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ABSTRACT

Tidal and seasonal fluctuations in the oligohaline reaches of estuaries may alter geochemical features that influence structure and function of microbial communities involved in sedimentary nitrogen (N) cycling. In order to evaluate sediment community responses to short-term (tidal) and long-term (seasonal) changes in different tidal regimes, nitrogen cycling rates and genes were quantified in three sites that span a range of tidal influence in the upper portion of the Cape Fear River Estuary. Environmental parameters were monitored during low and high tides in winter and spring. ^{15}N tracer incubation experiments were conducted to measure nitrification, denitrification, anaerobic ammonium oxidation (anammox), and dissimilatory nitrate reduction to ammonia (DNRA). Abundances of functional genes including bacterial and archaeal ammonia monooxygenases (*amo*A), nitrite reductases (*nir*S and *nrf*A), nitrous oxide reductase (*nos*Z), and hydrazine oxidoreductase (*hzo*) were measured using quantitative PCR assays. Denitrification rates were highest among the measured N cycling processes while bacteria carrying *nrf*A genes were most abundant. A discernable pattern in the short-term variation of N cycling rates and gene abundance was not apparent under the different tidal regimes. Significant seasonal variation in nitrification, denitrification, and anammox rates as well as bacterial *amo*A, *nir*S and *nos*Z gene abundance was observed, largely explained by increases in substrate availability during winter, with sediment ammonium playing a central role. These results suggest that the coupling of nitrification to N removal pathways is primarily driven by organic carbon mineralization and independent of tidal or salinity changes. Finally, changes in denitrification and nitrification activities were strongly reflectedby the abundance of the respective functional genes, supporting a linkage between the structure and function of microbial communities.

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1.0 INTRODUCTION

Estuaries are highly dynamic systems that form the transition between freshwater and marine environments. The interface between these two environments supports a tight coupling of biogeochemical activities that are important in regulating the amount of fixed nitrogen (N) in coastal systems. Microbially mediated N recycling processes such as nitrification and dissimilatory nitrate reduction to ammonium (DNRA) occur simultaneously with the removal processes of denitrification and anaerobic ammonium oxidation (anammox) in estuarine sediments (Capone et al., 2008). Each pathway can be influenced by a wide range of interacting factors including: temperature, salinity, dissolved oxygen (DO), substrate availability (nitrate + nitrite; $NO_3^- + NO_2^-$), ammonium (NH_4^+), dissolved organic carbon (DOC), and hydrogen sulfide (H_2S;(Cornwell et al., 1999; Dalsgaard et al., 2005; Seitzinger et al., 2006). These environmental factors change over short and long temporal scales in estuarine ecosystems and thus, differentially influence the activities and structure of N cycling microbial communities on these time scales.

Tidal fluctations in the oligohaline reaches of aquatic systems result in short-term changes in sediment and porewater chemistry, primarily as a result of movement of the water masses during the ebb and flood of the tide (Mortimer et al., 1999; Rocha and Cabral, 1998; Usi et al., 1998). Such changes in the supply of oxygen and salinity influence the availability of organic material as well as the species and distribution of N, creating a bottom up control on biogeochemical cycling. Expansion of the oxic zone as a result of tidal forcing may support higher rates of remineralization and nitrification in intertidal estuarine sediments (Mortimer et al., 1999; Rocha and Cabral, 1998). Enhanced nitrification can provide substrate for denitrification when NO_3^- diffuses to anoxic zones; however, inhibitory effects on denitrification

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in intertidal sediments can also be sustained through prolonged oxygen exposure during ebb tide or flushing of nutrients during flood tide (Thompson et al., 1995; Usi et al., 1998). In addition, distinct differences in microbial community structure have been observed between high and low tides, linked to variability in environmental conditions (Chauhan et al., 2009; Kara and Shade, 2009; Olapade, 2012). Temperature, light and nutrient availability contributed to greater bacterial diversity and abundance observed during high and outgoing tide (Olapade, 2012) while significant shifts in phylotype abundance occurred in response to elevated supply of dissolved organics during low tide (Chauhan et al., 2009). These studies primarily examine the effects of tidal fluctuation on planktonic microbial communities. However, little is known about the effects of tidal exchange on benthic communities in the oligohaline reaches of estuaries, where a major portion of N cycling takes place. Variations in N cycling activities and microbial community structure are likely to occur as a result of changes in sediment chemistry and the movement of different water masses with changing tides, greatly influencing estuarine N cycling.

Longerscale temporal shifts corresponding to seasonality influence sedimentary N cycling processes in various ecosystems (Berounsky and Nixon, 1990; Dunn et al., 2013; Eriksson et al., 2003; Jorgensen and Sorensen, 1985; Lisa et al., 2014; Rysgaard et al., 1995). Literature reports of peak N cycling activity vary both for season and for the reaction described; peaks often coincide with temperature as well as oxygen dynamics (Dunn et al., 2013; Rysgaard et al., 1995) or weather events that provide additional sources of substrate to support elevated processes (Eriksson et al., 2003; Lisa et al., 2014). Significant shifts in microbial community composition and metabolism in have been observed seasonally (Desnues et al., 2007; Kristensen, 1993). This trend is also evident in autotrophic microbial communities where particular ammonia oxidizing archetypes are more prevalent during specific seasons and correlated with temperature,

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DON and NH_4^+ concentrations (Bouskill et al., 2011). The seasonal reoccurrence of ammonia oxidizing organisms suggests the return of indigenous communities following large perturbations in more stable systems.

The greatest changes in geochemical conditions occur in oligohaline reaches of estuaries where fluctuations in salinity and nutrients occur tidally and seasonally, yet little is known about the response of sedimentary N cycling activities and sediment microbial communities to short or long-term changes under these environmental conditions. The extent to which temporal variability in the environment influences both activities and structure of microbial communities together has largely escaped attention and can have implications for coupling of sedimentary N cycling processes and the overall ability to remove N from the ecosystem. It is therefore important to consider the effects of dynamic environmental conditions on microbial community structure and function when examining the temporal effects on estuarine biogeochemical cycling over short and long-term scales. The objective of this study was to investigate the linkage between the abundance and activities of microbial communities responsible for N cycling processes under short and long term changes in a tidal estuarine ecosystem. We examined the effects of temporal changes on nitrification, denitrification, anammox, and DNRA activities and respective functional gene abundance in subtidal estuarine sediments at three tidal sites in the oligohaline reaches of the Cape Fear River Estuary, NC, USA.

