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Combining Techniques to Conceptualise Denitrification Hot Spots and Hot Moments in Estuaries

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Abstract

Degradation of aquatic ecosystems from nutrient pollution is a global issue, and quantifying nutrient removal in coastal ecosystems is a topic of interest for coastal managers worldwide. Analysing relationships between natural nitrogen removal processes, such as denitrification, and environmental variables from an ecological (rather than biogeochemical) perspective may help to identify and predict biogeochemically important habitat patches (hot spots). However, in situ measurements of denitrification that are coupled with ecosystem variables are rare. In this study, we analysed a dataset encompassing 18 estuaries, broad environmental gradients, and two methods of measuring denitrification (denitrification enzyme activity (DEA) and in situ N_2 flux quantification) to better understand natural estuarine nitrogen removal processes and to rationalise methods. Generally poor relationships between denitrification measures and environmental variables suggest strong context dependency, with different activation or limiting reactants affecting denitrification rates differentially in space and time. This research illustrates how biogeochemically important habitat patches may develop and demonstrates that single-method studies have the potential to miss hot spots or hot moments of nitrogen removal. A two-method approach that integrates both long-term (DEA) and short-term (in situ N_2 flux) conditions is more likely to lead to the identification of biogeochemically important habitat patches. A better understanding of natural nitrogen removal processes in estuaries will clarify assimilative capacity questions and feed into eutrophication mitigation management efforts in these highly valued freshwater–coastal interface areas.

Key words: ecosystem control points; nitrogen removal; environmental drivers; sediment properties; benthic macrofauna; denitrification enzyme activity; N_2 flux.

Highlights

- Ex situ denitrification enzyme activity and in situ N_2 flux were poorly correlated
- Combining time-integrative and short-term assessment tools is most useful
- Variance explained by environmental gradients differed by method

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Introduction

Nitrogen is an element essential for all life; however, excessive nitrogen inputs and other anthropogenic pressures continue to degrade valued coastal ecosystems (Howarth and Marino 2006). Excessive nitrogen and organic matter inputs to estuaries cause eutrophication, resulting in disproportionate growth of primary producers, decreased water and sediment oxygen concentrations, reduced water quality, loss of species, and loss of ecosystem integrity (Kennish and Townsend 2007; Nixon 1995). Therefore, it is increasingly important to progress understanding of nitrogen cycling, especially quantifying natural processes of nitrogen removal. One of the key mechanisms of nitrogen removal from estuaries is denitrification, which offers ecosystem resilience to eutrophication and associated degradation. Denitrification is the reduction of biologically available nitrate (NO_3^-) to relatively biologically inert dinitrogen gas (N_2), mediated by microbes that occur naturally within marine sediments (Ward 2013). Anammox (anaerobic ammonium oxidation to N_2) is another microbially mediated nitrogen removal pathway that can occur alongside or in competition with denitrification (Brandes and others 2007). The potential for coastal sediments to aid in mitigating the effects of excess nutrient inputs through denitrification, and to a lesser extent anammox, may be substantial (Brin and others 2014; Seitzinger and others 2006; Seitzinger 1988), but empirical in situ measurements that can be linked to patterns in local environmental characteristics (for example, sediment properties, benthic macrofaunal communities) are sparse in many parts of the world. Moreover, the bulk of research on estuarine nitrogen removal and knowledge of environmental drivers is from studies of eutrophic northern hemisphere estuaries (Vieillard and others 2020).

Most studies aimed at quantifying denitrification rates in coastal sediments have been laboratory studies (using core incubations) with few studies conducted in situ. Studies using intact core incubations (using the isotope pairing technique (IPT) or direct N_2 flux measurements) make up a large amount of denitrification literature and have been used successfully, resolving questions about nitrogen cycling across locations, habitats, seasons, (Eyre and others 2011a; Gongol and Savage 2016; Smyth and others 2013), influences of fauna (Bonaglia and others 2014; Lunstrum and others 2017; Pelegri and others 1994), benthic microalgae (Risgaard-Petersen and others 1994) and macrophytes (Caffrey and Kemp 1990; Eyre and others 2011b), and links with key environmental drivers of denitrification such as organic matter (Caffrey and others 1993) and nitrate (Hellemann and others 2017). However, the ability of laboratory incubations to capture real-world variability in nitrogen removal processes that can be linked more generally to environmental variability may be limited. For example, core sizes may limit representation of the benthic macrofaunal community, in particular the influence of large organisms which can significantly influence flux rates (Lohrer and others 2004). Pre-incubation of cores can also affect macrofaunal survival and behaviour, and transport of cores can change important biogeochemical gradients. Furthermore, the widely used IPT technique requires enrichment of the overlying water column with $^{15}NO_3^-$ which may artificially enhance denitrification, especially in low nutrient systems where water column nitrate is naturally low. It also assumes uniform transport of the solute to the denitrification zone, unlikely in natural heterogeneous bioturbated sediments; thus, denitrification may be underestimated (Cornwell and others 1999; Eyre and others 2002). Although chamber incubations may also alter macrofaunal behaviour and hydrodynamic influences on flux rates (Glud and others 1996; Huettel and others 2003), in situ incubations may be more representative of natural conditions than laboratory incubations.

