NITRATE RECYCLING VERSUS REMOVAL IN THE CAPE FEAR RIVER ESTUARY

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A Thesis Submitted to the
University of North Carolina Wilmington in Partial Fulfillment
of the Requirements for the Degree of
Master of Science

Center for Marine Science

University of North Carolina Wilmington

2008

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ABSTRACT

The Cape Fear River Estuary (CFRE) drains the largest watershed in North Carolina. Anthropogenic inputs of nutrients to this estuary have increased due to the rise in population and subsequent demand for agricultural products in southeastern North Carolina. Dissolved inorganic nitrogen (DIN) enters from tributaries, wet deposition, groundwater and wastewater runoff. Typical of many estuarine systems in the southeastern United States, increased nitrogen loading is coincident with upstream salinity encroachment resulting from fresh drinking water withdrawals and sea level rise. Nitrate is the dominant form of DIN in oxidized waters and was the primary area of focus for this study. Benthic nitrate recycling (DNRA) and removal (ANAMMOX and denitrification) mechanisms were studied in the CFRE, North Carolina. A rapid and real time method using a ^{15}N tracer was developed to simultaneously quantify rates of denitrification, anaerobic ammonium oxidation (ANAMMOX) and dissimilatory nitrate reduction to ammonium (DNRA) in a single sediment sample. Rates were assessed along the estuarine axis seasonally, as the salinity front migrated up and downstream. The ANAMMOX and denitrification rates were generally highest upstream at lower salinities, whereas the DNRA rates were always highest at elevated salinities. A strong, positive correlation was found between ANAMMOX and denitrification rates. A combined approach of laboratory measurements with fresh and transplanted sediment incubations were done in conjunction with geochemical monitoring of porewaters. Rates of ANAMMOX and denitrification tended to be highest when sulfide concentrations were lowest (upstream). Conversely, DNRA was highest when sulfide concentrations were elevated (downstream).

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INTRODUCTION

In the past fifty years, increases in population density coupled with changes in land use have accelerated the delivery of nutrients (primarily nitrogen) to coastal waters and sediments (Galloway et al. 2003). Coastal waters, particularly estuaries, are susceptible to these nitrogen inputs which have altered the biological assemblage and subsequent patterns of nitrogen cycling (Galloway et al. 2003). Increased inputs of nitrogen can increase primary productivity which can trigger eutrophication in high amounts (Cloern 1999). The nitrogen (N) cycle is complex and the balance of nitrogen removal versus recycling reactions in part determines ecosystem susceptibility to nitrogen loading. DIN is highly reactive and tightly conserved. It is transformed by myriad redox reactions including: mineralization of organic nitrogen, nitrification, denitrification, nitrate ammonification (ie. dissimilatory nitrate reduction to ammonium; DNRA), assimilatory nitrate uptake, and anaerobic ammonium oxidation (ANAMMOX) (Herbert 1999 and Dalsgaard et al. 2005, Figure 1, Table 1).

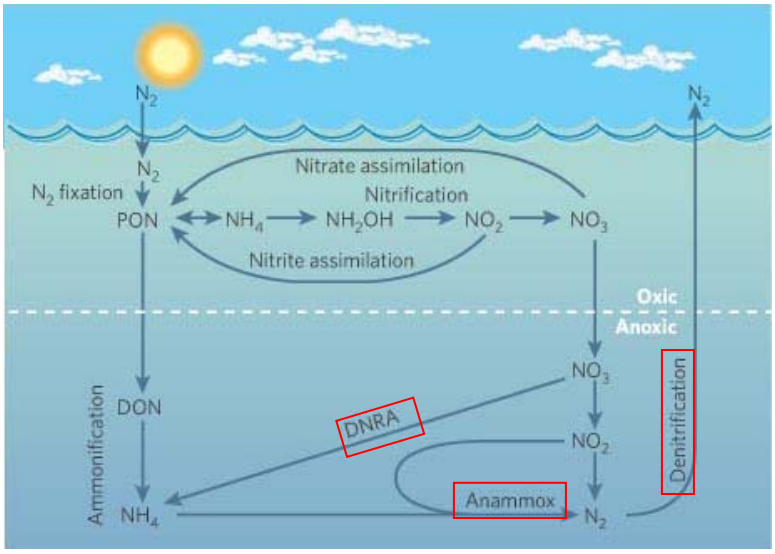

Figure 1: Nitrogen cycle, highlighting the NO_3^- transformations measured in this study, modified from Arrigo (2005).

Process	Nitrogen Half Reactions	Pathway
Ammonification	$ON \rightarrow NH_4^+$	Recycling
Nitrification	$NH_4^+ \rightarrow NO_3^-$	Recycling
DNRA	$NO_3^- \rightarrow NH_4^+$	Recycling
Denitrification	$NO_3^- \rightarrow N_2O/N_2$	Removal
Assimilation	$NH_4^+, NO_3^-, NO_2^- \rightarrow ON$	Removal
ANAMMOX	$NO_2^- + NH_4^+ \rightarrow N_2 + 2H_2O$	Removal

Table 1: Important reactions in the nitrogen cycle

Reactions in the N-cycle can roughly be spilt into those that recycle and those that remove nitrogen. Of the aforementioned processes, only denitrification and ANAMMOX truly remove nitrogen from the system (as N_2), whereas the other mechanisms transform the N-species.

Nitrate Removal

Denitrification is a reductive process that primarily occurs in the sub-oxic portion of sediments. A broad diversity of heterotrophic bacteria utilize NO_3^- as the terminal electron receptor and reduce it to either nitrous oxide (N_2O) or N_2 which evade to the atmosphere (Cornwell et al. 1999, Herbert 1999). Organic carbon (OC) is the dominant electron donor in estuaries, although Fe^{2+}, H_2S, and Mn^{2+} can also be sources of electrons (Cornwell et al. 1999). There is a wide diversity of denitrifying bacteria that operate either as facultative or obligatory anaerobes. The most commonly isolated denitrifying bacteria are of the genus *Pseudomonas* (Herbert 1999). Denitrification rates are the highest when nitrate and electron donors (usually as labile OC) are in ample supply under anoxic conditions but can become hindered when the concentrations of sulfide are high, which can interrupt denitrification pathways (An and Gardner 2002, Senga et al. 2006).

Denitrification was thought to be the sole removal mechanism of nitrate until fairly recently, when Mulder et al. (1995) discovered a bacterium in a wastewater treatment plant that anaerobically oxidized ammonia to N_2 while reducing nitrite. To date five genera of ANAMMOX are known: *Brocadia*, *Kuenenia*, *Scalindua*, *Anammoxoglobus*, and *Jettenia* (Dalsgaard et al. 2005, Kartal et al. 2007, and Quan et al. 2008). ANAMMOX bacteria rely on the reduction of NO_3^- to NO_2^-, which readily occurs in most anoxic marine environments, and combines this nitrite with ammonium in the following reaction:

$$NO_2^- + NH_4^+ \rightarrow N_2 + 2H_2O$$

ANAMMOX bacteria have been found in almost all marine and fresh water reducing environments and may account for up to 80% of the N_2 production in some environments (Dalsgaard et al. 2005). Reported estuarine rates of ANAMMOX are typically less than 20% of N_2 production (Dalsgaard et al. 2005), one recent study in the Cape Fear River Estuary (CFRE) has found that ANAMMOX may be responsible for up to 15.5% of the NO_3^- conversion to N_2 (Dale et al. 2008). This has lead to further investigation of the importance of ANAMMOX as a mechanism for nitrate and ammonium removal in the coastal sediments.

Nitrate Recycling

Nitrate ammonification or more commonly, dissimilatory nitrate reduction to ammonium (DNRA) recycles N, converting nitrate into another biologically usable form of DIN. DNRA is likely fueled by electrons from organic carbon but may draw on other electron sources to facilitate the process (Gardner et al. 2006). An and Gardner (2002) have proposed that a principle electron donor in the DNRA process is sulfide, although

most other studies point to DOC as the main electron source. DNRA generally increases in the presence of sulfide. However, it is still debatable whether the presence of sulfide has a direct effect of increasing DNRA or indirectly hinders denitrification so that more nitrate may be reduced via DNRA. High DNRA rates are also seen when sulfate reducing bacteria are present as these bacteria tend to have DNRA capacity as a secondary metabolism (Rysgaard et al. 1996). Given these potential linkages to sulfur cycling, DNRA would be expected to be elevated in the presence of high sulfate/sulfide levels found in the saltier sediments in the downstream estuarine reaches.

Geochemical Controls on Denitrification, ANAMMOX and DNRA

Estuarine salinity changes seasonally, with the tides and from precipitation events and can occur over short or long time scales. As salinity changes so does the ionic strength of the porewaters and the distribution of porewater solutes. A principal effect of higher salinity is the delivery of sulfate. Sulfate (SO_4^{2-}), which is a major anion in seawater, increases as the salinity increases. In the presence of ample organic matter, sulfate increases hydrogen sulfide in the sediment due to sulfate reduction and ferrous iron (Fe^{2+}) decreases due to savaging by sulfide (Howarth and Teal 1979 and Taillefert et al. 2000). A few studies have addressed the link between salinity and porewater parameters and nitrate recycling and removal processes. Some positive correlations have been found between sulfide/sulfate and rates of DNRA (Herbert 1999, Gardner et al. 2006) and some negative effects of hydrogen sulfide on denitrification have been seen (Joye and Hollibaugh 1995, Senga et al. 2006). Less is known about the relationship between these processes and other porewater analytes whose concentrations also covary with salinity. We suggest that the following porewater chemical species influence the

balance between denitrification, ANAMMOX and DNRA in estuaries: hydrogen sulfide (H_2S; electron donor for DNRA, inhibitor of denitrification), ammonium (NH_4^+; fuel for ANAMMOX and nitrification), salinity (effects availability of NH_4^+), sulfate (SO_4^{2-}; fuel for H_2S), nitrite/nitrate (NO_2^-/NO_3^-; fuel for denitrification, ANAMMOX and DNRA), dissolved organic carbon (DOC; electron source for all reactions) and ferrous iron (Fe^{2+}; electron source for all reactions). These changes in the porewater analytes are largely governed by the overlying fluctuations in the estuary's salinity at any given point. The geochemistry interacts with the microbial community to ultimately influence the reaction rates and solute balances in the estuary. This thesis aims to examine how the rates of nitrate recycling and removal rates, and the balance between them, are impacted by these changing porewater parameters in the Cape Fear River Estuary (CFRE).

The CFRE drains the largest watershed in North Carolina. Anthropogenic inputs of nutrients, primarily nitrogen, to this estuary have increased due to the rise in population and larger inputs from agricultural sources. Dissolved inorganic nitrogen (DIN) enters estuaries from tributaries, wet deposition, groundwater and waste water runoff (Cloern 1999, Gardner et al. 2006, Dafner et al. 2007). The CFRE is impacted by all of these nitrogen delivery pathways. Although currently less impacted than some other NC estuaries (e.g. Neuse, New River), the CFRE lies in one of the fastest growing regions in the southeastern United States. This project examines the removal versus recycling mechanisms in the sediments of the CFRE for nitrate. Specifically, denitrification, ANAMMOX and the recycling process of DNRA. Nitrate typically represents the dominant form of DIN in estuarine waters, including the CFRE. All of these reactions are subject to control at the microbial and geochemical levels.

In this thesis we examined the seasonal distribution of denitrification, ANAMMOX and DNRA in the CFRE (all of which process nitrate), and investigate the extent of geochemical controls on the patterns and rates. This project focused on the nitrate recycling and removal processes that occur in the sediment. We did not consider nitrate assimilation by phytoplankton in our assessment because of light limitation hindering primary productivity in the CFRE. The CFRE is a darkly colored, organic rich estuary with very high levels of light attenuation. Therefore, chlorophyll a and primary production rates are low (Dafner et al. 2007). Due to these conditions, dissimilatory reactions of denitrification, ANAMMOX and DNRA are hypothesized to be the primary mechanisms for removal and recycling of nitrate in the CFRE. The work was done by integrating field sampling with controlled laboratory experiments. We focused on the role of salt (i.e. ionic strength), DOC, DIN, H_2S and Fe^{2+} in porewaters as possible rate regulators.

METHODS

Study Area

The CFRE is defined as the 35 mile section between the city of Wilmington and the Atlantic Ocean. In order to address the nitrate recycling and removal rates in the CFRE three sites encompassing the salinity gradient were chosen: oligohaline (0 -5 ppt), mesohaline (5 – 18 ppt) and polyhaline (18 -35 ppt). For the mesocosm portion of the experiment (see below) these sites were HB, M61 and M35 (Figure 2, Table 2). The fresh/transplant sediment rate determination portion as well as the porewater monitoring and rate inhibition/enhancement experiments utilized the DT, RR and FF sites (Figure 2,

Table 2). These sites had sediment that was similar chemical composition and appearance (Table 3). Two continental shelf sites close in proximity to the mouth of the CFRE (CFP2 and MC-4A; Figure 2) were sampled in the summer of 2008 to estimate removal rates (ANAMMOX and denitrification) for comparison to the removal rates in the CFRE.

Figure 2: Cape Fear region of North Carolina, enlarged to show the sampling stations on the CFRE and continental shelf

	Mesocosms	Fresh Sediments	Transplanted Sediment	Rate Inhibition-Enhancement	Continetal Shelf
Sample Sites	HB, M61, M35	DT, RR, FF	DT, RR, FF	DT, RR, FF	CFP2, MC-4A
Rate Measurements	ANAMMOX, Denit	ANAMMOX, Denit, DNRA	ANAMMOX, Denit, DNRA	ANAMMOX, Denit, DNRA	ANAMMOX, Denit
Porewater Analytes	Salinity	H_2S, NH_4, SO_4, NO_3, DOC, Fe^{2+}	H_2S, NH_4, SO_4, NO_3, DOC, Fe^{2+}	H_2S, NaCl, Fe^{2+}	

Table 2: Sample sites and analysis conducted for each portion of the experiment

Site	C:N	Sed. Description
DT	12.3 (0.7)	Fine grain sand, trace silt
RR	10.4 (0.6)	Fine grain sand, trace silt
FF	10.4 (1.5)	Fine grain sand, trace silt

Table 3: Sediment characteristics from the CFRE sample sites, parentheses represent standard deviation

General Approach

A five pronged approach was used to assess rates and link those rates with changing porewater chemistry. The five methods were: (1) mesocosm sediment incubations; (2) porewater chemistry monitoring; (3) reaction rate determination on fresh sediments; and (4) reaction rate determination on transplanted sediments; (5) controlled solute induced changes on sediment rates. Reaction rates of denitrification, ANAMMOX and DNRA will be determined using isotopic tracer (^{15}N) techniques.

The first principle approach used in this study was porewater chemical analysis. Porewaters were sampled with diffusion techniques similar to those used in the Hesslein (1976) study. Inorganic analytes were assayed using a wet chemistry/spectrophotometric methods and DOC measured by high temperature combustion (Table 4).

Analyte	Method	Reference
H_2S	Spectrophotmetric/Methylene Blue	Cline (1969)
NH_4^+	Spectrophotmetric/Phenol Hypochlorite	Solorzano (1969) and Parsons (1984)
SO_4^{2-}	Turbidimetric	Eaton (1995)
NO_3^-/NO_2^-	Spectrophotmetric/Cd Reduction Azodye	Gordon (1993)
DOC	High Temperature Catalytic Oxidation	Hansell (1993)
Fe^{2+}	Spectrophotmetric/Ferrozine	Stookey (1970), Dawson (1990) and Lovely (1987)

Table 4: Porewater analytes and their methods and references

The second principle approach incorporated the use of a stable isotope pairing technique. A ^{15}N tracer was used to measure ANAMMOX, denitrification and DNRA pathways as follows. For ANAMMOX and denitrification, one gram of wet sediment (0 -3 cm, homogenized surface sediment) was taken and placed in an exetainer (Labco) and flushed with helium for 10 minutes and immediately capped and left overnight. This allowed the sediment to consume any residual oxygen that may have been entrained in the vial and allowed the vial to become anoxic. The exetainers were then flushed with helium (> 100 mls min^{-1}) for 10 minutes. The sediment was then inoculated with 0.25 ml of either $^{15}NO_3^- + {}^{14}NH_4^+$ or $^{15}NH_4^+$ (to a final porewater N concentration of 25 μM N) then monitored at times 0 minutes and three subsequent time points thereafter (up to 315 minutes) to measure the production of N_2 gas. The isotopically labeled N_2 was analyzed on an isotope ratio mass spectrometer (IRMS; Thermo Electron Delta V). This isotope pairing method is modeled after the Thamdrup and Dalsgaard (2002) method, which allows the user to distinguish between ANAMMOX and denitrification based on the mass of the N_2 gas produced form the $^{15}NO_3^- + {}^{14}NH_4^+$ incubation (Figure 3). Denitrification

uses two $^{15}NO_3^-$ molecules to produce $^{30}N_2$ and ANAMMOX uses one $^{15}NO_3^-$ molecule and one $^{14}NH_4^+$ molecule to produce $^{29}N_2$ gas. The area of these $^{29}N_2$ and $^{30}N_2$ peaks are integrated and normalized to the mass of a N_2 air standard, accounted for any atmospheric leakage and normalized to one gram of wet sediment. This value is then plotted versus time and the linear regression of that production is used to calculate rates of ANAMMOX and denitrification, respectively. An example of these chromatograms can be seen in Figure 4. The $^{15}NH_4^+$ incubation is used as a baseline measurement to ensure that no coupled nitrification/denitrification occurs, which would produce $^{29}N_2$ in the $^{15}NO_3^-$ + $^{14}NH_4^+$ incubation and thus be erroneously counted as ANAMMOX production. To measure the recycling of nitrate simultaneously with removal rate determination, a unique approach was developed. DNRA was measured on the same exetainers in which the removal rates were conducted. After these exetainers were assayed for ANAMMOX and denitrification they were uncapped and inoculated with 7 ml of 40 ppt NaCl, 0.15 g MgO, 200 μl 5 mM NH_4^+ (carrier) and an acidified ($KHSO_4^{2-}$) GF/D filter in a Teflon sandwich. This allows the ammonium to volatilize to ammonia ($NH_4^+(l) \rightarrow NH_3^+(g)$) and become trapped on the acidified filter disc. These ammonia laden discs were later run on the elemental analyzer coupled to the IRMS (Figure 3). This method is modified from the Holmes et al. (1998) method.

