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Nutrient removal processes in saltmarsh and adjacent habitats: Overview and A Preliminary Study of Denitrification in the Solent, UK.

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1 Contents

2 Policy Summary

Saltmarsh and adjacent coastal habitats (e.g. mudflat, seagrass) can provide functions valued by people i.e. ecosystem services. As part of the marine Natural Capital and Ecosystem Assessment (mNCEA) program, the UK Centre for Ecology and Hydrology (UKCEH) and Bangor University, on behalf of the Environment Agency, conducted a pilot study to assess denitrification in coastal habitats, which can contribute to water quality improvement.

Denitrification can be provided by saltmarsh and adjacent habitat. It is one process from a broader suite of nutrient removal processes (e.g. sediment burial, plant uptake) provided by coastal habitats that together constitute the ecosystem service of water quality improvement. Complete denitrification permanently removes nitrate from the water column/sediment, transforming this compound to the environmentally benign nitrogen gas. Saltmarsh ecosystems, due to their position within the intertidal zone, are considered to have potential to provide high denitrification rates.

Previous studies highlight how environmental conditions, such as temperature, substrate supply and oxygen status, lead to high variation in denitrification rates across space and over time. However, limited evidence on denitrification rates is available from intact inter-tidal coastal systems in the UK.

The pilot study, conducted within Chichester Harbour Site of Special Scientific Interest, and using two laboratory handling methods, confirmed high variation across space and season (autumn vs winter) in potential denitrification rates. This high variation and the necessarily limited sampling precluded statements of statistical significance or the robust estimation of effect size. A tidal core method, where intact cores are subjected to realistic tidal cycles, showed trends for higher denitrification rates in the autumn than winter in upper and pioneer/low marsh zones, and a consistent estimate in the mid-marsh. A slurry method indicated that denitrification rates generally declined with depth, irrespective of season or saltmarsh zone, except for samples in the pioneer/low marsh zone in winter. Seagrass and mudflat habitats, that could only be sampled in autumn due to logistical constraints, had generally low denitrification rates, irrespective of method.

This study confirmed the utility of a tidal core method for estimating denitrification rates to compare among and within inter-tidal habitats and provides a first benchmark for denitrification rates in intact saltmarsh and adjacent inter-tidal habitat in the UK. Given the variability, and the expectation that such variation would be amplified when different estuaries are considered, scaling up understanding to the national level requires further work.

Short-term priorities include comparing among marshes within and across estuaries in different locations and with variation in underlying sediment; intensive seasonal sampling; and deriving benchmarks for sites undergoing managed realignment as well as sites that constitute intact coastal habitat. Results from such studies will provide foundational data for model development (e.g. Combined Phytoplankton Macroalgae model of CEFAS) and assist economic valuation assessments.

Longer-term efforts will help determine the absolute and relative role of denitrification as one process within a broader suite of nutrient removal processes in these coastal habitats; how to scale to nationwide estimates in a cost-effective manner (e.g. through eDNA and/or remote sensing approaches); and, the extent to which nutrient removal exhibits synergies or trade-offs with initiatives to tackle biodiversity and climate emergencies. Such research will need to include quantifying the potential of inter-tidal habitats to capture and remove other critical nutrients, such as phosphorus, as well as nitrogen.

Ultimately, this and allied work within the Land Sea Interface project, and elsewhere, will help achieve the UK Government's commitment to "secure clean, healthy, productive and biologically diverse seas and oceans".

3 Executive Summary

Context

The Environment Agency are running the Land-Sea Interface (LSI) Project as part of Year 2 of the Marine Natural Capital and Ecosystem Assessment Programme (mNCEA). The LSI Project aims to improve available evidence in relation to the ecosystem services provided by key estuarine and coastal habitats, including saltmarsh, mudflat, seagrass, and shellfish beds.

The UK Centre for Ecology and Hydrology (UKCEH), in conjunction with Bangor University, was tasked with providing an overview of a key ecosystem service from saltmarsh and adjoining mudflat and seagrass habitats: nutrient removal.

In addition, we were asked to conduct a pilot study quantifying seasonal and spatial variation in one aspect of nutrient removal: denitrification, complemented by a comparison among denitrification rate estimation methods. Denitrification is the stepwise, microbially mediated conversion of an environmentally harmful form of nitrogen (N) (i.e. nitrate (NO_3^-)) into the environmentally benign dinitrogen gas (N_2). Denitrification can sometimes be coupled with nitrification, which transforms ammonium (NH_4^+) into NO_3^-.

Nutrient Cycling in Saltmarsh and Adjacent Systems: Overview

In contrast to typical terrestrial systems, saltmarsh and adjacent ecosystems have an open nutrient cycle for both N and phosphorus (P) associated with tidal flooding cycles, overland surface flow from landward systems, and groundwater exchange. There may be additional N and P inputs from atmospheric deposition and, for N, from free-living or plant-associated N_2-fixing bacteria. Internal mineralization processes convert plant-unavailable organic forms of N and P into plant-available forms. Plant-available P supply can be further buffered through kinetic reactions associated with less-available inorganic P forms attached to iron, aluminium, or calcium molecules in the sediment. Nutrient cycling processes interact due to balancing of chemical elements (stoichiometric constraints), which may mean P cycling depends on N supply and *vice versa*.

Nutrient removal processes operate over a range of timescales, from plant uptake of the available nutrient forms, fixation through dissimilatory nitrate reduction to ammonium (DNRA), sediment burial, leaching losses of dissolved or particulate forms with a range of availabilities, and, for N, gaseous loss. Gaseous N loss occurs through biotically-driven denitrification *sensu stricto*, but also anaerobic ammonium oxidation (anammox), coupled nitrification-denitrification, and co-denitrification. Of these processes, denitrification is generally assumed to be most important in the saltmarsh system, but there is limited quantitative evidence as to its absolute and relative importance.

Long-term sediment burial and gaseous loss of N_2 can be considered ‘environmentally benign’. In contrast, plant uptake of polluting amounts of available nutrient forms can be associated with undesirable community change and unwanted biodiversity consequences e.g. biotic homogenization. Nitrification and denitrification processes can lead to the loss of nitrous oxide (N_2O), a potent greenhouse gas, while leaching losses and any gaseous ammonia or NO_x losses may have detrimental consequences for receiving environments.

Given societal demand for nutrient removal but the potential for unwanted environmental consequences, it is important to accurately quantify the importance of different nutrient removal processes in saltmarsh and adjacent coastal habitats. Further, it is important to understand the environmental factors that can influence nutrient removal quantities and partitioning amongst different forms and processes.

A Denitrification Pilot Study and Methodological Comparison

As a first step to providing this understanding, we conducted a pilot study on seasonal variation in denitrification rates across different saltmarsh vegetation zones at Thorney Island, in the wider Chichester Harbour Site of Special Scientific Interest. Simultaneously, we provided an overview of the strengths and weaknesses of methodological approaches to estimating denitrification rates.

Methodologically, denitrification is difficult to measure. At least in part, this is due to high atmospheric background concentrations of N_2 gas – meaning it is hard to pick up the signal of N_2 release from denitrification itself. Methods therefore range from the relatively inexpensive, and scalable, acetylene blocking of the full denitrification process using samples processed in a lab, to *in situ* isotopic labelling with expensive equipment and complicated post-processing needed to discriminate among N compounds, and chamber systems with purged and modified atmospheres to accurately quantify subsequent N_2 release.

Acetylene blocking is popular but suffers from methodological artefacts including incomplete inhibition of denitrification. However, due to its potential to allow high throughput of multiple samples relatively cheaply, we adopted it in our pilot study. Further, we compared two laboratory handling methods:

(i) a **slurry method** which allows potential denitrification rate estimation across separate depths using disturbed samples; and,

(ii) a **core method** (pioneered by Blackwell et al. 2010), which allows sediment samples to remain intact within extracted cores and with integrated estimates of denitrification across depth under realistic tidal cycles.

In autumn, the tidal core method provided mean denitrification estimates ranging from 0.19 to 0.04 mg N_2O-N m^{-2} h^{-1} across the 20 cm depth of core. The mid-marsh provided consistent estimates in autumn and winter (0.04 and 0.05 mg N_2O-N m^{-2} h^{-1} respectively). Positive denitrification rates in the low and upper marsh in winter could not be estimated, likely reflecting depleted nitrate supply. Mudflat and seagrass areas were only sampled in autumn and showed lower mean denitrification rates than saltmarsh (0.02 mg N_2O-N m^{-2} h^{-1} and <0.01 mg N_2O-N m^{-2} h^{-1}, respectively).

Mean average denitrification rates from the 1:1 substrate-water slurries showed — irrespective of season or saltmarsh zone — a general decline in denitrification rates with sample depth, apart from winter samples from the low marsh. Neither the mudflat nor the seagrass samples showed a similar decline in denitrification rates with depth. The largest mean average denitrification rate (58.19 ng N_2O-N g^{-1} h^{-1}) was at a depth of 5 cm from the mid marsh in autumn. The lowest mean rate (2.38 ng N_2O-N g^{-1} h^{-1}) was from a depth of 10 cm from the mid marsh slurries in winter.

High variability among samples even within seasons and zones precluded statements in relation to statistical significance i.e. the results communicated above show the trends in the data. This high variability is to be expected, with earlier work across ecosystems highlighting the presence of ‘hotspots’ of denitrification and orders-of-magnitude in variation across space.

Extensions and Recommendations

The denitrification rate, seawater concentrations and porewater concentrations will allow extensions to the Combined Phytoplankton and Macroalgae (CPM) model, developed by the Centre for Environment, Fisheries and Aquaculture Science (CEFAS). This may involve considering separate compartments for nitrate and ammonium, currently considered together as ‘inorganic N in porewater’.

We make the following short-term recommendations to enable upscaling for national estimates of denitrification. Using the same laboratory estimation methods as the pilot study, and to quantify spatial and temporal variation in denitrification rates:

(i) Estimate potential denitrification rates across saltmarsh zones in two contrasting regions and sediment types e.g. muddy and sandy;

(ii) Estimate seasonal variation by monthly sampling throughout the year at a single saltmarsh site;
(iii) Estimate variation between natural and restored saltmarshes through investigations of a temporal chronosequence in the same area as (ii).

In the medium to longer term, to gain predictive understanding of nutrient removal processes, including denitrification, in varying environments, we recommend:

(i) *Ex situ* and *in situ* nutrient removal estimations across multiple saltmarsh locations along environmentally orthogonal gradients to disentangle causation and to understand the relative importance of different nutrient removal processes in different environmental contexts;
(ii) Investigations in relation to nutrient removal, especially denitrification, across a range of saltmarsh restoration approaches (e.g. dredging, managed realignment) given likely microbial and vegetation community differences feeding through to variation in the quantity of N and P removed;
(iii) Investigations on the impact of nitrate pollutant load and variation in other environmental variables on denitrification rates and N_2O yield, given the global warming potential of N_2O;
(iv) Investigations into the integration of remote sensing and ground-truthing nutrient removal estimates to allow nationwide scaling;
(v) Investigations into the use of proxy variables, including rapid biodiversity assessments e.g. eDNA approaches, as a means to estimate denitrification and other nutrient removal processes, without intensive field and laboratory work;
(vi) Investigations into the trade-offs and synergies among multiple ecosystem services provided by marsh systems to understand when crediting nutrient remediation is a sustainable environmental approach.

Overall, this and ongoing work within the LSI project will contribute to a better understanding of the full range of services and benefits provided by saltmarsh natural capital assets, complementing existing understanding of the blue carbon aspects of this habitat. Further, saltmarsh and adjacent habitats, as well as being priorities under European directives, need protecting to help the UK Government's 25-year Environmental Plan and its associated commitment to "secure clean, healthy, productive and biologically diverse seas and oceans".

4 Acknowledgements

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5 Introduction

5.1 Context

The Environment Agency (EA) are running the Land-Sea Interface (LSI) Project, as part of Year 2 of the marine Natural Capital and Ecosystem Assessment Programme (mNCEA). The LSI Project aims to improve available evidence in relation to the ecosystem services provided by key estuarine and coastal habitats, including saltmarsh, mudflat, seagrass, and shellfish beds.

One key ecosystem service provided by coastal habitats is nutrient removal (de Groot et al. 2012). This removal is regarded as particularly important, given coastal ecosystems now persist in an era of heightened nutrient pollution arising from urbanization, agriculture and industry (e.g. Deegan et al. 2012). Without the nutrient removal provided by buffering coastal habitats, the marine environment could suffer from eutrophication, subsequent deoxygenation and consequent loss of marine biodiversity e.g. fish kills (Diaz and Rosenberg 2008).

Despite the expectation that coastal habitats provide nutrient removal services, underlying evidence in the UK-context remains scarce (e.g. Blackwell, Yamulki, and Bol 2010; Koch et al. 1992). EA have therefore contracted the UK Centre for Ecology and Hydrology (UKCEH) to carry out a combined desk- and field-based study to improve the evidence base on nutrient removal rates in coastal habitats. The deliverables from the project will also be used to fill evidence gaps in an updated Combined Phytoplankton-Macroalgae (CPM) model, developed by the Centre for Environment, Fisheries and Aquaculture Science (CEFAS).

UKCEH have been asked to focus on two key coastal habitats: saltmarsh and shellfish (oyster) beds, reported separately in Fabra et al. 2024. We have also been asked to consider, in less detail, nutrient removal from adjacent mudflat and seagrass habitats. Ultimately, as well as filling evidence gaps in the CPM model, results will inform recommendations for England-wide studies of nutrient removal rates in coastal habitats, to be undertaken in subsequent phases of the mNCEA. This report fulfils EA's ultimate objective of recommendations for an England-wide study to capture variability in nutrient removal rates from saltmarsh (and, ultimately, adjacent seagrass and mudflat habitats).

5.2 Approach

'Nutrient removal' encompasses a number of pathways e.g. leaching, burial, denitrification, and could potentially cover a number of nutrients e.g. nitrogen (N), phosphorus (P), potassium (K), and micronutrients. However, N and P remain core nutrients in the context of nutrient pollution in the UK coastal environment (Maier et al. 2009). Consequently, our report focuses on these two nutrients. Furthermore, under the direction of the EA, we have focussed our initial field-based investigation on denitrification, as a key 'nutrient removal' pathway, in this case for N. To inform the CPM and potential analyses on drivers of denitrification, this pilot study included the measurement of porewater and tidal water (flood and ebb) N and P concentrations.