2.0 MATERIALS AND METHODS

2.1 Study System

The Cape Fear River Estuary (CFRE), located in southeastern North Carolina (Figure 1), makes up a 72 km long portion of the lower Cape Fear River proper and empties directly into

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Long Bay (Mallin et al. 1999, Dafner et al. 2007). The Cape Fear River proper is a sixth-order

brown water stream originating in the Piedmont (Mallin et al., 1999). Two fifth-order blackwater

tributaries originating in the coastal plains, The Black and Northeast Cape Fear Rivers, also

empty into the lower portion of the Cape Fear River proper.

The CFRE watershed encompasses 23,310 km^2, the largest in North Carolina, and

supports one fifth of the population of the state. One half of the land within the basin is forested

while the remaining fifty percent is dedicated to cropland and pastureland or is urbanized (Lin et

al., 2006). Within the Cape Fear River basin, the most industrialized river basin in the state, are

641 licensed point discharges, a harbor, and state port in Wilmington (Mallin et al., 2000, 1999).

High levels of inorganic nutrients enter the system through these point discharges and additional

nonpoint sources such as runoff from urban, suburban, and livestock facilities.

2.2 Seasonal Sampling of the CFRE

Sampling was conducted during two seasons (winter and spring) over the course of a tidal

cycle (high and low tide) in the upper portion of the estuary in February and May of 2012.

Sampling sites were selected based upon tidal salinity variation. Salinity, a conservative

environmental parameter often used as a proxy for changes in other geochemical conditions, was

used to monitor the magnitude of tidal influence on our study sites. Sites included, Indian Creek

(IC; 34.2842N, 77.9981W), where salinity is <0.1and invariant with tidal stage. Two other sites,

Navassa (NAV; 34.2589N, 77.9846W) and Horseshoe Bend (HB; 34.2422N, 77.9681W), had

larger changes in salinity throughout the tidal cycle, ranging from 0 to15. At each site,

samplingwas conducted in the channel and at thewest bank (subtidal) during low and high tide.

This sampling scheme allowed for seasonal comparison between winter and spring at each site

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under the two extreme tidal conditions. The design enabled us to deconvolute the effects of short and long- temporal variation in geochemical conditions on N cycling activities and community structure, seasonally and tidally.

2.3 Environmental Parameter Measurements

Environmental parameters including water column depth, temperature, salinity and dissolved oxygen (DO), were measured within 1 meter of the bottom at the time of sampling using a 6820 multi parameter YSI data sonde (YSI Incorporated, Yellow Springs, OH). Water samples were 0.7µm (GF/F) filtered and stored on ice prior to nutrient analysis. Ammonium and NO_3^- concentrations were measured spectrophotometrically on a Bran Luebbe segmented flow nutrient auto analyzer using phenol hypochlorite and Cd-reduction/azo dye methods, respectively following modified standard EPA methods (Long and Martin, 1997). Samples designated for DOC and TDN analysis were stored in glass vials, preserved with H_3PO_4 and refrigerated until analysis. The samples were analyzed within one week on a Shimadzu 5050A analyzer following standard operating procedures.

Sediment samples were collectedusing a petite ponar grab (Wildco, Buffalo NY). The top 2 cm of sediment were collected and divided into aliquots to be characterized for solid phase sediment properties, N cycling rate determinations, and microbial molecular analyses. Sediment percent organic content was determined by loss on ignition (LOI) of dried sediments (500ºC for 4 hours). Sediment NH_4^+ (free plus extractable) was measured by phenol hypochlorite following KCl extraction using a 1:1 ratio of 2M KCl to sediments (Mackin and Aller, 1984). Sediments from each site were stored at -80°C for molecular analysis.

2.4 N cycling rates

Nitrification rates were determined under aerobic conditions using an isotope pool dilution method modified from Wessel & Tietema (1992). Incubations were conducted using 20g of sediment incubated in 80mL filtered site water amended with 70µM NO_3^- at 0.7 15N atom% (Carini et al. 2010). Initial and final samples were taken at time (t) t_i and t_f from the same incubation container and conducted for 24 hours in triplicate. $^{15}NO_3^-$ was measured on an Elemental Analyzer Isotope Ratio Mass Spectrometer (EA-IRMS; Delta V, Thermo Fisher Scientific) following $^{15}NO_3^-$ isolation using the Devarda's reduction and alkaline acid trap diffusion technique (Sigman et al. 1997, Holmes et al. 1998). Dilution of the isotope pool from t_i to t_f was used to calculate the rates of nitrification (Tobias et al. 2003).

Sediment slurry incubation experiments with ^{15}N tracer were conducted to measure potential rates of denitrification and anammox following the method of Lisa et al. (2014). Production of $^{29}N_2$, and $^{30}N_2$ was measured on a Gas Bench - Isotopic Ratio Mass Spectrometer (GB-IRMS; Delta V Plus, Thermo Fisher Scientific, Waltham, MA) and used to calculate the rates of anammox and denitrification following the method of Thamdrup & Dalsgaard (2002) as modified by Song and Tobias (2011). Percent anammox (%anammox) was estimated based on the rates of anammox and total N_2 production in each sample.

The accumulation of $^{15}NH_4^+$ in the same incubations was measured to calculate potential rates of DNRA (Tobias et al. 2001) according to the modifications of Song et al. (2014). Briefly, NH_4^+ was isolated from the slurry by alkaline acid trap diffusion following the addition of 7 mL of 40 ppt NaCl solution, 0.15 g MgO, and 3 µmoles of unlabeled NH_4^+ carrier. DNRA rate calculations were based on the concentration and ^{15}N mole fraction excess of extractable NH_4^+ as well as the $^{15}NO_3^-$ mole fraction (Tobias et al. 2001). Percent DNRA (%DNRA) was estimated

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based on the contribution of DNRA to total NO_3^- reduction (denitrification + anammox +

DNRA).

2.5 Quantitative PCR Assays of functional genes

Sediment DNA was extracted following the cetyltrimethylammonium bromide (CTAB)

method (DeAngelis et al., 2010; Griffiths et al., 2000) with modifications. Modifications

included the increase of wet sediment from 0.5 to 0.75 g and the use of a Thermo Savant Fast

Prep FP 120 Cell Disrupter (Qbiogene Inc. Carlsbad, CA) for cell disruption. DNA extractions

were carried out on IC and HB sites from winter and spring sampling events only.

