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ARTICLE

Resource Availability Effects on Nitrate-Reducing Microbial Communities in a Freshwater Wetland

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Abstract Microbial communities in freshwater wetland soils process nitrate via denitrification (DNF) and dissimilatory nitrate reduction to ammonium (DNRA). Because the processes generate different end products (N-gas versus NH_4^+), the relative dominance of DNF versus DNRA has implications for ecosystem nitrogen cycling, greenhouse gas production, and downstream eutrophication. To examine how resource availability affects these two microbial groups, wetland soil was supplemented with labile (compost) or recalcitrant (wood) organic matter (OM) and/or potassium nitrate fertilizer. Following a three-month *in situ* incubation, the abundance and composition of the DNF- and DNRA-capable microbes were examined via quantitative polymerase chain reaction (qPCR) and terminal restriction fragment length polymorphism (T-RFLP) using process-specific functional genes (DNF: *nir*S qPCR, *nos*Z T-RFLP; DNRA: *nrf*A qPCR and T-RFLP). Denitrifer abundance was positively related to OM lability and simultaneous nitrate amendment enhanced OM effects, while DNRA abundance varied little across treatments. For both groups, community structure showed an interactive response to OM type and nitrate availability, even when abundances did not change. This work highlights the importance of considering co-varying resource gradients, and the differential responses of DNF and DNRA communities to resource manipulation provides insight into the environmental regulators of ecosystem nitrate removal in wetlands.

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Introduction

It has been estimated that approximately 60 % of fertilizer nitrogen (N) used in agriculture is never incorporated into plants, and instead washes out of the soil into rivers or groundwaters, primarily as nitrate (Canfield et al. 2010). Freshwater wetlands are important targets for conservation due to their ability to mitigate downstream nitrogen transport via microbial nitrate reduction—particularly the processes of denitrification (DNF) and dissimilatory nitrate reduction to ammonium (DNRA) (Fisher and Acreman 2004, Ma and Aelion 2005, Erler et al. 2008, Koop-Jakobsen and Giblin 2009, 2010). Both DNF and DNRA are anaerobic processes, typically coupled to organic matter (OM) oxidation, wherein nitrate (NO_3^-) is used as a terminal electron acceptor for microbial respiration to either N_2 and N_2O (DNF) or NH_4^+ (DNRA). The relative dominance of these two processes has implications for downstream eutrophication and greenhouse gas production (Conrad 1996; An and Gardner 2002; Fisher and Acreman 2004), but considerable uncertainty remains as to the biogeochemical regulators that determine the mechanism and extent of microbially-mediated nitrate transformations.

Presumably because of competition and overlapping resource needs, the balance of DNF and DNRA has been found to vary depending upon OM and nitrate availability, though scientists have yet to develop a predictive understanding of these relationships (Hill and Cardaci 2004; Scott et al. 2008; Sutton-Grier et al. 2009; Koop-Jakobsen and Giblin 2010; Nizzoli et al. 2010). Early work led to the development of a hypothesis that DNRA is favored when there is high availability of OM relative to nitrate, whereas DNF is favored under low OM to nitrate ratios (Tiedje 1988;

Burgin and Hamilton 2007). This theory, however, has not been uniformly supported in subsequent examinations of wetland nutrient processing (Scott et al. 2008, Koop-Jakobsen and Giblin 2010). For example, a wetland fertilization study by Koop-Jakobsen and Giblin (2010) found addition of nitrate to increase the activity of both processes by roughly equal amounts, despite the resultant change in OM to nitrate ratio. Other recent work suggests that carbon quality is also a necessary component of conceptual models that consider how OM and nitrate interactively regulate microbial nitrate-reduction in wetlands (Hill and Cardaci 2004; Burgin and Hamilton 2007; Lou et al. 2007; Dodla et al. 2008). For instance, DNF potential in wetlands has been linked with OM lability (e.g., Dodla et al. 2008) and the ratio of cellulose to lignin content (Lou et al. 2007).

Studies that simultaneously consider DNF and DNRA in wetlands have focused heavily on process rate measurements, but have rarely considered microbial community composition (An and Gardner 2002; Scott et al. 2008; Koop-Jakobsen and Giblin 2010). However, given the growing body of evidence that microbial community structure may be important for understanding ecosystem functions (Philippot and Hallin 2005; Reed and Martiny 2007; Fuhrman 2009; Dimitriu et al. 2010), an examination of the microbial ecology underlying these biogeochemical processes is warranted. In the case of DNF, both community composition and abundance of DNF-capable organisms has been found to co-vary with activity measurements (Wolsing and Prieme 2004; Magalhaes et al. 2008; Dang et al. 2009; Attard et al. 2011). In contrast, research into DNRA is limited, and very little is known about how environmental conditions affect community composition, or the relationship between community composition/abundance and process rates (Mohan et al. 2004; Smith et al. 2007; Lam et al. 2009).

In this study, we address this knowledge gap by examining how resource availability influences the abundance and composition of DNF- and DNRA-capable organisms in a tidal freshwater wetland. *In situ* manipulations of OM type and nitrate concentration were conducted using a modified litterbag approach, and subsequent molecular genetic analysis targeted functional genes specific to each nitrate-reduction pathway. Two types of OM were used: wood shavings, representing a more recalcitrant material with a low nutrient content, and compost, which is relatively more labile and nutrient rich (Moore et al. 2005; Antil et al. 2011). These amendments are comparable to those used to increase soil OM during wetland restoration and construction (Davis 1995; Bruland et al. 2009; Sutton-Grier et al. 2009; Warneke et al. 2011). The simultaneous addition of various levels of potassium nitrate fertilizer, mimicking porewater concentrations up to 30 mg L^{-1}, provided a model system with which to study the interaction of OM quality and nitrate availability as co-regulators of wetland nitrogen cycling.

Methods

Experimental Design

This research was conducted in a 30-ha tidal freshwater wetland along the James River at Virginia Commonwealth University's Walter and Inger Rice Center for Environmental Life Sciences, located in Charles City County, Virginia (37° 19′38″ N, 77°12′13″ W). Experimental manipulations took place within a 10 ×10 m square plot near the center of the wetland, in an area that was dominated by obligate wetland vegetation including *Leersia oryzoides*, *Juncus effusus*, and *Polygonum arifolium*. Soils were continually saturated, with a low OM content (~ 6 %) and a C:N ratio of 14 (by mass). Soil texture was classified as silt loam, with approximately 30 % sand, 55 % silt, and 15 % clay. Soil pH varied between 5 and 6, and cation exchange capacity was ~8 meq (100 g)$^{-1}$.

