Seagrass as a coastal filter: Investigating the role of seagrass meadows in mediating nitrogen cycling in shallow coastal lagoons

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Abstract

Seagrass meadows are highly productive ecosystems that are widely distributed in coastal waters throughout the world. In shallow coastal lagoons, seagrass meadows provide an important ecosystem function by acting as a nitrogen filter. Nitrogen (N) that enters the lagoon is temporarily retained in seagrass biomass and is removed from the system through burial and denitrification in sediments. This filter function contributes to the health of seagrass ecosystems by reducing nitrogen in the water column, slowing rapid N cycling through algal biomass, and reducing the total nitrogen load exported from the ecosystem. However, seagrass meadows are declining worldwide; with the loss of seagrass meadow area, the N filter function is also lost. Restoration may offer a pathway to restore this important ecosystem function, but to date the effectiveness of the nitrogen filter in restored seagrass meadows has not been evaluated.

In this dissertation, I assessed the return of the nitrogen filter function within a successful seagrass restoration project in the Virginia coastal bays. I measured a suite of N cycling processes in the seagrass meadow and in adjacent bare sediments to understand the effects of the restoration. To assess retention and removal of nitrogen, I measured denitrification, burial, and assimilation into seagrass biomass. To evaluate the importance of the N filter function, I compared retention and removal to inputs of nitrogen to the lagoon from external N loading (from atmospheric and terrestrial sources) and from N fixation within the meadow. Finally, I compared the magnitude of N removal in the seagrass meadow to predicted changes in N loading from future development scenarios in this ecosystem in order to put the restored filter function in context within the landscape.

My results showed that the N filtration processes were enhanced in the restored meadow compared to adjacent unvegetated sediments. Through the development of a novel in situ

incubation method (the push-pull method), I measured denitrification rates that were 4x greater in the restored seagrass sediments compared to bare sediments. Rates measured using this push-pull method were also significantly greater than rates measured using a conventional core technique, indicating that further research is needed to understand denitrification in seagrass sediments. N burial in sediments was 10x greater in the seagrass meadow than in bare sediments, and assimilation of N into seagrass biomass was the largest measured flux of nitrogen. The restored meadow had therefore regained both the retention and removal components of the coastal N filter. N removal in the meadow was comparable to 37% of current N loading from watershed and atmospheric sources, showing the importance of this restored function at the ecosystem scale. Future development scenarios were predicted to lead to increased N loads, but continued expansion of the seagrass meadow will also increase N removal. The maximum predicted meadow extent could offset >68% of the enhanced N load from future development scenarios, potentially limiting harmful effects from higher N loading. Overall, the seagrass restoration has reestablished the N filter function and has increased the capacity of the lagoon to withstand anthropogenic perturbations to the coastal nitrogen cycle.

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Chapter One: Introduction to the dissertation

Background

Widely distributed in shallow waters throughout the world, seagrass meadows influence both physical and biological processes in coastal ecosystems through high productivity rates and changes to the structural environment. These impacts are especially important in mediating coastal nitrogen (N) cycling. In coastal bays, meadows may act as a nitrogen filter by enhancing retention and removal of nitrogen within the meadow compared to unvegetated, bare sediments (McGlathery et al. 2007). Retention includes uptake of nitrogen into seagrass biomass, where it is temporarily unavailable to the coastal N cycle (Pedersen et al. 2004). Turnover and decomposition of seagrass biomass are slow relative to other marine primary producers (e.g. phytoplankton, macroalgae), so retention of N in seagrass biomass can slow the rate of coastal N cycling (Banta et al. 2004). Removal processes include long-term burial of nitrogen in seagrass sediments, where N may remain for stored for decades or longer in some seagrass meadows (Mateo et al. 1997, Middleburg et al. 2004), and denitrification, which leads to permanent release of nitrogen in the form of inert dinitrogen gas (Risgaard-Petersen 2004, Romero et al. 2006). The combination of these retention and removal processes in seagrass meadows provides a nitrogen filter function that helps maintain high water clarity in areas where seagrass meadows are present.

The presence of seagrass can also stimulate internal N loading processes, i.e. remineralization and N fixation within the meadow. The high productivity of the seagrass provides large amounts of biomass for remineralization, and in sediments, root exudates can stimulate heterotrophic N fixation. Seagrass leaves can also be colonized by epiphytic autotrophic N fixers (McGlathery 2008). Thus, the meadows can play a dual role – the filtering

processes (denitrification, burial, and retention in biomass) contribute to low water column chlorophyll and nutrient levels, while the internal N loading processes (remineralization and fixation) support the high seagrass growth rates. Both these sets of processes are critical to seagrass health; water clarity improves light availability at the benthos, and seagrass growth requires fixed nitrogen. Thus, the combination of the nitrogen filter function and internal nitrogen loading within the meadow enable seagrass meadows to thrive.

Many coastal areas face increasing pressures from anthropogenic disturbances, including increased levels of nitrogen loading and development driven by high human populations in the coastal zone (Small & Nicholls 2003, Seitzinger & Harrison 2008). These human impacts contribute to seagrass declines (Orth et al. 2006, Ralph et al. 2006); worldwide, approximately 1/3 of historical seagrass extent has been lost over the last century, and these losses are accelerating (Waycott et al. 2009). With the loss of seagrass meadows, important ecological processes, including the coastal nitrogen filter function, are also lost. However, restoration efforts can successfully reverse some of these declines in seagrass meadow area, leading to the renewal of seagrass meadow ecosystems and, potentially, the return of ecological functions and services. Previous research in restored seagrass meadows indicates that ecosystem metabolism and carbon sequestration can occur at rates comparable to natural meadows, indicating that ecological functions can be regained following restoration (Greiner et al. 2013, Rheuban et al. 2014, Oreska et al. 2017). However, the nitrogen filter function of restored seagrass meadows has yet to be assessed.

Understanding the coastal N filter function in seagrass meadows requires knowledge of both physical processes (e.g. sediment accretion rates) and biological processes (e.g. seagrass productivity). In particular, microbial processes occurring in seagrass sediments play an

important role in N cycling and affect the N filter function. Denitrification, the microbial transformation of nitrate into inert dinitrogen gas, is an important loss of fixed nitrogen from aquatic ecosystems (Seitzinger et al. 2006). At the same time, N fixation, the microbial transformation of inert dinitrogen gas into biologically available ammonium, can be an important source of nitrogen in seagrass meadows (Welsh 2000). These processes, along with numerous other N transformations, occur in subsurface sediments and may be stimulated by exudation of organic carbon and/or oxygen from living seagrass roots. However, current methods of measuring these N transformations do not generally target subsurface sediments and may underestimate in situ rates. Advances in the methods used to measure N cycle processes in seagrass sediments are needed to improve our knowledge of the coastal N filter.

The goal of this dissertation was to assess the coastal N filter function in a large-scale, successful seagrass restoration and to compare filtration processes with external and internal N loading processes. This work included measuring processes underlying the N filter in an established seagrass meadow and in neighboring unvegetated, bare sediments in order to understand how the restoration altered N cycling within the lagoon. The following chapters report on the development of a new method for measuring N transformations in subsurface sediments, differences in N cycling between bare sediments and seagrass sediments, and the contribution of specific N cycle processes to the coastal filter function. The final research chapter considers the ecosystem service of water filtration provided by the seagrass N filter function and describes how the magnitude of this service, both in terms of the ecological processes and the economic value, compares to external N loading rates under current and future development scenarios.

Site description

The study site for this body of work was the Virginia Coast Reserve Long-Term Ecological Research site (VCR LTER), a 110-km stretch of Virginia's Eastern Shore peninsula that encompasses a series of shallow bays bordered by barrier islands. In the Virginia coastal bays, seagrass (*Zostera marina*) were historically present until the 1930s when a combination of a fungal infection (*Labyrinthula* sp.) and physical disturbance from a hurricane in 1933 led to the disappearance of seagrass within the bays (Orth & McGlathery 2012). After the discovery of a small patch of seagrass in the late 1990s, large-scale restoration was begun in 2001 by broadcasting *Z. marina* seeds in replicate 0.4 ha plots in four of the coastal bays (Orth et al. 2012). Over time, extensive (>25 km^2) meadows have developed, and long-term monitoring shows that seagrass characteristics (e.g. productivity rates, shoot densities) have achieved a mature steady-state approximately one decade after restoration (McGlathery et al. 2012). Recent work has highlighted the impact of the seagrass restoration on numerous ecological processes, including wave attenuation, sediment transport, metabolism, and carbon storage (Hansen & Reidenbach 2012, Greiner et al. 2013, Rheuban et al. 2014, Oreska et al. 2017). This study site therefore represents an ideal location to assess the return of the coastal N filter function following seagrass restoration.

Long-term monitoring of water quality parameters at the VCR has shown that water quality has not declined over the past twenty years, in contrast to many nearby locations (McGlathery & Christian 2017). Nutrient loading in this system is also quite low, with the majority of nitrogen entering the lagoons from atmospheric deposition (Anderson et al. 2010). These conditions have enabled the success of the restoration, as seagrass tend to be sensitive to water quality. However, these conditions may change in the near future. Although the upland counties that comprise the watersheds of the VCR are relatively undeveloped, there is a potential

for both residential development and changes in agriculture that could lead to greater nutrient loading (Giordano et al. 2011). Coastal lagoons north of the VCR, where there is greater development and poultry farming is more widespread, face higher nutrient loads and degraded water quality (Boynton et al. 1996, Stanhope et al. 2009). Understanding nitrogen processes under current, unpolluted conditions at the VCR will therefore provide a baseline of comparison, either for other locations that experience greater nitrogen loads or for potential future scenarios in the VCR.

Approach

The objectives of my dissertation were as follows:

(1) To develop a new in situ incubation method appropriate for measuring subsurface rates of nitrogen transformations in seagrass sediments;

(2) To compare rates of N inputs and N removal and retention processes within the restored seagrass meadow in order to asses the coastal N filter function;

(3) To compare rates of N transformations and the N filter function in seagrass sediments and adjacent unvegetated sediments;

(4) To assess the magnitude and value of the seagrass N filter function under current and future N loading scenarios.

I addressed these objectives in five research chapters, each written and formatted for publication in peer-reviewed journals. Chapter 2, "Push-pull incubation method reveals the importance of denitrification and dissimilatory nitrate reduction to ammonium in seagrass root zone" describes the development of a new incubation method and was published in *Limnology and Oceanography: Methods* in 2017. Chapter 3, "Resoration enhances denitrification and DNRA in the subsurface sediments of *Zostera marina* seagrass meadows," applies the novel

push-pull method developed in Chapter 2 to measure nitrate reduction processes in seagrass and bare sediments; this chapter is in review at *Marine Ecology Progress Series*. Chapter 4, "High rates of N fixation in seagrass sediments measured via direct $^{30}N_2$ push-pull method", adapts the push-pull method to measure N fixation rates and again compares rates in seagrass and bare sediments. Chapter 5, "Seagrass restoration reestablishes the coastal nitrogen filter" synthesizes data from Chapters 3 and 4 along with measurements of additional N cycle processes to evaluate the coastal N filter function in comparison to external and internal N loading. Chapter 6, "Nitrogen removal in restored seagrass meadows under future development scenarios," places the seagrass N filter function in context compared to N loading from current and future development. The final chapter of the dissertation summarizes the major research findings and explores potential directions of future work. Chapters 4-6 will be submitted for publication in the near future; the respective target journals for these three chapters are *Marine Ecology Progress Series*, *Limnology and Oceanography*, and *Estuaries and Coasts*.

References

Anderson IC, Stanhope JW, Hardison AK, McGlathery KJ (2010) Sources and fates of nitrogen in Virginia coastal bays. In: Coastal Lagoons: Critical habitats of environmental change. CRC Press, Boca Raton, FL, p 43–72

Banta GT, Pederson MF, Nielsen SL (2004) Decomposition of marine primary producers: Consequences for nutrient recycling and retention in coastal ecosystems. In: Estuarine nutrient cycling: the influence of primary producers. Kluwer Academic Publishers, Dordrecht, The Netherlands, p 187–216

Boynton WR, Murray L, Hagy JD, Stokes C, Kemp WM (1996) A comparative analysis of eutrophication patterns in a temperate coastal lagoon. Estuaries 19:408–421

Giordano JCP, Brush MJ, Anderson IC (2011) Quantifying annual nitrogen loads to Virginia's coastal lagoons: Sources and water quality response. Estuaries Coasts 34:297–309

Greiner JT, McGlathery KJ, Gunnell J, McKee BA (2013) Seagrass restoration enhances "blue carbon" sequestration in coastal waters (J Cebrian, Ed.). PLoS ONE 8:e72469

Hansen J, Reidenbach M (2012) Wave and tidally driven flows in eelgrass beds and their effect on sediment suspension. Mar Ecol Prog Ser 448:271–287

Mateo MA, Romero J, Pérez M, Littler MM, Littler DS (1997) Dynamics of millenary organic deposits resulting from the growth of the Mediterranean seagrass *Posidonia oceanica*. Estuar Coast Shelf Sci 44:103–110

McGlathery KJ, Christian R (2017) Water quality sampling - integrated measurements for the Virginia Coast, 1992-2017. Virginia Coast Reserve Long-Term Ecological Research Project Data Publication doi:10.6073/pasta/8783dd64be7cae3a36a864ac5b5129e1

McGlathery K, Reynolds L, Cole L, Orth R, Marion S, Schwarzschild A (2012) Recovery trajectories during state change from bare sediment to eelgrass dominance. Mar Ecol Prog Ser 448:209–221

McGlathery KJ (2008) Seagrass habitats. In: Nitrogen in the marine environment. Elsevier, New York, p 1037–1072

McGlathery K, Sundbäck K, Anderson I (2007) Eutrophication in shallow coastal bays and lagoons: The role of plants in the coastal filter. Mar Ecol Prog Ser 348:1–18

Middleburg JJ, Soetaert K, Herman PMJ, Boschker HTS, Heip CR (2004) Burial of nutrient in coastal sediments: The role of primary producers. In: Esutarine Nutrient Cycling: The Influence of Primary Producers. Kluwer Academic Publishers, Dordrecht, The Netherlands, p 217–230

Oreska MPJ, McGlathery KJ, Porter JH (2017) Seagrass blue carbon spatial patterns at the meadow-scale (J Cebrian, Ed.). PLOS ONE 12:e0176630

Orth RJ, Carruthers TJB, Dennison WC, Duarte CM, Fourqurean JW, Heck KL, Hughes AR, Kendrick GA, Kenworthy WJ, Olyarnik S, Short FT, Waycott M, Williams SL (2006) A global crisis for seagrass ecosystems. BioScience 56:987–996

Orth R, McGlathery K (2012) Eelgrass recovery in the coastal bays of the Virginia Coast Reserve, USA. Mar Ecol Prog Ser 448:173–176

Orth R, Moore K, Marion S, Wilcox D, Parrish D (2012) Seed addition facilitates eelgrass recovery in a coastal bay system. Mar Ecol Prog Ser 448:177–195

Pedersen MF, Nielsen SL, Banta GT (2004) Interactions between vegetation and nutrient dynamics in coastal marine ecosystems: An introduction. In: Estuarine nutrient cycling: the influence of primary producers. Kluwer Academic Publishers, Dordrecht, The Netherlands, p 1–16

Ralph PJ, Tomasko D, Moore K, Seddon S, Macinnis-Ng CMO (2006) Human Impacts on Seagrasses: Eutrophication, Sedimentation, and Contamination. In: Seagrasses: Biology, Ecology, and Conservation. Springer, Dordrecht, Netherlands, p 567–593

Rheuban JE, Berg P, McGlathery KJ (2014) Ecosystem metabolism along a colonization gradient of eelgrass (*Zostera marina*) measured by eddy correlation. Limnol Oceanogr 59:1376–1387

Risgaard-Petersen N (2004) Denitrification. In: Estuarine nutrient cycling: the influence of primary producers. Kluwer Academic Publishers, Dordrecht, The Netherlands, p 263–280

Romero J, Lee K, Pérez M, Mateo MA, Alcoverro T (2006) Nutrient dynamics in seagrass ecosystems. In: Seagrasses: Biology, ecology, and conservation. Springer, The Netherlands, p 227–254

Seitzinger SP, Harrison JA (2008) Land-Based Nitrogen Sources and Their Delivery to Coastal Systems. In: Nitrogen in the Marine Environment, 2nd edn. Elsevier, New York, p 469–510

Seitzinger S, Harrison JA, Böhlke JK, Bouwman AF, Lowrance R, Peterson B, Tobias C, Drecht GV (2006) Denitrification across landscapes and waterscapes: A synthesis. Ecol Appl 16:2064–2090

Small C, Nicholls RJ (2003) A global analysis of human settlement in coastal zones. J Coast Res 19:584–599

Stanhope JW, Anderson IC, Reay WG (2009) Base Flow Nutrient Discharges from Lower Delmarva Peninsula Watersheds of Virginia, USA. J Environ Qual 38:2070

Waycott M, Duarte CM, Carruthers TJ, Orth RJ, Dennison WC, Olyarnik S, Calladine A, Fourqurean JW, Heck KL, Hughes AR, others (2009) Accelerating loss of seagrasses across the globe threatens coastal ecosystems. Proc Natl Acad Sci 106:12377–12381

Welsh DT (2000) Nitrogen fixation in seagrass meadows: Regulation, plant–bacteria interactions and significance to primary productivity. Ecol Lett 3:58–71

Chapter 2: Push-pull incubation method reveals the importance of denitrification and DNRA in seagrass root zone

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Abstract

In this study, we developed a push-pull incubation method to measure denitrification and dissimilatory nitrate reduction to ammonium (DNRA) in subsurface sediments of subtidal seagrass meadows. This subtidal push-pull technique, adapted from a push-pull method developed for intertidal salt marshes, used mini-piezometers to directly sample porewater in the root zone of the seagrass during an in situ incubation. The porewater was amended with $^{15}NO_3^-$ and $Ar_{(g)}$ as a tracer gas, and the denitrification products (^{28}, ^{29}, and $^{30}N_{2(g)}$) were measured with membrane inlet mass spectrometry (MIMS), using the Ar tracer to correct for dilution and gas loss. Production of $^{15}NH_4^+$ was also measured using hypobromite oxidation and MIMS to determine rates of DNRA. Using this new technique, subsurface rates of denitrification and DNRA were determined to be 17.5 μmol N m^{-2} h^{-1} and 14.7 μmol N m^{-2} h^{-1} respectively for a restored *Zostera* meadow. Rates showed substantial spatial variability, likely due to both heterogeneous conditions in the root zone sediment and variable in situ conditions. When compared to traditional core and slurry incubations, push-pull rates were greater and more variable, suggesting that the push-pull technique more accurately captures heterogeneity and the natural range of denitrification and DNRA in subsurface sediments under field conditions. In vegetated systems with low water column nitrate concentrations, the majority of denitrification and DNRA occurs below the sediment surface, and the subtidal push-pull technique provides an effective method to assess these subsurface rates.

Introduction

Shallow coastal bays are critical sites for nitrogen cycling. Denitrification, the microbially mediated transformation of nitrate into dinitrogen gas, has long been considered the dominant dissimilatory process of nitrate removal from coastal ecosystems. However, dissimilatory nitrate reduction to ammonium (DNRA) can be an important competing pathway of nitrate reduction (Risgaard-Petersen 2004, Burgin & Hamilton 2007, Giblin et al. 2013, Murphy et al. 2016). DNRA is performed by heterotrophs via a fermentative pathway, where nitrate reduction is paired with the oxidation of reduced carbon, and by chemolithoautotrophs, which pair nitrate reduction with sulfide or iron oxidation. Sulfate-reducing bacteria may also use nitrate as an alternate electron acceptor, thus performing DNRA as a respiratory process (Dalsgaard & Bak 1994). Since DNRA conserves reactive nitrogen as ammonium while denitrification leads to the release of inert nitrogen (N_2) gas, the balance between these two processes affects the net flux of fixed nitrogen in sediments and in turn the net impact of nitrogen inputs on coastal systems.

Seagrass meadows are potential hotspots for both denitrification and DNRA. Previous studies have found a range of denitrification rates in seagrass meadows, from low rates of 0-5 μmol N m^{-2} h^{-1} at some sites (e.g. Ottosen et al. 1999, Welsh et al. 2001) to rates as high as 500 μmol N m^{-2} h^{-1} at other sites (e.g. Eyre et al. 2011, Smyth et al. 2013). DNRA rates have been less commonly measured but appear to have a similar range to denitrification (An & Gardner 2002, Smyth et al. 2013). A suite of factors, including temperature, C:N ratio, and sulfide concentration influence the relative importance of these competing nitrate reduction pathways in seagrass meadow sediment.

Conditions in the subsurface sediments of the seagrass root zone are critical drivers of both denitrification and DNRA. Seagrass roots oxygenate the rhizosphere (Borum et al. 2006, Frederiksen & Glud 2006), creating oxic microzones conducive to nitrification and potentially increasing the supply of nitrate for both denitrification and DNRA (Caffrey & Kemp 1990, Soana et al. 2015). Radial oxygen loss from roots also decreases sediment sulfide concentrations (Pagès et al. 2012), potentially enhancing denitrification and decreasing chemolithoautotrophic DNRA (Brunet & Garcia-Gil 1996). Seagrass ecosystems accumulate sediment carbon through increased sedimentation and the release of carbon exudates from living roots (Kaldy 2012, Greiner et al. 2013). This sediment carbon may support nitrate reduction since reduced carbon substrate is necessary for both denitrification and fermentative DNRA, and a high C:N ratio may enhance both fermentative and chemolithoautotrophic DNRA (Tiedje et al. 1982, Burgin & Hamilton 2007).

Despite the importance of subsurface sediments, current methods do not effectively measure subsurface nitrate reduction processes under field conditions. Techniques used to measure nitrate reduction in seagrass meadows include slurry incubations (Caffrey & Kemp 1990, Hou et al. 2012), static core incubations (Rysgaard et al. 1996, Welsh et al. 2000, 2001, Russell et al. 2016), flow-through cores (An & Gardner 2002, Gardner et al. 2006), perfusion cores (Risgaard-Petersen et al. 1998, Ottosen et al. 1999), and benthic chambers (Risgaard-Petersen et al. 1998, Eyre et al. 2011, 2013). Isotope additions of either $^{15}NO_3^-$ or $^{15}NH_4^+$ are commonly used; the isotope pairing technique or IPT (Nielsen 1992) uses $^{15}NO_3^-$ additions to differentiate between denitrification of nitrate derived from the water column and from coupled nitrification in the surface sediments. Measurements of net N_2 fluxes without isotope additions are also used with both cores and chambers (Ferguson et al. 2004, Smyth et al. 2013, Eyre et al.

2013). Each method has its own strengths and weaknesses (see Cornwell et al. 1999 and Steingruber et al. 2001 for more complete comparisons); however, none of these techniques both preserve conditions in the field and directly account for subsurface rates.

Slurry incubations can be used to directly measure subsurface rates but only under non-ambient conditions. In a slurry incubation, a small amount of sediment is sealed in a glass vial, labeled nitrate ($^{15}NO_3^-$) is added and changes in N_2 concentrations over time are used to measure denitrification rates. However, the mixing of the slurry increases nitrate availability, removes biogeochemical gradients, and eliminates processes such as seagrass root activity, bioturbation, and porewater advection. Nitrate reduction rates measured in slurries are therefore considered potential rates under optimal conditions for denitrifiers and DNRA-capable microbes, rather than representative of in situ rates (Behrendt et al. 2013). These potential rates are useful, especially for relative comparisons, but have limited applicability to field conditions.

In contrast, benthic chambers do preserve some field conditions, such as the sediment structure and gradients, but do not directly measure subsurface rates. When nitrate reduction occurs at depth, ammonium from DNRA will adsorb to sediment particles and N_2 produced via denitrification may not be transported to the surface water within the incubation period. An endpoint sediment slurry is therefore necessary when deploying benthic chambers but is not always feasible (Steingruber et al. 2001). Additionally, when using chambers in conjunction with the IPT, a long equilibration time is required to allow the $^{15}NO_3^-$ to diffuse from the surface water into subsurface denitrification zones (Nielsen & Glud 1996). Furthermore, benthic chambers necessarily disrupt factors such as the flow regime that alter sediment conditions and in turn microbial activity. For example, benthic chambers equipped with rotors to simulate ambient flow conditions create artificial radial pressure effects that alter oxygenation of

macrofauna burrows (Webb & Eyre 2004). Thus, although they preserve the structural integrity of the seagrass meadow, chambers do alter field conditions and do not fully capture rates in the subsurface sediments.

Like benthic chambers, core incubations preserve some field conditions compared to slurries but do not effectively measure rates in the subsurface sediments. As with chambers, the use of isotope tracers added to the water column in core incubations presents a challenge, given the long time period that must elapse for $^{15}NO_3^-$ additions to diffuse into the subsurface sediments to the depth of the root zone (Cornwell et al. 1999, Groffman et al. 2006). Perfusion cores, in which the porewater is extracted under a vacuum, amended with the isotope addition, and perfused back into the sediment, have been used to address this issue (Risgaard-Petersen et al. 1998), but other limitations introduced by core incubations remain. The act of taking a core, for example, can damage belowground biomass, leading to the release of DOC, which may serve as a substrate for nitrate reduction (Hansen & Lomstein 1999, Gribsholt & Kristensen 2002). In addition, the altered (and typically reduced) flow available in core incubations may significantly affect photosynthetic activity by the seagrass (Koch et al. 2006), again leading to changes in the biogeochemical conditions in the root zone (Koch 1999). Flow-through systems, in which cores are incubated under a continuous flow of water, maintain better flow compared to static core incubations, and subsurface nitrate reduction products will eventually equilibrate with surface concentrations if the incubation is run for sufficient time (Steingruber et al. 2001). However, seagrass shoots are often defoliated or excluded from flow-through core incubations (e.g. Gardner et al. 2006), and the collection of cores for flow-through incubations will also damage belowground biomass. Cores thus provide an imperfect measure of subsurface nitrate reduction rates because they exclude or underestimate plant effects.

Koop-Jakobsen and Giblin (2009), building on work by Addy et al. (2002) and Nielsen (1992), developed the push-pull isotope pairing technique (push-pull) for measuring subsurface denitrification in the root zone of intertidal salt marshes. A mini-piezometer is inserted into the sediment and porewater is pumped out using a peristaltic pump, amended with $^{15}NO_3^-$ and dissolved argon (Ar) as a tracer gas, returned to the sediment, and allowed to incubate in situ. Several samples are taken over time to measure rates of N_2 production, and isotope pairing equations are used to calculate denitrification rates. The push-pull technique demonstrates several advantages over traditional techniques. First, by amending and sampling the porewater directly, the technique captures denitrification rates in the subsurface sediment. Second, there is no interference with field conditions such as flow and light availability. The minimal disturbance maintains the natural effects of vegetation on biogeochemical conditions in the root zone. Calculated rates are therefore more representative of true in situ conditions compared to traditional techniques.

These advantages make the push-pull technique particularly suited to the subtidal seagrass environment. Hydrodynamic flow is a critical physical driver of metabolism in seagrass meadows (Koch et al. 2006) and is difficult to replicate in laboratory incubations. The push-pull technique effectively maintains natural hydrodynamic flow and its effects on porewater advection and seagrass photosynthesis. Similarly, minimal disruption to the seagrass reduces impacts on sediment biogeochemistry. The push-pull technique is especially appropriate for seagrass meadows in low-nutrient environments since the dominant pathway of denitrification is coupled to nitrification in sediments (Cornwell et al. 1999, Soana et al. 2015).

However, the push-pull technique requires refinement in two areas in order to be effective in seagrass sediments. First, in sandy coastal sediments, advective transport dominates,

compared to diffusive transport in muddy marsh sediment (Huettel et al. 2014). Numerous processes, including bioirrigation, flow-topography interactions, wave pumping, current shear, and gas bubble upwelling drive advective porewater exchange on the centimeter scale (Santos et al. 2012), potentially limiting the recovery of the Ar tracer and denitrification products. Second, the greater depth of overlying water in subtidal compared to intertidal systems presents a practical challenge requiring modifications to the experimental set-up. In addition to these modifications, the original push-pull technique did not provide a way to measure DNRA in the subsurface sediments. Development of an appropriate push-pull technique for subtidal, sandy sediments will allow researchers to accurately measure both denitrification and DNRA in the seagrass root zone, ultimately improving our understanding of nitrogen cycling in these important coastal ecosystems.

The purpose of this study was to develop the push-pull technique for use in subtidal seagrass sediments and to expand the technique to include measurements of DNRA. Field tests of a modified push-pull technique were conducted in a restored *Zostera marina* meadow in the Virginia coastal bays, and results were compared with rates measured in traditional slurry and core incubations.

Methods

Study site

Field measurements were conducted in a restored seagrass meadow in South Bay, a shallow lagoon in the Virginia Coast Reserve Long-Term Ecological Research Site (VCR LTER) on Virginia's Eastern Shore. South Bay has a semidiurnal tidal cycle, with a mean tidal range of 1.2 m and a mean water depth of approximately 1.4 m (Fagherazzi & Wiberg 2009). Nutrient loading to the VCR coastal bays is low (McGlathery & Christian 2017). Large-scale seagrass

restoration was begun in these bays in 2001 with the broadcasting of 10^6 seeds in 0.4 ha plots (Orth et al. 2010, 2012). In South Bay, these original plots eventually coalesced into a contiguous subtidal meadow, roughly 6.8 km^2 in 2015. Sediments are sandy with low organic matter content, although carbon, nitrogen, and organic matter content of the sediment are higher in the seagrass meadow compared to adjacent unvegetated sediment (McGlathery et al. 2012).

Denitrification and DNRA were measured using the push-pull technique during summer of 2014 and in June 2015. Measurements were made in the interior of the meadow, at one of the original 0.4 ha plots in 2014 and at three of the original plots in 2015. Up to four individual push-pull incubations were conducted within an area approximately 3 m^2 during each deployment; multiple deployments were carried out to collect 10 replicate incubations each summer. All push-pull incubations were conducted during a six-hour window bracketing low tide; the time of the deployments therefore varied with the tidal cycle from 08:00-14:00 to 11:00-17:00.

Subtidal push-pull technique

The experimental set-up and procedures for the subtidal push-pull method are similar to the original push-pull technique. The goal of this study was not to retest the details of the original technique but to make suitable adjustments for using the technique in subtidal sediments. The procedures outlined below therefore focus on adaptations of the original method. Detailed descriptions of the original push-pull method can be found in Koop-Jakobsen and Giblin (2009).

Experimental set-up

For each subtidal push-pull incubation, a miniature piezometer (2.4 mm outer diameter, 1.8 mm inner diameter) was inserted into the sediment to a depth of 5 cm, which was the depth of maximum seagrass root biomass. The piezometers were made of stainless steel tubing, with

one end closed off and a series of holes (0.38 mm diameter) drilled in the lower 2 cm of the closed end. The small piezometer size minimized damage to plant roots and rhizomes. An acrylic plate (15 x 15 x 2.5 cm) was used to stabilize each piezometer in the sediment. Each plate had four legs (small acrylic supports) attached to the corners; these legs were inserted into the sediment and held the plate in place at the sediment surface (Berg & McGlathery 2001). A set of holes (3 mm diameter) drilled in the center of the plate allowed the piezometer to be inserted through the plate into the sediment. A small piece of tubing, snuggly fitting on the piezometer but too large to fit through the holes in the plate, was used as a depth marker (Berg & McGlathery 2001). By placing the tubing 7.5 cm from the tip of the piezometer, the piezometer could be inserted to a depth of 5 cm and was prevented from moving deeper into the sediment.

The piezometers were connected via low-permeability Viton tubing (2.79 mm inner diameter) to a peristaltic pump (Masterflex C/L Dual Channel). The pump was held at the surface on an inflatable raft, which was anchored in place horizontally but allowed to move vertically. Sufficient tubing (approximately 180 cm) was used to allow for the movement of the raft (and pump) on the falling and rising tides without disturbing the piezometers.

Porewater extraction and amendment

Approximately 220 mL of porewater was pumped out of the sediment into a 250 mL graduated cylinder that served as a reservoir (Figure 2.1), with an additional 4 mL of porewater held in the tubing. This volume was sufficient for the collection of duplicate 15 mL samples over six time-points. In the seagrass meadow, the sediment porosity was 0.53 (volume of porewater/volume of sediment), so 220 mL corresponded to 415 cm^3 of sediment. Under ideal conditions, the extraction of porewater in the push-pull technique would correspond to a sphere centered at the injection point; with a volume of 415 cm^3, the radius of the sphere would be 4.63

cm. This radius is less than the 5 cm depth of the piezometer tip, thus the extraction captured the porewater in the root zone but avoided pulling in surface water.

During the extraction, the pumping speed was maintained at a slow 4 mL min^{-1} to minimize disturbance to hydraulic conductivity gradients; the total extraction time was about 1 h. A layer of 20 mL of castor oil was added to the reservoir at the beginning of the extraction to prevent exchange between the porewater and the atmosphere. Immediately after porewater extraction, duplicate background samples were collected by overfilling 12 mL Exetainer vials. The vials were filled from the bottom and allowed to overfill by 25% (3 mL) in order to minimize gas exchange with the atmosphere. Samples were fixed with 50 μL of $ZnCl_2$ (100% m/v), capped with gas-tight septa, and stored underwater. An additional 10 mL background sample was collected for analysis of dissolved nitrate; this sample was filtered (0.45 μm) and stored on ice.

After background sampling, 10-20 mL of artificial seawater spiked with $^{15}NO_3^-$ (99% ^{15}N, Cambridge Laboratories) were added to the reservoir and mixed with the porewater, bringing the concentration of NO_3^- in the porewater to approximately 100 μM. The spike solution was saturated with Ar gas, which is biologically inert and served as a gas tracer; the concentration of Ar in the porewater after spiking was approximately 40 μM, compared to 11 μM in the background samples. Spike solutions were prepared in the laboratory by bubbling with Ar and were transported to the field in gas-tight vials stored underwater. Duplicate post-spike samples were again collected in overfilled 12 mL Exetainer vials and fixed with 50 μL of $ZnCl_2$ (100% m/v), as well as a post-spike nitrate sample, after which the remaining ~150 mL of porewater, corresponding to 283 cm^3 of sediment, were slowly (~4 mL min^{-1}) pumped back into the sediment over 30-40 min and then allowed to incubate in situ.

Incubation

Duplicate samples were collected at half-hour intervals, beginning 30 minutes after the completion of the "push" of the spiked porewater into the sediment. Four sets of duplicate samples were collected during the incubation, which lasted 1.5-2 hours. In a standard incubation, in which ~150 mL of amended porewater was returned to the sediment, a total of 120 mL was recovered. In order to minimize exposure to the atmosphere, samples were slowly pumped into 30 mL syringes and then transferred to the Exetainers when sufficient volume had been recovered. Each set of duplicate samples corresponded to 56.6 cm^3 of sediment. The first 4 mL pumped out, corresponding to the volume of the tubing and piezometer, were discarded before each set of samples was collected. The full set of samples was stored underwater below the field temperature to prevent bubble formation for 4-6 weeks until analysis for dissolved ^{28}, ^{29}, and $^{30}N_2$ and Ar gas concentrations, using membrane inlet mass spectrometry (MIMS; see below).

The length of the incubation was primarily limited by tracer dilution. The Ar tracer measures dilution due to mixing with unspiked porewater and loss from gas exchange across seagrass roots and rhizomes. Dye tests in the lab showed some channeling of the injected porewater in the non-homogeneous sediment. Recovery of the argon tracer diminished rapidly (Figure 2.2), due to the uneven pattern of spread and large area of mixing and tracer dilution. Incubation times were therefore limited by the need for a tracer concentration above background levels to correct for dilution of gaseous N_2 products of denitrification.

Although the 1.5-2 h incubation time is less than in the original push-pull method (~6-7 h), production of ^{29} and $^{30}N_2$ was observed in this time frame which allowed for calculation of denitrification rates. Corrections to the production rates for gas loss and for changes in the background N_2 concentration due to advection and other factors increased the linearity and

magnitude of the production rates (Figure 2.3; the corrections are explained in detail below). Tests showed a delay in production between the first and second samples, caused by the time required to "push" the spiked porewater into the sediment. The initial sample (taken at time zero, immediately after adding the spike and before the "push") was therefore excluded from the calculation of production rates.

Sediment sampling for DNRA

Since ammonium produced via DNRA from the $^{15}NO_3^-$ spike was expected to be adsorbed to sediment particles, as well as dissolved in porewater, a sediment sample was collected at the end of the incubation for $^{15}NH_4^+$ analysis. The piezometer and acrylic plate were removed and a small sediment core (2.54 cm diameter, 10 cm depth) was collected from the point of injection. The depth of the core exceeded the maximum depth of the incubation, in order to fully capture any DNRA occurring at depth (see below for additional discussion of incubation depth). The core was frozen for $^{15}NH_4^+$ analysis using the oxidation/MIMS (OX/MIMS) method, as described below.

Sample analysis and calculations

Membrane inlet mass spectrometry (MIMS)

Samples collected from the subtidal push-pull incubations were analyzed for concentrations of $^{28}N_2$, $^{29}N_2$, $^{30}N_2$, and Ar gases using membrane inlet mass spectrometry (Kana et al. 1994, 1998). In the MIMS set-up, a vacuum pump extracted the dissolved gases from the water samples across a silicone membrane; the gas analyte then passed through a liquid nitrogen cryotrap to remove carbon dioxide and water vapor, a copper reduction column heated to 500 ºC to remove oxygen (Eyre et al. 2002), and a second cryotrap before ionization and detection by a quadropole mass spectrometer (Pfeiffer Balzers Prisma QMS 200). Oxygen removal via the

copper reduction column was critical due to interference with other gas signals that can cause overestimation of denitrification rates calculated from isotope-pairing equations (Eyre et al. 2004, Lunstrum & Aoki 2016). Analytical precision for the MIMS measurements was high (CV<0.07%).

Corrections to $^{29}N_2$ and $^{30}N_2$ concentrations

Production rates of $^{29}N_2$ and $^{30}N_2$ were calculated from the changes in concentration of the gases over the time period of the incubation ($\Delta[^{29}N_2]$ and $\Delta[^{30}N_2]$). However, $\Delta[^{29}N_2]$ included contributions not only from denitrification but also from mixing with ambient porewater, impurities in the spike, and gas loss through diffusion and root transport. Equations from Koop-Jakobsen and Giblin (2009) were therefore used to account for these non-denitrification components of $\Delta[^{29}N_2]$. Similarly, $\Delta[^{30}N_2]$ was corrected for gas loss, but not for mixing, as changes in $[^{30}N_2]$ due to denitrification exceeded changes due to mixing by at least an order of magnitude. Impurities in the spike also did not affect $\Delta[^{30}N_2]$. The calculations used to correct $\Delta[^{29}N_2]$ and $\Delta[^{30}N_2]$ were derived in detail by Koop-Jakobsen and Giblin (2009) and are summarized briefly below.

To isolate the changes due to mixing of the amended porewater with the ambient, unlabeled porewater, $[^{29}N_2]$ was compared to $[^{28}N_2]$. Because ambient nitrate in the system was very low (<0.04 μM) and the input of $^{15}NO_3^-$ was high (~100 uM), denitrification in the amended porewater was assumed to predominantly affect $^{29}N_2$ and $^{30}N_2$. Changes in the concentration of $^{28}N_2$ in the amended porewater due to denitrification were assumed to be negligible. Therefore, the measured concentration of $^{29}N_2$ was corrected for mixing as follows:

$$\Delta[^{29}N_2]_{corr1} = [^{29}N_2]_{sample} - [^{29}N_2]_{T0} \times \frac{[^{28}N_2]_{sample}}{[^{28}N_2]_{T0}} \quad (1)$$

where the "T0" subscript refers to the measured concentration of the gas immediately after the addition of the spike and the "sample" subscript refers to the measured concentration of the gas at each subsequent time point.

The value of $\Delta[^{29}N_2]_{corr1}$ was then further corrected to account for impurities in the spike. While 99% of the added nitrate was $^{15}NO_3^-$, 1% was $^{14}NO_3^-$, which would support the production of $^{29}N_2$ in the amended porewater. The contribution of impurities to the net change in $^{29}N_2$ was corrected according to the follow equation:

$$\Delta[^{29}N_2]_{corr2} = \Delta[^{29}N_2]_{corr1} - \left[\frac{(2\ x\ f^{14}N x f^{15}N)}{(f^{15}N)^2} x[^{30}N_2]_{sample}\right] \quad (2)$$

where $f^{15}N$ and $f^{14}N$ are the fractions of ^{15}N and ^{14}N in the spike material and $[^{30}N_2]_{sample}$ is the measured concentration of $^{30}N_2$ at each sampling time point. This correction relies on the assumption of random mixing between ^{15}N and ^{14}N derived from the spike in the amended porewater.

