

## Changes in denitrification rate from the maritime forest to the shallow sub-tidal in natural and altered salt marsh systems

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**Salt marshes have been significantly degraded due to human activities. As we continue to alter this valuable habitat, it is important to understand its role in mitigating excess nitrogen loading. Nitrogen removal via denitrification may be affected by habitat degradation in terms of both the quantity (total denitrification rates) and quality (fungal vs bacterial denitrification) of this process. Denitrification (DEN) potentials were measured by acetylene block technique in the Rachel Carson (RCR) and Currituck Banks (CBR) NC Estuarine Research Reserves to assess the function of sub-habitats within and adjacent to the marsh including the maritime forest, high marsh, low marsh, and subtidal. Additional measured parameters included porosity, organic matter, and nutrient concentrations. DEN potentials were much higher in the RCR than CBR. Fungal DEN potentials were a greater component of DEN in CBR than the RCR. Elevation played an important role in DEN rate in CBR. No elevation associated trend was observed in RCR where high DEN rates were detected. Porosity and sediment organic matter showed similar trends with elevation within a site. However, the trends were reversed between sites. In the RCR, both parameters were lowest in the high marsh and increased both landward and seaward. In CBR, both parameters were highest in the high marsh and decreased both landward and seaward.**

Human modification of the salt marsh has been extraordinary and multifaceted.<sup>1</sup> Construction, agricultural waste, concentrated animal feeding operations (CAFOs), storm releases of raw sewage from sewage treatment plants, and urban run-off are significant contributors of nitrogen loading to watersheds.<sup>2,3</sup> In addition, anthropogenic alterations of global climate will impact coastal zones through predicted sea level rise. The duration of inundation and area inundated will both be significantly altered. Unless sediment accretion can keep pace with the rising sea, low marsh systems will drown. Development upland significantly alters hydraulic regimes, habitat structure, species distribution, and reduces available natural habitat. This squeezing effect at both upper and lower edges of the marsh will have a significant impact on the nutrient cycling in the ecosystem, primarily through habitat loss. As we continue to alter the ecosystem it is important to understand how the changing topography and available habitat will affect nitrogen cycling in the marsh, particularly processes that can mitigate excess nitrate ( $\text{NO}_3^-$ ), such as denitrification. Denitrification (DEN) is the biological process by which  $\text{NO}_3^-$  (biologically active) is converted to  $\text{N}_2$  (essentially biologically inactive). Previously, DEN, especially in coastal systems has been thought to be predominately a bacteria-mediated process (B-DEN). However, it has been recently discovered that fungi-mediated DEN (F-DEN) can be a significant contributor to nitrogen processing in wetland ecosystems.<sup>4,5,6</sup> F-DEN has been studied mostly in freshwater and terrestrial systems.<sup>7</sup> However, few studies have investigated the capacity of F-DEN in marine systems, particularly intertidal zones.<sup>8,9</sup> According to a recent study by Mohamed and Martiny (2011), fungal diversity and abundance in salt marshes is comparable to freshwater systems, including types of fungi capable of DEN.<sup>10</sup> Therefore, F-DEN may be an important contributor to total DEN in coastal systems. Currently, data on DEN rates measured continuously from the maritime forest to the shallow subtidal is limited. Most estimates of DEN focus on low marsh and shallow subtidal leaving the high marsh and maritime forest relatively unstudied. In addition, current estimates of DEN in salt marsh systems have not distinguished F-DEN from B-DEN. While many people agree that F-DEN can be significant in terrestrial systems and some anoxic marine sediment, no one has investigated changes in the ratio of F-DEN to B-DEN from the high marsh to the low marsh.