The experiment was conducted using a modified litterbag approach. First, soil from 5 to 15 cm below the soil surface was collected from the field and homogenized in the laboratory. Soil treatments consisted of a partial factorial design of nitrate and/or OM amendments, wherein nitrate was examined at 4 levels and OM additions were made at the lowest and highest levels of nitrate addition. Nitrate (as KNO_3) was added in the form of temperature-controlled slow-release fertilizer pellets (Polyon, Agrium Advanced Technologies, CAS# 7757-79-1, Loveland, CO) to achieve amendment levels of 0, 0.5, 2, and 4 mg N g^{-1} wet sediment; preliminary incubations using the 4-mgN treatment yielded porewater concentrations of ~30 mg L^{-1} following a 3 month field incubation. Organic amendments consisted of 30 % dry weight additions of either compost (commercially available organic blend containing 26 % OM, C:N=18, 0.5 % total N, 0.5 % P_2O_5 and 0.5 % K_2O) or wood shavings (untreated pine, 99 % OM content). Compost and wood were homogenized to ensure they were of similar particle sizes (0.2–5 mm diameter). Both nitrate and OM amendments were removed directly from the manufacturer's containers, weighed, and mixed into soils using clean sterile tools. Neither amendment was sterilized because such treatment would have altered OM and fertilizer quality (e.g., heat degradation of OM, UV polymer complexation, and compromised fertilizer pellet coating).

Sediment bags (15 cm ×15 cm) were constructed using polyester thread and 0.5-mm Nitex mesh (Wildlife Supply Company, Buffalo, NY), and filled with 250-ml of control or augmented soil. Three sediment bags of each type were then buried between 5 and 15 cm below the sediment surface; this depth was necessary to ensure anoxia throughout the long-term incubation and to protect the bags from disturbance during high flow or precipitation events. Within each plot, bag placement was randomized and locations were marked with a flag. Sediment bags were incubated *in*

situ from June until September 2010, at which time they were harvested, placed in air-tight plastic bags, and returned to the laboratory at ambient temperature. Three replicate sediment bags were recovered for each treatment (N=3), except the 'unamended OM with 4N,' where one bag was lost. Upon reaching the laboratory, sediment bags were immediately homogenized and sub-sampled for sediment characterization; ~5 g subsamples were archived at −20 °C for genetic analysis.

Sediment and Porewater Analysis

For each homogenized subsample, redox potential and pH were measured using a Hanna Combo pH and ORP probe (QA Supplies Norfolk, VA), and soil moisture content was analyzed gravimetrically (100 ± 5°C for 72 h). Sediment organic matter (%) was calculated as the mass loss on ignition following combustion at 500°C for 4 h. Total carbon and nitrogen content was determined using a Perkin Elmer CHNS/O Analyzer (Waltman, MA) following acidification of samples using 10 % hydrochloric acid.

Porewater was extracted from 50-ml soil samples by centrifugation at 3,000×g for 15 min, filtered using a 0.45-μm pore-size mixed cellulose ester syringe filter, and stored at −20 °C. Porewater samples were subsequently analyzed for nitrate concentration via ion chromatography (Dionex ICS-1000, Sunnyvale, CA).

Molecular Analyses

Whole-community DNA was extracted from 0.5-g subsamples of soil using the MoBio Power Soil DNA kit (Carlsbad, CA) and then stored at −20 °C. DNA purity and concentration were analyzed using Nanodrop ND-1000 (Thermo Scientific, Willmington, DE). All DNA extracts and PCR products were verified using agarose gel (1.5 %) electrophoresis and ethidium bromide staining.

Functional Gene Abundance via qPCR

Functional gene abundance was determined using quantitative polymerase chain reaction (qPCR). Triplicate reactions were performed for each sample using SYBR GreenER qPCR Supermix for iCycler (Invitrogen, Grand Island, NY) and results were reported as the $\log_{10}$ of the number of gene copies g^{-1} wet soil after averaging technical replicates. As part of methods development, the lack of quenching effects on qPCR analyses were verified using serial dilutions of DNA extracted from wetland samples.

For DNF, the *nirS* gene was targeted using the primers cd3aF (5′GTS AAC GTS AAG GAR ACS GG′3) and R3cd (5′GAS TTC GGR TGS GTC TTG A 3′) (Throback et al. 2004). Genomic DNA from *Paracoccus denitrificans* (Strain #17741, ATCC, Manassas, VA) was used to establish the standard curve (average efficiency=102 % and correlation coefficient r^2=0.99). Reactions (25 μL) were performed with 10 ng DNA template and 0.1 μM concentrations of each primer; thermal cycling conditions were: 50 °C for 2 min, 95 °C for 8.5 min, and 50 cycles of 30 s at 94 °C, 30 s at 56 °C, and 75 s at 72 °C (Biorad iCycler, Hercules, CA).

For DNRA, the abundance of the *nrfA* gene was quantified using the primers nrfA6F (5′GAY TGC CAY ATG CCR AAA GT 3′) and nrfA6R (5′GCB KCT TTY GCT TCR AAG TG′3) (Takeuchi 2006). Genomic DNA from *Escherichia coli* (Strain #11775, ATCC, Manassas, VA) was used to establish the standard curve (average efficiency 85 % and correlation coefficient r^2=0.98). Reactions (25 μL) were performed with 10 ng DNA template and 0.3 μM concentrations of each primer; thermal cycling conditions were: 50 °C for 2 min, 95 °C for 8.5 min, and 50 cycles of 20 s at 94 °C, 40 s at 54.5 °C, and 10 s at 72 °C.