In situ measurements have not been taken across broad spatial, temporal, or environmental gradients or with adequate replication, limiting knowledge of what controls or drives variation in nitrogen removal processes in the real world. In order to scale up measurements of net nitrogen removal by sediments, definitions and quantifications of the relationships between the many factors controlling denitrification and actual denitrification rates are needed (Kulkarni and others 2015). Denitrification is especially difficult to measure, primarily because its end product, N_2 gas, comprises 78% of the atmosphere. Thus, measuring release of N_2 from sediment, soil, or water is challenging and has resulted in various scientific approaches to quantify it either directly or indirectly (Groffman and others 2006). The nitrogen cycle itself is complex with many potential reactants and products, and numerous potential pathways were carried out by both chemical and biological processes. Therefore, measurement of an increase in one product (for example, N_2) cannot be inferred as the result of one single process (for example, denitrification) because other contributing (for example, anammox) or competing processes (for example, nitrogen fix-

ation) may also be responsible. Because of the difficulty in measuring denitrification, characterising and quantifying relationships between measures of denitrification and environmental drivers can help us to understand and generalise nitrogen removal at broader scales.

Nitrogen removal is highly variable in both space and time, and has been described in terms of 'hot spots' and 'hot moments' (Groffman and others 2009, 1999; McClain and others 2003). Hot spots are patches with disproportionately high reaction rates relative to surrounding areas, and hot moments are short periods of time with disproportionately high reaction rates compared with longer intervening time periods (McClain and others 2003). These hot spots and hot moments may be responsible for the majority of nitrogen removal for a given area and are therefore important to capture in order to quantify meaningful nitrogen removal values (Groffman and others 2009).

Both hot spots and hot moments can occur at a range of scales. In marine sediments, a pocket of organic matter or a macrofaunal burrow may be the site of a small-scale (cm^2) hot spot, a landscape-scale (m^2) hot spot within an estuary may occur near to a nutrient-laden river input or a habitat patch such as a shellfish bed, and a whole estuary that has higher than average rates may be a regional-scale (km^2) hot spot. Hot moments may occur at the microscale (seconds to minutes) as a result of rapid changes in solute concentrations and oxic conditions brought about by macrofaunal activity and burrowing (Volkenborn and others 2012). Hot moments over the scale of minutes to hours may occur with different tidal phases as changes in dissolved oxygen, temperature, and animal behaviours alter the conditions for microbial reactions. Season and a water body's residence time can strongly influence denitrification and other nitrogen removal pathways by driving differences in temperature and time of exposure to particular concentrations of nutrients (Kieskamp and others 1991; Smith and others 2015), creating longer-term (weeks–months) hot moments. The environmental conditions that characterise areas or habitats within estuaries may help to explain where and why hot spots and hot moments occur, that is, the conditions creating habitat patches that are biogeochemically important.

Hot spots and hot moments are not necessarily independent; thus, it is important to integrate the spatial and temporal components of ecosystem biogeochemical processes, rather than simply defining areas and points in time as 'hot or not' (Bernhardt and others 2017). Bernhardt and others (2017) introduced the Ecosystem Control Point (ECP) concept, which incorporates spatial and temporal dynamics as well as the environmental drivers of biogeochemical processes, providing an alternative approach to studying uncommon but biogeochemically important habitat patches. Here, we hypothesise that estuarine sediments are either (1) 'cold spots' for denitrification, (2) 'Activated Ecosystem Control Points' which are landscape or habitat patches (hot spots) where high transformation rates occur only when the delivery rates of limiting reactants and abiotic conditions are optimised (that is, during hot moments), or (3) 'Permanent Control Points', that is, landscape or habitat patches (hot spots) where conditions for denitrification occur most of the time (Bernhardt and others 2017). Due to the inherent spatial and temporal heterogeneity of estuary ecosystems, we expect the 'activation' variables (that is, the ecosystem components that drive or limit denitrification) to be different in different places. Activation variables may include environmental or ecological characteristics that influence the supply of substrates such as sediment properties or benthic macrofaunal characteristics.