We have adopted the following approach to achieve the EA's deliverables:

(i) Introduce background to general nutrient cycling processes, and then a focus highlighting key differences from the general understanding for saltmarsh and adjacent coastal habitats (mudflats, seagrass). This is to understand how two focal nutrients (N and P) can cycle and be removed via different pathways;

(ii) Provided background information on what can drive variation in nutrient removal, both spatially and temporally across different scales e.g. within and among habitats and locations, within and across years;

(iii) Liaised with CEFAS to understand how denitrification, and nutrient cycling in general, is simulated in the CPM model and its planned extensions;

(iv) Compared and contrasted the methods (strengths, weaknesses, indicative costs) of a variety of denitrification rate estimation approaches, both *in situ* and *ex situ*, through a literature review;

(v) Carried out laboratory assays of potential denitrification rates in collaboration with Bangor University. This provides a preliminary understanding of spatial and seasonal variability in denitrification rates in saltmarsh and adjacent habitats from one estuary: Chichester Harbour, The Solent, UK;

Integrated and synthesized findings from (i) to (v) to provide short- and medium- to long-term recommendations to quantify and enable prediction of variation in nutrient removal rates, including through denitrification, in saltmarsh (and, where relevant, adjacent) habitats.

6 Nutrient Cycling Overview

6.1 Ecosystem Nutrient Cycling: A Short Primer

Nutrient cycles, especially in mature, relatively intact, non-agricultural, terrestrial systems, tend to be considered “closed”. This means there are limited inputs and losses, with any losses that there are tending to be dominated by nutrients in forms unavailable for plant uptake (Hedin, Vitousek, and Matson 2003). Inputs can come from long distances through dry and wet atmospheric deposition (Bauters et al. 2021; Tipping et al. 2014; Galloway et al. 2004), while the presence of nitrogen-fixing microbes in or outside of plants can fix N_2 gas from the atmosphere into plant-available nitrogen (N) forms (Schlesinger and Bernhardt 2020).

In such systems, plant nutrient demand beyond that supplied from inputs is supported by nutrients made available through the microbial community mineralizing organic matter in an ‘internal cycle’, where net mineralization is the balance between gross mineralization from organic matter and gross immobilization in microbial biomass. Nutrient (and water) uptake can be facilitated by the presence of mycorrhizal fungi (Kivlin, Hawkes, and Treseder 2011) and influence plant community composition and subsequent nutrient cycling processes through functional traits (see references in Farrer et al. 2022), while other microbes (e.g. saprotrophs, N transformers, phosphate solubilizing bacteria) can further influence decomposition and nutrient cycling (Farrer et al. 2022).

Other than generally small, dynamic pools of plant-available N (e.g. nitrate (NO_3^-), ammonium (NH_4^+) and rarely simple molecules of dissolved organic N (DON) (Näsholm, Kielland, and Ganeteg 2009)), most N is bound up in the organic matter, with NH_4^+ forming complexes with clay minerals on occasion (Nieder, Benbi, and Scherer 2011). Phosphate (PO_4^{3-}) is the available nutrient form of phosphorus (P) taken up by plants. Like N, P can also be bound in organic matter in many forms including through phosphate being attached to organic complexes, as well as P molecules within an organic moiety (George et al. 2018). Phosphate also has a propensity to adsorb on to mineral surfaces, forming stable complexes with aluminium (Al) and iron (Fe), as well as calcium (Ca) (e.g. McLaughlin, Ryden, and Syers 1981), the presence of which will depend on environmental context. These complexes provide a buffer to the available P pool (Perring et al. 2008), allow sustained release of P over different timescales, and tend to form during ecosystem succession, together with organic matter and more occluded (unavailable) P forms and with the loss of primary PO_4^{3-}-containing minerals (e.g. apatite) (Walker and Syers 1976; Wardle, Walker, and Bardgett 2004).

The processing of different nutrient forms occurs through relationships amongst them, especially in plants. The characteristic ratios in plant structures, and the need to support physiological processes, leads to a ‘biological stoichiometry’ (Elser et al. 2000). Thus, the ability of a plant to take up N will depend on there being sufficient P in the environment, as well as other essential macro-nutrients (such as potassium (K)) and micro-nutrients (such as boron (B)) (Kumar, Kumar, and Mohapatra 2021). In theory, for plant nutrient uptake and subsequent growth, this means both N and P pollution can be remediated in a slightly different form, as suggested for a heathland system by Marrs (1985).

These interactions also mean that the dynamics of ecosystem nutrient pools don’t only depend on the given nutrient’s cycling processes, but also relationships with supplies of other nutrients (and vice versa). For instance, the total amount of P in an ecosystem undergoing N addition depends on the relative rates of loss from plant unavailable and available loss pathways, the kinetics and size of the buffering P pool, and the limitation status of plant growth (Perring et al. 2008). Furthermore, the dynamics of microbial P processes can be affected by N inputs, for instance due to plants releasing phosphatase enzymes to encourage organic matter decomposition (Margalef et al. 2017), while N dynamics may be influenced by P supply (Wang et al. 2022). Indeed, McGill and Cole (1981) suggested a conceptual model where N is mineralised *biologically*, as a by-product in the search for energy and coupled with the release of carbon dioxide (CO_2). On the other hand, P

is mineralized *biochemically*, such that enzymes cleave the nutrient from organic matter without releasing CO_2 (McGill and Cole 1981). Enzyme production requires sufficient N supplies, providing another means linking the dynamics of N and P (Wang, Houlton, and Field 2007). The processes associated with N mineralization in particular, i.e. denitrification, are explored further below.

6.2 Nutrient Cycling in Saltmarsh and Adjacent Systems: Additional Considerations

The general nutrient cycling processes introduced above, and interactions amongst nutrients, pertain to saltmarsh systems and those adjoining coastal systems i.e. seagrass and mudflat (Bowen et al. 2023). However, contrary to the typical terrestrial situation, intact saltmarsh and their adjacent systems have more open nutrient cycles, linked to daily tidal exchanges and groundwater exchange (Xin et al. 2022), and given their high capacity for N_2 gas loss through denitrification (Ashok and Hait 2015). Inputs of nutrients in plant-available N and P forms are frequent through tidal inundation and occasional through surface flow from the landward boundary, although the availability of P can be influenced by the salinity and hydrodynamic characteristics of a given estuary (Statham, 2012). Unlike N, oxidation-reduction processes are thought to play a minor role in the P cycle; instead, river-derived dissolved and particulate P that enters the estuarine system can be modified by a suite of inorganic and biological interactions (Statham, 2012). In the case of N particularly, and to a lesser extent for P (Newman 1995), inputs can be complemented by atmospheric deposition (Bowen et al. 2023). An N-fixing component to the vegetation is rare, but not unknown, while free-living diazotrophs (N-fixing organisms) can be abundant in saltmarsh systems coming from diverse novel bacterial and archaeal lineages (Farrer et al. 2022) (**Figure 1**). Another difference from 'typical' terrestrial systems is that mycorrhizal symbioses may be inhibited by the salinity of the marsh system, such that some vegetation that is abundant on coasts can be non-mycorrhizal (e.g. glassworts: *Salicornia* sp.) or typically have low colonization (e.g. sedge: *Carex* sp.) (see references in Farrer et al. 2022). On the other hand, pathogenic organisms are thought to play a major role in the ecology of coastal habitats and can create die-off events (e.g. ergot (*Claviceps purpurea*) epidemics in common cordgrass (*Spartina anglica)* marshes in the UK (Raybould 1998 as cited in Farrer et al. 2022)) that would have implications for nutrient removal capacity and coastal resilience.

After entering the marsh ecosystem, nutrients can be intercepted through plant uptake (potentially through gaseous uptake in the leaves as well as through roots, although the presence/magnitude of this process requires further investigation) and microbial biomass production or captured through sediment burial and adsorption. These processes will 'lock-up' nutrients for different periods of time, depending on subsequent dynamics – for instance, sediment burial would be expected to lock-up previously available forms for a substantial period in the absence of erosion. Plant uptake will only sequester nutrients for the time that they reside in plant biomass and are transferred to and retained as organic matter. With mineralisation, they will be re-released for continued internal cycling or for loss in plant-available forms from the system. Even without mineralisation, particulate and dissolved organic matter losses can occur, especially with tidal flow. Creek drainage, leaching, and groundwater exchange provide a means for both N and P to be lost (in particulate and dissolved, as well as plant-available and plant-unavailable, forms), while N can also be lost through nitrogen-containing gases (as explored in more detail in the remainder of this report).

The ability of saltmarshes and other coastal systems to sequester nutrients or process them into benign forms (in the context of not causing harm to the environment) has captured the attention of those considering how to remediate high levels of nutrient pollution (e.g. Billah et al. 2022). Indeed, saltmarshes around the globe tend to be located in areas with large nutrient loads, particularly N (Deegan et al. 2012), and the United Kingdom is no exception. Despite efforts to reduce nutrient losses from agriculture and industrial/urban wastewater, nutrient pollution will likely remain a threat to ecosystem integrity and biodiversity (IPBES 2019), especially where it enters the marine ecosystem. Adding nutrients to the marine environment can lead to algal blooms, subsequent

deoxygenation and the death of organisms including economically-important fish species – as seen with the dead zone of the Mississippi delta and elsewhere (Diaz and Rosenberg 2008). This drives the interest in using coastal ecosystems, such as saltmarsh, oyster beds, seagrass, and kelp, as a means to intercept nutrient pollution prior to it causing harm in the wider marine environment. This can include the use of extant marshes, or through habitat restoration (e.g. Billah et al. 2022). Coastal systems are viewed as particularly promising as they are considered to have a high N_2 -production potential in comparison to other systems – for instance, forests and grasslands are only considered to have a low N_2 production potential (Ashok and Hait 2015).

Thus, to understand the nutrient remediation capacity of saltmarshes and adjacent systems we need to quantify the different nutrient stocks and nutrient flows that characterise the saltmarsh, mudflat, and seagrass system, as well as how these properties, and transfers amongst these habitats, respond under nutrient addition and other stressors e.g. climate change, sea level rise. In other words, nutrient cycling processes and their magnitudes under historic low levels of available nutrients may be perturbed by additional nutrients, as discussed in general above, complicating efforts to quantify remediation capacity. Processes may be further altered by droughts and flooding, changing salinity and other environmental factors, while the characteristic vegetation zonation of saltmarsh (with 'typical' plant communities at upper, mid, and low/pioneer marsh zones (Boorman 2003)), may influence nutrient removal capacity. Finally, there may also be nutrient cycling process rate differences in 'new' and 'developing' marshes created by various restoration techniques (Billah et al. 2022) and/or extant marshes. Given that the area of recreated marsh in the UK was recently quantified as being insufficient to replace that lost by erosion and other processes (Boorman and Hazelden 2017), it will be important, in due course, to quantify differences among these marsh types and over time.
The different saltmarsh vegetation communities, and their associated biodiversity can be affected by nutrients (e.g. Penk et al. 2020; Penk, Perrin, and Waldren 2020; Redelstein et al. 2018). This means that the use of saltmarshes as a nutrient remediation strategy also needs to account for impacts on associated biodiversity, especially where features have statutory conservation importance and there is a need to maintain favourable conservation status.

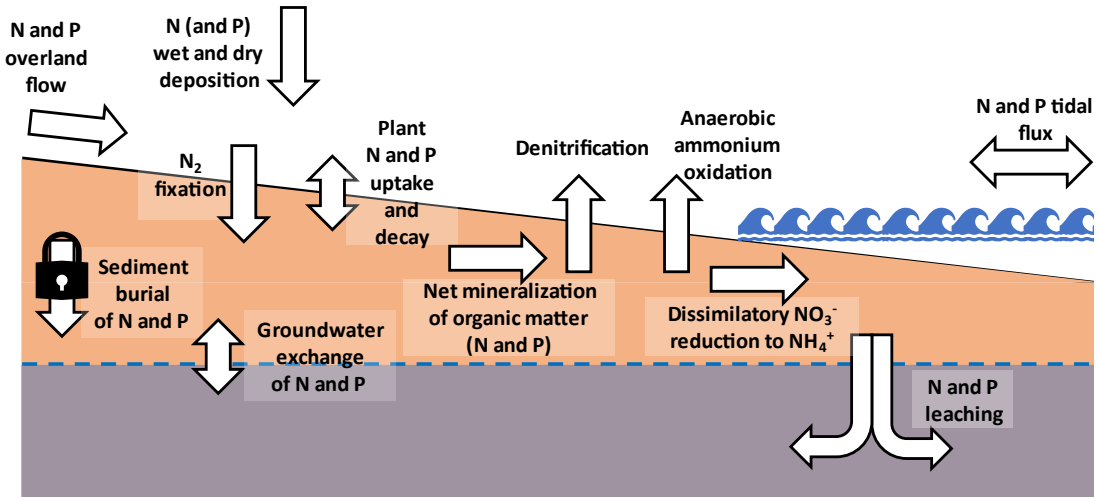

Figure 1: **Key nitrogen (N) and phosphorus (P) cycling processes in saltmarsh and adjacent systems.** Note the relationships between nutrients (stoichiometry) is not shown here; this could mean that rates of particular processes depend on supply of the other nutrient and vice versa. See main text for further details and for information on the potential presence of an inorganic pool of P that can buffer plant-available P pools.

6.3 Quantifying Saltmarsh Nutrient Cycling Processes

Since quantification of nutrient removal capacity is a relatively 'new' subject, there is limited available evidence to inform the Environment Agency as to the magnitude of the different nutrient removal processes across saltmarsh vegetation zones and environmental contexts e.g. sand or mud sediment. Indeed, a recent review in the context of saltmarsh restoration (Billah et al. 2022) emphasized that functional indicators that have typically been measured were sediment organic matter, sediment carbon (C) % and sediment N content. The authors argued that these are good proxies to understand energy flow and nutrient cycles but did not provide further information on relationships with the actual quantities of N (or P) cycling processes (Billah et al. 2022). This reiterates the importance of targeted research to enable the scaling-up of nutrient bioremediation as a nature-based solution (NbS), especially if it is to be used for the selling of nutrient credits.