Community Composition via T-RFLP

Microbial community composition was analyzed using Terminal Restriction Fragment Length Polymorphism (T-RFLP) targeting DNF- and DNRA-specific functional genes. For DNF, the *nosZ* gene was amplified using the primers Nos661F (fluorescently labeled, 5′**FAM**-CGG CTG GGG GCT GAC CA A 3′) and Nos1773R (5′ ATR TCG ATC ARC TGB TCG TT 3′) (Magalhaes et al. 2008). PCR reactions (50 μL) consisted of 0.25 μM concentrations of each primer, 100 μg BSA (bovine serum albumin; Roche Inc., Nutley, NJ), 50 ng DNA template, and GoTaq Green Master Mix (Promega, Madison WI). Cycling parameters were: 95°C for 3 min, 37 cycles of 30 s at 95 °C, 30 s at 59.7 °C, 90 s at 72 °C, followed by 72 °C for 8 min. The DNRA functional gene *nrfA* was amplified using F1*b* (fluorescently labeled, 5 ′**FAM**-GCN TGY TGG WSN TGY AA 3′) and R1*b* (5′TWN GGC ATR TGR CAR TC 3′) (Takeuchi 2006). Those PCR reactions (50 μL) consisted of 10 ng DNA template, 10 mMTrisHCl (pH 8.3), 50 mMKCl, 3 mM $MgCl_2$, 200 μM of each dNTP, 0.4 μM of each primer, 30 μg BSA, and 2.5 units of AmpliTaq DNA polymerase (reagents obtained from Applied Biosystems, Foster City, CA). Touchdown thermal cycling parameters were as follows: 94 °C for 5 min, 30 cycles of 60 s at 95 °C, 60 s at 60 °C (−0.5 °C $cycle^{-1}$), 90 s at 72 °C, followed by 30 cycles of 30 s at 95 °C, 30 s at 45 °C, 90 s at 72 °C with a final elongation step of 72 °C for 10 min. PCR products were purified using the MinElute 96 UF PCR purification kit (Qiagen, Valencia, CA) prior to restriction enzyme digest. The *nosZ* digests used 10 units of *Hin*P1I, 1X buffer #4 (New England Biolabs, Ipswich, MA), and 130 μM spermidine (Sigma-Aldrich, St. Louis, MO); incubations were at

37 °C for 6 h, followed by 20 min at 95 °C. The *nrfA* digests used 20 units of *Rsa*I in 1X buffer #4 (New England Biolabs, Ipswich, MA) at 37°C for 6 h, followed by 20 min at 65 °C. Digested amplicons were purified using the MinElute kit, recovered in molecular-grade water, and detected using capillary electrophoresis with a MegaBACE 1000 DNA Analysis System. An aliquot of 70–100 ng of purified, digested PCR product was combined with 0.5 μL of MapMarker 400 ROX ladder (Bioventures, Murfreesboro, TN) plus 4.75 μL injection buffer (0.1 % Tween-20). Samples were injected at 3 kV for 100 s, and electrophoresed using genotyping filter set 1 for 100 min at 10 kV. T-RFLP fragments between 70 and 400 bp were analyzed using Fragment Profiler software (Version 1.2; Amersham Biosciences, Buckinghamshire, UK) using a 1-bp size differential and a 40 relative fluorescent unit peak height threshold. Peaks accounting for less than 2 % of total sample fluorescence were removed prior to data analysis.

Data Analysis

Prior to statistical analysis, a Shapiro-Wilk test was applied to evaluate the distribution of data describing sediment characteristics, porewater nitrate concentrations, and gene copy number (qPCR). All data were normally distributed except the qPCR results, which required a log transformation and were subsequently analyzed as $\log_{10}$ of the functional gene copy number g^{-1} wet soil. To test for treatment effects due solely to nitrate amendment, a one-way analysis of variance (ANOVA) was performed, considering four levels of N addition: 0, 0.5, 2, and 4 mg N g^{-1} sediment (N=11, df=10), followed by a Tukey's HSD post-hoc test. A two-way ANOVA was performed to evaluate potential interactive effects of nitrate amendment and OM type using a subset of samples in a full factorial design (N levels: 0 and 4 mg N g^{-1} sediment, crossed with OM treatments: unamended, added labile, and added recalcitrant; N=17, df=16). One-way ANOVA and Tukey's HSD post-hoc test were used to identify significant differences between OM types within nitrate addition levels. Finally, t-tests were used to assess differences between the two nitrate addition levels within OM type. Analyses were performed using the JMP statistical software (Version 8.0.2, Cary, NC; Sall 2005) and evaluated using a 0.05 significance level.

The T-RFLP assay yielded 57 unique terminal restriction fragments (corresponding to putative taxonomic groups) for the DNF *nosZ* gene and 63 distinct fragments for DNRA *nrfA*. These results were recorded as a binary data matrix describing the presence or absence of each fragment in each sample. This matrix was then converted to a set of Jaccard coefficients that quantifies the relative similarity between each pair of samples, which was used for subsequent ordination analysis and determination of statistically significant treatment effects. Specifically, visualization of overall similarity between communities was achieved using non-metric multidimensional scaling (NMDS) performed using the PAST statistical software package (Version 2.10; Hammer 2001). One-way non-parametric multivariate analysis of variance (NP-MANOVA) was used to test for significant effects of nitrate amendment considering the four levels of addition: 0, 0.5, 2, and 4 mg N g^{-1} sediment (N=11, df=10). Interactive effects of OM type and nitrate amendment on community composition were evaluated using the two-way NP-MANOVA in R version 2.15.0 (Oksanen et al. 2012) using the adonis function of the vegan package (N levels: 0 and 4 mg N g^{-1} sediment, crossed with OM treatments: unamended, added labile OM, and added recalcitrant OM; N=17, df=16).

Results

Individual Effect of Nitrate Amendments

Soil Properties

Soils with higher amendments of nitrate had significantly greater redox potential (F=11.6, p<0.01) and OM content (F=4.8, p=0.04), and a nearly significant reduction in C:N ratio (F=4.4, p=0.05) as determined using one-way ANOVA (Fig. 1A, C, E). Porewater nitrate concentrations also increased (Fig. 1G), but the change was not statistically significant (F=3.4, p=0.08). In contrast, soil pH (mean ± S.E.; 6.0±0.1) and gravimetric moisture content (%, 46.5± 2.5) were not affected by these treatments (results not presented, pH: F=0.02, p=0.99; Moisture: F=0.5, p=0.69).

Microbial Community

Nitrate amendment did not significantly alter the abundance of either functional group (ANOVA for DNF: F=1.8, p= 0.24; DNRA: F=1.2, p=0.37; Fig. 2A, C) and did not exhibit a consistent impact on community composition. No nitrate-amendment effect on DNF community composition was detected (NP-MANOVA: F=1.0, p=0.40), and the effect on the DNRA community was small (F=1.4, p=0.02). Post-hoc pair-wise comparisons of the DNRA community across nitrate levels did not reveal any significant differences (all F<2.1, p>0.9).