Combining multiple approaches to estimating denitrification rates may provide a way of identifying Ecosystem Control Points and the variables that activate them. Denitrification Enzyme Activity (DEA), a type of acetylene block incubation, is conducted under optimal conditions for denitrification: unlimited carbon and nitrate, complete anoxia, and constant mixing (Smith and Tiedje 1979), and in this study, we use it as an enzymatic proxy for nitrogen removal. It does have some methodological caveats, specifically the inhibition of nitrification (which is coupled to and fuels denitrification in many natural systems) (Groffman and others 2006). This enzymatic proxy can complement direct measurements of N_2 flux because it provides an integration of the history of denitrification conditions from a given sample, which will be reflected in the composition of the microbial denitrifier community (Parsons and others 1991; Schipper and others 1993; Tiedje and others 1989). Therefore, it may be useful for identifying ECPs/hot spots of nitrogen removal. It may also encompass hot moments of nitrogen removal; denitrifying bacteria can persist in the sediments for several months and possibly years even if substrates for denitrification are absent for prolonged periods (Martin and others 1988; Smith and Parsons 1985), and can 'switch on' after being dormant and begin denitrifying relatively quickly, in response to favourable denitrification conditions (Kana and

others 1998) (that is, conditions provided in the DEA assay). Therefore, DEA can provide a time-integrated measure of denitrification unlike N_2 flux measurements that are taken over timescales of just a few hours, potentially missing hot moments (or conversely, overestimating denitrification rates if the incubation occurs during a hot moment).

The N_2 flux methodology used in this study measures the *net* N_2 flux which is the balance between denitrification and nitrogen fixation that determines net nitrogen removal. Although nitrogen fixation can be comparable to denitrification in some estuaries (Newell and others 2016; Russell and others 2016), we did not expect this to be the case in our study estuaries because the concentrations of dissolved inorganic nitrogen typically measured are predominantly in the form of ammonium. In such systems, nitrogen fixation rates are expected to be orders of magnitude less than denitrification (Eyre and others 2011a) so we assume N_2 flux is a good proxy for denitrification. Microbes are central to organic matter remineralisation and nitrogen transformation in marine sediments, and the DEA assay essentially provides an index of the abundance of microbes with denitrifying enzymes. The actual *rates* of N_2 emission however, may be controlled by a more complex set of factors that influence the delivery of solutes to denitrifying microbes. Bioturbation and bioirrigation by benthic macrofauna, for example, are instrumental in moving particles and solutes up and down across biogeochemical interfaces that exist at various depths in the sediment column. This is thought to have a profound, but difficult to generalise, effect on microbially mediated transformations such as denitrification (Stief 2013). Benthic microalgae, macrophytes, and seagrass can also have a strong influence on nitrogen removal processes through competition for bioavailable nitrogen, influencing oxygen gradients and the bacterial community (Bartoli and others 2012; Cook and others 2004; Decleyre and others 2015; Sundback and Miles 2000; Zarnoch and others 2017).

Here we use relationships between two common methods of assessing nitrogen removal (N_2 flux and DEA) and analyse the ecosystem components that drive each, in order to extend and scale up our knowledge of marine sediment nitrogen removal and its heterogeneity in the real world. The variables that control denitrification may operate at different spatial and temporal scales, and the two measures of nitrogen removal examined here may capture these different scales and provide further insight to the temporal and spatial heterogeneity of denitrification. We collated data from 18 different estuaries and looked for patterns between the two denitrification measures and environmental variables. Unlike many other denitrification studies, our approach is from an ecological rather than biogeochemical perspective, using in situ measures to capture real-world variability that includes hot spots and hot moments. In other words, we took an approach that would encompass as much spatial, environmental, and biological variability as possible, rather than a more reductive type approach that attempts to control variability. By combining these two measures and analysing differences in what drives them, we aim to provide a more holistic understanding of estuarine nitrogen removal processes that may provide more certainty in scaling up measurements, a critical step towards more effective management of coastal nutrients.

Methods

Measurements of N_2 flux from in situ chamber incubations (n = 139, dark) and DEA assays (n = 239) were collated from several studies conducted in 18 New Zealand estuaries in austral summer and autumn months (November–April) between 2013 and 2019 (n = 84 of which were paired samples shared between datasets) (Table S1). The estuaries were ocean-dominated and shallow with diurnal tides (range 2–4 m) and large soft sediment intertidal areas. Study sites ranged from clean, coarse sands to eutrophic, muddy sediments. All sites were unvegetated, except one study estuary which had some seagrass (*Zostera mulleri*, Kaipara Harbour) (Table S1). Some data were from manipulative experiments where only control plot data were used. The datasets included paired environmental and ecological variables, as well as bentho-pelagic O_2 and N_2 fluxes and/or DEA values (that is, each N_2 flux/DEA sample had unique paired environmental and macrofaunal samples collected from the same 1 × 1 m area). Paired variables included sediment organic matter (Org) and mud content (Mud), microphytobenthic biomass (Chla) and benthic macrofaunal community variables; number of individuals (Ninds), number of taxa (Taxa), *Austrovenus stutchburyi* abundance (Austro), and *Macomona liliana* abundance (Mac). Samples for sediment properties (Mud, Org, Chla; 5 cores pooled, 2.3 cm dia., 2 cm depth) and macrofaunal community composition (1 × 13 cm dia., 15 cm depth core, sieved on 500 μm mesh) were analysed using standard protocols (see O'Meara and others 2020). In some studies, cores were also collected for

analysis of pore water nutrient concentrations (5 cores pooled, 2.3 cm dia., 2 cm depth) (see Douglas and others (2016) for details); however, these data were not used in the analysis.