Burden et al. (2013) compared rates of N cycling among a natural saltmarsh at high- and low-shore locations, claimed arable land on a former high-shore marsh, and a managed realignment site, at high- and low-shore locations, 15 years after tidal inundation. The natural high-shore site had the highest soil organic matter concentrations and belowground biomass, while (unsurprisingly) the agricultural site had highest N contents, both in terms of extractable N and through the lowest soil C to N ratio. Interestingly, the aboveground biomass, extractable N and substrate mineralisation at the high-shore managed realignment site had values similar to natural saltmarsh but less dynamic properties remained more similar to the agricultural site (Burden et al. 2013).

Adams et al. (2012) examined the rates of burial of C, N and P in both natural saltmarshes and those re-created following managed re-alignment. They concluded that saltmarshes, despite being net sources of methane (CH_4) and nitrous oxide (N_2O), can sequester C and reduce estuarine nutrient loads. Specifically, they stated that managed realignment areas in the Blackwater estuary in the United Kingdom (comprising both saltmarsh (29.5 km^2) and intertidal mudflat (23.7 km^2)) could bury nearly 700 tonnes (t) of N per year with a further 476 t N per year denitrified. Saltmarsh managed realignment would sequester 139.4 tonnes of P per year. They suggest that similar areas in the Humber estuary, comprising 74.95 km^2 could bury 180 t N per year with a further 442 t N per year denitrified (Adams, Andrews, and Jickells 2012).

Adams et al.'s (2012) findings can be contrasted with those of Blackwell et al. (2010) from the River Torridge in Devon. In natural saltmarsh, Adams et al. (2012) found that N_2O flux had a mean of 0.03 g N_2O m^{-2} yr^{-1} (ranging from -0.39 to 0.33 g N_2O m^{-2} yr^{-1}) while Blackwell et al. (2010) showed a greater mean flux of 3.72 g N_2O m^{-2} yr^{-1}. Managed realignment had an even greater flux of 8.94 g N_2O m^{-2} yr^{-1} (figures from Table 5 of Adams et al. (2012)). Denitrification rates in Blackwell et al.'s (2010) study were orders of magnitude greater than N_2O production rates and remained higher in managed realignment sites; in their analysis units, denitrification rates of 2.88 mg N_2O-N m^{-2} h^{-1} in the natural saltmarsh sediment and 3.39 mg N2O-N m^{-2} h^{-1} in the managed realignment sites. Blackwell et al. (2010) extended their analysis by adding nitrate to the tidal water that they exposed cores to and within which they measured N_2O production and denitrification. This exposure showed that natural saltmarsh sites were more sensitive than managed realignment sites since natural saltmarsh assays increased their denitrification and nitrous oxide production rates with this additional N more than was observed in managed realignment assays, although only in the non-flood periods of the experiment. Blackwell et al. (2010) suggested that this was due to managed realignment sites having decomposing vegetation within the accreting sediments that supplied N and thus made process rates less dependent on external supplies.

In a classic study of denitrification rates using cores extracted at low tide from the River Torridge in Devon, Koch et al. (1992) examined upper and lower mudflats and sea purslane (*Atriplex portulacoides)* marsh. They showed that mudflat sites had low rates of denitrification over the course of a year, between 0.52 and 5.78 µmol N_2 m^{-2} h^{-1} in the lower mudflat, and 1.28 to 4.36 µmol N_2 m^{-2} h^{-1} in the upper mudflat. In contrast, marsh sediment denitrification rates varied between 2.51 and 59.00 µmol N_2 m^{-2} h^{-1}. When amending cores with river water, denitrification was stimulated 10-fold in the mudflat cores and doubled in the saltmarsh sediment cores. Koch et al.

(1992) conclude that when calculating annual denitrification rates in tidal estuaries there needs to be a consideration of several factors including seasonal nitrate concentrations in tidal water, tidal flooding duration and amplitude, and the depth of the aerobic and anaerobic zone in the sediment. Note that Aziz et al. (1986), in their early study of nitrogen cycling in the Colne estuary, concluded that "*Denitrification does not lead to any great loss of nitrogen from the saltmarsh*".

The remainder of the report concentrates on N removal from saltmarsh ecosystems. After introducing N removal processes, with a particular focus on denitrification, drivers of its variation, and methods of its estimation, we then present the results from a pilot study on potential denitrification rates across saltmarsh vegetation zones complemented by estimates from a co-located seagrass bed and mudflat. Ultimately, we make recommendations as to next steps in how to characterise in a robust and efficient manner N removal via denitrification in a UK context. **However, we also emphasize that a full understanding of saltmarsh nutrient removal capacity requires quantification of other processes, including sediment burial and long-term vegetation removal, together with consideration of other pollutants, especially dynamics associated with P.**

6.4 Nitrogen Removal from Saltmarsh Systems

With current understanding, there are four main processes through which available forms of N can be removed from ecosystems through microbial processing. Processes include denitrification *sensu stricto*, co-denitrification (which is only partially microbial), coupled nitrification and denitrification, and anaerobic ammonium oxidation (anammox). All these processes produce gaseous N molecules, typically through respiratory processes of microbes. Such gases can then be lost from the marsh system, thus preventing marine nutrient pollution. It should be noted that in addition to respiration-driven processes, there is also the possibility to lose N gases through other mechanisms. For instance, some fungal pathogens produce NO gas when infecting plants (Aldossari and Ishii 2021). Microbes can also process nitrate through dissimilatory nitrate reduction to ammonium (DNRA) which can fix N in the saltmarsh system and is believed to be more important than previously thought at preventing nitrate loss (Bowen et al. 2023) (**Figure 2**).

Nitrogen removal from a saltmarsh system can also occur through leaching and/or surface flow of dissolved and/or particulate forms, and can be made unavailable in the longer term, and thus essentially 'lost' through sediment burial. Loss of available forms through leaching and/or surface flow would be undesirable in the context of marine nutrient pollution, while subsequent processing of unavailable forms downstream may also prove detrimental for receiving ecosystems. **Thus, for nutrient remediation purposes, processes that create 'benign' forms of N or lead to long-term storage would be preferred.** However, to better characterise saltmarsh (or other system) N removal capacity, it would be useful to estimate the magnitude and absolute/relative importance of all these processes. Understanding the underlying drivers and the environmental contexts that lead to variation in the contributions of the different processes would also be beneficial and allow transferability of N removal estimates.

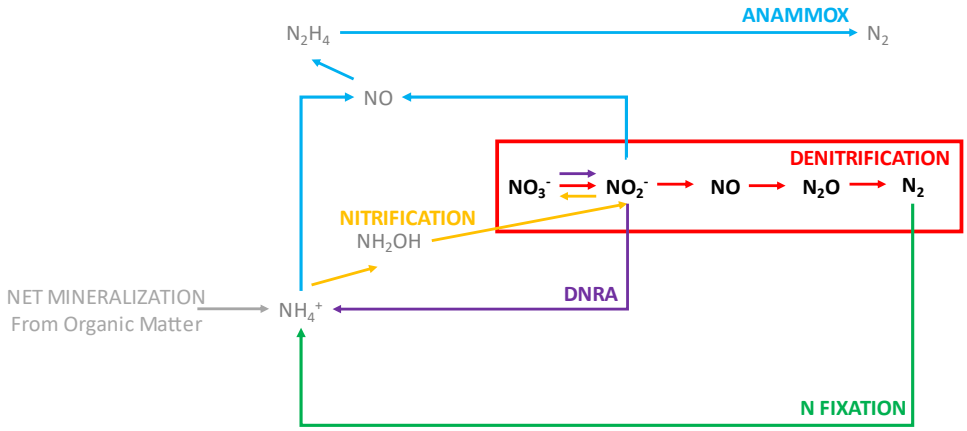

Figure 2: Overview of microbial nitrogen cycling processes in a saltmarsh and adjacent systems. The blue arrows represent the process of anaerobic ammonium oxidation (anammox) – an autotrophic process where oxidation of ammonium to dinitrogen gas is carried out using nitrite as an electron acceptor. The green arrow represents the fixation of dinitrogen gas in mineral form. The orange arrows represent nitrification while the red box and arrows represent denitrification, which is explored in more detail later in the report. The purple arrows represent dissimilatory nitrate reduction to ammonium (DNRA), where autotrophic and heterotrophic organisms convert nitrate to ammonium and retain fixed N in marshes where it can used to support primary production. Figure slightly modified from Bowen et al. (2023); the original is © Trends in Microbiology. See main text for a description of co-denitrification (not shown on this figure), where a mix of microbial and abiotic processes lead to the formation of N_2 gas.

Denitrification is typically considered a stepwise microbially-mediated process eventually leading to the production of N_2 gas, or nitrous oxide (N_2O) and/or nitric oxide (NO) where incomplete denitrification occurs (Groffman et al. 2006). However, co-denitrification can also lead to the production of hybrid forms of N_2 or N_2O species, through the combination of N in NO_2^- or NO and other N-containing compounds such as amines. This is conceptualised as a semi-microbial process and has also been termed 'BioNitrosation' (see references in Aldossari and Ishii 2021). Importantly, the differentiation of this process from anammox is complex, as both have the same isotopic labelling signature (Aldossari and Ishii 2021). Co-denitrification may be important in some marsh systems; for instance, chemo-denitrification (the abiotic reduction of NO_2 and NO) is facilitated under acidic or metal-rich conditions and can be a significant source of N_2O to the environment where ferrous iron (Fe^{2+}) or organic matter is abundant (Aldossari and Ishii 2021). Thus, microbes may cause the reduction of NO_3^- to NO_2^-, but the high reactivity of NO_2^- and NO can then lead to subsequent abiotic reduction – Aldossari and Ishii (2021) therefore caution that not all N_2O produced during the incubation of microbial strains arises from biological denitrification *sensu stricto*.

Denitrification can also be coupled with nitrification (the sequential transformation of ammonium to nitrate via nitrite). For a long time, this was presumed to occur through ammonia oxidizing bacteria (AOBs) but recent years have led to the discovery of ammonia oxidizing archaea (AOAs). These groups of organisms show diverging patterns according to salinity in estuarine systems, with patterns and process rates that differ from pelagic systems (Bernhard and Bollmann 2010). The composition could be important in determining process rates and the characteristics of by-products: aerobic AOB are chemolithoautotrophic while there are question marks over whether AOA are heterotrophic, autotrophic or mixotrophic (Bernhard and Bollmann 2010).

Coupled nitrification - denitrification can be stimulated by rhizodeposition under otherwise more anaerobic conditions (see Penton et al. 2013 and Reddy et al. 1989 as cited in Farrer et al. 2022). As also noted in Farrer et al. (2022), in flooded, anaerobic and wetland sediments bacterial symbionts in the rhizosphere perform the vast amount of nitrification (oxidation of ammonium to nitrate that can be leached, taken up by plants or denitrified) because it is an aerobic process that can be fuelled by oxygen flux through plant roots.

As well as the range of denitrification processes, anammox can process N into benign nutrient forms. Anammox is carried out by chemolithoautotrophic bacteria where ammonium is oxidized under anoxic conditions using nitrite as an electron acceptor. The end-product of anammox is dinitrogen gas (as for denitrification) and it directly reduces nitrite to N_2 gas while producing nitrate as a by-product (Weralupitiya et al. 2021). Thus, unlike denitrification, it avoids N_2O production and associated global warming potential. However, at least in New England where saltmarshes span a salinity gradient and nitrogen loading, it has low importance (maximum 3%) compared to denitrification (Koop-Jakobsen and Giblin 2009). More broadly, it appears that anammox can be triggered by ascending groundwater and could prove to be of great importance in remediating aquifers contaminated with nitrogen oxides (Wang et al. 2020).

Although not a focus of the report, the nitrification process may also be important to understand, as its end product (NO_3^-) is the substrate for denitrification. There are few studies of nitrification in saltmarshes, although order-of-magnitude differences in rates were found depending on whether vegetation communities were dominated by tall- or short-form saltmarsh cordgrass, *Spartina alterniflora* (Dollhopf et al. 2005 as cited in Bernhard and Bollmann 2010). However, others found no influence of saltmarsh grass species on potential nitrification rates (Moin et al. 2009 as cited in Bernhard and Bollmann 2010). Other studies tend not to report vegetation, and although there are expectations that vegetation would stimulate nitrification (due to oxygenating the sediments through roots as noted by Farrer et al. (2022)) others report inhibitory effects (see references in Bernhard and Bollmann 2010). Indeed, Bernhard and Bollman (2010) concluded that "…*our understanding of nitrification in salt marshes and the microorganisms responsible is severely lacking at present, despite the importance of nitrification in regulating nitrogen in these systems*".

Bernhard and Bollmann's (2010) review notes how tidal cycles, types of vegetation or distance from vegetation are all critical factors that may significantly impact the communities that are present and/or active. While salinity variation is expected to play a role in variability in the community of nitrifiers, they also presented evidence from Japan that suggested organic loading may be more important in their regulation (Urakawa et al. 2006 as cited in Bernhard and Bollmann 2010). Their review noted that abundance of particular groups did not necessarily scale to process rates either, which may be a 'real' result or may be a function of inaccurate characterisation of abundance, rates or both (Bernhard and Bollmann 2010). An accurate and robust estimation of rates and potential drivers is a key concern, especially for any future use of proxy variables to denote denitrification and/or in relation to the selling of nutrient credits from inter-tidal habitats. Variability in edaphic (soil) and biotic conditions also has implications for sampling and estimating nitrification rates. Bernhard and Bollmann (2010) suggest variation may affect the ability to collect comparable samples and thus obtain robust nitrification estimates. These same considerations will apply to denitrification. Correctly characterising other N cycling processes, in addition to denitrification may be important in understanding the absolute ability of the marsh system to remove N in the long term and not, for instance, contribute to further nitrate loss through the creation of plant-available nutrient forms.