The value of $\Delta[^{29}N_2]_{corr2}$ was then finally corrected for gas loss using the recovery of the Ar gas tracer as follows:

$$\Delta[^{29}N_2]_{corr3} = \Delta[^{29}N_2]_{corr2} \times \frac{([Ar]_{T0} - [Ar]_{background})}{([Ar]_{sample} - [Ar]_{background})} \quad (3)$$

where $[Ar]_{T0}$ is the concentration of Ar after the addition of the spike, $[Ar]_{background}$ is the concentration of Ar in the ambient porewater, and $[Ar]_{sample}$ is the concentration of Ar at each sampling time point. Following these corrections, the values of $\Delta[^{29}N_2]_{corr3}$ for each time point were used to calculate the production rate, p_{29}.

As stated above, $\Delta[^{30}N_2]$ was corrected for gas loss only, using the equation:

$$\Delta[^{30}N_2]_{corr} = \Delta[^{30}N_2]_{sample} \times \frac{([Ar]_{T0} - [Ar]_{back\square round})}{([Ar]_{sample} - [Ar]_{background})} \quad (4)$$

Values of $\Delta[^{30}N_2]_{corr}$ over time were used to calculate the production rate, p_{30}.

Calculation of denitrification rates

Standard isotope pairing equations (Nielsen 1992) were used to calculate denitrification rates from the production of ^{29} and $^{30}N_2$ (p_{29} and p_{30}) as follows:

$$D_{15} = p_{29} + (2 \times p_{30}) \quad (5)$$

$$D_{14} = D_{15} \times \frac{p_{29}}{(2 \times p_{30})} \quad (6)$$

In the IPT equations, D_{15} corresponds to the denitrification of $^{15}NO_3^-$ from the spike while D_{14} is the denitrification of $^{14}NO_3^-$. D_{15} can also be considered the potential denitrification rate, or the maximum denitrification rate with excess nitrate availability. In contrast, D_{14} is considered the ambient rate, or the underlying rate of denitrification in the absence of the $^{15}NO_3^-$ spike. The $^{14}NO_3^-$ that supports ambient denitrification may be present in porewater or produced via nitrification. In this system, porewater concentrations of nitrate were undetectable (<0.04 μM), suggesting that denitrification in the subsurface sediments was coupled to nitrification. Nitrate concentrations in the overlying water were also very low (Table 2.1), and so diffusion into subsurface sediments during the incubation period was not a factor. Therefore additional isotope pairing calculations that differentiate between nitrate supplied from the water column and from coupled nitrification were not applied.

Because ambient denitrification in this system is coupled to nitrification, the push-pull technique relies on the assumption that the introduction of the $^{15}NO_3^-$ spike does not alter the ambient nitrification rate, particularly through the addition of oxygen. Although the castor oil cap and low permeability Viton tubing were used to minimize diffusion of oxygen into the porewater during handling, the graduated cylinder used as a reservoir was not gas-tight and it is likely that oxygen was enhanced in the amended porewater relative to background levels. This

would lead to enhanced nitrification rates and could artificially enhance the ambient denitrification measured via IPT. However, this oxygenation effect was likely small. Nitrate concentrations in the amended porewater diminished rapidly during the incubation and returned to the undetectable background levels partway through the incubation (Figure 2.4). Oxygen concentrations were not measured, so the exact magnitude of this effect could not be quantified; however, the lack of measurable nitrate (at a detection level of 0.04 μM) suggests that nitrification was not greatly enhanced.

The rapid depletion of nitrate was temporally offset from the production of denitrification products, which may indicate the presence of colorless sulfur bacteria in the seagrass sediment. These bacteria, which can couple the reduction of nitrate to the oxidation of sulfide, include the so-called "big bacteria" (e.g. *Beggiatoa* spp.) which store nitrate in their vacuoles and migrate between oxic and anoxic zones in the sediment, allowing them to conduct a form of denitrification far from sources of nitrate (Jorgensen & Postgate 1982, Schulz & Jørgensen 2001, Burgin & Hamilton 2007). The presence of these bacteria could explain the offset between the depletion of the nitrate spike (Figure 2.4) and the production of N_2 (Figure 2.3). Alternatively, uptake of nitrate by competitors, such as benthic microalgae, could explain the rapid removal of nitrate from the porewater.

Previous studies have shown that IPT measurements can underestimate denitrification rates when compared to direct measurements of $^{28}N_2$ fluxes. This underestimation is typically attributed to incomplete diffusion of the $^{15}NO_3^-$ tracer into nitrate reduction zones during IPT core incubations (van Luijn et al. 1996, Ferguson & Eyre 2007). In the push-pull method, unlike traditional IPT incubations, the tracer is thoroughly mixed with the porewater and redistributed throughout the sediment; therefore, underestimation due to incomplete diffusion is likely not an

issue. In fact, it is more likely that the push-pull rates overestimate true denitrification rates, as microbes throughout the sediment matrix are exposed to the nitrate spike. However, as noted above, the undetectable levels of nitrate during the incubation suggest nitrification was not strongly enhanced, and therefore the ambient rates of coupled denitrification were also not likely strongly enhanced. Unfortunately, a comparison between the push-pull rates and direct $^{28}N_2$ fluxes was not possible in this study, as the addition of the Ar-sparged spike diluted the background $^{28}N_2$ concentrations. However, additional discussion of the accuracy of the ambient push-pull rates is included in the Assessment section below.

Conversion to areal rates

D_{15} and D_{14} rates from the IPT equations were calculated in μmol N L^{-1} hr^{-1}. These rates were multiplied by the sediment porosity to produce a bulk sediment denitrification rate in μmol N cm^{-3} hr^{-1}. This bulk rate applies to the volume of sediment affected by the spiked porewater, which can be calculated from the volume of spiked porewater injected and the sediment porosity. Because the corrections for gas loss and mixing account for tracer loss as well as changes in the background concentration of N_2, the bulk denitrification rate applies regardless of the distribution of the spiked porewater in the sediment.

However, in order to convert the bulk rate to an areal rate, the bulk rate must be integrated over the depth of sediment affected by the spiked porewater. Under ideal circumstances, the porewater distribution corresponds to a sphere centered at the injection point, and the total depth would be 5 cm, the depth of the piezometer, plus the radius of the ideal sphere (r_{ideal}), calculated from the volume of porewater returned to the sediment. Sediment heterogeneity likely led to non-ideal conditions, so dye tests were conducted in the laboratory to assess the porewater distribution and the depth of the incubation.

For the dye tests, sediment cores were collected in the seagrass meadow to a depth of 20 cm using acrylic cores with a 10 cm inner diameter. In the lab, 50 mL of artificial seawater colored with rhodamine dye were injected into the cores using the piezometer, tubing and pumps as in the field measurements. The sediment was then extruded and sliced vertically at the injection point to observe the pattern of dye distribution. Some horizontal channeling of the dye was observed, with rapid dye movement to the edges of the core; however, the dye did form a roughly spherical plume at the injection point with a radius approximately 0.7 x r_{ideal}. This result was used to determine the depth of each push-pull incubation; r_{ideal} was calculated from the volume of amended porewater returned to the sediment, and the depth was calculated as 5 cm plus 0.7 x r_{ideal}. Bulk rates were then converted to areal rates by integrating over this depth for each push-pull incubation.

Compared to the conditions in the field, the conditions of the dye tests may have enhanced horizontal channeling. The rigid bottom of the core likely reduced dye travel downward from the injection point and instead encouraged horizontal movement and channeling to the surface. Some loss of dye to the water column was observed during the tests, and this was also likely enhanced compared to the field measurements, due to the lack of the stabilizing plate at the sediment surface, which would serve as a barrier to loss to the water column. The dye test result of a plume radius of 0.7 x r_{ideal} is therefore a conservative estimate of the spiked porewater distribution in the field. The conversion to areal rates using 0.7 x r_{ideal} therefore likely underestimate true areal rates.

Anammox and DNRA interference with IPT

Prior to the push-pull field tests, slurry incubations were used to test for the presence of anaerobic ammonium oxidation (anammox). The IPT equations require modifications if

anammox and denitrification co-occur, since both processes produce $^{29}N_2$ (Risgaard-Petersen et al. 2003). Recent work also suggests that if both anammox and DNRA contribute significantly to nitrate reduction, additional corrections may be needed (Song et al. 2016). However, in this study system, anammox rates were found to be less than 1% of denitrification rates (data not shown). Corrections due to anammox and/or DNRA interference were therefore not included and the original IPT equations were applied in the push-pull measurements.

It is also possible that high rates of potential DNRA (reduction of the added $^{15}NO_3^-$) would enrich the ammonium pool in $^{15}NH_4^+$, reducing the probability of nitrification of the ambient $^{14}NH_4^+$ and therefore underestimating the ambient D_{14} rate. Ammonium concentrations in the porewater were on average 12.3 μM, which was about three times the amount of $^{15}NH_4^+$ produced from DNRA over the course of an incubation. Enrichment of the ammonium pool was therefore possible. However, no relationship was observed between the rate of potential DNRA and D_{14} or between the bulk concentration of ammonium and D_{14}. Ammonium enrichment was therefore considered negligible.

OX/MIMS

To measure DNRA, we modified the OX/MIMS method developed by Yin et al. (2014). In the original OX/MIMS method, $^{15}NH_4^+$ production is measured by oxidizing $^{15}NH_4^+$ to ^{29} and $^{30}N_2$ through the addition of hypobromite (BrO^-). The $^{29}N_2$ and $^{30}N_2$ concentrations are then measured using MIMS and converted to $^{15}NH_4^+$ concentrations by comparison with a standard curve. However, this approach requires the degassing of samples before oxidation to remove any ambient $^{29}N_2$ and $^{30}N_2$. To avoid this step, which becomes laborious with multiple samples, we used a paired vial approach. Replicate vials were prepared; half the vials received the BrO^- reagent and half remained unoxidized. The concentrations of $^{29}N_2$ and $^{30}N_2$ in both sets of vials

were then measured on the MIMS. The excess $^{29}N_2$ and $^{30}N_2$ in the oxidized vials was assumed to result from $^{15}NH_4^+$ as the product of DNRA.

To prepare samples for the modified OX/MIMS analysis, frozen sediment samples from the push-pull incubations were thawed and extracted with 90 mL of 2 M KCl. Samples were then centrifuged and the supernatant was filtered (0.45 μm) into five replicate 12 mL Exetainer vials. Three vials received 0.2 mL of BrO^- solution, prepared as in Yin et al. (2014). The two additional vials were not oxidized. All five samples were analyzed using MIMS; excess ^{29} and $^{30}N_2$ concentrations were calculated as the average of the unoxidized vials subtracted from the average of the oxidized vials. Variation between replicate vials was low (CV <2%) for both the oxidized and unoxidized vials. The total $^{15}NH_4^+$ concentration was then calculated according to equation 3:

$$[^{15}\mathrm{NH}_4^+] = [^{29}N_2]_{excess} + (2\times[^{30}N_2]_{excess}) \quad (7)$$

Calculation of DNRA rates

The production of $^{15}NH_4^+$ was considered to be the potential DNRA rate ($DNRA_{15}$, the maximum rate with unlimited $^{15}NO_3^-$ availability) and was calculated from the final concentration of $^{15}NH_4^+$, the porosity of the sediment, mass of the sediment core, and the duration of the incubation. The ambient DNRA rate ($DNRA_{14}$) was then calculated from $DNRA_{15}$ and the potential and ambient denitrification rates (D_{15} and D_{14}) for that incubation, according to the following relationship:

$$DNRA_{14} = DNRA_{15}\times\frac{D_{14}}{D_{15}} \quad (8)$$

This relationship relies on the assumption that the probability of reducing ^{14} or $^{15}NO_3^-$ is the same for both denitrification and DNRA (Christensen 2000). Bulk DNRA rates were then integrated over the depth of the sediment core to determine the areal DNRA rate.

Nutrient analysis

Frozen porewater samples were thawed and nitrate concentrations (combined NO_3^- and NO_2^-) were analyzed using cadmium reduction on a Lachat QuikChem 8500 with QuikChem Method 31-107-04-1-E, equivalent to EPA method 353.4 (Zhang et al. 1997). The detection limit for nitrate was 0.04 μM.

Comparison with slurry and core incubations

The push-pull measurements were compared to two traditional incubation techniques, slurry and core incubations, in which $^{15}NO_3^-$ was added and isotope pairing equations were used to calculate denitrification rates.

Slurry incubations

Three replicate sediment cores (7 cm inner diameter) were collected from one of the original 0.4 ha plots in the South Bay meadow in May 2014. Cores were transported on ice to the laboratory and were processed in an argon-filled glove bag in order to prevent oxidation of subsurface sediments. The upper 5 cm of each core were homogenized and sediment slurries were prepared by adding 30 g of sediment and ~45 mL of artificial seawater (adjusted to in situ salinity and bubbled with argon gas to remove oxygen) to 60 mL vials. Vials were capped with rubber septa and crimped with aluminum caps. Slurries were pre-incubated overnight to allow for the consumption of any remaining oxygen.

The following morning, a spike of $^{15}NO_3^-$ and excess unlabeled ammonium was added to 15 vials (5 from each of the 3 slurries). After additions, the nutrient concentrations in the vials were 30 μM for nitrate and 45 μM for ammonium. Incubations lasted 48 h, with sampling at time zero and every 12 h thereafter. At each sampling interval, 30 mL were withdrawn from each of 3 vials (one for each slurry), and 12-mL Exetainers were overfilled, fixed with 50 μL of $ZnCl_2$

(100% m/v), capped and stored underwater. The remaining sample was filtered (0.45 μm) and frozen for nutrient analysis.

Samples were analyzed using MIMS (as above) to determine the concentrations of $^{28}N_2$, $^{29}N_2$, and $^{30}N_2$. Rates of denitrification were calculated according to the equations described by Trimmer et al. (2003) and Thamdrup & Dalsgaard (2002). All slurry rates were considered potential, as conditions in the slurries are optimized for denitrification.

Core incubations

Seven sediment cores (10 cm inner diameter, 15 cm sediment depth, 15 cm overlying water) were collected in June 2015. In the laboratory, the cores were uncapped and pre-incubated overnight in a reservoir of site water held at the field temperature and bubbled with air.

The following day, four cores were wrapped in aluminum foil to be incubated in the dark, while the remaining three cores were incubated under a bank of halogen aquarium lights. Light levels were monitored using HOBO data loggers. A spike of $^{15}NO_3^-$ (dissolved in artificial seawater) was added to the overlying water, leading to a final concentration of 37 μM. The cores were left uncapped for 30 min to allow for the diffusion of the isotope label into the upper mm of sediment. The cores were then capped, and initial samples were collected to measure the concentration of N_2 gas in the overlying water. Samples were collected by overfilling 12 mL Exetainer vials and were fixed with 50 μL $ZnCl_2$ (100% m/v). Cores were incubated until oxygen levels were depleted to 70% of the initial value (approximately 5 hours). During the incubation, the overlying water was stirred by suspended magnets driven by an external set of magnets.

At the end of the incubation, the cores were opened and the upper 5 cm of sediment were rapidly mixed with the water column for 30 sec to release any N_2 gas trapped in the sediment

(Steingruber et al. 2001). Water column samples were then collected to measure denitrification rates, again by overfilling 12 mL Exetainers and fixing with $ZnCl_2$. Sub-cores of sediment (2 cm inner diameter, 15 cm depth) were collected using an acrylic tube and frozen for DNRA analysis.

As with the push-pull incubations, concentrations of ^{29} and $^{30}N_2$ gases were measured via MIMS. Denitrification rates were calculated by equations 1-2 above, and D_{14} was then divided into D_n (coupled denitrification in the surface sediment) and D_w (denitrification of water column nitrate) according to the following IPT equations:

$$D_w = D_{15} \times \frac{[^{14}NO_3^-]_w}{[^{15}NO_3^-]_w} \quad (9)$$

$$D_n = D_{14} - D_w \quad (10)$$

where $[^{14}NO_3^-]_w$ is the concentration of unlabeled nitrate in the water column and $[^{15}NO_3^-]_w$ is the concentration of labeled nitrate. Sediment sub-cores were analyzed via OX/MIMS and DNRA rates were calculated by equations 3-4 above.

Assessment

Ambient nitrate reduction rates

The push-pull technique was successfully implemented to measure both denitrification and DNRA rates in the root zone of the seagrass meadow. Integrated areal rates of in situ denitrification showed a wide range, from 0 to 106.3 μmol N m^{-2} h^{-1} over summer 2014 and 2015 (Figure 2.5). Mean rates did not vary between the two summers (Welch T-test, p =0.68), and the overall mean rate of 17.5 μmol N m^{-2} h^{-1} was significantly greater than zero (one-sample t-test, p < 0.005). Ambient DNRA rates had a similar range to denitrification, from 1.8 to 91.3 μmol N m^{-2} h^{-1}, and on average DNRA accounted for 45% of total subsurface nitrate reduction. As with denitrification, mean DNRA did not vary between the two summers (Welch T-test, p=0.99) and

the overall mean DNRA rate, 14.7 μmol N m^{-2} h^{-1}, was significantly different from zero (one-sample t-test, p <0.05).

In contrast, rates measured with the core incubations were much lower (Figure 2.5). No difference was observed between the light and dark core rates, and light intensity data from the HOBO loggers showed that the aquarium lights produced less than 10% of the light available in the seagrass meadow. The data from all cores were therefore treated as dark. In this case, the lack of difference in the light and dark cores was due to insufficient light; however, some previous studies have also found no difference in light and dark core incubations (Welsh et al. 2000, An & Gardner 2002, Soana et al. 2015), perhaps because cores do not capture subsurface activity.

When compared with the 2015 push-pull rates (the closest sampling date), ambient denitrification was significantly lower in the cores (Welch t-test, p <0.05), although core denitrification was still greater than zero (mean rate of 0.12 μmol N m^{-2} hr^{-1}; one-sample t-test, p<0.0005). The majority of denitrification in the cores was coupled to nitrification, with D_n on average 0.09 umol N m^{-2} h^{-1} compared to 0.03 μmol N m^{-2} h^{-1} for D_w. There was no significant difference between the ambient DNRA for the cores and push-pull rates (Welch t-test, p =0.13) and core DNRA rates were not different from zero (one-sample t-test, p = 0.13); however, there is a clear pattern of lower DNRA rates measured in the core incubations (average rate of 0.56 μmol N m^{-2} hr^{-1}).

The extremely low rates of denitrification and DNRA measured with the cores are surprising but not unreasonable compared to literature values for denitrification measured using static cores incubated in the dark. Rysgaard et al. (1996) measured mean rates as low as 0.88 μmol N m^{-2} h^{-1} in *Z. noltii* cores, while Welsh et al. (2000) measured rates of 2-6 μmol N m^{-2} h^{-1}, similar to the 1.5-5 μmol N m^{-2} h^{-1} reported by Ottosen et al. (1999). The constraints on the core

technique (slow diffusion of $^{15}NO_3^-$ into sediment, reduced seagrass activity and porewater advection) are likely to lead to underestimations of the total nitrate reduction rates. Furthermore, the light treatment in this study had no effect, suggesting the core data are a measurement of dark rates, which are likely lower than light rates. Thus, it is not surprising to measure very low rates using the static core method.

In contrast, the push-pull rates measured here are greater than previous measurements of subsurface nitrate reduction. Risgaard-Peterson et al. (1998) used perfusion cores to measure subsurface denitrification and found rates of 3.8 μmol N m^{-2} h^{-1} in April and undetectable rates in August. Welsh et al. (2001) used an injection technique to label subsurface porewater in cores and measured rates of 1.3 μmol N m^{-2} h^{-1}. Given the limitations of static cores, it is likely that these published rates underestimate ambient subsurface denitrification. However, the higher rates measured via push-pull may instead overestimate ambient subsurface denitrification because the push-pull method distributes the spiked porewater throughout the sediment matrix and thus introduces the nitrate spike to microzones where nitrate was not present under undisturbed conditions.

The possibility of the push-pull measurements overestimating ambient rates is difficult to ascertain. As noted above, the nitrate spike did not persist in the sediment for long, and the rapid turnover of interstitial nutrients in seagrass sediments likely allowed for the re-establishment of redox and nutrient gradients (Iizumi et al. 1980, Boon et al. 1986a, McGlathery et al. 1998). In addition, no relationship was observed between the concentration of the nitrate spike (which ranged from 76-146 μM) and the measured D_{14} rates, which would be expected if the introduction of excess nitrate had stimulated ambient denitrification. Similarly, there was no relationship between the background concentration of ammonium in the porewater and D_{14},

again suggesting that the introduction of oxygen through the amended porewater did not stimulate nitrification. In fact, in some incubations, no accumulation of $^{29}N_2$ was observed, suggesting no ambient denitrification occurred, an unlikely outcome if the push-pull method systemically enhanced nitrification. Finally, although potential rates are discussed in more detail below, it is important to note here that the potential rates exceeded the ambient rates by an order of magnitude in both the push-pull and core incubations, and the potential push-pull rates showed good agreement with the potential slurry rates. The ambient push-pull rates are thus clearly not "potential" rates in the sense of maximum rates unlimited by nitrate availability; instead, the ambient push-pull rates are an order of magnitude lower than these maximum rates. Nevertheless, the ambient push-pull rates likely represent the upper limit of true rates, while previous subsurface rates measured with static cores likely represent the lower limit.

The push-pull and core incubation techniques reflect nitrate reduction in different areas of the sediment; cores primarily measure surface rates and push-pull measures subsurface rates. In this study, the much lower core rates show that subsurface nitrate reduction can substantially outweigh the surface processes, likely due to low water column nitrogen concentrations at this site as well as the effects of seagrass activity on subsurface biogeochemistry. Previous work in the Virginia coastal bays has shown that denitrification is an important nitrogen sink compared to allocthonous nitrogen loading to the bays, estimated at 1.4 g N m^{-2} y^{-1} (Anderson et al. 2010). In the context of this low nitrogen loading, the push-pull measurements suggest that subsurface denitrification removes a substantial fraction, perhaps up to 1/2, of the external nitrogen inputs to the meadow, while subsurface DNRA retains a slightly smaller fraction, about 1/3 of the external inputs. Total subsurface nitrate reduction therefore could account for more than ¾ of the external

nitrogen inputs, significantly affecting the balance of nitrogen sources and sinks in the seagrass meadow.

A recent compilation of denitrification rates in temperate seagrass meadows suggested a range of 0-50 μmol N m^{-2} h^{-1}, based primarily on measurements of static core incubations (Murray et al. 2015). However, the range of denitrification measured via push-pull was approximately double this range, up to 106 μmol N m^{-2} h^{-1}. This discrepancy suggests that literature values underestimate total denitrification rates in temperate seagrass meadows by not accounting for subsurface rates. Future measurements of nitrate reduction processes in seagrass meadows should account for these subsurface processes.

Relatively few studies of DNRA rates in seagrass meadows have been published (Boon et al. 1986b, Rysgaard et al. 1996, An & Gardner 2002, Gardner et al. 2006, Smyth et al. 2013). However, there is evidence to suggest that DNRA may be of equal or greater magnitude to denitrification in shallow coastal sediments, including seagrass meadows (Giblin et al. 2013). The rates derived via the push-pull technique show that substantial DNRA occurs at depth in seagrass meadows, accounting for 41% of total nitrate reduction in summer 2014 and 48% in summer 2015. While the proportion of DNRA measured in the cores was greater (59%), the mean push-pull rate of DNRA was more than 25x the core rate. These findings suggest that DNRA is more important in the reduced subsurface sediments relative to the surface sediments, and subsurface DNRA rates must therefore be accounted for in future studies.

Potential nitrate reduction rates

Potential nitrate reduction rates (reduction of the excess $^{15}NO_3^-$ spike) were an order of magnitude greater than ambient nitrate reduction rates (Figure 2.6). Despite high variability, the potential push-pull rates were greater than zero (one sample t-tests, $p<0.05$), except

denitrification in 2015 ($p=0.14$). The high potential rates suggest that the subsurface sediments in the seagrass meadow have the capacity for substantially greater denitrification than the current ambient rates. The potential rates measured in the cores were similarly about an order of magnitude greater than ambient rates, although only denitrification was greater than zero in the cores ($p<0.0005$). The similar difference between ambient and potential rates suggests that the microbial communities that process nitrate responded comparably to the $^{15}NO_3^-$ spike in the surface and subsurface sediments.

When integrated on an areal basis, potential rates measured with the slurries were on average 112 μmol N m^{-2} h^{-1} compared to potential push-pull rates of 198 and 153 μmol N m^{-2} h^{-1} in 2014 and 2015 respectively (Figure 2.6). These fall within the range of potential rates measured using slurries in a previous study (67-205 μmol N m^{-2} h^{-1}, Caffrey and Kemp, 1990). The difference between the slurry and push-pull rates is primarily due to the different integration depths; the slurries were integrated over a 5 cm depth while the push-pull rates were integrated over depths of 6.8-8 cm. However, the two methods showed good agreement when comparing bulk sediment rates (μmol N kg $sediment^{-1}$ h^{-1}), which does not require depth integration. The push-pull rates can be converted to bulk sediment rates by dividing D_{15} by the sediment density after multiplying by porosity. The bulk rates calculated from the slurries and from the push-pull match more closely than the areal rates (Figure 2.7). This agreement suggests that the push-pull method is effective in distributing the $^{15}NO_3^-$ spike, creating an optimized environment for potential denitrification comparable to the slurry method.

Variability in push-pull measurements

Comparing the different techniques, it is clear that there is greater variability in nitrate reduction rates measured with push-pull than with cores or slurries, despite a larger sample size

for the push-pull measurements (Table 2.2). Analytical error in the push-pull technique was low; individual samples analyzed with MIMS had high precision (CV<0.07 %), and the time series of push-pull samples were linear with high r^2 values (0.72-0.99). The greater variability in push-pull measurements was therefore likely driven by heterogeneity in field conditions, particularly the dynamic effect of in situ light and flow conditions on seagrass activity and porewater advection. The heterogeneity of the root zone sediment is another important source of variability. Although the push-pull method rates were integrated over a fairly large volume of sediment, rates measured in adjacent push-pull incubations varied substantially, sometimes by an order of magnitude. This spatial variability was not captured with either the core or slurry incubations, suggesting the push-pull technique provides a better measure of denitrification and DNRA rates under field conditions.

Applicability of subtidal push-pull

The results of this study show that the push-pull technique can be employed effectively in subtidal seagrass meadows. In this study system, the depth and tidal range provided a window of approximately 6 hours when the push-pull system could be deployed, which was sufficient time for an incubation of 1.5-2 hrs. The incubation time was further limited by the tracer dissipation; after 2 hours, the tracer recovery was typically less than 5%. The loss of the gas tracer is likely due to a combination of hydrodynamics (flow driving porewater advection) and sediment conditions (especially porosity and grain size). In a system with muddier sediments, it is likely the incubation time could be increased.

The relative importance of subsurface nitrate reduction in a given system likely depends on nitrate availability. Seagrass root zone denitrification rates reported here were similar to rates measured in the root zone of a New England salt marsh (Koop-Jakobsen & Giblin 2010). In both

studies, denitrification in the root zone substantially outweighed surface and water column denitrification (measured with cores) at sites with low nitrate concentrations. However, at a marsh site with enhanced water column nitrate concentrations (>70 μmol), the surface denitrification dominated total denitrification. Thus, in systems with high nitrate concentrations, push-pull measurements should be used in concert with another method such as cores or benthic chambers in order to fully account for both surface and subsurface rates.

Limitations

The in situ deployment of the push-pull technique is a major advantage over traditional methods. However, that aspect also generates a number of practical limitations. Deployment of the technique is limited by tidal range, and would be impractical at depths much deeper than this study site. Similarly, capturing nitrate reduction over the full diel cycle with this method would be difficult, and push-pull deployments during the winter months would be challenging in temperate locations, potentially leaving a gap in seasonal or annual measurements. These practical limitations, as well as the labor-intensive nature of the method, should be considered in any future studies that employ the push-pull method.

The direct sampling of porewater to measure subsurface rates is another advantage that comes with limitations. As explained above, the depth integration of the push-pull rates uses a conservative estimate of depth based on the volume of amended porewater returned to the sediment. However, because the piezometer re-samples from the same point throughout the incubation, the sediment immediately surrounding the sampling point is over-represented compared to sediment further away. The calculated rates are therefore not truly depth-integrated rates but are most representative of the sediment surrounding the 5 cm insertion depth. This bias most likely leads to underestimation of the true rates, as seagrass biomass decreases below 5 cm.

However, there may also be overestimation, depending on the exact distribution of the amended porewater and the vertical heterogeneity of the root zone. Regardless, other drivers of variability in the rates (i.e. field conditions, lateral heterogeneity) likely overwhelm this effect and the depth integration described here is a reasonable approximation of true rates.

Finally, it is important to note that the push-pull technique will require further modifications in systems with high levels of anammox. In these systems, the modified isotope pairing technique presented by Risgaard-Petersen and others (2003) would need to be used. This modified technique relies on using slurries to measure ra, the contribution of anammox to N_2 production; slurry incubations could be conducted in conjunction with the push-pull technique. Additionally, in sediments where anammox is more than 20% of total N_2 production and DNRA is more than 20% of total nitrate reduction, interactions between anammox and DNRA may lead to overestimations of denitrification (Song et al. 2016). In this case, additional measurements and calculations will be needed to accurately determine the ambient denitrification, DNRA, and anammox rates.

Conclusions

The subtidal push-pull technique provided realistic estimates of in situ rates of subsurface denitrification and DNRA that could not be measured using traditional techniques. The results presented here showed that subsurface nitrate reduction rates were significant, and that DNRA was of the same order of magnitude as denitrification in the seagrass root zone. Furthermore, the push-pull rates revealed high spatial variability compared to the core and slurry rates. These findings highlight the importance of in situ measurements of nitrate reduction rates within the root zone and suggest that current literature values do not fully account for root zone nitrate reduction and consequently underestimate total rates. In light of these findings, root zone nitrate

reduction should be assessed in additional seagrass systems. In particular, meadows with greater belowground biomass, such as *Posidonia* meadows, may have even greater rates of root zone nitrate reduction than those presented here. Application of the push-pull technique in additional systems will generate important data to further our understanding of coastal nitrogen cycling in these highly productive ecosystems.

References

Addy K, Kellogg DQ, Gold AJ, Groffman PM, Ferendo G, Sawyer C (2002) In situ push–pull method to determine ground water denitrification in riparian zones. J Environ Qual 31:1017–1024

An S, Gardner WS (2002) Dissimilatory nitrate reduction to ammonium (DNRA) as a nitrogen link, versus denitrification as a sink in a shallow estuary (Laguna Madre/Baffin Bay, Texas). Mar Ecol Prog Ser 237:41–50

Anderson IC, Stanhope JW, Hardison AK, McGlathery KJ (2010) Sources and fates of nitrogen in Virginia coastal bays. In: Coastal Lagoons: Critical habitats of environmental change. CRC Press, Boca Raton, FL, p 43–72

Behrendt A, Beer D de, Stief P (2013) Vertical activity distribution of dissimilatory nitrate reduction in coastal marine sediments. Biogeosciences 10:7509–7523

Berg P, McGlathery K (2001) A high-resolution pore water sampler for sandy sediments. Limnol Oceanogr 46:203–210

Boon PI, Moriarty DJW, Saffigna PG (1986a) Rates of ammonium turnover and the role of amino-acid deamination in seagrass (*Zostera capricorni*) beds of Moreton Bay, Australia. Mar Biol 91:259–268

Boon PL, Moriarty DJW, Saffigna PG (1986b) Nitrate metabolism in sediments from seagrass (*Zostera capricorni*) beds of Moreton Bay, Australia. Mar Biol 91:269–275

Borum J, Sand-Jensen K, Binzer T, Pedersen O, Greve TM (2006) Oxygen movement in seagrasses. In: Seagrasses: Biology, ecology, and conservation. Springer, Dordrecht, The Netherlands, p 255–270

Brunet RC, Garcia-Gil LJ (1996) Sulfide-induced dissimilatory nitrate reduction to ammonia in anaerobic freshwater sediments. FEMS Microbiol Ecol 21:131–138

Burgin A, Hamilton S (2007) Have we overemphasized the role of denitrification in aquatic ecosystems? A review of nitrate removal pathways. Front Ecol Environ 5:89–96

Caffrey JM, Kemp WM (1990) Nitrogen cycling in sediments with estuarine populations of *Potamogeton perfoliatus* and *Zostera marina*. Mar Ecol Prog Ser 66:147–160

Christensen PB (2000) Sediment mineralization, nutrient fluxes, denitrification and dissimilatory nitrate reduction to ammonium in an estuarine fjord with sea cage trout farms. Aquat Microb Ecol 21:73–84

Cornwell JC, Kemp WM, Kana TM (1999) Denitrification in coastal ecosystems: methods, environmental controls, and ecosystem level controls, a review. Aquat Ecol 33:41–54

Dalsgaard T, Bak F (1994) Nitrate reduction in a sulfate-reducing bacterium, *Desulfovibrio desulfuricans*, isolated from rice paddy soil: Sulfide inhibition, kinetics, and regulation. Appl Environ Microbiol 60:291–297

Eyre BD, Ferguson AJP, Webb A, Maher D, Oakes JM (2011) Denitrification, N-fixation and nitrogen and phosphorus fluxes in different benthic habitats and their contribution to the nitrogen and phosphorus budgets of a shallow oligotrophic sub-tropical coastal system (southern Moreton Bay, Australia). Biogeochemistry 102:111–133

Eyre BD, Maher DT, Squire P (2013) Quantity and quality of organic matter (detritus) drives N_2 effluxes (net denitrification) across seasons, benthic habitats, and estuaries. Glob Biogeochem Cycles 27:1083–1095

Eyre BD, Rysgaard S, Dalsgaard T, Christensen PB (2002) Comparison of isotope pairing and N_2:Ar methods for measuring sediment denitrification: Assumption, modifications, and implications. Estuaries 25:1077–1087

Eyre BD, Rysgaard S, Dalsgaard T, Christensen PB (2004) Reply to comment on our paper "Comparison of isotope pairing and N_2:Ar methods for measuring sediment denitrification." Estuaries 27:177–178

Fagherazzi S, Wiberg PL (2009) Importance of wind conditions, fetch, and water levels on wave-generated shear stresses in shallow intertidal basins. J Geophys Res 114:F03022

Ferguson A, Eyre B (2007) Seasonal discrepancies in denitrification measured by isotope pairing and N_2:Ar techniques. Mar Ecol Prog Ser 350:19–27

Ferguson A, Eyre B, Gay J (2004) Nutrient cycling in the sub-tropical Brunswick estuary, Australia. Estuaries 27:1–17

Frederiksen MS, Glud RN (2006) Oxygen dynamics in the rhizosphere of *Zostera marina*: A two-dimensional planar optode study. Limnol Oceanogr 51:1072–1083

Gardner WS, McCarthy MJ, An S, Sobolev D, Sell KS, Brock D (2006) Nitrogen fixation and dissimilatory nitrate reduction to ammonium (DNRA) support nitrogen dynamics in Texas estuaries. Limnol Oceanogr 51:558–568

Giblin A, Tobias C, Song B, Weston N, Banta G, Rivera-Monroy V (2013) The importance of dissimilatory nitrate reduction to ammonium (DNRA) in the nitrogen cycle of coastal ecosystems. Oceanography 26:124–131

Greiner JT, McGlathery KJ, Gunnell J, McKee BA (2013) Seagrass restoration enhances "blue carbon" sequestration in coastal waters (J Cebrian, Ed.). PLoS ONE 8:e72469

Gribsholt B, Kristensen E (2002) Impact of sampling methods on sulfate reduction rates and dissolved organic carbon (DOC) concentrations in vegetated salt marsh sediments. Wetl Ecol Manag 10:371–379

Groffman PM, Altabet MA, Böhlke JK, Butterbach-Bahl K, David MB, Firestone MK, Giblin AE, Kana TM, Nielsen LP, Voytek MA (2006) Methods for measuring denitrification: Diverse approaches to a difficult problem. Ecol Appl 16:2091–2122

Hansen JW, Lomstein BA (1999) Leakage of ammonium, urea, and dissolved organic nitrogen and carbon from eelgrass Zostera marina roots and rhizomes during sediment handling. Aquat Microb Ecol 16:303–307

Hou L, Liu M, Carini SA, Gardner WS (2012) Transformation and fate of nitrate near the sediment–water interface of Copano Bay. Cont Shelf Res 35:86–94

Huettel M, Berg P, Kostka JE (2014) Benthic exchange and biogeochemical cycling in permeable sediments. Annu Rev Mar Sci 6:23–51

Iizumi H, Hattori A, McRoy CP (1980) Nitrate and nitrite in interstitial waters of eelgrass beds in relation to the rhizosphere. J Exp Mar Biol Ecol 47:191–201

Jorgensen BB, Postgate JR (1982) Ecology of the bacteria of the sulphur cycle with special reference to anoxic-oxic interface environments. Philos Trans R Soc Lond B Biol Sci 298:543–561

Kaldy J (2012) Influence of light, temperature and salinity on dissolved organic carbon exudation rates in *Zostera marina* L. Aquat Biosyst 8:19

Kana TM, Darkangelo C, Hunt MD, Oldham JB, Bennett GE, Cornwell JC (1994) Membrane inlet mass spectrometer for rapid high-precision determination of N_2, O_2, and Ar in environmental water samples. Anal Chem 66:4166–4170

Kana TM, Sullivan M., Cornwell JC, Groszkowski KM (1998) Denitrification in estuarine sediments determined by membrane inlet mass spectrometry. Limnol Oceanogr 43:334–339

Koch EW (1999) Preliminary evidence on the interdependent effect of currents and porewater geochemistry on *Thalassia testudinum* Banks ex König seedlings. Aquat Bot 63:95–102

Koch EW, Ackerman JD, Verduin J, Keulen M van (2006) Fluid dynamics in seagrass ecology: From molecules to ecosystems. In: Seagrasses: Biology, ecology, and conservation. Springer, Dordrecht, The Netherlands, p 193–225

Koop-Jakobsen K, Giblin AE (2009) New approach for measuring denitrification in the rhizosphere of vegetated marsh sediments. Limnol Oceanogr Methods 7:626–637

Koop-Jakobsen K, Giblin AE (2010) The effect of increased nitrate loading on nitrate reduction via denitrification and DNRA in salt marsh sediments. Limnol Oceanogr 55:789

Luijn F van, Boers PC, Lijklema L (1996) Comparison of denitrification rates in lake sediments obtained by the N_2 flux method, the ^{15}N isotope pairing technique and the mass balance approach. Water Res 30:893–900

Lunstrum A, Aoki LR (2016) Oxygen interference with membrane inlet mass spectrometry may overestimate denitrification rates calculated with the isotope pairing technique. Limnol Oceanogr Methods 14:425–431

McGlathery KJ, Christian R (2017) Water Quality Sampling - integrated measurements for the Virginia Coast, 1992-2017. Virginia Coast Reserve Long-Term Ecological Research Project Data Publication doi:10.6073/pasta/8783dd64be7cae3a36a864ac5b5129e1

McGlathery K, Reynolds L, Cole L, Orth R, Marion S, Schwarzschild A (2012) Recovery trajectories during state change from bare sediment to eelgrass dominance. Mar Ecol Prog Ser 448:209–221

McGlathery KJ, Risgaard-Petersen N, Christensen PB (1998) Temporal and spatial variation in nitrogen fixation activity in the eelgrass *Zostera marina* rhizosphere. Mar Ecol Prog Ser 168:245–258

Murphy AE, Anderson IC, Smyth AR, Song B, Luckenbach MW (2016) Microbial nitrogen processing in hard clam (*Mercenaria mercenaria*) aquaculture sediments: The relative importance of denitrification and dissimilatory nitrate reduction to ammonium (DNRA). Limnol Oceanogr 61:1589–1604

Murray RH, Erler DV, Eyre BD (2015) Nitrous oxide fluxes in estuarine environments: Response to global change. Glob Change Biol 21:3219–3245

Nielsen LP (1992) Denitrification in sediment determined from nitrogen isotope pairing. FEMS Microbiol Lett 86:357–362

Nielsen LP, Glud RN (1996) Denitrification in a coastal sediment measured in situ by the nitrogen isotope pairing technique applied to a benthic flux chamber. Mar Ecol Prog Ser 137:181–186

Orth RJ, Marion SR, Moore KA, Wilcox DJ (2010) Eelgrass (*Zostera marina* L.) in the Chesapeake Bay region of mid-Atlantic coast of the USA: Challenges in conservation and restoration. Estuaries Coasts 33:139–150

Orth R, Moore K, Marion S, Wilcox D, Parrish D (2012) Seed addition facilitates eelgrass recovery in a coastal bay system. Mar Ecol Prog Ser 448:177–195

Ottosen LDM, Risgaard-Petersen N, Nielsen LP (1999) Direct and indirect measurements of nitrification and denitrification in the rhizosphere of aquatic macrophytes. Aquat Microb Ecol 19:81–91

Pagès A, Welsh DT, Robertson D, Panther JG, Schäfer J, Tomlinson RB, Teasdale PR (2012) Diurnal shifts in co-distributions of sulfide and iron(II) and profiles of phosphate and ammonium in the rhizosphere of *Zostera capricorni*. Estuar Coast Shelf Sci 115:282–290

Risgaard-Petersen N (2004) Denitrification. In: Estuarine nutrient cycling: the influence of primary producers. Kluwer Academic Publishers, Dordrecht, The Netherlands, p 263–280

Risgaard-Petersen N, Dalsgaard T, Rysgaard S, Christensen PB, Borum J, McGlathery K, Nielsen LP (1998) Nitrogen balance of a temperate eelgrass *Zostera marina* bed. Mar Ecol-Prog Ser 174:281–291

Risgaard-Petersen N, Nielsen LP, Rysgaard S, Dalsgaard T, Meyer RL (2003) Application of the isotope pairing technique in sediments where anammox and denitrification coexist. Limnol Ocean Methods 1:63–73

Russell DG, Warry FY, Cook PLM (2016) The balance between nitrogen fixation and denitrification on vegetated and non-vegetated intertidal sediments. Limnol Oceanogr 61:2058–2075

Rysgaard S, Risgaard-Petersen N, Sloth NP (1996) Nitrification, denitrification, and nitrate ammonification in sediments of two coastal lagoons in Southern France. Hydrobiologia 329:133–141

Santos IR, Eyre BD, Huettel M (2012) The driving forces of porewater and groundwater flow in permeable coastal sediments: A review. Estuar Coast Shelf Sci 98:1–15

Schulz HN, Jørgensen BB (2001) Big bacteria. Annu Rev Microbiol 55:105–137

Smyth AR, Thompson SP, Siporin KN, Gardner WS, McCarthy MJ, Piehler MF (2013) Assessing nitrogen dynamics throughout the estuarine landscape. Estuaries Coasts 36:44–55

Soana E, Naldi M, Bonaglia S, Racchetti E, Castaldelli G, Brüchert V, Viaroli P, Bartoli M (2015) Benthic nitrogen metabolism in a macrophyte meadow (*Vallisneria spiralis* L.) under increasing sedimentary organic matter loads. Biogeochemistry 124:387–404

Song GD, Liu SM, Kuypers MMM, Lavik G (2016) Application of the isotope pairing technique in sediments where anammox, denitrification, and dissimilatory nitrate reduction to ammonium coexist: DNRA and isotope pairing. Limnol Oceanogr Methods

Steingruber SM, Friedrich J, Gachter R, Wehrli B (2001) Measurement of denitrification in sediments with the ^{15}N isotope pairing technique. Appl Environ Microbiol 67:3771–3778

Thamdrup B, Dalsgaard T (2002) Production of N_2 through anaerobic ammonium oxidation coupled to nitrate reduction in marine sediments. Appl Environ Microbiol 68:1312–1318

Tiedje JM, Sexstone AJ, Myrold, DD, Robinson, JA (1982) Denitrification: Ecological niches, competition, and survival. Antonie Van Leeuwenhoek 48:569–583

Trimmer M, Nicholls JC, Deflandre B (2003) Anaerobic ammonium oxidation measured in sediments along the Thames Estuary, United Kingdom. Appl Environ Microbiol 69:6447–6454

Webb AP, Eyre BD (2004) The effects of two benthic chamber stirring systems on the diffusive boundary layer, oxygen flux, and passive flow through model macrofauna burrows. Estuaries 27:352–361

Welsh DT, Bartoli M, Nizzoli D, Castaldelli G, Riou SA, Viaroli P (2000) Denitrification, nitrogen fixation, community primary productivity and inorganic-N and oxygen fluxes in an intertidal *Zostera noltii* meadow. Mar Ecol Prog Ser 208:65–77

Welsh D, Catadelli G, Bartoli M, Poli D, Careri M, Wit R de, Viaroli P (2001) Denitrification in an intertidal seagrass meadow, a comparison of ^{15}N-isotope and acetylene-block techniques: Dissimilatory nitrate reduction to ammonia as a source of N_2O? Mar Biol 139:1029–1036

Yin G, Hou L, Liu M, Liu Z, Gardner WS (2014) A novel membrane inlet mass spectrometer method to measure $^{15}NH_4^+$ for isotope-enrichment experiments in aquatic ecosystems. Environ Sci Technol 48:9555–9562

Zhang J-Z, Ortner PB, Fischer CJ (1997) Method 353.4 Determination of nitrate and nitrite in estuarine and coastal waters by gas segmented continuous flow colorimetric analysis. U.S. Environmental Protection Agency, Washington, D.C.