Sampling and assays for DEA measurements were taken according to Douglas and others (2017) using a chloramphenicol amended acetylene inhibition technique adapted for marine sediments (Groffman and others 2006, 1999; Tiedje and others 1989). For each DEA sample, five cores (5.3 cm dia. 5 cm depth) were collected from a 1 × 1 m sampling plot, pooled, and transported on ice to the laboratory. Sediment was homogenised, all visible macrofauna and macrophytes were removed, and 60 mL of the pooled sample was used for each DEA assay. All samples were processed and analysed to strictly consistent protocols in the same temperature controlled (20 °C) laboratory by the same person.

N_2 fluxes were quantified using benthic chamber incubations and analysed using membrane inlet mass spectrometry (MIMS) (Kana and others 1994). Chambers consisted of 0.25 m^2 metal bases pushed 5 cm into the sediment with Perspex lids to enclose approximately 40 L of water. Intertidal chambers were deployed at low tide, with incubations initiated after inundation on the incoming tide (Jones and others 2011). Water samples were drawn from each chamber using gastight syringes or peristaltic pumps (drawing water directly into exetainers) at the beginning and end of incubations (normally 4 h over midday high tides). From each syringe, three replicate 12-mL exetainers were filled to overflowing, excluding air bubbles, and a drop of preservative (ZnCl or HgCl) was added to the top of each before capping. Vials were stored upright in racks, partially submerged in water to maintain a constant temperature, until transported to the laboratory (within 12 h) where they were stored at 4 °C until analysis. For MIMS laboratory analysis protocols see O'Meara and others (2020). O_2 concentration measurements taken from samples extracted at the beginning and end of incubations using a handheld YSI ProODO Optical Dissolved Oxygen probe (O'Meara and others 2020) were used to estimate O_2 flux (a measure of benthic community metabolism).

Statistical Analyses

Pearson's correlation coefficients and biplots were used to explore relationships between variables and investigate drivers of DEA and N_2 flux. Multiple regression analyses (DistLM, Primer 7, PERMANOVA + , Anderson and others (2008)) were conducted to reveal the variability in DEA and N_2 flux explained by the measured ecosystem components in each dataset (N_2 flux, DEA). Although DistLM is not restrictive based on normality and homogeneity of variance, data were transformed (log(x + 1) for DEA, N_2 flux and environmental variables, and square root for macrofaunal variables) in order to increase linearity of relationships and improve model fit. Variables were normalised prior to building individual Euclidean similarity matrices for DEA and N_2 flux; these were used to run separate DistLMs for each.

Firstly, marginal tests were used to identify significant individual predictors of DEA and N_2 flux (Table 1). The overall best model for each denitrification measure was obtained using the backwards selection procedure and the corrected Akaike information criterion (AICc) (Table 2) with 9999 permutations. Predictor variables were analysed for covariance (Pearson's $r > 0.7$); where this occurred, the predictor explaining the least amount of variation in the response variable was excluded from the subsequent model.

Results

The dataset spanned a range of estuaries from very muddy (96% mud), organic-rich sediments (10% organic content) to sandy (0% mud) organic-poor sediments (0.3% organic content). Macrofaunal communities varied widely among sites (3–599 individuals $core^{-1}$, 2–29 taxa $core^{-1}$), especially the abundance of the bivalves *Austrovenus stutchburyi* (0–133 $core^{-1}$) and *Macamona liliana* (0–28 $core^{-1}$) (core size 0.05 m^2) (Table S1).

For the separate DEA and N_2 flux datasets, the measured ecosystem components (that is potential drivers or 'activation' variables) explained 62% of the variation in DEA, but only 12% of the variation in N_2 flux (Table 1). Sediment mud and organic content were the primary predictor variables for

Table 1. Multiple Regression Results for Variables Predicting DEA and N_2 Flux

DEA (n = 239)		
AICc	R^2	Predictors
86.4	0.62	Org + Chla + Ninds
N_2 flux (n = 139)		
AICc	R^2	Predictors
49.6	0.12	Chla + Ntaxa + Mac

Overall best DistLM model fit obtained using the 'Backwards' selection procedure. Abbreviations: Sediment organic content (Org), chlorophyll a (Chla), number of individuals (Ninds), number of taxa (Ntaxa), Mac (Macomona liliana abundance).