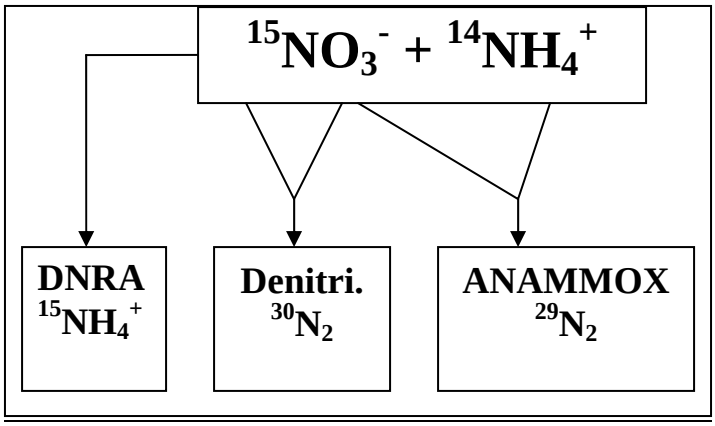

Figure 3: Denitrification produces mass 30 nitrogen gas and ANAMMOX produces mass 29 nitrogen gas. DNRA converts $^{15}NO_3^-$ to $^{15}NH_4^+$.

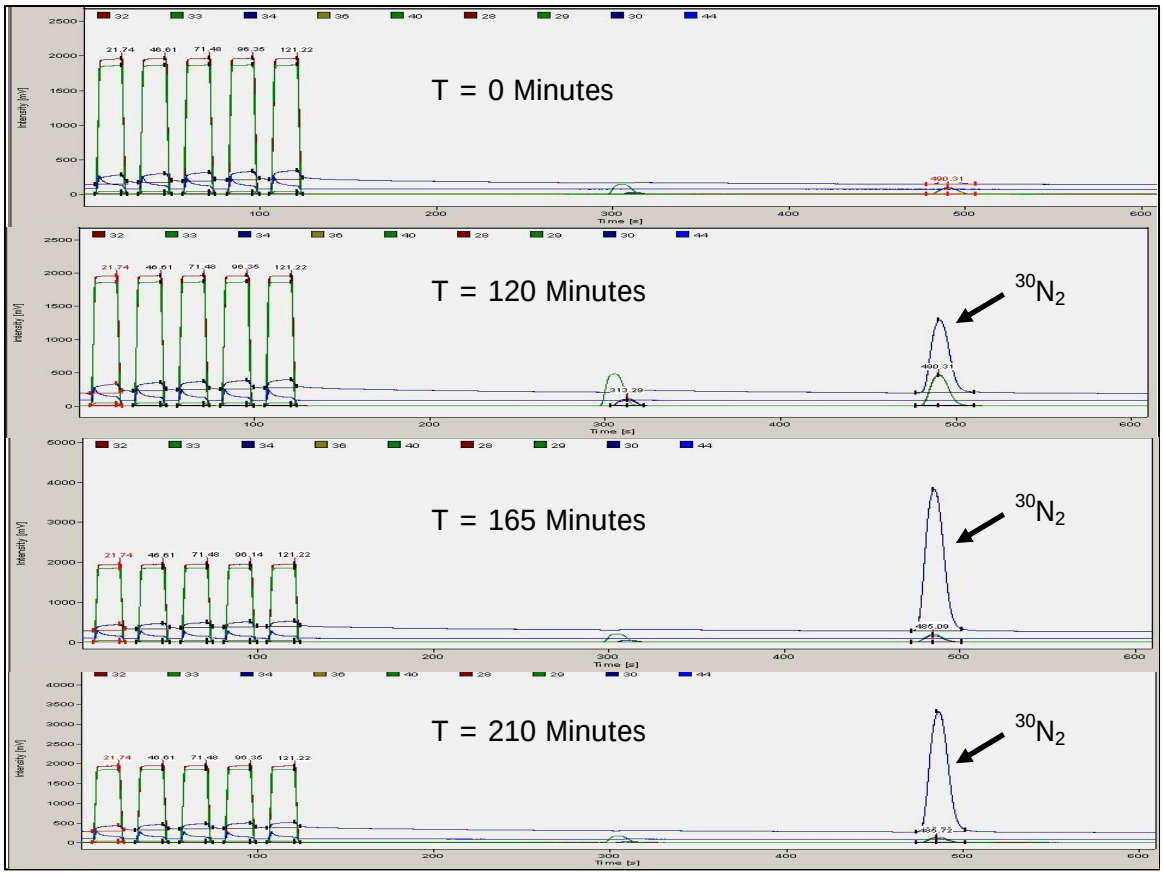

Figure 4: Example of the chromatograms from one sediment removal rate determination, time zero and three subsequent time points, showing the evolution of mass 30 nitrogen gas.

Mesocosm Sediment Incubations: Range of Nitrate Cycling Rates and Gross Salinity Effects

The mesocosms in this study were used to observe any general trends with salinity, such as the range of denitrification and ANAMMOX at high or low salinities. This work was conducted during the summer and fall of 2007 and used to guide the design of the subsequent field study. The mesocosm approach is designed to assess the effects of salinity on the reactions. However, no attempt was made to distinguish between the ionic strength effects and specific analyte effects that covary with salinity. Advantages of the mesocosm approach are that the salinity can be very carefully manipulated and offers ease of sampling. Drawbacks are a decreased ability to mimic the dynamic water chemistry that occurs in an estuary, as well as a pronounced long term sediment storage effect.

The HB (oligohaline, salinity = 5), M61 (mesohaline, salinity = 14) and M35 (polyhaline, salinity = 26) sites in Figure 2 were chosen as representative of the salinity gradient. At these sites, sediment and overlying water were collected and transferred into mesocosms and incubated for a period of six months. Sediments were collected aboard the R/V *Cape Fear*, using a hand operated "grab" corer and the overlying water was obtained using the ship's surface water lines. Sediment from each site was divided into three equal portions and arranged in a setup so that the overlying water from each site is circulated over each of the three sediment types (Figure 5). The upstream sediment and low salinity water would correspond to the HB site, the intermediate sediment and medium salinity water would correspond to the M61 site and the downstream sediment and high salinity water would correspond to the M35 site. The sediment was sampled at intervals of 15, 21, 54 and 85 days. Overlying water was continually monitored for

salinity and the sediment was sampled at predetermined intervals and incubated with ^{15}N tracer to determine rates of ANAMMOX and denitrification, as described above.

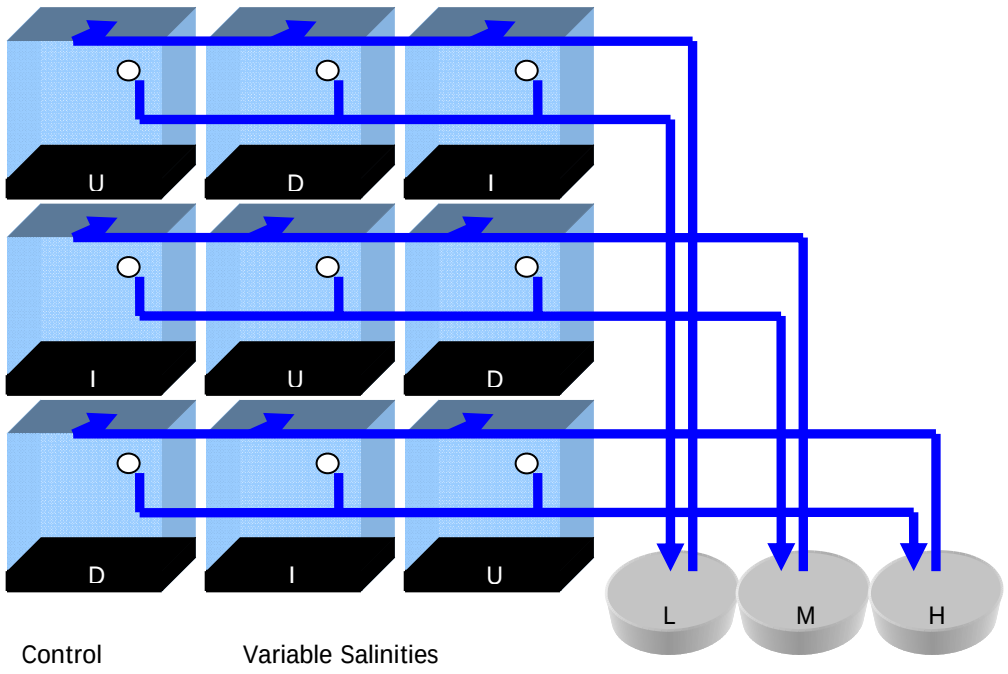

Figure 5: Mesocosm diagram, sediment samples taken from the three CFRE sampling sites (U: Upstream, I: Intermediate, D: Downstream) with in situ water taken from the sampling site (L: low salinity, M: medium salinity, H: high salinity).

Porewater Chemistry Monitoring: Seasonal Porewater Solute Concentrations in the CFRE

Two methods were employed to generate profiles of porewater analytes at each site. First, profiles were created using a porewater diffusion sampler (hereto after known as a Peeper), which is a block of PVC that has been machined to house diffusion vials vertically (Figure 6). Diffusion vials consist of a glass scintillation vial filled with deoxygenated, deionized water and capped with a 0.2 micron nylon membrane. This design of Peeper allows for large volume, duplicate porewater profiles, with diffusion vials on either side and is modified from the Hesslein (1976) design. Peepers were

placed at each of the DT, RR and FF sampling locations (Figure 2) and collected within a week of the fresh and transplanted sediment collection (see below). Once sampled, the diffusion vials were replaced and the Peeper reinserted into the sediment. Porewater in these vials was assayed for hydrogen sulfide (H_2S), ammonium (NH_4^+), salinity, sulfate (SO_4^{2-}), nitrite/nitrate (NO_2^-/NO_3^-), dissolved organic carbon (DOC) and iron (Fe^{2+}) (Table 4). Peepers allow for integration of porewater diffusing into the vials over a long period of time (months) where the equilibration time was on the same time scale as sampling of the fresh and transplanted sediment for rate determination. The Peeper provided profiles from 0 up to 35 cm below the surface. The second method provided "snapshots" of the porewater chemistry and was done with a minipoint sampler. This sampler consists of six one-eighth inch stainless steel pieces of tubing, measured to varying depths and affixed to a plate that is lowered into the sediment (Figure 6). The sampler design follows that of Duff et al. (1998). The stainless steel lines had tygon tubing attached to the ends which were connected to a peristaltic pump that draws the water from the sediment at a rate of two ml min^{-1}. Porewater analytes measured were the same as those measured in the Peepers. The minipoint sampler provided profiles from 0 to 12 cm. Porewater samples were filtered (0.2 micron) in the field for Fe^{2+}, H_2S, SO_4^{2-}, DOC; preserved with H_3PO_4 and stored in a refrigerator and analyzed within 3 weeks. Samples for NO_3^-/ NH_4^+ were stored frozen and analyzed within 3 weeks.

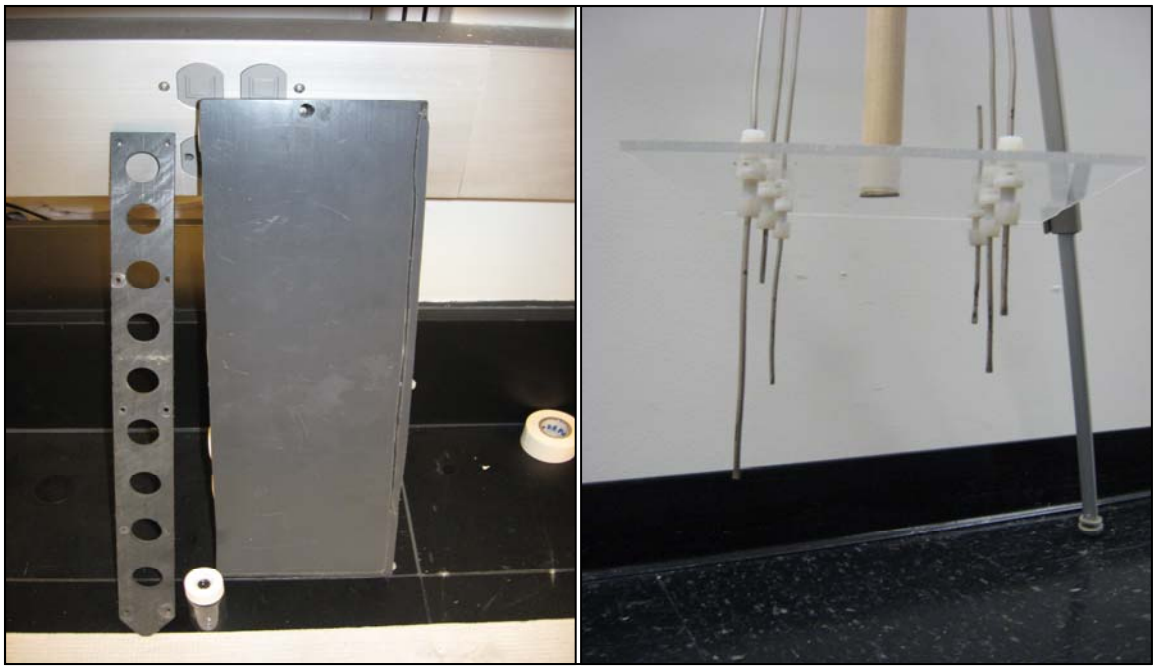

Figure 6: Peeper, with diffusion vial in foreground (L). Minipoint sampler (R).

Reaction Rate Determination in Fresh Sediments

Rate determinations of ANAMMOX, denitrification and DNRA in the fresh surface (0 – 2 cm depth) sediments experiment provided a more realistic scenario than the mesocosms, so the nitrate recycling and removal rates in the CFRE could be monitored under true estuarine conditions. Fresh sediments were collected at the DT (oligohaline: 0.5 – 5.0 ppt), RR (mesohaline: 5 – 18 ppt) and FF (polyhaline: 18 – 30 ppt) sample sites (Figure 2) which encompassed a seasonally variable salinity gradient. The rates were measured according to the previously described methods.

Reaction Rate Determination in Transplanted Sediments

For the transplanted portion, surface (0 – 2 cm depth) sediment was collected from the DT, RR and FF sites in August of 2007 and stored under site water until it was

able to be transferred into the sediment environment manipulators (SEMs;). These SEMs consisted of 0.2 micron nylon membrane packets filled with sediments from the three different sites and then heat-sealed closed. Each packet was then placed in a slotted PVC screen manifold and buried in the top four centimeters of sediment at each estuarine station (Figure 7).

Figure 7: Sediment in membrane packet being inserted into SEM

Each of these SEMs permits porewater solute exchange but prevents immigration or emigration of the microbial community inhabiting the enclosed sediment. These packets were made up the day of deployment to minimize the amount of time that the sediment was in contact with air. On each SEM, diffusion samplers, similar to the Peeper vials, were placed to sample the porewater. Each SEM contained 4 sediment packets from each of the three sampling locations and 4 diffusion vials. The SEM was buried in the sediment for a total of one year. After three months of equilibration and every three months after, one sediment packet from each tube on the SEM and all of the diffusion

vials were collected, new diffusion vials were replaced and the SEM was reburied (Figure 8).