The primary distinction between F-DEN and B-DEN is the redox potential most conducive to the process and the rate of  $\text{N}_2\text{O}$  production.<sup>4</sup> DEN rates are affected by a balance of oxidation and reduction. B-DEN is an anaerobic process and occurs when marsh sediments are inundated (creating a reduced environment), but  $\text{NO}_3^-$  will only be produced via nitrification in aerobic

conditions. Therefore, the tidal cycles of wet and dry are highly conducive to B-DEN. Low levels of  $\text{NO}_3^-$  will cause B-DEN to halt, but abundant  $\text{NO}_3^-$  (as well as other biologically active forms of N) can make the process of B-DEN less efficient. As a result, B-DEN is not always complete and produces more intermediate products ( $\text{N}_2\text{O}$ , a harmful greenhouse gas).<sup>11, 12</sup> F-DEN, alternatively, is enhanced by the presence of oxygen and readily occurs in aerobic conditions. Fungi are capable of concurrent respiration and DEN.<sup>6</sup> Because anaerobic conditions are not necessary for the process to occur, F-DEN may dominate in high marsh. In addition, while some fungi are capable of complete DEN (end product,  $\text{N}_2$ ), most F-DEN results in  $\text{N}_2\text{O}$ .<sup>6</sup> Excess N does not cause the increase in production of intermediates because  $\text{N}_2\text{O}$  is already the primary product. Organic-N, such as amino acids, aniline, or azides can be N-sources for F-DEN.<sup>6</sup> B-DEN usually requires  $\text{NO}_3^-$  present in the water column or supplied by the slow process of nitrification. Therefore, F-DEN has the potential to be a significant contributor to DEN in systems which are N-limited, such as salt marshes. Data collected from Bogue Sound have shown that DEN rate (F and B combined) was similar in marshes of varied widths and marshes with shoreline stabilization. However,  $\text{N}_2\text{O}$  concentrations were not measure and F-DEN rates were not quantified. In addition, DEN rates were not determined for the maritime forest or shallow sub-tidal which could have a significant effect due to the duration of inundation.

### Hypotheses

I hypothesized that F-DEN and B-DEN will be inversely related with F-DEN dominating in the high marsh and B-DEN dominating in the low marsh. In addition, I would expect natural versus altered systems to exhibit different ratios of F- to B-DEN.

### Methods

#### Sites

Two sites were chosen from Rachel Carson NCNERR and the Currituck Banks NCNERR to collect data. The Rachel Carson Reserve (RCR) site is located on the south east tip of Carrot Island (near Beaufort, NC). The RCR is a historic dredge spoil island with full salinity due to the Islands connectivity/close proximity to the Atlantic Ocean. The primary marsh vegetation is *Spartina alterniflora* and the maritime forest is sparse. Horses are common on the island and can significantly impact marsh vegetation density, local sediment compaction, and nitrogen concentrations.



Figure 1: Study site location within the Rachel Carson NC Estuarine Research Reserve.

The Currituck Banks Reserve (CBR) is located north of Corolla, NC on the soundside near the end of Highway 12. The CBR has natural sediment characteristics and low salinity. Natural vegetation cover has changed due to the invasion of *Phragmites* in this normally *Juncus gerardi* dominated marsh. In addition, horses can also be found at this site. However, they spend most of their time in the maritime forest unlike the horses at the RCR.



**Figure 2: Study site location within the Currituck Banks NC Estuarine Research Reserve.**

#### *Elevation surveys*

Each site was surveyed using an Auto Level apparatus to determine elevation along core transects. HOBO water level loggers were deployed to monitor water depth during tidal cycles. The combined data will produce information on the area inundated and the duration of inundation. Therefore, we can measure rates of DEN for dry (oxic) and wet (anoxic) sediment and determine more accurate yearly DEN rates. Currently, elevation surveys have been completed. However, HOBO data is still being collected to understand seasonal effects on tidal inundation.