Figures

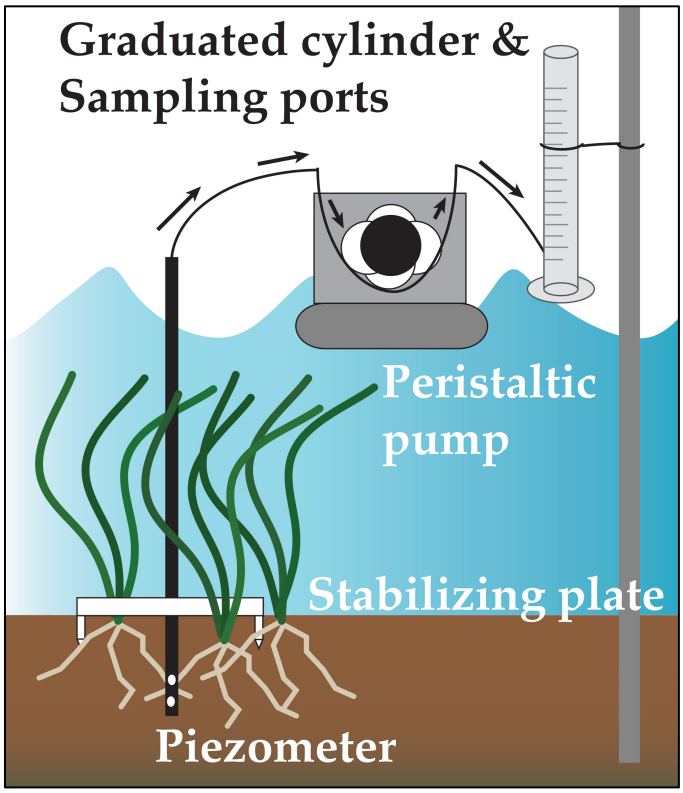


Figure 2.1 Conceptual diagram of push-pull equipment (not to scale).

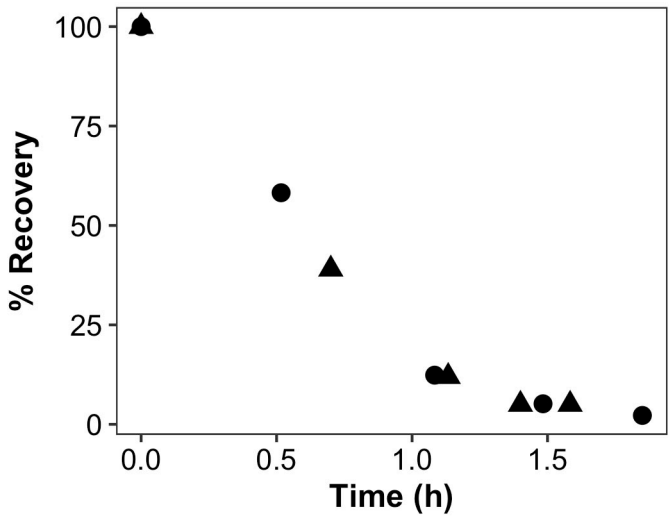


Figure 2.2 Recovery of argon tracer during the incubation showed a rapid decrease, with less than 5% of the tracer recovered after two hours. Circles and triangles represent individual tracer dilution curves from push-pull tests conducted in the seagrass meadow.

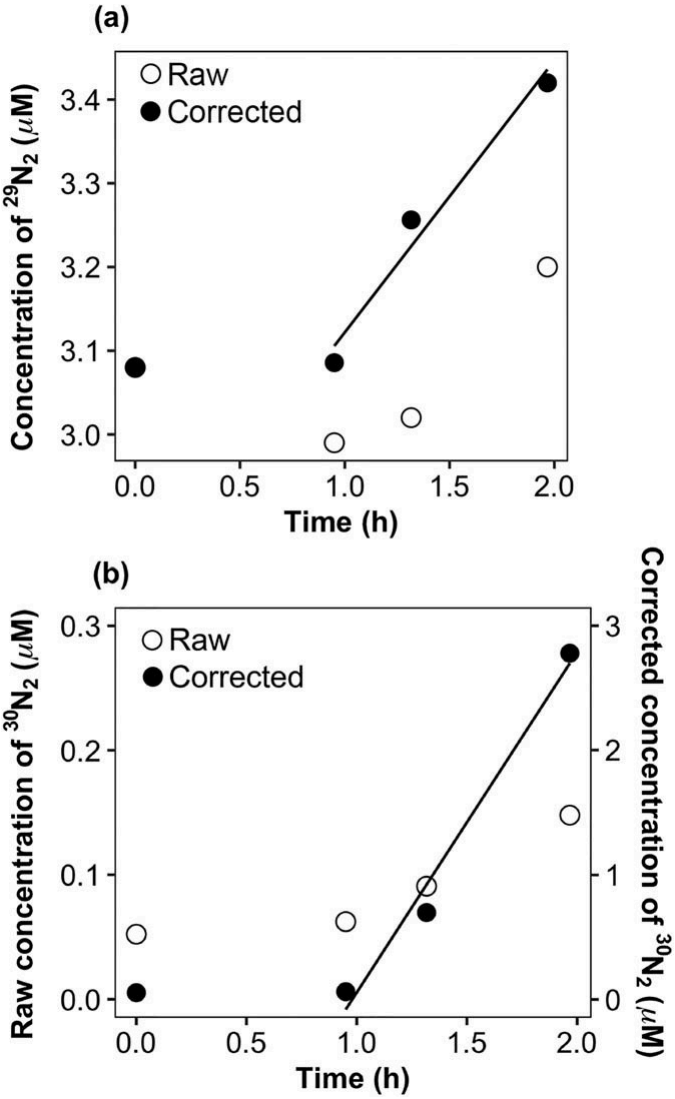


Figure 2.3 Samples collected over the course of a push-pull incubation showed a linear increase in porewater concentrations of both $^{29}N_2$ (a) and $^{30}N_2$ (b) after corrections for changes in background N_2, impurities, and gas loss using the argon tracer. Open circles represent the raw concentrations and filled circles show the corrected concentrations; lines show the linear regressions used to calculate production rates of ^{29} and $^{30}N_2$. Note the different scales for raw and corrected concentrations of $^{30}N_2$ in (b).

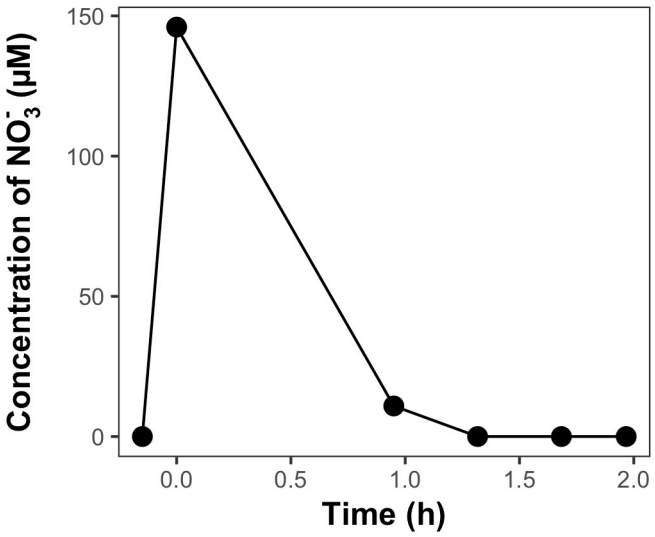


Figure 2.4 Nitrate concentrations in the porewater were undetectable before the addition of the $^{15}NO_3^-$ spike at time zero and declined rapidly over the incubation period.

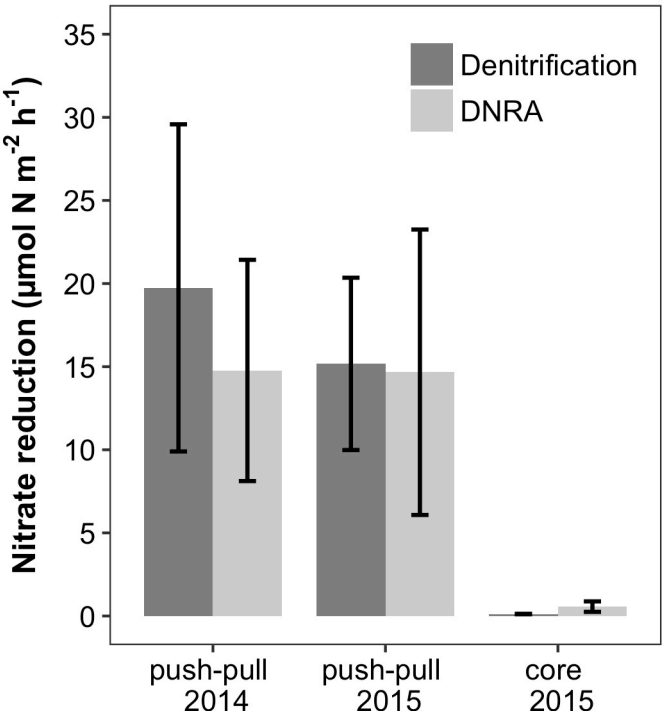


Figure 2.5 Ambient rates of denitrification and DNRA measured using the push-pull technique were greater and more variable than rates measured in core incubations. Error bars are +/- SE; n=10 for push-pull rates, and n=7 for core rates.

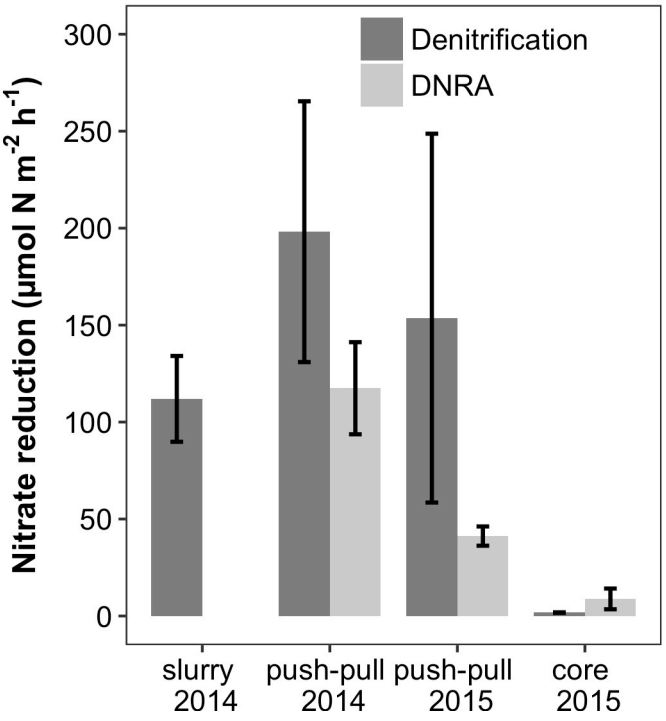


Figure 2.6 Areal potential rates of denitrification and DNRA measured using push-pull were up to two orders of magnitude greater than core rates and were about 2x the areal slurry rates (note that integration depths were different for the push-pull and slurry measurements). Error bars are +/- SE; n=3 for slurry rates, n=10 for push-pull rates, and n=7 for core rates.

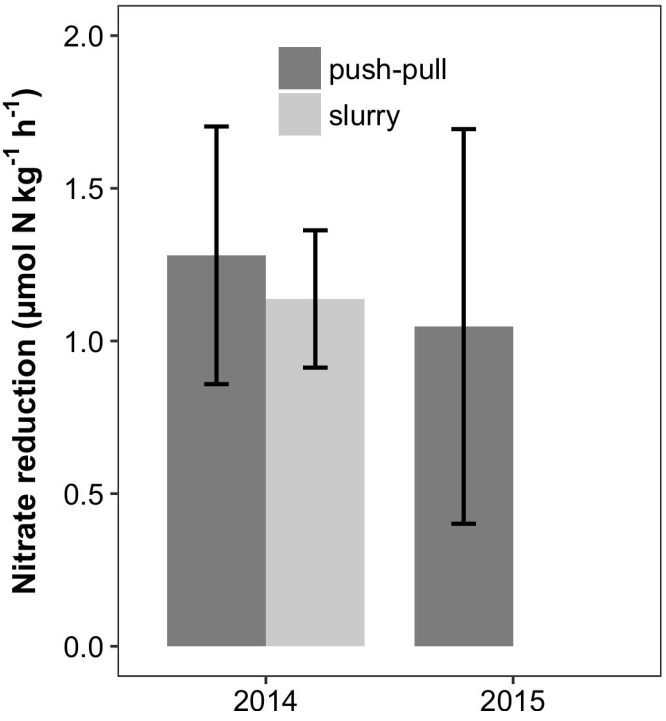


Figure 2.7 Bulk potential denitrification rates (μmol N per kg sediment per hour) measured with the slurry and push-pull techniques showed good agreement. Error bars are +/- SE, n=3 for slurry rates, n=10 push-pull rates.

Tables

Table 2.1 Nitrate concentrations before and after $^{15}NO_3^-$ additions for each technique.

Technique	Reservoir receiving nitrate addition	Ambient nitrate (μM)	Spiked nitrate (μM)
Push-pull	Porewater	<0.04	76-146
Cores	Surface water	0.67	37
Slurries	Slurry	n/a	31

Table 2.2 Mean denitrification rates and variability (μmol N m^{-2} hr^{-1}) measured using different techniques.

Technique	Number of replicates	Mean rate	Standard deviation	Standard error
Ambient				
Push-pull 2014	10	19.7	31.1	9.8
Push-pull 2015	10	15.2	16.4	5.2
Cores	7	0.12	0.04	0.01
Potential				
Push-pull 2014	10	198.1	212.6	67.2
Push-pull 2015	10	154.6	300.7	95.1
Slurries	3	112.0	38.3	22.5

Chapter 3: Restoration enhances denitrification and DNRA in subsurface sediments of *Zostera marina* seagrass meadows

In review at Marine Ecology Progress Series

Abstract

Seagrasses exude oxygen and labile carbon into the sediment, which can stimulate microbial activity. However, it is not clear how seagrasses impact competing nitrate reduction processes, including nitrogen removal through denitrification and nitrogen retention through dissimilatory nitrate reduction to ammonium (DNRA). Using an in situ push-pull incubation method, we measured denitrification and DNRA rates in the root zone of a restored *Zostera marina* meadow, in adjacent unvegetated sediments, and in experimentally cleared plots within the meadow. Denitrification and DNRA rates in the meadow sediments were highly variable and contained "hotspots" where maximum rates exceeded median rates by more than an order of magnitude. Hotspots were not observed in bare sediments, leading to average rates 4x greater in vegetated sediments than in bare sediments. In the meadow sediments, denitrification dominated over DNRA except in fall, during seagrass senescence, and after the experimental removal of seagrass. Extrapolated rates of annual nitrate removal via denitrification were greater in the vegetated sediments compared to bare sediments (0.62 g N m^{-2} y^{-1} compared to 0.16 g N m^{-2} y^{-1}) and accounted for 44% of annual N loading to the system. Similarly, annual DNRA rates were greater in the vegetated compared to bare sediments (0.45 g N m^{-2} y^{-1} and 0.12 g N m^{-2} y^{-1} respectively). The restoration of the seagrass meadow thus increased both nitrogen removal and recycling, but removal via denitrification was the dominant process. The dominance of

denitrification demonstrates how seagrass restoration can enhance the filter function of shallow coastal systems.

Introduction

Anthropogenic eutrophication in coastal ecosystems is a major environmental challenge (National Research Council 2000, Howarth & Marino 2006). As increasing amounts of reactive nitrogen enter the biosphere, much of that nitrogen will ultimately travel to coastal ecosystems, leading to nutrient over-enrichment and associated negative effects, including algae blooms, anoxia, and fish kills (Galloway et al. 2004, Seitzinger et al. 2006, Howarth 2008). The impact of increased nitrogen loading on coastal and estuarine systems will depend in part on the capacity of these areas to filter incoming nitrogen (Cloern 2001). Seagrass meadows are one coastal ecosystem that have the potential to serve as an effective nutrient filter. Temporary accumulation of nitrogen in seagrass biomass and more permanent storage in meadow sediment are two important pathways through which seagrass enhance nitrogen removal from the water column (McGlathery et al. 2007). In addition, seagrass can stimulate biogeochemical cycling in meadow sediments, potentially leading to the removal of nitrogen.

Denitrification, the microbially mediated transformation of nitrate into inert dinitrogen gas, requires a supply of nitrate, reduced carbon substrate, and anoxic conditions. In sediments below the sediment-water interface, the nitrate to support denitrification is typically produced via nitrification, an aerobic process that converts ammonium into nitrate. Coupled nitrification-denitrification is common in low nutrient ecosystems; in seagrass meadows, this coupled process is generally linked to plant metabolism. Seagrass roots exude both oxygen and labile organic carbon into the subsurface sediments, creating oxidized microzones and steep redox gradients that support coupled nitrification-denitrification (Frederiksen & Glud 2006, Jovanovic et al. 2015). Oxygenation via roots may also reduce sulfide concentrations in sediments (Pagès et al. 2012), in turn reducing sulfide-inhibition of denitrification (Brunet & Garcia-Gil 1996). Seagrass

meadows may also influence denitrification rates by increasing sedimentation of organic matter and thus enhancing the supply of reduced carbon in meadow sediments.

Despite the altered biogeochemical conditions in seagrass sediment, it is not clear whether seagrass meadows stimulate denitrification relative to unvegetated sediments. Several studies have measured low rates of denitrification in seagrass meadows (Rysgaard et al. 1996, Risgaard-Petersen & Ottosen 2000, Welsh et al. 2000, Russell et al. 2016), in some cases lower than in adjacent unvegetated areas (Risgaard-Petersen et al. 1998, Ottosen et al. 1999). These low rates are often attributed to competition for nitrate from benthic microalgae. However, other studies have found that denitrification rates in seagrass meadows greatly exceeded rates in adjacent unvegetated tidal flats (Eyre et al. 2011, Piehler & Smyth 2011, Smyth et al. 2013). These higher rates in seagrass meadows were observed in systems with low nutrient loading, where competition for nitrate would be high. Thus, there is uncertainty in the literature over the net effect of seagrass on denitrification rates. Methodological differences may explain some of these patterns; low rates of denitrification have been measured mainly using the isotope pairing technique (e.g. Welsh et al. 2000, Risgaard-Petersen et al. 1998, Russell et al. 2016) whereas higher rates have been measured using the N_2:Ar technique (e.g. Smyth et al. 2013, Eyre et al. 2011). However, methodology does not entirely explain these patterns; a recent study using the N_2:Ar method has also found low rates of denitrification in seagrass sediments, comparable to the rates measured with isotope pairing (Zarnoch et al. 2017). Moreover, it is important to note that the N_2:Ar measurements of enhanced rates in seagrass meadows have relied primarily on incubations conducted under dark conditions, which would alleviate competition for nitrate from autotrophs, and could overestimate daily and annual rates. Further study of denitrification rates in seagrass meadows is therefore needed to clarify whether seagrass stimulate denitrification.

Denitrification also competes with dissimilatory nitrate reduction to ammonium (DNRA). Like denitrification, DNRA requires nitrate, reduced carbon (or sulfide), and anoxic conditions. Partitioning between DNRA and denitrification depends on factors including the relative availability of nitrate and organic carbon, the presence of sulfides, and the quality of the carbon substrate (Burgin & Hamilton 2007, Hardison et al. 2015). In contrast to denitrification, DNRA retains nitrogen in the sediment as biologically available ammonium; thus, the balance between these competing processes may alter net nitrogen removal. Relatively few studies of DNRA have been conducted in seagrass meadows to date; in some studies, DNRA was low relative to denitrification (Boon et al. 1986, Smyth et al. 2013), while in others DNRA was equal to or greater than denitrification (Rysgaard et al. 1996, An & Gardner 2002, Gardner et al. 2006). This variation suggests that further study is needed to better understand partitioning between these two nitrate reduction processes (Giblin et al. 2013).

Uncertainty surrounding the magnitude and partitioning of denitrification and DNRA rates in seagrass meadows may be related in part to limitations of traditional sampling methods. Conventional methods rely on laboratory incubations of cores or sediment slurries that typically do not capture rates under in situ conditions of light and flow that are linked to plant activity (Koch et al. 2006, Rheuban et al. 2014) or fully capture subsurface rates or plant effects. Collection of cores may also damage belowground biomass, leading to release of dissolved organic carbon and ammonium that can stimulate microbial processes (Hansen & Lomstein 1999, Gribsholt & Kristensen 2002). In contrast, a new push-pull method can be used in the field, where miniature piezometers inject isotopically labeled $^{15}NO_3^-$ into seagrass sediments while maintaining the complex sediment matrix and without disturbing the hydrodynamic flow, light availability, or other drivers of seagrass activity (Koop-Jakobsen & Giblin 2009). In a

comparison with traditional core incubations, this push-pull method measured higher rates of both denitrification and DNRA, as well as greater variability in those rates, that were attributed to sediment heterogeneity, natural variation in field conditions, and the irregular effects of plant exudation (Aoki & McGlathery 2017). The push-pull method has limitations as well; notably, implementation of the method is constrained by practical considerations, and because the method targets subsurface processes, it is not sufficient in systems where microbial activity at the sediment surface dominates total denitrification and DNRA rates. However, by targeting subsurface processes and therefore capturing the plant effects on redox gradients and labile carbon supply, the push-pull method is particularly appropriate for measurements of denitrification and DNRA in the complex sediment matrix of the seagrass root zone.

Accurate measurements of the seagrass effect on nitrate reduction processes is critical to understanding how seagrass restoration affects the coastal nutrient filter. As a large-scale and well-established restoration project, our study site in the Virginia coastal bays is an ideal system to test for these impacts. Seagrass seeding in the Virginia coastal bays has transformed over 25 km^2 of unvegetated benthos into seagrass meadow since 2001. Work at this site has shown for the first time that seagrass restoration reinstates the capacity to sequester carbon in both biomass and sediments (McGlathery et al. 2012, Greiner et al. 2013, Oreska et al. 2017), but the impacts of seagrass restoration on nutrient filtration are not yet known. By measuring nitrate reduction rates in the restored meadow and in adjacent bare sediment, we can determine for the first time whether the restoration enhanced denitrification and therefore enhanced the nutrient filter function of the seagrass meadow.

In this study, we used the push-pull method to compare nitrate reduction rates at vegetated sites within the restored meadow to rates in unvegetated sediment outside the meadow.

In addition to the external bare site comparison, we wanted to isolate the effect of seagrass presence on sediment conditions and consequently on denitrification and DNRA rates. The external bare sites experienced different environmental conditions compared to the meadow sites (i.e. deeper water column, higher flow velocities, larger sediment grains) which may impact rates. We therefore conducted a removal experiment within the meadow in which we compared rates measured in the meadow sediments to rates measured in plots within the seagrass meadow that experienced identical environmental conditions where we experimentally cleared above- and below-ground seagrass biomass. Finally, we conducted seasonal measurements within the seagrass meadow in order to understand patterns in nitrate reduction rates over time.

Methods

Site description

South Bay is a shallow lagoon located on the Atlantic coast of the Eastern Shore of Virginia. The mean water depth is 1.4 m and the mean tidal range is 1.2 m (Fagherazzi & Wiberg 2009). Seagrass were historically present in South Bay, and other Virginia coastal bays, until the mid-1930s, when a combination of the pandemic wasting disease (*Labyrinthula* sp.) and a severe hurricane caused a local extinction (Orth & McGlathery 2012). A landscape-scale restoration experiment was begun in 2001; over 7.5×10^6 *Zostera marina* seeds were broadcast in replicate 0.2 and 0.4 ha plots beginning in 2001. In South Bay, the original plots coalesced into a contiguous meadow that has continued to spread, covering approximately 680 ha in 2015 (Orth et al. 2012, Oreska et al. 2017). Sediments in South Bay are predominantly fine sands (McGlathery 2016). Long-term monitoring has shown a shift in sediment characteristics, with smaller grain sizes and increased organic matter content in the restored meadow (McGlathery et al. 2012), and recent work has shown that the restored meadow has achieved carbon storage

capacities on par with natural meadows (Greiner et al. 2013). Nutrient loading to South Bay is quite low compared to coastal lagoons throughout the world, approximately 1.4 g N m^{-2} y^{-1} (McGlathery et al. 2007, Anderson et al. 2010) and water quality is high, with dissolved inorganic nitrogen concentrations frequently undetectable in surface water.

At this site, dissimilatory nitrate reduction occurs predominantly in subsurface sediments. Denitrification and DNRA measured in surface sediments using a traditional isotope pairing core incubation were exceedingly low (approximately 0.1 μmol m^{-2} h^{-1} in both seagrass and bare sediments) and were 34-135x less than rates measured using the push-pull method (Aoki and McGlathery 2017). In this system, it is therefore appropriate to rely on the push-pull method to measure denitrification and DNRA. In other systems with greater contributions from surface rates, fully capturing the dissimilatory nitrate reduction rates would require combining the push-pull method with another method targeting surface rates.

Subsurface rates of denitrification and DNRA were expected to be low in the bare sediments. However, previous work has shown that bare sediments in these lagoons are sufficiently permeable that advective transport can dominate over porewater diffusion (Huettel & Gust 1992, Rheuban et al. 2014). Tidally driven advection of oxygen into the upper mm-cm of the bare sediments could therefore support nitrification below the surface, supplying nitrate to denitrification and DNRA that were captured with the push-pull method. Oxygenation of macrofauna burrows could also support subsurface rates (Pelegri et al. 1994, Wenzhöfer & Glud 2004, Meysman et al. 2010).

Experimental design

The sampling design for the three components of this study is summarized in Table 3.1. For all three components, denitrification and DNRA rates were measured using the push-pull

method, described below. All push-pull measurements were conducted during the day (i.e. with ambient sunlight available); light and flow conditions varied naturally over the course of each 6-hour push-pull deployment and between deployments conducted on different days. Additional samples were collected to measure sediment characteristics, porewater chemistry, and seagrass metrics; details are included below. All sampling within the meadow was conducted within the areas of the initial seeding (three replicate 0.4 ha plots) in order to ensure that all seagrass plots were the same age (13 years since restoration in 2014 and 14 years in 2015).

External bare site comparison

Denitrification and DNRA were measured in situ using the push-pull technique throughout summer 2014 in order to gain data representative of the seagrass growing season. Rates were measured at one seagrass site in the interior of the meadow, and at one unvegetated, bare site located adjacent to the meadow edge. Between 2 and 4 push-pull measurements of nitrate reduction were made at both the seagrass and bare sites in June, July, and August for a total of 8-10 measurements at each site across the seagrass growing season (Table 3.1). Some environmental parameters influencing microbial activity remained constant over the summer (e.g. sediment temperature, see Table 3.2 below), but other parameters, especially seagrass biomass, varied (see Table 3.3 below). Temporal variability in these environmental parameters likely contributed to the overall variability in the compiled summer nitrate reduction rates. However, variability was also driven by root exudations and non-uniform accumulation of particulate organic matter, leading to heterogeneous sediment conditions on short temporal and small spatial scales. Replicate push-pull measurements conducted simultaneously within ~3 m^2 could vary by an order of magnitude during all summer months.

Porewater samples were collected during the push-pull measurements for dissolved inorganic nitrogen (DIN) and sulfide analysis. DIN samples were filtered (0.45 μm) and frozen until analysis. NH_4^+ and NO_3^- concentrations were measured on a Lachat QuikChem 8500 using standard colorimetric techniques (Zhang et al. 1997). Detection limits were 1.12 μM for NH_4^+ and 0.87 μM for NO_3^-. Sulfide samples were trapped with zinc acetate in the field and stored at 4 ºC until spectrophotometric analysis following Cline (1969).

At both the seagrass and bare sites, sediment samples were collected to determine porosity, organic matter, carbon, and nitrogen content of the sediment. A cut-off plastic syringe (2.5 cm inner diameter, ID) was used to collect 5 sediment samples to a depth of 5 cm at each site. Sediment samples were dried at 60 ºC to a constant weight; dry and wet weights were used to calculate sediment porosity. Organic matter was calculated based on loss on ignition after 6 hours in a 500ºC muffle furnace. Carbon and nitrogen content of sediments were measured on a Carlo Erba Elemental Analyzer with a 1020°C combustion tube, 650°C reduction tube, and helium as a carrier gas. Sediment samples were also collected to measure the concentration of chlorophyll *a* as a proxy for benthic microalgae abundance. A small cut-off syringe (1 cm ID) was used to collect 5 replicate surface sediment samples (2 cm depth) at each site. Samples were kept in the dark on ice and frozen on return to the laboratory. For analysis, thawed samples were extracted overnight in a 45:45 methanol:acetone solution and analyzed spectrophotmetrically after Lorenzen (1967).

At the seagrass site, shoot densities were measured by counting individual shoots in ten haphazardly distributed 0.25 m^2 quadrats. Seagrass biomass was measured in triplicate cores (15.24 cm ID, 15 cm depth); cores were sieved through 1 mm mesh and seagrass biomass was

sorted into aboveground and belowground fractions. Biomass samples were dried to constant weight at 60ºC.

Removal experiment

In summer 2015, a removal experiment was conducted in the meadow interior in order to compare denitrification and DNRA in sediments exposed to identical environmental conditions except for the presence of seagrass. Experimental sub-plots (4 m^2) were established at three of the original meadow plots. Plastic lawn edging was used to delineate the sub-plots and was inserted into the sediment to a depth of 8 cm. Denitrification and DNRA rates were measured in these sub-plots, and in surrounding seagrass sediments, before the removal of seagrass shoots (Figure 3.1). There was no statistical difference between rates in the sub-plots and surrounding sediments (Mann-Whitney U test, $p>0.05$ for denitrification and DNRA). Sediment samples were also collected to compare bulk sediment properties in the sub-plots and the surrounding sediments as above. The experiment was then begun by removing seagrass shoots within the sub-plots by hand; rhizomes in the surface sediments were also removed. Approximately 97% of living rhizome mass occurred in the upper 2 cm of sediment (based on belowground biomass in sediment cores segmented by 2 cm increments, data not shown); by removing these surface rhizomes and attached roots, we eliminated the majority of conduits for products of plant metabolism to deeper sediments. The cleared sub-plots were then left to equilibrate and re-establish sediment redox gradients for two weeks after clearing. The plastic lawn edging was left in place in order to prevent re-colonization of the cleared sub-plots by the surrounding seagrass

Two weeks after the removal, denitrification and DNRA rates were again measured in the cleared sub-plots and in the surrounding seagrass sediments; these measurements were repeated 4 weeks after removal. The cleared sub-plots remained bare during the four weeks of the

experiment. Samples for porewater DIN and sulfide and for sediment properties were collected and analyzed as above. There was no statistical difference in rates between weeks 2 and 4 (Mann Whitney U test, $p>0.05$ for both seagrass and cleared plots), so the rates were pooled. Analyses were then conducted to compare the rates in three datasets: 1) seagrass pre-removal, i.e. rates measured in sediments with seagrass present before the removal occurred, 2) seagrass at weeks 2-4, i.e. rates measured in sediments with seagrass present during weeks 2 and 4, and 3) cleared, i.e. rates measured in the experimentally cleared plots during weeks 2 and 4.

Seasonal monitoring

Additional measurements of denitrification and DNRA were made in seagrass sediments during October 2014 and April 2015. These measurements were combined with the summer 2014 and summer 2015 measurements at seagrass sites to complete a seasonal dataset for seagrass sediments only. Rates were measured at one meadow plot in October 2014 (n=7 total) and at three plots in April 2015 (n=9 total). Porewater samples for DIN and sulfide and seagrass density and biomass samples were measured as above.

Push-pull incubation technique

In the experiments described above, a new push-pull incubation technique was used to measure denitrification and DNRA in the seagrass and unvegetated sediment. Building on work by Koop-Jakobsen & Giblin (2009) and Addy et al. (2002), the push-pull technique is a non-destructive approach to measuring nitrate reduction in subsurface sediments under field conditions. Details of the technique are described in Aoki & McGlathery (2017), and it is summarized briefly below.

To measure dissimilatory nitrate reduction using the push-pull technique, a miniature piezometer (1.8 mm ID) was inserted into the sediment to a depth of 5 cm. Viton tubing

connected the piezometer to a graduated cylinder that served as a reservoir. A peristaltic pump was used to slowly (~4 mL min^{-1}) pump ~200 mL of porewater out of the sediment into the graduated cylinder; a 20 mL layer of castor oil in the cylinder was used to prevent exchange between the porewater and the atmosphere. Duplicate 12 mL samples of porewater were collected in Exetainers and fixed with 50 μL of $ZnCl_2$ (100% m/v) and stored in a water bath. An additional 10 mL sample was filtered (0.45 μm) and stored on ice for DIN analysis, and two 1 mL samples were fixed with 0.01 M zinc acetate for sulfide analysis.

The porewater was then amended with a spike of artificial seawater containing $^{15}NO_3^-$ (99% ^{15}N, Cambridge Isotope Laboratories) and saturated with argon gas (Ar). After spiking, the concentration of nitrate in the porewater was approximately 100 μM. Duplicate samples were again collected, fixed, and stored in a water bath. The spiked porewater was then pumped ("pushed") into the sediment and allowed to incubate in situ. Additional samples were retrieved ("pulled") at half-hour intervals over the next 2 hours to produce a time series. After the final porewater sample was collected, a small sediment core (2.54 cm ID, 10 cm depth) was collected from the injection point and frozen for ammonium extraction and DNRA analysis.

Porewater samples were held in the water bath at or below the field temperature until analysis using membrane inlet mass spectrometry (MIMS) within 6 weeks. MIMS was used to determine the concentrations of denitrification products ($^{28}N_2$, $^{29}N_2$, $^{30}N_2$) and Ar in the samples (Kana et al. 1994). A copper reduction column heated to 500 ºC was included inline with the MIMS to remove oxygen from the gas analyte before analysis. Previous work has shown that oxygen can interfere with detection of other gas signals, leading to overestimation of denitrification using the IPT equations (Eyre et al. 2004, Lunstrum & Aoki 2016). Ar concentrations were used to correct for diffusion and gas loss; $^{29}N_2$ concentrations were also

corrected to account for mixing with ambient porewater and impurities in the $^{15}NO_3^-$ spike (Aoki and McGlathery 2017, Koop-Jakobsen and Giblin 2009). Linear production rates (p_{29} and p_{30}) were calculated from the corrected time series of $^{29}N_2$ and $^{30}N_2$. Isotope pairing equations (Eqn. 1 and 2) were then used to calculate D_{14}, the denitrification of ambient nitrate, and D_{15}, the denitrification of the amended $^{15}NO_3^-$ nitrate (Nielsen 1992):

$$D_{15} = p_{29} + (2 \times p_{30}) \quad (1)$$

$$D_{14} = D_{15} \times \frac{p_{29}}{(2 \times p_{30})} \quad (2)$$

These rates were converted from units of μM h^{-1} to areal rates (μmol N m^{-2} h^{-1}) using the sediment porosity and integrating over the depth of the incubation (calculated from the volume of amended porewater returned to the sediment, see Aoki and McGlathery 2017 for details).

DNRA analysis was conducted using a modified OX/MIMS method Yin et al. (2014). The frozen sediment cores were thawed and ammonium was extracted with 90 mL of 2 M KCl. After extraction, each sample was centrifuged, and five replicate Exetainers were filled with the supernatant. A hypobromite solution, prepared as in Yin et al. (2014), was added to three of the five Exetainers, causing the ammonium to oxidize to N_2. All five vials were then analyzed using MIMS for ^{29} and $^{30}N_2$ concentrations. Excess ^{29} and $^{30}N_2$ in the oxidized vials compared to the unoxidized vials was assumed to result from the oxidation of $^{15}NH_4^+$, the product of DNRA in the sediment. $DNRA_{15}$, the reduction of the $^{15}NO_3^-$ spike, was calculated as the production of $^{15}NH_4^+$ over time. $DNRA_{14}$ was then calculated from Equation 3, which assumes that the probability of reducing ^{14} NO_3^- or $^{15}NO_3^-$ is the same for DNRA as for denitrification (Christensen 2000):

$$DNRA_{14} = DNRA_{15} \times \frac{D_{14}}{D_{15}} \quad (3)$$

Again, rates were integrated over the depth of the incubation to determine areal rates.