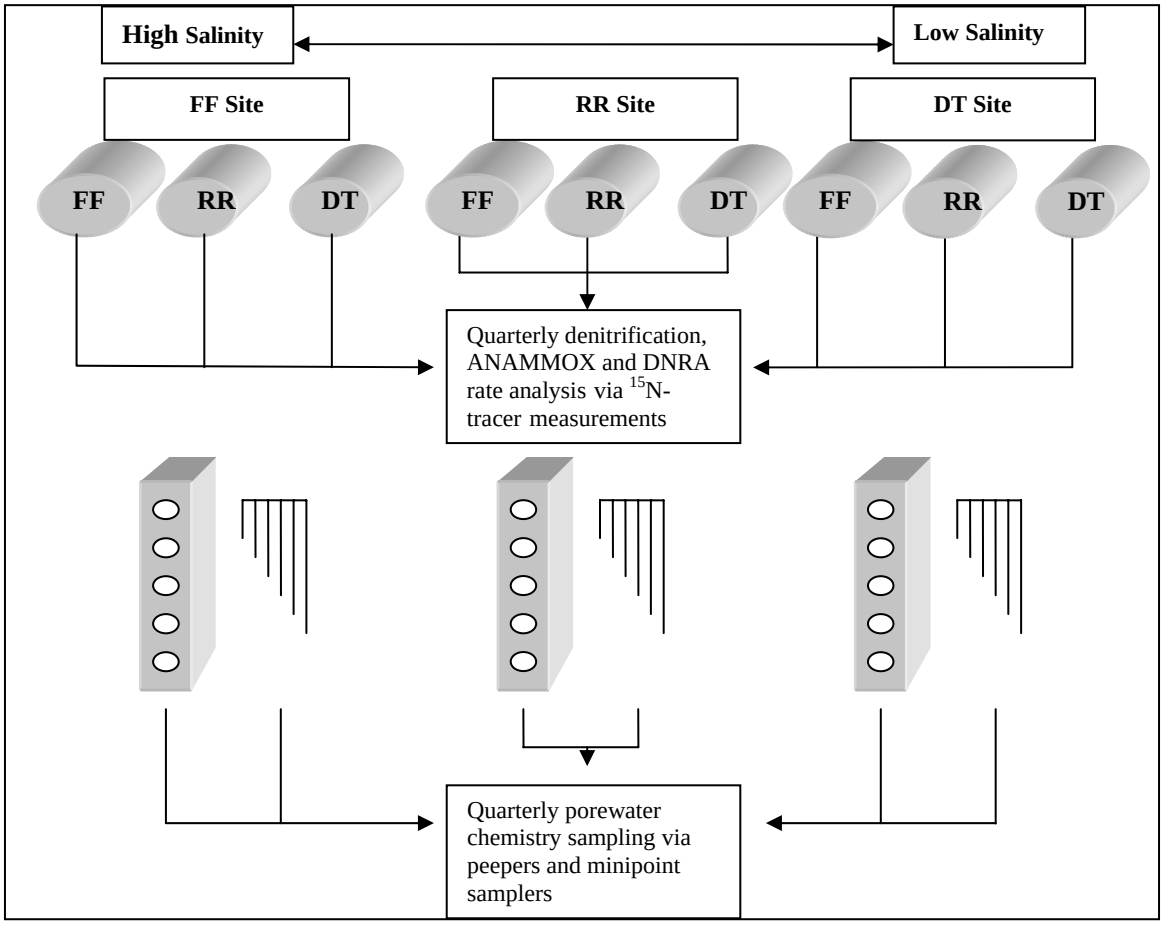

Figure 8: In situ transplant experimental design

This process occurred 4 times during the year to encompass yearly fluctuations in salinity and geochemistry (Figure 8) and was sampled along with fresh sediment collection and within a week of the Peeper sample collection. For the transplanted sediment, rates for ANAMMOX, denitrification and DNRA were determined with the $^{15}NO_3^-$ + $^{14}NH_4^+$ and $^{15}NH_4^+$ incubations as described earlier.

Controlled Solute Induced Changes on Sediment Rates: Specific Analyte Effect on Nitrate Recycling and Removal

The controlled solute induced changes on sediment rates experiment was designed to examine the inhibition or enhancement of each porewater analyte on ANAMMOX, denitrification and DNRA rates in February 2007. In this experiment, fresh sediment from each of the three sites was incubated in exetainers with varying additions of either Fe^{2+} ($FeCl_2$), H_2S (NaS), or salt (NaCl) to monitor how the sediment reacts to the different concentrations of the solutes (Table 5). Final analyte concentrations were (1) ambient solute concentrations, (2) two times ambient and (3) four times the ambient concentration for a given site and incubated up to 400 minutes. For all treatments except the ionic strength treatment, final solute concentrations were achieved by adding solute. For the NaCl incubation at the FF site, since the porewater was close to full strength seawater, 50% and 25% ambient concentrations (diluted with deionized water) were used to modify ionic strength. All sediments were then assayed for ANAMMOX, denitrification and DNRA according to the methods previously described.

	[Fe^{2+}] μM			**[H_2S] μM**			**[NaCl] ppt**		
DT	A	800 (2X)	1600 (4X)	A	5 (2X)	10 (4X)	A	32 (2X)	64 (4X)
RR	A	200 (2X)	400 (4X)	A	200 (2X)	400 (4X)	A	40 (2X)	80 (4X)
FF	A	50 (2X)	100 (4X)	A	20 (2X)	40 (4X)	A	25%	50%

Table 5: Schematic of the three sediments incubated with either ambient (A), two (2X) or four times (4X) ambient concentrations of the porewater analytes

RESULTS

Mesocosm Sediment Incubations: Range of Nitrate Cycling Rates and Gross Salinity Effects

Incubations were done on oligohaline (HB) and polyhaline (M35) sites only. The M61 sediment data is omitted due to a lack of water to circulate in the mesocosms at time of collection. Salinities in the mesocosms were 5, 14 and 26, for the low, medium and high salinity treatments. Higher rates of denitrification (up to 6.62 ± 0.11 nmol N g wet sed.$^{-1}$) and ANAMMOX (up to 0.92 ± 0.01 nmol N g wet sed.$^{-1}$) occurred in the upstream HB sediment that was incubated under the medium salinity and high salinity waters (Figure 9). Lowest values of denitrification and ANAMMOX occurred in the M35 sediment (0.77 and 0.10 nmol N gram wet sed^{-1}, respectively) under medium and low salinity treatments. There was an abrupt drop in activities in both sediments under all salinity treatments from days 15 to 21, with a rebound in activity at the day 54 time point in all but the HB high salinity treatment which steadily declined over time. In all treatments, ANAMMOX rates were approximately 10 fold less than denitrification rates. With the exception of the HB high salinity treatment, no clear salinity effect was observed on the removal rates in any of the treatments. The HB high salinity treatment showed steady decrease in ANAMMOX throughout the experiment, whereas denitrification rebounded slightly on the last sample period.

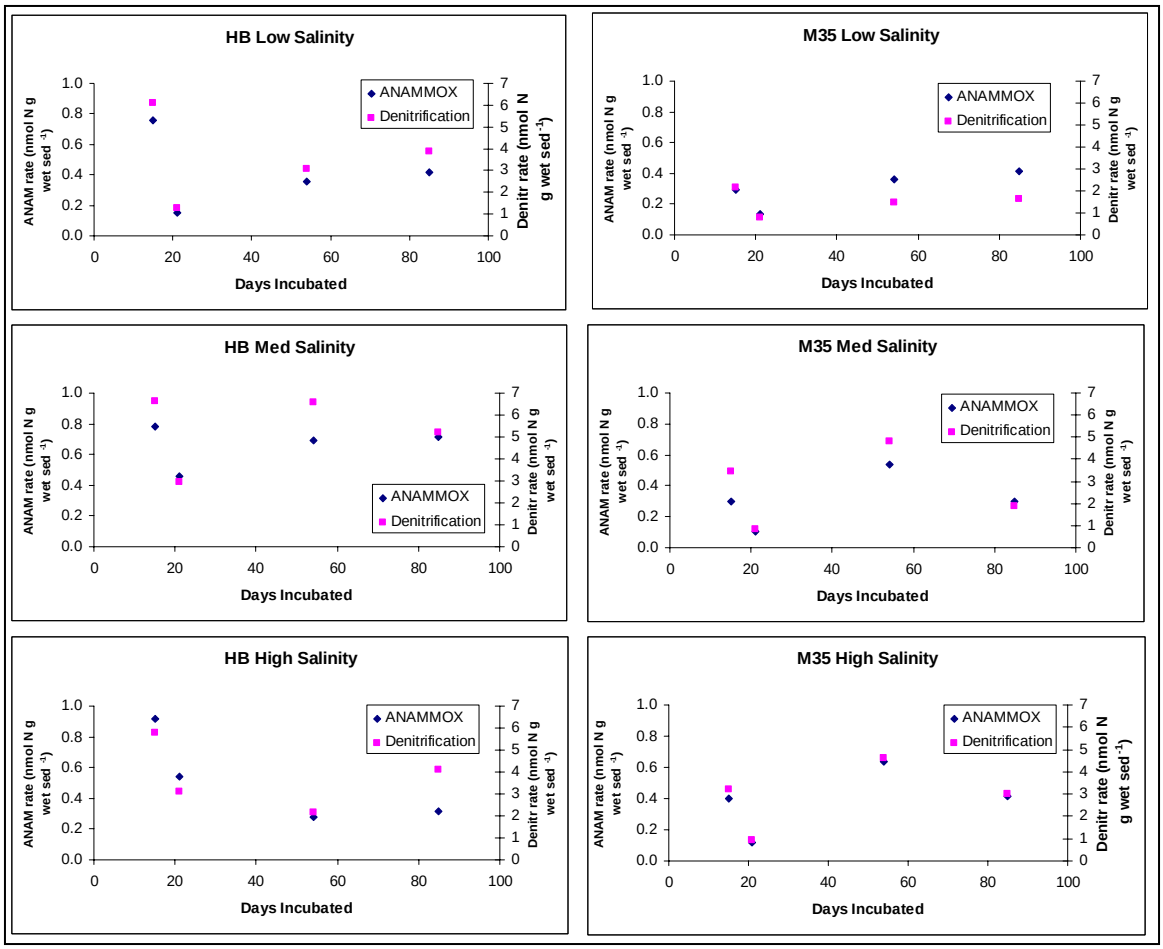

Figure 9: Mesocosm denitrification and ANAMMOX rates from two sediments (HB and M35) incubated with a control and two different water treatments

Porewater Chemistry Monitoring: Seasonal Porewater Solute Concentrations in the CFRE

Results in this section are reported from both porewater techniques, the Peeper and minipoint sampling methods. The minipoint concentrations differ from the Peeper concentrations mainly in the predominant form of reduced species at a given sample site (ie. Fe^{2+} for the DT site or H_2S for the FF site). This difference can be attributed to the variations in sampling methods. The Peepers sample via diffusion versus the minipoint method of macropore sampling. These differences have been previously observed by Harvey et al. (1993). We consider the Peeper data as the most indicative of the solute

pools most available for bacterial communities responsible for nitrogen cycling. Thus, only the Peeper data will be used in subsequent analysis of relationships between rates and porewater chemistry.

Fort Fisher (FF) was the most saline site (19 -37 ppt), River Road Park (RR) was the intermediate site (10 – 37 ppt) and a site just north of downtown Wilmington (DT) had the lowest salinity (2 - 26 ppt). Porewater analytes were measured seasonally, for comparison to the rate measurements, by Peepers and minipoint profilers. Peepers were analyzed within a week of sediment harvesting (November 2007, February 2008, May 2008 and August 2008). Minipoint profiles were generated three times, interspersed within the Peeper sampling (September 2007, January 2008 and June 2008). Seasonal porewater salinities pooled from both sampling methods were highest in September 2007 and lowest in May 2008 for all three sites (Figure 10).

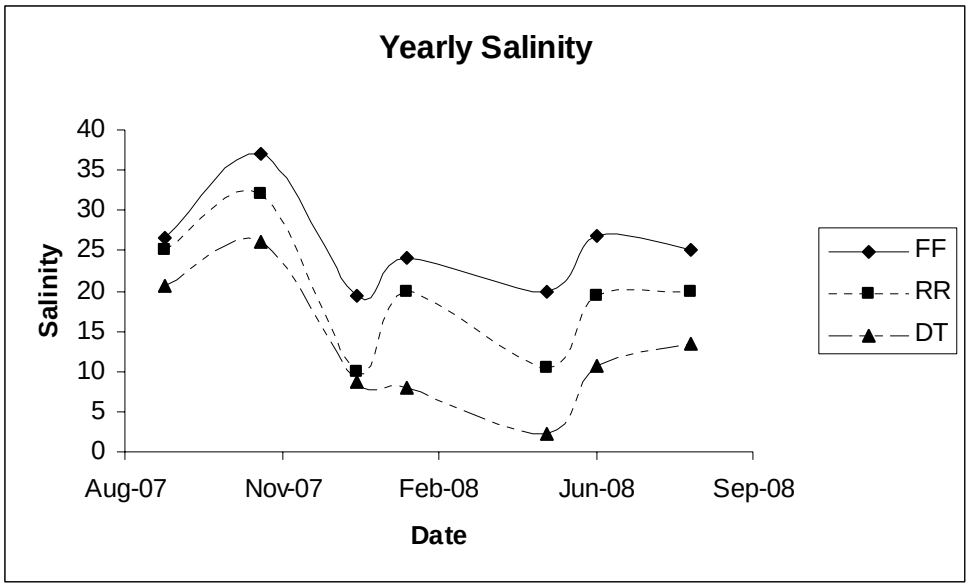

Figure 10: Yearly salinities from Peeper and minipoint 4 – 6 cm depths.

Profiles for the various analytes measured seasonally are shown in Figure 11 - Figure 17. Mean salinity increased, as expected, down the estuary. The range of salinity

variation decreased down-estuary. Porewater salinity was reset more quickly further upstream as is evident by discrete non-overlapping profiles with season (Figure 11) as observed at the DT site. While near surface porewater salinity changed discretely at the RR and FF sites, there was a more consistent salinity below a depth of about 5 cm and the deeper seasonal salinity profiles overlap at these depths more so than at the DT site.

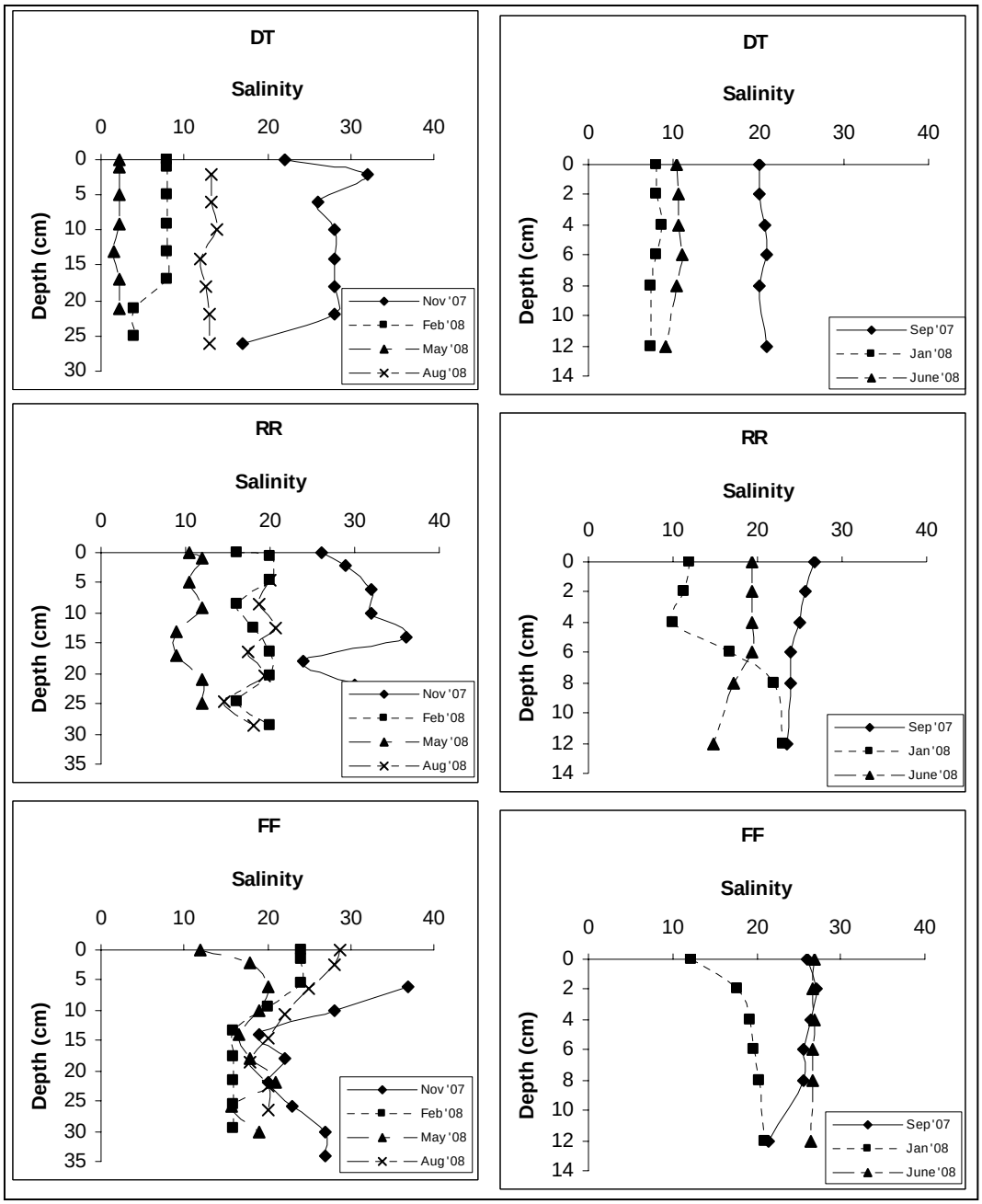

Figure 11: Yearly salinity profiles from the Peeper (L) and minipoint (R) samplers