Real time PCR assays were used to quantify abundance of functional genes in order to

obtain a quantitative measurement of the genetic potential a site has to carry out a particular

biogeochemical reaction. Primers targeting genes encoding the catalytic subunits of relevant

enzymes were used to quantify the abundances of microorganisms capable of N cycling

processes. Thermal cycling, fluorescent data collection, and data analysis were carried out using

the ABI 7500 Fast Real Time PCR System (Version 1.4). Assays were carried out in a volume of

20 μL containing 0.5 to 1.0 ng of template DNA and SYBR green using Go-Taq qPCR Master

Mix (Promega Corporation, Madison, WI). PCR specificity was confirmed using gel

electrophoresis to ensure amplification of the desired target and monitored by analysis of

dissociation curves. Standards were derived through a serial dilution of plasmids carrying

respective gene targets obtained from environmental samples. Standards were quantified using

Agilent 2200 TapeStation System (Agilent Technologies, Santa Clara, CA) following a digestion

with ECoR1. Information on PCR efficiency, R^2 valuesare reported below for each functional

gene. Efficieny of each qPCR reaction was calculated from the slope of the standard curve using the equation Efficiency = $-1+10^{(-1/\text{slope})}$.

Abundances of ammonia oxidizing bacteria and archaea were quantified by targeting ammonia monooxygenase (*amo*A) genes under the following PCR conditions: 95°C for 10 m; 50 cycles consisting of 95°C for 15 s, 53°C for 45 s, 72°C for 30 s, and 80°C for 30s (data acquisition); followed by dissociation step consisting of 95°C for 15 s, 60°C for 1 m, 95°C for 15 s, 60°C for 15 s. Bacterial *amo*A gene fragments were amplified using the PCR primers amoA-1F and amoA-2R (Gao et al., 2014; Rotthauwe et al., 1997) and archaeal *amo*A gene fragments were detected with Arch-amoAF and Arch-amoAR (Francis et al., 2005; He et al., 2007). Efficiencies for AOB and AOA were 85.09% and 98.03%, respectively. R^2 values were 0.99 and 0.98 for AOB and AOA, respectively.

Denitrifying bacterial abundance was assessed by quantifying nitrite reductase (*nir*S) and nitrous oxide reductase (*nos*Z) genes. Primers used to quantify *nir*S genes were nirS1F (Braker et al., 1998) and nirS-q-R (Mosier and Francis, 2010). The thermal cycling conditions were modified as follows: 95°C for 15 m; 38 cycles of 95°C for 15 s, 62.5°C for 30 s, 72°C for 30 s, and 84°C for 35 s (data acquisition), with a dissociation step of 95°C for 15 s, 60°C for 1 m, 95°C for 15 s, 60°C for 15 s. qPCR of *nos*Z genes were carried out using the primers nosZ2F and nosZ2R (Henry et al., 2006) under the following modified conditions: 95°C for 10 m; 50 cycles of 95°C for 15 s, 55°C for 45 s, 72°C for 35 s, and 75°C for 35 s (data acquisition), and a dissociation step of 95°C for 15 s, 60°C for 1 m, 95°C for 15 s, 60°C for 15 s. Efficiencies for *nir*S and *nos*Z were 83.30% and 74.17%, respectively. R^2 values were 0.99 for both *nir*S and *nos*Z.

Abundance of anammox bacteria was quantified using qPCR primers HZOQPCR1F and HZOQPCR1R following the methods of Long et al. (2013). Efficiency and R^2 values for *hzo* qPCR were 72.57% and 0.99, respectively. DNRA bacterial abundance was determined by quantifying*nrfA* gene abundance following the method of Song et al. (2014). Efficiency and R^2 values for the *nrf*A qPCR reaction were 60.29% and 0.99.

2.6 Statistical Analysis

Data were examined for normality using the Shapiro-Wilk Test and log transformed to meet the assumptions of statistical inference for parametric tests as well as reduce the range of the data. Paired-T Tests were used to identify differences among activities and gene abundance between tides and seasonally between paired samples. Pearson's product moment correlation analysis was used to investigate correlations between environmental conditions, potential N cycling activities and functional gene abundance. Linear regression analyses between N transformation rates andrespective bacterial functional gene abundanceswere conducted to determine if relationships between the variables exist. Due to the robust nature of the analyses, $\alpha=<0.05$ was retained to delineate significant relationships between all response and explanatory variables. All statistical analyses were conducted in R (version 2.15.3 Copyright 2013 The R Foundation for Statistical Computing).

3.0 RESULTS

3.1 Environmental Parameters

Changes in salinity were negligible at the IC site ranging from 0.01 to 0.10 during both seasons (Supplementary Table 1). Changes in salinity during the winter sampling event were

greater at the two downstream sites, with salinity varying from 0.1 to 6.4 and up to 8.0 within the

6-hour tidal exchange at NAV and HB, respectively. The overall salinity at the downstream sites

was higher during the spring low tide sampling, accompanied by lower amplitude of change

between tidal stages. Bottom water temperature was constant throughout the sites and reflected

typical seasonal conditions. DO in bottom water remained >6 mg L^{-1} during all sampling events.

Water column dissolved nutrients remained spatially uniform at the three sites within a

tide and season, but were quite variable between tidal stage and season (Supplementary Table 2).

Bottom water NO_3^- levels were elevated in the winter but remained relatively constant between

tidal stages. Seasonal and tidal trends for bottom water NH_4^+ were not observed. DOC and TDN

levels were both highest in winter. DOC was highly variable with the incoming tide while TDN

consistently decreased with the incoming tide. The ratio of bottom water Carbon:Nitrogen

$(DOC:NO_x)$ was also higher during the winter and elevated at high tide at all sites.

Sediment %organics were variable across tidal stage and season and increased with

downstream movement towards NAV and HB (Supplementary Table 3). Sediment extractable

NH_4^+ was consistently low for both seasons. The greatest increase in sediment extractable NH_4^+

was also observed downstream at NAV and HB, with the incoming tide where the largest

changes in salinity occurred.

3.2 N Cycling Rates & Their Correlation to Environmental Parameters

Tidal variations in all process rates - nitrification, denitrification, anammox, and DNRA -

between low and high tide were not significantly different from one another ($p>0.05$; Figure 2)

nor where the differences between bank and channel (data not shown). These data were pooled

for subsequent analysis. Seasonal variations were specific to particular N cycling processes.