For both denitrification and DNRA, the reduction of $^{14}NO_3^-$ (D_{14} and $DNRA_{14}$) was considered the ambient rate, or the underlying rate under natural conditions. Because nitrate concentrations in this system were very low (consistently below the detection limit of 0.87 μM in porewater), the ambient rates refer to low-nitrate conditions. In contrast, the reduction of the added $^{15}NO_3^-$ spike (D_{15} and $DNRA_{15}$) was considered the potential rate, or the rate under high-nitrate conditions.

Statistical analysis

The denitrification and DNRA rates measured in the seagrass sites were often non-normal, with maximum rates exceeding the median value by an order of magnitude, and log-transformations did not achieve normality. Conservative non-parametric methods were therefore used to compare the datasets, and boxplots were used to assess differences in the distributions. Mann Whitney U tests were used for the comparison with the external bare site, and Kruskal-Wallis tests were used for the removal experiment and the seasonal data. Statistical analyses were conducted in R 3.3.3 (R Core Team 2017).

Results

External bare site comparison

Ambient denitrification and DNRA rates were on average four times greater at the seagrass site compared to the bare site (mean denitrification and DNRA rates were 19.7 μmol N m^{-2} h^{-1} and 12.2 μmol N m^{-2} h^{-1} respectively at the seagrass site compared to 4.9 μmol N m^{-2} h^{-1} and 3.1 μmol N m^{-2} h^{-1} at the bare site). The rates measured at the seagrass site were also characterized by extreme rates that exceeded median rates by an order of magnitude, whereas extreme rates were not observed at the bare site (Figure 3.2). Due to the high variability in the

seagrass rates, the differences between sites had low statistical significance (Mann-Whitney U tests, $p = 0.10$ for denitrification, $p = 0.09$ for DNRA).

Dissimilatory nitrate reduction at both the seagrass and bare sites was limited by nitrate availability. Concentrations of nitrate in the porewater were undetectable at both sites (Table 3.2), suggesting that all dissimilatory nitrate reduction was coupled to nitrification. Potential rates (measured as reduction of the excess $^{15}NO_3^-$ spike) were significantly greater than ambient rates across both sites (Mann-Whitney U test, $p<0.0005$ for both denitrification and DNRA), indicating a nitrate limitation under ambient conditions (Figure 3.3). There was no significant difference in potential rates between the sites (Mann-Whitney U tests, $p = 0.48$ for denitrification and $p = 0.30$ for DNRA). Comparing the distributions, the potential DNRA distributions were very similar between the two sites, whereas potential denitrification had a higher median value and greater spread at the seagrass site. Spatially and temporally variable competition for nitrate from the seagrass likely contributed to the greater spread in potential denitrification rates at the seagrass site compared to the bare site. However, the minimum and maximum potential rates were higher at the seagrass site. At the bare site, multiple incubations produced undetectable potential denitrification rates (i.e. no measureable production of $^{30}N_2$ or $^{29}N_2$), and the maximum rate was about half the maximum rate at the seagrass site. These differences suggest that seagrass presence did have a stimulation effect on denitrification, despite additional competition for nitrate.

Removal experiment

In the seagrass removal experiment, denitrification and DNRA showed contrasting patterns following removal (Figure 3.4). Specifically, mean denitrification rates declined from 15.2 μmol N m^{-2} h^{-1} in seagrass plots before removal to 11.1 μmol N m^{-2} h^{-1} in the seagrass plots

at weeks 2-4 and 5.3 μmol N m^{-2} h^{-1} in the cleared plots at weeks 2-4 (Kruskal-Wallis test, p = 0.11). In contrast, mean DNRA rates were relatively constant between the treatments, at 11.8 μmol N m^{-2} h^{-1} in the pre-removal seagrass plots, 13.7 μmol N m^{-2} h^{-1} in the seagrass plots at weeks 2-4, and 15.4 μmol N m^{-2} h^{-1} in the cleared plots (Kruskal-Wallis test, p = 0.74). Consequently, while DNRA accounted for only 45% of total dissimilatory nitrate reduction in the pre-removal seagrass plots, DNRA dominated in both the seagrass plots at weeks 2-4 and the cleared plots, accounting for 61% and 71% of total dissimilatory nitrate reduction respectively. These contrasting patterns suggest the seagrass removal altered conditions in the sediment to favor DNRA over denitrification. A decrease in nitrification could have led to that change by creating high-carbon low-nitrate conditions favorable to DNRA. The presence of extreme outliers throughout the dataset again suggests that these effects on the sediment were heterogeneous over small spatial scales.

Comparing the seagrass rates before removal and at 2-4 weeks is complicated by the fact that the meadow experienced a die-back event after the removal experiment was initiated, likely caused by high surface water temperatures. Shoot densities declined from over 350 shoots m^{-2} in the pre-removal seagrass plots to 150 shoots m^{-2} in the seagrass plots at the end of the experiment. With lower seagrass densities, the effects of seagrass activity on sediment biogeochemistry were likely reduced compared to the pre-removal seagrass plots. The comparison of measurements in the cleared plots with the seagrass plots at 2-4 weeks is therefore a conservative estimate of the seagrass effects on nitrogen removal.

Changes in porewater chemistry were also observed following the seagrass removal in the cleared plots, where porewater ammonium concentrations increased by an order of magnitude, possibly indicating the lack of plant uptake (Table 3.4). A similar effect may have

occurred in the seagrass plots at 2-4 weeks, where seagrass shoot densities declined rapidly in response to the high-temperature event. Sulfide concentrations were similar in the seagrass plots throughout the experiment but were slightly elevated in the cleared plots. The seagrass removal may have increased sulfide concentrations by eliminating the transfer of oxygen from roots to the sediment; however this effect was limited as sulfide concentrations in the cleared plots remained low compared to coastal ecosystems with highly sulfidic (100-1000 μM) sediments such as marshes.

Under high-nitrate conditions, potential rates in the removal experiment were significantly greater than ambient rates (Mann-Whitney U test, $p<0.005$ for both denitrification and DNRA) and followed similar patterns as the ambient rates under low-nitrate conditions (Figure 3.5). Specifically, potential denitrification dominated in the pre-removal seagrass plots and declined following removal in the seagrass plots and cleared plots whereas potential DNRA was constant before and after removal in all plots. This pattern again suggests either greater carbon availability or greater nitrification in the pre-removal seagrass plots, although the trends in potential rates were not statistically significant (Kruskal-Wallis tests, $p = 0.24$ for denitrification and $p = 0.12$ for DNRA).

Denitrification rates were similar in the cleared sediments within the meadow and the external bare sediments outside the meadow (5.3 and 4.9 μmol m^{-2} h^{-1} respectively). In contrast, DNRA rates were higher in the cleared sediments compared to the external bare sediments (15.4 and 3.1 μmol m^{-2} h^{-1} respectively). Nitrate availability was low in both the cleared and bare plots (ambient nitrate concentrations were undetectable and the nitrate spike produced significantly higher potential rates). However, the cleared plots in the removal experiment had higher bulk organic matter and bulk carbon content than the bare plots (Table 3.5). Some amount of

belowground biomass was also likely present in the cleared plots, despite efforts to remove rhizomes from the surface sediments, and any remaining roots could have leached organic carbon into the sediments. Thus, more organic carbon was likely available at the cleared plots, creating low-nitrate, high-carbon conditions that favor DNRA over denitrification (Burgin and Hamilton, 2007).

Seasonal patterns in nitrate reduction

Measurements of nitrate reduction in the meadow from June 2014-June 2015 showed that denitrification was on average greater than DNRA during spring and summer (Figure 3.6). Denitrification showed a seasonal pattern, with the highest mean rates in the summer and the lowest mean rates in the spring (Kruskal-Wallis test, $p = 0.13$). DNRA also showed peak rates in summer, but there was no trend between seasons (Kruskal-Wallis test, $p = 0.48$). Low nitrate reduction rates in spring may indicate competition for nitrate from rapidly growing seagrass; although porewater nitrate levels were undetectable throughout the year, porewater ammonium concentrations were at a minimum in spring, suggesting greater plant uptake of nitrogen (Table 3.2). Lower mineralization rates in spring might also account for the low porewater ammonium concentrations.

As noted above, in summer 2014, the maximum rates of both denitrification and DNRA were roughly an order of magnitude greater than the median rates. This pattern was also evident in spring and summer 2015 for DNRA and in spring 2015 for denitrification. These maximum rates indicate that within the heterogeneous sediment matrix, conditions existed to support very high rates of dissimilatory nitrate reduction during spring and summer. In contrast, maximum rates in the fall were approximately 2x the median rates for both denitrification and DNRA,

suggesting conditions were less conducive to supporting high dissimilatory nitrate reduction rates.

Under high-nitrate conditions, both potential denitrification and potential DNRA were significantly enhanced across all seasons compared to the ambient rates (Mann-Whitney U-test, $p<0.005$, Figure 3.7). Significant differences were observed between summer 2014 and spring 2015 for potential denitrification and between summer 2014 and summer 2015 for potential DNRA (Kruskal-Wallis test, $p < 0.05$). More interestingly, the pattern of extreme rates was evident for potential denitrification across the seasons, with maximum rates that exceeded median rates by 4-47x. In contrast, potential DNRA rates were not as strongly enhanced by the nitrate spike, with maximum potential rates no more than 3x the potential median rates across all seasons. Thus, while extreme rates of both DNRA and denitrification were possible under the low-nitrate, ambient conditions, the addition of the excess nitrate spike enhanced the maximum rates of denitrification compared to DNRA.

Extrapolations to daily and annual rates

Given the presence of extreme values and consequent non-normal distribution of the data, we used bootstrapping to verify that the arithmetic mean rates of denitrification and DNRA were representative before scaling to daily and annual rates. Combining the two summers, we had a total of 20 individual rate measurements in seagrass sediments during summer (Figure 3.6). The arithmetic mean rates of denitrification and DNRA over those 20 measurements were 17.5 and 12.0 μmol m^{-2} h^{-1} respectively. We subsampled with replication over 1000 bootstrap replicates to calculate bootstrapped mean rates; over 10 repeated analyses, bootstrapped mean rates varied from 17.2-17.6 μmol m^{-2} h^{-1} for denitrification and from 11.8-12.2 μmol m^{-2} h^{-1} for DNRA. As these bootstrapped means agreed very well with the arithmetic means, we were confident in

scaling up the summer rates from the hourly arithmetic means. The sample sizes for the fall and spring rates were too small to apply bootstrapping (n=7 and n=9 respectively). However, the fall and spring rates had fewer extreme values and smaller ranges (Figure 3.6), so we concluded that the arithmetic means were reasonable to scale up.

Calculating daily rates required consideration of denitrification and DNRA under dark conditions, since the push-pull measurements were conducted only during the day. Under dark conditions, the seagrass effects from root exudation will be reduced but not eliminated; radial oxygen loss from root tips of *Z. marina* declined by approximately 70% in the dark compared to saturated light conditions but did not fall to zero (Jovanovic et al. 2015). Thus root exudation could continue to support some level of denitrification and DNRA even in the dark. Additionally, previous work has shown higher rates of coupled nitrification-denitrification in surface sediments under dark conditions; the enhanced rates were attributed to decreased competition for nitrate from the plants (Welsh et al. 2000). Therefore, it is not unreasonable to expect some amount of dissimilatory nitrate reduction under dark conditions. However, the high hotspot rates observed in the seagrass sediments would likely not occur in the dark. For comparative purposes, we therefore calculated a range of daily rates. For the minimum predicted daily rates, we assumed that no denitrification or DNRA occurred in the dark and scaled the daytime rates by 12 hours. For the maximum predicted daily rates, we removed the outliers from the datasets and used the median of the remaining points as the dark rate; we scaled the daylight and dark rates by 12 hours each.

Based on these assumptions, we predicted that the daily denitrification rates would fall between 53-109 μmol N m^{-2} d^{-1} in the fall, 80-81 μmol N m^{-2} d^{-1} in spring, and 209-351 μmol N m^{-2} d^{-1} in summer. Daily DNRA rates would range from 60-116 μmol N m^{-2} d^{-1} in fall, 48-63

μmol N m^{-2} d^{-1} in spring, and 144-191 μmol N m^{-2} d^{-1} in summer. We further hypothesized that rates were minimal during winter due to low sediment temperatures and decreased seagrass presence (data not shown); we therefore estimated winter rates as half of the fall rates, based on seasonal differences in other seagrass meadows (Eyre et al. 2013, Russell et al. 2016). Using the range of daily rates for each season, we estimated annual denitrification and DNRA in the meadow sediments as 34-54 mmol N m^{-2} y^{-1} and 26-39 mmol N m^{-2} y^{-1} respectively. We estimated annual rates in the bare sediments as a percentage of the annual rates in seagrass sediments, based on the ratio of bare to seagrass rates in summer; bare rates were 9-14 and 7-10 mmol N m^{-2} y^{-1} for denitrification and DNRA.

Discussion

Denitrification hotspots in seagrass sediment

This study provides important evidence for the presence of denitrification hotspots in subtidal seagrass sediments. Extreme rates were consistently measured in the vegetated sediments but not in the bare sediments, suggesting the presence of localized denitrification hotspots and/or hot moments (i.e. temporal hotspots) associated with seagrass presence. These hotspots likely indicated areas and times where the seagrass strongly altered nitrate and/or labile carbon availability. This effect was heterogeneous over small spatial scales (<1 m^2) and was variable over time, as many of the measured rates in seagrass sediments were low and similar to rates in the unvegetated sediments. Overall, there was a clear pattern of enhanced and more variable denitrification rates measured in the vegetated sediments, driven by the extreme rates occurring in hotspots and hot moments.

The presence of these hotspots and hot moments in subsurface sediments highlights the importance of accounting for subsurface denitrification and DNRA rates and raises questions

about scaling these rates both spatially and temporally. Our measurements suggest that sediment heterogeneity on small spatial scales (i.e. m^2) is comparable to heterogeneity at larger scales (i.e. between 0.4 ha plots). The mean rates presented here may therefore be broadly applicable within the seagrass meadow, even though spatial coverage was limited to three plots. However, in this particular meadow, sediment conditions and seagrass metrics show spatial patterns at the meadow scale (km^2) (Oreska et al. 2017), and it remains to be seen whether these differences influence the variability of denitrification and DNRA rates. Areas near the edge of the meadow, where seagrass shoot densities are lower, may have lower and/or less variable rates. In terms of temporal variability, extreme denitrification and DNRA rates were measured in spring and summer, but not in fall, indicating the importance of the seagrass growing season in supporting these hotspots. Additional measurements of subsurface denitrification and DNRA in other seagrass meadows and across seasons are needed to establish the general importance of subsurface hotspots.

The push-pull method used in this study improves on conventional core methods by conducting the incubation in situ and thus capturing the variability in rates driven by heterogeneous field conditions in subsurface seagrass sediments (Aoki & McGlathery 2017). Previous studies using core incubations have shown mixed impacts of seagrass on sediment denitrification, with some measuring higher rates in vegetated sediments (Eyre et al. 2011, Piehler & Smyth 2011, Smyth et al. 2013) and others showing higher rates in bare sediments (Risgaard-Petersen et al. 1998, Ottosen et al. 1999), no significant difference (Russell et al. 2016), or contrasting site-specific effects (Zarnoch et al. 2017). Differences in nutrient status do not explain the mixed findings, as studies that found no enhancement in seagrass include both low (e.g. Russell et al. 2016) and high nutrient sites (e.g. Ottosen et al. 1999). However, none of

these studies showed a hotspot effect in vegetated sediments, likely due to the more constrained conditions in core incubations that do not replicate hydrodynamic flow and the interactions of light and flow that can alter seagrass activity (Koch et al. 2006, Rheuban et al. 2014). The push-pull method also directly measures subsurface processes, in contrast to isotope pairing core incubations that rely on diffusion of the isotope tracer from surface water into the sediments. These earlier studies may therefore have underestimated coupled denitrification rates and may have minimized the difference between vegetated and bare rates. More widespread application of the push-pull incubation method would help to better understand how seagrass affects denitrification rates.

Although this study showed the presence of denitrification hotspots in the restored meadow, the areal denitrification rates were low (19.7 μmol m^{-2} h^{-1} in summer) compared to most recent measurements in subtidal seagrass meadows (28-824 μmol m^{-2} h^{-1}; Eyre et al. 2011, 2013, Piehler & Smyth 2011, Smyth et al. 2013). These studies used the N_2:Ar method, rather than isotope pairing, and there is some concern that methodological differences between the two techniques lead to higher rates in N_2:Ar studies (Eyre et al. 2013). However, another recent study using N_2:Ar measured rates comparable to this study (Zarnoch et al. 2017), which suggests that methodology is not the only source of difference in measurements of seagrass denitrification rates. Furthermore, it is critical to note that the higher rates of denitrification were measured primarily under dark conditions, which alleviate competition for nitrate from autotrophs. Under light conditions, Eyre et al. (2011) reported denitrification rates of <20 μmol N m^{-2} h^{-1} in a *Zostera capricorni* meadow measured via N_2:Ar, which is comparable to the mean rate of 19.7 μmol N m^{-2} h^{-1} reported here. The agreement between these two studies suggests that the push-

pull isotope pairing method is an effective alternative to N_2:Ar and also raises the possibility that much higher rates of denitrification might be measured via push-pull under dark conditions.

Relative importance of DNRA

In the seagrass sediments, DNRA rates were in general lower than denitrification rates, but the relative importance of DNRA fluctuated between seasons. In spring and summer, during periods of peak seagrass growth, DNRA was between 38-48% of total nitrate reduction, whereas in the fall, the relative importance of DNRA increased to 57%, making DNRA the dominant dissimilatory nitrate reduction process during seagrass senescence. Of the previous studies comparing DNRA and denitrification in seagrass meadows, Gardner et al. 2006 measured comparable rates, while others have found dominance of denitrification (Smyth et al. 2013) or DNRA (Boon et al. 1986, Rysgaard et al. 1996, An & Gardner 2002). The results of this study suggest that the dominance of DNRA versus denitrification can vary seasonally, following seasonal patterns in seagrass growth (Table 3.3).

The removal experiment results provide additional evidence that seagrass activity modulates the relative importance of DNRA in this system. Denitrification decreased and DNRA increased slightly in the cleared plots, increasing the relative importance of DNRA following seagrass loss. The pattern was more dramatic than the seasonal shifts observed above, with DNRA accounting for 71% of total nitrate reduction in the cleared plots, compared to only 45% in the pre-removal seagrass plots. DNRA importance also increased to 61% in the seagrass plots during weeks 2-4, when the seagrass suffered shoot losses following a high-temperature event. Overall, these results indicate that seagrass presence supports an environment more favorable to denitrification than DNRA.

The shift toward increased dominance of DNRA following the removal of seagrass could have been caused by a decrease in nitrification. Porewater concentrations of both ammonium and sulfide were enhanced in the cleared plots compared to the pre-removal seagrass plots (Table 3.4). Higher sulfide concentrations suggest more reduced conditions and therefore lower nitrification rates, while the increase in ammonium concentration could indicate either decreased nitrification or decreased uptake of nitrogen by the seagrass following the removal. Under low-nitrate conditions, DNRA-capable microbes are known to outcompete denitrifiers if sufficient carbon substrate is available (Burgin & Hamilton 2007, Hardison et al. 2015). The changes in porewater chemistry therefore suggest that nitrification was more limited following seagrass removal, leading to the shift toward DNRA dominance. Likewise, these changes also suggest that the presence of seagrass enhanced denitrification by supporting nitrification.

Ambient vs. potential nitrate conditions

Given an abundant supply of labile carbon, as in the seagrass meadow sediments, DNRA-capable microbes are predicted to out-compete denitrifiers if nitrate availability is low, whereas denitrifiers will dominate if nitrate availability is high (Tiedje et al. 1982, Burgin & Hamilton 2007). Differences between the ambient nitrate reduction rates (reduction of the ambient $^{14}NO_3^-$, reflecting low nitrate conditions) and the potential rates (reduction of the $^{15}NO_3^-$ spike, reflecting high nitrate conditions) in the seagrass sediments support this hypothesis. Ambient rates of both denitrification and DNRA included extreme values in hotspots that were an order of magnitude greater than median values. However, the potential rates included extreme values only for denitrification, not for DNRA. This pattern was observed across all the datasets, and it suggests that with higher nitrate availability in seagrass sediments, maximum denitrification rates will outweigh maximum DNRA rates.

Differences in the distributions of potential denitrification rates between the seagrass and bare sediments suggest the importance of labile carbon supplied by seagrass exudates. The excess nitrate available in the spike should have relieved nitrate limitations on the potential denitrification rates in both the seagrass sediments and the bare sediments. However, maximum and median potential denitrification rates were still higher in the seagrass sediments compared to both the external bare site and the cleared sediments from the removal (Figures 3.3 and 3.5). This difference may indicate that the seagrass enhanced labile carbon availability and thus boosted the maximum potential rates. However, more data, such as porewater DOC concentrations in the seagrass and bare plots, would be needed to fully support this conclusion.

The observed pattern of enhanced denitrification under high-nitrate conditions, as well as the increased dominance of DNRA following seagrass loss, provide insight into the possible trajectories of nitrate reduction in seagrass sediments experiencing increasing nutrient loading. As long as seagrass growth is undisturbed by higher nutrient loads, a greater availability of nitrate should lead to increased denitrification. Increased denitrification would in turn serve as a buffer against higher nutrient loading (up to a point) by removing reactive nitrogen from the system. In contrast, if higher nutrient loads impair seagrass growth or cause loss of seagrass, for example by increasing phytoplankton in the water column, epiphytes on seagrass leaves, or macroalgae, and reducing light availability, DNRA is likely to increase relative to denitrification, leading to greater retention of reactive nitrogen. This shift could drive a positive feedback, with increased porewater ammonium concentrations that negatively affect seagrass growth and contribute to seagrass loss (Figure 3.8).

Implications for restoration

The results of this study suggest that the seagrass restoration had a pronounced effect on nitrate reduction rates because vegetated sediment can support hotspots with much higher rates of both denitrification and DNRA than unvegetated sediment. This increase in dissimilatory nitrate reduction is important in the context of very low nitrogen loading to the Virginia coastal bays. Recent work has estimated loading rates of 1.4 g N m^{-2} yr^{-1} to the bays from allochthonous sources (atmospheric and terrestrial) (Anderson et al. 2010). Spatial and temporal variability in the measured rates introduce uncertainty into extrapolated daily and annual rates, but our data clearly show that denitrification peaked in summer. Using the assumptions described above (e.g. scaling hourly daytime rates by a 12-hour day), denitrification in the meadow would remove 19% of allochthonous nitrogen inputs per m^2 during the fall and 76% during the summer. In comparison, nitrogen removal via denitrification in bare sediments would be only 21% of allochthonous nitrogen inputs in the summer. The effect of the restoration on nitrate removal in the lagoon is thus non-trivial and serves to enhance the nutrient filtering capacity of the lagoon. At the same time, nitrate retention through DNRA was also enhanced by the restoration. Internal recycling is known to be an important source of nitrogen to the Virginia coastal bays, providing as much as 77% of total nitrogen inputs (Anderson et al. 2010). DNRA may therefore play an important role in supporting high rates of productivity in the restored meadow by recycling nitrate into more bioavailable ammonium. Removal of nitrate via denitrification was greater than recycling via DNRA in spring and summer, whereas recycling was greater than removal during the fall. Because maximum rates of both processes occurred during summer, denitrification outweighed DNRA on an annual basis. The net effect of the restoration on nitrate reduction was therefore to enhance nitrogen removal.

The effects of the seagrass restoration on nitrogen cycling extend beyond enhanced nitrate reduction processes. Seagrass assimilation of nitrogen in biomass, as well as burial of particulate nitrogen in the meadow sediments, likely outweigh nitrate reduction fluxes by an order of magnitude (McGlathery 2008). Nevertheless, nitrate removal via denitrification helps maintain positive feedbacks that support continued seagrass growth. Given the global declines in seagrass meadow area, as well as increasing anthropogenic N loading to coastal waters, the enhanced nutrient filter observed in this restored seagrass meadow highlights one important ecological benefit of restoration and provides motivation to protect and restore seagrass ecosystems.

References

Addy K, Kellogg DQ, Gold AJ, Groffman PM, Ferendo G, Sawyer C (2002) In situ push–pull method to determine ground water denitrification in riparian zones. J Environ Qual 31:1017–1024

An S, Gardner WS (2002) Dissimilatory nitrate reduction to ammonium (DNRA) as a nitrogen link, versus denitrification as a sink in a shallow estuary (Laguna Madre/Baffin Bay, Texas). Mar Ecol Prog Ser 237:41–50

Anderson IC, Stanhope JW, Hardison AK, McGlathery KJ (2010) Sources and fates of nitrogen in Virginia coastal bays. In: Coastal Lagoons: Critical habitats of environmental change. CRC Press, Boca Raton, FL, p 43–72

Aoki LR, McGlathery KJ (2017) Push-pull incubation method reveals the importance of denitrification and dissimilatory nitrate reduction to ammonium in seagrass root zone. Limnol Oceanogr Methods 15:766–781

Boon PL, Moriarty DJW, Saffigna PG (1986) Nitrate metabolism in sediments from seagrass (*Zostera capricorni*) beds of Moreton Bay, Australia. Mar Biol 91:269–275

Brunet RC, Garcia-Gil LJ (1996) Sulfide-induced dissimilatory nitrate reduction to ammonia in anaerobic freshwater sediments. FEMS Microbiol Ecol 21:131–138

Burgin A, Hamilton S (2007) Have we overemphasized the role of denitrification in aquatic ecosystems? A review of nitrate removal pathways. Front Ecol Environ 5:89–96

Christensen PB (2000) Sediment mineralization, nutrient fluxes, denitrification and dissimilatory nitrate reduction to ammonium in an estuarine fjord with sea cage trout farms. Aquat Microb Ecol 21:73–84

Cline JD (1969) Spectrophotometric determination of hydrogen sulfide in natural waters. Anal Chem 21:1005–1009

Cloern JE (2001) Our evolving conceptual model of the coastal eutrophication problem. Mar Ecol Prog Ser 210:223–253

Eyre BD, Ferguson AJP, Webb A, Maher D, Oakes JM (2011) Denitrification, N-fixation and nitrogen and phosphorus fluxes in different benthic habitats and their contribution to the nitrogen and phosphorus budgets of a shallow oligotrophic sub-tropical coastal system (southern Moreton Bay, Australia). Biogeochemistry 102:111–133

Eyre BD, Maher DT, Squire P (2013) Quantity and quality of organic matter (detritus) drives N_2 effluxes (net denitrification) across seasons, benthic habitats, and estuaries. Glob Biogeochem Cycles 27:1083–1095

Eyre BD, Rysgaard S, Dalsgaard T, Christensen PB (2004) Reply to comment on our paper "Comparison of isotope pairing and N_2:Ar methods for measuring sediment denitrification." Estuaries 27:177–178

Fagherazzi S, Wiberg PL (2009) Importance of wind conditions, fetch, and water levels on wave-generated shear stresses in shallow intertidal basins. J Geophys Res 114:F03022

Frederiksen MS, Glud RN (2006) Oxygen dynamics in the rhizosphere of *Zostera marina*: A two-dimensional planar optode study. Limnol Oceanogr 51:1072–1083

Galloway JN, Dentener FJ, Capone DG, Boyer EW, Howarth RW, Seitzinger SP, Asner GP, Cleveland CC, Green PA, Holland EA, Karl DM, Michaels AF, Porter JH, Townsend AR, Vorosmarty CJ (2004) Nitrogen cycles: Past, present, and future. Biogeochemistry 70:153–226

Gardner WS, McCarthy MJ, An S, Sobolev D, Sell KS, Brock D (2006) Nitrogen fixation and dissimilatory nitrate reduction to ammonium (DNRA) support nitrogen dynamics in Texas estuaries. Limnol Oceanogr 51:558–568

Giblin A, Tobias C, Song B, Weston N, Banta G, Rivera-Monroy V (2013) The importance of dissimilatory nitrate reduction to ammonium (DNRA) in the nitrogen cycle of coastal ecosystems. Oceanography 26:124–131

Greiner JT, McGlathery KJ, Gunnell J, McKee BA (2013) Seagrass restoration enhances "blue carbon" sequestration in coastal waters (J Cebrian, Ed.). PLoS ONE 8:e72469

Gribsholt B, Kristensen E (2002) Impact of sampling methods on sulfate reduction rates and dissolved organic carbon (DOC) concentrations in vegetated salt marsh sediments. Wetl Ecol Manag 10:371–379

Hansen JW, Lomstein BA (1999) Leakage of ammonium, urea, and dissolved organic nitrogen and carbon from eelgrass *Zostera marina* roots and rhizomes during sediment handling. Aquat Microb Ecol 16:303–307

Hardison AK, Algar CK, Giblin AE, Rich JJ (2015) Influence of organic carbon and nitrate loading on partitioning between dissimilatory nitrate reduction to ammonium (DNRA) and N_2 production. Geochim Cosmochim Acta 164:146–160

Howarth RW (2008) Coastal nitrogen pollution: A review of sources and trends globally and regionally. Harmful Algae 8:14–20

Howarth RW, Marino R (2006) Nitrogen as the limiting nutrient for eutrophication in coastal marine ecosystems: Evolving views over three decades. Limnol Oceanogr 51:364–376

Huettel M, Gust G (1992) Solute release mechanisms from confined sediment cores in stirred benthic chambers and flume flows. Mar Ecol Prog Ser 82:187–197

Jovanovic Z, Pedersen M, Larsen M, Kristensen E, Glud R (2015) Rhizosphere O_2 dynamics in young *Zostera marina* and *Ruppia maritima*. Mar Ecol Prog Ser 518:95–105

Kana TM, Darkangelo C, Hunt MD, Oldham JB, Bennett GE, Cornwell JC (1994) Membrane inlet mass spectrometer for rapid high-precision determination of N_2, O_2, and Ar in environmental water samples. Anal Chem 66:4166–4170

Koch EW, Ackerman JD, Verduin J, Keulen M van (2006) Fluid dynamics in seagrass ecology: From molecules to ecosystems. In: Seagrasses: Biology, ecology, and conservation. Springer, Dordrecht, The Netherlands, p 193–225

Koop-Jakobsen K, Giblin AE (2009) New approach for measuring denitrification in the rhizosphere of vegetated marsh sediments. Limnol Oceanogr Methods 7:626–637

Lorenzen CJ (1967) Determination of chlorophyll and pheo-pigments: spectrophotometric equations. Limnol Oceanogr 12:343–346

Lunstrum A, Aoki LR (2016) Oxygen interference with membrane inlet mass spectrometry may overestimate denitrification rates calculated with the isotope pairing technique. Limnol Oceanogr Methods 14:425–431

McGlathery KJ (2008) Seagrass Habitats. In: Nitrogen in the Marine Environment, 2nd edn. Elsevier, New York

McGlathery K (2016) Sediment grain size in seagrass restoration plots in the Virginia coastal bays, 2010-2016. Virginia Coast Reserve Long-Term Ecological Research Project Data Publication doi:10.6073/pasta/b842a04cb9f54e16ecc4b3f266828515

McGlathery K, Reynolds L, Cole L, Orth R, Marion S, Schwarzschild A (2012) Recovery trajectories during state change from bare sediment to eelgrass dominance. Mar Ecol Prog Ser 448:209–221

McGlathery K, Sundbäck K, Anderson I (2007) Eutrophication in shallow coastal bays and lagoons: The role of plants in the coastal filter. Mar Ecol Prog Ser 348:1–18

Meysman FJ, Galaktionov OS, Glud RN, Middelburg JJ (2010) Oxygen penetration around burrows and roots in aquatic sediments. J Mar Res 68:309–336

National Research Council (2000) Clean coastal waters: Understanding and reducing the effects of nutrient pollution. National Academies Press, Washington, D.C.

Nielsen LP (1992) Denitrification in sediment determined from nitrogen isotope pairing. FEMS Microbiol Lett 86:357–362

Oreska MPJ, McGlathery KJ, Porter JH (2017) Seagrass blue carbon spatial patterns at the meadow-scale (J Cebrian, Ed.). PLOS ONE 12:e0176630

Orth R, McGlathery K (2012) Eelgrass recovery in the coastal bays of the Virginia Coast Reserve, USA. Mar Ecol Prog Ser 448:173–176

Orth R, Moore K, Marion S, Wilcox D, Parrish D (2012) Seed addition facilitates eelgrass recovery in a coastal bay system. Mar Ecol Prog Ser 448:177–195

Ottosen LDM, Risgaard-Petersen N, Nielsen LP (1999) Direct and indirect measurements of nitrification and denitrification in the rhizosphere of aquatic macrophytes. Aquat Microb Ecol 19:81–91

Pagès A, Welsh DT, Robertson D, Panther JG, Schäfer J, Tomlinson RB, Teasdale PR (2012) Diurnal shifts in co-distributions of sulfide and iron(II) and profiles of phosphate and ammonium in the rhizosphere of *Zostera capricorni*. Estuar Coast Shelf Sci 115:282–290

Pelegri SP, Nielsen LP, Blackburn TH (1994) Denitrification in estuarine sediment stimulated by the irrigation activity of the amphipod *Corophium volutator*. Mar Ecol Prog Ser:6

Piehler MF, Smyth AR (2011) Habitat-specific distinctions in estuarine denitrification affect both ecosystem function and services. Ecosphere 2:art12

R Core Team (2017) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/

Rheuban JE, Berg P, McGlathery KJ (2014) Ecosystem metabolism along a colonization gradient of eelgrass (*Zostera marina*) measured by eddy correlation. Limnol Oceanogr 59:1376–1387

Risgaard-Petersen N, Dalsgaard T, Rysgaard S, Christensen PB, Borum J, McGlathery K, Nielsen LP (1998) Nitrogen balance of a temperate eelgrass *Zostera marina* bed. Mar Ecol-Prog Ser 174:281–291

Risgaard-Petersen N, Ottosen LDM (2000) Nitrogen cycling in two temperate *Zostera marina* beds: seasonal variation. Mar Ecol Prog Ser 198:93–107

Russell DG, Warry FY, Cook PLM (2016) The balance between nitrogen fixation and denitrification on vegetated and non-vegetated intertidal sediments. Limnol Oceanogr 61:2058–2075

Rysgaard S, Risgaard-Petersen N, Sloth NP (1996) Nitrification, denitrification, and nitrate ammonification in sediments of two coastal lagoons in Southern France. Hydrobiologia 329:133–141

Seitzinger S, Harrison JA, Böhlke JK, Bouwman AF, Lowrance R, Peterson B, Tobias C, Drecht GV (2006) Denitrification across landscapes and waterscapes: A synthesis. Ecol Appl 16:2064–2090

Smyth AR, Thompson SP, Siporin KN, Gardner WS, McCarthy MJ, Piehler MF (2013) Assessing nitrogen dynamics throughout the estuarine landscape. Estuaries Coasts 36:44–55

Tiedje JM, Sexstone AJ, Myrold, DD, Robinson, JA (1982) Denitrification: Ecological niches, competition, and survival. Antonie Van Leeuwenhoek 48:569–583

Welsh DT, Bartoli M, Nizzoli D, Castaldelli G, Riou SA, Viaroli P (2000) Denitrification, nitrogen fixation, community primary productivity and inorganic-N and oxygen fluxes in an intertidal *Zostera noltii* meadow. Mar Ecol Prog Ser 208:65–77

Wenzhöfer F, Glud RN (2004) Small-scale spatial and temporal variability in coastal benthic O_2 dynamics: Effects of fauna activity. Limnol Oceanogr 49:1471–1481

Yin G, Hou L, Liu M, Liu Z, Gardner WS (2014) Supplementary info. Environ Sci Technol 48:9555–9562

Zarnoch CB, Hoellein TJ, Furman BT, Peterson BJ (2017) Eelgrass meadows, *Zostera marina* (L.), facilitate the ecosystem service of nitrogen removal during simulated nutrient pulses in Shinnecock Bay, New York, USA. Mar Pollut Bull 124:376–387

Zhang J-Z, Ortner PB, Fischer CJ (1997) Method 353.4 Determination of nitrate and nitrite in estuarine and coastal waters by gas segmented continuous flow colorimetric analysis. U.S. Environmental Protection Agency, Washington, D.C.

Figures

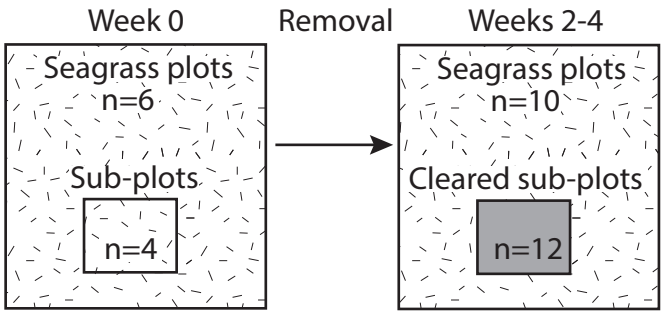


Figure 3.1 Experimental design of the removal experiment, showing the number of total push-pull measurements conducted in the seagrass plots (lines) and manipulated sub-plots (gray) before and after the removal of seagrass shoots.

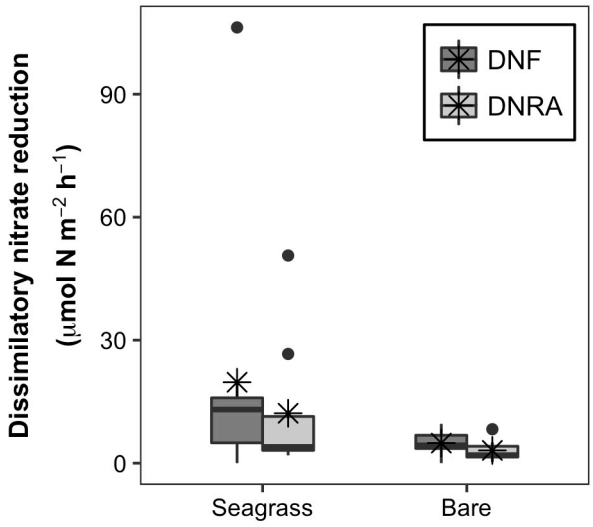


Figure 3.2 Ambient denitrification (DNF) and dissimilatory reduction to ammonium (DNRA) rates measured in the meadow interior during summer 2014 had higher mean values, greater variability, and extreme maximum values compared to rates measured at the external bare site. The box-and-whisker plots show the 25th to 75th quartiles in the box, with black bars at the median and stars at the mean rates. The whiskers denote maximum and minimum rates up to 1.5x the length of the box; outlier rates are shown individually as black dots.

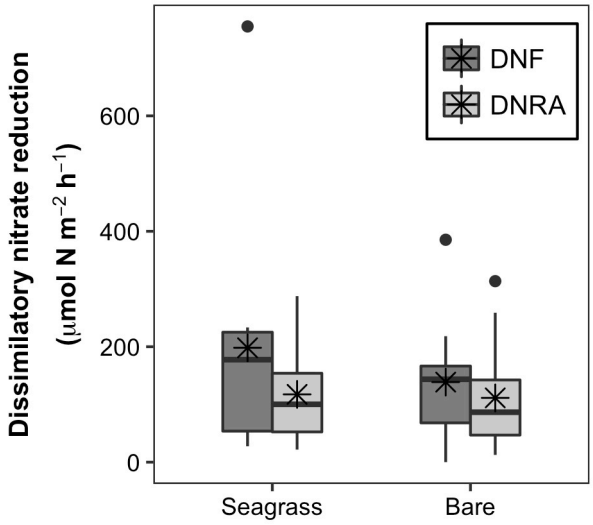


Figure 3.3 Potential nitrate reduction rates (rates under high nitrate conditions) measured in the meadow and external bare site in summer 2014 were an order of magnitude greater than ambient rates (shown in Figure 3.2). The box-and-whisker plots show the 25th to 75th quartiles in the box, with black bars at the median and stars at the mean rates. The whiskers denote maximum and minimum rates up to 1.5x the length of the box; outlier rates are shown individually as black dots.