Table 2. Ecosystem Components as Predictors of DEA and N_2 Flux

	DEA marginal tests n = 239				N_2 flux marginal tests n = 139			
	Pseudo-F	p	Prop	Direction	Pseudo-F	p	Prop	Direction
Mud	**297.6**	**0.001**	**0.56**	**+**	**3.73**	**0.047**	**0.026**	**+**
Org	**302.8**	**0.001**	**0.56**	**+**	**3.70**	**0.07**	**0.026**	**+**
Chla	**138.4**	**0.001**	**0.37**	**+**	**9.11**	**0.006**	**0.062**	**+**
Ninds	**65.2**	**0.001**	**0.22**	**+**	0.011	0.91	0.0001	-
Ntaxa	**8.08**	**0.006**	**0.03**	**-**	0.39	0.54	0.003	+
Austro	0.78	0.40	0.003	-	2.53	0.12	0.018	-
Mac	1.66	0.20	0.01	-	**3.04**	**0.09**	**0.02**	**-**
DO flux	NA	NA	NA	NA	0.029	0.88	0.0002	-

Marginal test results (DistLM); significant ($p < 0.1$) predictors are indicated in bold. Abbreviations: Sediment mud content (Mud), Sediment organic content (Org), chlorophyll a (Chla), number of individuals (Ninds), number of taxa (Ntaxa), Austro (Austrovenus stutchburyi abundance), Mac (Macomona liliana abundance), dissolved oxygen flux (DO flux).

DEA (individually each explaining 56% of the variation) (Table 2). Due to collinearity between sediment mud and organic matter content in both datasets (DEA: Pearson's $R = 0.78$, N_2 flux: Pearson's $R = 0.88$), these two variables could not be used in models together; mud content was excluded from subsequent models to avoid variance inflation issues. Secondary predictor variables for DEA were Chla and macrofaunal community variables; number of individuals, and the abundance of the venerid clam *Austrovenus stutchburyi* which is a large suspension feeding bivalve and key benthic bioturbator.

N_2 fluxes were usually highest in organic-poor sediments with low DEA values (Figures 1, 2) but unlike DEA, were not strongly predicted by sedimentary variables (Table 2, Figure 2). Chla was the predictor variable that individually explained the most variation in N_2 flux, although accounted for only 6% of the variation. Other predictors included in the full model were macrofaunal community measures that individually explained less than 3% of the variation in N_2 flux (Table 1, 2).

In the paired dataset, N_2 fluxes did not correlate with DEA rates (Figure 1 $R^2 = 0.12$, $p > 0.05$, $n = 84$). Across the sampled estuaries, DEA and N_2 flux data were scattered representing the full spectrum of biogeochemical activity with habitat patches characterised by all combinations of low and high rates of DEA and N_2 flux (Figure 1).

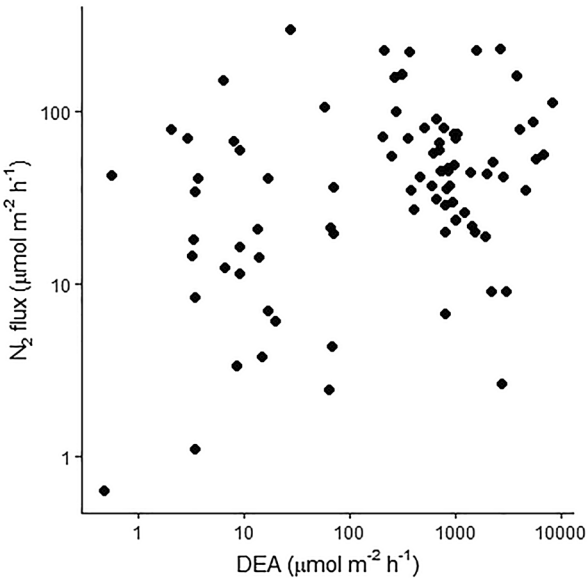

Figure 1. Relationship between DEA and N_2 flux (log scale) where both parameters were measured at the same place and time (that is, explicitly paired samples, $n = 84$).

Discussion

Results from our study, involving two nitrogen removal measurement methods and correlations with environmental variables, fall into three main conceptual categories. The categories include areas that were not favourable for nitrogen removal ('cold spots'), areas where most conditions for nitrogen removal were met ('activated Ecosystem Control Points'), and areas where conditions for nitrogen removal were possibly always met ('permanent Ecosystem Control Points'). Results also signal that both nitrogen removal assessment methods have the potential to miss biogeochemically important habitat patches and/or hot moments. Specifically, there were areas with low DEA but high N_2 flux, and areas with relatively low N_2 flux but high DEA.

Sediment organic matter content was the most important variable explaining DEA, supporting the notion that DEA provides a measure of the history of environmental conditions for nitrogen removal