#### *Acetylene Block Cores*

Acetylene block technique (ABT) methods were adapted from Thompson et al, 1995 to measure F-DEN, B-DEN, and combined DEN.<sup>13</sup> ABT cores were collected along transects spanning the maritime forest to the shallow subtidal. Larger cores were used to collect sediment from each site and returned to the lab to be subcored for each replicate. Samples were incubated in the lab to maintain consistency between sites for both light and temperature. Therefore, DEN potentials reported here are for samples at 25°C. Before subcoring for treatments, the larger cores rested for

12 hours to acclimate to ambient laboratory temperature. Fifty-four sub-cores (12mm diameter, 1cm depth) were collected for each habitat type to account for a full cross of pharmaceutical treatment (antifungal or antibiotic), oxygen presence, time point (Figure 3), and 3 replicates of each treatment. All cores were spiked with N (100 $\mu$ M glucose, 100 $\mu$ M NH<sub>4</sub>NO<sub>3</sub>) to determine maximum DEN potential and either purged of oxygen (to induce anoxia) or left with atmospheric oxygen concentrations. In addition, to isolate for F-DEN, B-DEN, or total DEN, each core was treated with an antibiotic solution (3 mg/L streptomycin in filtered site water), antifungal solution (2 mg/L cyclohexamide in filtered site water), or filtered site water (control) respectively. Acetylene was

O <sub>2</sub> Presence	Oxic			Anoxic		
	0	3	6	0	3	6
Control						
Antifungal						
Antibiotic						

**Figure 3. ABT core treatments. Different colored circles denote different pharmaceutical treatments to isolate bacteria, fungus, or neither (control, both present). Black rings show cores which have been purged of oxygen (i.e. anoxic). Finally, time point refers to hours of incubation before the core is sacrificed to analyze for N<sub>2</sub>O concentration.**

added to block the reduction of N<sub>2</sub>O to N<sub>2</sub> (i.e. force incomplete DEN).<sup>14</sup> Controls included cores of each treatment without acetylene. Duplicate cores were sacrificed at 0, 3, and 6 hours to analyze for N<sub>2</sub>O production using an electron capture detector (ECD).<sup>13</sup> DEN potential was determined by measuring increases in N<sub>2</sub>O concentrations over time.

#### *Additional environmental parameters*

We measured other parameters which can affect DEN including porosity, sediment organic matter, and nutrient content. Sediment samples for porosity and sediment organic matter content were collected from each habitat along transects in the field. Porosity was measured using equation 1:

$$P = 100 \left( \frac{V - V_{TG}}{V} \right) = 100 \left( 1 - \frac{V_{TG}}{V} \right) \quad (1)$$

where  $P$  is porosity,  $V$  is volume of wet sediment, and  $V_{TG}$  is volume of dried sediment. Sediment samples were collected using a 60 mL syringe to 1in to keep a constant wet volume and dried at 105°C for 4 hours. Dried sediment volume was measured by water displacement in a graduated cylinder.

Organic matter percentages were measured using loss on ignition. Samples were dried at 105°C for 4 hours and combusted at 525°C for 4 hours. Organic matter was determined using equation 2:

$$SOM\% = 100 \left( \frac{D - M}{D} \right) \quad (2)$$

Additionally, 50ml water samples will be collected from each site along with overlying sample water. Samples were filtered using Whatman GF/F filters with a pore size of 0.7  $\mu$ m. Nutrient samples will be analyzed with a Lachat Quick-Chem 8000 automated ion analyzer for NO<sub>3</sub><sup>-</sup>, NH<sub>4</sub><sup>+</sup>, PO<sub>4</sub><sup>3-</sup> and total nitrogen (TN).