Interactive Effects of Nitrate Amendment and OM Type

Soil Properties

No interactions among treatments were detected for the analysis of soil OM content or porewater nitrate concentration using the two-way ANOVA (OM: F=0.9, p=0.42,

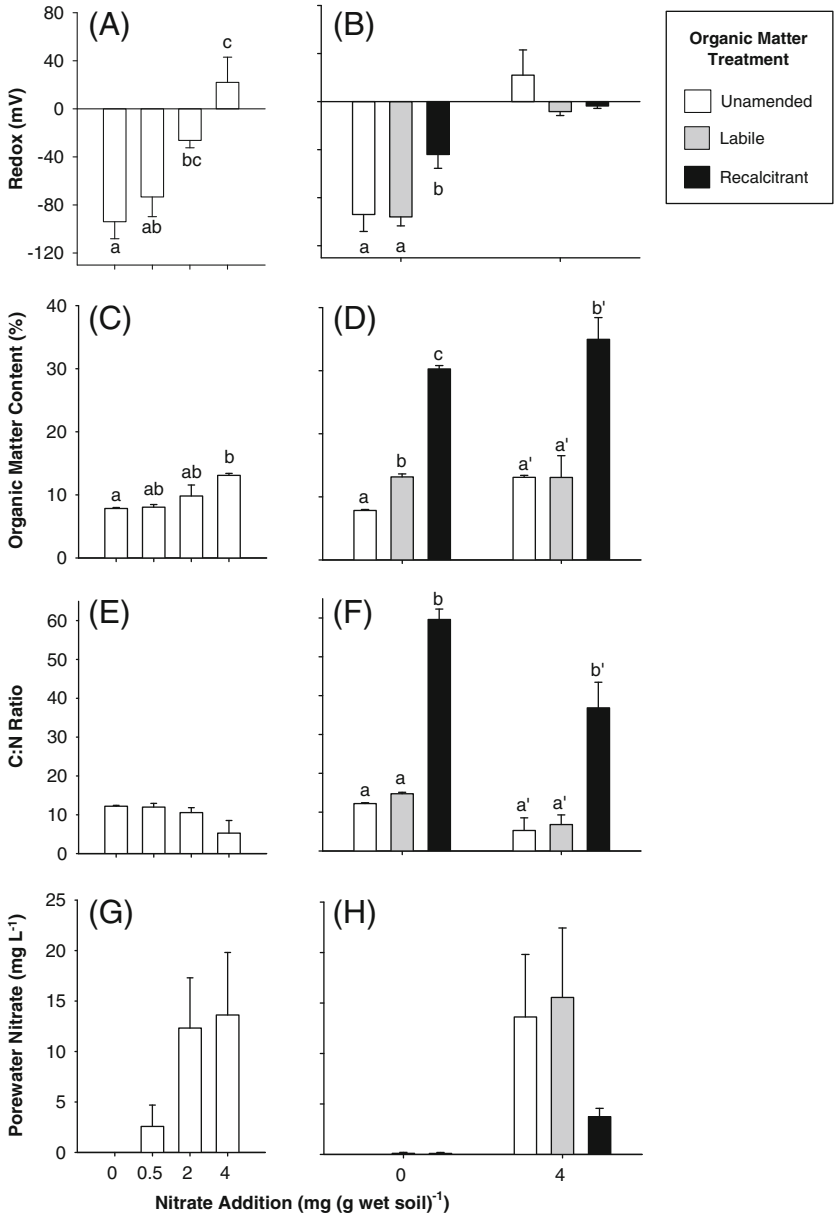

Fig. 1 Treatment effects (mean±1 standard error) on environmental parameters including: redox (**A**, **B**), OM content (**C**, **D**), C:N ratio (**E**, **F**), and porewater nitrate concentration (**G**, **H**). Panels in the left column display nitrate-amendments in the absence of added OM; letters denote significantly different subgroups as determined via one-way ANOVA and Tukey's HSD. Panels in the right column show combined treatment effects (OM and/or nitrate amendment). Different lower case letters (or letters with a prime (′)) represent statistically significant differences when one-way ANOVA and Tukey's HSD were performed on OM type at nitrate amendment levels of 0 and 4 mg N g^{-1} wet sediment, respectively

Porewater nitrate: F=1.7, p=0.21). However, there was a significant main effect of OM amendment on soil OM content (Fig. 1D; F=62.3, p<0.01), and of nitrate amendment on porewater nitrate concentration (Fig. 1H; F=14.3, p<0.01).

A significant interaction effect was observed in the two-way ANOVA of soil redox (F=6.6, p=0.01). In the absence of any added nitrate, the addition of recalcitrant OM increased redox potential relative to the unamended and labile OM types (Fig. 1B; F=6.7, p=0.03). In the presence of added nitrate, this differential response to OM was lost (F=3.1, p=0.13). Regardless of OM treatment, the addition of nitrate increased redox potential.

The interaction effect was not significant for soil C:N (F=3.4, p=0.07), but both nitrate addition and OM type had strong main effects (Fig. 1F). Specifically, nitrate amendment consistently decreased C:N (F=19.7, p<0.01) and the addition of recalcitrant OM increased C:N (F=86.2, p<0.01). No significant interactions or treatment effects were obtained for soil pH or gravimetric moisture content (results not presented; for pH: 5.8±0.2, F=3.0 and p=0.06; for moisture (%): 48.4±1.7, F=1.6 and p=0.23).

Microbial Community

When the combination of nitrate amendment and OM type was considered, DNF functional gene abundance was interactively affected (F=7.4, p<0.01; Fig. 2B). Regardless of nitrate addition, abundance was lowest when recalcitrant OM was added and highest when labile OM was added;

