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Saltwater Intrusion Modifies Microbial Community Structure and Decreases Denitrification in Tidal Freshwater Marshes

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Abstract

Environmental changes can alter the interactions between biotic and abiotic ecosystem components in tidal wetlands and therefore impact important ecosystem functions. The objective of this study was to determine how saltwater intrusion affects wetland nutrient biogeochemistry, with a specific focus on the soil microbial communities and physicochemical parameters that control nitrate removal. Our work took place in a tidal freshwater marsh in South Carolina, USA, where a 3.5-year saltwater intrusion experiment increased porewater salinities from freshwater to oligohaline levels. We measured rates of denitrification, soil oxygen demand, and dissimilatory nitrate reduction to ammonium (DNRA) and used molecular genetic techniques to assess the abundance and community structure of soil microbes. In soils exposed to elevated salinities, rates of denitrification were reduced by about 70% due to changes in the soil physicochemical environment (higher salinity, higher carbon:nitrogen ratio) and shifts in the community composition of denitrifiers. Saltwater intrusion also affected the microbial community responsible for DNRA, increasing the abundance of genes associated with this process and shifting microbial community composition. Though rates of DNRA were below detection, the microbial community response may be a precursor to increased rates of DNRA with continued saltwater intrusion. Overall, saltwater intrusion reduces the ability of tidal freshwater marshes to convert reactive nitrogen to dinitrogen gas and therefore negatively affects their water quality functions. Continued study of the interrelationships between biotic communities, the abiotic environment, and biogeochemical transformations will lead to a better understanding of how the progressive replacement of tidal freshwater marshes with brackish analogues will affect the overall functioning of the coastal landscape.

Key words: ecosystem ecology; global change; nitrogen cycle; salinization; sea level rise; wetland.

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Authors Contributions SCN, MFP, and RBF designed the study. All authors conducted the research and analyzed data. SCN wrote the manuscript with substantial contributions from MFP, ARS, and RBF. All authors edited the manuscript.

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Highlights

- Saltwater intrusion reduces the water quality functions of tidal freshwater marshes.
- Microbial community composition plays a role in regulating ecosystem process rates.
- Ecosystem responses to environmental change depend on both biotic and abiotic factors.

Introduction

Coastal freshwater wetlands around the world are being reshaped by saltwater intrusion, the upstream movement of seawater that increases salinities and alters water chemistry (Herbert and others 2015). Whether caused by sea level rise, decreased freshwater inflow, storm surges, or anthropogenic modification of coastal hydrology, saltwater intrusion can affect the ecosystem functions and services provided by tidal freshwater wetlands. For example, saltwater intrusion has converted tidal forests to herbaceous marshes (Hackney and others 2007), caused freshwater marshes to become increasingly dominated by salt-tolerant plants (Perry and Hershner 1999), and driven state change from wetland to open water (Nyman and others 1993; DeLaune and others 1994). These transitions can lead to lower rates of plant primary production and reduced accumulation of carbon (C) (Spalding and Hester 2007; Neubauer 2013; Weston and others 2014; Herbert and others 2018), negatively affecting the ability of these ecosystems to build soil volume and keep pace with rising sea levels. The loss of wetland area due to sea level rise—and especially the replacement of tidal freshwater wetlands with brackish marshes—can significantly reduce the ability of coastal wetlands to filter inorganic nitrogen (N) from tidal waters (Craft and others 2009). Saltwater intrusion can also affect the availability of inorganic N and phosphorus (P) (for example, van Dijk and others 2015), with implications for estuarine water quality and eutrophication.

Alterations to the soil physicochemical environment subsequently impact microbial and plant communities, leading to changes in biogeochemical functioning and ecosystem processes. For example, increased ionic strength can displace nutrients from soil particles (Weston and others 2010; Ardón and others 2013; Jun and others 2013). Elevated concentrations of sulfate in seawater can be transformed to sulfides by microbial sulfate reduction, stressing plants (Koch and others 1990; Munns and Tester 2008) and altering a variety of processes related to N and P biogeochemistry (for example, Joye and Hollibaugh 1995; Kostka and Luther III 1995; Brunet and Garcia-Gil 1996; Senga and others 2006; Jordan and others 2008). Microbial communities can respond to environmental perturbations like saltwater intrusion through changes in gene expression, abundance, and/or community composition. Changes in gene expression can lead to rapid biogeochemical responses (Weston and others 2006; Edmonds and others 2009), whereas shifts in abundance and community structure may manifest after longer periods, with responses that may depend on whether a site has previously experienced saltwater intrusion (Nelson and others 2015). Shifts in microbial community composition following saltwater intrusion can directly lead to changes in biogeochemical functioning (Jackson and Vallaire 2009), although the response may be modest if freshwater microbes are replaced by salt-tolerant microbes that fill a similar ecological niche (Hobbie 1988). A more complete understanding of the biogeochemical and microbial responses to saltwater intrusion will improve our ability to mechanistically predict how this global change stressor will affect ecosystem processes and the overall functioning of tidal freshwater marshes.

The objective of this study was to provide information on how sustained saltwater intrusion modifies soil microbial communities and the biogeochemical cycling of N and P, with a focus on processes involved in the retention and removal of nitrate (NO_3^-). Our work took place in a tidal freshwater marsh in South Carolina, USA, where an in situ saltwater intrusion experiment increased porewater salinities from freshwater to oligohaline levels. Using soil cores that were collected from the marsh after 3.5 years of sustained saltwater intrusion, we determined rates of denitrification [net dinitrogen (N_2) production], soil oxygen (O_2) demand, and nutrient fluxes using laboratory flow-through techniques; measured rates of dissimilatory nitrate reduction to ammonium (DNRA) via $^{15}NO_3^-$ isotope enrichment; and assessed the abundance and community structure of microbes (bacteria, archaea, and those capable of carrying out denitrification and DNRA) with molecular genetic techniques. These data were combined with environmental data (for example, porewater salinity, soil properties) to identify which biotic and abiotic factors were most important in determining N cycle responses to saltwater intrusion.

Materials and Methods

Study Site and Field Sample Collection

From June 2008 through November 2011, an in situ salinity and water addition experiment was conducted in a tidal freshwater marsh on the Waccamaw River, South Carolina (33°31.50′N, 79°5.51′W). The marsh, a 0.9-ha site at Brookgreen Gardens, is located roughly 25 km upriver of Georgetown, South Carolina, and experiences semi-diurnal flooding with freshwater (typical salinity ≤ 0.5 on the Practical Salinity Scale, which is functionally equivalent to units of parts per thousand; measured at United States Geological Survey station #021108125, ~ 5 km downstream of the study site; waterdata.usgs.gov). The experimental manipulations, which are described in more detail in Neubauer (2013), consisted of twice weekly additions of brackish water or freshwater to 0.37-m^2 marsh plots (+salt and +fresh treatments, respectively), along with a series of control plots (n = 5 per treatment). The freshwater added to the +fresh plots was from a groundwater well at the Baruch Marine Field Laboratory (BMFL), Georgetown, South Carolina. The brackish water added to the +salt plots had a salinity of ~ 10 and was a mixture of this groundwater with salt marsh creek water from the flow-through seawater system at the BMFL. Over the entire 3.5-year field manipulation, porewater in the control and +fresh plots was typical of the freshwater zone (salinity = 0.0–0.1, conductivity = 137–383 μS cm^{-1}; per-plot averages across more than 400 sampling dates at 10 and 25 cm soil depths), whereas porewater in the +salt plots resembled the oligohaline zone (salinity = 1.7–3.8, conductivity = 3188–6859 μS cm^{-1}). The experimental manipulations affected the species-rich plant community (Neubauer and Sutter, in prep), soil carbon biogeochemistry (Neubauer and others 2013), and ecosystem-scale primary production and respiration (Neubauer 2013).