#### *Statistics*

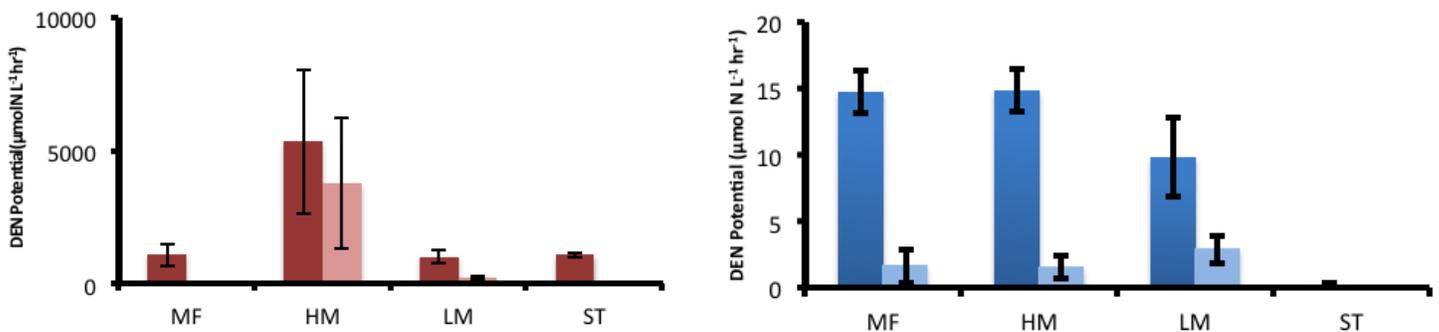
All statistics were run in R. Percentage data (SOM and porosity) were transformed using arcsin square root ( $\arcsin\sqrt{\text{proportion}}$ ). Homogeneity of variance was determined using Levene's test. ANOVAs were run for all data which fit the assumptions of the test and a Tukey post-hoc was used

to assess significant differences between treatments or habitats. If data required a non-parametric test, a Kruskal-Wallis was used with a Mann-Whitney U as a post-hoc.

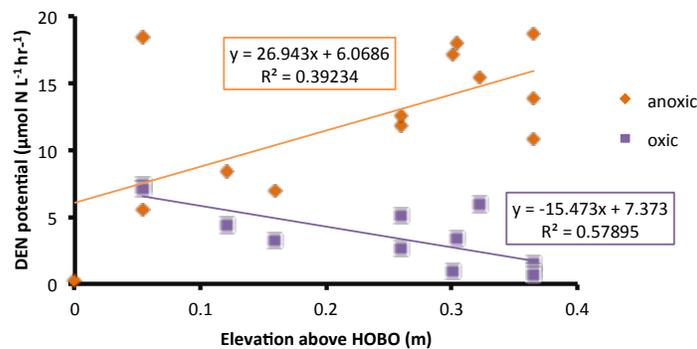
## Results

### Denitrification Potential

DEN potentials were greater in the RCR than the CBR (Figure 6). In some cases, DEN potentials were 400x higher in the RCR than the CBR at similar marsh elevations. Generally, potentials were higher for anoxic samples than oxic samples at both sites. DEN potential was not significantly impacted by habitat for the RCR or the CBR, but elevation was a key factor for both oxic ( $p < 0.005$ ) and anoxic ( $p < 0.05$ ) potentials in the CBR (Figure 7).



**Figure 6.** DEN potentials for both the RCR (red) and the CBR (blue). Note the differences in the scaling on the y-axes. Anoxic samples are denoted in dark colors and oxic samples are shown in light colors. MF=maritime forest, HM=high marsh, LM=low marsh, ST=subtidal



**Figure 7.** Correlation between DEN potential and elevation for anoxic and oxic samples in the CBR.

### Elevation Surveys

Elevation data was collected using a laser level and mapped with ArcMap10. Transects were terminated at the maritime forest when the vegetation was too dense for the laser to reach the transmitter on the elevation rod. Maps for each site are shown below (Figures 4-5). Slopes ranged from 0.003 to 0.016 in the RCR and 0.008-0.01 in the CBR. The overall elevation change for RCR was between 0.17m to 1.28m. Currituck Banks elevation change ranged from 0.31-0.39m. Integrating transect elevations, HOB0 (water level) data, and DEN potentials provided a basic estimate of yearly DEN potential for both reserves. Results are shown below in Table 2. Note that the estimates are based on the DEN rates of each site and are not standardized to the surface area of marsh transects.