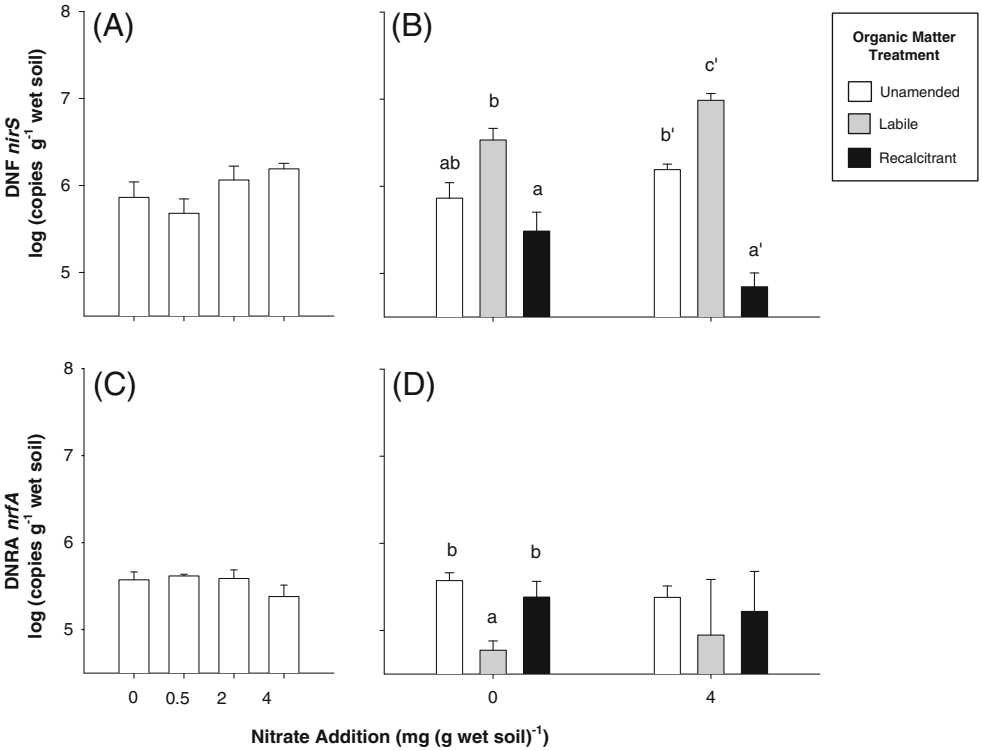

Fig. 2 Treatment effects (mean±1 standard error) on the abundance of DNF *nirS* (**A**, **B**) and DNRA *nrfA* (**C**, **D**) functional genes as determined via qPCR. Panels in the left column display nitrate-amendments in the absence of added OM. Panels in the right column show combined treatment effects (OM and/or nitrate amendment). Different lower case letters (or letters with a prime (′)) represent statistically significant differences when one-way ANOVA and Tukey's HSD were performed on OM type at nitrate amendment 0 and 4 mg N g^{-1} wet sediment, respectively

these differences were more pronounced when combined with nitrate addition. Within each OM treatment, t-tests revealed that nitrate fertilization significantly increased DNF abundance in the presence of labile OM (t=3.0, p=0.03) and decreased DNF in presence of recalcitrant OM (t=−2.4, p=0.04). In contrast, two-way ANOVA revealed no significant effects on DNRA functional gene abundance (all F<2.0, p>0.10), though DNRA abundance decreased when labile OM alone was added (F=10.1, p=0.01, Fig. 2D).

The NMDS of the T-RFLP data revealed interactive effects of nitrate and OM amendments on community composition. In ordination space, samples that are farther apart are less similar with regards to the presence/absence of terminal restriction fragments. For DNF, the greatest effect

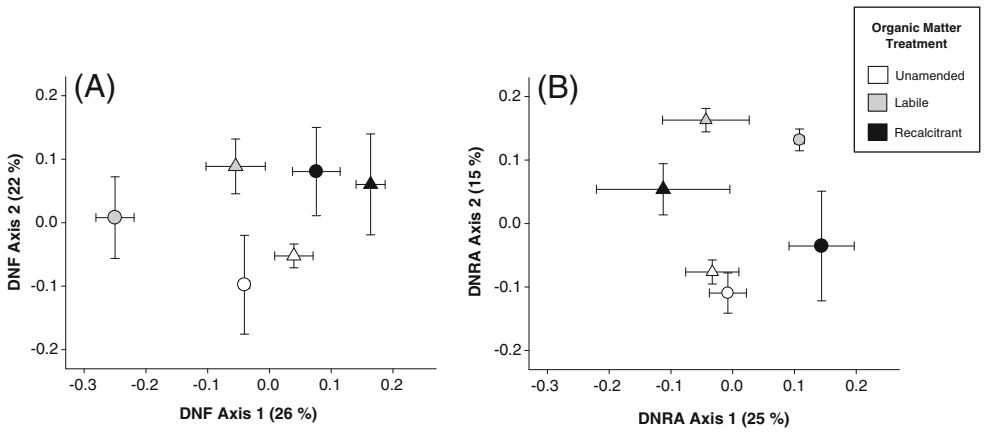

Fig. 3 Non-metric multidimensional scaling (NMDS) ordination diagrams derived from T-RFLP data for the DNF *nosZ* (**A**) and DNRA *nrfA* (**B**) genes. Stress values for the 3D solutions were 0.21 and 0.29 respectively. Numbers associated with each axis title correspond to the percent of variance explained by the axis. Points are centroids±1 standard error; circle (○) indicates no nitrate amendment and triangle (Δ) indicates 4N nitrate amendment. In the treatments with no added OM ("unamended"), the three nitrogen levels were not significantly different, so the results were pooled into a single point. Proximity of samples on these ordination diagrams reflects overall community similarity as determined using Jaccard coefficients applied to the presence/absence of each unique terminal restriction fragment in each community profile

was due to OM. This can be seen in Fig. 3A as a distinct separation of the three OM treatments in ordination space. Within each OM type, DNF community structure shifted toward the right on Axis 1 with the addition of nitrate, which suggests the amendment affected a consistent subset of community members regardless of OM treatment. The magnitude of the nitrate-induced shift was most dramatic in the presence of labile OM. NP-MANOVA confirmed this interaction effect was significant (F=2.9, p=0.01), as were the main effects of OM type (F=2.7, p=0.01) and nitrate addition (F=2.0, p=0.01).

OM type also had an effect on DNRA community composition, with the greatest difference being due to the addition of labile material (Fig. 3B). Further, simultaneous addition of nitrate caused a consistent shift in DNRA community composition, this time toward the left on Axis 1. NP-MANOVA detected a nearly significant interaction between OM type and nitrate level (F=1.3, p=0.05). This manifested on the NDMS plot as a small shift in community composition due to nitrate addition to native soil and a much larger shift when nitrate was added in concert with either form of OM (Fig. 3B). As with DNF, main effects on DNRA community composition were significant for both OM type (F=1.7, p<0.01) and nitrate amendment (F=1.6, p=0.03).

Discussion

Effect of OM and Nitrate Addition on Soil Properties

Analysis of soil characteristics demonstrated that the experimental manipulations altered OM concentration and porewater nitrate levels in a manner consistent with expectations (Fig. 1), while basic soil properties like pH and moisture content remained unchanged, thus creating a unique opportunity to examine microbial community responses to altered resource environments. Quality of soil OM, as estimated by C:N, was also affected by the treatments; this was especially evident when recalcitrant OM (wood) was added to the soil, which resulted in a large increase in C:N. In the case of the labile OM addition, C:N ratio was not appreciably changed (unamended native soil C:N=14; original compost C:N=18, which was diluted ~1/3 when treatments were prepared), though the composition and mineralization of compost OM has been shown to be distinct from native soils (Tuomela et al. 2000; Antil et al. 2011). The high-nitrate treatments had greater soil redox potential, which indicates a shift in the availability of terminal electron acceptors for microbial metabolism (Thullner et al. 2007; Reddy and DeLaune 2008). An unexpected response in the soil characteristics was a small but statistically significant increase in OM content caused by nitrate amendment (Fig. 1D). Because soil percent carbon did not change as a result of nitrate fertilization (data not shown), it is likely this result was an artifact caused by the combustion of fertilizer pellets during the assay for OM content. Taken together, these changes in soil OM quality and quantity, redox potential, and nitrate availability were expected to influence both the abundance and composition of the subset of the microbial community responsible for nitrate reduction.