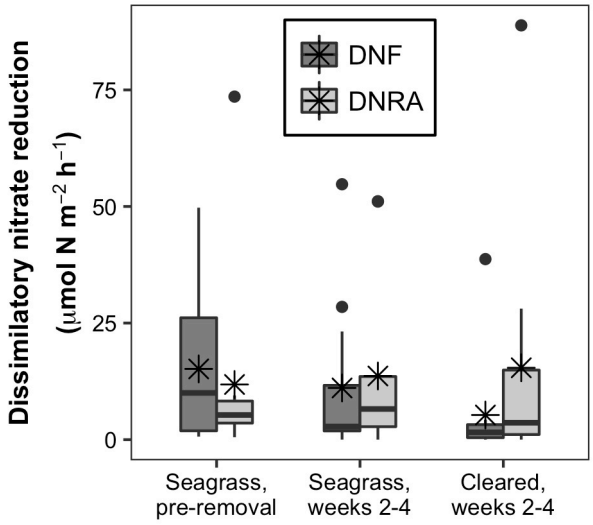


Figure 3.4 Ambient rates of denitrification (DNF) declined in the seagrass and cleared plots after removal, but rates of dissimilatory nitrate reduction to ammonium (DNRA) remained constant. The box-and-whisker plots show the 25th to 75th quartiles in the box, with black bars at the median and stars at the mean rates. The whiskers denote maximum and minimum rates up to 1.5x the length of the box; outlier rates are shown individually as black dots.

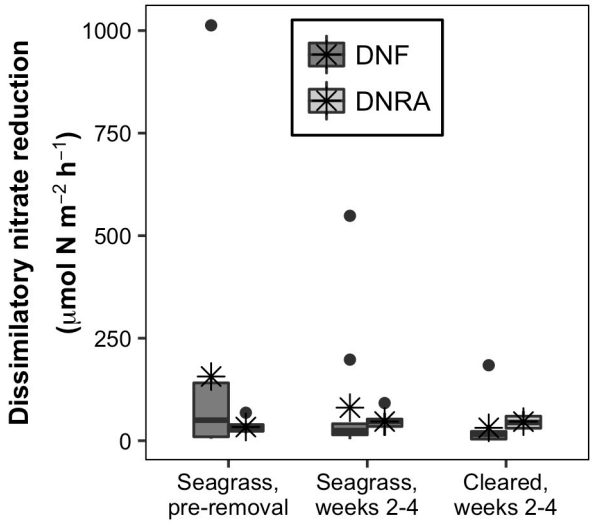


Figure 3.5 Potential nitrate reduction rates (rates under high nitrate conditions) in the removal experiment followed similar trends to the ambient rates in Figure 3.4. The box-and-whisker plots show the 25th to 75th quartiles in the box, with black bars at the median and stars at the mean rates. The whiskers denote maximum and minimum rates up to 1.5x the length of the box; outlier rates are shown individually as black dots.

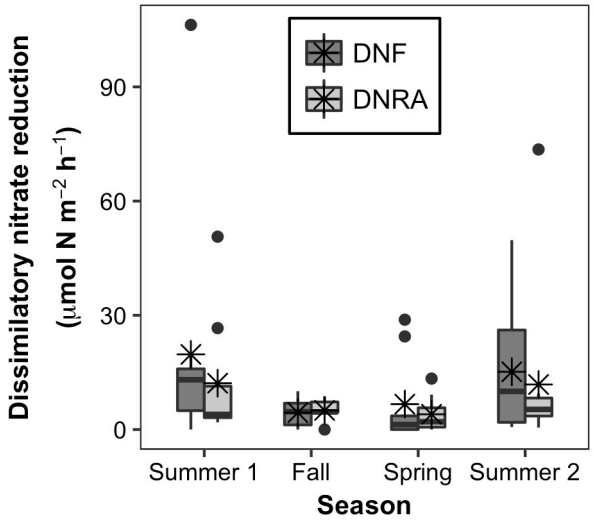


Figure 3.6 Seasonal monitoring of ambient denitrification (DNF) and dissimilatory nitrate reduction to ammonium (DNRA) rates in the meadow interior showed extreme rates throughout spring and summer. The box-and-whisker plots show the 25th to 75th quartiles in the box, with black bars at the median and stars at the mean rates. The whiskers denote maximum and minimum rates up to 1.5x the length of the box; outlier rates are shown individually as black dots.

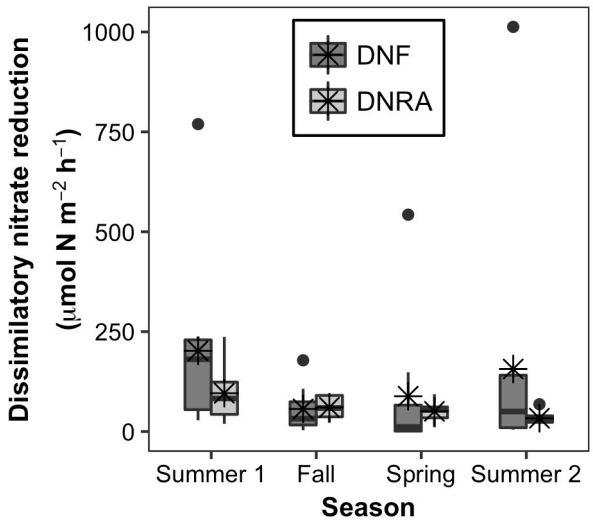


Figure 3.7 Seasonal monitoring of potential rates (rates under high nitrate conditions) in the meadow interior showed extreme rates for denitrification (DNF) but not dissimilatory nitrate reduction to ammonium (DNRA). The box-and-whisker plots show the 25th to 75th quartiles in the box, with black bars at the median and stars at the mean rates. The whiskers denote maximum and minimum rates up to 1.5x the length of the box; outlier rates are shown individually as black dots.

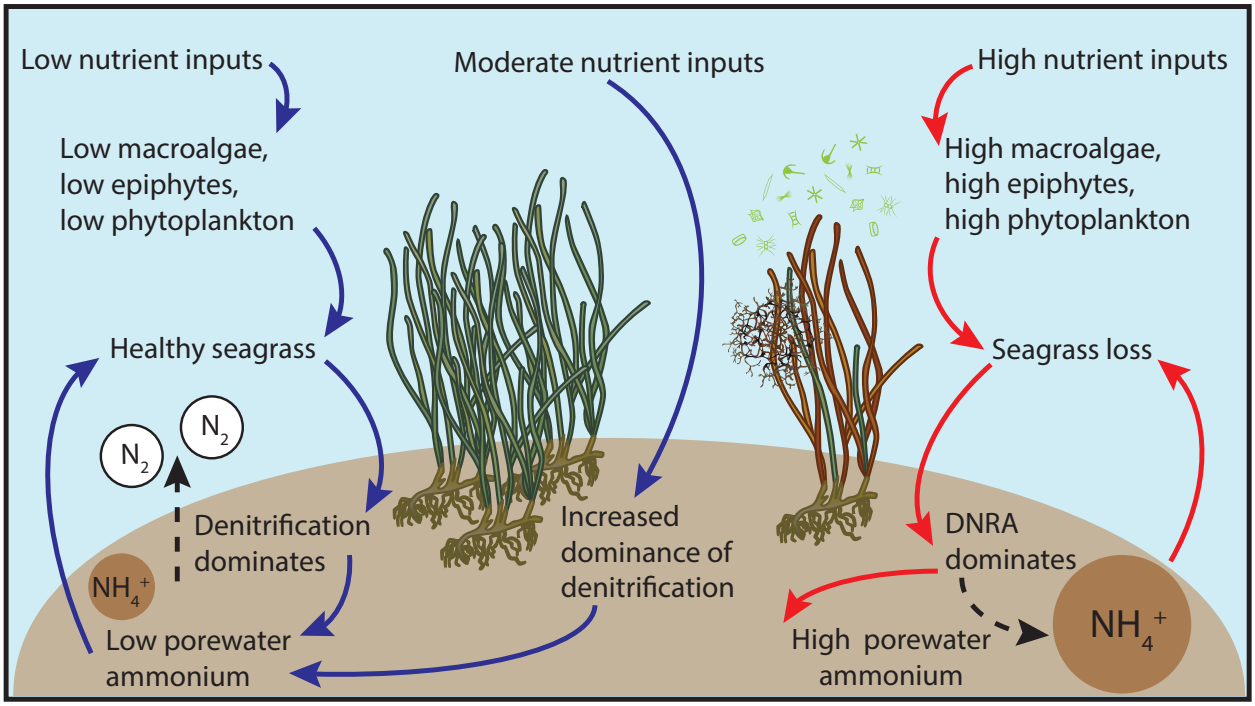


Figure 3.8 Conceptual model showing the possible positive feedbacks supporting denitrification dominance under low nutrient inputs, increased denitrification dominance under moderate nutrient inputs, and DNRA dominance under high nutrient inputs that cause seagrass loss.

Tables

Table 3.1 Sampling design of the study.

Study component	Sites	Sampling dates	Total push-pull measurements
External bare site comparison			
	1 meadow site	June-August 2014	10
	1 external bare site	June-August 2014	8
Removal experiment			
	3 meadow sites	June-July 2015	10, 12*
	3 cleared sub-plots	June-July 2015	10
Seasonal monitoring			
	1 meadow site	June-August 2014	10
	1 meadow site	October 2014	7
	3 meadow sites	April 2015	9
	3 meadow sites	June 2015	10

*10 replicate measurements before sub-plots were cleared, 12 replicate measurements during weeks 2-4 of the experiment.

Table 3.2 Sediment and porewater characteristics at the seagrass and external bare sites from June 2014-June 2015. Values are mean (SD), 'N.D.' indicates no data, '--' indicates months that the bare sites were not sampled. Porewater nitrate concentrations were below the detection limit (0.87 μM) across all sites and months.

	Sediment Temperature (ºC)		Salinity (ppt)		Porewater $[NH_4^+]$ (μM)		Porewater $[HS^-]$ (μM)	
	Seagrass	Bare	Seagrass	Bare	Seagrass	Bare	Seagrass	Bare
June 2014	29.0 (1.4)	29.0	32.5 (0.7)	N.D.	N.D.	N.D.	20.4 (13.2)	4.6 (5.4)
July 2014	28.0 (1.4)	28.0	33.5 (0.7)	33.0	N.D.	N.D.	2.5 (1.7)	1.5 (1.6)
Aug 2014	28.0	29.0	34.0	32.0	17.3 (12.0)	56.5 (15.1)	5.6 (6.8)	1.8 (1.2)
Oct 2014	21.3 (0.6)	--	34.3 (0.6)	--	56.6 (47.7)	--	4.9 (6.1)	--
April 2015	15.5 (1.5)	--	35.3 (0.6)	--	4.3 (3.8)	--	4.9 (2.2)	--
June 2015	26.5 (3.0)	--	32.3 (2.1)	--	10.1 (5.2)	--	8.5 (7.2)	--

Table 3.3 Seagrass shoot densities and biomass measured at meadow sites from June 2014-June 2015. Values are mean (SD), 'N.D.' indicates no data, '--' indicates months that the bare sites were not sampled.

	Shoot density (shoots m^{-2})	Aboveground biomass (g DW m^{-2})	Belowground biomass (g DW m^{-2})	Chlorophyll a (mg m^{-2})	
				Seagrass	Bare
June 2014	424 (76)	136.9 (53.5)	73.9 (13.3)	31.9 (10.4)	24.3 (4.6)
July 2014	638 (89)	167.4 (101.5)	208.2 (56.7)	19.1 (4.2)	N.D
August 2014	431 (101)	201.4 (64.3)	95.3 (28.8)	91.9 (70.0)	18.2 (4.9)
October 2014	205 (58)	65.1 (8.9)	51.5 (15.8)	25.0 (21.4)	--
April 2015	320 (54)	34.7 (20.0)	44.4 (25.8)	5.6 (4.0)	--
June 2015	346 (43)	50.5 (33.1)	55.1 (22.5)	11.6 (6.4)	--

Table 3.4 Porewater concentrations of ammonium and sulfide during the removal experiment. Values are mean (SD).

	Porewater $[NH_4^+]$ (μM)	Porewater $[HS^-]$ (μM)
Seagrass, pre-removal	10.9 (5.5)	8.5 (7.1)
Seagrass, weeks 2-4	175.6 (119.4)	11.0 (16.8)
Cleared, weeks 2-4	154.6 (105.5)	44.2 (74.1)

Table 3.5 Bulk sediment characteristics for seagrass, external bare, and cleared plots. Values are mean (SD).

Site and Year	Organic matter (%)	C content (%)	N content (%)	C:N	Bulk density (g cm^{-3})	Porosity (%)
Seagrass, 2014	2.53 (0.74)	0.57 (0.13)	0.04 (0.01)	13.3 (3.4)	1.45 (0.15)	0.52 (0.10)
External Bare, 2014	1.39 (0.21)	0.42 (0.16)	0.02 (0.002)	17.5 (4.3)	1.46 (0.36)	0.44 (0.10)
Seagrass, 2015	2.01 (0.45)	0.41 (0.10)	0.03 (0.01)	14.1 (2.2)	1.64 (0.20)	0.72 (0.06)
Cleared, 2015	2.00 (0.44)	0.47 (0.10)	0.03 (0.01)	13.7 (1.6)	1.55 (0.14)	0.67 (0.07)

Chapter 4: High rates of N fixation in seagrass sediments measured via direct $^{30}N_2$ push-pull method

Target journal: Marine Ecology Progress Series

Abstract

Highly productive seagrass meadows often occur in oligotrophic waters, and nitrogen (N) fixation may play a role in supporting the N demand of the meadows. To date, most studies of N fixation in seagrass sediments rely on proxy measurements via the indirect acetylene reduction technique. Recent measurements of N fixation in estuarine sediments using direct methods (e.g. N_2:Ar) have shown higher rates than acetylene reduction measurements; however, direct methods have not yet been applied to intact seagrass sediments. In this study, we used a new $^{30}N_2$ push-pull method to measure N fixation rates in seagrass sediments and we compared those rates to traditional acetylene reduction slurries. On average, hourly rates of N fixation measured via the $^{30}N_2$ push-pull method were more than an order of magnitude greater than the acetylene reduction rates during summer (389 μmol m^{-2} h^{-1} and 7.8 μmol m^{-2} h^{-1} respectively). These push-pull rates exceeded other published rates of N fixation in seagrass sediments measured via acetylene reduction but were within the range of reported rates for direct measurements of N fixation in estuarine sediments. These results indicate the need for further investigation of N fixation rates in seagrass sediments via direct measurements. In order to fully understand the role of seagrass meadows in coastal N cycling, it is critical to accurately determine the magnitude of N fixation that occurs in these highly productive ecosystems.

Introduction

Seagrass meadows are important sites for nitrogen (N) fixation in the coastal zone; seagrass N fixation rates typically exceed rates in surrounding benthic habitats (Eyre et al. 2011). Seagrass-associated N fixation can occur in epiphytes on seagrass leaves and in both surface and sub-surface sediments (McGlathery 2008). Both autotrophic and heterotrophic N fixers can be important, with sulfate-reducing bacteria in particular often accounting for a substantial portion of total N fixation in seagrass sediments (Welsh 2000). Understanding the magnitude and drivers of nitrogen fixation in seagrass meadows provides insight into their role as both productivity hotspots and coastal nutrient filters.

A wide range of N fixation rates are reported in the literature, although rates in temperate meadows are typically lower than tropical meadows. However, the majority of existing rates rely on indirect measurements via acetylene reduction. Because nitrogenase, the enzyme that fixes nitrogen, also reduces acetylene to ethylene, sediments can be incubated with acetylene (typically in slurries or cores) and ethylene production is used as a proxy for N fixation (Capone 1993). The acetylene reduction method is simple and affordable and has been widely adopted in studies of aquatic N fixation. However, acetylene reduction suffers from numerous drawbacks. Not only is there uncertainty surrounding the conversion ratio of ethylene production to N fixation (Capone 1988), but the presence of acetylene alters the microbial community in ways that affect the N fixation rate (Oremland & Taylor 1975, Payne 1984). Sulfate reducing bacteria, among others, may be stimulated or suppressed by the presence of acetylene, and ethylene may be produced or consumed by bacteria during the incubation. The acetylene reduction assay also inhibits N fixation by design, which can lead to N limitation and altered N fixation rates during

long incubations (Taylor 1983). Given these drawbacks, there is a clear need for alternative methods to measure N fixation in coastal sediments.

Recently, several studies have undertaken alternative methods to measure N fixation in coastal sediments (Gardner et al. 2006, Fulweiler et al. 2007, 2015, Newell et al. 2016). These studies all use direct methods, measuring changes to the dissolved N_2:Ar ratio to determine N fixation fluxes, in some cases after additions of $^{15}NO_3$ or $^{30}N_2$. In general, direct N_2:Ar methods produce higher N fixation rates than acetylene reduction assays. However, few studies have directly compared N fixation measured via acetylene reduction and an alternative method (Fulweiler et al. 2015). Furthermore, none of these direct methods have been conducted using seagrass sediments with intact shoots present, likely due to the challenges associated with using vegetated cores and the N_2:Ar method. The application of direct measurement of N fixation in seagrass sediments will provide important information to our knowledge of coastal N cycling.

In this study, we compared N fixation rates measured with the traditional acetylene reduction assay and a new, direct $^{30}N_2$ push-pull incubation method. This push-pull method was adapted from a technique that was developed to measure denitrification and DNRA in subtidal seagrass sediments (Aoki & McGlathery 2017). Rather than removing sediment cores to the laboratory for incubation, this technique allows for in situ incubations after the addition of isotopically labeled nitrogen. The natural conditions of flow and light availability are undisturbed, and the measured rates therefore capture variability driven by field conditions and plant effects. Previously, we measured significantly higher rates of denitrification and DNRA using this technique compared to traditional static core incubations (Aoki and McGlathery 2017), and we hypothesized that similarly high rates of N fixation would be measured by applying the push-pull method to $^{30}N_2$ incubations in seagrass sediments.

Methods

Study site

This study was conducted in a restored seagrass meadow in South Bay, Virginia, one of four meadows seeded in a landscape-scale restoration project begun in 2001 (Orth & McGlathery 2012). Long-term monitoring has shown that the meadow reached a mature steady-state after 9 years (McGlathery et al. 2012); this study took place 15-16 years after seeding. Measurements were made at the original seed plots of the restoration, i.e. the most mature areas in the interior of the meadow. South Bay is shallow, with a mean water depth of 1.4 m and a tidal range of 1.2 m (Fagherazzi & Wiberg 2009), and water quality is high, with low concentrations of dissolved nutrients and chlorophyll (McGlathery & Christian 2017).

Acetylene reduction

Nitrogen fixation rates from sediments and epiphytes were measured using the acetylene reduction technique (Capone 1993) in June (epiphytes only), August and October 2016, and in April and August 2017. For the sediment incubations, four small cores (2 cm inner diameter (ID), 5 cm depth) were collected from bare sediments at each of three experimentally cleared plots (4 m^2) within the meadow during each sampling month; four additional cores were collected from the seagrass sediments surrounding each plot. Sediment cores were transported on ice to the laboratory and immediately prepared for incubation. For the epiphyte incubations, twelve seagrass shoots were collected from each of three 0.4 ha plots during each sampling month. Shoots were held in site water and transported on ice to the laboratory; the shoots were then held overnight on ice in site water bubbled with air and prepared for incubation the following day.

Sediments were transferred to glass vials (37.5 mL) by sub-sampling within the 2 cm core using a 5 cm^3 cut-off plastic syringe. The sediments were massed and added to the vials with 1 mL of filtered (0.5 μm) site water; vials were sealed with silicone septa and aluminum crimp tops. The vials were sparged with argon gas for 5 min to remove oxygen before the addition of 10 mL of acetylene gas, after which the vials were shaken for 10 sec and then vented to atmospheric pressure. Vials were then placed in a temperature-controlled water bath for incubation. A full-spectrum light was used to provide saturating light conditions in the incubation tank and half of the vials were covered in aluminum foil to provide a dark treatment (except in October 2016 when no light treatment was used and all incubations were dark). After 6 hours, the vials were shaken for 30 seconds and the headspace was transferred to a pre-evacuated Exetainer using a double-ended needle.

Epiphyte incubations were prepared by selecting at random 6 intact shoots from the 12 shoots collected at each site. The number of leaves on each shoot was recorded, and the upper 15 cm of leaves 2 and 4 were transferred to 72 mL glass vials filled with 50 mL of filtered (0.5 μm) site water. An additional 10 mL of acetylene-saturated filtered site water were added to the vials, and the vials were sealed with septa and aluminum crimps, shaken vigorously for 30 sec, and placed in the incubation chamber under the grow-light. Half the vials were covered with aluminum foil to test the effect of light (except in August and October 2016 when no light treatment was used and all incubations were dark). After 4 hours, the vials were removed, shaken for 30 sec, and the headspace was transferred to pre-evacuated Exetainers using a double-ended needle.

Gas samples from all incubations were analyzed on a Shimadzu GC-14A at the Smithsonian Environmental Research Center in Edgewater, MD, within 3 weeks of the

incubation. The analysis was conducted using a 1.83 m x 0.32 cm x 0.22 cm column filled with Poropak N 80/100 connected to the flame ionization detector. Ethylene production rates were converted to nitrogen fixation rates using the theoretical 3:1 molar ratio of ethylene to nitrogen (Seitzinger & Garber 1987). For sediments, nitrogen fixation rates were converted to areal rates using the wet weight, volume, and depth of the sediment core. For the epiphyte rates, the average of leaves 2 and 4 (young and old leaves respectively) was scaled up to an areal rate based on the number of leaves per shoot and the shoots per square meter, measured in ten replicate 0.25 m^2 quadrats at each plot in the meadow.

$^{30}N_2$ push-pull

We used a $^{30}N_2$ push-pull method to measure N fixation directly at the same seagrass and experimentally cleared bare plots where sediments were collected for the acetylene incubations in summer 2017. Four replicate push-pull incubations were conducted simultaneously, with two incubations in bare sediments and two incubations in seagrass sediments; eight total push-pull incubations were conducted over two deployments. Details of this push-pull method, as developed to measure denitrification, are available elsewhere (Aoki and McGlathery 2017). The general method and adaptations to measure N fixation are summarized here.

For each $^{30}N_2$ push-pull incubation, a mini-piezometer (1.8 mm ID) was inserted into the sediment to a depth of 5 cm. The piezometer was connected via Viton tubing to a graduated cylinder that served as a reservoir. Approximately 220 mL of porewater were slowly (4 mL min^{-1}) extracted from the sediment using a peristaltic pump; a layer of castor oil in the graduated cylinder served as a barrier to atmospheric exchange during the pumping processes. Duplicate background samples of the porewater were collected in 12 mL Exetainers (overfilled by 3 mL to prevent atmospheric exchange) and fixed with 50 μL of $ZnCl_2$ (100% m/v). These background

samples were stored underwater until analysis using membrane inlet mass spectrometry (MIMS) within 4 weeks.

Approximately 20 mL of artificial seawater saturated with $^{30}N_2$ gas were then added to the reservoir. The $^{30}N_2$–saturated seawater was prepared in the laboratory by first bubbling artificial seawater with Ar gas to strip ambient N_2 and O_2, transferring the seawater to a gas-tight Tedlar bag, adding $^{30}N_2$ (Cambridge Isotope Laboratory, 98% purity), and shaking overnight. The $^{30}N_2$-seawater was transported to the field in gas tight glass vials stored underwater. After the addition of the $^{30}N_2$-seawater to the reservoir, a second set of samples was collected in Exetainers and fixed with $ZnCl_2$. These samples marked the beginning of the incubation (time-zero) and were also stored underwater until analysis via MIMS within 4 weeks. The isotopically labeled porewater was then pumped back into the sediment and allowed to incubate in situ for two hours. At the end of the incubation, a small sediment core was collected at the injection site (2.5 cm ID, 10 cm depth) and frozen until analysis.

The background and time-zero porewater samples were analyzed using a paired vial approach. Of the duplicate background and time-zero porewater samples, one duplicate was analyzed via MIMS without modification, and the second duplicate was first oxidized with hypobromite (prepared as in Yin et al. 2014) and then analyzed via MIMS. The hypobromite converts any $^{15}NH_4^+$ in the sample to N_2, increasing the concentration of $^{29}N_2$ and $^{30}N_2$ compared to the unoxidzed sample. The background porewater samples were used to verify the low ambient concentration of $^{30}N_2$ and to calculate the background concentration of $^{15}NH_4^+$ (always very low). The time-zero porewater samples were used to calculate the concentrations of dissolved $^{28}N_2$, $^{29}N_2$, and $^{30}N_2$ at the beginning of the incubation (Figure 4.1).

The paired vial approach also allowed for the measurement of the $^{15}NH_4^+$ produced in the sediments during the incubation. To measure $^{15}NH_4^+$, the frozen sediment cores were defrosted and mixed with 90 mL of 2 M KCl to extract ammonium. The sediments were then centrifuged and the supernatant of each core was filtered (0.45 μm) and transferred to a set of three Exetainers. One vial was analyzed via MIMS without modification and the remaining two vials were oxidized with hypobromite and then analyzed. The higher concentrations of $^{29}N_2$ and $^{30}N_2$ in the oxidized vials compared to the unoxidized vial corresponded to the concentration of $^{15}NH_4^+$ in the sediments at the end of the incubation, denoted as $[^{15}NH_4^+]_{fixed}$.

The $[^{15}NH_4^+]_{fixed}$ was produced via fixation of a portion of the $^{30}N_2$ label added to the porewater and the ambient $^{29}N_2$, according to Equation 1:

$$[^{15}NH_4^+]_{fixed} = 2 \times [^{30}N_2]_{fixed} + [^{29}N_2]_{fixed} \quad (1)$$

In order to solve for the quantities of $[^{30}N_2]_{fixed}$ and $[^{29}N_2]_{fixed}$, we assumed that the dissolved $^{30}N_2$ and $^{29}N_2$ were well-mixed in the porewater and were fixed randomly; therefore, the relative amount of each species that was fixed corresponded to the relative concentration of that species in the porewater according to Equation 2:

$$[^{30}N_2]_{fixed} / [^{29}N_2]_{fixed} = [^{30}N_2]_{aqueous} / [^{29}N_2]_{aqueous} \quad (2)$$

The two terms $[^{30}N_2]_{aqueous}$ and $[^{29}N_2]_{aqueous}$ were known quantities from the analysis of the time-zero porewater samples. Equations 1 and 2 could therefore be solved for $[^{30}N_2]_{fixed}$ and $[^{29}N_2]_{fixed}$, i.e. the concentrations of $^{29}N_2$ and $^{30}N_2$ that underwent fixation during the incubation.

In addition to $[^{29}N_2]_{fixed}$ and $[^{30}N_2]_{fixed}$, a significant proportion of the nitrogen gas undergoing fixation was expected to be $^{28}N_2$. The analysis of the time-zero porewater samples showed that after adding the $^{30}N_2$ label to the porewater, the concentration of $^{30}N_2$ increased by more than 100x compared to the background levels, from approximately 0.02 μM to 4 μM. This

concentration was sufficient to produce measureable production of $^{15}NH_4^+$; however, only a small portion of the N_2 pool was labeled. The concentration of $^{28}N_2$ by contrast was around 400 μM. Therefore, we used Equation 3 to calculate the total concentration of N_2 that underwent fixation during the incubation, again based on the assumption that all species of N_2 were well-mixed in the porewater and thus the relative amount of $^{30}N_2$ that was fixed corresponded to the relative concentration of $^{30}N_2$ in the porewater:

$$[^{30}N_2]_{fixed} / [^{total}N_2]_{fixed} = [^{30}N_2]_{aqueous} / [^{total}N_2]_{aqueous} \quad (3)$$

Having calculated $[^{total}N_2]_{fixed}$, the total concentration of N_2 that underwent fixation during the incubation, we used the depth of the sediment core and the porosity of the sediment to determine the volume of porewater in the core and therefore the total amount of N_{2fixed} during the incubation. Using the duration of the incubation, we calculated the N fixation rate in μmol N_2 cm^{-3} sediment hr^{-1}. By integrating over the depth of the sediment core, we converted the rate into an areal N fixation rate.

Statistical analysis

For the acetylene reduction incubations, individual sediment and leaf incubations were pooled to produce plot-level averages (n=3 replicate plots). The effects of the light treatment, seagrass cover, and season on N fixation rates were tested using a repeated measures ANOVA. Post-hoc contrasts were performed for significant effects. The effect of seagrass cover on the push-pull rates was determined using a t-test; push-pull and acetylene reduction rates were also compared using a t-test. All analyses were conducted in R 3.3.3 (R Core Team 2017).

Results

Acetylene reduction

Hourly N fixation rates measured from acetylene reduction in the sediments peaked in August and were low in April and October, ranging from 0-18.8 μmol N_2 m^{-2} h^{-1} across all seasons (Figure 4.2). The repeated measures ANOVA showed a significant seasonal effect ($F_{3,24}$=6.135, p=0.003) as well as a significant interaction between season and cover ($F_{3,24}$=3.909, p=0.02). However, the main effects of light and cover were not significant. Post-hoc contrasts showed that the N fixation rates were significantly greater in both summers compared to the spring and fall rates, and in summer 2017, the bare rates were significantly greater than the seagrass rates.

Hourly nitrogen fixation rates measured from acetylene reduction in the leaf incubations were generally low, between 0 and 16.9 umol N_2 m^{-2} h^{-1}. Rates were undetectable in April and October and peaked in August, when the seagrass blades were more heavily colonized by epiphytes (Figure 4.3). There was no significant effect of light (repeated measures ANOVA, $F_{1,10}$=0.78, p=0.39), indicating the dominance of heterotrophic N-fixers. However, in August 2017 individual leaves incubated in the light showed rates up to 10x higher than the leaves incubated in the dark, indicating the presence of autotrophic N-fixers.

$^{30}N_2$ push-pull

Hourly N fixation rates calculated from the $^{30}N_2$ push-pull incubations were significantly greater than acetylene reduction rates (t-test, p<0.0005). Both bare and seagrass $^{30}N_2$ push-pull rates were more than an order of magnitude greater than the acetylene reduction rates, on average 389 and 239 μmol N_2 m^{-2} h^{-1} in the seagrass and bare sediments respectively (Figure 4.4). As with the sediment slurry incubations, there was no significant difference in the $^{30}N_2$ push-pull rates between seagrass and bare sediments (t-test, p=0.10), although the $^{30}N_2$ push-pull rates had limited replication (n=4).

Discussion

The acetylene reduction incubations confirmed earlier findings from this site that heterotrophic bacteria dominate N fixation in both sediments and leaf epiphytes (Cole & McGlathery 2012). Heterotrophic N fixation is commonly observed in seagrass meadows and is likely stimulated by the release of photosynthetic exudates (Welsh et al. 1996, McGlathery et al. 1998, Hansen et al. 2000). However, unlike in previous studies, we found no effect of seagrass cover on N fixation rates. This result likely comes from the similar conditions in the vegetated and unvegetated sediments used in this study. Unlike in previous studies, the unvegetated sediments sampled here were experimentally-cleared plots located within the seagrass meadow, first cleared in 2015 and re-cleared in 2016 and 2017 following limited re-colonization by seagrass. Organic matter content, a known driver of N fixation rates, did not differ between the bare sites and surrounding seagrass sediments in 2017 (data not shown), and because the bare sites were situated in the center of the meadow, they were subject to the same seagrass-influenced flow and light regime as the surrounding seagrass sediments. Furthermore, the sediment slurries could not capture the full effects of seagrass presence, as intact shoots were excluded from the incubations. It is therefore not surprising to find a lack of difference between the seagrass and bare sediments in the sediment slurry incubations.

The areal rates of N fixation in seagrass sediments calculated from the acetylene reduction slurries (2-19 μmol N_2 m^{-2} h^{-1}) show good agreement with previous studies using acetylene reduction (Table 4.1). However, the areal rates calculated from the $^{30}N_2$ push-pull incubations were substantially higher than the acetylene reduction rates. Multiple different factors likely contributed to this large difference. First, the $^{30}N_2$ push-pull incubations were a direct measurement of N fixation, in contrast to the proxy rates measured with acetylene

reduction. Acetylene is known to alter the microbial community, and in particular can both increase and decrease the abundance of species of sulfur and sulfate reducing bacteria over short time scales (Fulweiler et al. 2015). Although we did not assess the role of sulfate reducing bacteria in this study, previous work has clearly shown the importance of these microbes as heterotrophic N fixers in marine environments, including seagrass sediments (e.g. McGlathery et al. 1998, Welsh et al. 1996). Recent studies that used direct methods (e.g. N_2:Ar) have also found rates that exceed the acetylene reduction rates and include values similar to the $^{30}N_2$ push-pull results (Table 4.1). Thus, by using a direct method instead of a proxy method, the $^{30}N_2$ push-pull incubations provided what may be a more realistic assessment of N fixation rates.

Second, the push-pull method improves on the slurry method used for the acetylene reduction measurements by maintaining the heterogeneous sediment structure and capturing the effects of intact seagrass on sediment biogeochemistry. Seagrass root exudation and rapid root turnover can stimulate microbial activity, including N fixation (McGlathery 2008); field conditions that affect seagrass productivity, such as temperature and light availability may in turn affect root exudation. By conducting the incubations in the field, with minimal disturbance to the seagrass and sediments, the push-pull method can better capture these seagrass effects than the slurry incubations. The push-pull measurements show this seagrass effect in the difference between the bare and seagrass rates (Figure 4.4). Although this difference had low statistical significance ($p=0.097$), the pattern of higher seagrass rates was much more evident than in the acetylene slurries, where the seagrass and bare sediment rates were very similar (Figure 4.2). The push-pull rates also captured the full variability of rates under field conditions, which exceeded the variability in the laboratory slurry incubations. This variability was likely driven by heterogeneity in the intact seagrass sediments as well as variable field conditions. Overall, the in

situ nature of the push-pull incubation method likely contributed to the measurement of higher rates, in addition to the use of $^{30}N_2$ substrate rather than acetylene.

The magnitude of N fixation in seagrass sediments has important implications for understanding coastal N cycling. Seagrass thrive in low-nutrient waters, and the high rates of seagrass productivity are typically supported by remineralization of organic matter (McGlathery 2008). In temperate *Z. marina* meadows, N fixation is thought to support a relatively low percentage of total N demand by seagrass (5-20%, Welsh et al. 1996, McGlathery et al. 1998, Cole and McGlathery 2012). However, if N fixation rates have been significantly underestimated through widespread use of acetylene reduction, we may need to update our understanding of N dynamics in seagrass meadows. More broadly, high rates of N fixation will be important to balancing coastal nitrogen budgets. For example, recent N budgets for three shallow sub-tropical estuaries found large N deficits that could only be partially accounted for by inputs of oceanic nitrogen; unmeasured N fixation may have supplied the missing N (Eyre et al. 2016). In this study, if we take the most conservative approach that the hourly push-pull rates should be scaled by a 12-hour day, since measurements were only made during daylight hours, the total N fixed during the month of June would exceed the annual N fixation rate as measured by the acetylene reduction slurries. If we apply the findings from the light treatment of the slurries, that is, that N fixation was driven by heterotrophic bacteria, and scale the push-pull rates by a 24-hour day, the discrepancy only increases. Further investigation of these patterns is needed in order to advance our understanding of N fixation in shallow coastal sediments.

While acetylene reduction remains an affordable and simple technique to implement, the divergence between acetylene reduction rates and rates measured with direct methods ($^{30}N_2$, N_2:Ar) suggest that investigators should pursue these direct techniques. The $^{30}N_2$ push-pull

method has the additional advantage of being conducted in situ, so that the sediment conditions are not disturbed. Additional implementation of the $^{30}N_2$ push-pull method is needed to confirm the findings presented here, and, in general, additional measurements of N fixation using direct methods will give us a better understanding of the magnitude and variability of N fixation in seagrass sediments.

References

Aoki LR, McGlathery KJ (2017) Push-pull incubation method reveals the importance of denitrification and dissimilatory nitrate reduction to ammonium in seagrass root zone. Limnol Oceanogr Methods 15:766–781

Capone DG (1988) Benthic nitrogen fixation. In: Nitrogen cycling in coastal marine environments. John Wiley & Sons Ltd, p 85–123

Capone DG (1993) Determination of nitrogenase activity in aquatic samples using the acetylene reduction procedure. In: Handbook of methods in aquatic microbial ecology. Lewis Publishers, Boca Raton, p 621–632

Cole L, McGlathery K (2012) Nitrogen fixation in restored eelgrass meadows. Mar Ecol Prog Ser 448:235–246

Cook P, Evrard V, Woodland R (2015) Factors controlling nitrogen fixation in temperate seagrass beds. Mar Ecol Prog Ser 525:41–51

Eyre BD, Ferguson AJP, Webb A, Maher D, Oakes JM (2011) Denitrification, N-fixation and nitrogen and phosphorus fluxes in different benthic habitats and their contribution to the nitrogen and phosphorus budgets of a shallow oligotrophic sub-tropical coastal system (southern Moreton Bay, Australia). Biogeochemistry 102:111–133

Eyre BD, Maher DT, Sanders C (2016) The contribution of denitrification and burial to the nitrogen budgets of three geomorphically distinct Australian estuaries: Importance of seagrass habitats. Limnol Oceanogr 61:1144–1156

Fagherazzi S, Wiberg PL (2009) Importance of wind conditions, fetch, and water levels on wave-generated shear stresses in shallow intertidal basins. J Geophys Res 114:F03022

Fulweiler RW, Heiss EM, Rogener MK, Newell SE, LeCleir GR, Kortebein SM, Wilhelm SW (2015) Examining the impact of acetylene on N-fixation and the active sediment microbial community. Front Microbiol 6:418

Fulweiler RW, Nixon SW, Buckley BA, Granger SL (2007) Reversal of the net dinitrogen gas flux in coastal marine sediments. Nature 448:180–182

Gardner WS, McCarthy MJ, An S, Sobolev D, Sell KS, Brock D (2006) Nitrogen fixation and dissimilatory nitrate reduction to ammonium (DNRA) support nitrogen dynamics in Texas estuaries. Limnol Oceanogr 51:558–568

Hansen JW, Udy JW, Perry CJ, Dennison WC, Lomstein BA (2000) Effect of the seagrass *Zostera capricorni* on sediment microbial processes. Mar Ecol Prog Ser 199:83–96

McGlathery KJ (2008) Seagrass habitats. In: Nitrogen in the marine environment. Elsevier, New York, p 1037–1072

McGlathery KJ, Christian R (2017) Water quality sampling - integrated measurements for the Virginia Coast, 1992-2017. Virginia Coast Reserve Long-Term Ecological Research Project Data Publication doi:10.6073/pasta/8783dd64be7cae3a36a864ac5b5129e1

McGlathery K, Reynolds L, Cole L, Orth R, Marion S, Schwarzschild A (2012) Recovery trajectories during state change from bare sediment to eelgrass dominance. Mar Ecol Prog Ser 448:209–221

McGlathery KJ, Risgaard-Petersen N, Christensen PB (1998) Temporal and spatial variation in nitrogen fixation activity in the eelgrass *Zostera marina* rhizosphere. Mar Ecol Prog Ser 168:245–258

Newell SE, McCarthy MJ, Gardner WS, Fulweiler RW (2016) Sediment nitrogen fixation: A call for re-evaluating coastal N budgets. Estuaries Coasts 39:1626–1638

Oremland RS, Taylor BF (1975) Inhibition of methanogenesis in marine sediments by acetylene and ethylene: validity of the acetylene reduction assay for anaerobic microcosms. Appl Microbiol 30:707–709

Orth R, McGlathery K (2012) Eelgrass recovery in the coastal bays of the Virginia Coast Reserve, USA. Mar Ecol Prog Ser 448:173–176

Payne WJ (1984) Influence of acetylene on microbial and enzymatic assays. J Microbiol Methods 2:117–133

R Core Team (2017) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/

Seitzinger SP, Garber JH (1987) Nitrogen fixation and $^{15}N_2$ calibration of the acetylene reduction assay in coastal marine sediments. Mar Ecol Prog Ser 37:65–73

Taylor BF (1983) Assays of microbial nitrogen transformations. In: Nitrogen in the marine environment, 1st edn. Academic Press, New York, NY, p 809–837

Welsh DT (2000) Nitrogen fixation in seagrass meadows: Regulation, plant–bacteria interactions and significance to primary productivity. Ecol Lett 3:58–71

Welsh DT, Bourgues S, De Wit R, Herbert RA (1996) Seasonal variations in nitrogen-fixation (acetylene reduction) and sulphate-reduction rates in the rhizosphere of *Zostera noltii*: Nitrogen fixation by sulphate-reducing bacteria. Mar Biol 125:619–628

Yin G, Hou L, Liu M, Liu Z, Gardner WS (2014) A novel membrane inlet mass spectrometer method to measure $^{15}NH_4^+$ for isotope-enrichment experiments in aquatic ecosystems. Environ Sci Technol 48:9555–9562

Figures

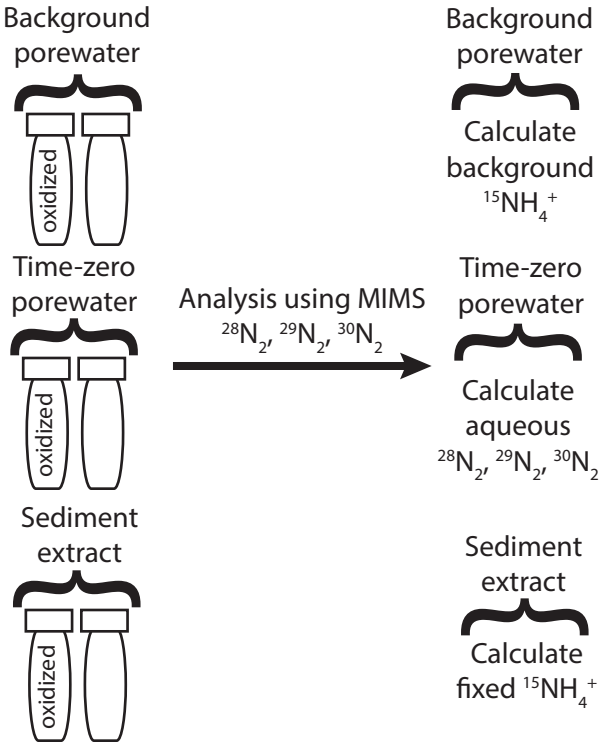
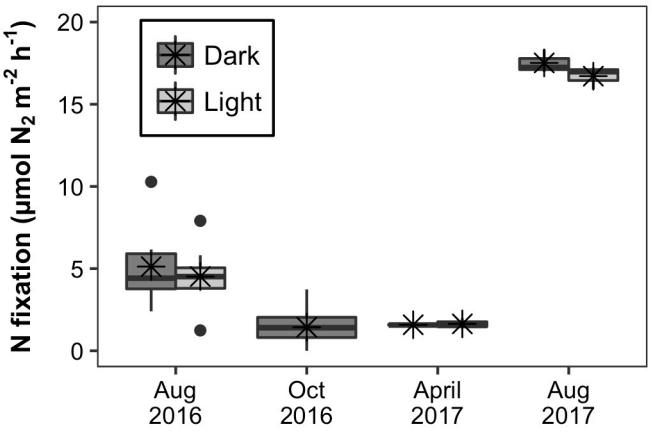


Figure 4.1 Schematic showing the duplicate samples collected during the $^{30}N_2$ push-pull incubation method. Background porewater was sampled before the push-pull incubation began, time-zero porewater was sampled immediately after adding the $^{30}N_2$ spiked seawater, and the sediment extract samples were prepared through KCl extraction of sediments collected at the end of the 2-hour incubation. One duplicate of each sample set was oxidized with hypobromite, and all samples were analyzed using membrane inlet mass spectrometry (MIMS) in order to calculate the concentrations of N_2 and NH_4^+ used in Equations 1-3.