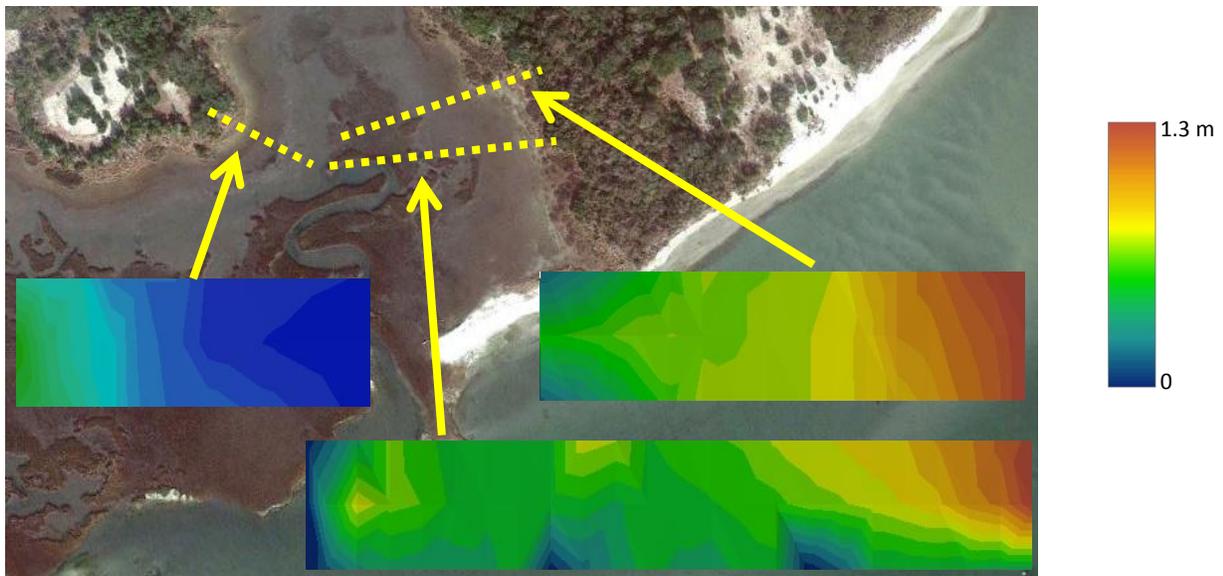


Figure 4. Elevation surveys from the RCR. Blue denotes low elevation and red is high elevation. All elevations are determined in relation to the HOB0.

