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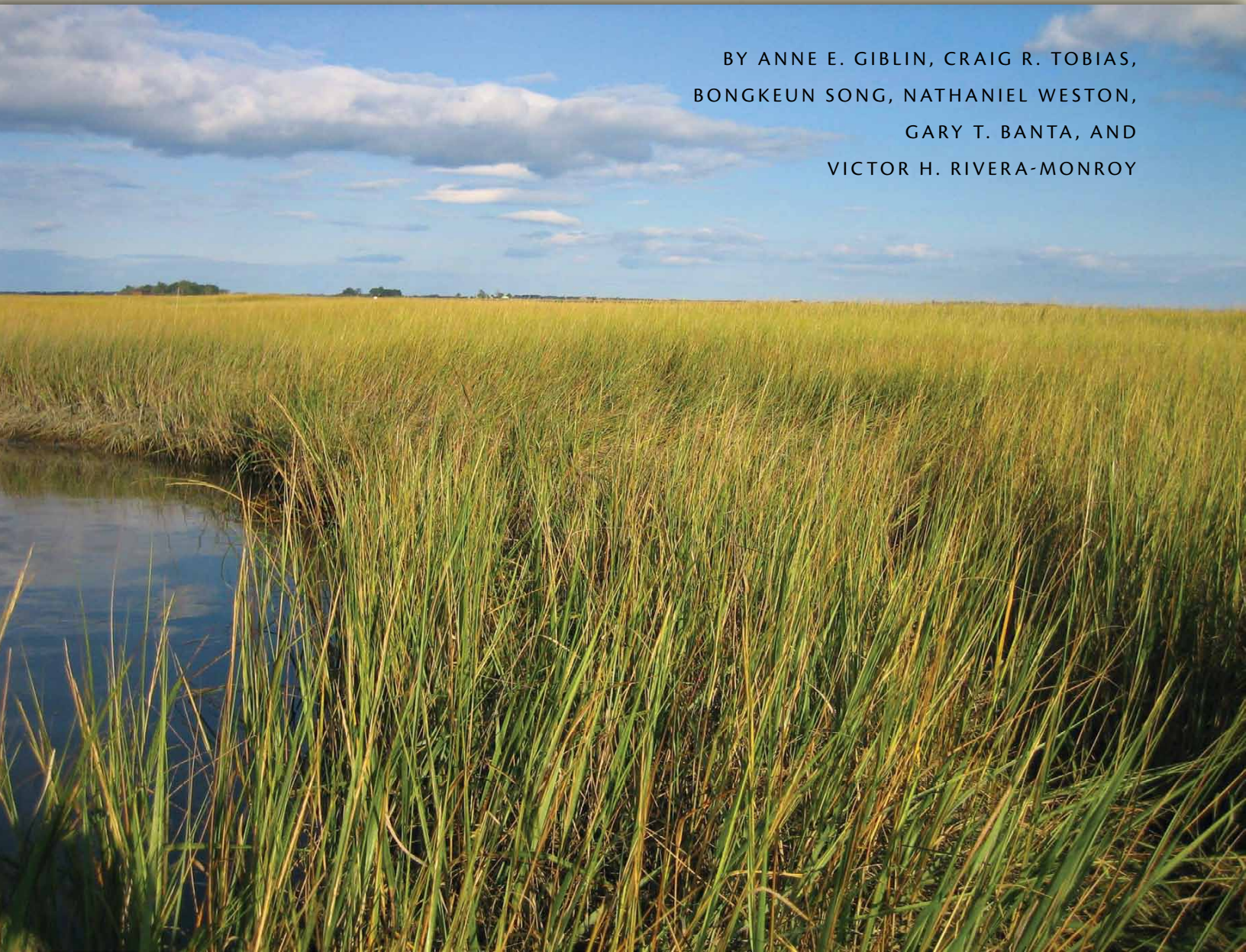
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# The Importance of Dissimilatory Nitrate Reduction to Ammonium (DNRA) in the Nitrogen Cycle of Coastal Ecosystems