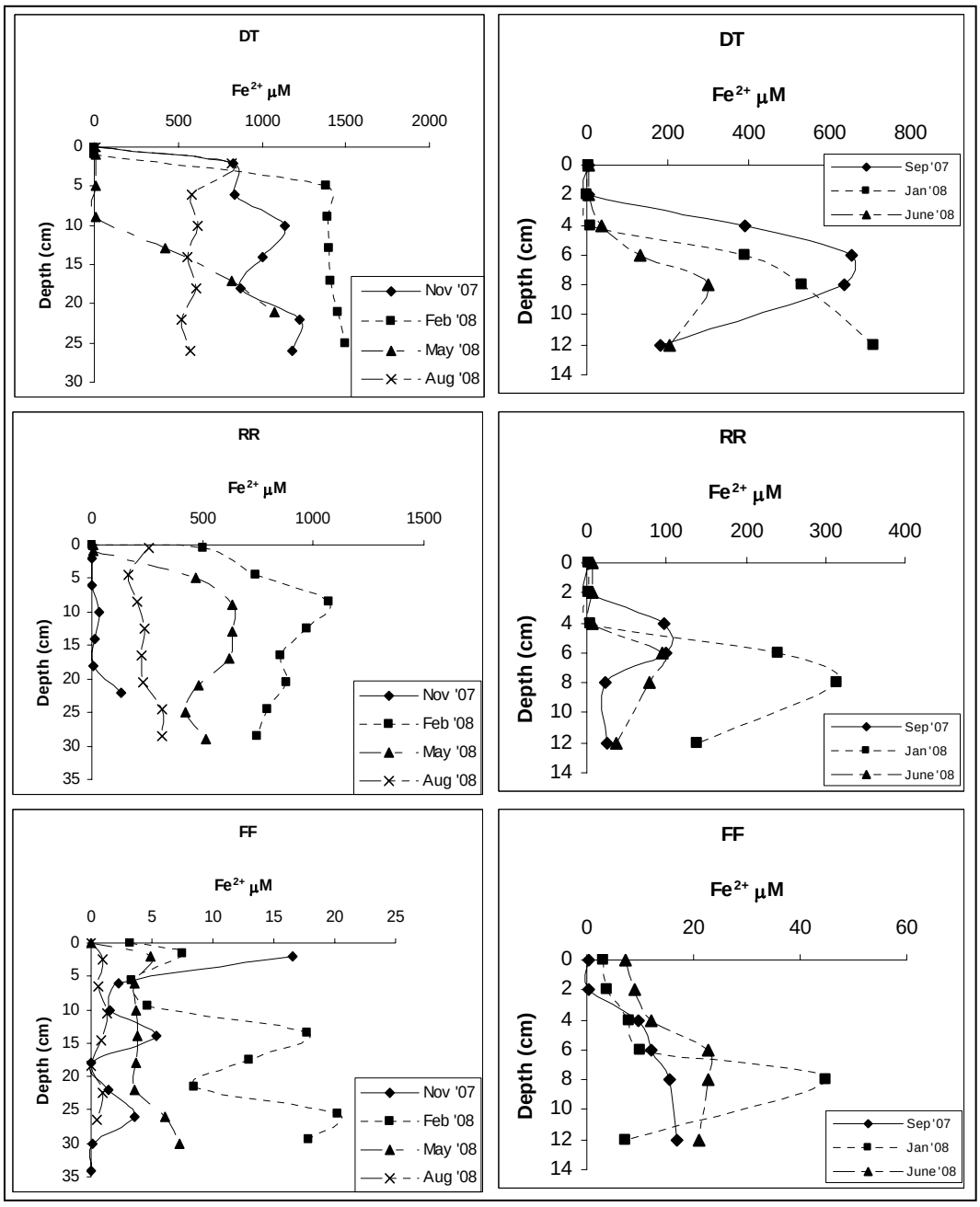

Figure 12: Peeper (L) and minipoint (R) profiles of Fe^{2+} from all three sites depicting yearly fluctuations

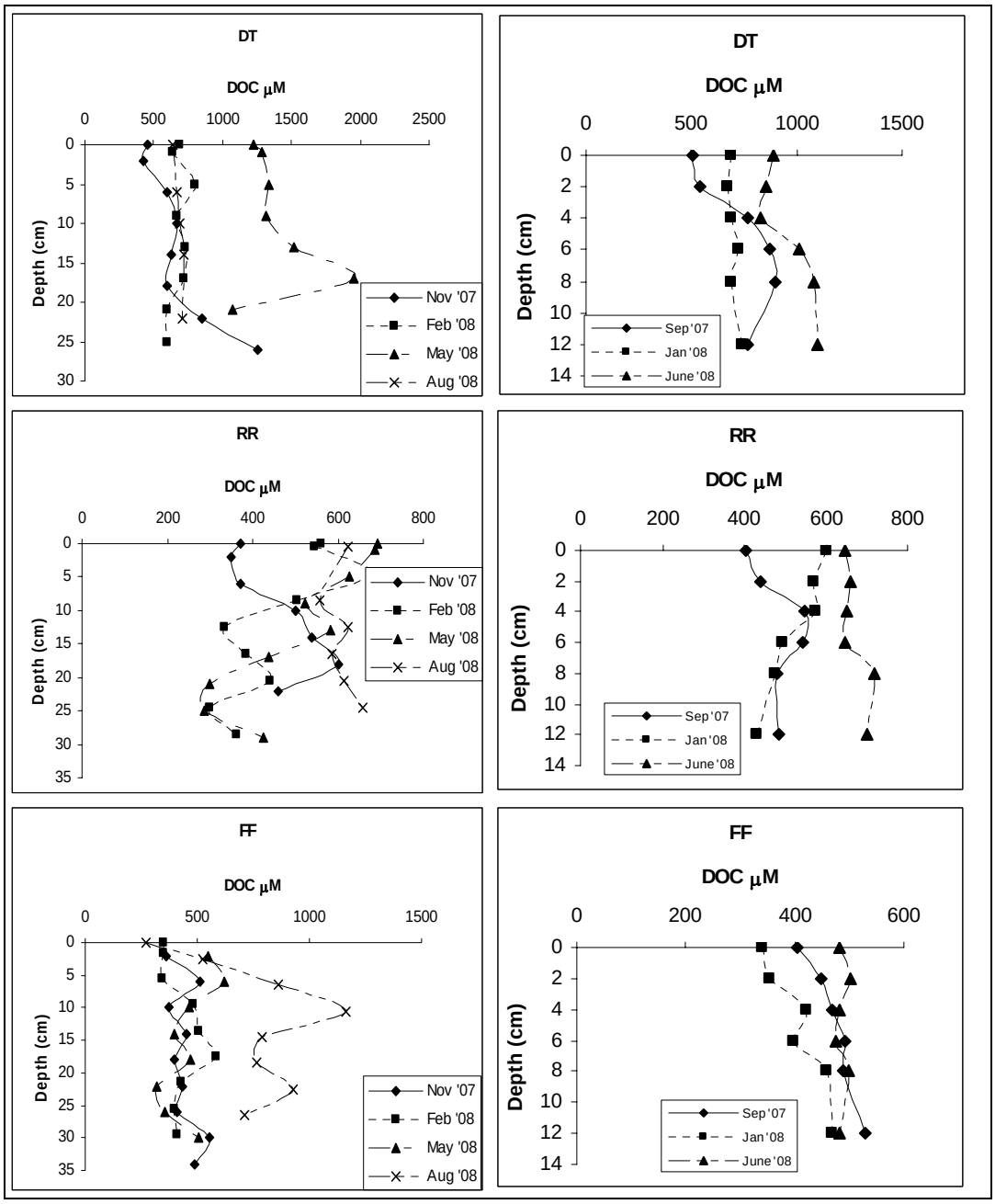

Figure 13: Yearly DOC profiles from the Peeper (L) and minipoint (R) samplers

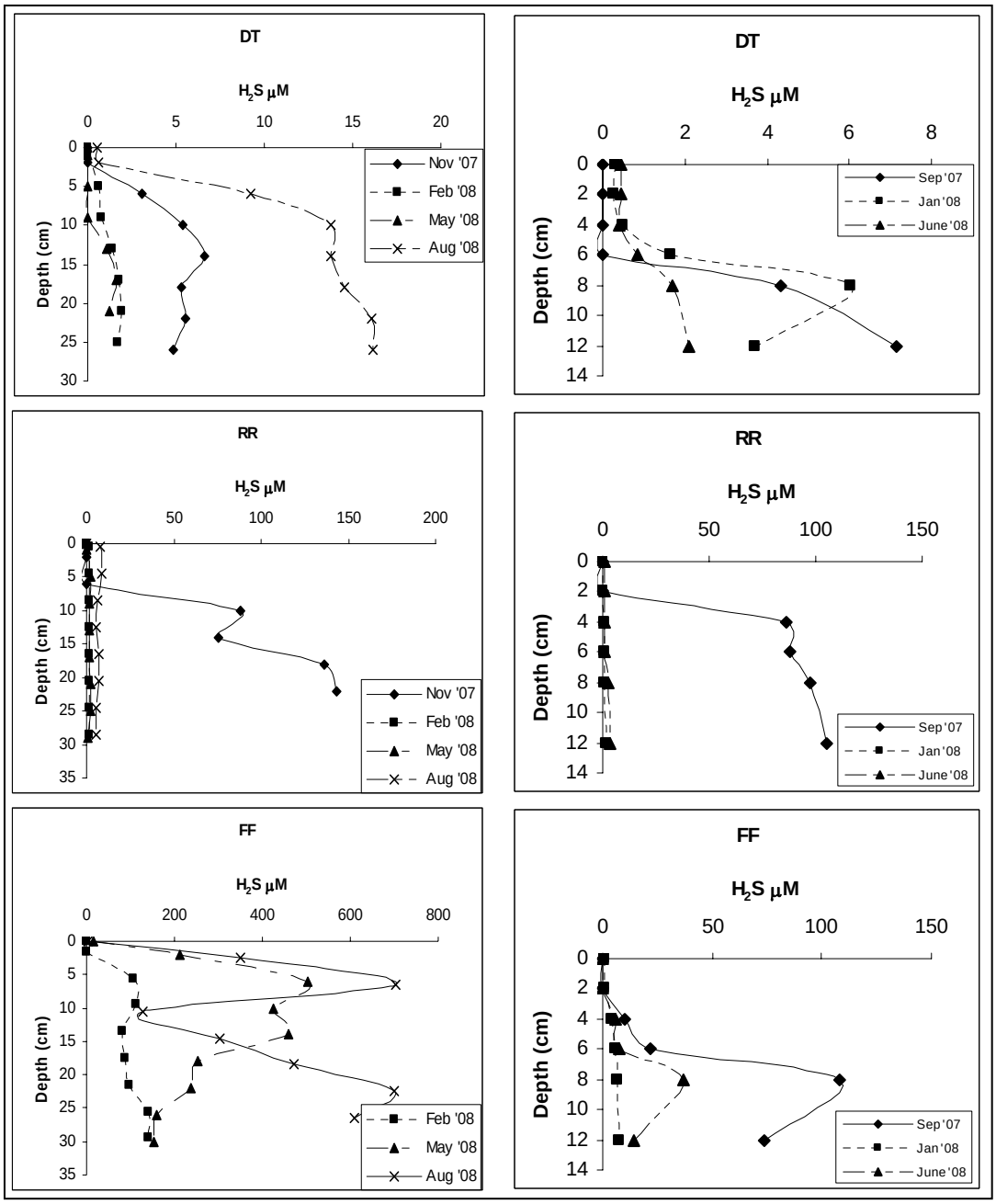

Figure 14: Yearly hydrogen sulfide profiles from the Peeper (L) and minipoint (R) samplers