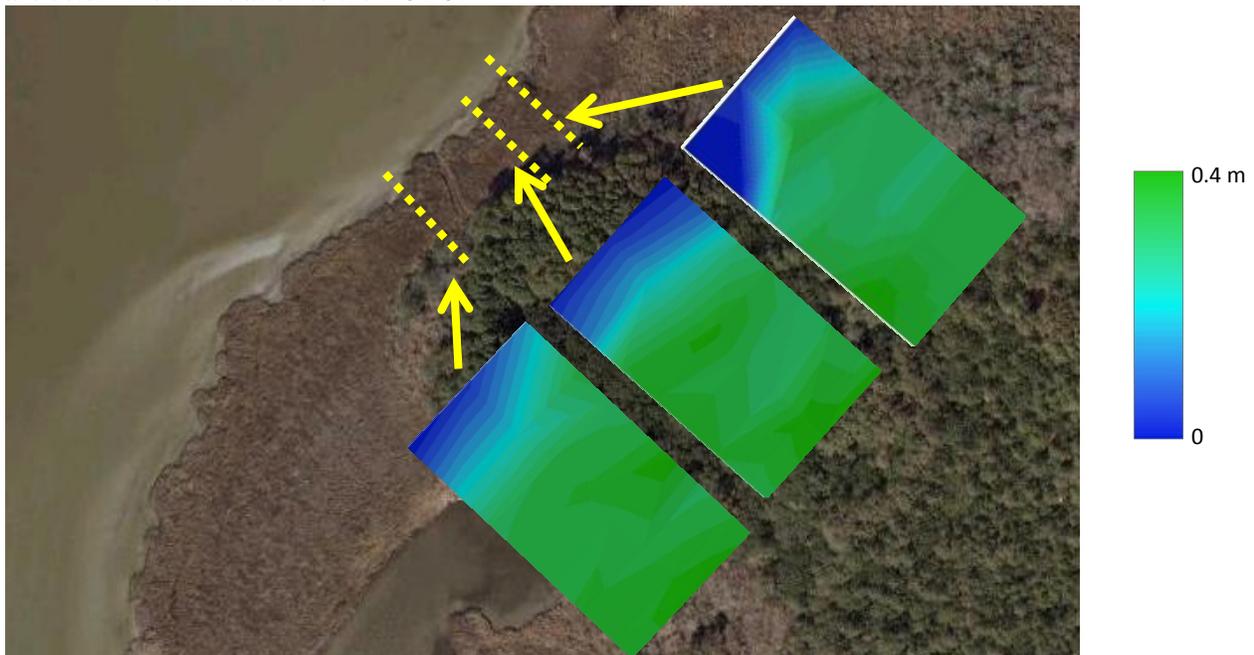


Figure 5. Elevation surveys from the CBR. Blue Denotes low elevation and red is high elevation. All elevations are determined in relation to the HOB0.

Reserve	Transect	Area (m <sup>2</sup> )	N removal (kg N yr <sup>-1</sup> )
Rachel Carson	South East	2258.45	10.02
	North East	1522.29	5.88
	West	1100.01	5.91
Currituck Banks	North	686.66	1.28
	Central	701.22	1.32
	South	729.19	1.26

Table 2. Results from HOB0, DEN, and elevation data.

*Fungal vs Bacterial Denitrification*

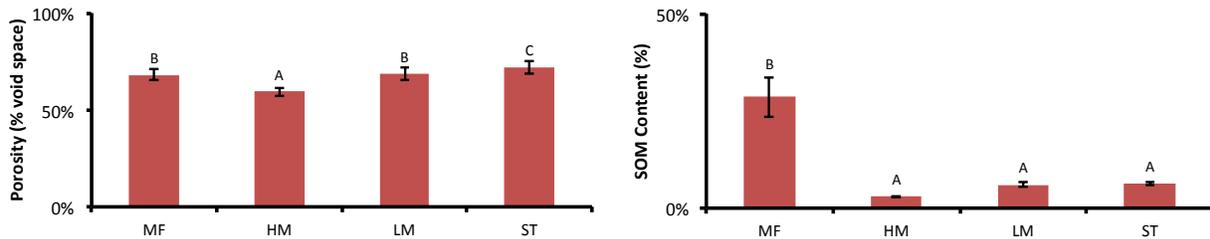
F-DEN and B-DEN values did not always sum to pharmaceutical-free treatment or control DEN values. Therefore, when calculating the relative importance of each microbe in terms of DEN potential, I used the sum of the F-DEN and B-DEN values rather than the control DEN values. As shown in Table 2, bacteria are the dominant microbe in RCR except in the low marsh during anoxic conditions in which the F-DEN potential is slightly greater than the B-DEN potential. The CBR has a much more even distribution of F-DEN to B-DEN. For most of the habitats, F-DEN contributions are approximately equal to B-DEN contributions. However, again, there is a slightly higher fungal contribution in the anoxic low marsh treatment.

	Rachel Carson				Currituck Banks			
	Anoxic		Oxic		Anoxic		Oxic	
	B-DEN	F-DEN	B-DEN	F-DEN	B-DEN	F-DEN	B-DEN	F-DEN
MF	80.8 %	19.2 %	55.0 %	45.0 %	46.9 %	53.1 %	42.8 %	57.2 %
HM	58.9 %	41.1 %	59.9 %	40.1 %	47.0 %	53.0 %	44.6 %	55.4 %
LM	45.5 %	54.5 %	64.5 %	35.5 %	38.7 %	61.3 %	61.2 %	38.8 %
ST	68.1 %	31.9 %			54.9 %	45.1 %		