Independent Effects of OM and Nitrate Addition on Microbial Community Composition

When only nitrate availability was manipulated, no significant differences were observed in the abundance of either DNF- or DNRA-capable organisms (Fig. 2A, C). Likewise, the effect of nitrate amendment on community composition was small (Fig. 3). This suggests that nitrate levels were not limiting population growth of either functional group. In contrast, when soil OM was altered, DNF and DNRA communities changed in both abundance and composition. The addition of labile OM to the soil resulted in higher abundance of the DNF functional gene (Fig. 2B), which is consistent with prior studies that have found labile OM to benefit DNF communities (Hill and Cardaci 2004; Ullah and Faulkner 2006; Dodla et al. 2008; Sutton-Grier et al. 2009). The simultaneous effect on composition of the DNF community (Fig. 3A) demonstrates that this change in abundance was not simply the result of increased population size, but also a shift in the relative abundance of the various DNF populations. This provides evidence that genetically distinct populations of DNF-capable organisms vary in their resource preferences, and suggests that certain groups may be especially well poised to take advantage of the labile OM. In contrast, the addition of labile OM resulted in lower abundance of the DNRA functional gene (Fig. 2D), which implies that DNRA microbes may be less effective competitors than DNF in the presence of labile resources. When recalcitrant OM was added, DNF abundance decreased (Fig. 2B), and community composition shifted for both functional groups (Fig. 3). Such effects could result from decreased levels of carbon availability in the sediment that was amended with recalcitrant OM (i.e., “dilution of resources”). This reduction could depress DNF abundance by decreasing access to their preferred labile substrates. An alternative explanation is that the addition of OM may have diluted the abundance/biomass of the native microbial community, and competition during regrowth could have influenced community structure. There is a possibility that microbial communities associated with the unsterilized OM amendments could persist and influence the abundance and composition of the functional groups measured. However, the *in situ* incubation time of 3 months was likely

sufficient for the environmental influence on microbial communities to surpass any small initial bioagumentation or dilution effects.

The effect of OM type on the composition of DNF and DNRA communities is consistent with prior work, and suggests that the changes in community composition we observed are likely a result of selection based on the differential ability of organisms to utilize the different components of the soil OM pool. Previous research has shown that both individual populations and whole communities of bacteria can have distinct substrate utilization profiles (Doutereol et al. 2010; Yadav et al. 2011), which likely occurs within the diverse set of organisms that comprise the DNF and DNRA community. For instance, Peralta et al. (2010) found both bacterial community composition (evaluated using the *16S rRNA* gene) and variations in the DNF *nosZ* gene to be structured by soil C:N and total OM. It is worth noting that, as with all molecular microbial ecology studies, our results may be biased by our choice of target functional genes and associated primer sequences. For example, not all DNF bacteria contain the *nosZ* gene (Jones et al. 2008) and there is considerable selection bias across *nosZ* primer sets (Throback et al. 2004). The primers we used, 661F and 1773R, have been employed in a variety of studies (Magalhaes et al. 2008; Krishnani 2010; Baxter et al. 2012) but some researchers have found them to be ineffective at amplifying *nosZ* sequences from particular denitrifying strains (Throback et al. 2004). Overall, we do not see this as a severe limitation in the current study due to the fact that our molecular assays detected changes in community composition and abundance *despite* these methodological limitations. Ultimately, analysis of additional genes and use of alternate primer sets could increase the resolution of the community analysis, and may be useful in future studies to determine finer-scale controls on DNF and DNRA response to resource conditions.

Interactive Effect of OM and Nitrate Addition on Microbial Community Composition

Combined addition of OM and nitrate demonstrated the ability of resources to interactively regulate microbial communities. In the DNF community, nitrate addition alone had no effect on abundance (Fig. 2A), but magnified the individual effects of OM type (Fig. 2B). This suggests that DNF abundance was more limited by OM than nitrate availability in this system, which is consistent with previous research that has similarly found microbial biomass and DNF activity to be carbon-limited in anaerobic wetland soils (Sutton-Grier et al. 2009). The effect of nitrate amendment on DNF and DNRA community composition was also greater when combined with OM addition than in isolation (Fig. 3). With respect to the DNF community, previous results have been equivocal, with some studies concluding that nitrate structures communities whereas others report that it is not an important environmental determinant (Jones and Hallin 2010; Peralta et al. 2010; Tang et al. 2010; Song et al. 2011). The current study helps resolve this inconsistency by emphasizing the synergistic effects of nitrate and OM type, suggesting that nitrate's effect on community composition may be strong when accompanied by particular OM characteristics (e.g., labile OM) but weak under other soil OM conditions (e.g., unamended OM; Fig. 3). One mechanism by which nitrate concentration could impact composition of DNF and/or DNRA organisms is by differential selection of organisms based on enzyme affinity for nitrate. Community composition shifts associated with enzyme affinity for a terminal electron acceptor have been demonstrated in other heterotrophic microbial communities (Kiesel et al. 2008).

This study garnered little evidence for direct resource competition between the DNF- and DNRA-capable microorganisms. A population increase in one group did not necessarily correspond to a decrease in the other, and abundances were not significantly correlated (Spearman's $\rho = -0.31$, $p = 0.15$). However, abundance was affected by resource availability in a manner that suggests these two groups employ contrasting metabolic strategies for resource utilization. Specifically, the abundance of DNF organisms was consistent with an ecological classification as "copiotrophs" in that they were strong competitors in environments with abundant available nutrients, and weak competitors under resource-poor conditions (Fierer et al. 2007). This preference derives from the fact that copiotrophic organisms typically have high growth rates, high maintenance requirements, and low enzyme affinity (Button 1993; Kovárová-Kovar and Egli 1998). Conversely, the pattern of DNRA abundance was more consistent with an oligotrophic classification; relative to denitrifiers, DNRA organisms thrived in resource poor environments and were not effective competitors under resource rich conditions. In addition to changes in abundance, resource manipulation resulted in differences in community composition for both groups. Further study is necessary to determine the functional significance of such changes, but microbial community composition has previously been linked to ecosystem processes rates (see Allison and Martiny 2008, and citations therein), and likely plays an important role in resource utilization associated with microbially-mediated nitrate transformations.