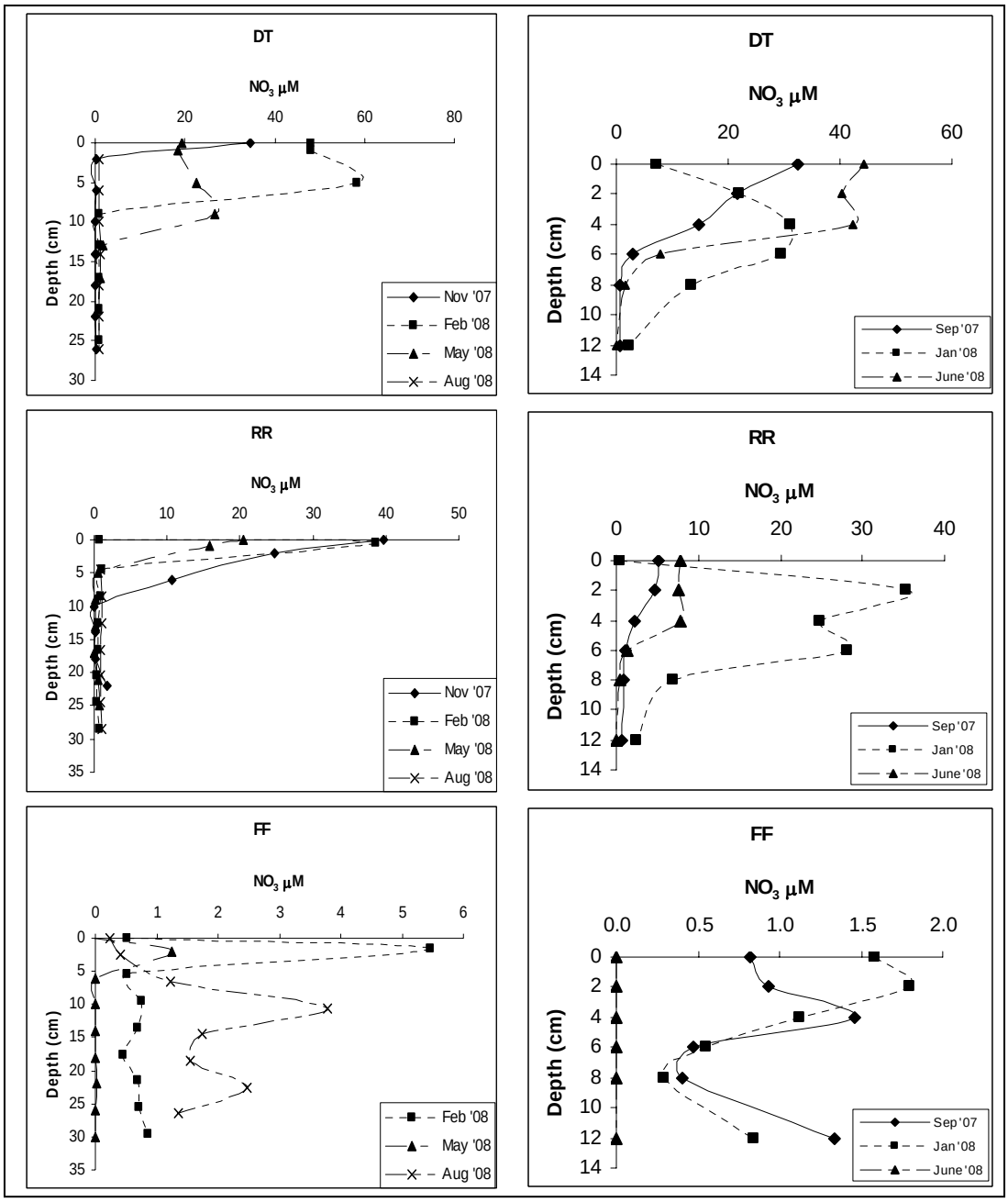

Figure 15: Nitrate profiles from the Peeper (L) and minipoint (R) samplers

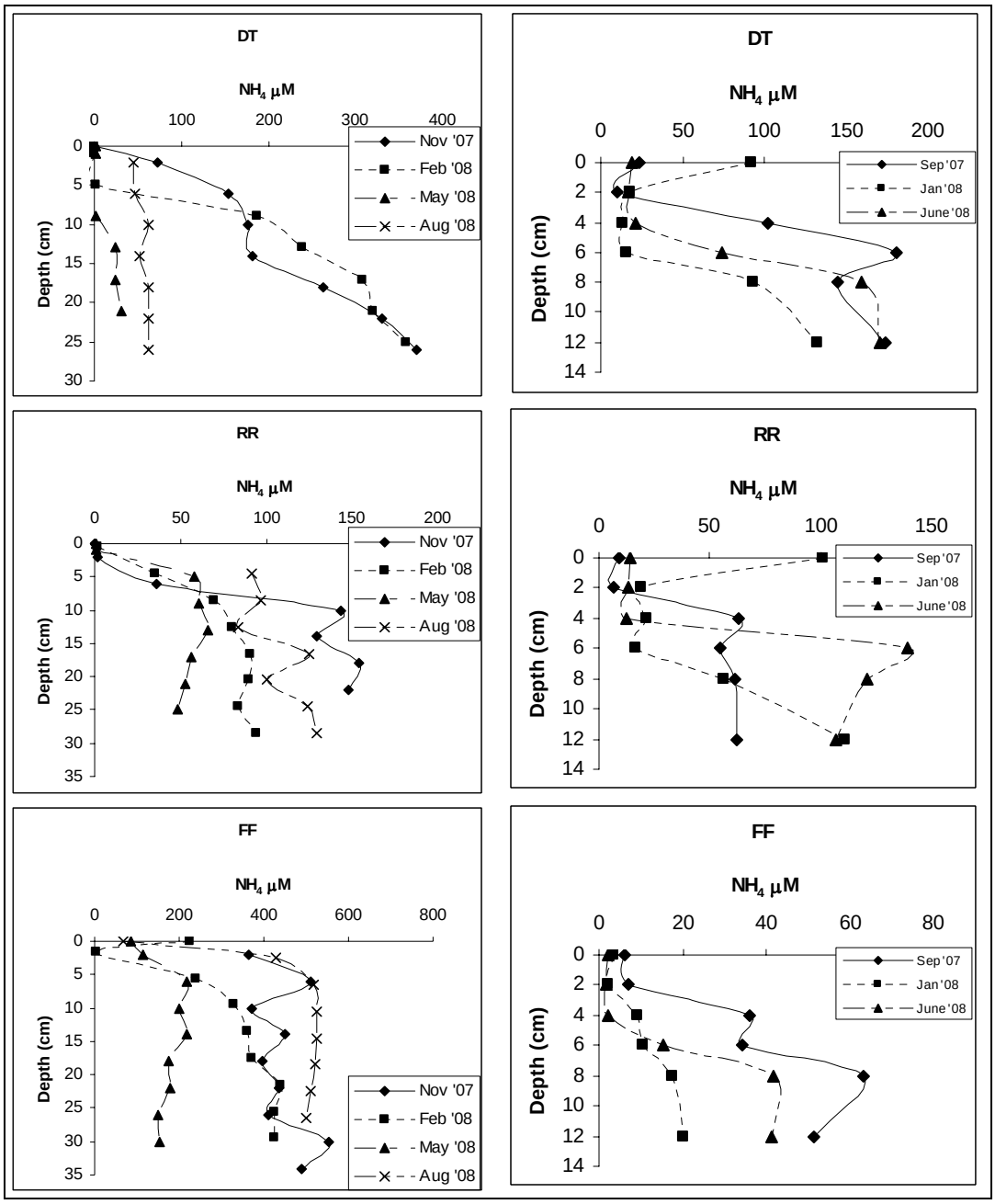

Figure 16: Ammonia profiles from the Peeper (L) and minipoint (R) samplers

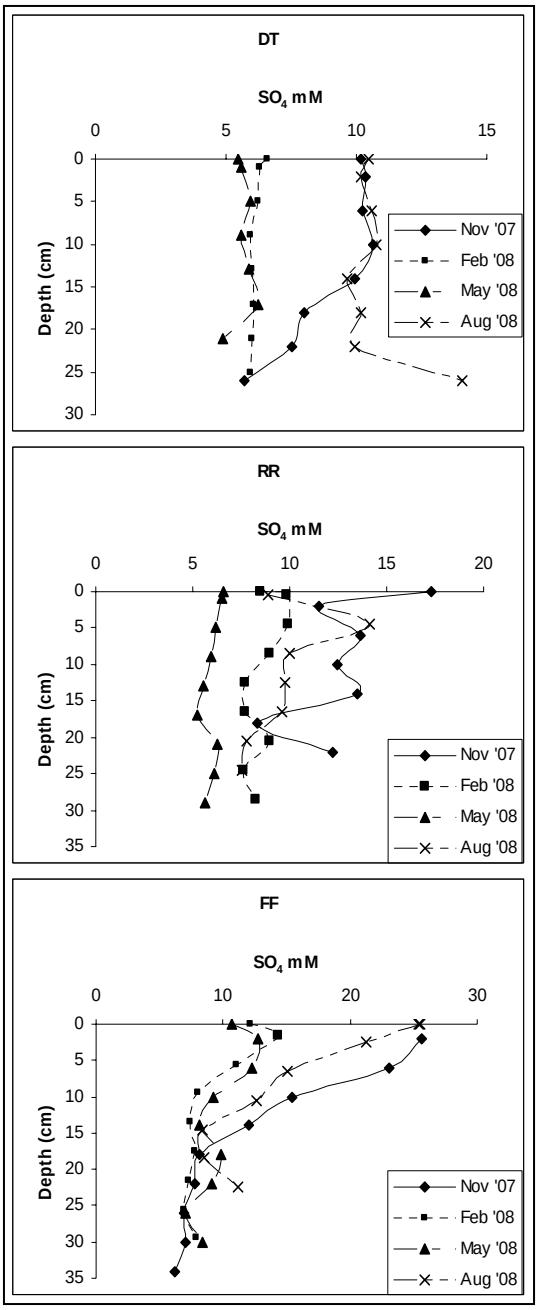

Figure 17: Yearly sulfate profiles from the Peeper sampler at all three sites

The Downtown site is characterized by the highest values of the three sites for Fe^{2+}, DOC, and nitrate, lowest values of the three sites for salinity, hydrogen sulfide and sulfate and variable concentration for ammonium. The salinity ranged from 1.5 to 32 (mean value of 12.8), with the minimum occurring in May of 2008 and the maximum in

November 2007 (Figure 11). The Fe^{2+} concentrations ranged from 0 μM to 1495 μM. The lowest values of Fe^{2+} were always near the surface and increased with depth, some seasons showed a mid-depth maximum while others did not. The highest Fe^{2+} concentrations were seen in the February 2008 profile, when salinities were relatively low and lowest concentration during the May and June 2008 profiles (Figure 12). Porewater DOC in the DT site ranged from 430 to 1957 μM. The lowest values occurred near the surface and had maximum values in the minipoint profiles in the 4 – 6 cm depth and the depths greater than 16 cm in the Peeper profiles. The highest concentrations of DOC were seen in May of 2008 the lowest values were during the November 2007 period (Figure 13). Nitrate is reduced very quickly in this environment, as is evident in the DT profiles, exhibiting maximum values (58 μM) near the surface and essentially disappearing below depths of 10 cm. The highest nitrate concentrations were seen in February 2008 and lowest values during August 2008 (Figure 15). Hydrogen sulfide was generally very low at this site ranging from undetectable to 16 μM. The month with the lowest hydrogen sulfide was May 2008 and the highest was during August 2008 (Figure 14). As would be expected, this same pattern is seen in sulfate with concentration ranges from 4.9 to 14.1 mM, which was about half saltwater concentration (minipoint sulfate was omitted due to oxidation of H_2S, which produced elevated SO_4^{2-} values). Ammonium generally was very low near the surface and increased with depth. This site displayed concentration ranges from 0 to 370 μM (Figure 16). The lowest concentrations were seen in May 2008 and maximum concentrations during the February 2008 period.

The mesohaline River Road site is characterized by intermediate concentrations of all porewater analytes compared to the Downtown and Fort Fisher sites. The RR site had

maximum and minimum salinities at the same times as the DT site, with values ranging from 9 to 36 with a mean value of 19.5 (Figure 11). The Fe^{2+} concentrations ranged from 1.2 to 1072 μM. The highest values were found in February 2008 and lowest values in November 2007 (Figure 12). DOC at the RR site ranged from 288 to 720 μM, with the lowest values occurring in February 2008 and highest values in June 2008 (Figure 13). The nitrate at the RR site peaked at the November 2007 period and was lowest at the August 2008 sample date, ranging from 0 to 40 μM (Figure 15). Hydrogen sulfide had a distinct maximum during the fall of 2007 (143 μM) and very low concentrations during the summer 2008 (1 μM). As with the DT site, sulfate at the RR site mimicked the hydrogen sulfide pattern (Figure 17). The highest values were observed during November 2007 (17.3 mM) and lowest concentrations during May 2008 (5.3 mM). Ammonium ranged from 0 to 154 mM, with the highest values occurring in November 2007 and lowest values in May 2008 (Figure 16).

The polyhaline Fort Fisher site had the highest values of salinity, hydrogen sulfide, sulfate and ammonium and lowest values of Fe^{2+}, nitrate and DOC. The FF site was the most saline throughout the year and also exhibited the same seasonal maximum and minimum measured at the other two sites, ranging from 12 to 37, but the highest mean salinity of 25.5 (Figure 11). The Fe^{2+} concentrations at this site ranged from 0 to 45 μM, with the highest values occurring in February 2008 and the lowest values in August 2008 (Figure 12). DOC had a minimum concentration of 342 μM in January 2008 and maximum concentration of 1162 μM in August 2008 (Figure 13). Nitrate at the FF site never exceeded 6 μM with the highest concentration in February 2008 (5.5 μM). Hydrogen sulfide at the FF site was undetectable in near surface porewaters but exceeded

700 μM in samples below 6 cm (Figure 14). The largest values of H_2S were found in August 2008 when temperatures were high, while the lowest were found in January 2008. Similarly, sulfate maxima and minima mirrored that of sulfide, ranging from 6.1 to 25.6 mM (Figure 17). The FF site showed the highest ammonium ranging from 2 to 550 μM with the highest values occurring in August 2008 and lowest values in January 2008 (Figure 16).

Seasonal variations in porewater analytes can primarily be attributed to the fluctuations in salinity. The highest concentrations of salinity usually coincided with maximums of hydrogen sulfide, sulfate and ammonium. The lowest concentrations of salinity generally coincided with maximums of DOC, nitrate and iron. When these conditions were not met, the maximums or minimums of any analyte lagged behind the corresponding maximum or minimum of salinity by no more than one sample period (three months). Profiles of the analytes maintained their general shape throughout the year for all species measured, indicating that redox zonation is a static feature at each site. Concentrations and trends in data were made for comparison with the recycling and removal rates measurements. Results showed that recycling (through DNRA) rates were highest downstream where concentrations of hydrogen sulfide, sulfate and ammonium were highest, whereas the removal rates (both ANAMMOX and denitrification) were greatest upstream where the DOC, iron and nitrate concentrations were highest.

Reaction Rate Determination in Fresh Sediments

Activities were measured on fresh sediments from each site during the course of the experiment, August 2007 to August 2008 (Figure 18). The range of ANAMMOX rates for oligohaline, mesohaline and polyhaline were 0.05-0.36, 0.11-0.48 and 0.01-0.14

nmol N g wet sed^{-1}, respectively. The annual ANAMMOX rates were highest at the mesohaline and oligohaline sties, (0.23 ± 0.16 and 0.15 ± 0.12 nmol N g wet sed^{-1}, respectively). The range of denitrification rates for the DT, RR and FF were 0.89-3.96, 0.80-4.66 and 0.24-1.56 nmol N g wet sed^{-1}, respectively. Annual rates of denitrification were greatest at the RR and DT sites (2.27 ± 1.55 and 2.16 ± 1.45 nmol N g wet sed^{-1}, respectively). The range of DNRA rates for the oligohaline, mesohaline and polyhaline sites were 0.34-2.30, 0.86-2.81 and 2.19-3.59 nmol N g wet sed^{-1}. Annually, for ANAMMOX and denitrification, rates at the DT and RR sites were statistically higher (Student t-test, p = 0.04 and 0.08, respectively) than the FF site, however they were not statistically significant from one another. The pattern of DNRA was opposite that of ANAMMOX and denitrification, with the rates of DNRA increasing with increasing salinity (up to 2.94 nmol N g wet sed^{-1}, annually, at the FF site). Generally, lower salinity sites (DT and RR) showed two fold higher rates of ANAMMOX and denitrification rates than the rates at the highest salinity site (FF). In contrast, annual averaged DNRA rates were two times higher at the FF site than at the RR and DT sites. These rates were also more pronounced during the summer time when water temperatures and salinity were highest. For all rate measurements, denitrification and DNRA were within a factor of five of each other whereas ANAMMOX was typically 10% that of denitrification.

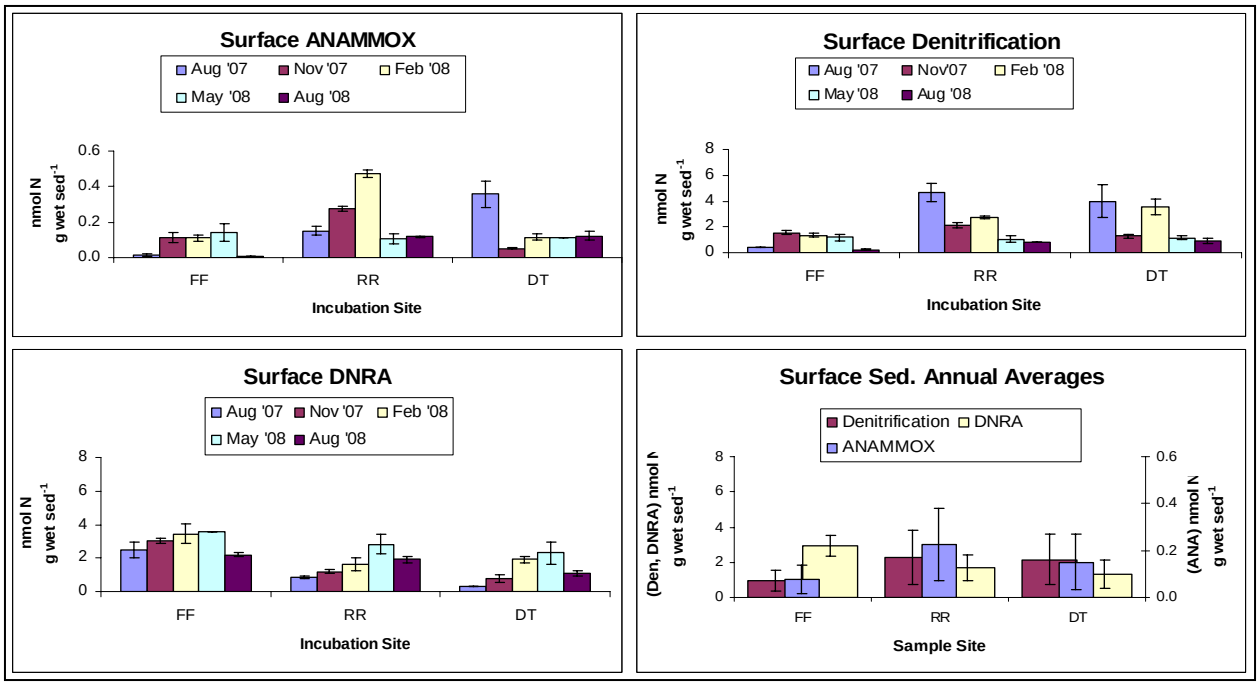

Figure 18: Fresh surface sediment rates for ANAMMOX, denitrification and DNRA, collected from each sample location. Error bar denote standard errors.

The annual pattern of recycling and removal rates shift moving up and down the estuary. Rates of removal (ANAMMOX and denitrification) decreased with down-estuary distance (Figure 19) while recycling rate increased with down-estuary distance. Removal rates accounted for 64% of the nitrate reduced at the DT site and accounted for only 26% at the FF site. Conversely, recycling rates accounted for 74% at the FF site decreasing to 36% at the DT site. However, the highest ANAMMOX contribution occurred at the RR site (5%).

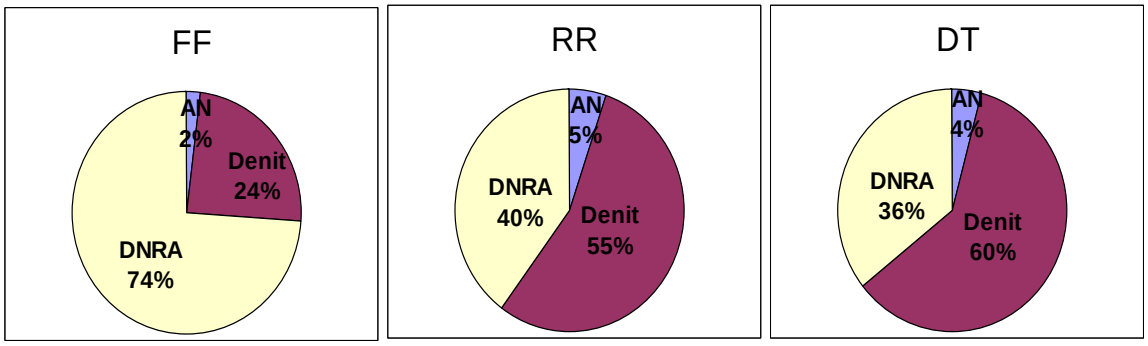

Figure 19: Pie charts representing 100% of the annualized reduced nitrate at the three CFRE locations.

Seasonal rates of the fresh surface sediment varied greatly between the recycling and removal rates. For most seasons, ANAMMOX either increased upstream or showed a mid estuary peak at the RR site. The mid estuary peak was observed most strongly in February 2008, but also occurred in November 2007. No estuarine gradient was seen in the ANAMMOX rates for May 2008. For all other times, the RR and DT sites were greater than the FF site, but were not statistically different from one another. Seasonality in ANAMMOX rates was seen in the RR site with a maximum occurring in February 2008. The FF site showed constant rates throughout the year with the exception of very low rates in August 2007 and 2008. In contrast, the DT ANAMMOX peaked in August 2007, but showed no discernable seasonality from then on. The estuarine gradient was strongest for denitrification in August 2007. With the exception of May 2008, the FF site was lower than the RR and DT sites, but the DT site was not greater than the RR site. Like ANAMMOX in November 2007, although to a lesser extent, denitrification showed a small mid estuary maximum. Otherwise, the mesohaline (RR) and oligohaline (DT) sites were not distinguishable from one another. As was seen in the ANAMMOX rates, the FF site showed some seasonality in denitrification whereby the November 2007, February 2008 and May 2008 well exceeded the low rates that were measured in August

2007 and 2008. No seasonal patterns in denitrification could be inferred for the RR and DT sites. The DNRA rates showed the strongest estuarine gradient in August 2007 and November 2007 when the FF rates were greater than the RR rates which were greater than the DT rates. Some seasonality was exhibited in DNRA rates at the RR and DT sites when the February 2008 and May 2008 rates were higher than the other months.

Reaction Rate Determination in Transplanted Sediment

The sediment taken from the three estuarine sites and incubated at its native location in the SEMs (ie. FF sediment incubated at the FF site) served as a test of artifacts within the SEMs (Figure 20), when compared with the fresh surface sediment. Like the fresh surface sediments, the native sediment shows the highest annual rates of ANAMMOX and denitrification at the oligohaline site (0.12 ± 0.05 and 2.84 ± 1.86 nmol N g wet sed^{-1}, respectively) and lowest value at the polyhaline site (0.07 ± 0.02 and 1.74 ± 0.45 nmol N g wet sed^{-1}). Similar to the fresh sediment rates, the highest annual rates of DNRA were found at the FF site (2.42 ± 1.26 nmol N g wet sed^{-1}) and decreased with decreasing salinity. In 7 of the 9 comparisons of native versus fresh sediments, the annual rates of the native sediment at each incubation site were not statistically significant (Student t-test, $p < 0.05$) from the annual fresh sediment rates which suggest that there is are minimal incubation artifacts. However, seasonal signals in the native incubation rates were not always consistent with the fresh sediment rates, the potential causes of which will be discussed later. The FF August 2008 sample point is omitted due to the SEM being vandalized prior to the final collection time.