A



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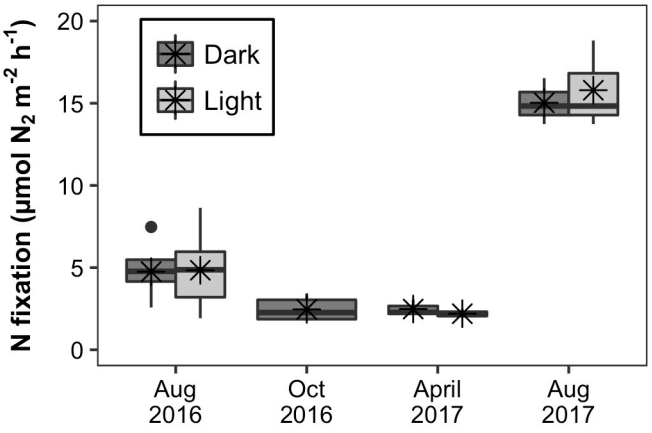

Figure 4.2 N fixation rates measured via acetylene reduction were comparable in (A) bare sediments and (B) seagrass sediments, showed no significant effect of light, and peaked in summer. The box-and-whisker plots show the 25th to 75th quartiles in the box, with black bars at the median and stars at the mean rates. The whiskers denote maximum and minimum rates up to 1.5x the length of the box; outlier rates are shown individually as black dots.

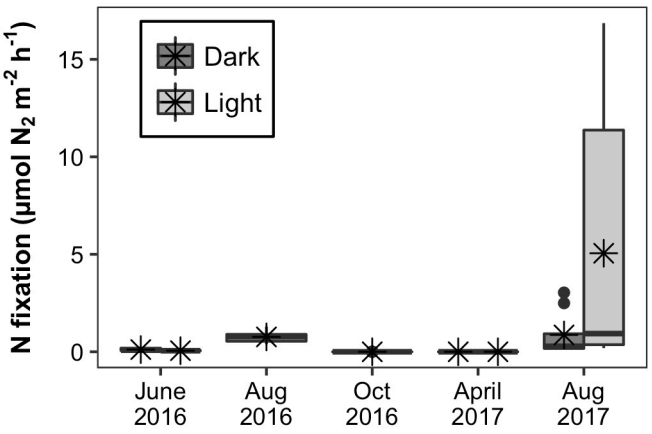

Figure 4.3 N fixation measured rates via acetylene reduction on seagrass epiphytes were low and showed no significant effect of light, although peak rates in the light in August 2017 suggested the presence of autotrophic N-fixers. The box-and-whisker plots show the 25th to 75th quartiles in the box, with black bars at the median and stars at the mean rates. The whiskers denote maximum and minimum rates up to 1.5x the length of the box; outlier rates are shown individually as black dots.

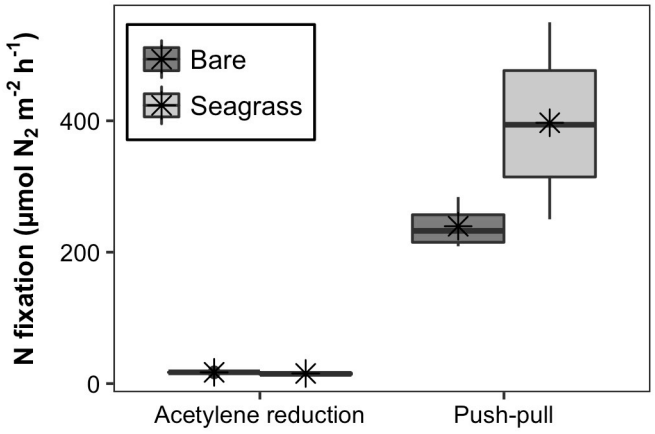

Figure 4.4 N fixation rates measured via $^{30}N_2$ push-pull were significantly greater than rates measured using acetylene reduction in summer 2017 (t-test, $p<0.0005$); however, the difference between $^{30}N_2$ push-pull rates in seagrass and bare sediments had low statistical significance (t-test, $p=0.10$). The box-and-whisker plots show the 25th to 75th quartiles in the box, with black bars at the median and stars at the mean rates. The whiskers denote maximum and minimum rates up to 1.5x the length of the box; outlier rates are shown individually as black dots.

Tables

Table 4.1 N fixation rates measured with acetylene reduction are typically lower than rates measured with direct techniques.

N fixation rate (μmol N_2 m^{-2} h^{-1})	Site	Method	Citation
6-22	*Z. noltii*, Summer, Arcachon Bay, France	Acetylene reduction, added to headspace of cores	Welsh et al. 1996
4-20	*Z. marina,* Summer, Limfjord, Denmark	Acetylene reduction, added to porewater of cores via perfusion	McGlathery et al. 1998
3-90	*Z. mulleri*, *Z. nigicalaous*, Spring and Summer, Port Philip Bay, Australia	Acetylene reduction, injected into core sediments	Cook et al. 2015
0-97	Estuarine sediment, all seasons, TX estuaries	N_2:Ar cores, with $^{15}NO_3$ addition	Gardner et al. 2006
25-650	Estuarine sediment, Summer, Narragansett Bay, RI	N_2:Ar cores	Fulweiler et al. 2007
49-103	Estuarine sediment, Fall, Waquoit Bay, RI	N_2:Ar cores, with $^{15}NO_3$ and $^{30}N_2$ additions	Newell et al. 2016
250-550	*Z. marina*, Summer, South Bay, VA	$^{30}N_2$ push-pull	This study
2-19	*Z marina*, Summer, South Bay, VA	Acetylene reduction, sediment slurries	This study

Chapter 5: Seagrass restoration reestablishes the coastal nitrogen filter

Target journal: Limnology and Oceanography

Abstract

Large-scale restoration has established >25 km^2 of seagrass meadows in Virginia's coastal bays since 2001. These restored meadows may act as a coastal filter for nitrogen (N) through temporary retention of N in seagrass biomass and long-term removal via burial and denitrification. We assessed the impact of the restoration on the coastal filter function by comparing N inputs (loading and fixation) to N removal (denitrification and burial) within one restored meadow (6.9 km^2) and in adjacent bare sediments. We also measured N assimilation into seagrass biomass to assess the magnitude of temporary retention. N removal was 8x greater in the meadow than in the bare sediments (2.57 and 0.33 g N m^{-2} y^{-1} respectively), and N removal outweighed N inputs in the meadow but not in bare sediments (N inputs were 2.16 and 1.97 g N m^{-2} y^{-1} respectively). These findings indicate that the restoration enhanced N removal thus reestablishing the filter function. Temporary retention was similar in magnitude to N removal (2.62 g N m^{-2} y^{-1}). N recycling within the meadow likely provided an unmeasured internal source to support this high biomass N demand. In situ N fixation rates were likely underestimated by the acetylene reduction method and may have been a large source of N. High rates of temporary retention contributed to the coastal N filter by slowing the transport of N loads to the coastal ocean. This model system demonstrates how seagrass restoration can reestablish the coastal filter, an important function in the face of anthropogenic impacts on shallow coastal ecosystems.

Introduction

Seagrass meadows are highly productive ecosystems that provide a wide array of ecosystem services, ranging from supporting tourism and recreation to regulating climate via carbon sequestration (Barbier et al. 2011). Through their high productivity, seagrass can strongly influence the nitrogen (N) cycle in coastal waters. Large amounts of N are assimilated into seagrass biomass, which turns over slowly compared to algal biomass, leading to temporary retention of nitrogen (on the order of weeks to months) (Banta et al. 2004, McGlathery et al. 2007). Seagrass meadows can also more permanently remove nitrogen from coastal waters by increasing N burial in seagrass sediments. The seagrass canopy reduces wave energy, leading to increased sedimentation and reduced resuspension, which contributes to high N burial rates (Hansen & Reidenbach 2012); buried N can remain stored for years to decades or longer in some seagrass sediments (Mateo et al. 1997, Middleburg et al. 2004). In addition to burial, permanent N removal occurs via denitrification, the microbial transformation of biologically available nitrate into inert dinitrogen gas. Seagrass roots exude oxygen and labile carbon which can stimulate coupled nitrification-denitrification in subsurface sediments (Iizumi et al. 1980, Aoki and McGlathery, in review, Chapter 3). Taken together, these retention and removal processes form a coastal "filter" for nitrogen that slows and decreases the movement of nitrogen inputs from watersheds to the coastal ocean.

Previous measurements of N removal processes in seagrass meadows have focused primarily on denitrification. Recent studies have generally found elevated rates of denitrification in seagrass meadows compared to other estuarine habitats (Eyre et al. 2011, 2013, Smyth et al. 2013), although this is not always the case (Russell et al. 2016, Zarnoch et al. 2017). In contrast, measurements of N burial in seagrass meadows are few and focus mainly on peat-forming

Posidonia species (Mateo & Romero 1997, Cebrian & Duarte 2001, Gacia et al. 2002) and tropical meadows (Pedersen et al. 1997, Pérez et al. 2001). However, a recent study by Eyre et al. (2016) found elevated rates of both denitrification and N burial within the seagrass communities relative to subtidal flats in three sub-tropical lagoons; these N removal rates contributed significantly to N removal in the lagoons. Further work is needed to better characterize N burial in temperate seagrass meadows and to assess the contributions of burial and denitrification to net N removal.

Unlike long-term N removal processes, N retention in seagrass biomass is temporary and subject to multiple recycling processes that occur over different timescales. Seagrass N demand, measured from shoot specific productivity and tissue N content, typically peaks during the summer growing season in temperate meadows (Pedersen & Borum 1993, Risgaard-Petersen et al. 1998, Park et al. 2013). That demand can be met from external inputs of N from watershed N loads and by internal N loading within the meadow, including remineralization, N fixation in meadow sediments, and N reclamation within seagrass shoots (relocation from senescent to new tissue and/or translocation of stored N) (Hemminga et al. 1999, Romero et al. 2006). Large amounts of seagrass biomass may be exported from the meadow throughout the growing season, and the fate of exported biomass is generally unknown. Seagrass tissue is more recalcitrant than algae, but seagrass likely also leach significant amounts of DON from both roots and leaves (Jørgensen et al. 1981). Despite these complexities, measurements of N demand give some indication of the magnitude and seasonal patterns in N retention. In non-eutrophic estuarine systems, the N demand typically exceeds N loading (Pedersen et al. 2004), indicating that most N entering these systems passes through the primary producer pool (McGlathery et al. 2007).

Placing N retention in context compared to N removal and N inputs is therefore important to fully conceptualizing the magnitude of the coastal N filter.

It is also important to consider the effects of seagrass on the coastal N filter within the context of other nitrogen cycle processes. Internal loading of nitrogen within the system through remineralization and N fixation can be enhanced in seagrass meadows compared to unvegetated sediments (Russell et al. 2016, Eyre et al. 2011). This enhanced availability of fixed nitrogen is important to supporting the high rates of productivity in seagrass meadows (McGlathery 2008); since the physical structure of the seagrass canopy drives N removal via burial, the availability of fixed nitrogen to support seagrass growth is also linked to the magnitude of N removal. Thus, the presence of seagrass affects both N filtering processes and other N cycle processes. However, the filtering processes in seagrass meadows overall result in a net reduction of dissolved and particulate nitrogen in the water column (Gurbisz et al. 2017), which maintains the water clarity and helps prevent negative effects such as algal blooms (McGlathery et al. 2007). This filter function therefore plays an important role in supporting ecosystem health alongside the concurrent effects of seagrass presence on N fixation, remineralization, and other N transformations.

The coastal N filter function performed by seagrass meadows is lost when seagrass meadows decline. Anthropogenic impacts, including dredging and eutrophication, contribute to accelerating declines in seagrass coverage worldwide (Orth et al. 2006a, Waycott et al. 2009). However, human intervention can lead to successful restoration of seagrass meadows and the reestablishment of natural meadow processes. Recent work has shown that restored meadows can sequester carbon at rates on par with natural, undisturbed meadows (Greiner et al. 2013, Oreska et al. 2017a). We expect that restoration will also reestablish the coastal filter function of

seagrass meadows to a level comparable to natural meadows; however, this effect has yet to be examined.

The purpose of this study was to assess the return of the coastal nitrogen filter in a restored *Zostera marina* (eelgrass) meadow located in a shallow coastal lagoon in Virginia, and to compare the magnitude of N filtering processes to external and internal N loading rates. Historically, eelgrass dominated the Virginia coastal bays but a combination of wasting disease and the impacts of a hurricane in 1933 lead to a local extinction (Orth & McGlathery 2012). Restoration was begun in 2001 by the Virginia Institute of Marine Sciences (VIMS) through seeding efforts in four of the coastal bays, which now support thriving, mature eelgrass meadows. This restoration provides a unique opportunity to assess the impact of seagrass presence on retention and removal of nitrogen in the lagoon. By comparing measurements made in the mature restored meadow to measurements in adjacent, unrestored ("bare") sediments, we were able to directly assess the nitrogen cycle in the lagoon with and without seagrass. We collected detailed measurements of biogeochemical fluxes and seagrass productivity in order to understand how the restoration affected the coastal N filter and sources of N to the meadow.

Methods

Study site

This study was conducted at the restored *Z. marina* meadow in South Bay, a shallow lagoon located on the Eastern Shore of Virginia. The South Bay meadow is one of four restored seagrass meadows in Virginia that were seeded as part of a landscape-scale restoration project (Orth et al. 2012). In South Bay, seeds were broadcast in 0.4 ha plots in 2001; these original seed plots eventually coalesced into a contiguous meadow that has continued to spread (Figure 5.1). In 2015, the restored meadow was approximately 6.9 km^2.

Nitrogen cycle processes

To assess the magnitude of the coastal filter in the restored seagrass meadow, we compared N inputs, removal, and retention measured in the meadow and in adjacent bare sediments. N inputs included loading from terrestrial and atmospheric sources and N fixation occurring within the meadow. N removal consisted of burial and denitrification, and N retention was estimated by measuring N demand, i.e. N assimilation in seagrass biomass. Burial and assimilation rates were measured directly in this study, and fixation and denitrification data were compiled from recent studies in the South Bay meadow (Chapters 3 and 4). N loading data were adapted from a nitrogen budget prepared by Anderson et al. (2010) for a neighboring coastal lagoon. Fixation, denitrification, and assimilation were measured seasonally in spring, summer, and fall; in order to determine annual rates, winter rates were estimated as half of the rates during fall. The annual rates were compared in units of g N m^{-2} y^{-1}; standard errors for each rate were propagated using the approach and equations described by Lehrter & Cebrian (2010). Bare measurements were made in sediments outside of the established seagrass meadow and, for denitrification and fixation, at experimentally cleared sub-plots (4 m^2) in the meadow interior. These sub-plots were first cleared in 2015 and were re-cleared in 2016 and 2017 following limited colonization by surrounding seagrass. By measuring rates in the bare sub-plots we attempted to isolate the direct effects of seagrass growth, i.e. oxygen and carbon exudation, and to eliminate the effects of confounding factors outside the meadow such as varying water depths and flow regimes on sediment biogeochemistry.

N loading

Inputs of nitrogen to South Bay from terrestrial and atmospheric sources were assessed based on a study by Anderson and others (2010). Anderson et al. studied nitrogen cycling in Hog

Island Bay, a coastal lagoon located approximately 20 km north of South Bay, with a similar ratio of watershed area to lagoon area and similar level of development in the watershed. Terrestrial and atmospheric inputs to Hog Island Bay were therefore considered to be broadly similar to South Bay (Cole 2011). The nitrogen sources evaluated in the Anderson study were base flow, surface water run-off, groundwater discharge, and atmospheric deposition. Base flow was calculated from stream discharge and nitrate concentrations; N loading from run-off and groundwater discharge were estimated as 40% and 33% of base flow loading respectively, based on a combination of direct measurements and modeling (see Anderson et al. 2010 for details). Atmospheric deposition (wet and dry) was calculated from samples collected at a meteorological tower located on Hog Island, the barrier island adjacent to Hog Island Bay, from 1990-1999, and corrected for sample preservation error. For the South Bay meadow budget, the loading rates determined in the Anderson study (kg N ha^{-1} y^{-1}) were scaled based on the area of the South Bay lagoon and watershed, as determined by Hayden & Porter (2001).

N fixation

N fixation rates in sediments and in seagrass epiphytes were measured using the acetylene reduction method, as reported by Aoki et al. (in prep, Chapter 4). Sediment samples and seagrass shoots were collected at three of the original seed plots in the interior of the meadow; sediments were also collected at the experimentally cleared sub-plots. The samples were incubated in glass vials held in a temperature-controlled water bath under a grow light that provided saturating light conditions; half the vials were wrapped in aluminum foil to provide a dark treatment. The sediment vials were injected with acetylene, and the leaves were incubated in acetylene-saturated site water. Incubations lasted 4-6 hours, and at the end of the incubation the vial headspace was transferred to a pre-evacuated Exetainer using a double-ended needle and

stored underwater until analysis on a Shimadzu GC-14A at the Smithsonian Environmental Research Center in Edgewater, MD, within 3 weeks. Acetylene reduction incubations were conducted in June, August, and October 2016 and in April and June 2017. Ethylene production rates were converted to N_2 fixation rates using the theoretical 3:1 ratio (Seitzinger & Garber 1987, Welsh 2000); areal fixation rates were calculated based on the depth of the sediment cores and the seagrass shoot density, measured in 8-10 replicate 0.25 m^2 quadrats scattered haphazardly at each plot. Four replicate sediment cores and six replicate shoots were incubated for each plot during each sampling period and rates were averaged at the plot level. No significant light effect was observed (Chapter 4), so the hourly light and dark rates were pooled and scaled by 24 hours to calculate daily rates in each season.

Denitrification

Hourly denitrification rates were measured using a push-pull incubation method as reported by Aoki & McGlathery (in review, Chapter 3). The push-pull method uses a mini-piezometer and peristaltic pump to label the porewater with ^{15}N and to sample the porewater over a 2-hour, in situ incubation. For the denitrification incubations, $^{15}NO_3$ was added to the porewater, denitrification products ($^{29}N_2$ and $^{30}N_2$) were measured via MIMS (Kana et al. 1994), and isotope pairing equations were applied to calculate denitrification rates (Nielsen 1992). Full details of the push-pull technique are available elsewhere (Aoki & McGlathery 2017, Chapter 1). Measurements were made at three of the original seed plots in the meadow interior in June, July, August, and October 2014 and in April and June 2015. Measurements were also conducted in adjacent bare sediments outside the meadow in summer 2014 and in experimentally cleared sub-plots within the meadow in June 2015. In Chapter 3, we calculated minimum and maximum daily rates of denitrification based on different assumptions about dark rates (the push-pull

measurements were made only during daylight hours). Here, we adopted the mean daily rate, which assumes a low level of denitrification in the sediments under dark conditions compared to light conditions. There is some evidence that denitrification rates in seagrass meadows may be greater under dark conditions compared to light conditions, due to the lack of competition for nitrate from autotrophs (Welsh et al. 2000, Eyre et al. 2011, 2013). The rates reported here may therefore underestimate daily denitrification. Fall and spring rates of denitrification in the bare sediments were estimated based on the ratio of bare rates to seagrass rates during summer.

Burial

N burial rates in seagrass sediments and bare sediments were calculated from sediment accretion rates and the N content of the sediment. Accretion rates (cm yr^{-1}) were provided in a previous study by Greiner et al. (2013) that used ^{210}Pb dating of sediment cores to a 20 cm depth. To determine N content, small sediment cores (2.5 cm diameter, 6 cm depth) were collected in 2013 from 16 sites distributed across the meadow and 2 bare sediment sites outside the meadow, with 4 replicate cores per site. An additional 6 sites clustered in the interior of the meadow and an additional bare site outside the meadow were sampled to a depth of 5 cm in 2014, with 5 replicates per site. Sediments were dried to a constant weight at 60ºC, and bulk density was calculated from the dry weight and wet volume of the sample. N content (%N) was measured using a Carlo Erba NA 2500 Elemental Analyzer, bulk N content (mg cm^{-3}) was calculated from %N and bulk density, and N burial rates (g N m^{-2} y^{-1}) were calculated from bulk N content and accretion rates.

As the South Bay meadow has expanded from the original seed plots, different areas of the meadow have been restored for varying amounts of time, i.e. the meadow age varies spatially. Long-term monitoring has shown a 5-year lag between the initial seeding and changes

to sediment characteristics; after 9 years, the meadow had achieved a mature, steady-state (McGlathery et al. 2012). The accretion rates measured by Greiner et al. (2013) showed the same pattern of a slight increase in accretion over the first five years of restoration, followed by a more rapid increase over years 5-9. We therefore calculated separate burial rates for three age brackets of restored meadow: 1-5 years, 5-9 years, and >9 years. Aerial photographs from an annual survey by the Virginia Institute of Marine Science (http://web.vims.edu/bio/sav/) allowed us to determine the age of the 22 plots sampled for burial within the meadow and to calculate mean N content and burial rates for each age bracket. We also used the aerial photography (digitized in ArcGIS) to determine the extent of meadow coverage within each age bracket in 2015 in order to calculate a weighted average burial rate for the entire meadow.

Assimilation

Assimilation of N into seagrass biomass was calculated from seagrass productivity rates and seagrass tissue N content. Productivity was measured using the leaf-marking technique (Short & Duarte 2001) in June, August, and October 2016 and in April and June 2017. Replicate 20 x 10 cm wire frames were anchored in the seagrass sediment, and all shoots within the frame were marked by puncturing the sheath bundle with a 25.5-gauge needle. After 10-15 days, the shoots, including rhizomes, were carefully harvested. Each shoot was separated into new growth (unmarked new leaves and leaf tissue below the needle scar) and old growth (tissue above the scar); new and old aboveground tissue was dried to a constant weight to determine shoot specific growth rates over the marking period (g shoot^{-1} d^{-1}). The plastochrone interval was calculated based on the number of new leaves that appeared during the marking period. The total rhizome length and average internode length were measured and rhizomes were also dried to a constant weight in order to calculate a length-to-weight ratio for belowground biomass. Because *Zostera*

marina produces a new rhizome node for each new leaf, the belowground biomass growth rates (g shoot^{-1} d^{-1}) could be calculated from the average internode length, length-to-weight ratio, and the plastochrone interval. Four productivity frames were deployed at each of 3 plots in each sampling month except in June when frames were deployed at 6 plots; on average, 68 shoots were marked and recovered during each marking period. Above- and belowground productivity rates were converted to N assimilation rates using the N content of triplicate seagrass biomass samples that were collected simultaneously with the productivity samples at two plots. Biomass samples were sorted into live and dead above and belowground biomass, dried to a constant weight at 60ºC, pulverized using a Biospec Products MiniBeadBeater, and analyzed for N content on a Carlo Erba NA 2500 Elemental Analyzer. Seagrass shoot density was also measured concurrently with productivity in 8-10 replicate 0.25 m^2 quadrats scattered haphazardly at each plot, and shoot-specific assimilation rates were scaled to areal assimilation rates by shoot density.

Results

N inputs

Table 5.1 shows the N loading values from the Anderson et al. study scaled to the lagoon and watershed areas to calculate the total N load to the South Bay meadow. On an areal basis, the lagoon received a nitrogen load of 1.23 g N m^{-2} y^{-1}. Atmospheric deposition was the dominant source of nitrogen, accounting for 77% of the external N inputs. Since these external inputs were not affected by the presence of seagrass in the lagoon, the external N load was equivalent for both the seagrass and bare sediment.

N fixation measured via acetylene reduction peaked in August both in sediments and leaf epiphytes (Figure 5.2). Sediment rates dominated epiphyte rates and showed substantial inter-

annual variability, with peak rates in summer 2017 up to 6x the peak rates in summer 2016. Bare and seagrass sediments had comparable N fixation rates, likely due to the similar organic matter content (Aoki et al. in prep, Chapter 4). The annual N fixation rate was 0.93 g N m^{-2} y^{-1} in the seagrass meadow and 0.74 g N m^{-2} y^{-1} in the bare sediment.

N removal

Denitrification rates measured via the push-pull method were on average 4x greater in seagrass sediments than in adjacent bare sediments (Figure 5.3). Rates peaked in summer and were at a minimum during fall. The annual denitrification rate based on these measurements was 0.62 g N m^{-2} y^{-1} in seagrass sediments and 0.16 g N m^{-2} y^{-1} in bare sediments.

As the seagrass meadow has matured over time, burial rates have increased due to increased sedimentation rates and increased sediment N content (Figure 5.4). Burial rates within each age bracket ranged from 0.17 g N m^{-2} y^{-1} in bare sediments to 3.52 g N m^{-2} y^{-1} in the oldest (>9 years old) sediments (Table 5.2). Compared across age brackets, burial rates varied significantly (ANOVA, $F_{3,21}=5.22$, $p<0.05$), and post-hoc contrasts showed that the >9 year old sediments had significantly higher rates than both the bare and recently colonized (1-5 year old) sediments (Tukey test, $p<0.05$). The total area of the seagrass meadow has also expanded steadily through 2015 (Figure 5.5), and the oldest areas of the meadow accounted for only a small proportion of the total meadow area in 2015 (Table 5.2). The average burial rate over the total 2015 meadow area, weighted based on the proportion of the meadow area in each age bracket, was 1.95 g N m^{-2} y^{-1}.

N retention

Shoot-specific aboveground productivity peaked in June (Figure 5.6), leading to peak N assimilation rates in summer (Figure 5.7). Shoot counts were highest in June 2017; however, N

content of aboveground tissue was highest in October 2016. Belowground tissue N content and belowground biomass growth rates showed little variation across seasons. Assuming a winter assimilation rate of half the fall assimilation rate (based on winter shoot densities measured in other years, data not shown), the annual assimilation rate was 2.62 g N m^{-2} y^{-1}, which was the largest flux of nitrogen in the seagrass meadow.

Discussion

N removal in seagrass sediments

Differences in the N removal and N inputs in the seagrass and bare sediments showed that the seagrass restoration led to the reestablishment of the N filter function. In bare sediments, net N removal accounted for only 15% of N inputs, whereas in the seagrass sediments, net N removal was roughly equal to N inputs from watershed loading and fixation (Table 5.3). Before restoration, the unvegetated sediment did not act as a substantial nitrogen filter, and indeed mainly contributed to the total N load via N fixation; the excess N load from the atmosphere, terrestrial sources, and fixation was likely exported to the coastal ocean. After restoration, the seagrass presence dramatically increased the magnitude of the nitrogen removal processes in the lagoon.

The meadow-wide burial rate in the restored meadow (1.95 g N m^{-2} y^{-1}) was slightly lower than previously reported rates in *Zostera* meadows (2.7-3.9 g N m^{-2} y^{-1}, Eyre et al. 2016). However, the meadow-wide rate in this study was calculated as a weighted average of burial rates in the different age classes (time since restoration) of the meadow. The maximum burial rate measured at the mature meadow sites was 3.52 g N m^{-2} y^{-1}, which agrees very well with previously reported rates. Thus, our analysis shows that within 10 years after seeding, the restoration has successfully reestablished N removal via burial to rates comparable to natural

meadows. As the restored meadow continues to mature, we expect that the burial rate will increase in younger areas of the meadow, leading to a meadow-wide burial rate on par with natural systems.

Areal rates of annual N removal via denitrification in seagrass meadows are scarce in the literature, but recent studies suggest a range of 7-19 g N m^{-2} y^{-1} (Smyth et al. 2013, Eyre et al. 2016). These values greatly exceed the rate reported here, 0.62 g N m^{-2} y^{-1}. Extremely low availability of nitrate in South Bay surface waters contributes to the low denitrification rates in this system (McGlathery & Christian 2017). Since nitrate is undetectable in surface waters throughout the year, denitrification occurs mainly in subsurface sediments, coupled to nitrification occurring in oxic microzones surrounding seagrass roots (Aoki and McGlathery, in review, Chapter 3). In other systems, higher levels of nitrate in surface waters can support higher denitrification rates in surface sediments. Additionally, in this study, since the in situ denitrification measurements were conducted only under natural light conditions, dark denitrification rates were conservatively assumed to be a fraction of light rates (see Chapter 3 for detailed discussion of the scaling assumptions). Light and dark comparisons using core incubations suggest that denitrification in seagrass sediments are similar or even higher in the dark, due to reduced competition for nitrate from autotrophs (Welsh et al. 2000, Eyre et al. 2011). Light and dark comparisons have yet to be made using the push-pull method employed in this study, and it is unclear if subsurface denitrification rates also increase under dark conditions. However, a recent study that found no difference between light and dark rates of coupled nitrification-denitrification in surface sediments of *Z. muelleri* cores suggests that it may be reasonable to extrapolate the daylight rates measured here to 24 hours (Russell et al. 2016). In

that case, the annual rate of N removal via denitrification would be 0.96 g N m^{-2} y^{-1}, still below other estimates and about half of the meadow-wide burial rate.

Overall, the restored meadow showed dramatic increases in N removal compared to the unrestored sediments, indicating the reestablishment of N filtering processes. Burial was the main driver of N removal, although annual denitrification may have been underestimated in this study. The net N removal rate was approximately 8x greater in the restored meadow compared to bare sediments (2.57 compared to 0.33 g N m^{-2} y^{-1}), and the burial rates agreed with the available literature values for *Z. marina* meadows. Fourteen years after the initial seeding, the restored South Bay meadow appears to have regained the filter function of a natural meadow.

N retention in seagrass biomass

In addition to the enhanced N removal in seagrass sediments, we measured a large N assimilation rate of 2.62 g N m^{-2} y^{-1}, the largest flux of N in the meadow (Table 5.3). This N assimilation rate likely underestimates N demand, since the leaf-marking technique used in this study does not account for leaf maturation (thickening and widening of the leaf) above the needle scar (Park et al. 2010). The large N assimilation rate, in excess of N inputs, indicates that N recycling processes played an important role in the meadow N cycle. N recycling includes both remineralization of seagrass-derived N in meadow sediments and internal recycling of N within seagrass shoots, sometimes known as N reclamation. We did not measure remineralization or reclamation, but previous studies indicate that each of these processes may meet as much as 50% of the seagrass N demand (Romero et al. 2006), and previous work has demonstrated that remineralization is important to meet benthic microalgal demand in this system (Anderson et al. 2003). Generally, tightly linked recycling processes allow seagrass to achieve extremely high rates of productivity in nutrient-poor waters, as was likely the case in our study system.

Enhanced N fixation within the meadow sediments provided additional N that could have helped to support the high rates of seagrass growth. N fixation rates were about 20% higher in the meadow than in the bare sediments (Table 5.3), and these measurements likely underestimate the true N fixation rate. The N fixation rates used in this study were measured indirectly using acetylene reduction, and agree well with literature values for N fixation in *Z. marina* meadows (e.g. Welsh et al. 1996, McGlathery et al. 1998). However, acetylene reduction has several known drawbacks (Welsh 2000, Fulweiler et al. 2015), and more recent studies using direct measurement techniques in estuarine sediments have found substantially higher N fixation rates compared to published acetylene reduction values (Gardner et al. 2006, Fulweiler et al. 2007, Newell et al. 2016). In the South Bay meadow, we recently used a direct $^{30}N_2$ push-pull method to measure N fixation and found rates that exceeded the acetylene reduction rates by more than an order of magnitude (Aoki and McGlathery in prep, Chapter 4). The $^{30}N_2$ measurements were conducted only in June 2017, so we cannot use these higher rates to calculate an annual average rate. However, the total amount of N fixed only in the month of June based on the $^{30}N_2$ push-pull rates would be approximately 4 g N m^{-2}, which would be sufficient to support the measured N assimilation of 2.62 g N m^{-2} y^{-1}. More than 70% of seagrass N assimilation occurred in summer, so it is possible that these high summer rates of N fixation coincided with the peak in seagrass N demand, but additional $^{30}N_2$ push-pull data are needed to fully explore these patterns. Overall, the preliminary summer data suggest that N fixation may have supplied the additional N needed to support high rates of N assimilation in the meadow.

Another possible source of N could have come from the import of dissolved and/or particulate nitrogen from surrounding systems, particularly the *Spartina alterniflora* marshes that border the seagrass meadow (Figure 5.1). Marsh outwelling of particulate and dissolved organic

matter has been studied and debated in the literature for decades (e.g. Nixon 1980, Childers et al. 2002), although little work has focused specifically on transfer of marsh nitrogen to adjacent seagrass meadows. In South Bay, recent analysis of the sediment organic matter in the seagrass meadow using stable isotopes showed that *Spartina* contributes only 10% of the sediment organic carbon (Oreska et al. 2017c); applying this percentage to the N burial rate suggests that the marsh could have supplied 0.21 g N m^{-2} y^{-1}, a small proportion of the seagrass N demand. We looked for evidence of marsh outwelling of dissolved nitrogen in summer 2016 by collecting surface water along transects from the marsh to the center of the meadow at different stages in the tidal cycle. Concentrations of inorganic nitrogen (ammonium and nitrate) were consistently low (<1 μM), including in samples collected adjacent to the marsh on the falling tide (data not shown). Export of dissolved nitrogen from the marsh therefore likely did not contribute substantially to the N supply in the seagrass meadow.

The fate of nitrogen assimilated into seagrass biomass is largely unknown. While a portion of the nitrogen that accumulates in seagrass biomass will be buried in seagrass sediments, an additional portion will be exported from the ecosystem. Leaves and shoots are lost throughout the growing season, and, in this system, accumulations of seagrass wrack have been observed on the ocean side of the barrier islands. Seagrass biomass may contribute to sediment organic matter in the neighboring marshes, and a recent study suggests that seagrass wrack may be buried on the continental shelf (Duarte & Krause-Jensen 2017). This physical transport of biomass nitrogen out of the seagrass meadow, coupled with the low rates of external N inputs from the watershed, underscores the importance of internal N loading from enhanced N fixation and remineralization.

Fully tracking the fate of nitrogen retained in seagrass biomass is beyond the scope of this study. However, it is clear that N assimilation is a dominant N flux in the restored meadow and N retention in seagrass biomass is similar in magnitude to N removal via burial and denitrification. The combination of these processes supports the high water quality in the seagrass meadow and continued seagrass health. By reducing water column concentrations of nitrogen, seagrass limit algal growth and associated light attenuation, thus creating a positive feedback for seagrass growth (Gurbisz et al. 2017). Through this positive feedback, the coastal filter function therefore supports the many other ecosystem services provided by seagrass meadows, such as habitat provisioning and carbon sequestration, in addition to limiting water column nitrogen.

Comparisons with other seagrass meadows

A few studies have combined measurements of microbial N transformations in concert with seagrass productivity and/or external N loading rates. Compared to the South Bay meadow, *Z. marina* meadows in Denmark had higher rates of productivity and similar rates of denitrification and N fixation; seagrass uptake of nitrogen was consequently even more dominant in these systems than in the South Bay meadow (Pederson and Borum 1993, Risgaard-Petersen et al. 1998). *Z. marina* are known to be sensitive to temperatures above 28ºC; these high temperatures occur frequently in the South Bay meadow during summer and may influence the productivity rates. At the southern end of its range, in Baja California (30ºN), *Z. marina* meadows showed similar productivity rates to those measured here (Ibarra-Obondo et al. 1997). If *Z. marina* productivity is strongly influenced by climate, the relative dominance of seagrass N assimilation in the meadow N cycle may vary along a latitude gradient.

Nutrient loading rates also influence relative rates of denitrification and N fixation in seagrass meadows (Herbert 1999). Denitrification rates in the South Bay meadow were likely limited by the very low availability of dissolved inorganic nitrogen in surface waters; surface water nutrient concentrations were very low, often < 1 μM, and porewater ammonium levels were below concentrations measured at other seagrass meadow sites (see Chapter 3 for more discussion). A recent study found higher rates of denitrification in intertidal *Z. muelleri* flats exposed to higher N loading rates than flats with lower N loading (Russell et al. 2016). In general, many previous studies have found that N fixation outweighs denitrification in seagrass meadows, including sites with moderate N loading (e.g. Welsh et al. 2000). However, other studies have used the N_2:Ar method to measure much higher net effluxes of N_2 from sediments, indicating dominance of denitrification over N fixation (Eyre et al. 2011, Smyth et al. 2013). This disparity between studies has yet to be resolved. In this study, under low N loading conditions, denitrification was about two-thirds of N fixation measured via acetylene reduction. However, the direct $^{30}N_2$ push-pull measurements of N fixation were much greater (Chapter 4). Further measurements of N fixation, especially comparing acetylene reduction and direct measurements, are needed to better understand the N fixation in seagrass sediments and to compare with denitrification rates.

As noted earlier, measurements of N burial in temperate seagrass meadows are rare. In this study, we found that burial was one of the dominant nitrogen cycle processes, on par with seagrass productivity. More estimates of N burial in seagrass meadows are needed to understand if N burial is generally a dominant process across different systems and seagrass species. In this study, our measurements showed the strong influence of the seagrass restoration on the upper 6 cm of the sediment; these measurements show the immediate effect of short-term deposition and

accumulation of N in seagrass sediments. This immediate effect contributes to the coastal filter function by removing particulate N from the water column. However, studies of persistent natural meadows are needed to understand the magnitude of N burial over longer timescales.

Meadow-wide N removal and retention

To fully assess the impact of the coastal filter function in the restored meadow, we compared N removal and retention at the meadow-scale with an equivalent area of bare sediment. In order to make this comparison, we scaled up the N removal and retention rates by the total area of the meadow in 2015 (6.9 km^2), which required consideration of spatial variability across the meadow. Burial, the larger component of N removal, incorporated spatial differences in accretion and N content and therefore could be directly scaled up to the total meadow area (see "Methods"). The denitrification rate, on the other hand, did not include spatial variability, as denitrification was measured only in the interior, mature areas of the meadow where high organic carbon content in the sediment may have supported elevated rates (Oreska et al. 2017a). Directly scaling up denitrification may therefore have overestimated the contribution to N removal. However, the annual denitrification rate accounted for a minor portion of the total N removal (~24% of total N removal). Overestimation of denitrification therefore likely had a small effect on total N removal estimates. Similarly, N retention in seagrass biomass was only measured in interior meadow plots, which might be expected to have greater productivity than younger areas of the meadow. However, shoot-specific productivity rates at this restoration site did not show a trend with meadow age since restoration (McGlathery et al. 2012), and shoot densities at six sites in the meadow interior were not significantly different from six sites distributed across the meadow in summer 2017 (McGlathery 2017). The N assimilation rates

measured in the meadow interior could therefore be scaled up to estimate N retention at the meadow scale.