6.5 Denitrification: The Process and Modifying Factors

Denitrification is a facultative anaerobic microbial respiration process in which nitrate (NO_3^-) or nitrite (NO_2^-) is reduced to nitric oxide (NO), nitrous oxide (N_2O) or nitrogen gas (N_2) in a stepwise manner (Zumft 1997; Wallenstein et al. 2006) (**Figure 3a**). Electrons taken from donors such as organic C flow through an 'electron transport chain' and are used to generate a proton gradient

across a membrane for ATP synthesis (Aldossari and Ishii 2021). Both fungal and bacterial denitrifiers occur, with some important differences in process and their relative importance in different systems (Aldossari and Ishii 2021). For instance, the enzyme 'nitric oxide reductase' (Nor) is structurally different between these organisms and unlike the bacterial system, fungal Nor is not directly associated with the membrane-bound electron transport chain – fungi receive electrons directly from NADH. This led Aldossari and Ishii (2021) to suggest that fungi receive benefits under anoxic conditions, as would be typical in marsh systems at different elevations and tidal states.

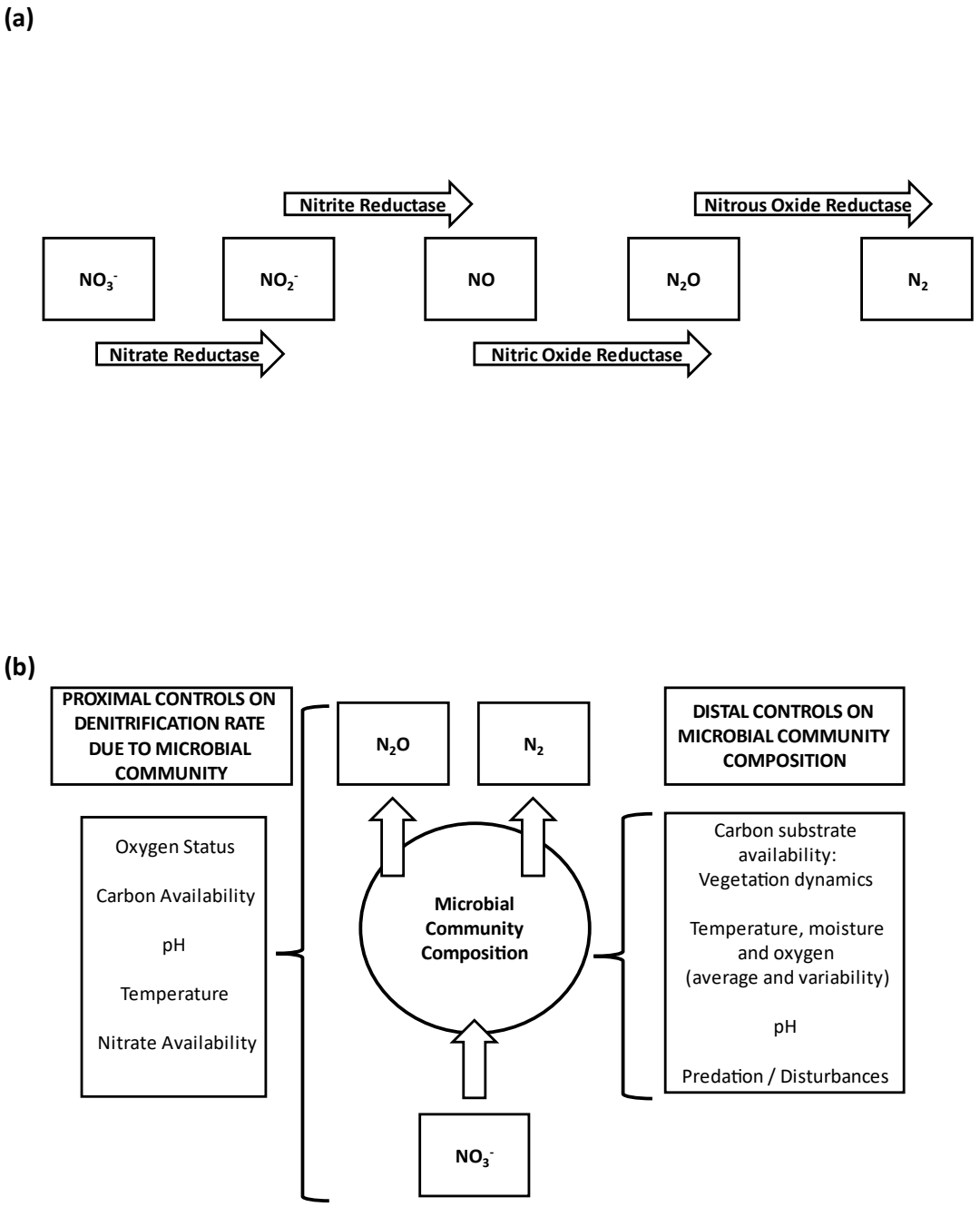

Figure 3: (a) Stepwise biochemical reactions involved in denitrification (after Choudhary, Muduli, and Ray 2022) and **(b) long-term 'distal' factors influencing denitrifier microbial community composition and short-term 'proximal' environmental influences on the instantaneous rate of denitrification** (after Wallenstein et al. 2006). Note that in some environmental situations denitrification can be incomplete leading to the release of the potent greenhouse gas nitrous oxide (N_2O).

Importantly, the enzyme 'nitrous oxide reductase' (Nos) can be lacking in some denitrifiers, leading to the release of N_2O rather than N_2. This has important implications for climate change mitigation as N_2O is a potent greenhouse gas with a global warming potential that is 265 to 298 times as high as that of one molecule of carbon dioxide (CO_2) (Makowski 2019), with a global trajectory of increasing atmospheric concentrations (Tian et al. 2020). Furthermore, N_2O can deplete the stratospheric ozone layer, while, where NO (nitric oxide) is released, formation of smog (and ground level ozone) can be encouraged (Almaraz, Wong, and Yang 2020). However, NO is typically considered a minor end-product of denitrification (Almaraz, Wong, and Yang 2020) although this would need confirming in saltmarsh systems. We can confidently state that the ratio (yield) of N_2:N_2O (calculated as: N_2 / (N_2 + N_2O)) is an important indicator of the extent to which 'full' denitrification has occurred. Furthermore, when combined with absolute amounts of N_2O released, it can be an important indicator of undesired consequences.

At the global scale, a recent meta-analysis indicates that denitrification rates tend to increase with temperature, precipitation, soil C and N contents and with microbial biomass C and N. However, it tended to decrease with increasing clay contents (Li et al. 2022). Li et al. (2022) also found that variables related to soil N content explained a greater amount of variation in denitrification rates as compared to climatic and edaphic variables such as soil pH. Interestingly, only two years earlier, Almaraz et al. (2020) suggested that available data were not suitable for a meta-analysis and instead highlighted biases in available data; Li et al.'s (2022) article used various assumptions to 'correct' experimental results, for instance through assuming an average Q_{10}. They also only considered papers that had used acetylene inhibition methods of estimation, with studies from the UK solely encompassing forest and grassland (Li et al. 2022).

At the UK-scale, it is instructive to consider what may modify denitrification rates and how these may scale up to nutrient removal. According to Wallenstein et al. (2006) (**Figure 3b**) there are immediate, proximal controls on denitrification rates, such as nitrate availability, oxygen (and thus redox conditions) and pH (see refs cited in Wallenstein et al. 2006). However, the response of denitrification rates to such changing resources and conditions will depend on the denitrifier community itself and how its metabolism is affected by them. The structure of the denitrifier community composition depends on more distant controls (termed 'distal' by Wallenstein et al. 2006), some of which feature in the proximal list but also others. For instance, these distal controls can include vegetation composition and function and its influence on carbon substrate availability, and other biotic interactions such as predation, conditions such as temperature, moisture and oxygen where average and variability is important, pH, and disturbances (Wallenstein et al. 2006).

The fact that these drivers vary over space and time, especially in saltmarshes where redox conditions change frequently (see also Bowen et al. 2023), leads to high variability in denitrification rates. There have also been discoveries of 'hotspots' of denitrification, even within single cores, where small areas account for a very large percentage of areal denitrification (Groffman et al. 2006). Such hotspots may be particularly prevalent in aquatic systems with ephemeral patches of biofilms, periphyton and decomposing leaves and stems (Groffman et al. 2006), which may characterise some saltmarshes, mudflats, and seagrass beds. Furthermore, such variation can create difficulty in incorporating denitrification into models (Groffman et al. 2009).

6.6 Denitrification: A Review of Methods

Given the potential importance of denitrification (and incomplete denitrification) to N pollution remediation and to global change, substantial efforts have been exerted to measure it (Groffman et al. 2006; Almaraz, Wong, and Yang 2020). However, since the end-member process of denitrification is N_2 gas, it can be a very difficult (and expensive) process to measure *in situ* since background concentrations of N_2 are so high in the atmosphere (Groffman et al. 2006). Indeed, Groffman et al. (2006) went so far as to state: "*It is a miserable process to measure*" and that despite progress, "*Concerns about methodological misery have grown*" (Groffman et al. 2006), especially when considering denitrification's high spatial and temporal variation (as explored above).

The most common method (at least for upland systems: Almaraz et al. 2020) is acetylene inhibition, where acetylene (C_2H_2) is injected or added to the headspace of a sealed soil/sediment core and N_2O accumulation is measured over time – as the addition of acetylene inhibits the final step of the denitrification process i.e. from N_2O to N_2. It also inhibits the continued supply of nitrate from ammonium oxidation and thus concerns were raised that it underestimates denitrification rates, especially where there are small and dynamic pools of nitrate, as characterises sediment (Groffman et al. 2006). This underestimation could be a concern for saltmarsh systems, especially when comparing different zones, if one zone had much smaller nitrate reserve than another. If acetylene inhibition is used at the same time as sediment is disturbed and a large supply of organic matter and/or nitrate is maintained (for instance through a slurry method), then overestimation of actual denitrification rates in the sediment may occur instead, as more, readily available, substrate is provided to the microbial population than would occur *in situ* (Almaraz, Wong, and Yang 2020). On the other hand, this method may be particularly applicable for large scale surveys and/or experimental treatments when trying to rapidly assess multiple samples and understand the relative importance of denitrification in different areas (Almaraz, Wong, and Yang 2020). However, if trying to characterise denitrification's contribution to NO, N_2O and/or N_2, and thus implications for climate change and/or ozone formation, Groffman et al. (2006) argue that physical problems and the general alteration of substrate and product flow by acetylene (C_2H_2) can lead investigations astray.

Another option for understanding denitrification is through ^{15}N tracer methods. This can include various methodological approaches, including isotope fractionation, isotope dilution, ^{15}N mass balances, and direct measurement of labelled gases upon addition of $^{15}NO_3^-$ and $^{15}NH_4^+$ (Groffman et al. 2006) (see **Table 1**). However, using these methods at scale can be limited due to expensive instrumentation, while addition of labelled gases may stimulate process rates (Almaraz, Wong, and Yang 2020). Some authors argue that approaches such as the ^{15}N gas flux method offer substantial promise for a more accurate quantification of *in situ* denitrification (Micucci et al. 2023); indeed Groffman et al. (2006) point out that "*…there is little doubt that it can be used to generate reliable estimates of denitrification rates within the sampled unit*". However, Groffman et al. (2006) state that this reliability may be compromised for aquatic systems, such as saltmarsh: "*Aquatic systems with a complex matrix of macrophytes, microphytes, and solid substrates might be an overwhelming challenge for ^{15}N tracer methods. In addition to the sediment, multiple more or less ephemeral hotspots of nitrification and denitrification could be present in biofilms, periphyton, and decomposing pieces of leaves and stems in these systems. Here the standard concentration test may indicate that the isotope pairing method is not applicable as the assumptions of homogeneous isotope mixing and linearity between denitrification rates and bulk water nitrate concentration will be far from valid. In these systems typical of many streams, wetlands and littoral zones, the C_2H_2 inhibition technique or other methods may be superior.*" Further, as emphasized by Almaraz et al. (2020) for upland soils, labelled substrate pool dilution techniques are not suitable for flooded soils because of restricted gas exchange (see **Table 1**).

Direct measurements of N_2 gas also provide a means of estimating denitrification rates, for instance through helium gas flow incubation (**Table 1**). For this method, either mixed or intact core samples are incubated under conditions of reduced atmospheric N_2 to allow N_2O and N_2 production from denitrification to be estimated – crucially without the addition of labelled substrates or inhibitors (Groffman et al. 2006). However, enclosure effects can be an issue (regardless of approach), and require the maintenance of oxygen, ammonium, and nitrate to be near *in situ* conditions to produce accurate estimates of *in situ* denitrification rates (Groffman et al. 2006). The requirement for gas tightness and the need to remove any N_2 stored in aggregates or pores of the soil prior to measurement make this difficult to deploy at scale, although the method itself is simple. This method may be useful for parameterisation experiments when trying to understand how variation in particular drivers (e.g. temperature) alters denitrification rates (Groffman et al. 2006).