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**ABSTRACT.** Until recently, it was believed that biological assimilation and gaseous nitrogen (N) loss through denitrification were the two major fates of nitrate entering or produced within most coastal ecosystems. Denitrification is often viewed as an important ecosystem service that removes reactive N from the ecosystem. However, there is a competing nitrate reduction process, dissimilatory nitrate reduction to ammonium (DNRA), that conserves N within the ecosystem. The recent application of nitrogen stable isotopes as tracers has generated growing evidence that DNRA is a major nitrogen pathway that cannot be ignored. Measurements comparing the importance of denitrification vs. DNRA in 55 coastal sites found that DNRA accounted for more than 30% of the nitrate reduction at 26 sites. DNRA was the dominant pathway at more than one-third of the sites. Understanding what controls the relative importance of denitrification and DNRA, and how the balance changes with increased nitrogen loading, is of critical importance for predicting eutrophication trajectories. Recent improvements in methods for assessing rates of DNRA have helped refine our understanding of the rates and controls of this process, but accurate measurements in vegetated sediment still remain a challenge.

## INTRODUCTION

Nitrogen (N) cycling has been intensively studied in coastal ecosystems for decades. Until recently, the major nitrogen pathways were believed to be well understood. Most coastal ecosystems, with the exception of coral reefs, have been found to be sinks for nitrate as a result of gaseous N losses through quite high denitrification rates. However, over the last decade, the widespread use of new  $^{15}\text{N}$  tracer techniques and microbial molecular methods has revealed new N cycling pathways. A series of recent papers provides an overview of our changing understanding of the nitrogen cycle in general (Canfield et al., 2010; Thamdrup, 2012) and nitrate reduction processes in particular (Burgin and Hamilton, 2007), but these papers do not focus specifically on their implications for coastal ecosystems. Here, we discuss the growing evidence that dissimilatory nitrate reduction to ammonium (DNRA) is an important, and sometimes the dominant, fate of nitrate in

many coastal systems, and consider its ecological implications as well as future research challenges.

DNRA is performed both by heterotrophic organisms, which use organic carbon as the electron donor (fermentative DNRA), and by chemolithoautotrophic organisms, which use nitrate to oxidize sulfide or other reduced inorganic substrates (Figure 1). Unlike heterotrophic or autotrophic denitrification to gaseous  $\text{N}_2$ , DNRA conserves nitrogen in the ecosystem as ammonium, a form of biologically reactive nitrogen that may be taken up by plants or bacteria, or oxidized back to nitrate. The environmental factors that determine the balance between DNRA, denitrification, and other pathways of nitrate uptake in aquatic ecosystems are not fully understood. The conservation of biologically available N by DNRA has major implications for our understanding of how coastal ecosystems will respond to increases in the nitrogen loads they receive from land.

## DNRA PATHWAY BIOCHEMISTRY

Recent biochemical and genetic studies yield a great deal of information about the enzymes and genes involved in DNRA and the organisms capable of carrying out fermentative DNRA. A periplasmic nitrate reductase complex (NapAB) mainly catalyzes the initial reduction of nitrate to nitrite. Nitrite reduction to ammonium is mediated by a pentaheme cytochrome C nitrite reductase (NrfA) and is carried out without producing any intermediate N compound (Einsle et al., 1999). NrfA can also use other compounds, including hydroxylamine, sulfite, and hydrogen peroxide as an alternative substrate (see the review of Simon et al., 2011; Figure 1). The functional gene *nrfA* is present in diverse bacteria, including *Proteobacteria*, *Planctomycetes*, *Bacteroides*, and *Firmicutes* (Mohan et al., 2004). While the *nrfA* gene is frequently targeted as a marker for DNRA, DNRA may not be restricted to the bacteria carrying the *nrfA* genes. A metal-reducing bacterium, *Shewanella oneidensis* MR-1, carries octaheme tetrathionate reductase (Otr), which also catalyzes nitrite reduction to ammonium (Atkinson et al., 2007). In addition, octaheme cytochrome c nitrite reductase (Onr) was found in *Thioalkalivibrio nitratireducens*, a nitrate-reducing obligate chemolithoautotrophic sulfur oxidizing bacterium (Tikhonova et al., 2006). Although purified Onr catalyzes the reduction of nitrite and hydroxylamine to ammonia, *T. nitratireducens* is not able to grow with nitrite as an electron acceptor under anaerobic conditions. Homologous