For the analyses described herein, we collected a single soil core (32.2 cm^2 × 10 cm deep) from each plot on 16 November 2011, the day after the final experimental water additions were made. We also collected approximately 100 l of water from the adjacent creek for nutrient analysis and use in the incubations. The cores were capped on top and bottom with rubber stoppers and transported to the University of North Carolina's Institute of Marine Sciences (IMS), Morehead City, North Carolina, where we quantified rates of denitrification, DNRA, fluxes of dissolved inorganic nutrients and dissolved organic nitrogen (DON) between the core and overlying water, and soil O_2 demand. Following these measurements, the top 2 cm of each core was homogenized and a frozen subsample was shipped to Virginia Commonwealth University for storage (− 80°C) until DNA extraction and microbial community characterization.

Soil Nutrient Biogeochemistry

Continuous Flow Incubation

At IMS, the soil cores and water were placed in an environmental chamber (Bally, Inc., Morehead City, NC, USA) set to in situ temperature (20°C). In the chamber, the cores were submerged in site water to mimic high tide conditions and sealed with gas-tight lids equipped with an inflow and outflow port (Ensign and others 2008). Because all of the field plots experienced tidal flooding from the same freshwater creek, we used water from that creek as the source water for the laboratory incubations for all soil cores, regardless of treatment. The entire duration of the laboratory experiments described herein was shorter than the typical 3–4-day interval between water additions to the field plots. Consequently, we did not add any brackish water to the cores after they were removed from the marsh. Unfiltered, aerated water from the reservoir was passed over the cores at a flow rate of 1 ml min^{-1}, creating a homogeneous water column (Miller-Way and Twilley 1996; Lavrentyev and others 2000). This flow rate allowed for a well-mixed water column without the washout that can occur when flow is too high. Dark conditions were maintained throughout the incubation to reduce the effects of photosynthetic algae (An and Joye 2001; Hochard and others 2010) and to prevent the formation of bubbles that would affect dissolved gas concentrations (Reeburgh 1969).

Following an 18-hour pre-incubation period (Eyre and others 2002), samples were collected from the outflow port of each core three times over the following 20-hour period to verify that steady-state conditions had been achieved (Miller-Way and Twilley 1996). Samples were also collected from a bypass line that flowed directly into sample vials and used to determine the concentration of dissolved constituents entering the cores. Successive measurements from each core were averaged to give core-specific values. A core that contained only site water was incubated along with the soil cores and used as a water blank correction.

Membrane Inlet Mass Spectrometry (MIMS)

Concentrations of dissolved gases in water were measured using a Balzers Prisma QME 200 quadrupole mass spectrometer (Pfeiffer Vacuum, Nashua, NH, USA; Kana and others 1994) and used to calculate fluxes of N_2 and O_2 (Ensign and others 2008). The MIMS coupled with continuous flow core incubations determines the net flux (that is, production–consumption) across the soil–water interface. This method does not discern between the sources [denitrification and anaerobic ammonium oxidation (anammox)] or sinks of N_2 (N_2 fixation); we use the term ''denitrification'' to refer to the net release of N_2 from the soil. We assume that denitrification is the major production pathway of N_2 in this study because anammox is only a small portion of the total N_2 flux in shallow water coastal systems (Koop-Jakobsen and Giblin 2009). Fluxes of O_2 directed into the soil were used to determine rates of soil O_2 demand (SOD; Kana and others 1994; Piehler and Smyth 2011).

Nutrients

Water samples (50 ml) were collected for nutrient analysis from the bypass line and the outflow port of each core 24 h after the incubation began. Water was filtered through Whatman GF/F filters (25 mm diameter, 0.7 μm nominal pore size) and analyzed for nitrate + nitrite after cadmium reduction ($NO_3^- + NO_2^-$, collectively NO_x), ammonium (NH_4^+), total N after persulfate digestion, and soluble reactive phosphorus (SRP). Nutrient concentrations were measured with a Lachat Quick-Chem 8000 automated ion analyzer (Lachat Instruments, Milwaukee, WI, USA) using protocols equivalent to EPA methods 303.2 (for NO_x), 350.1 (for NH_4^+ and total N), and 365.1 (for SRP). The detection limits were 0.04 μmol l^{-1} for NO_x, 0.18 μmol l^{-1} for NH_4^+, 2.53 μmol l^{-1} for total N, and 0.06 μmol l^{-1} for SRP. Dissolved organic nitrogen (DON) was calculated as the difference between total N and inorganic N (NO_x + NH_4^+).

Dissimilatory Nitrate Reduction to Ammonium (DNRA)

An isotopic enrichment experiment was conducted to determine potential rates of DNRA. Four hours after collecting the last samples for the denitrification and soil O_2 demand assays, the reservoir water was enriched with $Na^{15}NO_3$ to a final concentration of about 100 μmol l^{-1}. Incubations were continued and, after 24 h, samples were collected for DNRA and nutrient analysis. The concentration of $^{15}NH_4^+$ was measured by HPLC (Gardner and others 1995), with rates of DNRA calculated from $^{15}NH_4^+$ production as described in An and Gardner (2002).

Flux Calculations

Fluxes of dissolved gases and nutrients between the soil and water columns were calculated assuming steady-state conditions and a homogeneous water column (Miller-Way and Twilley 1996). Flux rates (μmol m^{-2} h^{-1}) were calculated as:

$$\text{Flux} = ([i_{\text{outflow}}] - [i_{\text{inflow}}]) \times \frac{F}{A}$$

where $[i_{\text{outflow}}]$ and $[i_{\text{inflow}}]$ are the concentrations (μmol l^{-1}) of dissolved constituents leaving and entering the core, respectively; F is the peristaltic pump flow rate (l h^{-1}); and A is the surface area of the core (m^2) (Miller-Way and Twilley 1996).

For denitrification and all dissolved nutrients, a positive flux rate indicates a net exchange from the soil to the water column. Conversely, a net exchange of O_2 in the reverse direction, from the water column into the soil, is reported as a positive soil O_2 demand.

Soil Microbial Community Characterization

DNA Extraction

Whole-community DNA was extracted from 0.5-g subsamples of soil using the MoBio PowerSoil DNA Isolation Kit (Carlsbad, CA, USA). DNA purity and concentration were analyzed using a Nanodrop ND-1000 (Thermo Scientific, Wilmington, DE, USA), and extracts were stored at − 20°C. Agarose gel (1.5%) electrophoresis and ethidium bromide staining were used to verify all DNA extracts and PCR products.