Denitrification Estimation Method	Strengths	Weaknesses	Recommended Applications	Caution in Data Interpretation
Acetylene Inhibition: N_2 production estimated as the difference between N_2O production in the absence and presence of acetylene.	Targets N_2 production from denitrification. High throughput so high capacity for samples. Broadly accessible: gas chromatograph, low cost and easy to learn.	Can estimate negative N_2 production rates due to soil heterogeneity between control and acetylene treated samples. Limited *in situ* capability.	Comparisons of instantaneous fluxes among sites or experimental treatments.	Measured N_2 production rates likely underestimates due to acetylene inhibition of nitrification and incomplete inhibition of N_2O reduction. Differences in soil moisture or texture affect acetylene diffusion leading to variability.
Direct Measurement: Helium gas flow incubation systems. Measures N_2O and N_2 production from intact soil cores incubated under an N_2-free headspace.	Direct measurement of N_2 and N_2O production from same core allowing accurate estimation of denitrification and N_2O yield. No substrate or inhibitor addition.	Low throughput and custom instrumentation. No *in situ* capability.	Comparisons of instantaneous fluxes among sites or experimental treatments.	N_2 and N_2O production cannot be attributed solely to denitrification because source partitioning not possible e.g. anammox could contribute to N_2 production. Measured rates may overestimate N_2O relative to N_2 production due to high surface area exposure to an aerobic headspace.
^{15}N-NO_3 tracer: Measures $^{15}N_2O$ and $^{15}N_2$ production rates by tracing ^{15}N label from soil NO_3^- pool into the N_2O and N_2 pools.	Targets N_2O and N_2 production from denitrification.	Low throughput and high cost of label. Requires isotope mass spectrometer and limited *in situ* capability due to requirement of homogeneous ^{15}N labelling.	Experiments in N-rich environments such as fertilized agricultural fields.	^{15}N addition may stimulate process rates leading to measured rates being overestimated. This may be more of an issue in environments with low background nitrate availability. Inhomogeneity in distribution of label may lead to bias in rate estimation.
^{15}N-N_2O pool dilution: estimates gross N_2O emission	Can be used for *in situ* measurements in the field; targets	Low throughput and high cost of label/gas. Requires an isotope ratio	Field measurements using surface flux chambers to obtain	Estimated gross N_2O uptake rates cannot be equated with N_2 production because of

and uptake rates from the isotopic dilution and disappearance of added ^{15}N-N_2O respectively.	N_2O reduction to N_2 by denitrification.	mass spectrometer interfaced with a trace gas preconcentration unit for sample analysis.	*in situ* estimates. But needs to be in soils which are not flooded to facilitate gas exchange between the chamber headspace and soil pores (e.g. ^{15}N-N_2O diffusion into the soil).	unknown N_2 production in isolated soil microsites. In the field, an unknown depth of the soil profile is probed by this method.
N_2:Ar: Estimates N_2 production rates from changes in the N_2:Ar ratio in the headspace of a surface flux chamber or from soil depth profiles of N_2:Ar ratios.	Can be used to measure *in situ* rates; does not require addition of substrates or inhibitors.	Does not target N_2 production from denitrification. Has a high detection limit and requires a dual inlet isotope ratio mass spectrometer and vacuum line for high precision analysis.	Not recommended (for upland soils) due to high detection limit.	Better to use N_2:Ar ratio as mass spectrometric measurements more precise for gas ratios. Measure net denitrification as balance between gross N denitrification and gross N fixation (Groffman et al. 2006).
Clumped isotopes of N_2: Estimates N_2 production rates based on soil depth profiles of Δ_{30} values representing the proportional deviation in $^{15}N^{15}N$ abundance from a random distribution of ^{14}N and ^{15}N isotopes in N_2.	Can be used to measure *in situ* rates. Does not require addition of substrates or inhibitors.	Does not target N_2 production from denitrification. Requires costly ultra-high resolution isotope ratio mass spectrometer for clumped isotope analyses.	Field measurements using soil depth profiles to obtain *in situ* estimates.	Estimated N_2 production rates depend on the assumptions used to estimate rates from soil depth profiles of Δ_{30}. This new method has not yet been evaluated across soil and ecosystem types so potential biases and artefacts are not fully understood.

Table 1: Comparison of empirical denitrification rate estimation methods with cell inputs based on Almaraz et al. (2020) except where otherwise stated.

7 Denitrification: A Pilot Study

7.1 Aim, Site Description and Survey Approach

To compare among potential laboratory handling methods and inform any potential nationwide denitrification sampling campaign, we trialled the collection and analysis of saltmarsh and adjacent coastal habitat denitrification samples. We also characterised vegetation for future analyses of potential driving variables of denitrification and to inform future survey time estimates.

Specifically, we surveyed vegetation and collected sediment/soil samples in the vicinity of Chichester Harbour Site of Special Scientific Interest (SSSI), in October 2023 (autumn sampling) and in January 2024 (winter sampling). Chichester Harbour is a semi-enclosed branching symmetrical tidal inlet with four channels (Chichester, Emsworth, Thorney, Bosham) and a connecting channel in the North to Langstone Harbour. The bedrock geology is mostly sedimentary and formed of clay, silt and sand from the Palaeogene period (London clay formation and Lambeth clay formation). There are superficial tidal deposits of clay, silt, sand, and gravel. The River Lavant, River Ems and various other small, low-flow streams provide some freshwater to the harbour (Campos, Teixeira Alves, and Walker 2020). The harbour has a tidal range of 4.2 m and covers 29.5 km^2 (Dohmen-Janssen and Hulscher 2007) approximately 79% of which is intertidal, including 91 ha of intertidal seagrass, 307 ha of saltmarsh and 2008 ha of mudflat (extents estimated from the Magic Map tool: https://magic.defra.gov.uk/MagicMap.aspx; saltmarsh extent from 2016 Environment Agency report of Chichester Harbour Waterbody (Environment Agency 2022.)).

We specifically focussed our sampling on the large (22 ha) eastern area of saltmarsh at the tip of Thorney Island, and its adjacent 55 ha area of mudflat (**Figure 4**). The marsh is situated on the southeast side of the island behind the Pilsey gravel barrier. Thorney Island is owned by the Ministry of Defence and has a very small population as it is mostly used for military activity. The marsh sits approximately 4 km downstream from both Thornham sewage treatment plant, which has a consented dry weather flow of 6565 m^3 day^{-1} and Bosham sewage treatment works which has a consented dry weather flow of 1221 m^3 day^{-1} (Campos, Teixeira Alves, and Walker 2020). To sample a sufficient area of seagrass, we sampled across the channel from Thorney Island, to the west of Itchenor (**Figure 4**). West Itchenor also has marsh areas (approximately 27 ha) and is split down the middle by a creek which winds its way from Bosham channel to the foreshore, through 3.7 ha of *Zostera noltei* (Dwarf eelgrass) beds. The adjacent mudflat is approximately 60 ha including the channels, with varying composition of sand, silt and clay. The area is approximately 2 km from Bosham sewage treatment works (Campos, Teixeira Alves, and Walker 2020). Our sampling was close to oyster beds/reefs that were also assessed for potential denitrification rates, in the complementary Environment Agency project (Fabra et al. 2024), where denitrification rates from *Crassostrea gigas* high density reef, *C. gigas* low density reef, and mixed sediment with *C. gigas* and *Ostrea edulis* present but not forming reef were compared with rates from control mudflats).

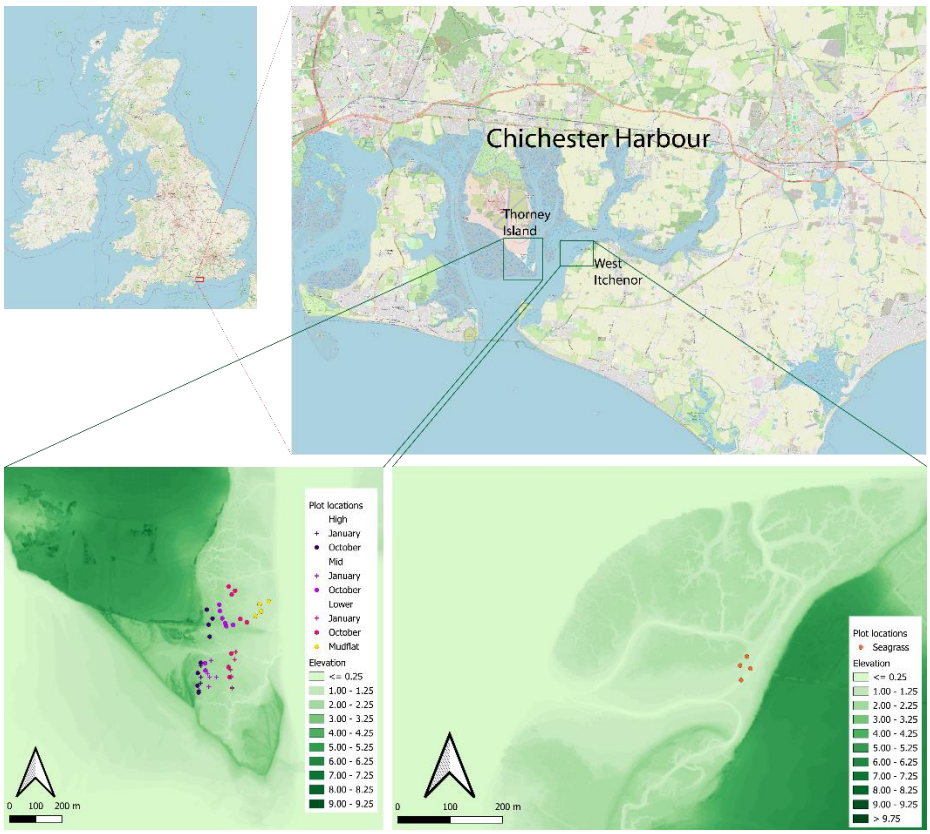

Figure 4: Sampled saltmarsh, dwarf eelgrass (Zostera noltii) and mudflat locations in Chichester Harbour, The Solent, United Kingdom. Saltmarsh sites were sampled in autumn 2023 and winter 2024, as noted in the legend. Logistical constraints restricted seagrass and mudflat sampling to autumn 2023 only.

For the autumn sampling, we located, at random and with an approximate minimum distance of 20 m between them, eight 1 x 1 m quadrats in each of the high, mid, and low marsh zones. We avoided creek lines and pools, and locations were influenced by ease of access and exit e.g. in case of needing to exit the marsh in an emergency; they were not chosen prior to arrival at the marsh. Due to resource and tidal time constraints, we located four 1 x 1 m quadrats on a mudflat without algal cover, and a further four 50 x 50 cm quadrats on the seagrass bed. Quadrats were smaller for the seagrass sampling because they were complementing an existing project being undertaken by Portsmouth University. There is no impact of this decision on the comparison of denitrification rates among habitats due to the same core/slurry sampling approach being adopted regardless of habitat (see below).

For the winter sampling and using five 1 x 1 m quadrats in each of the high, mid and low marsh zones, we sampled the saltmarsh. Resource and laboratory constraints prevented sampling at the mudflat and seagrass beds and reflected the initial request to only provide limited mudflat/seagrass sampling. Although quadrats continued to be placed at random across the zones and within the same areas as the autumn sampling, we stratified our sampling to only include similar vegetation within each zone. For instance, rather than sampling in low marsh in the presence of *Salicornia*, and then elsewhere in the low marsh in the presence of *Spartina*, we focussed on *Spartina* stands alone. This was because initial analysis of the autumn data showed high variation in denitrification rates and we wished to understand whether part of this variation may relate to vegetation composition. By restricting the winter sampling to similar vegetation, we attempted to control for this, notwithstanding that this would need further testing with a more appropriate design to consider whether variation may also relate to season of sampling. We did not sample in precisely the same location as autumn due to the potential for legacy effects of any initial disturbance.

For sampling in autumn and winter at each of these quadrat locations (or adjacent, in the case of seagrass sediment samples) we:

- Recorded the date and time of sampling, and the duration of sampling;
- Photographed the quadrat;
- Recorded its location according to the Ordnance Survey UK grid reference system (10 km square reference, and location to 10 digits);
- Gathered sediment samples (see methods below) to assay potential denitrification via different methods in the laboratory;
- Characterised the vegetation;
- Gathered porewater samples (in all quadrats in the mudflat and seagrass; only in half of the saltmarsh samples i.e. 4 of 8 quadrats per zone in autumn, 4 of 5 quadrats per zone in winter)

The latter two elements were collected to examine purported drivers of denitrification (along with seasonality) in due course, and aid parameterisation of the CEFAS CPM. We present collection and analysis methods and provided data to the EA and CEFAS, provide porewater results in an Annex but do not discuss them further herein due to the limited capacity to link them to the denitrification results. For the autumn sampling, we surveyed/sampled quadrats in the high marsh on 17th October 2023, mid marsh on 17th and 18th October 2023 and the mudflat and low marsh on 18th October 2023. The seagrass bed was surveyed/sampled on 19th October 2023. For the winter sampling, we surveyed/sampled the high and mid saltmarsh on 30th January 2024, and the low marsh on 31st January 2024. On each day of sampling, we also collected a 1-litre sample of tidal water on the flood and ebb tides – again to assist in parameterisation of the CEFAS CPM model as well as help characterise seawater nutrient profiles.

7.2 Sediment Sampling

Two methods of sediment sampling were employed, so that potential denitrification could be assessed through (i) the tidal core method (undisturbed cores) (results in mg N_2O-N m^{-2} hr^{-1}) and (ii) through the slurry method (disturbed samples) (results in ng N_2O-N g^{-1} of sediment hr^{-1}).
We extracted two undisturbed circular cores (depth 22 cm, diameter 68 mm) at two points within the quadrat (adjacent in the case of seagrass), and within 1 m of each other. Where vegetation was present, cores were paired such that the vegetation composition and biomass (approximately, and by eye) were identical at the surface of each core. Cores were chamfered and made of black plastic. They were hammered into the sediment where necessary, and 2 cm was left exposed i.e. sediment depth was 20 cm. In a few instances, there was a loss of sediment – this was noted for subsequent analyses. After extraction, cores were uniquely labelled and capped with black plastic at either end. In total, 8 pairs of cores were extracted per saltmarsh zone in autumn (i.e. 48 cores across the marsh as a whole), and five pairs per saltmarsh zone in winter (30 cores across the marsh in total). Four pairs of cores were extracted from each of the mudflat (8 cores in total) and seagrass bed (8 cores in total).

From the sides of one of the cores, we collected grab samples for assessment through the slurry method. These samples were collected at approximately 5, 10 and 15 cm depth, through collection from 2 – 7, 8 – 12 and 13 – 17 cm respectively. Where possible, sediment was formed into a satsuma-sized ball in a uniquely labelled plastic bag. The ‘core’ of the ‘satsuma ball’, assumed to provide the best protection for the microbial community, was used for the slurry method (detailed below).

7.3 Vegetation Characterisation

Prior to core sampling, we characterised the vegetation in the 1 x 1 m quadrat by recording percentage cover to genus level. At least two surveyors per quadrat agreed on cover values. The height of the vegetation can also influence biomass, which may influence subsequent denitrification in conjunction with plant species effects on the microbial community. For all genera with a cover greater than or equal to 15%, we recorded the uppermost vegetated height (to the nearest cm) of 6 randomly selected stems. Inflorescence height was not recorded. Stem height,

when combined with cover, may be informative for determining aboveground biomass without the need for destructive sampling. However, to generate these relationships initially, destructive sampling would be required. At the time of the sampling, we did not have permission for such destructive sampling; this may need to be a priority in future research.