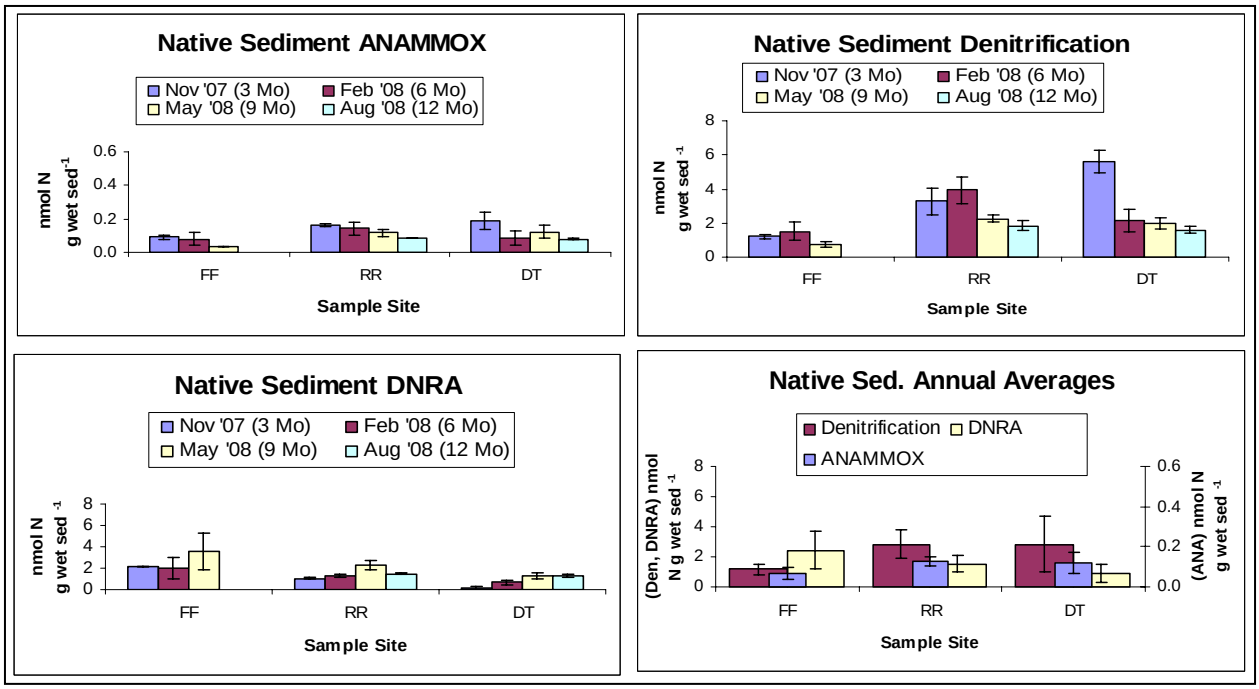

Figure 20: Native sediment, incubated in SEMs, rates for ANAMMOX, denitrification and DNRA, collected from each sample location. Error bars denote standard errors. This data is also shown in Figure 21, Figure 22 and Figure 23 as site sediment incubated at the same site.

Figure 21 through Figure 23 detail the transplant data at the DT, RR and FF sites throughout the course of the experiment. In some instances, the recycling and removal rates of transplanted sediment mimicked their incubation location. For example, the DT sediment incubated at the DT site had an initial (November 2007) ANAMMOX rate of 0.19 ± 0.05 nmol N g wet sed^{-1}(Figure 21) decreased when incubated at the FF site to 0.06 ± 0.02 nmol N g wet sed^{-1}. There were some seasonal effects observed, described below. Annually, there was no transplant effect on the aggregated recycling and removal rates, as is evident in the annual averages for the transplanted sediment for all three sites.

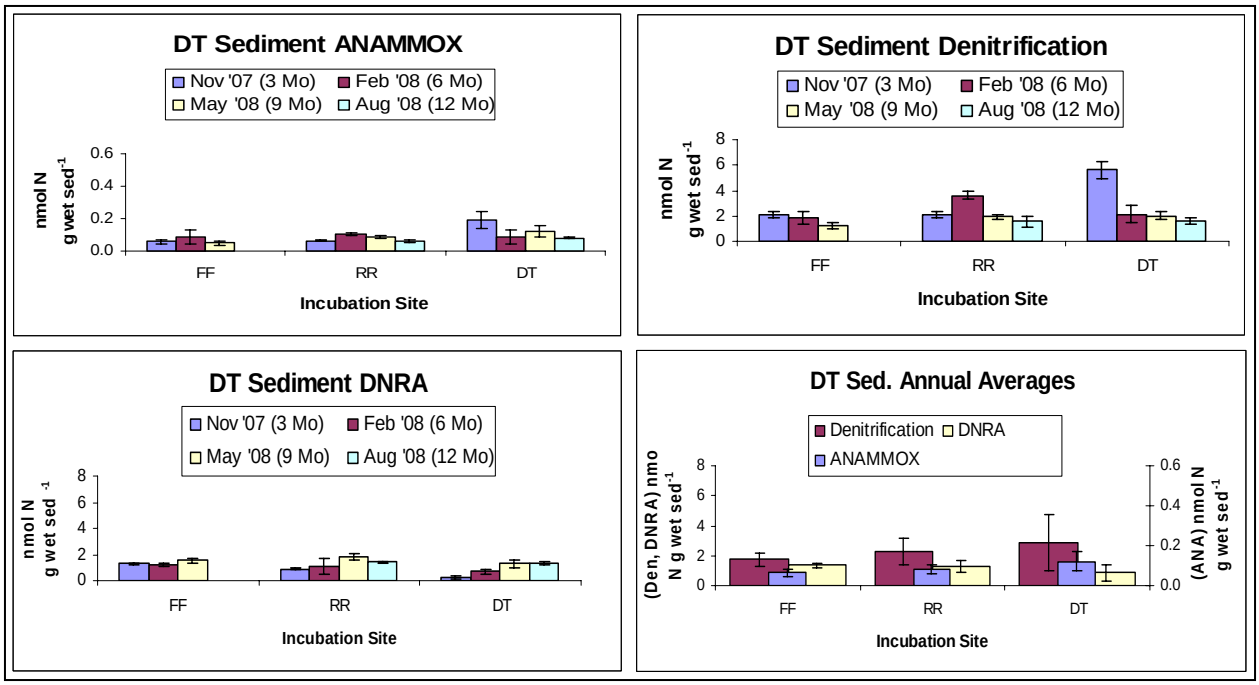

Figure 21: Downtown sediment, incubated in SEMs, rates for ANAMMOX, denitrification and DNRA, collected from each sample location. Error bars denote standard errors.

Seasonality for the DT transplanted sediment varied between reaction rates (Figure 21). There was a reduction in ANAMMOX rates in the initial (November 2007) rates from the DT to the FF sites. After the second sample period (February 2008) the ANAMMOX activity stabilized at all three locations. Similarly with ANAMMOX, the initial denitrification rates decreases from the native location to the downstream, FF site. With the exception of the February 2008 RR rate, denitrification rates stabilized after the second sample period. Conversely to patterns seen in the ANAMMOX and denitrification rates, DNRA rates increased during the initial sample period from the DT site to the FF site, after which, the sediment stabilized at all three locations.

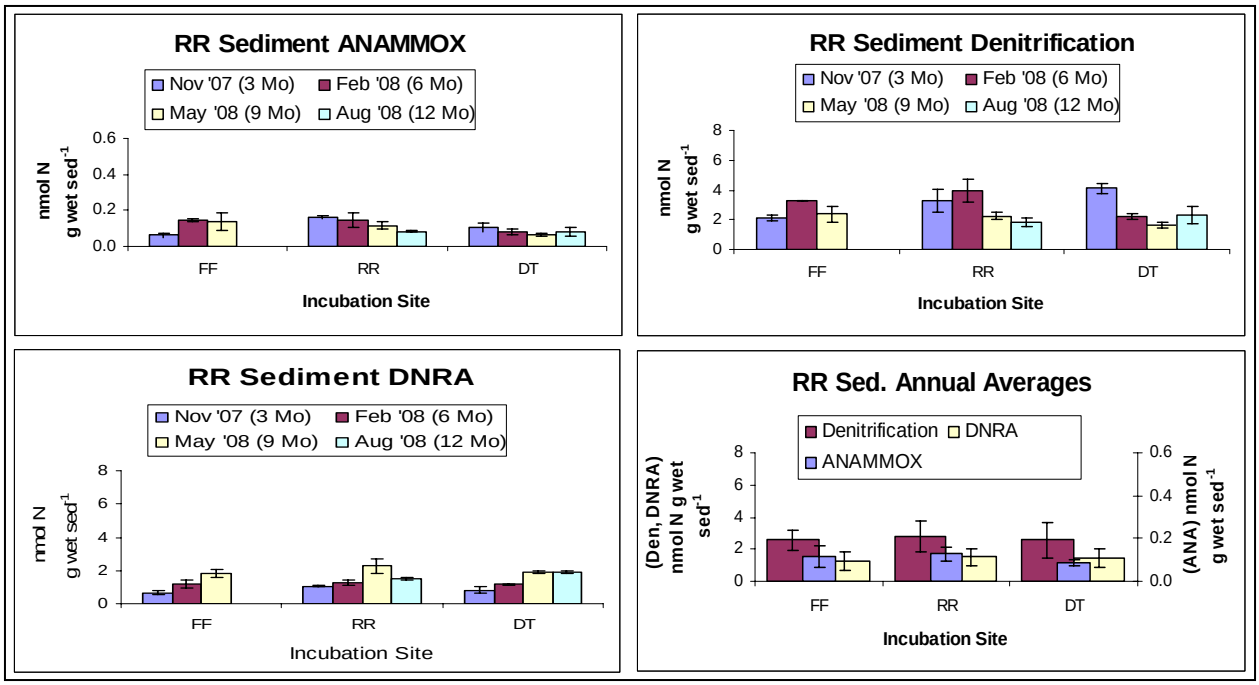

Figure 22: River Road sediment, incubated in SEMs, rates for ANAMMOX, denitrification and DNRA, collected from each sample location. Error bars denote standard errors.

The seasonality exhibited by the transplanted RR sediment was less distinctive than the DT sediment (Figure 22). The initial ANAMMOX rates decreased from the native location when incubated upstream or downstream, followed by an increase in the FF site incubation then a stabilization at all three sites from the May 2008 time point on. The initial RR denitrification rates decreased when incubated downstream (FF) and increased when incubated upstream (DT). Interestingly, the DT incubation for May 2008 showed the lowest rates of denitrification in all locations, before rebounding to rates similar to its native location in August 2008. The DNRA rates did not seem to exhibit any transplant effect throughout the experiment.

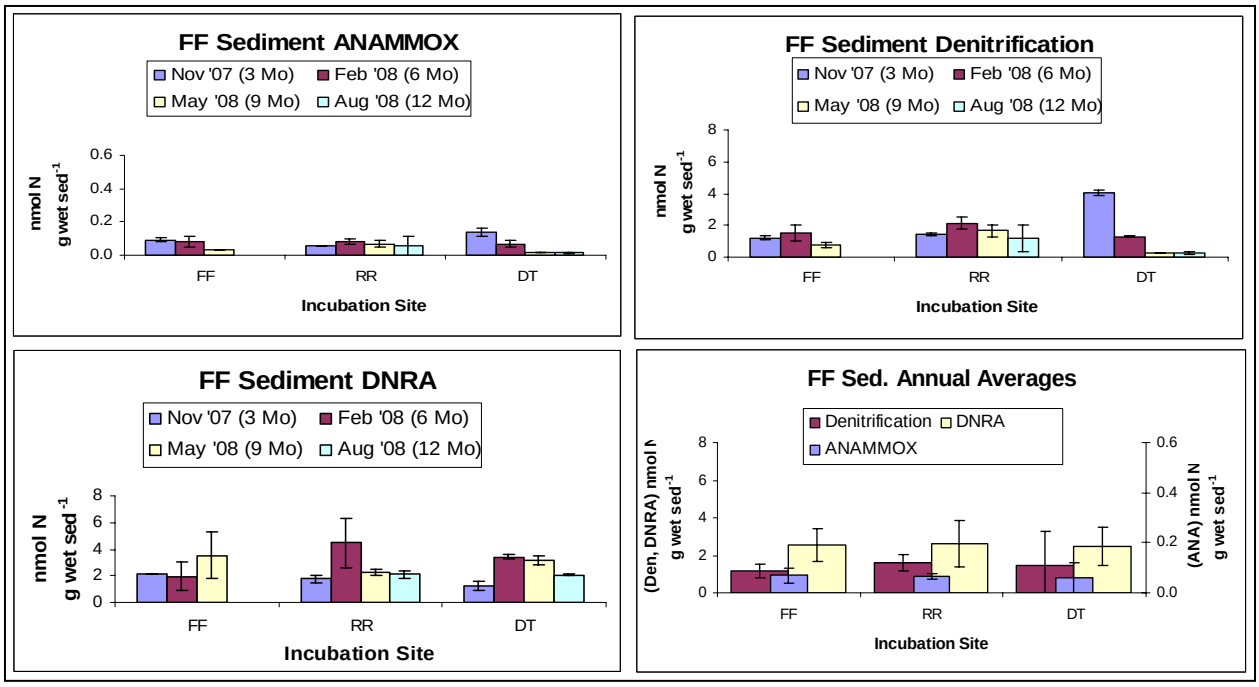

Figure 23: Fort Fisher sediment, incubated in SEMs, rates for ANAMMOX, denitrification and DNRA, collected from each sample location. Error bars denote standard errors.

The seasonality of the FF transplanted sediment were opposite those seen in the DT sediment. The initial ANAMMOX rates showed an increase at the DT incubation site, but not at the RR site, after which the rates stabilized for the February 2008 sample period, then dropped off from the May 2008 sample point onward. The initial denitrification rates increased from the native FF location to the DT incubation site. The rates increased during the February 2008 sample point for the FF and RR locations, but decreased at the DT site, after which the rate decreased in all incubation locations. The initial DNRA rates decreased from their native FF location moving upstream, but increased at these locations during the February 2008 sample period but decreased at the native location. From the May 2008 sample period onward, the DNRA rates appeared to stabilized for all locations.

The transplants examined the role of changing geochemistry while the import and export of the microbial community was held in place by the 0.2 micron membrane of the SEM. Rates in the transplants were still subject to both changes in geochemistry and in microbial community within the SEM. To address this issue, the controlled solute induced change in sediment rates experiment was designed to separate specific solute's direct and immediate impact on recycling and removal rates.

Controlled Solute Induced Changes on Sediment Rates: Specific Analyte Effect on Nitrate Recycling and Removal

This portion of the experiment was used to examine the individual solute effect on ANAMMOX, denitrification and DNRA rates. Because porewater analytes covary with salinity, ionic strength, ferrous iron and hydrogen sulfide were targeted as specific treatments. At the oligohaline site, the ANAMMOX rates were not affected by the addition of any of the three solutes and maintained a range of activity between 0.08 and 0.15 nmol N g wet sed^{-1} (Figure 24). Denitrification in DT sediment increased twofold from 1.8 ± 0.2 (ambient) to 3.6 ± 0.4 nmol N g wet sed^{-1} when Fe^{2+} was 4X ambient, consistent with our predictions. However, H_2S increased from 1.8 ± 0.2 to 3.7 ± 0.8 nmol N g wet sed^{-1} at 4X ambient concentrations, which was the inverse of our prediction. Higher denitrification in the DT incubations were seen in 2X ambient salt concentrations, increasing from 1.8 ± 0.2 to 4.4 ± 0.9 nmol N g wet sed^{-1}. DNRA rates for the DT sediment were not affected by changing solute concentrations and maintained a range of 0.8 to 1.9 nmol N g wet sed^{-1}.

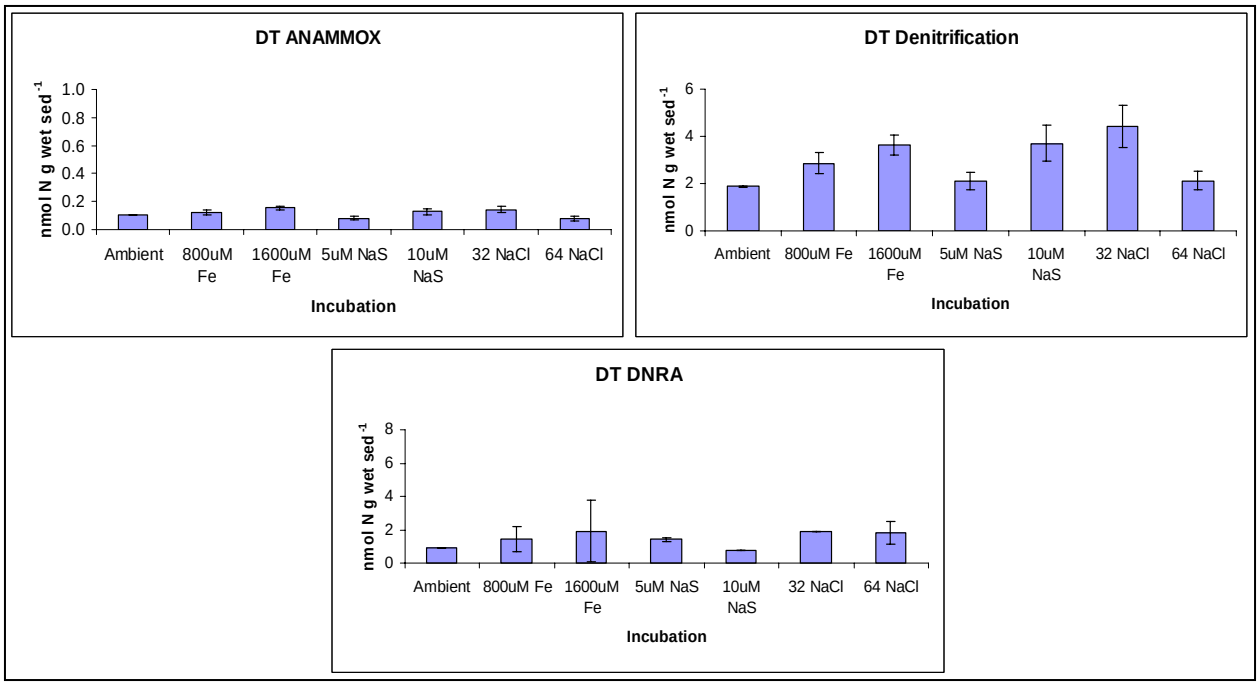

Figure 24: Synoptic Inhibitions for the DT sediment

For the mesohaline (RR) sediment, ANAMMOX rates were not affected by any solute or ionic strength incubations and were consistently 0.2 nmol N g wet sed^{-1} (Figure 25) in all NaCl treatments. For denitrification rates, all additions of the 2X ambient Fe^{2+}, H_2S and NaCl decreased the rates by almost 50% (from 3.3 ± 0.3 to 1.7 ± 0.1 nmol N g wet sed^{-1}). However, the 4X ambient concentrations appeared to have no change from the ambient rate (3.3 ± 0.3 nmol N g wet sed^{-1}). Rates of DNRA were unaffected by additions of iron, H_2S or sodium chloride and were consistent at about 3.3 nmol N g wet sed^{-1}.