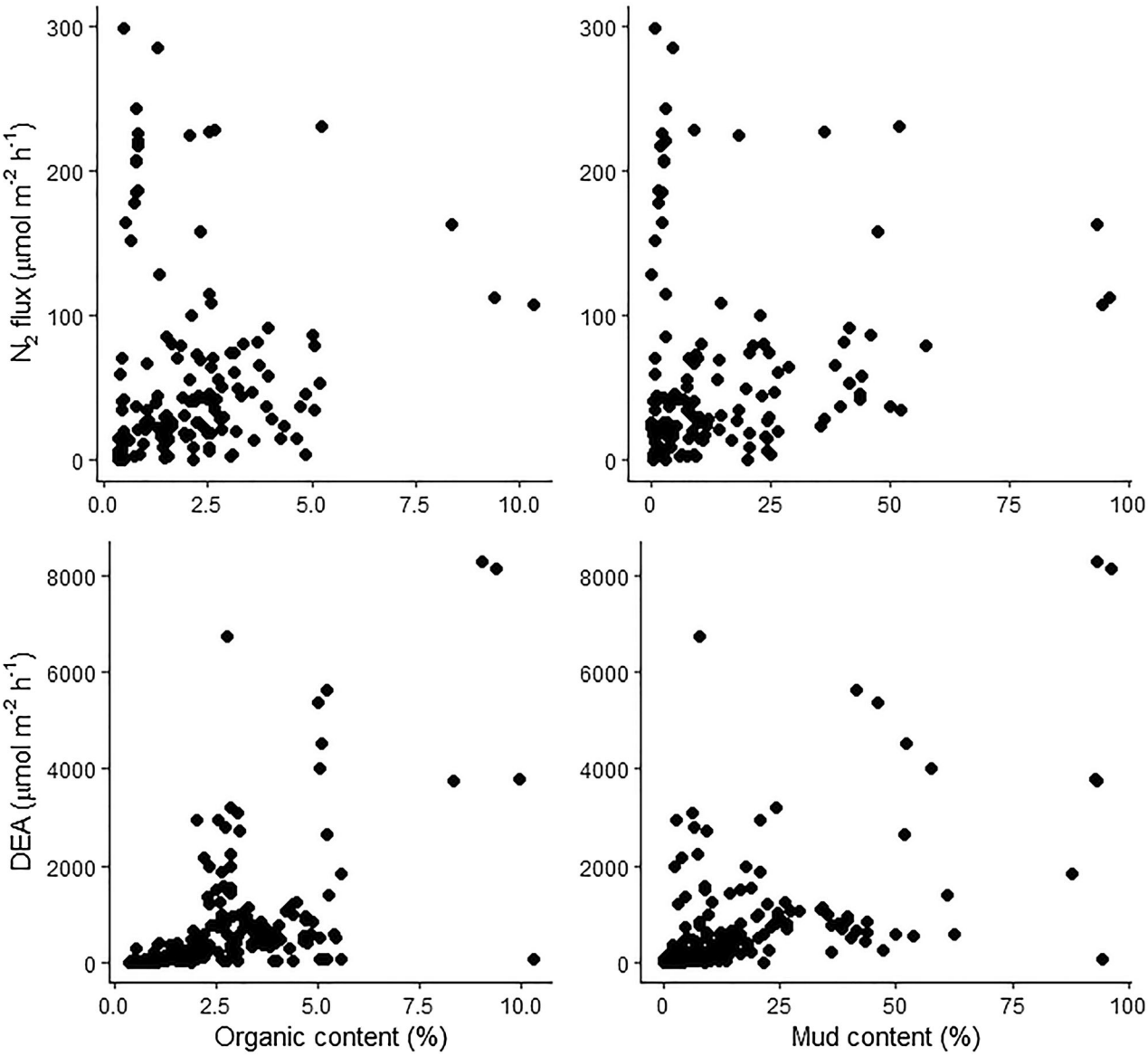

Figure 2. Relationship between sediment organic (left) and mud (right) content with N_2 flux (top) and DEA (bottom).

in the sampled sediments (Parsons and others 1991; Schipper and others 1993; Tiedje and others 1989), and an approximation of the active denitrifier population which can be stable and persistent for periods of at least two months (Martin and others 1988; Smith and Parsons 1985). DEA is often referred to as the 'potential' of the denitrifying community to denitrify when conditions are right; that is, the population reflects historical conditions and turns on when conditions are right. DEA may thus integrate or average across hot/cold moment phenomena, that is, representing a longer-term integrated value in which there may have been hot and cold moments. Sediments that are rich in organic matter likely contain the organic carbon and, after organic matter remineralisation (ammonification, nitrification), the nitrate required for nitrogen removal. In our study, organic-rich sediments had higher DEA, indicating that these sediments contained larger populations of microbial denitrifiers, but this was not where the highest N_2 release was measured. There may be a threshold in organic matter where further increases do not increase nitrogen removal (that is, another factor such as sediment permeability or hydraulic conductivity becomes rate limiting) even though a large but inactive denitrifier community persists above this threshold (that is, high DEA, low N_2 flux; Figure 2). Other studies have attributed a lack of relationship between denitrifier gene expression

and denitrification rates partly due to the high genetic diversity of organisms that are denitrifiers (even at small spatial scales) (Bowen and others 2014).