As noted above, the limited sampling prevents a robust analysis of relationships between vegetation and denitrification rates. In addition, the main aim of this section of the report was to conduct a trial of denitrification methods, so we do not present vegetation results in detail. In the interests of understanding context, our surveys showed low marsh samples were characterised by varying covers of *Salicornia* and *Spartina* sp. together with bare sediment sometimes covered with algae, mid marsh samples by *Armeria maritima, Limonium* spp., *Puccinellia* spp. and occasional *Salicornia*, and high marsh samples by *Armeria*, *Inula crithmoides*, and *Atriplex portulacoides*, together with Cyperaceae genera, possibly *Bolboschoenus* or *Carex/Scirpus* species plus occasional *Elymus* and *Festuca* species. As noted above, we stratified our random sampling in January 2024 by restricting sampling in the low marsh zone to *Spartina* stands (with varying amounts of bare ground and algal cover), in the mid marsh zone to *Armeria-Puccinellia-Limonium* communities, and in the high marsh zone to sites with *Cyperaceae* and *Poaceae*.

7.4 Porewater Sampling

In all seagrass and mudflat quadrats, and in four of the eight/five saltmarsh quadrats per zone in autumn/winter respectively, porewater samples were extracted from the paired core that had not been used for grab samples. For the autumn sampling, porewater extractions were taken from alternate quadrats except where tidal pressure to exit the marsh meant an earlier quadrat needed to be chosen. One 5 cm length rhizon, attached to a 50 ml syringe, was inserted at each of 5, 10 and 15 cm depths. A vacuum was created to draw the pore water from the sediment. Syringes were left attached until sufficient sample had been obtained or 30 minutes had passed (whichever was earlier), and then porewater in the syringe was placed into uniquely-labelled Falcon tubes. As explained above, these samples were gathered on request to assist the parameterisation of the CEFAS CPM model and may subsequently prove useful, when combined with additional results from other marshes, for understanding potential drivers of variation in denitrification rates. At this time, the number of samples precludes robust analysis to inform such understanding, so detailed consideration of results is not given herein, but Annex 1: Porewater Results provides a graph of results with brief summary.

7.5 Sample Storage

Sediment, porewater and seawater samples were placed in cool boxes as soon as practically possible after sampling and kept cool (< 5°C) prior to transfer for analysis at Bangor University.

7.6 Laboratory Protocols

7.6.1 The Slurry Method

Each sample was homogenised for 30 seconds, and any large roots and invertebrates were removed. Two subsamples of 10 g were weighed from each sample and placed into separate 50 mL plastic centrifuge tubes. One to undergo acetylene inhibition, and one for measurement of the control nitrous oxide (N_2O) flux.

Ten mL of artificial seawater was added to the centrifuge tube and mixed to create a 1:1 slurry. The tube was then capped with a SubaSeal-fitted lid and then flushed with oxygen-free N_2 for 10 minutes. Five mL of acetylene was then added to create a 0.1 N acetylene atmosphere and the sample was then mixed on a vortexer for 20 seconds (Figure 5). Control samples received no acetylene. The sample was then placed on an orbital shaker set at 100 rpm for a set incubation time. After

incubation, a 10 mL gas sample was taken from the centrifuge tube and transferred to a 5 mL gas-tight glass container.

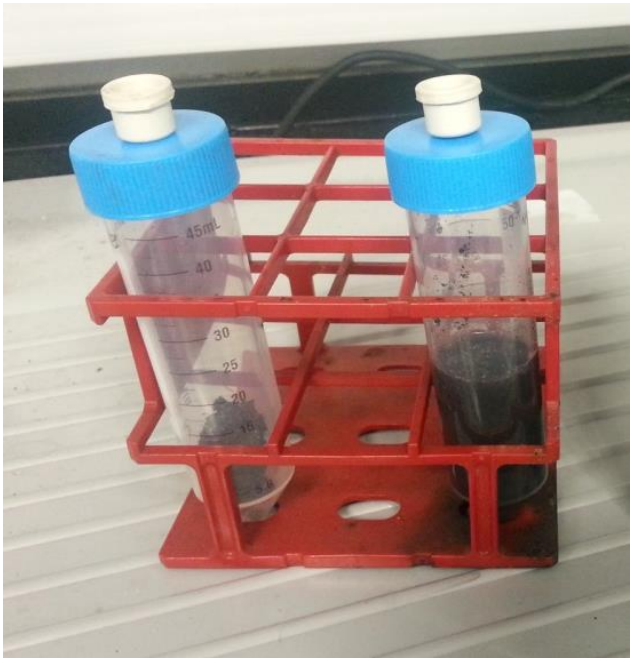

Figure 5 Set-up for acetylene blocking technique to analyse denitrification rates from sediment slurry samples.

Incubation time was decided by conducting a linearity test on a random sample from each site. Gas production was measured at one-, two-, three- and five-hour time periods for the selected sample. As noted above, denitrification rates are given in ng N_2O-N g^{-1} sediment $h^{-1,}$ which is not easily converted for use in the CEFAS CPM model.

7.6.2 The Core Method

Denitrification rates from the whole core samples were analysed using the custom-built Wetland Hydroperiod Simulator (WHS; Figure 6). The WHS consists of a chamber linked to a water reservoir via a system of pipework. The water reservoir is fitted to a raising platform which can alter the level of water in the cores. This level is controlled by the WHS, using a Raspberry Pi, coded to simulate the water level changes of a 24-hour neap tide.

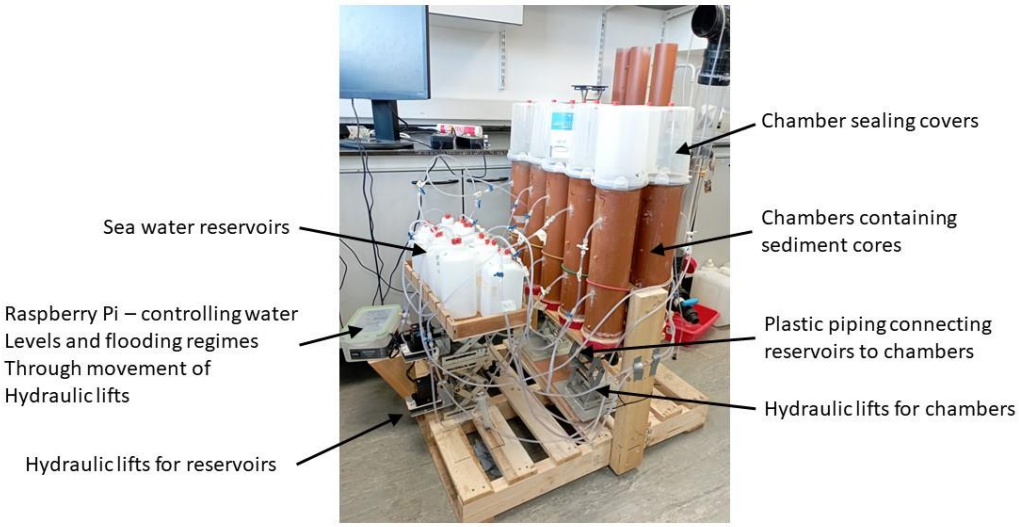

Figure 6 The components of Bangor University's Wetland Hydroperiod Simulator (WHS).

To calculate denitrification for one sample, two cores were taken, one to undergo acetylene inhibition and one to act as a control. Cores were placed in the chambers with a set volume of water, calculated based on the depth of cores and tidal depth required. Chambers were sealed and acetylene chamber atmospheres were then altered to a 0.1 N atmosphere of acetylene. After the chambers were connected to the reservoirs, via the pipework, the water reservoirs for the acetylene chambers were also spiked to a 0.1 N acetylene atmosphere. Once all chambers were connected, a 10 mL gas sample was taken and transferred to a 5 mL gas-tight glass container, this was referred to as a Time Zero sample (T0). The WHS then ran a full 24-hour tidal cycle. At the end of this cycle, a second gas sample was taken (T24).

Denitrification rates from the core method are presented in this report in mg N_2O-N m^{-2} hr^{-1}, though can be converted to other units for the CEFAS CPM model, or other relevant models.

7.6.3 Gas Analysis

Gas samples, collected from both the slurry and core methods, were analysed by gas chromatography using a Varian model 450 gas chromatograph (GC) instrument, equipped with an electron capture detector (ECD) for N_2O. Two mL of gas from the gas-tight glass containers (Exetainers®) containing the samples was injected via a 1041 on-column injector system, set at 40 ºC, onto a PoroPak QS (1.83 m x 3.18 mm) 80/100 column. The septum of this system was changed after approximately 500 injections. The column oven temperature was set to 40 ºC and the carrier gas, oxygen-free nitrogen, had a flow rate of 30 mL min^{-1}. The temperature of the ECD was 340 ºC with a constant flow of 20 mL min^{-1} of oxygen-free nitrogen. Injection of the samples was achieved with a Combi PAL headspace auto-sampler (CTC Analytics, Zwingen, Switzerland) equipped with a 5 mL syringe and specially constructed trays for holding 50 individual 5.9 mL Exetainers®. N_2O (retention time 3.26 minutes) was quantified by comparison of peak area with that of three standards of known concentration (0.3, 1.5 and 5 ppm), prepared by BOC (an industrial gases company) and used in the preparation of a standard curve, which - according to standard laboratory protocol - was only accepted if the correlation coefficient (R^2) value was greater than 0.98; indicating the strongest relationship between the variables.

7.7 Denitrification Rate Calculation

We used the following set of equations to calculate denitrification rate:

$$DR\ (mg\ m^{-2} s^{-1}) = \frac{TNP}{\delta t} \times (\frac{V \times M}{S \times V_{mol}}) \quad \textbf{Equation [1]}$$

where:

- *DR*: Denitrification rate (mg m^{-2} s^{-1})
- *TNP*: Total N_2O produced (mg)
- *δt*: Change in time between first and second measurement (seconds (s))
- *V*: Volume of headspace in chamber (m^3)
- *M*: Molecular weight of gas (mol)
- *S*: Area of core (m^2)
- V_{mol}: Volume of a mol of gas at a given temperature (m^3 mol^{-1})

where V_{mol} is given by:

$$V_{mol} = p \times (R \times K) \quad \textbf{Equation [2]}$$

- *p*: Pressure (kPa)
- *R*: Equal to ideal gas constant (8.314)
- *K*: Temperature (Kelvin)

where Total N_2O produced (*TNP*) is given by:

$$TNP = (Ksp \times m \times W{:}Hs) + m \quad \textbf{Equation [3]}$$

- *Ksp*: N_2O solubility
- *m*: Mass of N_2O in headspace (mg)
- *W:Hs*: Ratio of water to headspace

and, where mass of N_2O in headspace (*m*) is given by:

$$m = Hs \times N_A \times (A\delta C - C\delta C) \quad \textbf{Equation [4]}$$

- *m*: Mass of N_2O in headspace (mg)
- *Hs*: Headspace volume (ml)
- N_A: Avogradro constant (6.022×10^{23} mol^{-1})
- *AδC*: Change in the gas concentration in acetylene samples: T0-T24
- *CδC*: Change in the gas concentration in control samples: T0-T24

7.8 Sea- and porewater analyses

Seawater and porewater samples were analysed using colorimetric based methods. Nitrate (NO_3^-) was measured using a Vanadium reduction followed by a Griess reaction. Ammonium (NH_4^+) was measured using a buffered indophenol method. Phosphate (PO_4^{3-}) was measured using the molybdenum blue method. Detailed analyses were not conducted on these samples as this was not a key aim for the current work, instead these data were used to provide context to the focal denitrification study and foundations for future work.

7.9 Denitrification Results

Analysis of the complete cores in the tidal chambers (**Table 2**; **Figure 7**), created by the Wetland Hydroperiod Simulator (WHS), showed that in autumn (October) 2023 the saltmarsh cores from high marsh and low marsh had similar mean average denitrification rates: mean (*M*) = 0.19 mg N_2O-N m^{-2} h^{-1}, standard error (*SE*) = 0.11, and *M* = 0.15 mg N_2O-N m^{-2} h^{-1}, *SE* = 0.09, respectively. The denitrification rates from the mid marsh cores were around a quarter those of the high and low marsh sites (*M* = 0.04 mg N_2O-N m^{-2} h^{-1}, *SE* = 0.02), though due to the large variation in high and low marsh results, following analysis of the means of the groups using ANOVA (Analysis of Variance) this was not classed as statistically significant. Both the mudflat and seagrass cores had lower denitrification rates than saltmarsh zones (*M* = 0.02 mg N_2O-N m^{-2} h^{-1}, *SE* = 0.01, and *M* = <0.01 mg N_2O-N m^{-2} h^{-1}, *SE* = 0.01, respectively), with seagrass having negligible denitrification.

For the cores collected in winter (January) 2024, the mid marsh had a similar mean average denitrification rate to that from autumn sampling (*M* = 0.05 mg N_2O-N m^{-2} h^{-1}, *SE* 0.03). Both the high and low marshes reported negative denitrification rates in winter (*M* = -0.01 mg N_2O-N m^{-2} h^{-1}, *SE* = <0.01; *M* = -0.01 mg N_2O-N m^{-2} h^{-1}, *SE* < 0.01, respectively). This is due to the control flux having a higher N_2O flux than the acetylene flux. As previously mentioned in the method review, acetylene also partially inhibits nitrification, the conversion of ammonium to nitrate. Inhibition of this step by the acetylene could have prevented the pool of ammonium being converted to nitrite preventing denitrification from continuing once the available nitrite had been exhausted. High and low marsh also had significantly lower standard errors, attributed to improved sample collection as well as small mean values.