Quantitative PCR for Abundance

Quantitative polymerase chain reaction (qPCR) was used to determine the abundance of functional genes specific to nitrate reduction. For denitrification, we studied the genes that encode for nitrite reductase, which catalyzes the reduction of nitrite (NO_2^-) to nitric oxide (NO). This enzyme is found in all denitrifiers and can be one of the two types: *nirK* (copper-containing nitrite reductase) and *nirS* (cytochrome cd_1-containing nitrite reductase). We amplified the *nirS* gene using the primers cd3aF and R3cd following the protocols outlined in Morrissey and Franklin (2015). The *nirK* gene was amplified using the nirK-q-F and nirK1040 primers (Mosier and Francis 2010). For both assays, the total reaction volume was 15 μl, which included

2 ng of template DNA and each primer at 0.35 μmol l^{-1}. Thermal cycling conditions were: 95°C for 15 min, touchdown that included 9 cycles of 95°C for 15 s, 68°C (− 1°C per cycle) for 60 s, and 81.5°C for 30 s, followed by 29 cycles of 95°C for 15 s, 60°C for 60 s, and 81.5°C for 30 s. For DNRA, we studied *nrfA*, which encodes for the nitrite reductase that reduces NO_2^- to NH_4^+, using the primers nrfA6F and nrfA6R (Morrissey and others 2013). Reactions (15 μl) included 10 ng of template DNA and 0.3 μmol l^{-1} concentrations of each primer; thermal cycling conditions were: 50°C for 2 min, 95°C for 8.5 min, and 50 cycles of 94°C for 20 s, 58.5°C for 40 s, and 72°C for 10 s. Standard curves for *nirS*, *nirK*, and *nrfA* were produced using *Paracoccus denitrificans* (Strain #17741, ATCC, Manassas, VA, USA), *Pseudomonas nitroreducens* (Strain #1650, DSMZ, Braunschweig, Germany), and *Escherichia coli* (Strain #11775, ATCC), respectively. Triplicate reactions were performed for each sample and each assay using SYBR GreenER qPCR Supermix for iCycler (Invitrogen, Grand Island, NY, USA) and a Bio-Rad CFX 96 Real-Time System (Bio-Rad, Hercules, CA, USA). Results are reported as the number of gene copies per g of organic matter (OM), after averaging the technical replicates. Reaction efficiencies were 98% for *nirS*, 98% for *nirK*, and 90% for *nrfA*; correlation coefficients were all higher than 0.995.

TRFLP for Community Composition

Microbial community composition was analyzed using Terminal Restriction Fragment Length Polymorphism (TRFLP). Overall patterns for bacteria and archaea were assessed via the *16S rRNA* gene, and organisms associated with nitrate reduction were targeted using the same functional genes as above for qPCR. All reactions (50 μl) used AmpliTaq DNA polymerase (Applied Biosystems, Foster City, CA, USA) and primers obtained from IDT (Integrated DNA Technologies, Coralville, IA, USA) with a FAM label attached to each forward.

The bacterial and archaeal assays used the primer pairs 27F with 1492R (Lane 1991) and 21F with 958R (Cytryn and others 2000), respectively, and followed the PCR conditions outlined in Morrissey and others (2014). Amplification of *nirS* used the primers nirS1F and nirS6R following Morrissey and Franklin (2015), whereas *nirK* used nirK1F and nirK5R following Yin and others (2014). For *nrfA*, amplification used the primers F1b and R1b (Morrissey and others 2013). Reactions included 1X PCR Buffer II, 1.8 mM $MgCl_2$, 200 μM of each dNTP, 1.5 μg BSA, 0.2 μM of each primer, 2.5 units of AmpliTaq DNA polymerase, and 10–30 ng of template DNA. Thermal cycling conditions were: 94°C for 5 min, touchdown that included 30 cycles of 95°C for 60 s, 60°C (− 0.5°C per cycle) for 60 s, and 72°C for 90 s, followed by 30 cycles of 95°C for 30 s, 45 for 30 s, and 72°C for 90 s, and ending with a 10-min incubation at 72°C.

Amplicons were cleaned using the MinElute PCR purification kit (Qiagen, Valencia, California, USA) and then digested using either Hha1 (*16S rRNA*), HaeII (*nirS* and *nirK*), or HinP1I (*nfrA*) following the manufacturer's protocol for each restriction enzyme (New England Biolabs, Ipswich, MA, USA). Digests were cleaned using the MinElute PCR purification kit and analyzed using a MegaBACE 1000 DNA Analysis System (Amersham Biosciences, Buckinghamshire, UK). Aliquots (70–100 ng) of each purified digest were combined with 0.5 μl of MapMarker 400 ROX ladder (Bioventures, Murfreesboro, TN, USA) plus 4.75 μl injection buffer (0.1% Tween-20) and then injected at 3 kV for 100 s. Electrophoresis used genotyping filter set 1 for 100 min at 10 kV. TRFLP fragments between 70 and 400 base pairs (bp) were analyzed with Fragment Profiler software (version 1.2; Amersham Biosciences) set for a 1-bp size differential and a 50 relative fluorescent unit peak height threshold. Peaks accounting for less than 2% of total sample fluorescence were removed prior to data analysis.

Soil Physicochemical Properties

In addition to the soil cores used for dissolved gas, nutrient flux, and DNA analyses, a parallel core was collected from each plot. The upper 3 cm of this second core was analyzed for OM content (loss on ignition; combustion of dried sample at 550°C for 5 h) and soil C and N contents (ECS4010 elemental analyzer, Costech Analytical Technologies, Valencia, CA, USA). Ten-gram aliquots of these surface soil samples were centrifuged to extract porewater (10 min at 4000 rpm), which was analyzed for salinity using a YSI 3200 conductivity meter (YSI Inc., Yellow Springs, OH, USA). These soil data were previously reported in Neubauer and others (2013).

Additionally, on the day that the nutrient biogeochemistry cores were collected, porewater was collected from sippers in each plot (sampling depths of 10 and 25 cm). The salinity and conductivity of these samples were measured within 2 days of sample collection as described above.

Statistical Analysis

Shapiro–Wilk tests confirmed that the environmental data and process rates were normally distributed. Salinity, SRP flux, and gene abundance data required a $\log_{10}$ transformation prior to statistical analysis. For each variable, one-way ANOVAs were performed to test for treatment effects, followed by a Tukey's HSD test for post hoc comparisons. Pearson correlation (r) analyses were used to evaluate direct relationships among variables, and partial correlations ($r_{partial}$) were performed to compare process rates with gene abundances while controlling for shared environmental variation. Multivariate linear regressions were performed using stepwise selection (p entry = 0.10, p exclusion = 0.15, p for the final inclusion = 0.05) to identify suites of variables that were significant predictors of N_2 production. An F test was used to determine the significance of the change in r^2 following the inclusion of each new predictor variable, and model complexity was only increased when the associated $p < 0.05$. All of these statistical analyses used $\alpha = 0.05$ and were performed using SPSS statistical software (Version 24, IBM Corp., Armonk, NY, USA).

The TRFLP results were treated as a binary data matrix describing the presence or absence of each fragment (peak) in each sample. Bray–Curtis similarity was calculated between each pair of samples and visualized using nonmetric multidimensional scaling (NMDS). Permutational multivariate analysis of variance (PERMANOVA) was used to test for treatment effects. Mantel tests (r_M) were used to assess correlations between TRFLP profiles, environmental data, and process rates using the Bray–Curtis and Gower matrices, respectively; partial Mantel tests ($r_{M\text{-partial}}$) were used to compare community structure and process rates while controlling for environmental parameters. All community analyses were conducted using the PAST statistical package (Version 2.16, Hammer 2001).

Results

Nutrient Biogeochemistry

During the whole-core incubations, the rate of denitrification was significantly greater in cores collected from the control plots and +fresh plots than in cores from the +salt plots (Figure 1A; Table 1). The flux of dissolved oxygen into the cores (that is, soil O_2 demand) was highest in the cores from the control plots, intermediate in the +fresh cores, and lowest in the +salt cores (Figure 1A; Table 1). There was a significant and positive relationship between soil O_2 demand and N_2 efflux from the cores ($r = 0.88$, $p < 0.001$; Figure 1A). Rates of DNRA were zero (undetectable) in all cores.