**Table 2. Relative importance of B-DEN and F-DEN for each habitat in both the RCR and CBR sites.**

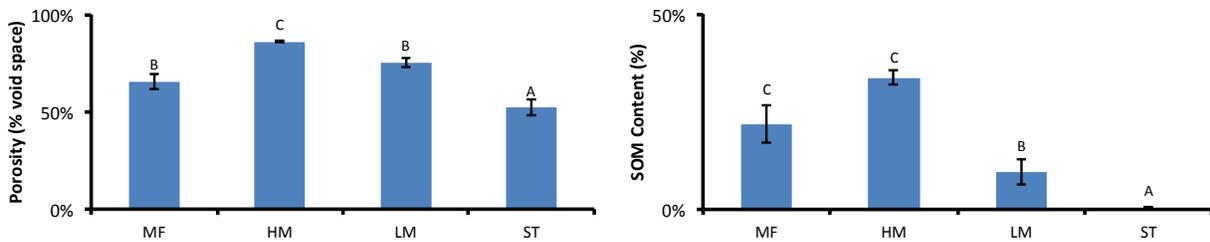
*Sediment Characteristics*

Porosity and SOM content followed similar trends within sites but not between sites. Porosity and SOM in the RCR were lowest in the high marsh and increase both landward and seaward of the habitat (Figure 8).



**Figure 8. Porosity and SOM values by site for the RCR. MF=maritime forest, HM=high marsh, LM=low marsh, ST=subtidal**

However, this is the opposite of the trend observed in the CBR where SOM and porosity peak in the high marsh and decline in either direction (towards the maritime forest and subtidal; Figure 9).



**Figure 9. Porosity and SOM values by site for the CBR. MF=maritime forest, HM=high marsh, LM=low marsh, ST=subtidal**

## **Discussion**

While more samples and experiments are required to reduce errors and increase statistical power, this project has given valuable insight into the denitrification rates of upland marine ecosystems as well as the abundance and dominance of fungal denitrification. Contrary to what we hypothesized, F-DEN proportion was not highest in areas of low inundation, high elevation but was most important in lower elevation habitats with a tidal influence. In addition, due to the abundance of studies on F-DEN in freshwater systems, I anticipated the highest F-DEN proportions in the CBR which was consistent with our data. Note that differences in proportion of F-DEN to B-DEN are not indicative of overall DEN potential. For example, the RCR may have a relatively low F-DEN contribution in the maritime forest, but the F-DEN potential in the RCR is still greater than the CBR potential. In addition, there were differences in the sites, but they could be attributed to differences in both porosity and SOM. The trends in SOM in the RCR and CBR are a factor of each site's history. Dredge spoil sediment is typically very sandy with low SOM content. The RCR, which is a dredge spoil island, exhibits these qualities. The CBR, on the other hand, has a natural sediment profile with a high nutrient content. However, with a limited number of data points, it is hard to say with certainty if the observed correlations are true.

Oxygen presence has different effects on the relationship between elevation and DEN potential. Under anoxic conditions, samples from higher elevations showed higher rates of DEN. However, the opposite was true of oxic samples which showed that DEN potential increased with decreasing elevation. If we consider DEN in the traditional sense, we can justify both trends. The tidal cycles of wet and dry are highly conducive to the process of DEN. DEN is an anaerobic process and most often occurs when marsh sediments are inundated (creating a reduced environment). However,  $\text{NO}_3^-$ , an oxidized form of N, will only be available under aerobic conditions. Therefore, providing the right oxygen conditions can change the redox potential in our samples and supply them with either substrate or anoxic conditions and increase DEN potential. For example, in the maritime forest where anoxic zones are lacking, but  $\text{NO}_3^-$  is plentiful, providing a low oxygen environment enhances DEN.

In the future, I hope to strengthen my data by continuing to collect data in the spring and summer of 2013 as well as complete the elevation modelling and nutrient analyses. Additionally, I would like to use an RTK to determine elevations relative to sea level so that we can accurately compare both the CBR and RCR. Elevation data may be extrapolated to the full area of each marsh using habitat map information from the Reserve websites. In addition, the calculations of N removal could be strengthened by running samples at different temperatures to account for changes. Additionally, it may be important to assess the microbial communities at each of the different habitats. Differences in vegetation, inundation, nutrient content, etc. can all impact microbial communities which will react differently to pharmaceutical treatment. Changes that we observe or the lack of a trend may be the results of shifts in microbial communities in different habitats.

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