Habitat	Season	Number of locations (paired cores)	Mean denitrification rate (mg N_2O-N m^{-2} h^{-1})	Standard error (mg N_2O-N m^{-2} h^{-1})
Saltmarsh – high (upper)	Autumn	8	0.19	0.11
Saltmarsh – mid	Autumn	8	0.04	0.02
Saltmarsh – low (pioneer)	Autumn	8	0.15	0.09
Seagrass	Autumn	4	0.01	< 0.01
Mudflat	Autumn	4	0.02	0.01
Saltmarsh – high (upper)	Winter	5	-0.01	< 0.01
Saltmarsh – mid	Winter	5	0.05	0.03
Saltmarsh – low (pioneer)	Winter	5	-0.01	< 0.01

Table 2: **Potential denitrification rates** in Chichester Harbour SSSI as estimated from intact cores within the Wetland Hydroperiod Simulator.

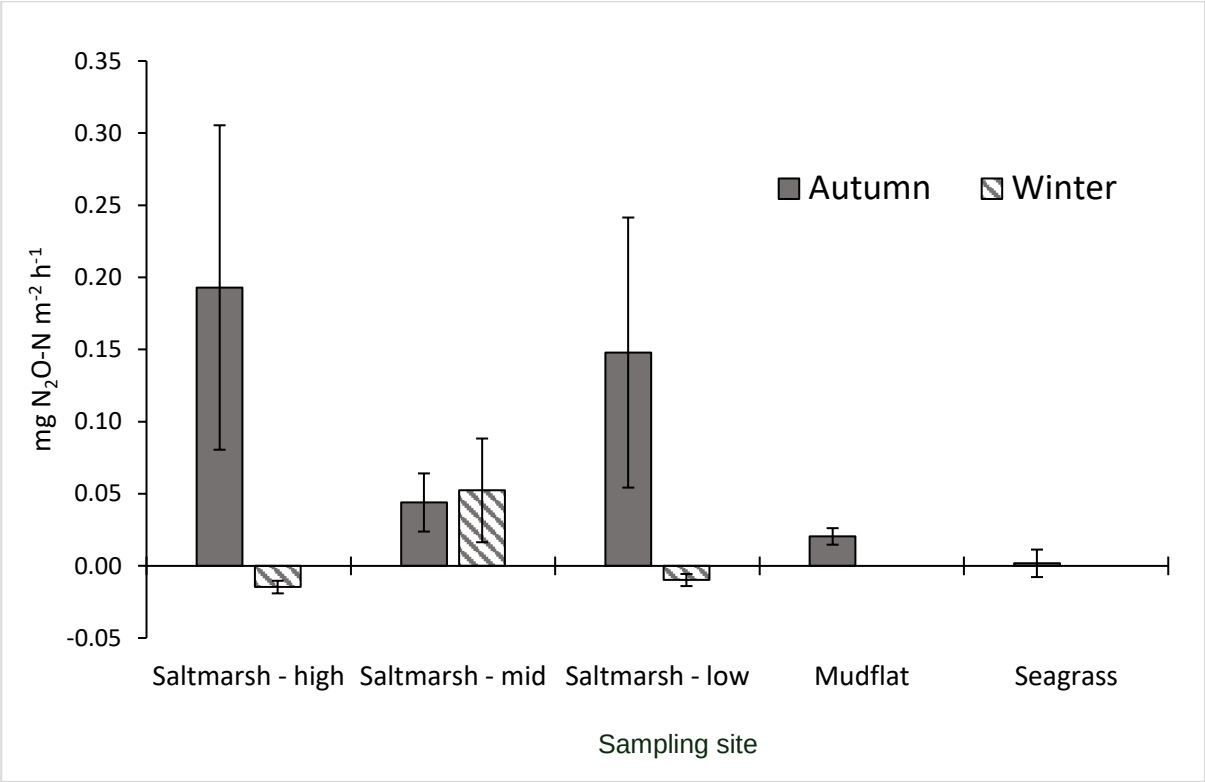

Figure 7: Denitrification from coastal-substrate cores. Mean averages of denitrification rates (mg N_2O-N m^{-2} h^{-1}) from cores collected in autumn (October) 2023 and winter (January) 2024 from different substrate types; analysed using an acetylene blocking technique in tidal chambers. Autumn cores: saltmarsh, n = 8; mudflat, n = 4; and seagrass, n = 4. Winter cores: saltmarsh, n = 5; mudflat and seagrass, n = 0.

Mean average denitrification rates (ng N_2O-N g^{-1} h^{-1}) from the 1:1 substrate-water slurries showed that — irrespective of season, or location — there was a general decline in denitrification rates with depth, apart from winter samples from the low marsh (**Figure 8**). Neither the mudflat nor the seagrass samples showed a similar decline in denitrification rates with depth.

The highest mean average denitrification rate was at a depth of 5 cm from the mid marsh in autumn (*M* = 58.19 ng N_2O-N g^{-1} h^{-1}, *SE* = 15.0). The lowest mean rate was from a depth of 10 cm from the mid marsh slurries in winter (*M* = 2.38 ng N_2O-N g^{-1} h^{-1}, *SE* = 1.13) – an order of magnitude lower.

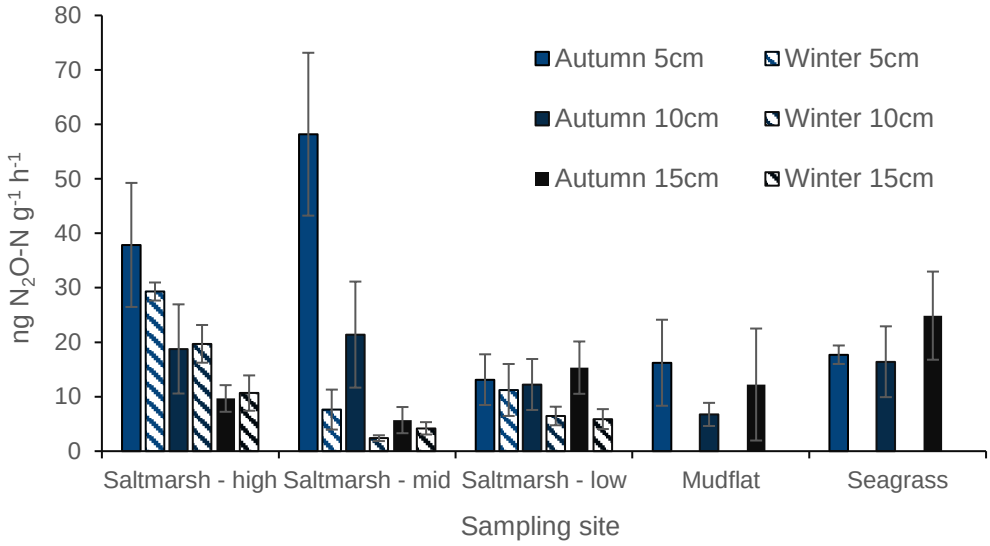

Figure 8: Denitrification from coastal-substrate slurries. Mean averages of denitrification rates (ng N_2O-N g^{-1} h^{-1}) from differing substrates and collected from various depths, made into a 1:1 aqueous slurry, and analysed using an acetylene blocking technique. Samples collected in autumn (October) 2023 and winter (January) 2024. Autumn cores: saltmarsh, n = 8; mudflat and seagrass, n = 4. Winter cores: saltmarsh, n = 5; mudflat and seagrass, n = 0.

Seawater and porewater ion concentrations were measured for use in the CEFAS Combined Phytoplankton Macroalgae (CPM) model, introduced later. Detailed statistical analysis was not conducted in this part of the report. It can be noted though, that ammonium (NH_4^+) and phosphate (PO_4^{3-}) concentrations remained at similar levels between seasons, and at the different locations, while nitrate (NO_3^-) concentrations varied. Nitrate concentrations were up to six-times higher at West Itchenor (the site of the seagrass sampling) than Thorney Island (location of the saltmarsh sites) in autumn. During the winter sampling, nitrate concentrations from Thorney Island were up to 10-times higher than the same location in Autumn: M = 1.95 mg/L NO_3^-, SE < 0.01 (winter, Day 2, ebb tide), compared to M = 0.2 mg/L NO_3^- , SE = 0.02 (autumn, Day 2, ebb tide), respectively. There was a general trend for flooding tides to be higher in nutrient concentrations than ebb tides across all locations and seasons (**Figure 9**).

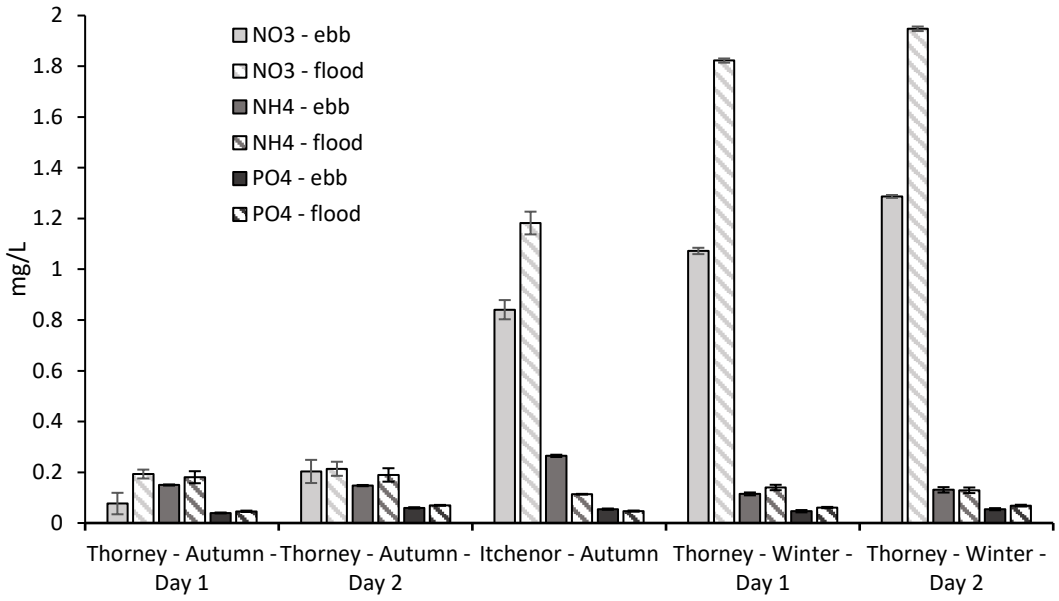

Figure 9: Nitrate (NO_3^-), Ammonium (NH_4^+) and Phosphate (PO_4^{3-}) concentrations of site seawater. Mean averages of seawater ion concentration (mg/L) from two locations during autumn and winter: Thorney Island (samples collected adjacent to saltmarsh) and West Itchenor (samples collected adjacent to mudflats and beyond seagrass beds). Two days of sampling were conducted on Thorney Island in both autumn and winter, and one day in West Itchenor in autumn, for budgeting and logistical reasons. Data are presented for both ebb and flood tides; n =4 for all samples.

7.10 Discussion

Our key result was to demonstrate the capability of a tidal core simulator method in conjunction with acetylene reduction to derive potential denitrification rate estimates, with the added value of being able to use results, together with those of porewater (Annex 1: Porewater Results) in the CPM model. The tidal core method, and the widely-adopted slurry method, both exhibited substantial variation in denitrification rate estimates, across space, within and between habitats, and across the two seasons, which was necessarily restricted to saltmarsh habitat only. We discuss potential reasons for this variation and the implications of using the methods below.

Overall, the variation exhibited by either approach confirms much of what previous research in this area has suggested (e.g. Blackwell, Yamulki, and Bol 2010; Koch et al. 1992), i.e. that there are many factors affecting denitrification rates from saltmarsh substrates, and these are likely to be inter-related in a complex manner (e.g. Wallenstein et al. 2006). However, our findings build on previous saltmarsh studies (e.g. Piehler and Smyth 2011 who also showed higher rates of denitrification in saltmarsh, submerged aquatic vegetation and oyster reefs compared to mudflats), and have helped develop a methodology to greatly increase our understanding of the processes. A recent terrestrial review (Pan et al. 2022) highlighted how (freshwater) wetlands had much higher rates of denitrification than other systems (e.g. 0.89 kg N ha^{-1} yr^{-1} cf. upland fields with 0.11 kg N ha^{-1} yr^{-1}), albeit with a small number of observations (n = 11) compared to some (e.g. 331 observations for grassland; and some grassland systems exhibited as high a denitrification rate as the wetlands). We have not attempted to scale our results to the same units as it is unclear how the meta-analysis carried this out across studies. Our estimated mean denitrification rates were lower than those found by Blackwell et al. (2010) in the saltmarshes of the River Torridge i.e. their 2.88 mg N_2O-N m^{-2} h^{-1}, emphasizing the high variability observed within systems.

The large variation in denitrification rates seen from the high and low marsh cores in the first sampling campaign (from October 2023 - autumn) is likely to have been caused by the decision to randomly sample the different areas, as well as from inherent variability alluded to before. This meant

there was uncontrolled variation in micro-topography, different vegetation structures and substrate formations. Given the relatively small sample size (n = 8), variation in the results was high. The mudflat and seagrass cores showed less variation, probably due to the more homogeneous structure of these habitats.

The second sampling campaign (from January 2024 - winter) was more targeted and cores were all taken from similar micro-topographical and vegetation locations within the different saltmarsh zones. This controlled for as many variables as possible in the absence of detailed characterisation prior to sampling. This resulted in less variation in denitrification rates in the winter sampling on the saltmarsh sites.

Negative denitrification rates reported during winter further stress the complexities of measuring nutrient processes in saltmarshes. Low porewater nitrate concentrations reduced the pool of available N for denitrification. Inhibition of nitrification by acetylene likely caused the large ammonium pool to be inaccessible by the acetylene cores causing a larger N_2O flux in the control. Unfortunately, we were unable to assess whether seasonal trends in the saltmarsh were also present in mudflat and seagrass due to logistical constraints.

It is important to point out the variation in results, from the saltmarsh cores, was not likely to be caused by length of time from core collection to analysis. Ongoing data analysis suggests the cores are stable for two weeks, from date of collection, if stored in the dark at 4 ^{o}C. This is an important factor to be borne in mind for future studies.

Using slurries may be a far quicker approach from a sampling and analysis standpoint. However, as pointed out in the methods review, such analyses likely promote denitrification through mixing and thus may not be reflective of real-world conditions. Our study further suggests there is too much variation in the results and too many factors that need to be considered, to use it as a reliable and robust indicator of whole-site denitrification rates. The equation used to calculate denitrification rates from the slurries is also problematic if required by the CPM model, due to the issue of converting a mass and/or volume of slurry to an area unit.