Nutrient concentrations in the inflowing water, which was collected from the tidal creek adjacent to the study site and used for the core incubations, had submicromolar concentrations of NO_x (0.74 μmol l^{-1}), NH_4^+ (0.39 μmol l^{-1}), and SRP (0.24 μmol l^{-1}). The DON concentration in the inflowing water was 14.7 μmol l^{-1}. There were no differences in the fluxes of NO_x, NH_4^+, DON, or SRP as a function of field treatment ($p = 0.16$–0.69). The NO_x fluxes were always directed out of the cores into the overlying water column, regardless of treatment, and averaged 6.1 ± 1.0 μmol N m^{-2} h^{-1} (average across all treatments ± standard error; Table 1). The fluxes of NH_4^+, DON, and SRP were more variable, with fluxes being directed into the soil for some cores and into the water column for others. Across all treatments, NH_4^+ fluxes averaged 0.30 ± 1.0 μmol N m^{-2} h^{-1}, DON fluxes were − 15.1 ± 10.8 μmol N m^{-2} h^{-1}, and SRP fluxes were − 0.1 ± 0.3 μmol P m^{-2} h^{-1} (positive numbers indicate flux from the soil into the water column; Table 1). Aside from the aforementioned relationship between dissolved N_2 and O_2 fluxes (Figure 1A), there were no significant relationships between any of the measured gas and nutrient flux rates ($|r| < 0.48$, $p = 0.07$–0.74).

Microbial Community Characterization

When considering the overall microbial community (*16S rRNA* gene), we found the structures of the bacterial and archaeal communities were significantly different in the +salt treatment compared to the control and +fresh treatments (Figure 2, both $p < 0.001$ for PERMANOVA). When focusing on microbes involved in denitrification (*nirS* and *nirK* genes) and DNRA (*nrfA* gene) in this marsh, the community of nitrate reducers is dominated by denitrifiers, with the abundance of *nirS* + *nirK* genes exceeding that of *nrfA* by four to five orders of magnitude [$\sim 10^9$ vs. 10^4–10^5 gene copies (g OM)$^{-1}$ for denitrification genes vs. DNRA genes, respectively; Figure 3]. Further, there was a much greater abundance of denitrifiers with the *nirS* gene [$\sim 10^9$ gene copies (g OM)$^{-1}$] over those with the *nirK* gene [$\sim 10^7$ gene copies (g OM)$^{-1}$].

There was a decrease in the abundance of *nirS*-type denitrifiers (Figure 3A) as well as a shift in *nirS* community structure (Figure 3B) in both the +fresh and +salt plots, relative to the control plots.

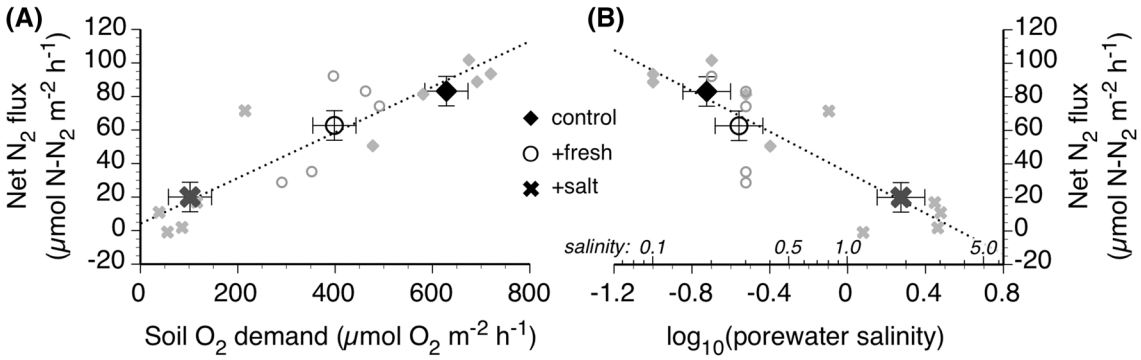

Figure 1. Relationships between (**A**) net N_2 fluxes (denitrification) and soil O_2 demand determined from whole-core incubations and (**B**) net N_2 fluxes and $\log_{10}$-transformed surface porewater salinity (0–3 cm depth). The small italicized numbers above the *x*-axis show nontransformed salinity values. In both panels, the large symbols and error bars show the mean fluxes (± standard error, $n = 5$). The smaller, lighter-colored symbols indicate data from individual cores. The dashed regression lines are fit to measurements from the fifteen individual cores, not to the treatment averages, and have equations of (**A**): N_2 flux = 0.14 × O_2 demand + 4.2; $r = 0.88$ and (**B**): N_2 flux = −60.8 × $\log_{10}$(salinity) + 34.9; $r = -0.83$.

Table 1. Soil Physicochemical Properties and Biogeochemical Flux Rates

Parameter	Control	+Fresh	+Salt	*p*
Soil properties				
Salinity†	0.2 ± 0.1[a]	0.3 ± 0.0[a]	2.1 ± 0.5[b]	< 0.001
OM (%)	71.0 ± 1.9	72.9 ± 1.0	68.6 ± 0.7	0.11
C (%)	33.8 ± 1.3[a]	34.2 ± 0.3[a]	31.1 ± 0.5[b]	0.03
N (%)	2.4 ± 0.1	2.4 ± 0.1	2.2 ± 0.1	0.07
C:N (molar)	16.4 ± 0.5	16.4 ± 0.4	16.8 ± 0.3	0.76
Biogeochemical fluxes				
Denitrification (μmol N-N_2 m^{-2} h^{-1})	83.2 ± 8.8[a]	62.7 ± 12.9[a]	20.1 ± 13.2[b]	0.008
SOD (μmol O_2 m^{-2} h^{-1})	628.7 ± 44.4[a]	398.5 ± 36.4[b]	101.9 ± 31.1[c]	< 0.001
NO_x flux (μmol N m^{-2} h^{-1})	7.4 ± 2.0	3.3 ± 0.8	7.7 ± 1.9	0.16
NH_4^+ flux (μmol N m^{-2} h^{-1})	2.1 ± 2.4	− 0.8 ± 1.0	− 0.3 ± 1.7	0.50
DON flux (μmol N m^{-2} h^{-1})	− 23.8 ± 15.0	− 1.4 ± 18.2	− 20.2 ± 24.5	0.69
SRP flux (μmol P m^{-2} h^{-1})†	− 0.4 ± 0.2	− 0.1 ± 0.3	0.4 ± 0.7	0.48

Values are treatment means (± standard error, n = 5). The N_2 fluxes (denitrification) are directed out of the soil, whereas soil O_2 demand (SOD) is an uptake of O_2 by the soils. For dissolved nutrient fluxes, positive values indicate a flux from the soil into the water column. Superscripted lowercase letters indicate significant differences among treatments as determined by ANOVA followed by Tukey's HSD post hoc tests. The soil physicochemical data were previously reported in Neubauer and others (2013).
OM = organic matter; DON = dissolved organic nitrogen; SRP = soluble reactive phosphorus.
†*ANOVA performed on $\log_{10}$-transformed data.*

These changes were greatest for the community from the +salt treatment, which was significantly different from both the control and +fresh communities (ANOVA for abundance: $p = 0.002$, PERMANOVA of community structure: $p < 0.0001$). In contrast, there were no significant differences across treatments for either *nirK* abundance (Figure 3C, ANOVA $p = 0.13$) or community composition (Figure 3D, PERMANOVA $p = 0.36$), though the NMDS plot does show a modest shift in community composition for the +salt treatment. For *nrfA*, we found significant treatment effects for both abundance (Figure 3E, ANOVA $p = 0.05$) and community composition (Figure 3F, PERMANOVA $p < 0.0001$). In general, *nrfA* abundance increased with additions of either freshwater or brackish water, but this increase was only significant for the +salt treatment.