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Several environmental parameters, including temperature, DO, and dissolved nutrients, showed strong seasonal variation and correlated with particular N cycling pathways, with sediment extractable NH_4^+ taking on a strong central role in connecting the N cycling activities.

Nitrification rates ranged from 0.02 to 5.72 nmols N g^{-1} wet sediment $hour^{-1}$ and were significantly higher in the winter ($p<0.001$; Supplementary Table 4a). Nitrification was negatively correlated with temperature, and positively correlated with DO levels, DOC, TDN, bottom water NH_4^+, as well as sediment extractable NH_4^+ (Table 1).

Denitrification was the highest of all N cycling processes, ranging from 2.50 to 44.21 nmols N g^{-1} wet sediment $hour^{-1}$, with significantly higher rates also occurring in the winter ($p=0.008$; Supplementary Table 4b). Temperature negatively correlated with denitrification rates (Table 1). Interestingly, increases in DO and sediment extractable NH_4^+ were accompanied by increases in denitrification activities.

Anammox activities were on the same order of magnitude as nitrification, ranging from 0.17 to 4.77 nmols N g^{-1} wet sediment $hour^{-1}$ (Supplementary Table 4c). Highest anammox rates were also observed during winter ($p<0.05$). Anammox rates were inversely correlated with temperature, but positively responded to increasing DO levels, DOC, TDN and bottom NH_4^+ (Table 1). Once again, sediment extractable NH_4^+ correlated with anammox N_2 production.

DNRA was the lowest of all N cycling processes measured throughout the study, ranging from 0.00 to 1.89 nmols N g^{-1} wet sediment $hour^{-1}$ (Supplementary Table 4d). Differences in DNRA both tidally and seasonally were not significant for DNRA ($p>0.05$). DNRA was positively correlated with salinity and negatively correlated with bottom water nitrate (Table 1). Sediment extractable NH_4^+ was also positively correlated with DNRA activities.

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Mean %anammox was 11.58 and overall %anammox spanned the broad range of 1.9 to

44.6% contribution to N_2 production. %DNRA also varied, ranging from 0 to 24.7% with a mean

of 2.56% contribution to NO_3^- reduction. Seasonal, spatial, and tidal variations and correlations

with environmental parameters were not significant for either %anammox or %DNRA (data not

shown).

3.3 Functional Gene Abundance & Its Correlation to Environmental Parameters

Variations in functional gene copy numbers showed no discernable pattern with respect

to the different tidal stages and were not significantly different from one another between low

and high tides (Figure 3). However, seasonal trends in gene abundance were evident and

correlated with particular environmental parameters.

Bacterial and archaeal *amo*A genes showed very similar distribution patterns in

abundance. Bacterial *amo*A gene abundance ranged from 1.60 x 10^3 to 3.78 x 10^5, while archaeal

*amo*A ranged from 2.31 x 10^3 to 2.53 x 10^5 gene copies g^{-1} wet sediment (Supplementary Table

5a and 5b). Bacterial *amo*A gene copy number was significantly higher in the winter (p=0.040),

while archaeal *amo*A gene abundance was not seasonally influenced (p>0.05). As a result,

bacterial *amoA* genes comprised a higher proportion of total *amo*A gene abundance during the

winter, but this trend did not hold during the spring. Abundance of AOB and AOA also

correlated with differing environmental parameters. Bacterial *amoA* gene abundance was

negatively correlated with temperature and positively correlated with DO (Table 1).

Alternatively, archaeal amoA gene abundance was correlated with DOC (Table 1). Positive

correlations with sediment extractable NH_4^+ were common in both AOB and AOA communities.

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Abundance of *nir*S genes ranged from 5.91 x 10^5 to 7.53 x 10^7 and *nos*Z genes ranged

from 1.82 x 10^6 to 7.10 x 10^7 copies g^{-1} wet sediment (Supplementary Table 5c and 5d). Both

*nir*S and *nos*Z genes were also most abundant during winter (*p*=0.031 and 0.045 respectively),

but did not differ tidally. Several environmental parameters were commonly correlated with both

*nir*S and *nos*Z gene abundance; these parameters included temperature, DO, and sediment

extractable NH_4^+ (Table 1). TDN was the only parameter that correlated with *nir*S gene

abundance.

Abundance of *hzo* genes ranged from 1.38 x 10^4 to 5.80 x 10^5 gene copies g^{-1} wet

sediment (Supplementary Table 5e). Seasonal variations in *hzo* gene abundance were not present

(*p*>0.05). Environmental parameters examined in this study showed no correlations with *hzo*

gene abundance (Table 1). Bacteria possessing *nrf*A were the most abundant out of all groups in

the examined communities, ranging from 5.79 x 10^6 to 1.34 x 10^9 gene copies g^{-1} wet sediment

(Supplementary Table 5f). Abundance of *nrf*A was not significantly different between two

seasons (*p*>0.05). Sediment organics and sediment extractable NH_4^+ positively correlated with

*nrf*A gene copy number (Table 1).

3.4 Relationships Among Rates and Functional Gene Abundance

Changes in N cycling activities strongly reflected changes in their respective functional

genes for denitrification and nitrification but not for anammox and DNRA (Figure 4).

Nitrification rates were positively and significantly correlated with both bacterial and archaeal

*amo*A gene abundances. Similarly, increases in denitrification rates significantly reflected

increases in both *nir*S and *nos*Z gene abundances. However, anammox and DNRA activities

were not correlated with abundance of their respective genes.

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4.0 DISCUSSION

Overall, sedimentary N cycling processes in term of the rates and gene copy numbers measured in this study were comparable topreviousstudies conducted in similar environments. Denitrification rates and gene copy numbers were consistent with that of other estuaries of similar trophic status (Dalsgaard et al., 2005; Dong et al., 2009; Henderson et al., 2010; Henry et al., 2006; Seitzinger et al., 2006; Smith et al., 2015, 2007). Nitrification rates were within the lower quartile of published values (Caffrey et al., 2007) but gene abundance (AOA and AOB) were not remarkably different from others (AOB (Bernhard et al., 2007; Stehr et al., 1995) and AOA (Moin et al., 2009; Mosier and Francis, 2008)). Anammox, %anammox and *hzo* gene copy number were also consistent with the range of activities (Dalsgaard et al., 2005; Nicholls and Trimmer, 2009) and gene abundance (Dang et al., 2010; Lisa et al., 2014) reported in other estuarine systems. DNRA activities,however, were substantially lower than those measured in other estuaries (Tobias et al. 2001, Gardner et al. 2006, McCarthy et al. 2008) despite the abundance of *nrf*A genes similar to those found in other estuaries (Dong et al., 2009; Smith et al., 2015; Song et al., 2014).The low rates of DNRA in this study are consistent with findings in oligohaline systems (Giblin et al., 2013) and suggest that the conditions necessary to support DNRA were not met. Instead, denitrification is favored as the primary dissimilatory NO_x^- reduction process in the tidal reaches of the CFRE. Denitrification tends to dominate under higher NO_3^- conditions while DNRA is favored under low NO_3^- and high labile organic carbon environments (Koop-Jakobsen and Giblin, 2010; Smith et al., 2015; Tobias et al., 2001).