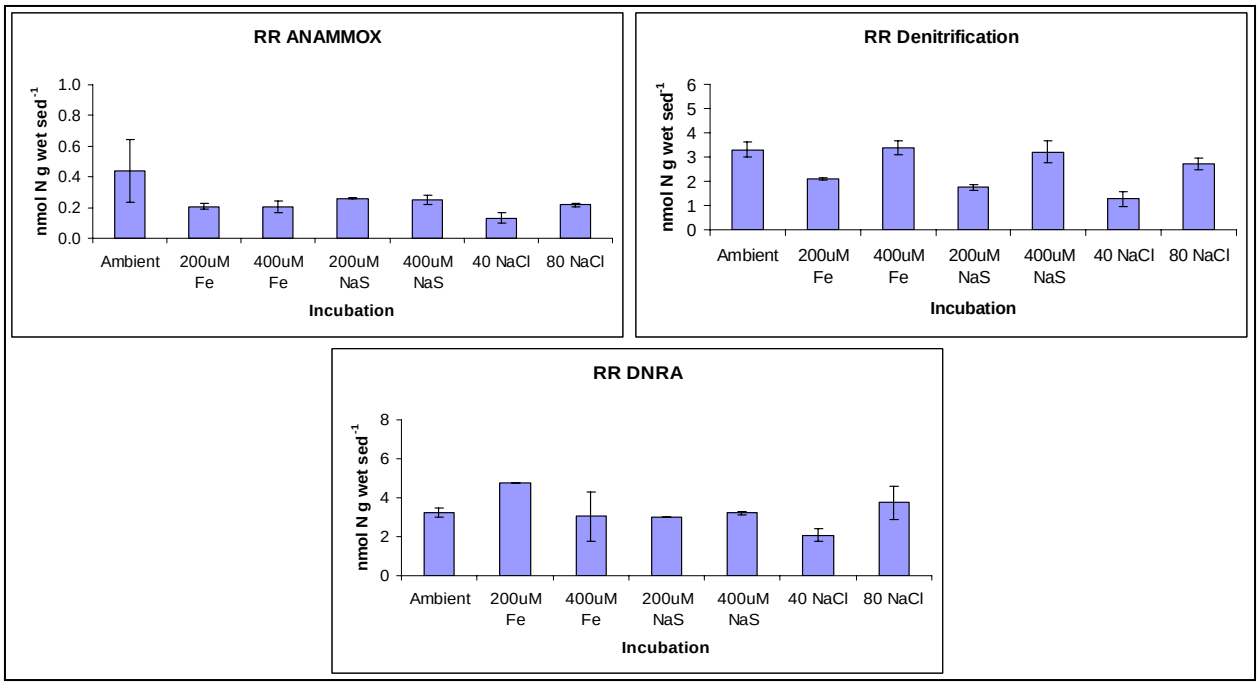

Figure 25: Synoptic Inhibitions for the RR sediment

For the polyhaline (FF) sediment incubations, rates of ANAMMOX were not affected by any of the additions and maintained rates around 0.6 nmol N g wet sed^{-1}. Rates of denitrification decreased from the ambient concentration value of 2.3 ± 0.7 nmol N g wet sed^{-1}in all treatments by 30 to 68%. Rates of DNRA were unaffected by the any solute additions and maintained values of 5.2 ± 0.8 nmol N g wet sed^{-1}.

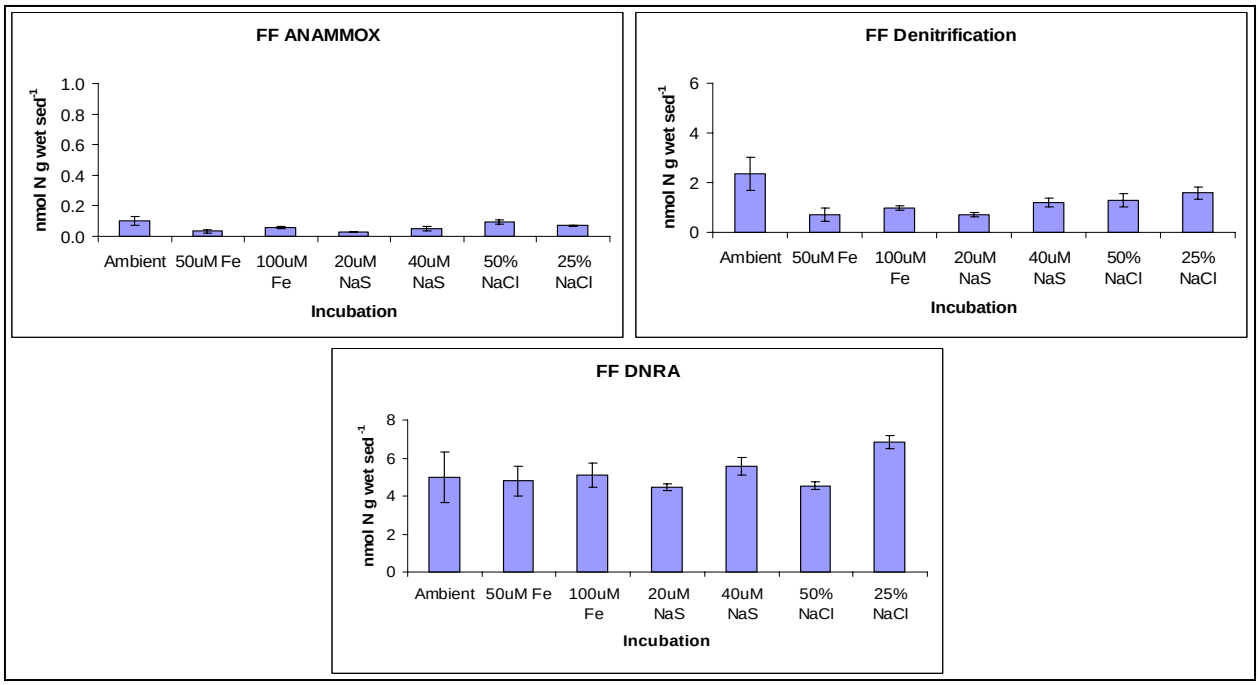

Figure 26: Synoptic Inhibitions for the FF sediment

Synoptic inhibition results exhibited some changes in the recycling and removal rates. However, these changes were not clearly caused by different concentrations of Fe^{2+}, H_2S or NaCl, as the 2X concentration may have induced a decrease in rate whereas the 4X treatment showed no effect (or a rate higher than the 2X rate). These results were determined to be inconclusive for reasons discussed below.

DISCUSSION

The upper and middle reaches of the CFRE showed a net removal of nitrate through the dominance of ANAMMOX and denitrification, and the lower estuary displayed a net recycling of nitrate through dominance of DNRA. Spatial differences in denitrification and DNRA not withstanding, these two reactions were responsible for roughly equal amounts of nitrate reduction, whereas ANAMMOX rates were about 10 fold less than those of denitrification and DNRA. Rates of denitrification and

ANAMMOX were linearly correlated over time and space (Figure 27). No clear seasonal variations in any of the reaction rates were found. Principle component analysis (PCA) indicated that denitrification covaried with NO_3^-, Fe^{2+} and DOC and DNRA covaried with H_2S, SO_4^{2-} and NH_4^+ but ANAMMOX showed only weak relationships to porewater chemistry (Figure 28). Despite the spatial connection between some rates and *in situ* porewater chemistry, rates were not uniformly affected by induced changes in porewater salinity, Fe^{2+} or H_2S in laboratory manipulations (controlled solute induced changes on sediment rates portion). Likewise, no annual changes in rates could by induced by transplanting sediments from one part of the estuary to another with different geochemistry.

Recycling and removal rates are principally governed by available substrates, potential inhibition caused by other porewater solutes, and the microbial community. Nitrate availability was a factor of ten larger in the upper estuary (Figure 15) compared to the lower estuary and nitrate limitation of denitrification has been demonstrated in numerous coastal environments (Lohse et al. 1996; Rysgaard et al. 1998, Jenkins and Kemp 1984). Cabrita and Brotas (2000) observed that denitrification in a Portuguese estuary was driven by river nitrate input. Similar results have been seen in wetland denitrification (Merrill and Cornwell 2000). High nitrate concentrations support denitrifying bacteria communities, and provide limiting substrate in the organic-rich sediments of the CFRE. The upper estuary, where denitrification was highest, also contains the lowest amount of sulfide. Joye and Hollibaugh (1995) reported that the high H_2S levels inhibit nitrifying bacteria that normally fuel denitrifying bacteria with nitrate. Senga et al. (2006) found that H_2S may directly inhibit denitrifying bacteria. In high

hydrogen sulfide environments such inhibition of denitrifying bacteria may allow the DNRA pathway to dominate overall nitrate reduction. The DNRA pathway was greatest in the lower estuary where concentrations of hydrogen sulfide were ten-fold higher than the upper estuary (Figure 14). ANNAMOX and denitrification were found to be linearly correlated over time and space, with the magnitude of ANAMMOX rates 5-10 % less than that of denitrification rates. This fractional contribution of ANAMMOX to N_2 production agrees with studies in similar estuarine environments (Dalsgaard et al. 2005). This correlation may be due to a codependence by ANAMMOX and denitrification for labile organic matter. Trimmer et al. (2003) found that rates of ANAMMOX were positively correlated with organic matter content in sediments. These same results of high organic matter coupled to high rates of denitrification were seen in an English estuarine study by Trimmer et al. (2000). Organic matter supplies the electrons for nitrate reduction in denitrification, but is also responsible for supplying ammonium to sediment porewater through its subsequent mineralization which may then supply ANAMMOX. High rates of mineralization accompanying high organic matter create a highly reducing, low oxygen environment necessary for the ANAMMOX pathway to function (Hamersley et al. 2007). Additionally, denitrification may serve as a source of nitrite (an intermediate in the denitrification process) to ANAMMOX bacteria which would explain an enhancement of ANAMMOX in the presence of denitrification.

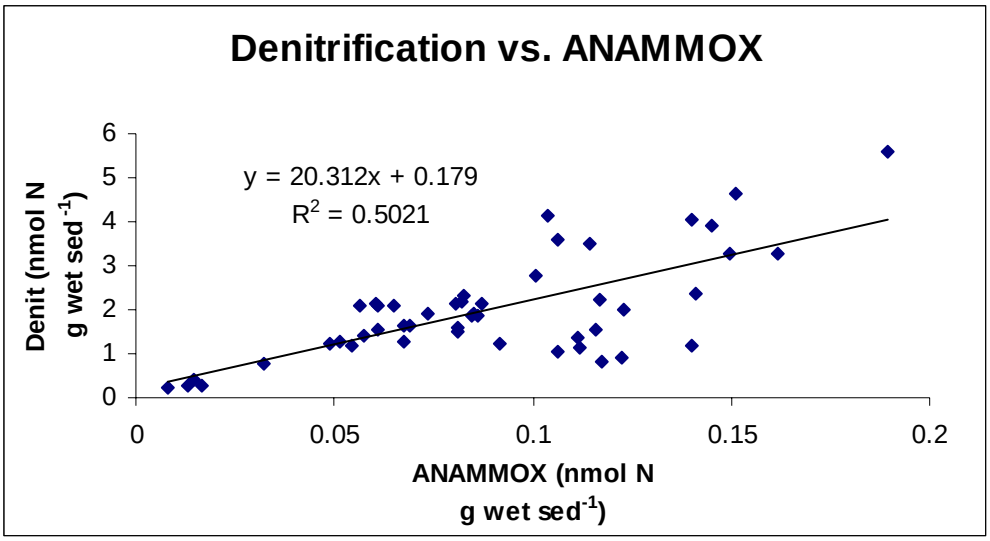

Figure 27: Correlation between denitrification and ANAMMOX rates for 48 of the 55 rate measurements run throughout the experiment ($p < 0.000001$).

PCA was used to determine any significant relationships between porewater analytes and recycling and removal rates (Figure 28). Surface rates determined from fresh sediment measurements over the four collection times were combined with the measurements from the Peeper depths of 4 – 6 cm for all of the analytes. Covariance was found between nitrate concentrations and denitrification rates (Figure 28); this agrees with the findings of many studies including Cabrita and Brotas (2000). Denitrification rates also covaried with concentrations of Fe^{2+} and DOC, which were coincident with higher nitrate concentrations in the upper CFRE. These elevated concentrations are typical of the CFRE, which is an organic-rich estuary with high allochthonous DOC levels in its upper reaches. The high Fe^{2+} in the sediment of the upper CFRE can serve as a direct electron source for denitrification (Appelo and Postma 1996), although its role in ANAMMOX is ambiguous. Denitrification exhibited negative covariance with sulfate, hydrogen sulfide and ammonium (Figure 28). Joye and Hollibaugh (1995) suggest that denitrification is indirectly inhibited by the effect of H_2S decreasing nitrification which

fuels nitrate for denitrification. PCA analysis showed positive covariance of DNRA with H_2S. In addition, DNRA correlated linearly with H_2S ($R^2 = 0.22$, $p = 0.056$), sulfate ($R^2 = 0.03$, $p < 0.0001$) and ammonium concentrations ($R^2 = 0.28$, $p = 0.01$). Gardner et al. (2006) found that DNRA rates increased with increasing salinity and the increased salinity would imply increased hydrogen sulfide and sulfate levels, which would explain this relationship. Elevated sulfate levels fuel sulfate reduction (under reducing conditions) and produce hydrogen sulfide as well as compete with denitrification for available electron donors. Ammonium is the end product of DNRA, and higher DNRA rates down estuary are consistent with higher ammonium concentrations at the FF site (Figure 16). Interestingly, ANAMMOX rates did not appear to covary in the PCA with the analytes measured. The estuary is a gradient with certain porewater species dominating near the mouth (NH_4^+, H_2S and SO_4^{2-}) whereas other species dominate in the lower salinity reaches (Fe^{2+}, NO_3^- and DOC). The lack of porewater solute maximums or minimums in the mesohaline region explains the lack of covariance with maximum ANAMMOX rates found there. This region provides an environment for microbial communities that operate best under moderate porewater concentration ranges but dynamic conditions. Thus, ANAMMOX may be best suited for non-extreme, mid estuarine conditions. An ANAMMOX maximum in the mid CFRE region was also seen in a study by Dale et al. (2008).

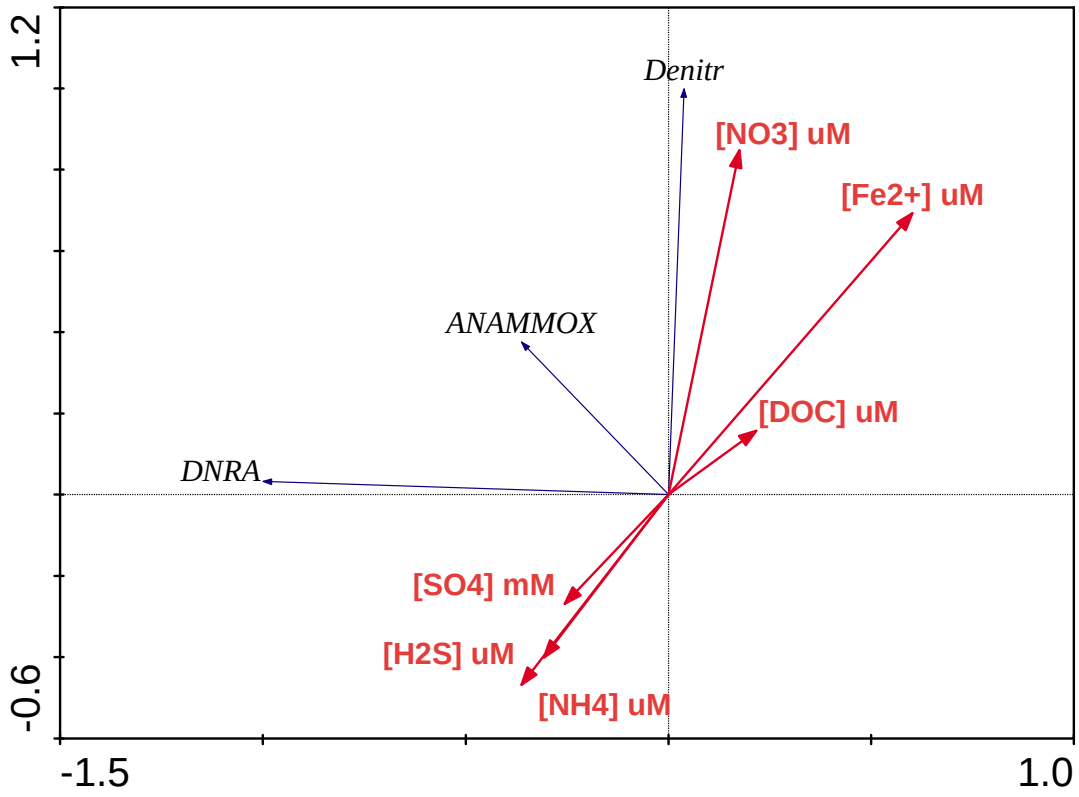

Figure 28: PCA plot of the seasonal surface denitrification, ANAMMOX and DNRA versus various parameters taken from the seasonal Peeper measurements.