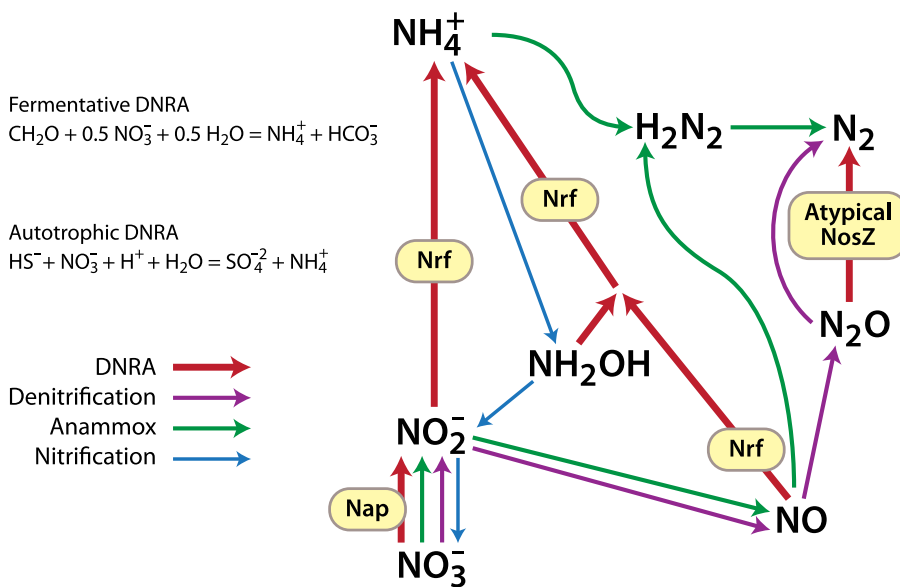


Figure 1. Some important aspects of the nitrogen cycle emphasizing the pathways important to dissimilatory nitrate to ammonium (DNRA). Some of the enzymes known to be involved in the DNRA process, or known to be associated with organisms carrying out DNRA, are shown in yellow. Nap = Periplasmic nitrate reductase. Nrf = Cytochrome C nitrite reductase. NosZ = Nitrous oxide reductase.

genes encoding *Onr* are found in various bacteria, but their physiological involvements in nitrite respiration are unknown. Future studies are required to identify the enzymes and genes involved in the chemolithoautotrophic DNRA pathway. The diverse genera and multiple pathways that characterize DNRA suggest the potential for wide prevalence in different habitats and plasticity of the reaction to changing environmental conditions.

An important ecological implication of the DNRA pathway as catalyzed by Nrf is the lack of intermediate products between nitrite and ammonium.

Therefore, DNRA should not result in the production of  $\text{N}_2\text{O}$ , a powerful greenhouse gas. However, there have been numerous reports suggesting that organisms carrying out DNRA can produce  $\text{N}_2\text{O}$  in both field and culture conditions. More recent evidence indicates that some bacteria may carry both *nrfA* and dissimilatory nitrite reductase (*nirK*) genes on the same genome, suggesting that the prevailing view that the DNRA and canonical denitrification are incompatible may not be correct (Sanford et al., 2012). Although this could explain the production of  $\text{N}_2\text{O}$  by organisms capable

of carrying out DNRA, there are many other possible mechanisms. Thus, the actual contribution of  $\text{N}_2\text{O}$  production by organisms carrying out DNRA is currently unresolved. It is significant that Sanford et al. (2012) identified atypical nitrous oxide reductase genes (*nosZ*) in soil DNRA bacteria, which may involve the reduction of  $\text{N}_2\text{O}$ , indicating that organisms carrying out DNRA can potentially consume  $\text{N}_2\text{O}$ .

### RATES OF DNRA IN ESTUARINE AND COASTAL ENVIRONMENTS

One of the earliest reports on the occurrence of DNRA in the environment came from a study of estuarine sediment using  $^{15}\text{N}$  tracers (Buresh and Patrick, 1978). However, over the next 20 years, there were only scattered reports of DNRA occurrence in coastal areas and few actual rates. While the situation has improved over the last decade, the number of studies is still low and in most cases derived from sediment slurries where potential rates were measured. These studies do suggest that DNRA is of major importance in most shallow coastal sediment under a variety of environmental conditions, as we briefly review below (Figure 2).