Short-term temporal variation in N cycling processes and functional gene abundance was observed in the upper CFRE during tidal exchange, however a significant, clear pattern was not

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found. These results are contrary to several studies that observed significant changes in activities

and structure of microbial communities in intertidal sediments exposed to the atmosphere during

low tide. The difference in findings could be a result of a reduced capacity for solute exchange

due to slower diffusion in continually inundated sediments of CFRE. Under the circumstances

of continual inundation observed in this study, advection or seepage is less likely to accelerate

solute exchange as it would in intertidal systems thereby not affecting N cycling communities or

resulting activities simply with the changing tide. The difference in findings of this study could

also have to do with the slow growth rate of some of the microbes that mediate the various N

cycling processes. We might expect rapid changes in the structure and activities of denitrifying

and DNRA communities relative to nitrifying and anammox communities due to the versatile

lifestyles of the organisms. Additionally, we would also expect to see the most changes reflected

in the expression of the functional genes, not necessarily at the DNA level.

Nitrification, denitrification, and anammox rates and respective gene abundance (with the

exception of *hzo*) did show significant changes over the long-term, with elevated values observed

during winter. Temperature had the strongest correlation with nitrification, denitrification, and

anammox rates, as well as functional gene abundance in ammonia oxidizing and denitrifying

communities in the CFRE; however, co-variation between temperature and nutrients supply is

the likely factor contributing to higher winter activities.

Generally, metabolic processes and functional gene abundance positively respond to

increases in temperature, as is sometimes the case with nitrification rates and AOB abundance

(Berounsky and Nixon, 1990; Cébron et al., 2003), denitrification and denitrifier abundance

(Nowicki, 1994; Szukics et al., 2010). However, the inverse relationships between nitrification,

denitrification and anammox with temperature in this study, as well as the studies conducted by

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Kemp et al. (1990) and Bernhard et al. (2007), suggest seasonally variable environmental factors other than temperature have a stronger control on activities and structure of microbial communities in the oligohaline reaches of CFRE.

The inverse relationships between N cycling processes and gene abundance with temperature in this study can be explained by a higher supply of nutrients, particularly DOC, TDN, and sediment extractable NH_4^+, to the upper reaches of the CFRE during the winter. Taking the relationship of the three variables into consideration, it is likely that elevated N supply during the winter and lack of competition for DIN is driving the availability of dissolved N and sediment extractable NH_4^+ ($r=0.82$, $p<0.001$; $r=0.47$, $p=0.024$, respectively). This elevated supply of NH_4^+ during the winter may be responsible for higher winter nitrification activities and AOB abundance that can in turn support elevated denitrification and anammox activities in the CFRE.

Although not directly measured in this study, our data suggest the abundance of ammonia oxidizers and nitrification activities are, at least in part, coupled to denitrification and anammox rates and respective communities. This conclusion is corroborated by an earlier study in the upper CFRE, which showed 43% of denitrification activities were coupled to nitrification (Hines et al., 2012) as well as a significant positive correlation between nitrification and denitrification rates in this study ($p<0.001$, $r=0.733$). These findings are also supported by the occurrence of substrate induced stimulation of coupled nitrification-denitrification and nitrification-anammox in other marine and estuarine systems (Caffrey et al., 2007; Cornwell et al., 1999; Crowe et al., 2012; Lam et al., 2007; Rysgaard et al., 1995; Seitzinger, 1994). Caffrey et al (2007) also noted that sediment NH_4^+ concentrations, as a result of organic matter remineralization, were highly central to nutrient cycling; results from this study suggest this is also the case in the CFRE.

Thisgenetic approach combined with the use of stable isotope tracer techniquesto examine multiple N transformation processes offers a unique and holistic view of microbial community dynamics in the CFRE sediments under varying environmental conditions. A strong positive relationship between denitrifier community abundance and potential denitrification rates was observed in this study. Similar relationships between denitrification rates and *nir*S gene abundance were observed in other estuarine sediments, with an increase in rates and gene abundance supported by higher substrate concentrations (Dong et al., 2009; Smith et al., 2015). Despite low nitrifying activities, the reflection of changes in nitrification rates with changes in AOB and AOA gene abundance, indicate that a large portion of the nitrifying community is active. Significant correlations between nitrification rates with *amo*A gene abundance have also been observed in other coastal systems, linked to changes in salinity and substrate concentrations(Bernhard et al., 2007; Caffrey et al., 2007; Petersen et al., 2012). In the CFRE sediments, sediment $NH_4^+$was the most important environmental factors correlated with denitrification and nitrification rates as well as functional gene abundance, highlighting the importance of organic carbon remineralization and the tight coupling of these metabolic processes with the abundance of their respective microbial communities.

A significant correlation between gene abundance and rates of anammox communities was not observed in this study, as in other estuarine sediments (Dong et al., 2009). Similarly, increases in the numbers of *nrfA* genes did correlate with increases in DNRA rates. While positive relationships between *nrfA* copies and DNRA rates have been observed (Dong et al. 2009, Song et al. 2014), decoupling between the genetic potential and rates for the process has been observed within the same system (Smith et al., 2015).

When we consider the what is known about the physiological nature of the organisms involved in N metabolisms and the environmental conditions of the system, we can begin to explain why genetic potential for a particular metabolic process may or may not predict the potential for that process. In the case of nitrification and denitrification, gene abundance was strongly correlated with activities, and largely a result of the favorable conditions of the system as well as the ability of the organisms to respond to these conditions. The supply of NH_4^+ supported populations of both bacterial and archaeal nitrifiers that were active despite the rapidly changing environment. These environmental conditions also supported a high abundance of denitrifiers, directly and possibly through the coupling of nitrification. The strong correlations between genetic potential and rates of nitrification and denitrification suggest that the microorganisms present in these dynamic environments are well adapted and poised to respond to the continually changing conditions in the tidal oligohaline reaches of the CFRE.