The present study included several commonly known drivers of denitrification, but these could only explain a relatively small amount (13%) of the variation in N_2 flux measurements. (Other techniques including boosted regression trees were used to test for nonlinearities and interactions, but did not significantly increase the explained variation.) This suggests that net N_2 flux is highly variable in space and time and supports our idea that measurements from incubations taken over relatively short time periods may not be representative of longer-term rates of net nitrogen removal. In contrast to DEA, sedimentary variables were not important regulators of N_2 flux, which was (partially) explained by Chla and macrofaunal variables. Chla may represent the influence of microphytobenthos on the availability of nitrate and ammonium through competition, or the effect of the oxygen produced by microphytobenthic photosynthesis, both of which could facilitate coupled nitrification–denitrification (Rysgaard and others 1995). Although N_2 fluxes were measured in the dark, the effects of microphytobenthos on local biogeochemistry will persist even when photosynthesis is not occurring. Microphytobenthos can also influence nitrogen fixation (Russell and others 2016) which may contribute to its role in influencing N_2 flux in this study, although nitrogen fixation has been shown to be low in oligotrophic estuaries (Eyre and others 2011a). Similarly, macrofauna may affect nitrogen removal rates by altering solute concentrations (dissolved oxygen and ammonium) and rapidly advecting solutes such as ammonium and nitrate across oxic and anoxic sediment interfaces, thus affecting sedimentary nitrification and denitrification (Kristensen and others 1991). Although N_2 flux measurements principally capture variability occurring in individual chambers while the incubations take place, the DEA assays do not because they are ex situ procedures on mixed sediment slurries that have been sieved free of macrofauna. Therefore, effects on DEA attributed to macrofauna likely represent the legacy of macrofaunal activities on denitrifying microbial populations, rather than real-time effects. In other words, DEA is integrating the temporal variability in rates that occurred over the past weeks or months, and this may be contributing to the higher explained variation.

Other known denitrification regulating variables not presented here, including temperature, pore water and water column nitrate concentrations, and the quality of carbon sources may help explain some of the variability (Eyre and others 2013; Knowles 1982). In the estuaries included in this study, denitrification is likely to be coupled to nitrification in the sediments (Gongol and Savage 2016) because water column nitrate concentrations in this study were low. Where measured, most nitrate values were below instrument detection limits (see Table S1), which is characteristic of many New Zealand estuaries (Dudley and Jones-Todd 2018; Plew and others 2020). Nitrogen removal may be partially reliant on the nitrification process (which is influenced by another suite of controlling factors), and this is another reason why nitrogen removal may be highly variable in space and time. The poor ability of the measured ecosystem components to explain variation in N_2 fluxes suggests either that key drivers of nitrogen removing processes were not measured, or that the spatial resolution of measurements, sampling effort, and duration of chamber incubations do not capture variability in nitrogen removal particularly well.

We observed that N_2 flux can be high even when DEA is low (Figure 1). Thus, DEA was not a universally good representation of denitrification potential (for which it is often used). This could have occurred if N_2 flux was dominated by anaerobic ammonium oxidation (anammox) rather than denitrification; however, conditions in this study are unlikely to favour anammox as a significant contributor to net N_2 flux, given low concentrations of water column and pore water nitrate (Vieillard and Thrush 2021). High net N_2 flux together with low DEA may also indicate habitat patches with very high denitrification efficiency, or that flux chamber incubations have coincidently captured a particularly 'hot moment' or a point in time where an ECP was 'activated'. Other studies have also found poor correlations between DEA and N_2 flux measurements and have suggested that DEA may be better interpreted as an estimate of the biomass of denitrifying bacteria rather than as a denitrification rate (Martin and others 1988), in other words DEA represents a measure of the resident denitrifying community in the sediments. In this study, the spatial scales at which data are collected for the two measures differs, which could have contributed to the poor correlation between methods. The chamber incubations measure net N_2 efflux from a 0.25 m^2 area of sediment, whereas small DEA cores were randomly collected and pooled from a 1 × 1 m sampling plot. Although both methods integrate spatial heterogeneity, the

internal area of the incubation chambers (0.25 m^2) is greater than the combined area of DEA cores paired with them (0.01 m^2).

Bioturbation by resident macrofauna is likely to positively influence both solute transport and microbial activity, and thus DEA and net N_2 flux (Aller 1988; Berg and others 2001; Henriksen and others 1983). However, the relationships between these functions (DEA and N_2 flux) and macrofaunal community variables were not consistent. Bioturbating shellfish species *Austrovenus stutchburyi* and the wedge shell *Macomona liliana* are known to play pivotal roles in benthic ecosystem functioning (Thrush and others 2006). Large bioturbating organisms typically accelerate N_2 flux from the sediments (Karlson and others 2007; Stief 2013; Webb and Eyre 2004), as do epifaunal shellfish (Hillman and others 2021; Newell and others 2002), but abundances of bioturbating shellfish were negatively correlated with N_2 flux in the dataset analysed here. The positive relationships between the number of taxa and N_2 flux agrees with other studies showing that a more biodiverse community facilitates ecosystem functions such as nitrogen removal (Thrush and others 2017; Vieillard and Thrush 2021). Activation of ECPs (or hot moments) may be enhanced in a biodiverse benthic sediment with increased spatial and temporal heterogeneity of the oxic–anoxic interface where coupled nitrification–denitrification occurs (Aller 1988).