Scaled over the extent of the meadow in 2015, burial and denitrification removed 17.6 t N y^{-1}; assimilation temporarily retained an equivalent 17.6 t N y^{-1}. In contrast, a comparable area of bare sediment would have removed only 2.3 t N y^{-1}. Because the N removal was driven by burial, and because burial increases with meadow maturation, the N removal rate will continue to increase as the meadow matures. In 2015, more than a third of the meadow was less than 5 years old. Assuming no additional expansion of the meadow from the 2015 area, by 2020, 84% of the meadow will be more than 9 years old, and 16% will be between 5 and 9 years (Figure 5.8A). Applying the burial rates measured for each age bracket in this study (Table 5.2), the average N burial in the meadow would be 3.31 g N m^{-2} y^{-1}, approximately 1.7x the current rate, and the total burial would be 23 t N (Figure 5.8B).

Additional burial in the South Bay lagoon will occur through meadow expansion. Beginning around 2013, seagrass began to colonize a large secondary area only minimally connected to the original meadow. This secondary meadow expanded rapidly, and by 2015 was roughly 2/3 the size of the original restored meadow (4.7 km^2, based on analysis of aerial photography from the VIMS survey, http://web.vims.edu/bio/sav/). If we assume that the burial rate we measured for newly colonized sediment, 0.53 g N m^{-2} y^{-1}, applies to this area, the meadow expansion would bury an additional 2.5 t N y^{-1} in 2015, about 3x more than would be buried in an equivalent bare area. Leaving aside the possibility of any additional N removal through enhanced denitrification in the meadow expansion, the enhanced burial demonstrates how rapidly the seagrass restoration can transform the N cycle in the lagoon.

We expect to see continued expansion of the seagrass meadow in South Bay, as well as expansion of the other restored meadows in the VCR. Preliminary analysis based of sediment characteristics and bathymetry suggest a total habitable area for eelgrass of 151 km^2 within the VCR coastal bays (Oreska et al. 2017b). This estimate represents the upper limit of possible seagrass expansion, but additional expansion beyond the current seagrass extent of 25 km^2 is likely, as seagrass were historically present throughout the coastal bays (Orth et al. 2006b) Assuming broadly consistent burial rates throughout the system, 151 km^2 of restored seagrass meadow could remove 80 t N y^{-1} over the first five years of restoration and 325 t N y^{-1} after five years, compared to 26 t N y^{-1} in a comparable area of unvegetated sediment. While these maximum removal rates are unlikely to be realized, continued expansion of the restored seagrass will translate into additional N burial and removal. Thus, a major effect of the restoration project on the lagoon has been to restore a coastal filter function that has been missing since the local disappearance of seagrass in the 1930s.

Conclusions

Overall, this study demonstrated for the first time how restoration of seagrass reinstates the costal filter function by increasing N removal and temporary N retention compared to unrestored sediments. Both denitrification and burial (N removal) increased in the restoration compared to bare sediments, and assimilation into seagrass biomass (N retention) was the largest measured flux of nitrogen. These filtering processes was important in this system in part due to the very low rates of N loading, which were among the lowest measured rates for shallow coastal bays worldwide (McGlathery et al. 2007). The high productivity of seagrass within the bay was therefore likely supported by internal recycling (remineralization and N reclamation) as well as N fixation, which may have been underestimated in this study. Additional direct measurements

of N fixation are needed to better assess the effect of the seagrass restoration on N cycling. Further study of N burial rates in non-peat-forming seagrass meadows will also help to characterize the magnitude of the N removal ecosystem service.

Although N loading to the Virginia coastal bays is currently very low, residential development and intensification of agriculture on Virginia's Eastern Shore are likely to cause increased N loading in the future (Giordano et al. 2011). The N removal service provided by the restored seagrass meadow will increase the ability of the coastal bays to filter increased N loads without suffering the negative effects of eutrophication. The success of this buffering capacity will depend on the magnitude of future N loads relative to the N removal rates in the seagrass meadow (de Wit et al. 2001). However, the detailed field measurements presented in this study show that the N removal rate has increased as the restored meadow has matured and expanded. Given continued availability of suitable habitat, we expect the meadow will continue to expand, allowing the total N removal to increase over time. The restoration thus represents a successful investment in the continued health of the coastal bays. By documenting the reestablishment of the coastal filter function in this restored meadow, this study provides motivation to restore and preserve other seagrass meadows threatened by human and natural impacts.

References

Anderson IC, McGlathery KJ, Tyler AC (2003) Microbial mediation of "reactive" nitrogen transformations in a temperate lagoon. Mar Ecol Prog Ser 246:73–84

Anderson IC, Stanhope JW, Hardison AK, McGlathery KJ (2010) Sources and fates of nitrogen in Virginia coastal bays. In: Coastal Lagoons: Critical habitats of environmental change. CRC Press, Boca Raton, FL, p 43–72

Aoki LR, McGlathery KJ (2017) Push-pull incubation method reveals the importance of denitrification and dissimilatory nitrate reduction to ammonium in seagrass root zone. Limnol Oceanogr Methods 15:766–781

Banta GT, Pederson MF, Nielsen SL (2004) Decomposition of marine primary producers: Consequences for nutrient recycling and retention in coastal ecosystems. In: Estuarine

nutrient cycling: the influence of primary producers. Kluwer Academic Publishers, Dordrecht, The Netherlands, p 187–216

Barbier EB, Hacker SD, Kennedy C, Koch EW, Stier AC, Silliman BR (2011) The value of estuarine and coastal ecosystem services. Ecol Monogr 81:169–193

Cebrian J, Duarte CM (2001) Detrital stocks and dynamics of the seagrass *Posidonia oceanica* (L.) Delile in the Spanish Mediterranean. Aquat Bot 70:295–309

Childers DL, Day JW, Mckellar HN (2002) Twenty more years of marsh and estuarine flux studies: revisiting Nixon (1980). In: Concepts and controversies in tidal marsh ecology. Springer, New York, NY, p 391–423

Cole L (2011) Inputs and fluxes of nitrogen in the Viriginia coastal bays: Effects of newly-restored seagrasses on the nitrogen cycle. Ph.D, University of Virginia

De Wit R, Stal LJ, Lomstein BA, Herbert RA, Van Gemerden H, Viaroli P, Cecherelli V-U, Rodríguez-Valera F, Bartoli M, Giordani G (2001) ROBUST: The ROle of BUffering capacities in STabilising coastal lagoon ecosystems. Cont Shelf Res 21:2021–2041

Duarte CM, Krause-Jensen D (2017) Export from seagrass meadows contributes to marine carbon sequestration. Front Mar Sci 4:13

Eyre BD, Ferguson AJP, Webb A, Maher D, Oakes JM (2011) Denitrification, N-fixation and nitrogen and phosphorus fluxes in different benthic habitats and their contribution to the nitrogen and phosphorus budgets of a shallow oligotrophic sub-tropical coastal system (southern Moreton Bay, Australia). Biogeochemistry 102:111–133

Eyre BD, Maher DT, Sanders C (2016) The contribution of denitrification and burial to the nitrogen budgets of three geomorphically distinct Australian estuaries: Importance of seagrass habitats. Limnol Oceanogr 61:1144–1156

Eyre BD, Maher DT, Squire P (2013) Quantity and quality of organic matter (detritus) drives N_2 effluxes (net denitrification) across seasons, benthic habitats, and estuaries. Glob Biogeochem Cycles 27:1083–1095

Fulweiler RW, Heiss EM, Rogener MK, Newell SE, LeCleir GR, Kortebein SM, Wilhelm SW (2015) Examining the impact of acetylene on N-fixation and the active sediment microbial community. Front Microbiol 6:418

Fulweiler RW, Nixon SW, Buckley BA, Granger SL (2007) Reversal of the net dinitrogen gas flux in coastal marine sediments. Nature 448:180–182

Gacia E, Duarte CM, Middelburg JJ (2002) Carbon and nutrient deposition in a Mediterranean seagrass (*Posidonia oceanica*) meadow. Limnol Oceanogr 47:23–32

Gardner WS, McCarthy MJ, An S, Sobolev D, Sell KS, Brock D (2006) Nitrogen fixation and dissimilatory nitrate reduction to ammonium (DNRA) support nitrogen dynamics in Texas estuaries. Limnol Oceanogr 51:558–568

Giordano JCP, Brush MJ, Anderson IC (2011) Quantifying annual nitrogen loads to Virginia's coastal lagoons: Sources and water quality response. Estuaries Coasts 34:297–309

Greiner JT, McGlathery KJ, Gunnell J, McKee BA (2013) Seagrass restoration enhances "blue carbon" sequestration in coastal waters (J Cebrian, Ed.). PLoS ONE 8:e72469

Gurbisz C, Kemp WM, Cornwell JC, Sanford LP, Owens MS, Hinkle DC (2017) Interactive effects of physical and biogeochemical feedback processes in a large submersed plant bed. Estuaries Coasts 40:1626–1641

Hansen J, Reidenbach M (2012) Wave and tidally driven flows in eelgrass beds and their effect on sediment suspension. Mar Ecol Prog Ser 448:271–287

Hayden B, Porter J (2001) Terrestrial-marine watershed boundaries on the Delmarva peninsula of Virginia 2001. Virginia Coast Reserve Long-Term Ecological Research Project Data Publication doi:10.6073/pasta/9a5052eb398458ad1d8dea50f1136fa1

Hemminga MA, Marba N, Stapel J (1999) Leaf nutrient resorption, leaf lifespan and the retention of nutrients in seagrass systems. Aquat Bot 65:141–158

Herbert RA (1999) Nitrogen cycling in coastal marine ecosystems. FEMS Microbiology Reviews 23:563–590

Ibarra-Obando SE, Boudouresque C-F, Roux M (1997) Leaf dynamics and production of a Zostera marina bed near its southern distributional limit. Aquatic Botany 58:99–112

Iizumi H, Hattori A, McRoy CP (1980) Nitrate and nitrite in interstitial waters of eelgrass beds in relation to the rhizosphere. J Exp Mar Biol Ecol 47:191–201

Jørgensen NOG, Blackburn TH, Henriksen K, Bay D (1981) The importance of *Poisidonia oceanica* and *Cymodocea nodosa* as contributors of free amino acids in water and sediment of seagrass beds. Mar Ecol 2:97–112

Kana TM, Darkangelo C, Hunt MD, Oldham JB, Bennett GE, Cornwell JC (1994) Membrane inlet mass spectrometer for rapid high-precision determination of N_2, O_2, and Ar in environmental water samples. Anal Chem 66:4166–4170

Lehrter JC, Cebrian J (2010) Uncertainty propagation in an ecosystem nutrient budget. Ecol Appl 20:508–524

Mateo MA, Romero J (1997) Detritus dynamics in the seagrass *Posidonia oceanica*: Elements for an ecosystem carbon and nutrient budget. Mar Ecol Prog Ser:43–53

Mateo MA, Romero J, Pérez M, Littler MM, Littler DS (1997) Dynamics of millenary organic deposits resulting from the growth of the Mediterranean seagrass *Posidonia oceanica*. Estuar Coast Shelf Sci 44:103–110

McGlathery K (2017) Density of seagrass in Hog Island Bay and South Bay, VA 2007-2017. Virginia Coast Reserve Long-Term Ecological Research Project Data Publication doi:10.6073/pasta/5a6ea442cf59cabb3112bb634a968ae5

McGlathery KJ, Christian R (2017) Water quality sampling - integrated measurements for the Virginia Coast, 1992-2017. Virginia Coast Reserve Long-Term Ecological Research Project Data Publication doi:10.6073/pasta/8783dd64be7cae3a36a864ac5b5129e1

McGlathery K, Reynolds L, Cole L, Orth R, Marion S, Schwarzschild A (2012) Recovery trajectories during state change from bare sediment to eelgrass dominance. Mar Ecol Prog Ser 448:209–221

McGlathery KJ, Risgaard-Petersen N, Christensen PB (1998) Temporal and spatial variation in nitrogen fixation activity in the eelgrass *Zostera marina* rhizosphere. Mar Ecol Prog Ser 168:245–258

McGlathery KJ (2008) Seagrass habitats. In: Nitrogen in the marine environment. Elsevier, New York, p 1037–1072

McGlathery K, Sundbäck K, Anderson I (2007) Eutrophication in shallow coastal bays and lagoons: The role of plants in the coastal filter. Mar Ecol Prog Ser 348:1–18

Middleburg JJ, Soetaert K, Herman PMJ, Boschker HTS, Heip CR (2004) Burial of nutrient in coastal sediments: The role of primary producers. In: Esutarine Nutrient Cycling: The Influence of Primary Producers. Kluwer Academic Publishers, Dordrecht, The Netherlands, p 217–230

Newell SE, McCarthy MJ, Gardner WS, Fulweiler RW (2016) Sediment nitrogen fixation: A call for re-evaluating coastal N budgets. Estuaries Coasts 39:1626–1638

Nielsen LP (1992) Denitrification in sediment determined from nitrogen isotope pairing. FEMS Microbiol Lett 86:357–362

Nixon SW (1980) Between coastal marshes and coastal waters: A review of twenty years of speculation and research on the role of salt marshes in estuarine productivity and water chemistry. In: Hamilton P, Macdonald KB (eds) Estuarine and Wetland Processes: With Emphasis on Modeling. Springer US, Boston, MA, p 437–525

Oreska MPJ, McGlathery KJ, Porter JH (2017a) Seagrass blue carbon spatial patterns at the meadow-scale (J Cebrian, Ed.). PLOS ONE 12:e0176630

Oreska MPJ, Truitt B, Orth RJ, Luckenbach MW (2017b) The bay scallop (Argopecten irradians) industry collapse in Virginia and its implications for the successful management of scallop-seagrass habitats. Mar Policy 75:116–124

Oreska MPJ, Wilkinson GM, McGlathery KJ, Bost M, McKee BA (2017c) Non-seagrass carbon contributions to seagrass sediment blue carbon. Limnol Oceanogr:S3–S18

Orth RJ, Carruthers TJB, Dennison WC, Duarte CM, Fourqurean JW, Heck KL, Hughes AR, Kendrick GA, Kenworthy WJ, Olyarnik S, Short FT, Waycott M, Williams SL (2006a) A global crisis for seagrass ecosystems. BioScience 56:987–996

Orth RJ, Luckenbach ML, Marion SR, Moore KA, Wilcox DJ (2006b) Seagrass recovery in the Delmarva coastal bays, USA. Aquat Bot 84:26–36

Orth R, McGlathery K (2012) Eelgrass recovery in the coastal bays of the Virginia Coast Reserve, USA. Mar Ecol Prog Ser 448:173–176

Orth R, Moore K, Marion S, Wilcox D, Parrish D (2012) Seed addition facilitates eelgrass recovery in a coastal bay system. Mar Ecol Prog Ser 448:177–195

Park SR, Kim YK, Kim SH, Lee K-S (2013) Nitrogen budget of the eelgrass, *Zostera marina* in a bay system on the south coast of Korea. Ocean Sci J 48:301–310

Park SR, Li WT, Kim SH, Kim J., Lee K. (2010) A comparison of methods for estimating the productivity of *Zostera marina*. J Ecol Field Biol 33:59–65

Pedersen MF, Borum J (1993) An annual nitrogen budget for a seagrass *Zostera marina* population. Mar Ecol-Prog Ser 101:169–169

Pedersen MF, Duarte CM, Cebrián J (1997) Rates of changes in organic matter and nutrient stocks during seagrass *Cymodocea nodosa* colonization and stand development. Mar Ecol Prog Ser 159:29–36

Pedersen MF, Nielsen SL, Banta GT (2004) Interactions between vegetation and nutrient dynamics in coastal marine ecosystems: An introduction. In: Estuarine nutrient cycling: the influence of primary producers. Kluwer Academic Publishers, Dordrecht, The Netherlands, p 1–16

Pérez M, Mateo MA, Alcoverro T, Romero J (2001) Variability in detritus stocks in beds of the seagrass *Cymodocea nodosa*. Bot Mar 44:523–531

Risgaard-Petersen N, Dalsgaard T, Rysgaard S, Christensen PB, Borum J, McGlathery K, Nielsen LP (1998) Nitrogen balance of a temperate eelgrass *Zostera marina* bed. Mar Ecol-Prog Ser 174:281–291

Romero J, Lee K, Pérez M, Mateo MA, Alcoverro T (2006) Nutrient dynamics in seagrass ecosystems. In: Seagrasses: Biology, ecology, and conservation. Springer, The Netherlands, p 227–254

Russell DG, Warry FY, Cook PLM (2016) The balance between nitrogen fixation and denitrification on vegetated and non-vegetated intertidal sediments. Limnol Oceanogr 61:2058–2075

Seitzinger SP, Garber JH (1987) Nitrogen fixation and $^{15}N_2$ calibration of the acetylene reduction assay in coastal marine sediments. Mar Ecol Prog Ser 37:65–73

Short FT, Duarte CM (2001) Methods for the measurement of seagrass growth and production. In: Global Seagrass Research Methods. Elsevier, p 155–182

Smyth AR, Thompson SP, Siporin KN, Gardner WS, McCarthy MJ, Piehler MF (2013) Assessing nitrogen dynamics throughout the estuarine landscape. Estuaries Coasts 36:44–55

Waycott M, Duarte CM, Carruthers TJ, Orth RJ, Dennison WC, Olyarnik S, Calladine A, Fourqurean JW, Heck KL, Hughes AR, others (2009) Accelerating loss of seagrasses across the globe threatens coastal ecosystems. Proc Natl Acad Sci 106:12377–12381

Welsh DT (2000) Nitrogen fixation in seagrass meadows: Regulation, plant–bacteria interactions and significance to primary productivity. Ecol Lett 3:58–71

Welsh DT, Bartoli M, Nizzoli D, Castaldelli G, Riou SA, Viaroli P (2000) Denitrification, nitrogen fixation, community primary productivity and inorganic-N and oxygen fluxes in an intertidal *Zostera noltii* meadow. Mar Ecol Prog Ser 208:65–77

Welsh DT, Bourgues S, De Wit R, Herbert RA (1996) Seasonal variations in nitrogen-fixation (acetylene reduction) and sulphate-reduction rates in the rhizosphere of *Zostera noltii*: Nitrogen fixation by sulphate-reducing bacteria. Mar Biol 125:619–628

Zarnoch CB, Hoellein TJ, Furman BT, Peterson BJ (2017) Eelgrass meadows, *Zostera marina* (L.), facilitate the ecosystem service of nitrogen removal during simulated nutrient pulses in Shinnecock Bay, New York, USA. Mar Pollut Bull 124:376–387

Figures

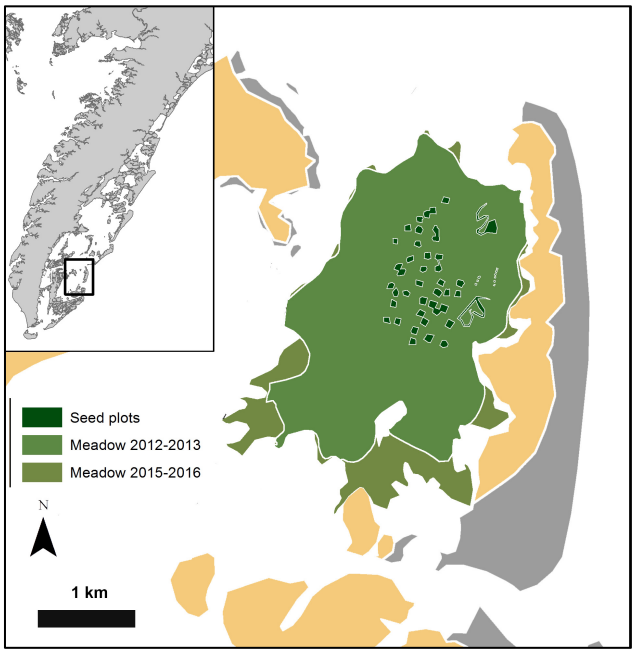

Figure 5.1 Map of the South Bay meadow showing the original seed plots and meadow expansion over time.

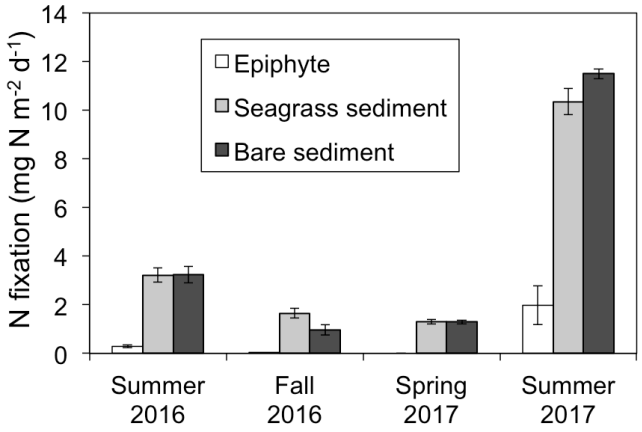

Figure 5.2 Daily rates of N fixation measured via acetylene reduction showed peak values in summer and dominance of sediment rates over leaf epiphyte rates. Errors are SE, n=6 except for summer 2016, n=18.

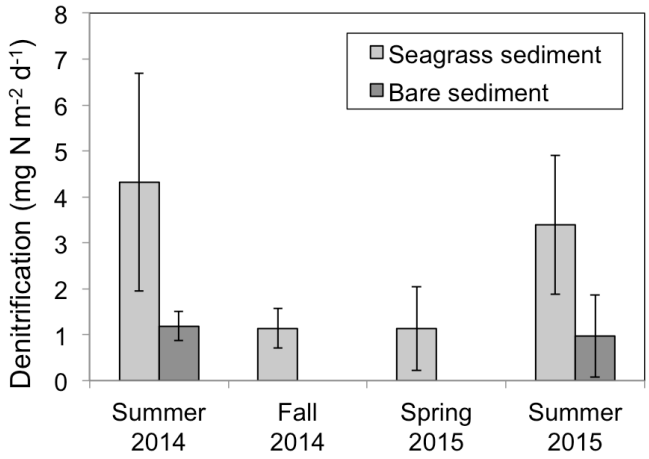

Figure 5.3 Daily denitrification rates, measured using push-pull incubations, peaked in summer, when rates were about 4x greater in seagrass sediments compared to bare sediments (no data for bare sediments in fall or spring). Errors are SE, n=10 (Summer 2014), 7 (Fall 2014), 9 (Spring 2015), 12 (Summer 2015).

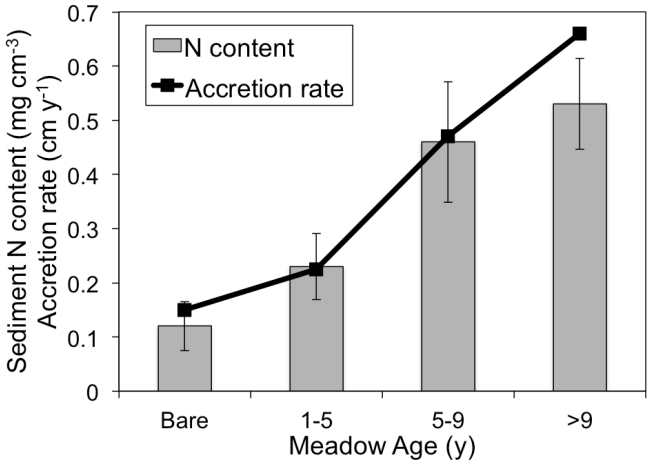

Figure 5.4 Accretion rates (from Greiner et al. 2013) and sediment N content increased with meadow age (time since restoration). Error bars are SE, n=3 (bare), 8 (1-5 years), 4 (5-9 years), 10 (>9 years).

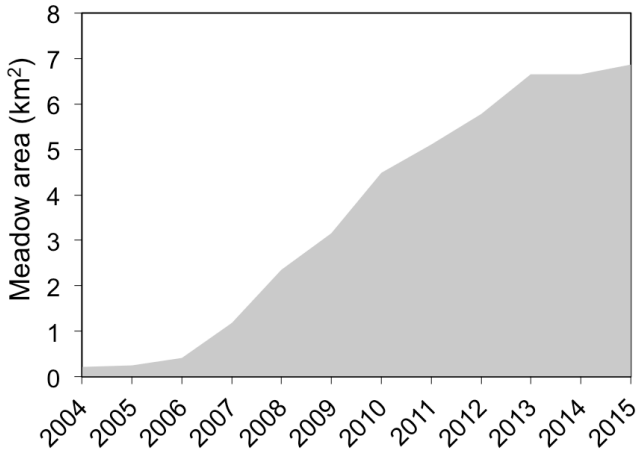

Figure 5.5 The restored meadow expanded rapidly through 2015, leading to spatial variability in the age (time since restoration) of the meadow.

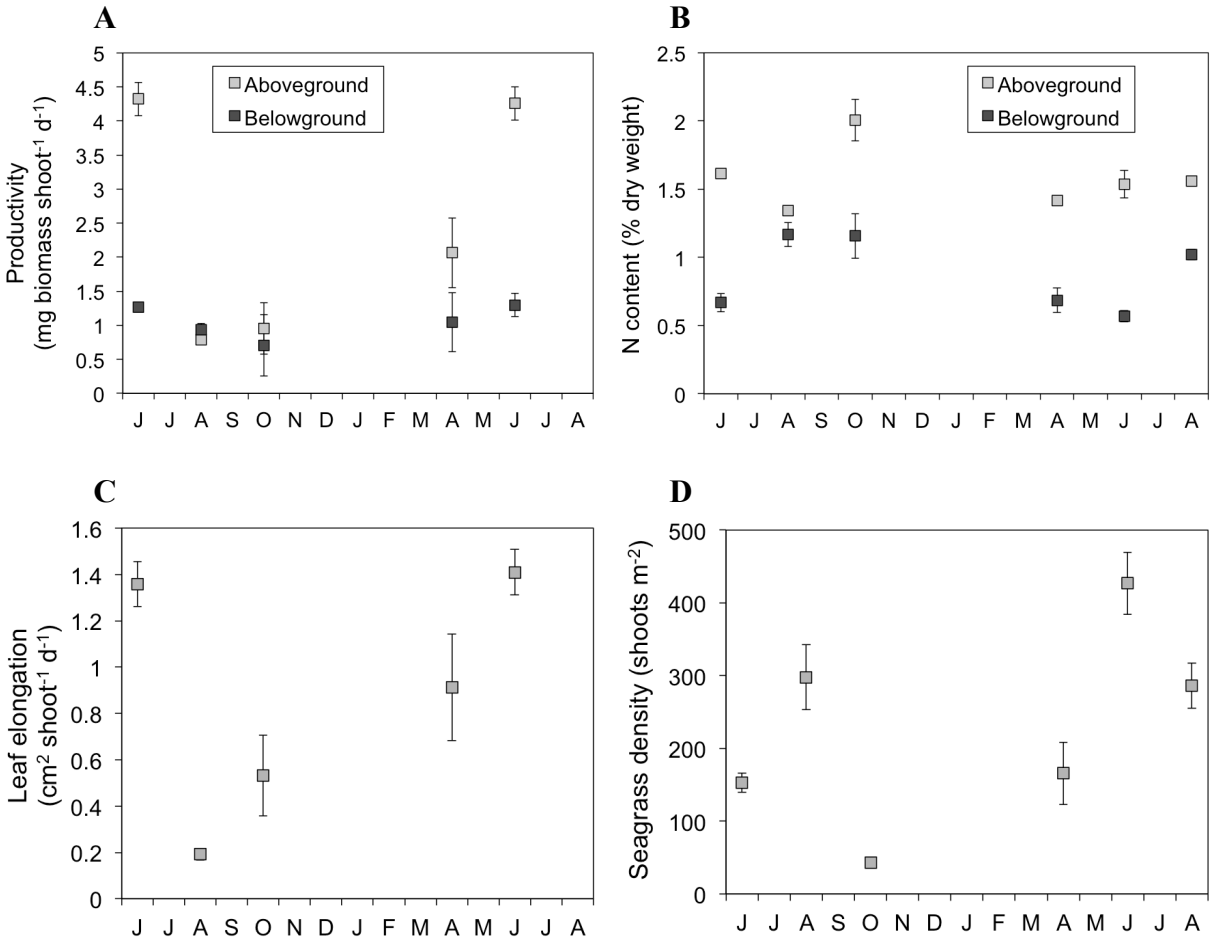

Figure 5.6 Shoot-specific productivity (A), seagrass tissue N content (B), shoot-specific elongation (C), and seagrass shoot density (D) were measured seasonally from June 2016-August 2017. Error bars are standard error, n=3 for Oct, April, August, n=6 for June measurements.

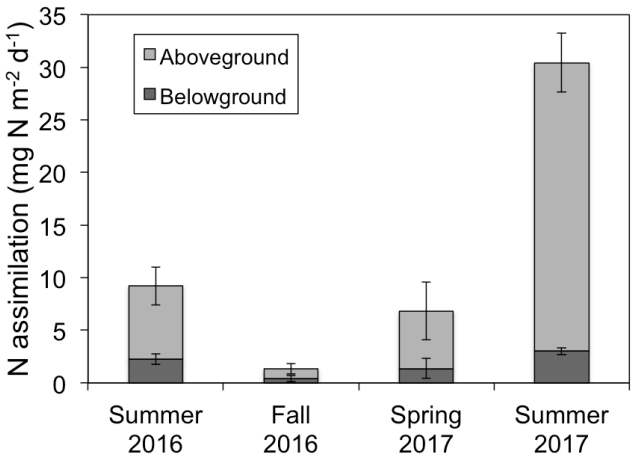

Figure 5.7 Areal N assimilation rates were calculated from the shoot-specific productivity, N content, and shoot density (shown in Figure 5.5). Error bars are standard error, n=6 in summer, n=3 in fall and spring.

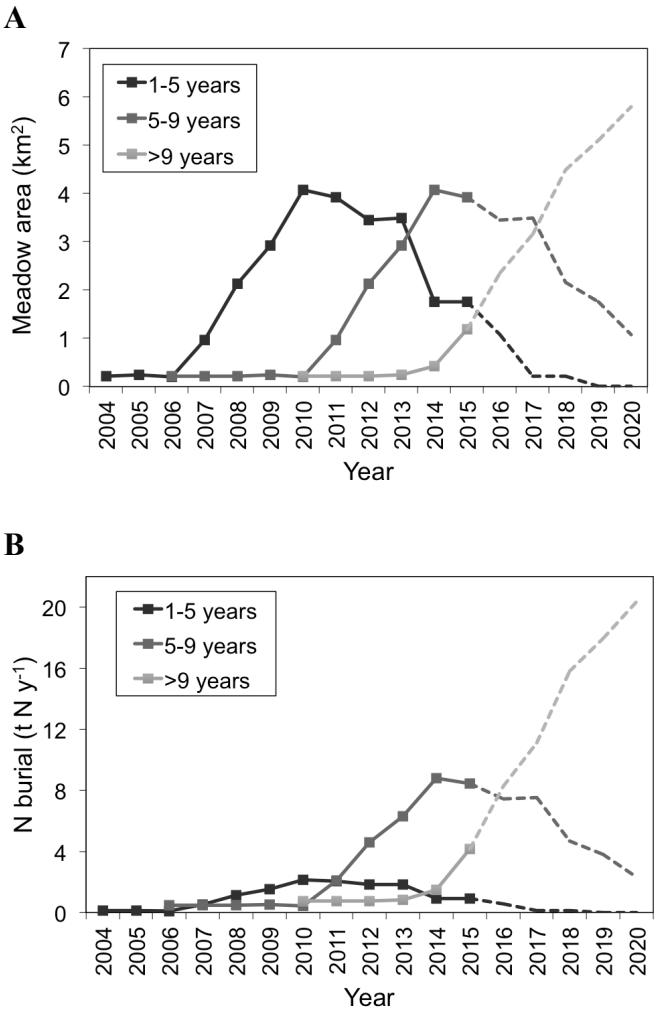

Figure 5.8 In 2015, when the meadow was on average 6 years old, meadow-wide burial was around 13 t N. In 2020, assuming no expansion of the meadow beyond the 2015 area, the meadow will be on average 11 years old and total burial will be around 23 t N.

Tables

Table 5.1 N Loading values were adopted from Anderson et al. 2010, and loads were calculated based on catchment and lagoon areas; loading rates are shown as mean (95% CI min-max).

	N Source	N loading (kg N ha^{-1} y^{-1})	Area (ha)	N load (kg N y^{-1})
Catchment			6037	47000
	Base Flow	4.59 (0.86-8.76)		
	Surface water runoff	1.84 (0.34-3.50)		
	Groundwater discharge	1.51 (0.28-2.89)		
Lagoon			16946	161000
	Atmospheric deposition	9.49 (7.97-11.01)		
Total				208000

Table 5.2 Burial rates were calculated from the accretion rate and N content for different ages of restored meadow; values are mean (SE, n=3 (bare), 8 (1-5 years), 4 (5-9 years), 10 (>9 years)).

	Bare sediment	1-5 years old	5-9 years old	>9 years old
Burial ($g\ m^{-2}\ y^{-1}$)	0.17 (0.07)	0.53 (0.14)	2.16 (0.52)	3.52 (0.55)
Proportion of meadow area in 2015	--	26%	57%	17%

Table 5.3 Comparison of annual N inputs, N retention, and N removal in the meadow and bare sediments*

	N flux	Mean rate ($g\ N\ m^{-2}\ y^{-1}$)	95% confidence limit
Seagrass			
	Loading	1.23	0.39
	Fixation	0.93	0.17
	Denitrification	-0.62	0.21
	Burial	-1.95	0.64
	Assimilation	-2.62	0.78
Bare			
	Loading	1.17	0.39
	Fixation	0.74	0.16
	Denitrification	-0.16	0.05
	Burial	-0.17	0.14

*Positive rates indicate N inputs, negative rates indicate N removal and retention.

Chapter 6: Nitrogen removal in restored seagrass meadows under future development scenarios

Target journal: Estuaries and Coasts

Abstract

Seagrass meadows remove nitrogen (N) from coastal waters through denitrification and burial, and these ecological processes support the important ecosystem service of water filtration. However, seagrass meadows are vulnerable to environmental stressors, including increases in anthropogenic nutrient loading, which may lead to seagrass declines and subsequent loss of the N removal ecosystem service. In this study, we compared N removal rates in a well-characterized restored seagrass meadow in the Virginia coastal bays to N loading rates under current conditions and future development scenarios. The future scenarios, including residential development (low to high impact), adoption of nitrogen-intensive crops (high impact), and increases in poultry farming (low to high impact), led to enhanced watershed loading up to 4x greater than the baseline scenario. Current N removal in the restored seagrass meadow was substantial only in comparison to the low-impact development scenarios; however, N removal at the bay scale is predicted to increase due to continued seagrass expansion. Maximum N removal could potentially account for 68% of the highest N loading scenario, suggesting that the seagrass meadow has the capacity to buffer future increases in anthropogenic N loading. Using a benefit transfer approach to economic valuation, we estimated the value of the current N removal service at $200,000 and the maximum projected N removal service at >$1 million. The majority of this value derived from N burial, which has generally been over-looked in valuations of N removal in seagrass meadows. Overall, the N removal service enhances the resilience of the coastal bay to

increased N loading, providing an added incentive to preserve and restore seagrass meadows in the face of increasing coastal development.

Introduction

Seagrass meadows are among the most productive ecosystems in the world and play an important ecological role as a filter for nutrients entering the coastal zone (Romero et al. 2006, McGlathery et al. 2007). By reducing and slowing the flow of nutrients, especially nitrogen (N), from terrestrial systems to the coastal ocean, seagrass meadows help maintain high water quality in coastal areas and avert negative effects associated with excess nitrogen loading, such as harmful algal blooms and hypoxia. The ecological process of N removal is thus directly linked to the ecosystem service of water filtration, which in turn enhances the value of various economic goods such as recreation and commercial fisheries production. Although it is difficult to fully capture the value of an ecosystem service like water filtration, the rate of the underlying biophysical process, in this case N removal, can be considered a direct stand-in for the magnitude of the ecosystem service (Cole & Moksnes 2016). Understanding N removal dynamics within a seagrass meadow is therefore an important step towards conceptualizing the value of the ecosystem.

N removal in seagrass meadows occurs via two main pathways: burial and denitrification. The seagrass canopy reduces water flow, leading to sedimentation of organic matter that is buried in the sediment on long time-scales (decadal or longer) (Mateo et al. 1997, Mcleod et al. 2011). Reduced flow also decreases resuspension of sediment and organic particles (Hansen & Reidenbach 2012). Seagrass root and rhizomes contribute to the sediment organic matter pool, and in some cases the sheltered flow environment may facilitate benthic microalgae production, which can also contribute to sediment organic matter (Oreska et al. 2017c). N burial in seagrass meadow sediment is thus a combination of physical and biological processes. Seagrass meadows may also be hotspots for denitrification, the microbial transformation of biologically available

NO_3^- into inert N_2, although denitrification rates are typically low compared to burial rates (McGlathery 2008). Temporary retention of nitrogen in seagrass biomass is an important short-term filtering mechanism; some portion of seagrass biomass will enter the sediment N pool, but large proportions will be either recycled in situ or exported (Duarte & Cebrian 1996). Burial and denitrification therefore represent the major pathways by which seagrass meadows filter and remove N from coastal waters over long time-scales.

Worldwide, seagrass meadows are declining in large part due to anthropogenic pressures including eutrophication and coastal development (Orth et al. 2006, Waycott et al. 2009). As seagrass meadows are lost, coastal ecosystems have a reduced ability to remove N and consequently to provide the ecosystem service of water filtration. Restoration of seagrass meadows has the potential to reinstate the N removal process and thus to return a valuable ecosystem service to coastal areas (Chapter 5). A positive feedback between seagrass growth and water quality may support this recovery; as degraded meadows recover, they increase rates of N removal, helping to speed their own recovery (Gurbisz & Kemp 2014). However, restored seagrass meadows remain vulnerable to anthropogenic impacts from coastal development. If anthropogenic N loading greatly exceeds rates of N removal within a seagrass meadow, seagrass are likely to experience negative effects, including algal shading, increased ammonium and sulfide concentrations in porewater, and increased sediment anoxia (Burkholder et al. 2007). If these conditions persist, seagrass loss is likely.

The goal of this study was to compare the N removal service within a restored seagrass meadow to expected N loads under future development scenarios. Our study site is a successful, landscape-scale (6.9 km^2) seagrass (*Zostera marina*) restoration project within the Virginia coastal bays. Detailed measurements of N cycling have shown that the meadow functions as a N

filter, with burial and denitrification rates that outweigh current rates of N loading from the rural, undeveloped watershed (Aoki et al. in prep, Chapter 5). However, increased residential development and changes to agricultural practices within the watershed are expected, potentially leading to much greater N loading rates in the future (Giordano et al. 2011). Here, we compared N loads from projected development scenarios to both current levels of N removal and projected N removal based on continued expansion of the seagrass meadow.

To put the N removal service under current and future N loading conditions in an economic context, we used market alternative approaches to estimate the monetary value of N removal. While the ecological processes that drive N removal in seagrass meadows have been the subject of numerous studies (for a review of N cycling in seagrass meadows, see McGlathery 2008; for a review of nitrate removal in aquatic ecosystems, see Burgin & Hamilton 2007), few studies have explicitly linked seagrass ecological processes to economic value. Ecosystem service valuations in seagrass meadows have focused mainly on habitat provisioning for fisheries (e.g. Jackson et al. 2001, Blandon & zu Ermgassen 2014, Bertelli & Unsworth 2014, Tuya et al. 2014), and carbon sequestration (Russell & Greening 2015, Campagne et al. 2015, Cole & Moksnes 2016, Reynolds et al. 2016). Of the handful of studies that have applied an economic valuation to N removal in seagrass meadows, most have not used site-specific measurements and/or have accounted for only one component of N removal (burial only: Cole & Moksnes, 2016; denitrification only: Russell & Greening 2015, Piehler & Smyth 2011, Zarnoch et al. 2017). To our knowledge, this study is the first to combine site-specific measurements of both burial and denitrification in order to estimate the value of total N removal within a seagrass meadow.