Alternatively, a decoupling between the genetic potential and activities of anammox and DNRA is in part due to the physiology of the microorganisms and/or unfavorable environmental conditions. Given the diversity of organisms capable of DNRA pathway (Kartal et al., 2007; Mohan et al., 2004; Rutting et al., 2011; Simon, 2002), it is not particularly surprising that a correlation between activities and *nrf*A gene abundance was not observed in the tidal reaches of the CFRE. On the other hand, anammox bacteria prefer stable environmental conditions with a continual and simultaneous low supply of substrate and oxygen. Thus, anammox bacteria are typically out-competed for substrate by heterotrophic processes such as denitrification and DNRA dynamic environments such as estuarine sediments (Dalsgaard et al., 2005; Dong et al., 2011). The slow growing nature of anammox, low genetic potential, in a rapidly changing

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environment put anammox at a disadvantage and resulted in the decoupling of gene abundance and observed anammox activities.

5.0 CONCLUSIONS

A unique experimental approach that combined the use of molecular and stable isotope techniques was used to examine multiple N transformation processes under varying environmental conditions. Denitrification activities were highest among the measured N cycling processes while bacteria capable of DNRA were most abundant. The strong centrality of sediment NH_4^+ levels and the potential for coupled nitrification-denitrification and nitrification-anammox supports the role of nutrients and substrate availability as drivers of these processes rather than temperature and salinity. Alternatively, salinity was found to positively influence DNRA activities. We observed significant correlations between nitrification activities and *amo*A gene abundances in both AOB and AOA, suggesting that both bacteria and archaea are equally important to NH_4^+oxidation in the CFRE. Similar trends occurred with denitrification activities and abundance of denitrifying bacterial communities possessing both *nir*S and *nos*Z genes. The strong correlation between nitrifier and denitrifier microbial communities and rate measurements implies that abundances of microbial members is important in determining the magnitude of nitrification and denitrification activities.

6.0 ACKNOWLEDGMENTS

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7.0 LITERATURE CITED

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8.0 Table and Figure Legends

Table 1. Correlation analysis of N cycling activities and gene abundance with environmental parameters. Bold values indicate significant correlations.

Supplementary Table 1. Water column physical parameters in the Cape Fear River Estuary.

Supplementary Table 2. Water column dissolved nutrients in the Cape Fear River Estuary.

Supplementary Table 3. Sediment characteristics of the Cape Fear River Estuary.

Supplementary Table 4. Tidal and seasonal fluctuations in sedimentary N cycling rates in the Cape Fear River Estuary. NA indicates Not Available. N cycling values are presented in nmol N g^{-1} sediment hr^{-1}. "SD" indicates standard deviation.

Supplementary Table 5. Abundance of relevant N cycling genes in the Cape Fear River Estuary during low and high tides. NA indicates Not Available.

Figure 1. Sampling sites in the Cape Fear River Estuary, NC, USA. Sampling sites in the upper, oligohaline portion of the CFRE include Indian Creek (IC), Navassa (NAV) and Horseshoe bend (HB).