Implications for Wetland Restoration

The results of this study are relevant to scientists interested in the restoration of natural wetlands and the construction of artificial wetlands. In both situations, OM amendments are

commonly employed to accelerate soil development, enhance bulkdensity, and modulate soil moisture fluctuations in wetlands (e.g., see references in Bruland et al. 2009), all of which are important determinants in the growth and survival of colonizing vegetation and thus restoration success. Further, because organic carbon is a key substrate for many microbial processes taking place in wetlands, OM amendments are often used to enhance biogeochemical activity in newly restored wetland soils. The results presented here demonstrate that different types of OM amendments will have different consequences in terms of nitrate removal and, further, that the response of an ecosystem to OM amendment will depend on the anticipated nutrients loads from the watershed (e.g., nitrate concentration). Given that addition of OM to the surface or sediment during wetland construction is a common way to enhance nitrate removal through DNF (Fleming-Singer and Horne 2002; Burchell et al. 2007; Kadlec 2012), the current study suggests the addition of labile OM will increase denitrifier abundance. In contrast, wetlands to which more recalcitrant OM has been added may retain more of the reactive nitrogen via DNRA conversion to ammonium, thus altering nutrient availability, which could influence both plant productivity and carbon mineralization.

Conclusions

This work demonstrates that both OM and nitrate have strong yet disparate effects on DNF and DNRA community structure, and highlights the importance of evaluating resource combination effects on microbial communities in wetlands. Specifically, DNF populations are favored and DNRA populations are reduced under high resource environments, which suggests different ecological strategies may be employed by each functional group of microbes. Because of these microbial community attributes, the types of OM additions made in wetland engineering efforts should be chosen carefully based on the ecosystem services desired.

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References

Allison SD, Martiny JBH (2008) Resistance, resilience, and redundancy in microbial communities. Proceedings of the National Academy of Sciences USA 105:11512–11519

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

Antil RS, Bar Tal A, Fine P, Hadas A (2011) Predicting nitrogen and carbon mineralization of composted manure and sewage sludge in soil. Compost Science Utilization 19:33–43

Attard E, Recous S, Chabbi A, De Berranger C, Guillaumaud N, Labreuche J, Philippot L, Schmid B, Roux L (2011) Soil environmental conditions rather than denitrifier abundance and diversity drive potential denitrification after changes in land uses. Global Change Biology 17:1975–1989

Baxter A, Johnson L, Edgerton J, Royer T, Leff L (2012) Structure and function of denitrifying bacterial assemblages in low-order Indiana streams. Freshwater Science 31:304–317

Bruland GL, Richardson CJ, Daniels WL (2009) Microbial and geochemical responses to organic matter amendments in a created wetland. Wetlands 29:1153–1165

Burchell MR II, Skaggs RW, Lee CR, Broome S, Chescheir GM, Osborne J (2007) Substrate organic matter to improve nitrate removal in surface-flow constructed wetlands. Journal of Environmental Quality 36:194–207

Burgin A, Hamilton S (2007) Have we overemphasized the role of denitrification in aquatic ecosystems? A review of nitrate removal pathways. Frontiers in Ecology and the Environment 5:89–96

Button D (1993) Nutrient-limited microbial growth kinetics: overview and recent advances. Anton Van Leeuwenhoek 63:225–235

Canfield D, Glazer A, Falkowski P (2010) The evolution and future of Earth's nitrogen cycle. Science 330:192–196

Conrad R (1996) Soil microorganisms as controllers of atmospheric trace gases (H_2, CO, CH_4, OCS, N_2O, and NO). Microbiology Reviews 60:609–640

Dang H, Wang C, Li J, Li T, Tian F, Jin W, Ding Y, Zhang Z (2009) Diversity and distribution of sediment *nirS*-encoding bacterial assemblages in response to environmental gradients in the eutrophiedJiaozhou Bay, China. Microbial Ecology 58:161–169

Davis L (1995) A handbook of constructed wetlands: A guide to creating wetlands for-agricultural wastewater, domestic wastewater, coal mine drainage, stormwater in the Mid-Atlantic Region. USDA-Natural Resources Conservation Service and the US Environmental Protection Agency, 55 pp

Dimitriu P, Lee D, Grayston S (2010) An evaluation of the functional significance of peat microorganisms using a reciprocal transplant approach. Soil Biology and Biochemistry 42:65–71

Dodla S, Wang J, DeLaune R, Cook R (2008) Denitrification potential and its relation to organic carbon quality in three coastal wetland soils. Science of the Total Environment 407:471–480

Doutereol I, Goulder R, Lillie M (2010) Soil microbial community response to land-management and depth, related to the degradation of organic matter in English wetlands: Implications for the *in situ* preservation of archaeological remains. Applied Soil Ecology 44:219–227

Erler DV, Eyre BD, Davison L (2008) The contribution of anammox and denitrification to sediment N_2 production in a surface flow constructed wetland. Environmental Science and Technology 42:9144–9150

Fierer N, Bradford M, Jackson R (2007) Toward an ecological classification of soil bacteria. Ecology 88:1354–1364

Fisher J, Acreman M (2004) Wetland nutrient removal: a review of the evidence. Hydrology and Earth System Science 8:673–685

Fleming-Singer MS, Horne AJ (2002) Enhanced nitrate removal efficiency in wetland microcosms using an episediment layer for denitrification. Environmental Science and Technology 36:1231–1237

Fuhrman J (2009) Microbial community structure and its functional implications. Nature 459:193–199

Hammer Ø (2001) PAST: Paleontological statistics software package for education and data analysis. Palaeontol Electron 4:1–9

Hill A, Cardaci M (2004) Denitrification and organic carbon availability in riparian wetland soils and subsurface sediments. Soil Science Society of America Journal 68:320–325

Jones C, Hallin S (2010) Ecological and evolutionary factors underlying global and local assembly of denitrifier communities. ISME Journal 4:633–642

Jones C, Stres B, Rosenquist M, Hallin S (2008) Phylogenetic analysis of nitrite, nitric oxide, and nitrous oxide respiratory enzymes reveal a complex evolutionary history for denitrification. Molecular Biology and Evolution 25:1955–1966

Kadlec R (2012) Constructed marshes for nitrate removal. Critical Reviews in Environmental Science and Technology 42:934–1005

Kiesel B, Balcke G, Dietrich J, Vogt C, Geyer R (2008) Microbial community shifts as a response to efficient degradation of chlorobenzene under hypoxic conditions. Biodegradation 19:435–446