#### Unvegetated Coastal Sediment

In unvegetated coastal sediment, the percent of nitrate reduction attributed to DNRA covers the full range from 0 to 100%. The importance of DNRA shows a fairly large seasonal variation in most coastal systems, often increasing during the summer. This increase has been attributed to temperature, which increases sediment oxygen consumption, thus creating more reduced conditions in the sediment (Ferrón et al.,

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2009; Gardner and McCarthy, 2009; Smyth et al., 2013). A number of studies have found DNRA to be favored over denitrification when salinity increases, especially when salinity changes are large (Gardner et al., 2006; Giblin et al., 2010). Others have argued that organisms that carry out DNRA are favored over denitrification at higher temperatures, possibly accounting for the large differences observed in the importance of DNRA across latitudes. For instance, increases in both salinity and temperature were linked with declines in denitrification and increases in DNRA in oligohaline estuarine sediment at the Plum Island Ecosystems Long Term Ecological Research (LTER) site in Massachusetts (Giblin et al., 2010). While DNRA has been reported to be of minimal importance in cold sediment, such as deep sediment in the St. Lawrence Estuary (Crowe et al., 2012), DNRA dominates in many tropical estuaries (Dong et al., 2009, and references therein).

The presence of benthic microalgae (BMA) in shallow sediment can alter nitrate reduction pathways. Temperate studies suggest that benthic algae are strong competitors for nitrate, and that while some of the nitrate can be denitrified, DNRA was not an important pathway (Tobias et al., 2003; Porubsky et al., 2008, 2009). In unvegetated creek bank sediment in the Georgia Coastal Ecosystems LTER, uptake of nitrate by BMA resulted in relatively low rates of both denitrification and DNRA (Porubsky et al., 2008). This contrasts with studies in systems with higher nitrate availability where DNRA is more important (Rysgaard et al., 1996). The predominance of DNRA can hold under both light and dark conditions

(Dunn et al., 2012), and, surprisingly, variations in DNRA rates and light show no consistent pattern.

### Seagrasses

There are only a handful of measurements of DNRA in submerged macrophyte beds. Rysgaard et al. (1996) first reported that DNRA exceeded denitrification in *Zostera noltii* dominated sediment in southern France. Boon et al. (1986) attributed up to 28% of nitrate reduction to DNRA in sediment populated with *Z. capricorni*. The highest overall nitrate reduction rates measured in south Texas estuaries were found in *Thalassia spp.* beds where DNRA dominated denitrification by a 3:1 margin (An and Gardner, 2002; Gardner et al., 2006).

The enhanced levels of DNRA observed during illuminated conditions, when photosynthetic carbon substrates would presumably be more available, were consistent with DNRA derived either from fermentation or sulfate reduction pathways. In contrast, relatively low rates of DNRA, accounting for no more than 5% of the total nitrate flux into the sediment, were reported for mixed *Halodule spp.* and *Z. marina* beds in North Carolina (Smyth et al., 2013).

### Marshes and Mangroves

Rivera-Monroy and Twilley (1996) were among the first to suggest that denitrification was not the major nitrate sink in mangrove sediment, and they proposed that immobilization and DNRA may be