Figure 2. Fluctuation of sedimentary nitrogen cycling rates at high and low tides. N cycling values are presented in nmol N g^{-1} sediment hr^{-1} for two of six total sites, the banks of Indian Creek (IC) and Horseshoe bend (HB) during winter. Rate measurements are presented as follows: A) nitrification, B) denitrification, C) anammox, and D) dissimilatory nitrate reduction to ammonium (DNRA). Error bars represent standard deviation.
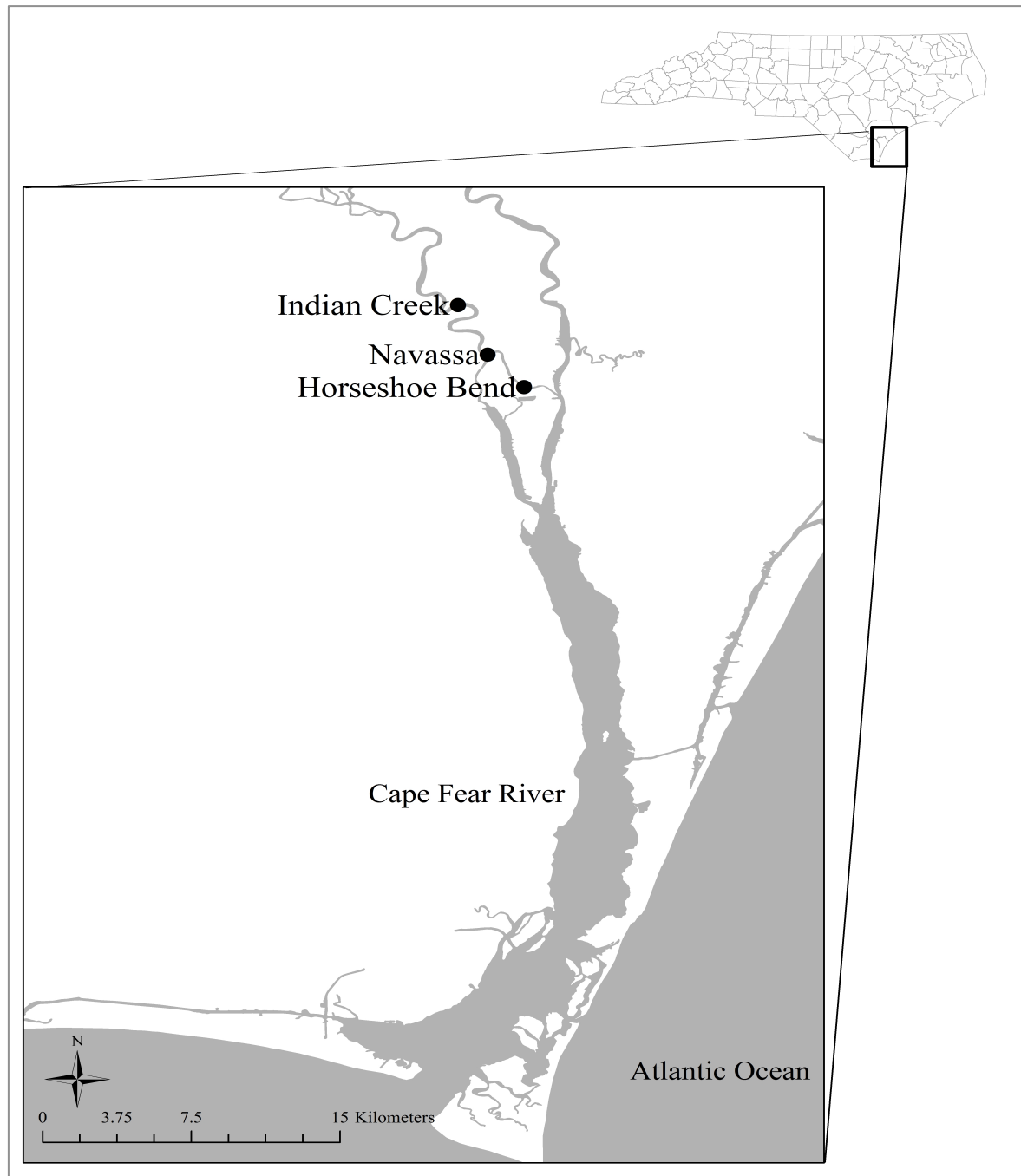
Figure 3. Sedimentary nitrogen cycling functional gene abundance during low and high tides.Gene copy numbers are presented for two of six total sites, the banks of Indian Creek (IC) and Horseshoe bend (HB) during winter only. Genes include: A) bacterial ammonia monooxygenase (Bacterial *amo*A), B) archaeal ammonia monooxygenase (Archaeal *amo*A), C) nitrite reductase (*nir*S), D) nitrous oxide reductase (*nos*Z), E) hydrazine oxidoreductase (*hzo*), and F) cytochrome C nitrite reductase (*nrf*A). Error bars represent standard deviation.
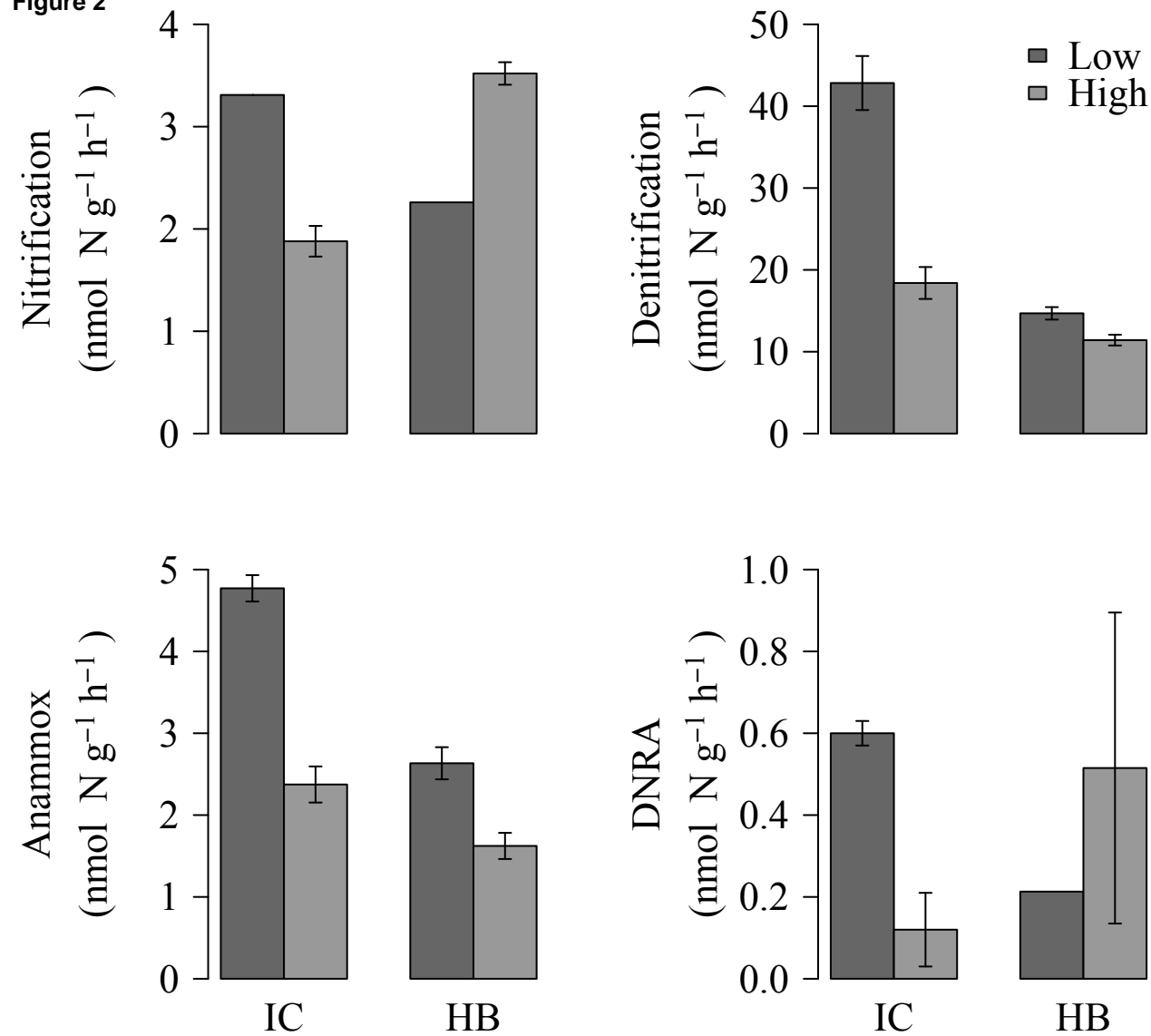
Figure 4. Correlation analyses of nitrogen cycling rates and respective functional gene abundance in the Cape Fear River Estuary. Correlations are presented for: A) nitrification and bacterial ammonia monooxygenase (Bacterial *amo*A), B) nitrification and archaeal ammonia monooxygenase (Archaeal *amo*A), C) denitrification and nitrite reductase (*nir*S), D) denitrification and nitrous oxide reductase (*nos*Z), E) anammox and hydrazine oxidoreductase (*hzo*), and F) dissimilatory nitrate reductase to ammonium (DNRA) and cytochrome C nitrite reductase (*nrf*A). Error bars represent standard deviation.
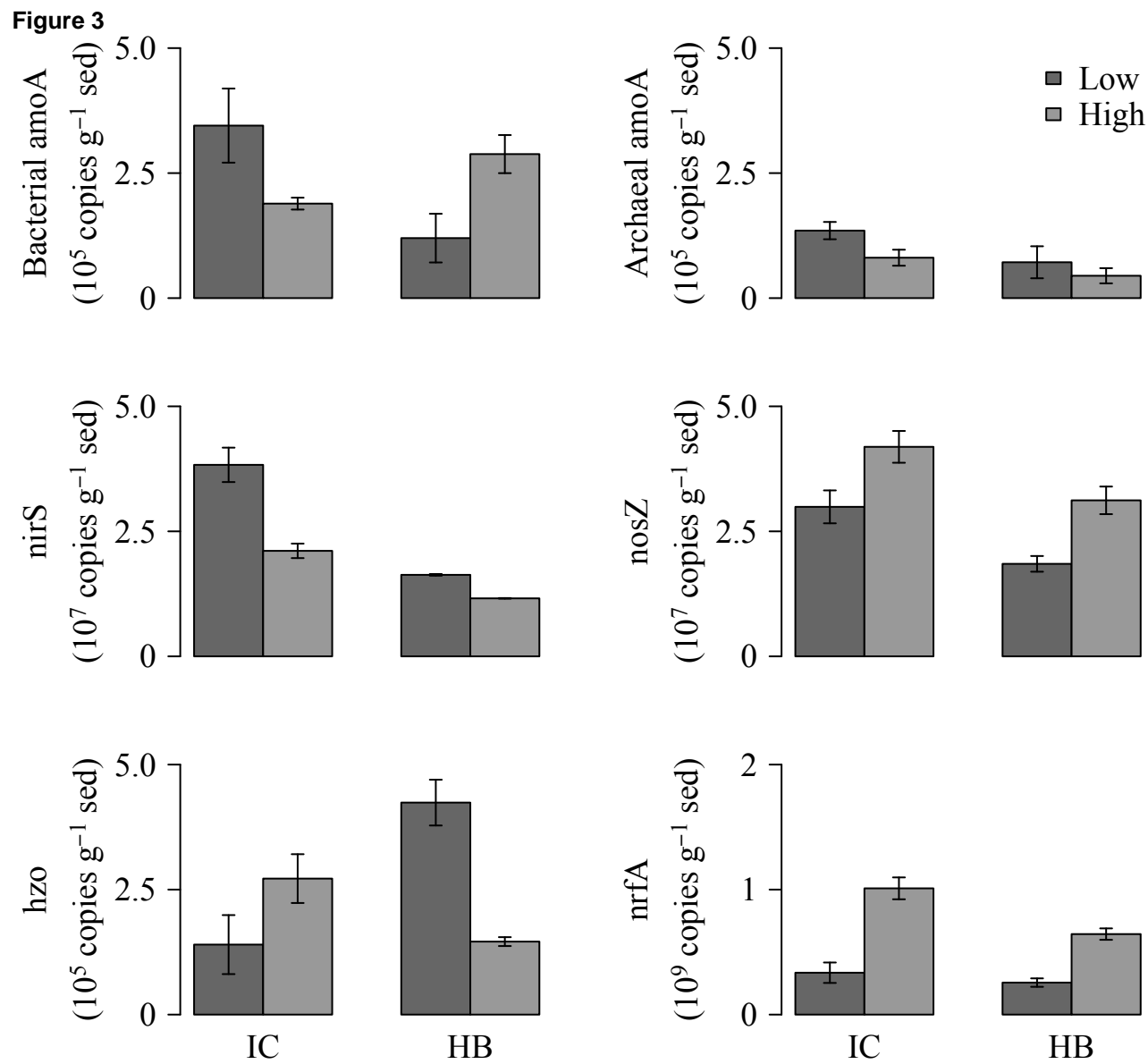
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Table 1