Our results suggest that denitrification rates of coastal habitat substrates vary with location and season, from high and low marshes having an autumn rate of 0.19 mg N_2O-N m^{-2} h^{-1} and a winter rate of 0.15 mg N_2O-N m^{-2} h^{-1}, going to -0.01 mg N_2O-N m^{-2} h^{-1}. Although not necessarily causal, it is noteworthy that winter nitrate seawater concentrations, in both the flood and ebb tides were much higher than those observed in autumn.

As part of the experimental design the same composition seawater was used for every core. The large difference between autumn and winter seawater concentrations shows the need for modelling seawater nutrient concentrations, during seasonal monitoring, using field data.
We recommend that whole cores are used to measure denitrification rates from saltmarsh habitats, with many more replicates being taken from not only each saltmarsh zone, but also well-characterised areas in those zones. Samples should be taken at least monthly over a 12-month period to get a fuller picture of the effects of seasonality (as also emphasized by Koch et al. (1992) more than thirty years ago.).

8 Nutrient Removal from Saltmarsh: Extensions and Recommendations

8.1 The Combined Phytoplankton Macroalgae (CPM) Model

The Combined Phytoplankton Macroalgae (CPM) model is used by the EA to predict the response of macroalgae and phytoplankton to changes in light conditions and nutrient loadings in UK estuaries. It can be used to guide decisions for managing nutrient inputs including, for instance, investments in improved wastewater treatment. In brief, in its characterisation of the benthic nutrient system, the CPM avoids complex process representation: for instance, it does not distinguish among N chemical species, rather modelling inorganic N as a total in porewater, and assumes there is a remineralization rate constant from one pool of organic matter i.e. there is a net mineralization flux from the processes of gross mineralization and immobilization (**Figure 10**).

Porewater N can be returned to the water column via porewater exchange or can be taken up by plants. Alternatively, it can be lost through denitrification, where denitrification flux is modelled as a first order rate process, being a function of nitrate concentration across a defined depth. Since inorganic N is the state variable, the model also uses a parameter α, to represent the ratio of nitrate to total N, where total N is assumed to comprise NO_3^- and NH_4^+, and the contribution of nitrite and other chemical N species is negligible. Since the ratio $NO_3^-/(NO_3^- + NH_4^+)$ could be variable through time, it may be necessary to extend the model by including separate state variables for porewater NH_4^+ and porewater NO_3^-.

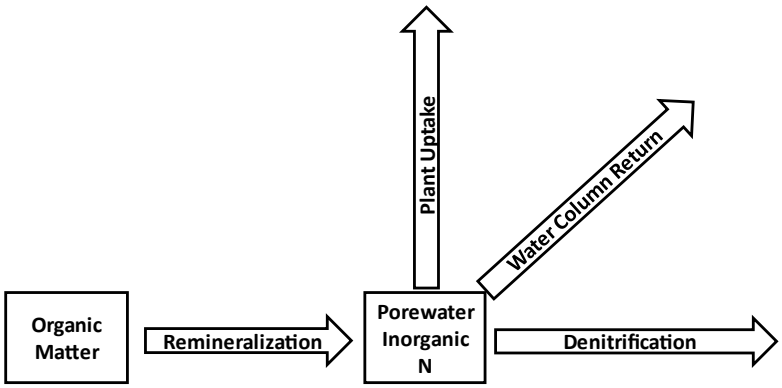

Figure 10: Current representation of sediment nutrient cycling processes in the CPM model. Denitrification flux (which we have estimated in our work; F_d), in units of mmol m^{-2} day^{-1} is assumed to be a first order rate process given by: $F_d = k \times [NO_3^-] \times d$, where k is the rate coefficient (day^{-1}), $[NO_3^-]$ is the nitrate concentration in the denitrification region (in mmol m^{-3}), and d (in m) is the depth of the denitrification layer. Our method of data collection from Thorney Island allows the estimation of k, the first order denitrification process rate as we have estimated denitrification flux, porewater nitrate concentration and the depth of the denitrification region. Return to the water column and plant uptake have equivalent units of mmol m^{-2} day^{-1}. These rates have not been estimated in our study and provide a logical extension for future work necessary to inform CPM model development.

The model has recently been extended to include seagrass and saltmarsh habitats. To predict the effect of these habitats on nutrient dynamics, removal of nitrogen by denitrification in the associated sediments is one of the key processes that needs to be quantified. The use of separate porewater ammonium and nitrate state variables mentioned above may also be necessary given the likelihood of coupled nitrification and denitrification processes in the potentially oxygenated rhizosphere associated with saltmarsh and seagrass roots, together with the variable concentrations associated with tidal flux. Results from our study will be used to set preliminary denitrification first order rate constants in the model for saltmarsh and seagrass habitats, relative to bare mudflats. Ultimately, and with further estimates from different environments, including macroalgal covered mudflats, the potential benefits of these habitats for nutrient management can be assessed.

8.2 Short Term Recommendations

The high variability and seasonal change shown by the pilot study at the Solent suggests a co-ordinated approach is required to enable upscaling and transferability of denitrification results at the national scale. We therefore recommend the following tasks for the next stage of the Land-Sea Interface project:

(i) Contrasting two representative marsh types (e.g. muddy sediment marsh from the south east with sandy sediment marsh in the north west) and respective vegetation zones. Detailed characterisation of vegetation, including biomass as well as cover, together with edaphic and climate factors.

(ii) Understand seasonal variation in denitrification rates through targeting a single natural marsh system. The work reported herein only shows that there is variation between autumn and mid-winter but characterising seasonality will help scale the magnitude of nutrient removal across a year.

(iii) Using the same marsh estuary as (ii), compare denitrification rates in natural and restored marshes. This will help understand how newly created marshes can contribute to nutrient removal during their establishment and maturation while providing direct comparison to natural marshes in the same environment.

(iv) To enable upscaling and projection, that observational and empirical work in (i) to (iii) liaises with process-based model capabilities to ensure appropriate contextual data are collected. Almaraz et al. (2020), for upland sites, suggest the following should be collected at minimum (beyond location): method used to measure N_2 production rates, soil sample treatment, control N_2 flux, control N_2O flux, soil ammonium concentration, soil nitrate concentration, soil total N concentration, soil organic C concentration, antecedent soil moisture, experimental soil moisture, headspace oxygen, soil temperature, soil pH in water, bulk soil density, soil texture and topographic position. We recommend this list is considered and amended as appropriate for saltmarsh sites and adjacent coastal systems e.g. porewater concentrations, and consideration given to characterising the vegetation community.

8.3 Medium to Longer Term Recommendations

The Short-Term approach outlined above will provide much needed information on magnitudes and drivers of potential denitrification rates in the UK environment. In the absence of resource constraints, it would also be useful to consider seasonality at multiple marsh types and locations to understand whether annual cycles interact with the type of sediment and/or climate of the area. In other words, although the design for (i) focuses on contrasting sediment type across two regions — a likely distal driver of community composition and thus denitrification rate (see Wallenstein et al. 2006) — other factors likely vary in those estuaries e.g. extent of nutrient pollution, climate. It

thus becomes difficult to disentangle causation which would be useful for prediction and upscaling. Thus, if multiple marsh sites can be chosen in the longer term, for instance by complementing other mNCEA projects, it may be possible to organise an orthogonal design to isolate what is driving variation in denitrification. This could be a focus of medium to longer term planning, together with the integration of remote sensing and/or rapid eDNA characterisations of the saltmarsh environment to ground-truth measures of denitrification. This would further enhance upscaling capabilities. It would also account for criticisms whereby treatment manipulations (sometimes adopted in laboratory methods) can help identify hotspots of denitrification but may be less relevant for understanding spatial (and temporal) variation in denitrification driven by environmental heterogeneity (Almaraz, Wong, and Yang 2020) – a key concern of the Environment Agency.

In addition, and given the interest in (a) nutrient removal more broadly, (b) interactions amongst nutrients and carbon storage, (c) need to understand actual/realised nutrient removal rates *in situ*, and (d) achieve a sustainably managed environment, in the medium to longer term we recommend:

(i) Exploration of capability and costs of *in situ* methods to characterise denitrification – where capability includes potential of equipment to operate in harsh conditions that characterise the saltmarsh environment and adjacent habitats;

(ii) Quantification of denitrification in saltmarshes restored via different pathways e.g. dredged sediment, managed realignment. This could be particularly crucial as some methods may lead to very different microbial communities and thus variation in denitrification (see also Farrer et al. 2022; Billah et al. 2022)

(iii) Quantification of nutrient removal processes beyond denitrification e.g. sediment burial, P uptake and the relative importance of different nutrient removal processes in different saltmarsh contexts;

(iv) Understanding of how nutrient removal processes evolve given changing environmental conditions. In particular, given the concern with incomplete denitrification and subsequent deleterious N_2O emissions, accurate quantification of N_2O : (N_2 + N_2O) yield. This is especially important, as in some systems additional nitrate can lead to a greater contribution of N_2O through incomplete denitrification (e.g. Senbayram et al. 2012).

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10 Annex 1: Porewater Results

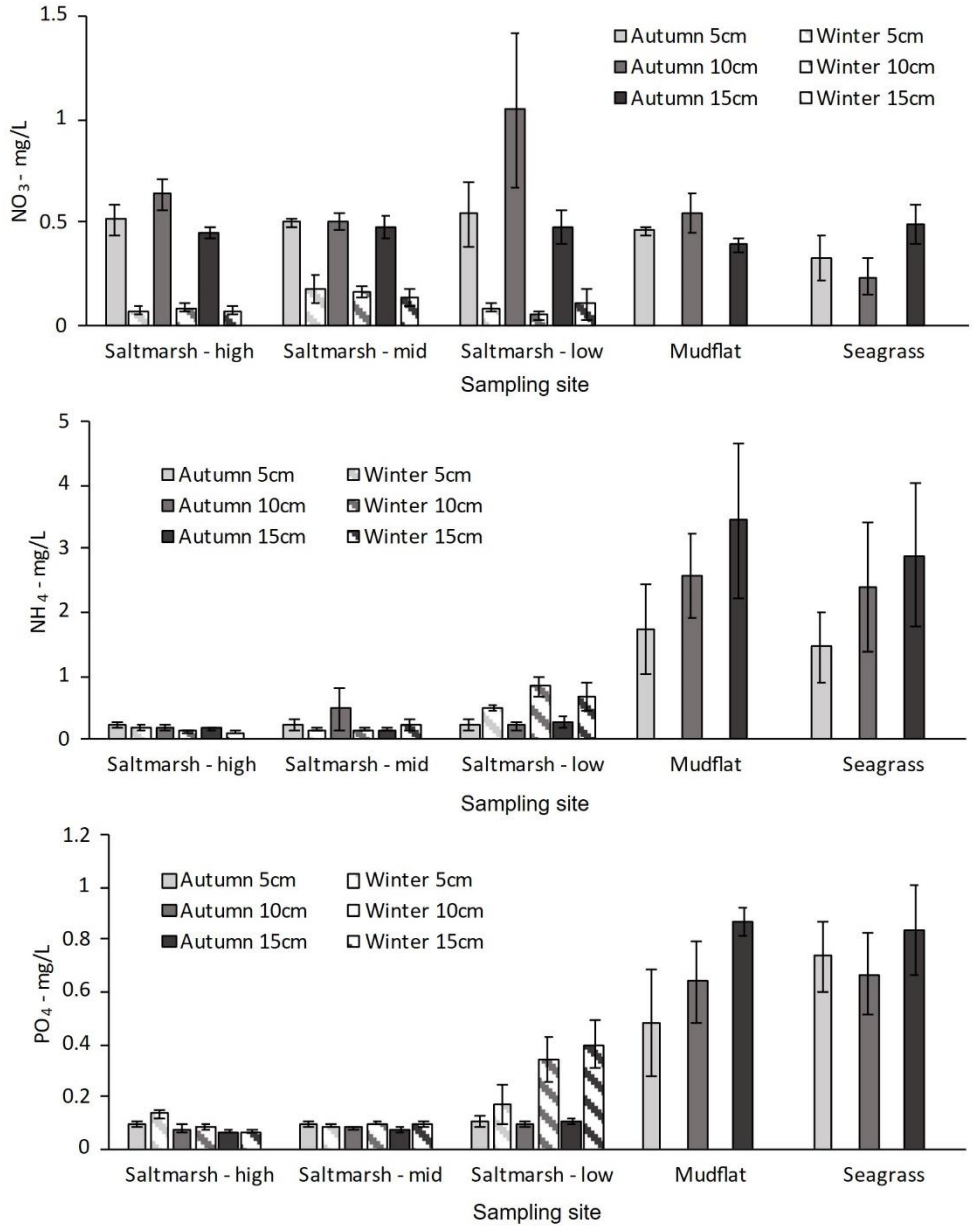

Figure 11: Substrate ion porewater concentrations. Mean average ion concentration (mg/L) of nitrate (top), ammonium (middle) and phosphate (bottom) in the porewater of sediment samples. Samples collected in autumn (October) 2023 and winter (January) 2024 across different substrate/habitat types; n = 4 for all porewater measurements in each substrate/habitat types.

Porewater ammonium and phosphate concentrations were generally low in either season in all saltmarsh habitats, except for the low marsh in winter, which exhibited an increase, especially at depth for phosphate. Mudflat and seagrass habitats showed higher but more variable porewater ammonium and phosphate, with mudflat exhibiting a tendency to increase with depth for both compounds. Seagrass exhibited such an increase for ammonium only.

Nitrate, the starting substrate for the denitrification process, showed very low porewater levels in winter in saltmarsh, while it was higher in the autumn with no obvious trends with depth in any of the vegetation zones. These very low levels in winter may explain the very low denitrification rate estimates in the main report at that time i.e. there was no substrate for denitrification. This may also relate to the high seawater nitrate levels that were observed in winter. Mudflat and seagrass nitrate porewater concentrations were around the same level as high and mid marsh zones in

autumn and showed no discernible trend with sampling depth. As explained in the main body of the report, no sampling took place on mudflat or seagrass in winter.