In terrestrial systems, denitrifying bacteria can lie dormant in (dried) non-denitrifying soils and 'switch on' and denitrify when conditions are right (Smith and Parsons 1985). Smith and Parsons (1985) found that fluctuations in conditions that stress denitrifying bacteria such as wetting/drying processes possibly enhance N_2 flux compared with soils not subject to such fluctuations. This phenomenon may hold true in marine sediments where other conditions fluctuate to facilitate rapid changes in nitrogen removal rates regardless of the existing denitrifier population. Fluctuations in oxygen content, sediment water content, and pore water nitrate concentrations occur regularly in intertidal estuary sediments due to tides, wave forces, and physical and biological disturbances. These as well as regular emergence/submergence of intertidal sediments may be important for nitrogen removal hot moments/activation. However, since benthic chamber incubations are only conducted when submerged, differences occurring throughout the tidal cycle may go undetected. Furthermore, chambers may reduce physically driven porewater exchange through dampening of waves and currents, and/or biologically driven porewater exchange through alteration of organism behaviour (Glud and others 1996; Huettel and Gust 1992).

The dataset analysed here came from several estuaries, and spanned gradients within estuaries, from relatively pristine to eutrophic sediments. Further understanding of local-scale patterns as well as inclusion of more potentially important controlling variables (pore water and water column nitrate concentrations, sediment oxygen concentration, sediment temperature, and so on) is likely to enhance future analyses. The high variability in the regulators of nitrogen removal processes may reflect the different limiting variables or 'activation' variables at the different sites. Differences in factors that limit nitrogen removal should be expected due to the breadth of environmental variability across the dataset. The relationships between regulators and the two nitrogen removal measures were different in the different estuaries included (preliminary exploratory analyses—not presented here), although sample sizes at the site level were generally not large enough to conduct robust site-specific analyses.

Despite the high degree of variability encompassed in this study, there were overarching trends in nitrogen removal. This work shows the potential of combining DEA, N_2 flux measurements, and site- and replicate-specific environmental characteristics, to identify biogeochemically important habitat patches and/or conditions (Ecosystem Control Points). It also highlights the potential for these to go undetected when studies employ only one technique for measuring nitrogen removal, that is, we found patches with low DEA/high N_2 flux and patches with high DEA/low N_2 flux. Thus, our results would suggest that by utilising both methods it is possible to identify the different types of ECPs. The conditions that make up cold spots, activated ECPs, and permanent ECPs will be dependent on the history of conditions for denitrification and variables that influence the concentration and transport of substrates for denitrification. The activation variable(s) (or what limits denitrification the most) may differ depending on the site and may change temporally as well.

A permanent ECP would be at a location with both high DEA (implying an abundant denitrifier population and a history of conditions that are favourable for denitrification) and a high net N_2 flux (showing a high instantaneous N_2 removal). In an activated ECP, there may be intermittently high N_2 fluxes, but only when 'activation' occurs (that is, on average the N_2 flux is low), which would be when something that is normally limiting the

nitrogen removal processes is available. The activation variables can, however, be different in different locations. For example, this may occur in a location with a high background denitrifier population (that is, high DEA) and plentiful substrate (that is, high organic matter content which supplies carbon and nitrogen) but insufficient transport of solutes between the nitrification and denitrification zones to sustain N_2 flux rates. Conversely, activated ECPs may also be where low DEA and high N_2 flux were measured, for example, due to the timing of the incubation capturing an above average period of N_2 flux, when all activation variables were favourable for nitrogen removal processes. Future investigations of activated ECPs using manipulative experiments or temporally repeated surveys may help to disentangle the context-dependent mechanisms behind ECP activation, and further the understanding of nitrogen removal hot spots and hot moments in estuarine ecosystems.

Increasingly managers request whole-of-ecosystem values for nitrogen removal processes to create nutrient budgets and set limits on discharges and nutrient use in catchments. This requires measurements to be generalised and scaled up to much larger areas which will be aided by integrating both local- and regional-scale patterns of the drivers of nitrogen removal. Measuring different elements of an ecosystem process (that is, DEA, the microbial element, and N_2 flux, the process element) revealed different patterns in relationships with ecosystem variables and environmental drivers. These patterns are scale-dependent, and this has implications for the way we use data to scale up measures of nitrogen removal. With mapping techniques and broad spatial surveys using relatively cheap, fast, time-integrative methods (for example, DEA, sensu Lohrer and others (2020)), studies can predict or detect where hot spots are likely to occur using predictive models based on known environment–process relationships. More sensitive but spatially and temporally restricted methods (such as N_2 flux incubations) can progress the understanding of the specific roles of macrofauna in particular places (or other potential limiting/activation variables) in order to understand hot moment phenomena. Ultimately combining such studies will support scaling up and modelling of nitrogen removal that incorporates hot spots and hot moments (Ecosystem Control Points), and progress knowledge of local-, landscape-, and regional-scale variability in nitrogen removal.

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Declarations

Conflict of interest The authors declare no competing interests.

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