| | Bottom Water | | | | | | | | | | | | | | | | Sediment | | | |
| | Salinity | | Temperature | | DO | | DOC | | TDN | | Nitrate | | Ammonium | | Organics | | Ammonium | |
	r	p-value	r	p-value	r	p-value	r	p-value	r	p-value	r	p-value	r	p-value	r	p-value	r	p-value
Nitrification	-0.07	0.747	**-0.82**	**<0.001**	**0.80**	**<0.001**	**0.78**	**<0.001**	**0.68**	**<0.001**	-0.27	0.205	**0.52**	**0.010**	0.14	0.526	**0.75**	**0.000**
Denitrification	0.19	0.384	**-0.45**	**0.027**	**0.43**	**0.037**	0.38	0.066	0.30	0.149	-0.40	0.052	0.32	0.122	0.27	0.215	**0.67**	**0.000**
Anammox	-0.08	0.722	**-0.57**	**0.003**	**0.55**	**0.005**	**0.49**	**0.016**	**0.49**	**0.015**	-0.14	0.525	0.32	0.128	0.01	0.950	**0.61**	**0.002**
DNRA	**0.48**	**0.034**	0.00	0.996	-0.03	0.898	-0.03	0.912	-0.14	0.560	**-0.74**	**<0.001**	-0.05	0.831	0.41	0.079	**0.49**	**0.034**
Bacterial *amo* A	0.17	0.569	**-0.68**	**0.008**	**0.66**	**0.011**	0.44	0.111	0.51	0.060	-0.30	0.305	0.37	0.199	0.18	0.529	**0.62**	**0.023**
Archaeal *amo* A	0.12	0.713	-0.52	0.081	0.53	0.073	0.40	0.194	0.42	0.169	-0.35	0.271	0.16	0.615	0.28	0.380	**0.64**	**0.035**
nir S	-0.27	0.332	**-0.65**	**0.009**	**0.67**	**0.006**	0.37	0.180	**0.59**	**0.021**	-0.13	0.641	0.29	0.298	-0.21	0.462	**0.69**	**0.006**
nos Z	0.25	0.368	**-0.59**	**0.021**	**0.55**	**0.033**	0.39	0.153	0.44	0.105	-0.39	0.154	0.36	0.194	0.30	0.284	**0.66**	**0.011**
hzo	-0.13	0.642	-0.47	0.080	0.49	0.061	0.41	0.125	0.45	0.094	-0.16	0.577	0.04	0.892	0.20	0.469	0.47	0.092
nrf A	0.39	0.156	-0.06	0.840	0.03	0.913	0.18	0.533	-0.09	0.756	-0.46	0.084	0.06	0.845	**0.82**	**<0.001**	**0.55**	**0.041**

Figure 1

Figure 2

Figure 3

Figure 4