Despite the spatial pattern in rates consistent with spatial differences in geochemistry, seasonal changes in rates did not coincide with seasonal changes in geochemistry. Temperature may play a role in rate regulation but was not measured in the field, however all rate determinations were conducted in the laboratory and measured at the same temperature. The porewater analytes measured throughout the course of this study exhibited seasonal fluctuations. The highest values of salinity were found during November 2007 for all sites and the lowest salinity occurred during May 2008. The general profile trends at the oligo, meso and polyhaline sites maintained the same form throughout the year for all analytes measured. Concentrations of these analytes shifted

due to variable penetration of saltwater into the estuary. Maximum values of hydrogen sulfide/sulfate coincided (or lagged by no more than a month) with peaks in salinity. Higher concentrations of hydrogen sulfide typically coincided with lower concentrations of Fe^{2+} or vice versa, as Fe^{2+} is scavenged by hydrogen sulfide to form iron monosulfides or pyrite (Taillefert et al. 2000). Salinity may also affect the concentration of ammonium in estuarine waters. Gardner et al. (1991) suggest that ion pairing in salt water promotes the release of ammonium from sediments. Desorption of ammonium has also been shown in resuspended sediment, subsequently releasing larger amounts of this solute in estuarine environments (Morin and Morse 1999). When these processes are coupled with higher rates of DNRA in more saline waters, concentrations of ammonium may be much higher than expected in downstream environments. Unusually high concentrations of nitrate were found at the RR and DT sites and may be attributed to high rates of nitrification as ammonium was sufficiently high enough to fuel nitrification at these sites, thus maintaining robust denitrification and ANAMMOX communities that can use this nitrate/nitrite.

The lack of seasonal changes in rates with seasonal changes in geochemistry could be attributed to differences in sediment type which influences microbial community, surface area and gas diffusion. However, sites were selected to normalize for similar grain size, and carbon to nitrogen ratios at different sites ranged from 10.4 to 12.3 which implies a similar sediment composition between sites. A simpler explanation may be that spatial differences in porewater chemistry between sites were much greater than seasonal fluctuations in chemistry at any given site. For instance, Fe^{2+} concentrations at the DT site had minimum values (below 8 cm depth) of about 500 μM

whereas the maximum concentrations at the FF site (below 8 cm depth) were 20 μM (Figure 12). This pattern was also seen for nitrate where the DT surface values had an annual minimum of 8 μM and the maximum surface values at the FF were 5.5 μM (Figure 15). The opposite pattern was exhibited in the hydrogen sulfide profiles where the minimum FF values (below 5 cm depth) were around 100 μM and the maximum values at the DT site (below 5 cm depth) were 15 μM (Figure 14). The mesohaline site's concentration ranges were always in between the oligohaline and polyhaline sites despite seasonal variation. These spatial differences in porewater solute ranges may serve as boundaries for certain microbial communities and cause heterogeneity of the bacterial species in the sediment.

Initial results of the transplanted sediment appeared to demonstrate that these microbial communities could adapt to and mimic the characteristics of sediment native to the location. These results were seen in the November 2007 sample period for upstream (DT) sediment incubated at a downstream (FF) location decreasing in ANAMMOX and denitrification capability and increasing in DNRA capability. Similar results were also seen when sediment from a downstream location (FF) was taken and incubated at an upstream site (DT) which showed increases in the ANAMMOX and denitrification rates and decreases in DNRA rates. However, these transplant effects appeared to wash out over time, and eventually showed characteristics of their native location (ie. high recycling rates for downstream sediment and high removal rates for up and mid stream sediment). This may be due to a bottle effect due to the behavior of the native sediments, which served as a control, showed this same effect.

Attempts to induce changes in rates by manipulating porewater (Fe^{2+}, H_2S and NaCl) proved unsuccessful as there were few changes in rates outside of the observed variances (Figure 24-Figure 26). These few differences could not be correlated to multiple concentration changes. Causes for this may be due to wrong choices of solutes, insufficient incubation time or concentrations of the solutes insufficient to induce an effect. The solutes used in this experiment have received the most attention in the literature, but other solutes may play a role in inhibiting or enhancing recycling and removal rates, such as manganese or nitrite. Luther et al. (1997) suggested that manganese may directly oxidize NH_4^+ to N_2 in sediments and therefore decrease nitrification and ANAMMOX rates due to less availability of reactive substrates. Strous et al. (1999) showed a complete inhibition of ANAMMOX bacteria in the presence of 0.1 g/L (~2.1 mM) nitrite. Concentrations were small relative to past studies, but realistic given observed changes in the estuary. Concentrations for the inhibition and enhancement were chosen from the initial SEM diffusion measurements which yielded low values for H_2S. Sulfide concentrations at the FF site had a maximum value of about 700 μM, therefore the 4X ambient concentration (40 μM) did not exceed the range that this site experiences throughout the year. However, the concentrations for the NaCl and Fe^{2+} trials should have been sufficiently high enough to induce a change in rates if they were true inhibitors. Direct geochemical control on recycling and removal rates would have been seen in an inhibition or enhancement in our experiments. However, if the geochemistry exhibits control on the microbial community, a much longer incubation time (days to weeks) would be needed to establish the new microbial community.

Tsushima et al. (2007) report ANAMMOX doubling time to be 3.6 days, which was much longer than our maximum incubation of 7 hours.

Spatial differences in rates suggest that geochemistry is important, but may be exerted through a change in the microbial community and not by impacting the reaction directly. Ultimately, a lack of clear response to changing geochemistry in the lab is similar to a lack of seasonal changes in rates when geochemistry varied seasonally and by the inability to change rates by transplanting sediments. We suggest that the microbial community is structured by the geochemistry and it is the community structure that ultimately regulates rates.

CONCLUSION

Our results indicate that the rates of recycling and removal of nitrate in the CFRE were inversely related throughout the estuary. Nitrate removal rates were greatest upstream at H_2S poor, nitrate rich environments. Nitrate recycling rates were highest when salinity levels were elevated and subsequently, H_2S and sulfate levels are elevated. ANAMMOX and denitrification were found to be directly correlated across the entire estuary. Rates of nitrate recycling and removal did not demonstrate a significant change when transplanted or display a clear seasonal signal with changing geochemistry. Most likely, rates of nitrate recycling and removal in the CFRE are governed by the microbial community throughout the estuary, which is established by salinity control on porewater speciation and availability.

REFERENCES

An, S. and Gardner, W.S., 2002. Dissimilatory nitrate reduction to ammonium (DNRA) as a nitrogen link, versus denitrification as a sink in shallow estuary (Laguna Madre/Baffin Bay, Texas). Marine Ecology Progress Series, 237: 41-50.

Appelo, C.A.J. and Postma, D., 1996. Geochemistry, groundwater and pollution. A. A. Balkema, Rotterdam, The Netherlands.

Arrigo, K.R., 2005. Marine microorganisms and global nutrient cycles. Nature, 437(7057): 349-355.

Cabrita, M.T. and Brotas, V., 2000. Seasonal variation in denitrification and dissolved nitrogen fluxes in intertidal sediments of the Tagus estuary, Portugal. Marine Ecology Progress Series, 202: 51-65.

Cline, J.D., 1969. Spectrophotometric Determination of Hydrogen Sulfide in Natural Waters. Limnology and Oceanography, 14(3): 454-458.

Cloern, J.E., 2001. Our evolving conceptual model of the coastal eutrophication problem. Marine Ecology Progress Series, 210: 223-253.

Cornwell, J.C., Kemp, W.M. and Kana, T.M., 1999. Denitrification in coastal ecosystems: methods, environmental controls, and ecosystem level controls, a review. Aquatic Ecology, 33(1): 41-54.

Dafner, E.V., Mallin, M.A., Souza, J.J., Wells, H.A. and Parsons, D.C., 2007. Nitrogen and phosphorus species in the coastal and shelf waters of Southeastern North Carolina, Mid-Atlantic U.S. coast. Marine Chemistry, 103(3-4): 289-303.

Dale, O., Tobias, C. and Song, B., 2008. Linking Community Structure of Anaerobic Ammonium Oxidizing (ANAMMOX) Bacteria to Their Activities in Cape Fear River Estuary. Environmental Microbiology, Submitted.

Dalsgaard, T., Thamdrup, B. and Canfield, D.E., 2005. Anaerobic ammonium oxidation (anammox) in the marine environment. Research in Microbiology, 156(4): 457-464.

Duff, J.H. et al., 1998. A Mini Drivepoint Sampler for Measuring Pore Water Solute Concentrations in the Hyporheic Zone of Sand-Bottom Streams. Limnology and Oceanography, 43(6): 1378-1383.

Eaton, A., Clesceri, L. and Greenberg, A. (Editors)1995. Sulfate: Turbidimeteric Method. In: Standard Methods for the Examination of Water and Wastewater, pp. 136-137.

Galloway, J.N. et al., 2003. The Nitrogen Cascade. Bioscience, 53(4): 341-356.

Gardner, W.S. et al., 2006. Nitrogen fixation and dissimilatory nitrate reduction to ammonium (DNRA) support nitrogen dynamics in Texas estuaries. Limnology and Oceanography, 51(1 part 2): 558–568.

Gardner, W.S., Seitzinger, S.P. and Malczyk, J.M., 1991. The Effects of Sea Salts on the Forms of Nitrogen Released From Estuarine and Freshwater Sediments: Does Ion Pairing Affect Ammonium Flux. Estuaries, 14(2): 157-166.

Hamersley, M.R. et al., 2007. Anaerobic ammonium oxidation in the Peruvian oxygen minimum zone. Limnology & Oceanography, 52(3): 923-933.

Hansell, D.A., 1993. Results and observations from the measurement of DOC and DON in seawater using a high-temperature catalytic oxidation technique. Marine Chemistry, 41(1-3): 195-202.

Harvey, J., Chambers, R. and Hoelscher, J., 1995. Preferential flow and segregation of porewater solutes in wetland sediment. Estuaries, 18(4): 568-578.

Herbert, R.A., 1999. Nitrogen cycling in coastal marine ecosystems. FEMS Microbiology Reviews, 23: 563-590.

Hesslein, R.H., 1976. An in Situ Sampler for Close Interval Pore Water Studies. Limnology and Oceanography, 21(6): 912-914.

Holmes, R.M., McClelland, J.W., Sigman, D.M., Fry, B. and Peterson, B.J., 1998. Measuring $^{15}N\text{-}NH_4^+$ in marine, estuarine and fresh waters; An adaptation of the ammonia diffusion method for samples with low ammonium concentrations. Marine Chemistry, 60: 235-243.

Howarth, R.W. and Teal, J.M., 1979. Sulfate reduction in a New England salt marsh. Limnology & Oceanography, 24: 999-1013.

Jenkins, M.C. and Kemp, W.M., 1984. The coupling of nitrification and denitrification in two estuarine sediments. Limnology & Oceanography, 29(3): 609-619.

Joye, S.B. and Hollibaugh, J.T., 1995. Influence of Sulfide Inhibition of Nitrification on Nitrogen Regeneration in Sediments. Science, 270(5236): 623-625.

Kartal, B. et al., 2007. *Candidatus* "Anammoxoglobus propionicus" a new propionate oxidizing species of anaerobic ammonium oxidizing bacteria. Systematic and Applied Microbiology, 30(1): 39-49.

Lohse, L., Kloosterhuis, H.T., van Raaphorst, W. and Helder, W., 1996. Denitrification rates as measured by the isotope pairing method and by the acetylene inhibition technique in continental shelf sediments of the North Sea. Marine Ecology Progress Series, 132: 169-179.

Luther, G.W., Sundby, B., Lewis, B.L., Brendel, P.J. and Silverberg, N., 1997. Interactions of manganese with the nitrogen cycle: Alternative pathways to dinitrogen. Geochimica et Cosmochimica Acta, 61(19): 4043-4052.

Merrill, J.Z. and Cornwell, J.C., 2000. The role of oligohaline marshes in estuarine nutrient cycling. In: M.P. Weinstein and D.A. Kreeger (Editors), Concepts and Controversy in Tidal Marsh Ecology. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp. 425-442.

Miller, A.E.J., 1999. Seasonal Investigations of Dissolved Organic Carbon Dynamics in the Tamar Estuary, U.K. Estuarine, Coastal and Shelf Science, 49(6): 891-908.

Morin, J. and Morse, J.W., 1999. Ammonium release from resuspended sediments in the Laguna Madre estuary. Marine Chemistry, 65: 97-110.

Mulder, A., van de Graaf, A.A., Robertson, L.A. and Kuenen, J.G., 1995. Anaerobic ammonium oxidation discovered in a denitrifying fluidized bed reactor. FEMS Microbiology Ecology, 16(3): 177-184.

Quan, Z.-X. et al., 2008. Diversity of ammonium-oxidizing bacteria in a granular sludge anaerobic ammonium-oxidizing (anammox) reactor. Environmental Microbiology, 10(11): 3130-3139.

Rysgaard, S., Risgaard-Petersen, N. and Sloth, N.P., 1996. Nitrification, denitrification, and nitrate ammonification in sediments of two coastal lagoons in Southern France. Hydrobiologia, 329(1): 133-141.

Rysgaard, S. et al., 1998. Seasonal carbon and nutrient mineralization in a high-Arctic coastal marine sediment, Young Sound, Northeast Greenland. Marine Ecology Progress Series, 175: 261-276.

Seitzinger, S.P., 1988. Denitrification in freshwater and coastal marine ecosystems: Ecological and geochemical significance. Limnology & Oceanography, 33(4 part 2): 702-724.

Senga, Y., Mochida, K., Fukumori, R., Okamoto, N. and Seike, Y., 2006. N_2O accumulation in estuarine and coastal sediments: The influence of H_2S on dissimilatory nitrate reduction. Estuarine, Coastal and Shelf Science, 67(1-2): 231-238.

Solorzano, L., 1969. Determination of Ammonia in Natural Waters by the Phenolhypochlorite Method. Limnology and Oceanography, 14(5): 799-801.

Stookey, L.L., 1970. Ferrozine---a new spectrophotometric reagent for iron. Anal. Chem., 42(7): 779-781.

Strous, M., Kuenen, J.G. and Jetten, M.S.M., 1999. Key Physiology of Anaerobic Ammonium Oxidation. Appl. Environ. Microbiol., 65(7): 3248-3250.

Taillefert, M., Bono, A.B. and III, G.W.L., 2000. Reactivity of freshly formed Fe(III) in synthetic solutions and (pore)waters: Voltametric evidence of an aging process. Environmental Science and Technology, 34: 2169-2177.

Thamdrup, B. and Dalsgaard, T., 2002. Production of N_2 through Anaerobic Ammonium Oxidation Coupled to Nitrate Reduction in Marine Sediments. Applied and Envrironmental Microbiology, 68(3): 1312-1318.

Trimmer, M., Nedwell, D.B., Sivyer, D.B. and Malcolm, S.J., 2000. Seasonal benthic organic matter mineralisation measured by oxygen uptake and denitrification along a transect of the inner and outer River Thames estuary, UK. Marine Ecology Progress Series, 197: 103-119.

Trimmer, M., Nicholls, J.C. and Deflandre, B., 2003. Anaerobic Ammonium Oxidation Measured in Sediments along the Thames Estuary, United Kingdom. Appl. Environ. Microbiol., 69(11): 6447-6454.

APPENDIX

Two continental shelf sites were sampled in a cruise that occurred in June 2008 aboard the *R/V Cape Hatteras*. The sites were sampled for sediment using a multicorer. Fresh sediment rates were determined from the continental shelf sites, MC4A and CFP2 (Figure 2), and showed ANAMMOX two times lower (0.08 N g wet sed^{-1}) than the FF site rate, the nearest estuarine site (Figure 29). Similarly, rates of denitrification at these shelf sites were lower than in the CFRE by a factor of eight (0.4 N g wet sed^{-1}). Despite lower rates, ANAMMOX rates at the shelf sites represented the same portion of N_2 production as at the CFRE sites (7 – 9%).

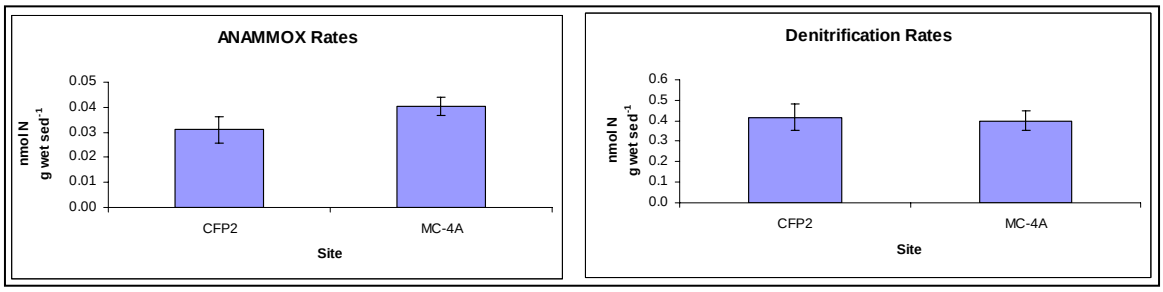

Figure 29: Rate measurements from June 2008 continental shelf sediments. Rates of ANAMMOX were: CFP2 = 0.03 ± 0.01, MC4A = 0.04 ± 0.00 nmol N g wet sed^{-1}. Denitrification rates were: CFP2 = 0.42 ± 0.07, MC4A = 0.40 ± 0.05 nmol N g wet sed^{-1}