Physicochemical Properties

In surface soils (0–3 cm), there was a small, but statistically significant decrease in soil C content with increased salinity (treatment means of 33.8–34.2% by weight in control and +fresh, vs. 31.3% in +salt; $p = 0.03$; Table 1). There were no significant differences among treatments for soil organic

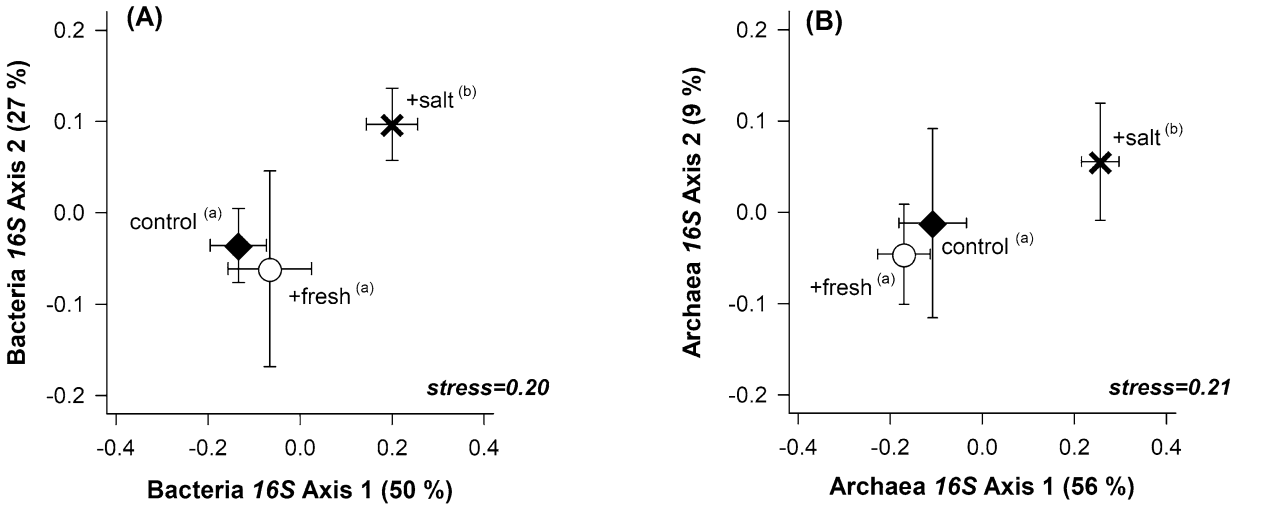

Figure 2. Nonmetric multidimensional scaling (NMDS) plots describing the composition of *16S rRNA* genes associated with bacteria (**A**) and archaea (**B**). Within each panel, lowercase letters indicate significant differences among treatments, as determined using PERMANOVA. Values are treatment means (± standard error, $n = 5$).

matter content, N content, or C:N ratios at the soil surface ($p = 0.07$–0.76; Table 1).

In surface soils, the average salinity and conductivity of porewater extracted by centrifugation were roughly an order of magnitude higher in the +salt plots (salinity = 2.1, conductivity = 4064 µS cm^{-1}) than in the control or +fresh plots (salinity = 0.2–0.3, conductivity = 556–683 µS cm^{-1}; Table 1). Porewater collected using sippers from 10 and 25 cm depth showed similar differences by treatment, with average salinity values of 2.5 (conductivity of 4611 µS cm^{-1}; average of samples collected from 10 and 25 cm depth) in the +salt plots and a salinity of 0.1 (conductivity of 402–409 µS cm^{-1}) in the control and +fresh plots (data not shown). Conductivity did not vary between the 10 and 25 cm depths ($p = 0.48$).

Biogeochemistry–Microbial–Physicochemical Relationships

Across all of the experimental plots, there were strong negative correlations between porewater salinity and rates of denitrification ($r = -0.76$, $p < 0.001$) and soil O_2 demand ($r = -0.79$, $p < 0.001$). Similarly, there were modest correlations between denitrification rates and soil N content ($r = 0.64$, $p = 0.01$) and C:N ratio ($r = -0.52$, $p = 0.04$), and between soil O_2 demand and soil C ($r = 0.58$, $p = 0.02$) and N contents ($r = 0.65$, $p = 0.009$). The relationship between the gas fluxes and soil salinity persisted even after controlling for soil %C, %N, %organic matter, and C:N ratios (N_2 $r_{partial} = -0.83$, $p = 0.001$; SOD $r_{partial} = -0.81$, $p = 0.002$), implying that changes in denitrification and soil O_2 demand were largely driven by the change in salinity (or, more broadly, water chemistry) rather than indirectly through changes in the quantity or composition of soil organic matter. However, when controlling for salinity, there was still a significant relationship between denitrification and soil C:N ratios ($r_{partial} = -0.61$, $p = 0.02$), likely reflecting the role of organic matter quality on denitrification. The best single predictor of rates of denitrification was $\log_{10}$ salinity (Figure 1B; adjusted $r^2 = 0.66$, $p < 0.001$), whereas the best multivariate model used $\log_{10}$ salinity and soil C:N ratio as predictors (adjusted $r^2 = 0.73$, $p < 0.001$). In each case, higher salinity and higher C:N ratio were associated with lower denitrification rates.

Although denitrifiers with the *nirS* gene were the most abundant group of nitrate reducers that we quantified, there was not a significant relationship between *nirS* abundances and rates of denitrification or soil O_2 demand after controlling for the physicochemical differences between plots (Table 2). However, the shifts in *nirS* community structure were strongly correlated with changes in process rates, even when the shared variance with environmental parameters was removed (partial Mantel tests, Table 2). There was also a strong relationship between salinity and community structure that remained significant after controlling for shared variation with soil organic properties (Table 2).

As with *nirS*, there were no significant direct or partial correlations between *nirK* abundance and gas flux rates (all $|r| < 0.30$, $p > 0.40$). Similar to