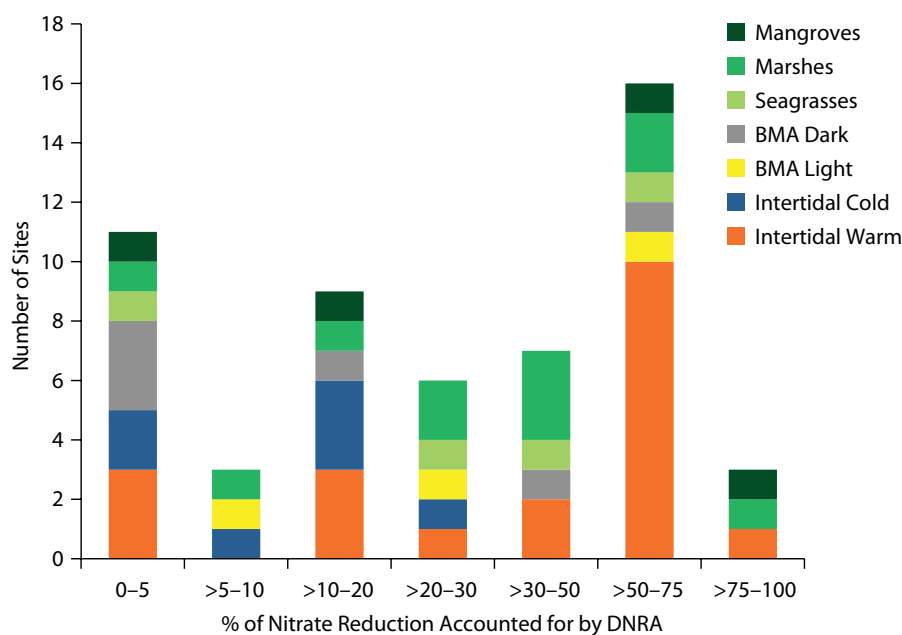


Figure 2. A compilation of the data from the literature cited in this paper showing the percent nitrate reduction attributed to different ecosystems or habitats. In cases of subtidal studies where there were seasonal studies, cold conditions ( $< 12^{\circ}\text{C}$ ) were separated from warm conditions. Data from benthic microalgae (BMA) were separated into light and dark measurements. When data from multiple sites were presented, each site was treated as a separate point. These studies do not include older data obtained by acetylene block methods (reviewed by Kelly-Gerreyn et al., 2001). These data span a similar range but were not included, as experience has shown that acetylene block may underestimate denitrification.

more important. Over the past decade, DNRA rates have been reported for just over a dozen different intertidal wetlands, including high- and mid-latitude *Spartina* spp. marshes (Poulin et al., 2009; Koop-Jackobsen and Giblin, 2010; Tobias et al., 2001a; Uldahl, 2011; Smyth et al., 2013), mangroves (Fernandes et al., 2012; Molnar et al., 2013), intertidal fresh marshes (Neubauer et al., 2005), and marshes receiving nitrogen delivery from tidal flooding or from groundwater (Tobias et al., 2001b; Porubsky et al., 2011; Viellard and Fulweiler, 2012). Studies that consider mineralization and DNRA indicate that while DNRA can be significant relative to denitrification, it typically remains small relative to  $\text{NH}_4$  produced from decomposition (Tobias et al. 2001a, 2003; Porubsky et al., 2011). As was the case for unvegetated sediment, the DNRA contribution to nitrate reduction ranges from < 3% to > 60–99%. To date, 30% of the studies reported DNRA rates in marshes and mangroves that exceed measured denitrification rates at some sites or times (Neubauer et al., 2005; Koop-Jackobsen and Giblin, 2010; Uldahl, 2011; Fernandes et al., 2012), and half the studies report DNRA rates that account for 25–50% of the total nitrate reduction. Given the small number of studies, no clear pattern has emerged that would allow generalizations about differences in DNRA among marsh and mangrove ecotypes. Similar to other N cycle reactions, including denitrification (e.g., Rivera-Monroy et al., 2010), maximum DNRA rates occur nearest the marsh surface where labile organic matter is most abundant (Tobias et al., 2001a; Koop-Jackobsen and Giblin, 2010). There have been few seasonal studies, but unlike

subtidal sediment where rates are highest in warmest months, Uldahl (2011) found that DNRA was higher in the fall as marsh grasses were senescing, as opposed to summer.

### IMPLICATIONS FOR COASTAL ECOSYSTEMS AND RESEARCH NEEDS

Denitrification is often viewed as an ecosystem service that removes reactive N from the ecosystem. Understanding how denitrification rates change with increased loading is of critical importance for understanding eutrophication trajectories (Rivera-Monroy et al., in press). Higher carbon loads and increased rates of sulfate reduction may favor DNRA over denitrification, leading to reduction of this removal service and to a negative feedback between increased N inputs and N removal in seagrass beds, as hypothesized by McGlathery et al. (2007). A similar shift toward DNRA has been proposed for unvegetated subtidal sediment and for marshes as a function of increasing sulfide and temperature (Gardner and McCarthy, 2009). Importantly, increased organic matter deposition from aquaculture has been demonstrated to stimulate DNRA in a wide variety of systems. Christensen et al. (2000) found rates of DNRA were three to seven times higher below fish cages than in nearby reference sediment. *Beggiatoa* mats were present below the fish cage and were presumed to be responsible for the high rates of DNRA. Absolute rates of DNRA were considerably higher in mangroves receiving effluent from shrimp ponds than in nearby sites, and DNRA was two to three times more important as a nitrate reduction pathway than

denitrification (Molnar et al., 2013).