Koop-Jakobsen K, Giblin A (2009) Anammox in tidal marsh sediments: the role of salinity, nitrogen loading, and marsh vegetation. Estuaries and Coasts 32:238–245

Koop-Jakobsen K, Giblin A (2010) The effect of increased nitrate loading on nitrate reduction via denitrification and DNRA in salt marsh sediments. Limnology and Oceanography 55:789–802

Kovárová-Kovar K, Egli T (1998) Growth kinetics of suspended microbial cells: From single-substrate-controlled growth to mixed-substrate kinetics. Microbiology and Molecular Biology Reviews 62:646–666

Krishnani K (2010) Detection and diversity of nitrifying and denitrifying functional genes in coastal aquaculture. Aquaculture 302:57–70

Lam P, Lavik G, Jensen MM, van de Vossenberg J, Schmid M, Woebken D, Gutierrez D, Amann R, Jetten MS, Kuypers MM (2009) Revising the nitrogen cycle in the Peruvian oxygen minimum zone. Proceedings of the National Academy of Sciences USA 106:4752–4757

Lou Y, Ren L, Li Z, Zhang T, Inubushi K (2007) Effect of rice residues on carbon dioxide and nitrous oxide emissions from a paddy soil of subtropical China. Water Air and Soil Pollution 178:157–168

Ma H, Aelion C (2005) Ammonium production during microbial nitrate removal in soil microcosms from a developing marsh estuary. Soil Biology and Biochemistry 37:1869–1878

Magalhaes C, Bano N, Wiebe W, Bordalo A, Hollibaugh J (2008) Dynamics of nitrous oxide reductase genes (*nosZ*) in intertidal rocky biofilms and sediments of the Douro River Estuary (Portugal), and their relation to N-biogeochemistry. Microbial Ecology 55:259–269

Mohan S, Schmid M, Jetten M, Cole J (2004) Detection and widespread distribution of the *nrfA*gene encoding nitrite reduction to ammonia, a short circuit in the biological nitrogen cycle that competes with denitrification. FEMS Microbiology Ecology 49:433–443

Moore T, Trofymow J, Siltanen M, Prescott C (2005) Patterns of decomposition and carbon, nitrogen, and phosphorus dynamics of litter in upland forest and peatland sites in central Canada. Canadian Journal of Forest Research 35:133–142

Nizzoli D, Carraro E, Nigro V, Viaroli P (2010) Effect of organic enrichment and thermal regime on denitrification and dissimilatory nitrate reduction to ammonium (DNRA) in hypolimnetic sediments of two lowland lakes. Water Research 44:2715–2724

Oksanen JF, Blanchet G, Kindt R, Legendre P, Minchin PR, O'Hara RB, Simpson GL, Solymos PM, Stevens HH, Wagner H (2012) vegan: Community Ecology Package. R package version 2.0–3

Peralta A, Matthews J, Kent A (2010) Microbial community structure and denitrification in a wetland mitigation bank. Applied and Environmental Microbiology 76:4207–4215

Philippot L, Hallin S (2005) Finding the missing link between diversity and activity using denitrifying bacteria as a model functional community. Current Opinions in Microbiology 8:234–239

Reddy R, DeLaune R (2008) Biogeochemistry of wetlands: science and applications. CRC Press Taylor and Francis Group, Boca Raton

Reed H, Martiny J (2007) Testing the functional significance of microbial composition in natural communities. FEMS Microbiology Ecology 62:161–170

Sall J (2005) JMP start statistics: a guide to statistics and data analysis using JMP and JMP IN software. SAS Institute Inc, Cary

Scott J, McCarthy M, Gardner W, Doyle R (2008) Denitrification, dissimilatory nitrate reduction to ammonium, and nitrogen fixation along a nitrate concentration gradient in a created freshwater wetland. Biogeochemistry 87:99–111

Smith C, Nedwell D, Dong L, Osborn AM (2007) Diversity and abundance of nitrate reductase genes (*narG*and *napA*), nitrite reductase genes (*nirS* and *nrfA*), and their transcripts in estuarine sediments. Applied and Environmental Microbiology 73:3612–3622

Song K, Lee S, Kang H (2011) Denitrification rates and community structure of denitrifying bacteria in newly constructed wetland. European Journal of Soil Biology 47:24–29

Sutton-Grier A, Ho M, Richardson C (2009) Organic amendments improve soil conditions and denitrification in a restored riparian wetland. Wetlands 29:343–352

Takeuchi J (2006) Habitat segregation of a functional gene encoding nitrate ammonification in estuarine sediments. Geomicrobiology Journal 23:75–87

Tang H, Yan K, Zhang L, Chi F, Li Q, Lian S, Wei D (2010) Diversity analysis of nitrite reductase genes (*nirS*) in black soil under different long-term fertilization conditions. Annals of Microbiology 60:97–104

Throback I, Enwall K, Jarvis A, Hallin S (2004) Reassessing PCR primers targeting *nirS*, *nirK* and *nosZ* genes for community surveys of denitrifying bacteria with DGGE. FEMS Microbiology Ecology 49:401–417

Thullner M, Regnier P, Van Cappellen P (2007) Modeling microbially-induced carbon degradation in redox-stratified subsurface environments: Concepts and open questions. Geomicrobiology Journal 24:139–155

Tiedje JM (1988) Ecology of denitrification and dissimilatory nitrate reduction to ammonium. In: Zehnder A (ed) Biology of anaerobic microorganisms. John Wiley and Sons, New York, pp 179–244

Tuomela M, Vikman M, Hatakka A, Itävaara M (2000) Biodegradation of lignin in a compost environment: a review. Bioresource Technology 72:169–183

Ullah S, Faulkner S (2006) Use of cotton gin trash to enhance denitrification in restored forested wetlands. Forest Ecology and Management 237:557–563

Warneke S, Schipper L, Matiasek M, Scow K, Cameron S, Bruesewitz D, McDonald I (2011) Nitrate removal, communities of denitrifiers and adverse effects in different carbon substrates for use in denitrification beds. Water Research 45:5463–5475

Wolsing M, Prieme A (2004) Observation of high seasonal variation in community structure of denitrifying bacteria in arable soil receiving artificial fertilizer and cattle manure by determining T-RFLP of *nir* gene fragments. FEMS Microbiology Ecology 48:261–271

Yadav S, Kaushik R, Saxena A, Arora D (2011) Diversity and phylogeny of plant growth-promoting bacilli from moderately acidic soil. Journal of Basic Microbiology 51:98–106