Methods

Study site

Our study site was a restored *Z. marina* meadow located in South Bay, VA, one of four coastal lagoons that was seeded as part of a landscape-scale restoration project (Orth et al. 2012, Orth & McGlathery 2012). Seeding in South Bay was conducted in 2001; seeds were broadcast in 0.4 ha plots that eventually coalesced into a contiguous meadow that was 6.9 km^2 in 2015 and has continued to expand. Long-term monitoring of the restored meadow has shown that the meadow has achieved a mature "steady-state" with consistent shoot density counts in summer (McGlathery 2017a). Carbon burial rates within the meadow are within the range of natural meadows (Greiner et al. 2013, Oreska et al. 2017a). The water quality in South Bay is oligotrophic; surface water ammonium concentrations are regularly <1 μM and nitrate concentrations are undetectable (McGlathery & Christian 2017).

The South Bay meadow is an ideal site for comparing N removal with predicted N loads. The species of interest, *Z. marina*, is a useful test case as it is widely distributed throughout the temperate zone (Moore & Short 2006). This restored meadow has been monitored since the initial seeding, creating a long-term record of meadow expansion. The N burial rates incorporated into the baseline N removal therefore account for the large differences in burial rates between recently colonized and mature meadow sediments (Chapter 5). The oligotrophic conditions in South Bay are among the lowest measured N loading rates to coastal bays in the world (McGlathery et al. 2007), but it is likely that development and changes to agricultural practice within the watershed will lead to higher N loading in the future, as has occurred in the nearby Maryland coastal bays (Glibert et al. 2014). Comparing N loading and N removal in this system can therefore produce insight into other shallow bay systems currently experiencing anthropogenic N loading. As population growth in the coastal zone continues worldwide (Small

& Nicholls 2003), the dynamics evident in the South Bay meadow will be useful to managers of both restored and natural seagrass meadows in other regions.

Baseline scenario

Current N removal and N loading rates evaluated by Aoki et al. (Chapter 5) were used for the baseline scenario (Table 6.1). N removal included denitrification and burial in sediments (long-term removal of reactive N from the bay) and excluded temporary storage of nitrogen in seagrass biomass. Annual denitrification, based on seasonal measurements using the isotope pairing technique, was 0.62 g N m^{-2} y^{-1}. Burial, based on ^{210}Pb dating of sediment cores and sediment N content, ranged from 0.53 g N m^{-2} y^{-1} in newly colonized sediments to 3.52 g N m^{-2} y^{-1} in mature meadow sediments (>9 years old); in 2015 the meadow-wide average burial rate was 1.95 g N m^{-2} y^{-1}. N loading was 1.23 g N m^{-2} y^{-1} and included atmospheric deposition, terrestrial loading from base flow, surface water runoff, and groundwater discharge. Loading rates were scaled over the area of the seagrass meadow in 2015, based on digitized aerial photography from the Virginia Institute of Marine Science SAV survey (http://web.vims.edu/bio/sav/), in order to calculate the total N load to the coastal bay. Burial was the dominant sink of N (76% of total N removal) and atmospheric deposition was the dominant source of N (77% of total N load).

Future development scenarios

Six potential future development scenarios were identified based on modeling and monitoring work done by Giordano et al. (2011). These scenarios included increases to residential development, increases to poultry farming within the lagoon watershed, and increases in tomato planting. Giordano et al. (2011) modeled the changes to the watershed nitrogen load under these development scenarios using a watershed nutrient loading model (Valiela et al. 1997)

for two of Virginia's coastal bays, Gargathy Bay and Burton's Bay. We used these results to calculate a multiplier for each development scenario, and we applied the multiplier to the baseline N load for South Bay in order to estimate the increase in N loading from each scenario (Table 6.2). These scenarios included relatively extreme scenarios (e.g. conversion of all cropland within the watershed to tomato plasticulture, a nitrogen-intensive crop). The highest resulting N loads are therefore indicative of maximum N loads to the coastal bay due to changes in the watershed. Atmospheric deposition to the bay surface area, the largest source of N in the baseline scenario, was considered constant within each scenario.

Future seagrass expansion

The restored seagrass meadow in South Bay has continued to expand, and recent modeling work by Oreska et al. (2017b) indicates that substantial additional area in the Virginia coastal bays may be suitable to support seagrass. We therefore anticipated future expansion of the restored seagrass meadows, which may occur in tandem with increased N loading from development and changes to agriculture. We calculated an additional future N removal based on projections of seagrass expansion in the South Bay area and current N removal rates. We compared the N loading rates for the future development scenarios to the current level of N loading and the future level of N loading given maximum seagrass expansion.

Valuation of N removal service

Finally, to estimate the economic value of the N removal service in the restored seagrass meadow, we used a multi-metric approach by analyzing replacement costs and damage costs avoided associated with the N removal ecosystem service. Replacement costs estimate the value of an ecosystem service based on the cost associated with using a man-made intervention to provide that magnitude of service. Damage costs avoided estimate the value of an ecosystem

service based on the cost of damages that would accrue in the absence of the service (Hussain & Gundimeda 2012). For example, coastal ecosystems including seagrass meadows provide shoreline protection; increased erosion would result from the loss of those ecosystems, and the costs of that erosion would be considered the damage costs. By using multiple metrics, i.e. replacement costs and damage costs avoided, to evaluate the N removal service, we provide multi-faceted quantitative information regarding the significance of the N removal service in the restored seagrass meadow (Birch et al. 2011).

For the replacement costs analysis, we applied abatement costs associated with atmospheric nitrogen deposition. In nearly all development scenarios, atmospheric loading directly to the coastal bay was the dominant source of nitrogen. We therefore used estimates of the costs of reducing atmospheric N inputs as an appropriate replacement cost for the value of the N removal performed by the seagrass meadows. Birch et al. (2011) estimated abatement costs of atmospheric N loading for nearby Chesapeake Bay to be \$14 kg^{-1} N for mobile sources of N, primarily NO_x. Costs were higher for industrial sources of NO_x and lower for electric utilities; however, these sources are limited on Virginia's Eastern Shore, so we focused on the abatement cost for mobile sources of N. For the damage costs avoided analysis, we used estimates from Sobota et al. (2015) of the marginal costs associated with coastal N loading; damages costs from loss of recreational use were \$6.38 kg^{-1} N and damage costs from declines in habitat quality were \$15.84 kg^{-1} N. These damage costs were additive, so the total damage costs avoided used in the analysis was \$22.22 kg^{-1} N.

Using these abatement costs and damage costs avoided, we calculated the value of N removal within the current extent of the seagrass restoration and the potential maximum extent. We assumed linear relationships between both replacement costs and damages costs avoided and

N removal rates. However, the true relationships may be non-linear and subject to threshold responses, especially with regard to damage costs avoided (Sobota et al. 2015, see Discussion below). We then compared these estimates of value to the costs of the restoration to date, estimated by Reynolds et al. (2016).

Results

The N load multipliers for each development scenario, and the resulting N loads, are shown in Table 6.2. The minimal increase between the baseline scenario and the moderate poultry scenario resulted from an assumption in the model (following practices on the Eastern Shore) that poultry waste would replace other sources of synthetic nitrogen fertilizer within the watershed until there was an excess of poultry waste supply compared to agricultural demand for nitrogen fertilizer (Giordano et al. 2011). Thus, although the addition of 5 million new poultry birds to the watershed would be a substantial increase over current levels, the effects on N loading would be relatively minimal. However, increases beyond 5 million birds would have a more pronounced effect on N loading, as poultry waste supply would outstrip demand for N fertilizer. Of all the scenarios, the tomato plasticulture scenario would have the greatest effect on the N load to the lagoon; the increased N load of 188 t N y^{-1} would exceed the current level of atmospheric loading (151 t N y^{-1}). In all the other scenarios, atmospheric loading would remain the dominant source for N to the lagoon.

Under current conditions, the seagrass meadow in South Bay removes approximately 17 t N y^{-1} through burial and denitrification (Aoki et al. in prep, Chapter 5). This removal would account for more than 100% of the enhanced N load resulting from the moderate poultry scenario and over 56% of low residential development scenario N load, but would account for only 12-41% of the N loads derived from the more intensive development scenarios (Figure 6.1).

Extrapolating the current N removal rate in the seagrass meadow, over 58 km^2 of mature restored seagrass meadow would be needed to completely balance out the increased N loads from the most intensive scenario (tomato plasticulture). Continued expansion of the seagrass restoration is likely; since 2013, a secondary meadow in South Bay has rapidly expanded, covering an area of 4.7 km^2 in 2015 (about 2/3 the size of the original restored meadow). However, the total extent of seagrass restoration will be limited based on the habitable area within the bays, as well as competing interests such as clam aquaculture. An estimated 151 km^2 of habitable area is available for seagrass colonization within the Virginia coastal bays (Oreska et al. 2017b), of which an estimated 38 km^2 occurs within the South Bay drainage basin. Extrapolating the current N removal rate in the restored meadow over 38 km^2 results in total N removal of 98 t N y^{-1}. This upper limit of N removal would potentially account for 68% of the tomato plasticulture N load. However, the development scenarios are also likely to occur concurrently, potentially leading to increased N loads beyond those shown in this analysis.

Valuations of current and projected N removal within the seagrass meadow are shown in Table 6.3. Replacement costs to achieve the current level of N removal in the restored meadow were approximately \$250,000 y^{-1}. Damage costs avoided associated with recreational use and habitat protection were higher (\$390,000 y^{-1}). Both the replacement costs and damage costs avoided increased substantially with increasing meadow area. The cost of the restoration project was estimated at \$2 million dollars, including funds and personnel time (Reynolds et al. 2016). Both replacement costs and damage costs avoided from seagrass N removal in 2015 were approximately 10% of the total project costs and the projected maximum N removal would exceed 50% of costs.

The predicted future valuations assume a consistent rate of N removal within the current meadow and any expansion. However, predicting and valuing future N removal is complicated by the fact that N removal rates will vary non-linearly with time. Recent analysis of N burial, the major component of N removal in the meadow, showed that burial rates were as much as 7x greater in mature (>9 years old) areas of the meadow compared to recently colonized sediments (Aoki et al. in prep, Chapter 5). Therefore, the value of N burial in the restored meadow does not track linearly with meadow expansion; instead, rates of N burial and value increase slowly with colonization and more rapidly as the meadow matures (Figure 6.2). This non-linear pattern is important to consider in estimates of the benefits derived from seagrass restoration. N removal via denitrification likely also increases as the meadow matures. We lack sufficient data to estimate denitrification as a function of meadow age; however, we note that burial was the major component of N removal in this system (Table 6.1) and changes in denitrification likely had a small impact on total N removal over time.

Discussion

Comparing current N removal rates and N loading in future scenarios, there is a clear potential for the seagrass meadow to offset increased N loads. Especially for the low-impact development scenarios (moderate poultry, low and moderate development), the N load increases are relatively modest and can be offset even by current N removal. As we expect the total N removal to increase as the seagrass meadow expands and matures, we will likely see a continued offset effect, which will help maintain the high water quality of the coastal bays. At the maximum extent of seagrass expansion within the lagoon (38 km^2), N removal at current rates would offset approximately 68% of the most extreme N loading scenario (tomato plasticulture).

Additionally, we may see an increase in the maximum areal removal rate if increased availability of nitrate stimulates denitrification in meadow sediments. Recent work using the isotope pairing method has shown that denitrification in these seagrass sediments can be as much as an order of magnitude higher with high nitrate availability (Aoki and McGlathery in review, Chapter 3). On an annual scale, this increase would make denitrification the dominant N removal term and could lead to the removal of 7.3 g N m^{-2} y^{-1} via denitrification compared to only 0.62 g N m^{-2} y^{-1} under current ambient conditions. If these potential denitrification rates were realized, the current meadow extent (6.9 km^2) would remove over 64 t N y^{-1}, substantially more than the current 17 t N y^{-1}. Those maximum potential rates would allow the current meadow area to offset increased N loading from the more intensive scenarios, and would offset nearly half of the N load enhancement in the most extreme scenario. Overall, the current N removal rates are significant compared to the low-impact development scenarios, and increased N removal from either seagrass expansion, enhanced denitrification, or both would lead to even greater removal rates.

At the same time, the effect of high levels of N loading on the N removal rate is unclear. While extensive seagrass expansion and/or enhanced denitrification may be able to offset higher inputs of N, at some point excess N loading will have deleterious effects on seagrass. *Z. marina* are known to be sensitive to high nutrient levels, and as nutrient loading increases, increased shading from phytoplankton and algae, as well as increased sediment anoxia will lead to seagrass declines. Eventually, much higher rates of N loading would likely lead to a shift from seagrass dominated state to a micro or macro-algae dominated state and consequently reduced N removal (McGlathery et al. 2007). Long-term monitoring of the meadow has shown that seagrass decline from other disturbances (e.g. marine heat waves) can lead to reduced burial of N and indeed loss

of N stored in sediment (McGlathery 2017b). Therefore, although we expect N removal in seagrass meadows to remain important under future N loading scenarios, there is likely a tipping point at which excess N loading will lead to seagrass decline and loss of the N removal service (Figure 6.3). Detecting that tipping point is a challenge; anthropogenic N loading is a known factor in seagrass meadow declines worldwide, but there are no established thresholds for TDN or DIN levels at which we would expect seagrass decline to occur (Burkholder et al. 2007). Site specific factors such as residence time are important in determining the response of a given seagrass system to increased nutrient loads (Tomasko et al. 1996).

Using both replacement costs and damage costs avoided, the current value of the N removal service provided by the seagrass meadow is over $200,000 and is expected to increase with N removal. However, this predicted increase assumes a linear relationship between the N removal rate and the indirect source of value, either replacement costs or damage costs avoided. With regards to damage costs avoided, the true relationship is likely non-linear. As noted above, increased levels of N loading in the future have the potential to lead to a tipping point that results in loss of the seagrass meadows. In that case, the damage costs may be considerably greater than the estimates used here; seagrass loss in the Gulf of Mexico due to eutrophication resulted in damages to fisheries of $56 kg^{-1} N (Compton et al. 2011), about double the $22.22 kg^{-1} N used in this analysis. More detailed data regarding the site-specific response of the meadow to increases in N loading would be needed to capture this threshold response. Qualitatively, by providing a buffer against the more damaging impacts of high N loading, the seagrass N removal may protect against greater damages than are included in this analysis.

Non-linear relationships between replacement costs and N removal rates are less concerning in this analysis. In general, the marginal costs of pollution abatement increase with

decreasing levels of pollution; however, in this case, the range of N removal rates, from 17 t N y^{-1} to 98 t N y^{-1} is almost certainly too small to produce this effect. However, the appropriate replacement cost in this system may change with increases in N loading. Currently, the abatement costs associated with reducing N loading from atmospheric deposition are the most appropriate choice to use as a proxy for value since atmospheric loading was the largest source of N to the coastal bays. However, in the future, if development scenarios lead to increasing terrestrial N loading, a different abatement cost might be more appropriate. In the neighboring Chesapeake Bay watershed, terrestrial and freshwater sources of nitrogen have varying abatement costs compared to atmospheric sources (Birch et al. 2011), so the value of future N removal based on replacement costs is difficult to predict.

In addition, high levels of N loading in the future might lead to regulatory programs that provide economic incentives to reduce N loading from various sources, which again could lead to alternative valuation. For example, Virginia's existing nutrient credit trading program for Chesapeake Bay might be modified for the coastal bays if widespread negative effects of N loading occur, in which case the value of a nitrogen credit might be a more appropriate proxy for value. Absent these environmental harms however, a regulatory framework is unlikely. Thus, while the valuations used here provide useful metrics for understanding the current N removal service, predictions of future value are decidedly uncertain.

Recent studies that included valuation of the N removal service in seagrass meadows have focused mainly on denitrification (e.g. Piehler & Smyth 2011, Russell & Greening 2015). These studies have found higher values for N removal via denitrification per unit area of seagrass meadow than the estimates presented here, mainly due to higher measurements of denitrification rates (Table 6.4). The denitrification measurements used in this study are at the low end of recent

measurements in the literature, likely due to the extremely low nutrient availability in the coastal bays (see Chapter 2 for more discussion). The variability in rates between different seagrass meadows indicates that N removal via denitrification is likely site-specific. The specific value of this ecosystem service may therefore not be easily transferable between sites, as is the case with other aquatic N removal services, such as from shellfish aquaculture (Rose et al. 2015). However, this study indicates that burial is an overlooked component of the N removal service in seagrass meadows. One valuation study by Cole and Mosknes (2016) did include N burial based on data from the same system examined here; other estimates of N burial in seagrass meadows focus mainly on peat-forming species and do not include valuation (e.g. Mateo & Romero 1997, Cebrian & Duarte 2001, Gacia et al. 2002). Our results indicate that burial can be the dominant process of N removal and therefore may provide additional value that is not accounted for in studies that only consider denitrification. Burial is also likely to vary between sites, and future studies evaluating the N removal service in seagrass meadows should include site-specific measurements of both burial and denitrification.

The intent of this valuation project was to conceptualize the N removal service provided by the meadow. The replacement costs approach allowed us to estimate the value of the service, but that value remains abstract; there is no straightforward choice between spending $200,000 on reducing atmospheric N pollution from mobile sources and planting a seagrass meadow that can provide a comparable service. Rather, by choosing to invest in the seagrass meadow restoration, we are investing in the health and resilience of the coastal bays. Because of the N removal provided by the seagrass meadow, these ecosystems may be better able to withstand pressure from anthropogenic N loading in the future and to continue to provide many other valuable services (carbon sequestration, biodiversity, fish habitat, recreation, aesthetic value). By

conceptualizing the N removal value in economic terms, we can describe this service within existing frameworks and provide a motivation to pursue similar restoration projects in the future. The trajectories for development on the Eastern Shore remain uncertain, as does the response of the seagrass meadow to increased N loading. However, this analysis gives us context to understand how possible scenarios will affect the current, successful restoration. The findings from this study show that restoration of seagrass meadows provides a valuable ecosystem service that increases the resilience of the ecosystem to perturbations from anthropogenic N loading.

References

Bertelli CM, Unsworth RKF (2014) Protecting the hand that feeds us: Seagrass (*Zostera marina*) serves as commercial juvenile fish habitat. Mar Pollut Bull 83:425–429

Birch MBL, Gramig BM, Moomaw WR, Doering, III OC, Reeling CJ (2011) Why metrics matter: Evaluating policy choices for reactive nitrogen in the Chesapeake Bay watershed. Environ Sci Technol 45:168–174

Blandon A, Ermgassen PSE zu (2014) Quantitative estimate of commercial fish enhancement by seagrass habitat in southern Australia. Estuar Coast Shelf Sci 141:1–8

Burgin A, Hamilton S (2007) Have we overemphasized the role of denitrification in aquatic ecosystems? A review of nitrate removal pathways. Front Ecol Environ 5:89–96

Burkholder JM, Tomasko DA, Touchette BW (2007) Seagrasses and eutrophication. J Exp Mar Biol Ecol 350:46–72

Campagne CS, Salles J-M, Boissery P, Deter J (2015) The seagrass *Posidonia oceanica*: Ecosystem services identification and economic evaluation of goods and benefits. Mar Pollut Bull 97:391–400

Cebrian J, Duarte CM (2001) Detrital stocks and dynamics of the seagrass *Posidonia oceanica* (L.) Delile in the Spanish Mediterranean. Aquat Bot 70:295–309

Cole SG, Moksnes P-O (2016) Valuing multiple eelgrass ecosystem services in Sweden: Fish production and uptake of carbon and nitrogen. Front Mar Sci 2

Compton JE, Harrison JA, Dennis RL, Greaver TL, Hill BH, Jordan SJ, Walker H, Campbell HV (2011) Ecosystem services altered by human changes in the nitrogen cycle: a new perspective for US decision making. Ecol Lett 14:804–815

Duarte CM, Cebrian J (1996) The fate of marine autotrophic production. Limnol Oceanogr 41:1758–1766

Eyre BD, Ferguson AJ (2002) Comparison of carbon production and decomposition, benthic nutrient fluxes and denitrification in seagrass, phytoplankton, benthic microalgae-and macroalgae-dominated warm-temperate Australian lagoons. Mar Ecol Prog Ser 229:43–59

Gacia E, Duarte CM, Middelburg JJ (2002) Carbon and nutrient deposition in a Mediterranean seagrass (*Posidonia oceanica*) meadow. Limnol Oceanogr 47:23–32

Giordano JCP, Brush MJ, Anderson IC (2011) Quantifying annual nitrogen loads to Virginia's coastal lagoons: Sources and water quality response. Estuaries Coasts 34:297–309

Glibert PM, Hinkle DC, Sturgis B, Jesien RV (2014) Eutrophication of a Maryland/Virginia coastal lagoon: A tipping point, ecosystem changes, and potential causes. Estuaries Coasts 37:128–146

Greiner JT, McGlathery KJ, Gunnell J, McKee BA (2013) Seagrass restoration enhances "blue carbon" sequestration in coastal waters (J Cebrian, Ed.). PLoS ONE 8:e72469

Gurbisz C, Kemp WM (2014) Unexpected resurgence of a large submersed plant bed in Chesapeake Bay: Analysis of time series data. Limnol Oceanogr 59:482–494

Hansen J, Reidenbach M (2012) Wave and tidally driven flows in eelgrass beds and their effect on sediment suspension. Mar Ecol Prog Ser 448:271–287

Hussain S, Gundimeda H (2012) Tools for valuation and appraisal of ecosystem services in policy making. In: The Economics of Ecosystems and Biodiversity in Local and Regional Policy and Management. Earthscan, Washington, D.C., p 57–98

Jackson EL, Rowden AA, Attrill MJ, Bossey SJ, Jones MB (2001) The importance of seagrass beds as a habitat for fishery species. Oceanogr Mar Biol 39:269–304

Mateo MA, Romero J (1997) Detritus dynamics in the seagrass *Posidonia oceanica*: Elements for an ecosystem carbon and nutrient budget. Mar Ecol Prog Ser:43–53

Mateo MA, Romero J, Pérez M, Littler MM, Littler DS (1997) Dynamics of millenary organic deposits resulting from the growth of the Mediterranean seagrass *Posidonia oceanica*. Estuar Coast Shelf Sci 44:103–110

McGlathery KJ (2008) Seagrass habitats. In: Nitrogen in the marine environment. Elsevier, New York, p 1037–1072

McGlathery K (2017a) Density of seagrass in Hog Island Bay and South Bay, VA 2007-2017. Virginia Coast Reserve Long-Term Ecological Research Project Data Publication doi:10.6073/pasta/5a6ea442cf59cabb3112bb634a968ae5

McGlathery K (2017b) Sediment carbon and nitrogen of seagrass restoration in Hog Island Bay and South Bay, VA 2007-2017. Virginia Coast Reserve Long-Term Ecological Research Project Data Publication doi:10.6073/pasta/bd02ce327caa3f24992b4388b2eeb8f9

McGlathery KJ, Christian R (2017) Water quality sampling - integrated measurements for the Virginia Coast, 1992-2017. Virginia Coast Reserve Long-Term Ecological Research Project Data Publication doi:10.6073/pasta/8783dd64be7cae3a36a864ac5b5129e1

McGlathery K, Sundbäck K, Anderson I (2007) Eutrophication in shallow coastal bays and lagoons: The role of plants in the coastal filter. Mar Ecol Prog Ser 348:1–18

Mcleod E, Chmura GL, Bouillon S, Salm R, Björk M, Duarte CM, Lovelock CE, Schlesinger WH, Silliman BR (2011) A blueprint for blue carbon: Toward an improved understanding of the role of vegetated coastal habitats in sequestering CO_2. Front Ecol Environ 9:552–560

Moore KA, Short FT (2006) *Zostera*: Biology, Ecology, and Management. In: Seagrasses: Biology, Ecology, and Conservation. Springer, Dordrecht, The Netherlands, p 361–386

Oreska MPJ, McGlathery KJ, Porter JH (2017a) Seagrass blue carbon spatial patterns at the meadow-scale (J Cebrian, Ed.). PLOS ONE 12:e0176630

Oreska MPJ, Truitt B, Orth RJ, Luckenbach MW (2017b) The bay scallop (Argopecten irradians) industry collapse in Virginia and its implications for the successful management of scallop-seagrass habitats. Mar Policy 75:116–124

Oreska MPJ, Wilkinson GM, McGlathery KJ, Bost M, McKee BA (2017c) Non-seagrass carbon contributions to seagrass sediment blue carbon. Limnol Oceanogr:S3–S18

Orth RJ, Carruthers TJB, Dennison WC, Duarte CM, Fourqurean JW, Heck KL, Hughes AR, Kendrick GA, Kenworthy WJ, Olyarnik S, Short FT, Waycott M, Williams SL (2006) A global crisis for seagrass ecosystems. BioScience 56:987–996

Orth R, McGlathery K (2012) Eelgrass recovery in the coastal bays of the Virginia Coast Reserve, USA. Mar Ecol Prog Ser 448:173–176

Orth R, Moore K, Marion S, Wilcox D, Parrish D (2012) Seed addition facilitates eelgrass recovery in a coastal bay system. Mar Ecol Prog Ser 448:177–195

Piehler MF, Smyth AR (2011) Habitat-specific distinctions in estuarine denitrification affect both ecosystem function and services. Ecosphere 2:art12

Reynolds LK, Waycott M, McGlathery KJ, Orth RJ (2016) Ecosystem services returned through seagrass restoration. Restor Ecol 24:583–588

Romero J, Lee K, Pérez M, Mateo MA, Alcoverro T (2006) Nutrient dynamics in seagrass ecosystems. In: Seagrasses: Biology, ecology, and conservation. Springer, The Netherlands, p 227–254

Rose JM, Bricker SB, Ferreira JG (2015) Comparative analysis of modeled nitrogen removal by shellfish farms. Mar Pollut Bull 91:185–190

Russell M, Greening H (2015) Estimating benefits in a recovering estuary: Tampa Bay, Florida. Estuaries Coasts 38:9–18

Small C, Nicholls RJ (2003) A global analysis of human settlement in coastal zones. J Coast Res 19:584–599

Sobota DJ, Compton JE, McCrackin ML, Singh S (2015) Cost of reactive nitrogen release from human activities to the environment in the United States. Environ Res Lett 10:025006

Tomasko DA, Dawes CJ, Hall MO (1996) The effects of anthropogenic nutrient enrichment on turtle grass (*Thalassia testudinum*) in Sarasota Bay, Florida. Estuaries 19:448–456

Tuya F, Haroun R, Espino F (2014) Economic assessment of ecosystem services: Monetary value of seagrass meadows for coastal fisheries. Ocean Coast Manag 96:181–187

Valiela I, Collins G, Kremer J, Lajtha K, Geist M, Seely B, Brawley J, Sham CH (1997) Nitrogen loading from coastal watersheds to receiving estuaries: new method and application. Ecol Appl 7:358–380

Waycott M, Duarte CM, Carruthers TJ, Orth RJ, Dennison WC, Olyarnik S, Calladine A, Fourqurean JW, Heck KL, Hughes AR, others (2009) Accelerating loss of seagrasses across the globe threatens coastal ecosystems. Proc Natl Acad Sci 106:12377–12381

Welsh D, Catadelli G, Bartoli M, Poli D, Careri M, Wit R de, Viaroli P (2001) Denitrification in an intertidal seagrass meadow, a comparison of ^{15}N-isotope and acetylene-block techniques: Dissimilatory nitrate reduction to ammonia as a source of N_2O? Mar Biol 139:1029–1036

Zarnoch CB, Hoellein TJ, Furman BT, Peterson BJ (2017) Eelgrass meadows, *Zostera marina* (L.), facilitate the ecosystem service of nitrogen removal during simulated nutrient pulses in Shinnecock Bay, New York, USA. Mar Pollut Bull 124:376–387

Figures

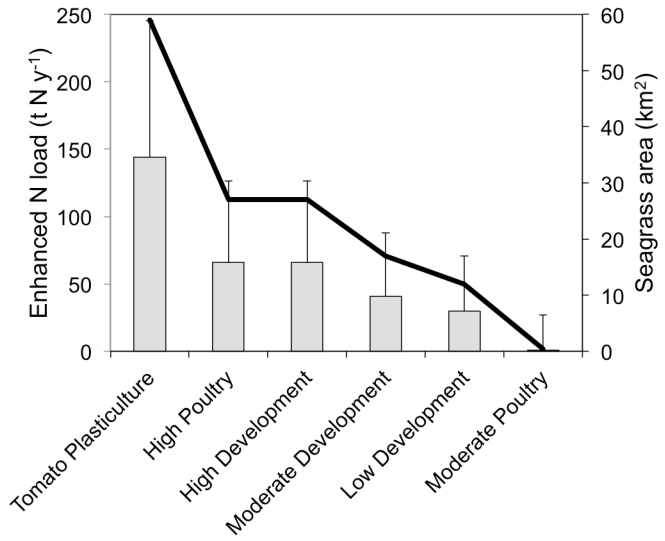

Figure 6.1 Enhanced N loads above the baseline for each development scenario are shown by the gray bars (error bars are 95% CI). The solid black line shows the area of seagrass meadow that would be needed to offset the increase in N load with N removal.

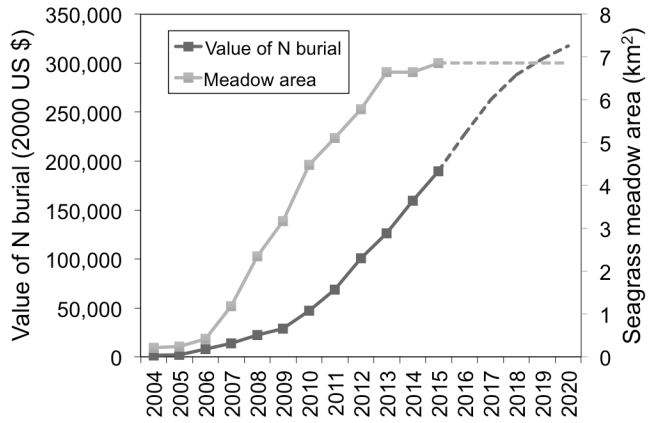

Figure 6.2 N burial rates increase non-linearly with meadow age, leading to non-linear increases in the value of N removal via burial as the meadow expands and matures (value estimated from replacements costs). The meadow area is shown in light gray; annual value of N burial is shown in dark gray. The dark dashed line shows the projected value of N burial within the 2015 meadow area as the meadow matures.

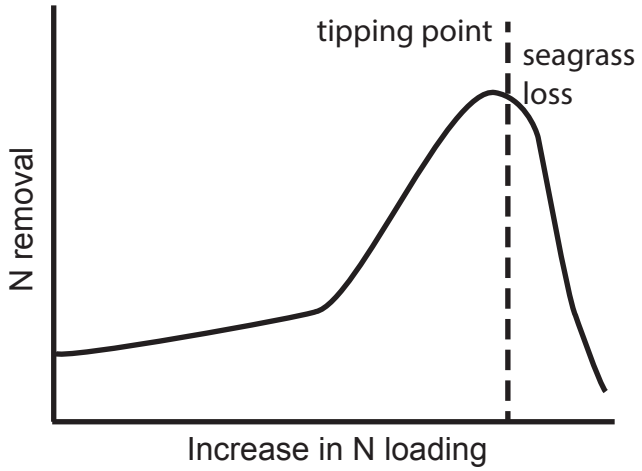

Figure 6.3 Conceptual diagram showing how N removal in a seagrass meadow will increase with N loading until some tipping point, at which point seagrass loss is expected and N removal will rapidly decline as the meadow transitions to an algae-dominated state (after McGlathery et al. 2007).

Tables

Table 6.1 N fluxes in the restored seagrass meadow (from Aoki et al., Chapter 5)

N flux	Annual rate ($g\ N\ m^{-2}\ y^{-1}$)	95% CL
Loading	1.23	0.39
Burial	-1.95	0.64
Denitrification	-0.62	0.21

Table 6.2 Summaries of future development scenarios modeled by Giordano et al. 2011 and resulting multipliers for watershed N loads.

Scenario	Summary	N load multiplier	Catchment N load (t N y^{-1})
Baseline	Current N loading	1.00	47
Moderate Poultry	5 million additional birds raised within watershed	1.02	48
High Poultry	20 million additional birds raised within watershed	2.38	112
Tomato Plasticulture	Convert all cropland within watershed to tomatoes	4.00	188
Low Development	Convert all cropland within watershed to residential development	1.62	76
Moderate Development	Convert half of all cropland and half of natural vegetation in watershed to residential development	1.85	87
High Development	Convert all natural vegetation to residential development	2.38	112

Table 6.3 Current and projected values of N removal in the restored seagrass meadow

	Seagrass meadow area (km^2)	N removal ($t\ N\ y^{-1}$)	Damage costs avoided value ($\$\ y^{-1}$)	Replacement costs value ($\$\ y^{-1}$)
Current (2015)	6.9	17.6	390,000	250,000
Projected maximum	38	97.7	2,200,000	1,400,000

Table 6.4 Comparison of valuation studies of N removal in seagrass meadows, ND indicates no data

Study	Denitrification (kg N km^{-2} y^{-1})	Burial (kg N km^{-2} y^{-1})	Total N Removal (kg km^{-2} y^{-1})	Cost of N removal ($ kg^{-1} N)	Seagrass Areal value ($ km^{-2} y^{-1})	habitat (km^2)	Total value ($ y^{-1})
This study	620	1,950	2,570	14[1]	36,000	6.9	250,000
Piehler and Smyth, 2011	5700	ND	5700	28.65[2]	163,000	74.0	12,000,000
Russell and Greening, 2015	9,000*	ND	9000	18[3]	162,000	133.1	22,000,000

**Denitrification values in Russell and Greening 2015 were calculated from literature rates (Welsh et al. 2001, Eyre & Ferguson 2002).*

[1]*Abatement cost for reduction of atmospheric NO_x from mobile sources, Birch et al. 2011*

[2]*Price of nitrogen credit in North Carolina Nutrient Offset Credit Program, circa 2012*

[3]*Abatement cost for reduction of point source nitrogen to freshwater, Birch et al. 2011*

Chapter Seven: Conclusions and Synthesis

This body of work provides several important insights into the influence of seagrass meadows on the coastal nitrogen cycle. One major contribution of this dissertation is to highlight the importance of new methods for measuring microbial processes in seagrass sediments. Building on work by Koop-Jakobsen and Giblin (2009) and Addy et al. (2002), the push-pull method developed in Chapter 2 shows that in situ measurements targeting subsurface microbial processes yield higher rates of denitrification, DNRA, and N fixation compared to traditional methods. This concept is not entirely novel; in the development of traditional methods, other authors have commented on the difficulties of assessing subsurface microbial processes in seagrass sediments, due to the complex redox environment created by root exudation (e.g. Nielsen 1992, Groffman et al. 2006). However, the results presented here (Chapters 3-4) show that these subsurface rates are in fact substantial and that traditional measurements of N transformations in seagrass meadows may overlook the contributions from subsurface processes. The higher variability of push-pull measurements compared to laboratory incubations also indicates the importance of collecting data under field conditions in order to capture the full range of rates driven by spatial and temporal heterogeneity in sediment conditions and plant effects. Additional measurements using the push-pull method in seagrass meadows with different species, sediment characteristics, ambient nitrate concentrations, and hydrodynamic environments would add to our general understanding of how these subsurface rates contribute to N cycling in seagrass meadows.

The limitations of the push-pull method should certainly be considered along with the promising results. Perhaps most importantly, the push-pull method is a seriously labor-intensive

technique. Relatively few replicates can be collected compared to laboratory incubations using cores or slurries, and given the high variability of push-pull measurements, greater replication is likely necessary compared to traditional techniques. Additionally, the push-pull method is constrained in that measurements under dark conditions are difficult to achieve, and the method does not provide measurements of additional processes, such as sediment oxygen demand and nutrient fluxes across the sediment-water interface, that are available from traditional core incubations. Thus, future studies of N cycling in seagrass sediments will likely continue to rely on traditional methods. However, these traditional methods should be supplemented by additional push-pull measurements. Especially in the case of N fixation, the push-pull rates suggest dramatically different results compared to traditional incubation methods, and there is a clear need for additional measurements to understand these subsurface processes.

Beyond the advances in methodology, this dissertation also demonstrates how seagrass restoration successfully reestablishes N filtration, an important ecological function. Both denitrification and N burial were enhanced in the seagrass meadow compared to bare sediments, demonstrating the increased N filtration capacity of the meadow following restoration. Much of the study of N removal in seagrass meadows to date has focused on denitrification (e.g. Piehler & Smyth 2011, Russell & Greening 2015); however, in this study system, burial was a more important N removal process than denitrification. Relatively few measurements of N burial in non-peat-forming seagrass species exist, and the results presented here (Chapter 5) show that in some cases burial drives the N filter function. Further investigation of N burial in seagrass meadows is therefore warranted in order to understand how this overlooked ecological process contributes to the N filter function.

A third conclusion from this dissertation is that the seagrass meadow restoration has enhanced the capacity of the lagoon to buffer future increases in N loading. Predicting how the lagoon will respond to increases in N loading from development in the watershed is difficult; however, a simple comparison suggests that the magnitude of N removal in the seagrass meadow is relevant compared to the enhanced N loads from future development (Chapter 6). Continued expansion of the restored meadow will continue to increase this ecosystem service and consequently the value we attach to N removal in the seagrass meadows. While we can quantitatively assess the value of the seagrass N removal using replacement costs and damage costs avoided, it is perhaps equally important to qualitatively show that the seagrass meadow has enhanced the resilience of the lagoon ecosystem by increasing the N filter capacity.

The analysis of N cycling in seagrass meadows presented here is extensive but not comprehensive. Study of additional N transformations in the meadow would enhance our understanding of how the restoration had altered N cycling in the lagoon. Specifically, measurements of remineralization would help to verify the hypothesis that recycling of N supports the high demand from seagrass biomass. Previous work has demonstrated rapid shuttling of macroalgal carbon and nitrogen into benthic microalgae and bacteria pools (Hardison et al. 2010); similar work tracing the fate of nitrogen fixed in seagrass biomass could illuminate how seagrass productivity supports N recycling in sediments. Export of seagrass biomass from the lagoon is a notoriously difficult flux to measure, but one trajectory of exported biomass could be traced by using stable isotopes to determine the contribution of seagrass biomass to sediment organic matter in the neighboring marshes. Finally, although large seagrass herbivores are absent in this system, understanding the effects of grazing on the nitrogen cycle remains an under-explored area. Grazing of seagrass epiphytes may provide a significant transfer of nitrogen to

upper trophic levels within the ecosystem; furthermore, control of epiphyte biomass via grazing may be important to maintaining seagrass health (Borowitzka et al. 2006). Clearly, numerous avenues of exploration remain to answer the question of how the seagrass restoration altered nitrogen cycling in the lagoon.

Overall, the findings from this dissertation show a dramatic and beneficial effect on N cycling following seagrass restoration. Moreover, the benefits of the restored N filter function extend beyond reduced nitrogen in the water column. The N filter function creates a positive feedback with seagrass growth, with high water clarity supporting high light availability at the benthos which in turn supports seagrass productivity. The numerous ecosystem functions and services associated with healthy seagrass meadows, such as carbon sequestration and habitat provisioning, are therefore indirectly supported by the N filter function, and the true value of this ecosystem service is only partially captured through the replacement cost approach used in Chapter 6. The recovery of the N filter function in restored seagrass meadows thus represents one mechanism that can help restored meadows to establish and thrive. By demonstrating the reestablishment of this important ecological function, this work can serve as motivation to protect and restore seagrass ecosystems in the face of widespread decline.

References

Addy K, Kellogg DQ, Gold AJ, Groffman PM, Ferendo G, Sawyer C (2002) In situ push–pull method to determine ground water denitrification in riparian zones. J Environ Qual 31:1017–1024

Borowitzka MA, Lavery PS, van Keulen, M. (2006) Epiphytes of Seagrasses. In: Seagrasses: Biology, Ecology, and Conservation. Springer, Dordrecht, Netherlands, p 441–461

Groffman PM, Altabet MA, Böhlke JK, Butterbach-Bahl K, David MB, Firestone MK, Giblin AE, Kana TM, Nielsen LP, Voytek MA (2006) Methods for measuring denitrification: Diverse approaches to a difficult problem. Ecol Appl 16:2091–2122

Hardison A, Canuel E, Anderson I, Veuger B (2010) Fate of macroalgae in benthic systems: carbon and nitrogen cycling within the microbial community. Mar Ecol Prog Ser 414:41–55

Koop-Jakobsen K, Giblin AE (2009) New approach for measuring denitrification in the rhizosphere of vegetated marsh sediments. Limnol Oceanogr Methods 7:626–637

Nielsen LP (1992) Denitrification in sediment determined from nitrogen isotope pairing. FEMS Microbiol Lett 86:357–362

Piehler MF, Smyth AR (2011) Habitat-specific distinctions in estuarine denitrification affect both ecosystem function and services. Ecosphere 2:art12

Russell M, Greening H (2015) Estimating benefits in a recovering estuary: Tampa Bay, Florida. Estuaries Coasts 38:9–18