Early researchers proposed that high organic carbon/nitrate ratios favor DNRA over denitrification (Tiedje et al., 1982). Recent experiments (Streminska et al., 2012) and models (Algar and Vallino, in press) support this general view, and they suggest that there is a positive covariance between anthropogenic organic carbon loads and DNRA (Burgin and Hamilton, 2007; Ferrón et al., 2009). For instance, high dissolved organic carbon to nitrate ratios favored DNRA over denitrification in sediment at the Georgia Coastal Ecosystems LTER (Porubsky et al., 2008). However, DNRA rates across different ecosystems, and within marshes (Tobias et al. 2001a) and mangroves (Rivera-Monroy et al., 1995; Molnar et al., 2013), cannot currently be predicted based upon carbon stocks alone. Shifts in nitrate reduction pathways are likely to be influenced less by carbon quantity as by quality or lability, which is more difficult to assess. In addition, chemolithoautotrophic DNRA might be favored by increased carbon loading, which produces more sulfide. The mechanisms controlling this process may be quite different, albeit likely correlated to fermentative DNRA. Increased nitrate should favor denitrification at the expense of DNRA. Although this shift has been observed in laboratory studies, the impact of increased environmental nitrate on N pathways in coastal systems has been mixed. At the Plum Island LTER site, a long-term whole system fertilization experiment exhibits increased overall rates of both denitrification and DNRA (Koop-Jakobsen and Giblin, 2010; Drake et al., 2009). However, in the early years of the study, the percent of nitrate reduction going through DNRA

was similar or lower in fertilized plots than in control plots (Koop-Jakobsen and Giblin, 2010). Uldahl (2011) also did not find any consistent relationship between the importance of DNRA and nitrate loading using slurry measurements. Recently, however, Viellard and Fulweiler (2012), using mass balance, calculated that DNRA was more important in fertilized than in control sites, although it was not directly measured.

The available data demonstrate that we need to better understand how changes in nitrate and carbon availability, as well as environmental conditions such as temperature and salinity, affect

both fermentative and chemolithoautotrophic DNRA. The construction of coupled biogeochemical models may be one way forward. These types of models are further needed to evaluate how hydrological restoration programs can affect landscape-level N cycling. For example, it is not clear how near-canal marshes across the Florida Coastal Everglades LTER site may act to buffer downstream ecosystems from potential N-loading events, or what are the specifics of how these marshes internally process N to determine subsequent N fate (Inglett et al., 2011). Another factor contributing to our lack of understanding may come

from the overwhelming predominance of studies using potential measurements made on slurries, which alter natural gradients and eliminate the normal activities of macrophytes (Figure 3). New approaches that combine  $^{15}\text{NH}_4$  isotope dilution techniques in a flow-through setting provide a powerful way to more fully describe ammonium dynamics in sediment (Lin et al., 2011) and are just beginning to be used more widely to measure DNRA. While a greater application of these techniques to coastal sediment will help advance our understanding of DNRA, numerous experimental challenges exist when




Figure 3. Vegetated sediment is extremely heterogeneous, and the roots may exert a strong but local control on N cycling pathways. (a) Sediment surrounding *S. alterniflora* roots growing against a glass plate in the lab. Disrupting the root-sediment system may disrupt nitrate-reducing pathways. Photo by Zoe Cardon (b) Mangrove sediment showing similar heterogeneity. Photo by Jane Tucker (c) White sulfur bacteria forming a mat near an area of porewater drainage in a *S. alterniflora* marsh. Such bacteria have been shown to be capable of nitrate reduction by sulfide and elemental sulfur, and they may be other active sites of DNRA. Plum Island Ecosystems Long Term Ecological Research Photo Gallery



trying to apply these techniques in intertidal or vegetated sediment. Finally, our lack of understanding of the full diversity and metabolic potentials of organisms carrying out both fermentative and chemolithoautotrophic DNRA greatly hampers our ability to predict how changing environmental conditions may impact N cycling pathways. Further application and development of molecular probes and tools will continue to add insights here.

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