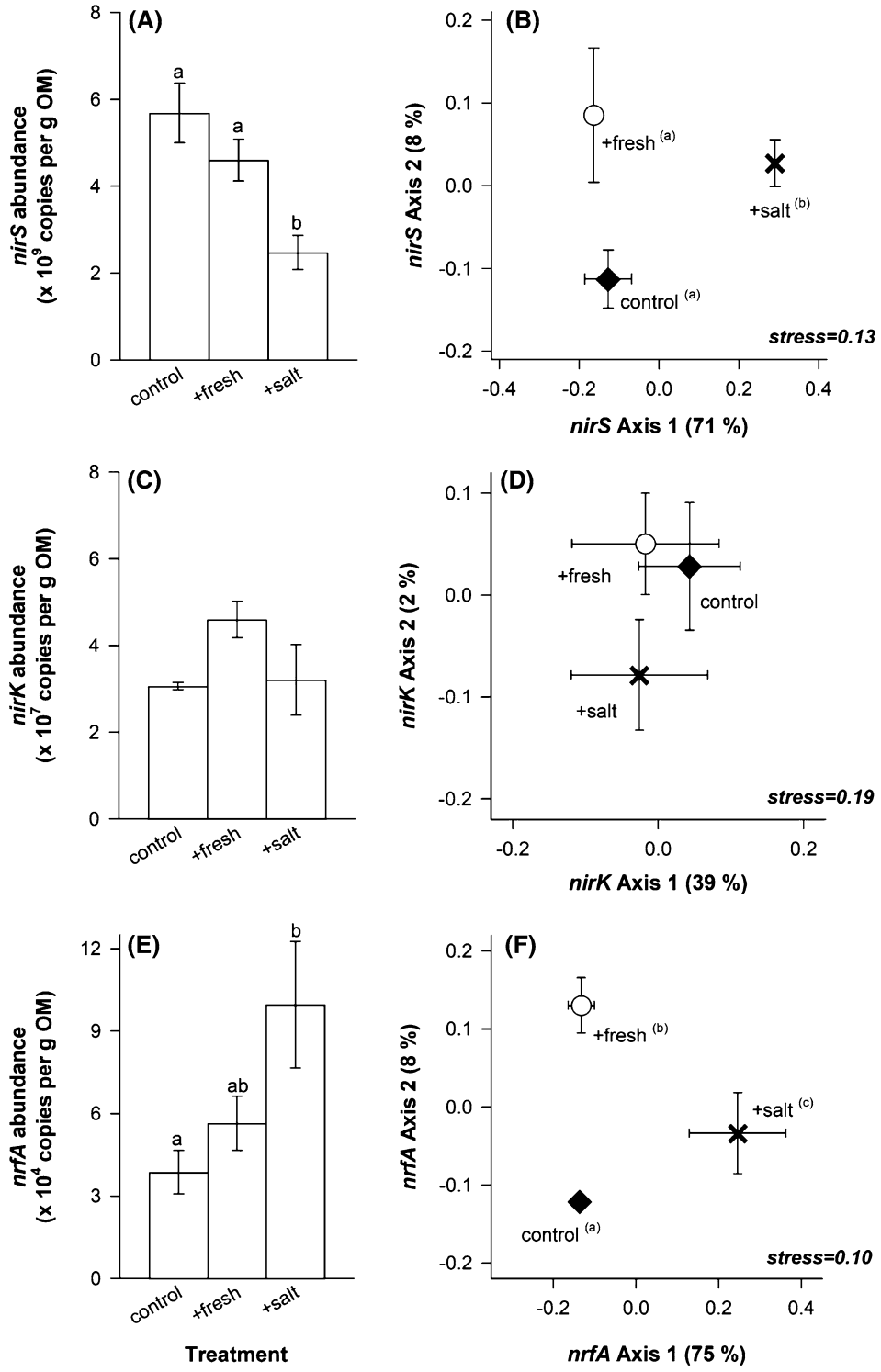

Figure 3. Abundance (**A**, **C**, **E**) and composition (**B**, **D**, **F**) of functional genes associated with denitrification (*nirS* and *nirK*) and DNRA (*nrfA*). Within each panel, lowercase letters indicate significant differences among treatments, as determined using Tukey's HSD tests (**A**, **C**, **E**) or PERMANOVA (**B**, **D**, **E**). Values are treatment means (± standard error, $n = 5$).

nirS, the community structure of *nirK*-denitrifiers was directly correlated with both denitrification ($r_M = 0.41$, $p = 0.003$) and soil O_2 demand ($r_M = 0.62$, $p = 0.0002$), and these relationships remained significant after removing the shared correlation with salinity and soil properties (Table 2).

Table 2. Relationships Between Microbial Genetic Traits and Environmental Data

Parameter	Gene	Salinity[†]		Denitrification[‡]		SOD[‡]	
		r	*p*	*R*	*p*	*r*	*p*
Abundance	*nirS*	− 0.42	0.21	− 0.06	0.86	0.61	0.06
	nirK	0.36	0.27	− 0.30	0.40	0.10	0.78
	nrfA	0.61	0.05*	− 0.28	0.44	− 0.40	0.26
Community structure	*nirS*	0.53	< 0.001*	0.29	0.01*	0.52	< 0.001*
	nirK	0.53	< 0.001*	0.29	0.02*	0.51	< 0.001*
	nrfA	0.43	0.004*	0.19	0.06	0.40	0.005*

Partial correlation analyses comparing denitrification (nirS, nirK) and DNRA (nrfA) functional gene abundance (Pearson correlations, qPCR results) and community structure (Mantel tests, TRFLP data) to salinity and gas flux rates. Additional tests (results not presented) showed no significant partial correlations with percent organic matter, C, N; C:N ratios; or any of the dissolved nutrient fluxes. Asterisks indicate correlations that are statistically significant at α = 0.05.
SOD = soil O_2 demand; OM = organic matter.
[†]*Partial correlation controlling for soil organic properties (%OM, %C, %N, and C:N).*
[‡]*Partial correlation controlling for all environmental data (salinity and organic properties).*

The only other variable that was correlated with *nirK* community composition was salinity (Table 2, all other $r_M < 0.14$, $p > 0.07$).

Rates of DNRA were too low to be detected so we were unable to examine relationships between the abundance/composition of the *nrfA* gene and the process of DNRA. After removing the shared variance with salinity and soil properties, the only significant relationship between the *nrfA* gene and the measured N_2 and O_2 fluxes was between community structure and soil O_2 demand (Table 2). Salinity was the only environmental variable that was related to the *nrfA* data. For *nrfA* abundance, there was a strong positive relationship with salinity (Table 2), but no significant direct correlations or partial correlations for any of the soil organic properties (all $|r| < 0.38$, all $p > 0.16$). Similarly, *nrfA* community structure was correlated with salinity (Table 2), but not with any other environmental variables (all other $r_M < 0.22$, $p > 0.09$).

There were no significant correlations between the nutrient flux rates and the exchanges of N_2 and O_2 ($|r| < 0.41$, $p > 0.13$), gene abundances ($|r| < 0.39$, $p > 0.14$), soil salinity ($|r| < 0.37$, $p > 0.17$), or soil properties ($|r| < 0.39$, $p > 0.15$).

Discussion

Tidal freshwater zones contribute significantly to riverine and estuarine N processing, in large part because they are highly active sites for denitrification (Ensign and others 2008; Hopfensperger and others 2009; Von Korff and others 2014). The research presented herein demonstrates that saltwater intrusion suppresses rates of tidal freshwater marsh denitrification and soil O_2 demand, changes that are driven by interactions between the physicochemical environment and the microbial communities involved in N processing. This new work complements our previous research at this site, which has shown that sustained saltwater intrusion leads to changes in the composition of plant (Neubauer 2013; Neubauer and Sutter in prep) and soil microbial communities (Figure 2), ultimately leading to reductions in rates of primary production, respiration, decomposition, and extracellular enzyme activity (Neubauer 2013; Neubauer and others 2013). Further, our work builds on the growing body of research summarized in Herbert and others (2015), which documented saltwater intrusion impacts across levels of ecological organization ranging from individuals to ecosystems and spatial scales extending from microbes to landscapes.

Experimental Considerations

One of the strengths of the current study is that we examined N cycle responses to sustained (3.5 years) in situ saltwater intrusion so the microbial and biogeochemical responses that we report herein represent more than the initial disturbance-type responses to elevated salinity. Also, manipulating salinity in the field maintains interactions between biotic (for example, plants, soil microbes) and abiotic ecosystem components (for example, soils, hydrology, weather) that can be lost in shorter-term laboratory or mesocosm experiments.

That said, there are several important experimental considerations that should be kept in mind. Firstly, due to analytical considerations, our measurements of denitrification, soil O_2 demand, DNRA, and nutrient fluxes were made on cores

that were first removed from the marsh and then subjected to artificial (always flooded) hydrology so our reported rates may differ from in situ rates, where water level fluctuations can be important (Ensign and others 2008; Von Korff and others 2014; Zheng and others 2016). Nonetheless, cores from all treatments were treated identically, so this should not affect the between-treatment differences that we reported (Figure 1, Table 1). Secondly, we urge caution in extrapolating N biogeochemistry results from our single sampling event to an entire year. This was a necessary experimental design decision as our field plots were too small to allow repeated sampling without compromising other measurements at the site. Given that salinity was consistently elevated in our +salt plots throughout the year, we expect our reported treatment effects would persist throughout the year, even as absolute rates may vary due to seasonal fluctuations in NO_3^- availability, plant productivity, and temperature. Lastly, we manipulated a ''pristine'' tidal freshwater marsh that is far enough upstream that it rarely experiences natural saltwater intrusion events. If saltwater intrusion had occurred steadily over decades, the initial freshwater plant and microbial assemblages might have been gradually replaced by those that were tolerant of brackish water. In contrast, our experimental design forced a step change in salinity and favored any salt-tolerant plants and microbes that were already present in the marsh; there would have been little opportunity for immigration of new species.

