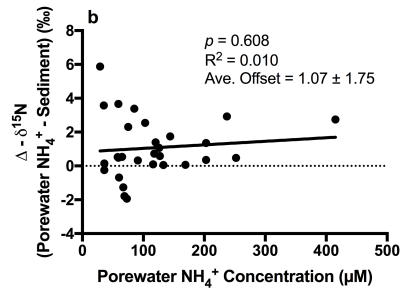
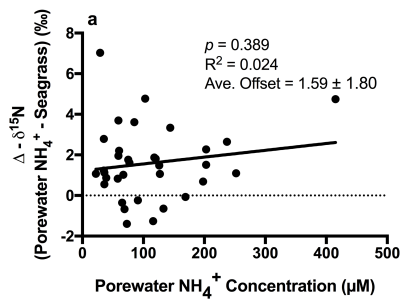


Figure 4: Plot of seagrass  $\delta^{15}\text{N}$  against sediment  $\delta^{15}\text{N}$ .

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Figure 5: Plot of porewater  $\text{NH}_4^+$  concentration against (a) the difference between the porewater  $\delta^{15}\text{N-NH}_4^+$  and the seagrass  $\delta^{15}\text{N}$  (b) the difference between the porewater  $\delta^{15}\text{N-NH}_4^+$  and the bulk sediment  $\delta^{15}\text{N}$ .

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