Despite these caveats, our research provides novel insights into how saltwater intrusion affects the tidal freshwater marsh N cycle. We expect that our chosen experimental and analytical methods have not affected our fundamental conclusion that saltwater intrusion reduces rates of denitrification and soil O_2 demand. Indeed, our findings of the importance of organic matter quality (C:N ratio) and the composition of the microbial community suggest that sustained saltwater intrusion, if accompanied by a shift from freshwater to salt-tolerant plants and microbes, could have even larger effects on rates of denitrification and soil O_2 demand than we report herein.

Nitrogen Cycle Responses to Saltwater Intrusion

Denitrification

Long-term saltwater intrusion reduced rates of denitrification in the Brookgreen tidal freshwater marsh. This mirrors patterns that are often observed experimentally in wetland soils (Osborne and others 2015) and across broad estuarine salinity gradients (Magalhães and others 2005; Craft and others 2009). Similarly, a meta-analysis of coastal ecosystems including freshwater and saline wetlands, grasslands, and unvegetated systems reported an overall decrease in denitrification due to increased salinity, although this change was not significantly different from zero (Zhou and others 2017) and a few individual studies have even found the opposite trend (for example, Marton and others 2012). Decreases in denitrification rates with increasing salinity are often explained as sulfide interference with denitrification and/or nitrification (Joye and Hollibaugh 1995; Brunet and Garcia-Gil 1996). At our study site, we observed that saltwater intrusion led to a significant decrease in denitrification and the concentration of soil C in surface soils (Table 1), suggesting that C limitation also may have played a role. Across the top 25 cm of the soil profile, soils from the +salt plots had significantly lower soil C and N contents and higher C:N ratios compared to the other treatments (Neubauer and others 2013). Our partial correlation analyses revealed a larger role of organic matter quality (C:N ratios) than organic matter quantity (soil %C) on rates of denitrification. Similarly, organic matter quality has been identified as an important driver of denitrification in other wetlands (Hume and others 2002; Dodla and others 2008).

There was a net flux of NO_x out of the marsh into the overlying water column, strongly suggesting that denitrification in this marsh is coupled with nitrification rather than supported by direct NO_3^- uptake from the water column. We assume that NO_x in our system is primarily NO_3^- since work in other tidal freshwater marshes has shown that the concentrations and/or fluxes of NO_x typically are dominated by NO_3^- rather than NO_2^- (for example, Gribsholt and others 2005; Seldomridge and Prestegaard 2012). The conclusion about the importance of coupled nitrification–denitrification is further supported by the relationship between rates of soil O_2 demand and denitrification observed here (Figure 1A) and elsewhere (Piehler and Smyth 2011). The nitrification of one mole of NH_4^+ to NO_3^- requires the consumption of two moles of O_2 while denitrification reduces two moles of NO_3^- to one mole of N_2 (neither stoichiometry considers losses to nitrous oxide), giving a net stoichiometry for coupled nitrification–denitrification of 2 mol O_2 + 1 mol NH_4^+ $\rightarrow$ 1 mol N–N_2 (Tobias

and Neubauer 2009). Thus, the slope of the denitrification versus soil O_2 demand (Figure 1A) implies that 28% of the soil O_2 demand is used to oxidize NH_4^+ to NO_3^-, which is subsequently denitrified. In other words, there is sufficient O_2 flux into the soil to support coupled nitrification–denitrification. Individual wetland studies (for example, Merrill 1999; Hamersley 2002; Greene 2005; Piehler and Smyth 2011) and a synthesis of coastal ecosystems (Fennel and others 2009) generally report somewhat lower fractions of soil/sediment O_2 demand supporting nitrification ($\sim$ 10–23%). This may be related to the low mineral content of our soil, which limits iron oxidation as a competitive sink for O_2.

The decrease in denitrification rates due to salinization mirrored the change in abundance of *nirS*-type denitrifiers (Figure 3A); however, this correlation was not statistically significant after controlling for covariation with the physicochemical data (Table 2). Salinization appeared to have no effect on the abundance of *nirK*-type denitrifiers (Figure 3C), and neither *nirK* nor total *nir* (*nirS* + *nirK*) copies were correlated with N_2 production. This may initially seem surprising; however, gene abundance is often imperfectly (or not at all) correlated with biogeochemical activity (for example, Enwall and others 2010; Bowen and others 2014; Lisa and others 2015; Babbin and others 2016). This is because DNA-based assays can only provide information about the genetic *potential* of a community to carry out a particular process like denitrification—realized biogeochemical activity depends on much more, including the actual synthesis of enzymes that catalyze a reaction and on environmental parameters (for example, availability of NO_3^- and labile C). We did find that denitrifier community structure was correlated with denitrification rates (Table 2), implying a link between microbial structure and biogeochemical function. This adds to a growing body of evidence that microbial community composition regulates ecosystem process rates (Reed and Martiny 2012; Foulquier and others 2013; Philippot and others 2013; Morrissey and Franklin 2015; Babbin and others 2016).

In our samples, the abundance of *nirK*-type denitrifiers was quite low, relative to *nirS*. Both *nir* genes have been used extensively to study the distribution and diversity of denitrifying microorganisms, and it is not uncommon to find this sort of marked difference in the abundance of the two groups. For example, Franklin and others. (2017) also reported a strong dominance of *nirS*-type denitrifiers in wetland systems similar to ours, whereas Yoshida and others (2009) and Azziz and others (2017) found *nirK* abundance to be 10- to 100-fold greater than *nirS*. In studying marine sediment communities, Braker and others (2000) found some sites contained a diverse mixture of both *nirS* and *nirK* types, whereas *nirK* were completely undetectable at other sites. These sorts of findings, along with the exclusivity of *nir* enzyme types, provide strong support for the idea of niche differentiation between these two groups of denitrifiers. In fact, several studies have shown that *nirS*- and *nirK*-type denitrifiers respond differently to environmental gradients (Santoro and others 2006; Smith and Ogram 2008; Enwall and others 2010), despite the fact that the *nirS* and *nirK* genes are functionally equivalent. The factors that drive differential habitat selection by the two groups remain poorly understood, but a meta-analysis by Jones and Hallin (2010) identified salinity as one of the most important factors. Our results suggest that *nirS*-type denitrifiers may be more strongly affected by salinity (or the soil properties that change with salinization), compared to *nirK* types. A more generalized understanding of the sensitivity of these two groups across a broad range of salinities is important for predicting wetland responses to saltwater intrusion.

Dissimilatory Nitrate Reduction to Ammonium

Regardless of treatment, any DNRA occurring in our soil cores was below detection. We are unsure why rates of DNRA were so low and suspect that it was not a methodological issue since we have previously used the same technique to measure DNRA in other coastal ecosystems (for example, Scott and others 2008; Gardner and McCarthy 2009; Smyth and others 2013). Other studies have reported that DNRA is favored versus denitrification in high sulfide environments when labile carbon concentrations are high and NO_3^- is limited (Tiedje 1988; Silver and others 2001; Burgin and Hamilton 2007). At our Brookgreen study site, approximately 30% of the soil is organic C (Table 1) and water column NO_x concentrations were low (< 1 μmol l^{-1}), conditions that should favor DNRA. However, the soil has high porosity that may lead to a relatively deep (micro)aerobic zone and therefore limit rates of DNRA. This is supported by the net NO_x efflux from the soil (Table 1), which indicates aerobic activity (that is, nitrification) in the marsh soils. The flux of NO_x out of the soil also suggests that the soils may be NO_3^--replete, which would tend to favor denitrification over DNRA,

despite low NO_x concentrations in tidal waters. Regardless of treatment, the abundance of *nrfA* genes was quite low [$< 10^5$ gene copies (g OM)$^{-1}$], but, presumably, DNRA-capable microbes would be more abundant if the environmental conditions were appropriate to support high(er) rates of DNRA. For example, Zheng and others (2016) reported substantial rates of DNRA ($\sim$ 10–60% of total NO_3^- removal) in a brackish marsh on the Yangtze River, China, along with about 10^7 *nrfA* gene copies (g soil)$^{-1}$, abundances that are roughly two orders of magnitude greater than we detected in our soils.

We had expected that rates of DNRA would increase following saltwater intrusion since sulfide inhibition of denitrification can drive nitrate reduction toward DNRA and DNRA-capable microbes can use sulfide as an electron donor (Brunet and Garcia-Gil 1996; Senga and others 2006; Plummer and others 2015). In seagrass beds and subtidal unvegetated sediments, enhanced sulfur cycling (greater sulfate reduction or higher sulfide concentrations) can lead to accelerated rates of DNRA (McGlathery and others 2007; Gardner and McCarthy 2009; Murphy and others 2016). Indeed, additional data from our study system (not shown) show a significant positive correlation between the abundances of genes for DNRA (*nrfA*) and sulfate reduction (*dsrA*), a relationship that persists even after controlling for the effects of salinity and suggests that the DNRA-capable microbes are responding to the increased sulfide that results from sulfate reduction. Similarly, across estuarine gradients, the importance of DNRA is often higher in more saline environments (Gardner and others 2006; Giblin and others 2010; Plummer and others 2015), although denitrification can still be the dominant process in high salinity estuarine waters (Smyth and others 2013). However, this effect could be offset to the extent that saltwater intrusion decreases plant productivity, leading to lower soil C availability (Neubauer and others 2013) and therefore a lower organic C : NO_3^- ratio. Additionally, the surface soils at our Brookgreen study site may have been oxidized, as discussed above, such that sulfides did not accumulate enough to contribute to a shift in NO_3^- removal from denitrification to DNRA. Although rates of DNRA were undetectable in our study—and therefore we could not determine how saltwater intrusion affected rates—the abundance of *nrfA* genes was higher in the plots experiencing saltwater intrusion (Figure 3E). Working in low salinity marshes in Virginia, USA, Franklin and others (2017) found a positive (albeit statistically nonsignificant) correlation between *nrfA* abundance and salinity and, as we did, an effect of salinity on the community composition of DNRA-capable microbes.

Soil Oxygen Demand and Nutrient Fluxes

Rates of soil O_2 demand were reduced due to sustained saltwater intrusion in our tidal freshwater marsh site. Soil O_2 demand integrates all processes that consume O_2, including aerobic respiration and the biological (for example, nitrification, methanotrophy) and abiotic processes (for example, chemical oxidation of sulfides and ferrous iron) that regenerate electron acceptors for use in anaerobic metabolism. As discussed above, about 28% of the O_2 flux into the soil was used to support coupled nitrification–denitrification; we did not calculate the rates of other processes that collectively contribute to total soil O_2 demand. The saltwater-induced reduction in soil O_2 demand reported herein is consistent with observed decreases in rates of soil carbon dioxide and methane production (that is, soil metabolism) and soil C concentrations at our site (Neubauer and others 2013) and may be partially driven by lower plant productivity (Caffrey and others 1993; that is, C loading, after Borsuk and others 2001).

There were no effects of our simulated multiyear saltwater intrusion on the soil–water fluxes of NO_x, NH_4^+, DON, or SRP. This finding contrasts strongly with previous experimental work, which has shown that salinization can lead to enhanced losses of NH_4^+ and either increased or decreased fluxes of phosphate (PO_4^{3-}) from tidal freshwater wetland soils (Noe and others 2012; Ardón and others 2013; Chambers and others 2013; Jun and others 2013; van Diggelen and others 2014; van Dijk and others 2015). Higher NH_4^+ fluxes and concentrations are typically attributed to ionic exchange of soil-bound NH_4^+ with saltwater cations (Seitzinger and others 1991; Weston and others 2010) and/or to enhanced rates of organic matter mineralization following saltwater intrusion (Weston and others 2011; Noe and others 2012; Jun and others 2013; Neubauer and others 2013), which would lead to enhanced mobilization of NH_4^+ from soil organic matter. Higher fluxes of PO_4^{3-} out of salinized soils and sediments are often related to interactions between the iron, sulfur, and phosphorus cycles. Phosphate has a strong affinity for iron oxides, but, in the presence of sulfides, which should be more abundant following saltwater intrusion, iron instead can bind to the sulfides, allowing PO_4^{3-} to diffuse out of the soil/sediment and into the overlying water

column (Jordan and others 2008). However, increased calcium (Ca) mobilization following saltwater intrusion can lead to greater Ca–P binding, thus lowering PO_4^{3-} availability (van Dijk and others 2015).

In our Brookgreen study, there may have been transient changes in both NH_4^+ and PO_4^{3-} fluxes following the initial salinization of our experimental plots (for example, Ardón and others 2013; Jun and others 2013; van Dijk and others 2015), driven by the mechanisms outlined above, but these effects could have dissipated after 3.5 years of saltwater intrusion prior to sample collection. Because of the small size of our experimental plots, we were unable to study temporal effects on nutrient dynamics. We also note that surface soil carbon mineralization rates at the time we conducted this study were up to 85% lower in our +salt soils due to decreased plant production and lower soil C availability (Neubauer and others 2013), suggesting that NH_4^+ production and fluxes may be suppressed following sustained saltwater intrusion. There was a trend toward lower NH_4^+ fluxes in the +salt cores (versus the control soils), but the same pattern also was observed for the +fresh cores (Table 1), suggesting that salinity was not the primary driver. We cannot discount the possibility that differences in soil properties between our site and those referenced above led to differences in nutrient responses to saltwater intrusion. For example, iron–sulfur–phosphorus interactions are likely to be considerably less important in our soil ($\sim$ 70% organic matter) versus a soil with a greater mineral (and iron) content; the ability of iron to bind to sulfides plays a key role in driving system responses to salinization (Schoepfer and others 2014).

Conclusions

Sustained saltwater intrusion in a tidal freshwater marsh reduced rates of denitrification by approximately 70%, a change that was driven by both abiotic and biotic drivers in the ecosystem. Saltwater intrusion increased porewater salinities, which had a strong negative effect on rates of denitrification that were independent of other changes in the environment (soil properties). After controlling for salinity, there was a significant effect of organic matter quality on denitrification. Also, denitrification rates were positively correlated with rates of soil O_2 demand, in part because nitrification was the likely source of NO_3^- for denitrifiers. The abundance of *nirS* and *nirK* denitrification genes did not have a strong effect on denitrification rates, although there were correlations between the community structure of denitrification genes and rates of denitrification, implying a strong link between microbial community composition and biogeochemical function. Rates of DNRA were too low to quantify, but the increased abundance of *nrfA* genes following saltwater intrusion suggests potential for an (eventual) enhancement of DNRA in this oligotrophic system. The reduction in denitrification due to saltwater intrusion can lead to a loss of water quality functions provided by tidal wetlands, an alteration in ecosystem functioning that is independent of any future changes in marsh area due to wetland submergence from sea level rise.

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