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Bacterial community structure of *nir*K-bearing denitrifiers and the development of properties of soils in created mitigation wetlands

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ABSTRACT

We investigated the abundance and genetic heterogeneity of bacterial nitrite reductase genes (*nir*) and soil structural properties in created and natural freshwater wetlands in the Virginia piedmont. Soil attributes included soil organic matter (SOM), total organic carbon (TOC), total nitrogen (TN), pH, gravimetric soil moisture (GSM), and bulk density (D_b). A subset of soil attributes were analyzed across the sites, using euclidean cluster analysis, resulting in three soil condition (SC) groups of increasing wetland soil development (i.e., SC1 < SC2 < SC3; less to more developed or matured) as measured by accumulation of TOC, TN, the increase of GSM, and the decrease of D_b. There were no difference found in the bacterial community diversity between the groups ($p = 0.4$). *Nir*K gene copies detected ranged between 3.6×10^4 and 3.4×10^7 copies g^{-1} soil and were significantly higher in the most developed soil group, SC3, than in the least developed soil group, SC1. However, the gene copies were lowest in SC2 that had a significantly higher soil pH (~6.6) than the other two SC groups (~5.3). The same pattern was found in denitrifying enzyme activity (DEA) on a companion study where DEA was found negatively correlated with soil pH. Gene fragments were amplified and products were screened by terminal restriction fragment length polymorphism (T-RFLP) analysis. Among 146 different T-RFs identified, fourteen were dominant and together made up more than 65% of all detected fragments. While SC groups did not relate to whole *nirK* communities, most soil properties that identified SC groups did significantly correlate to dominant members of the community.

1. Introduction

Mitigation wetlands are created and/or restored as a result of the national policy of 'no net loss,' which mandates the amelioration of the loss of wetland services through creation, replacement or enhancement (National Research Council, 2001). Previously, many mitigation projects have been unsuccessful in meeting the performance criteria that have been legally mandated (Zedler, 1996; National Research Council, 2001; Ballantine and Schneider, 2009). Even successful cases of mitigation wetlands often fail or turn out to be slow in developing soil properties that are critical for the development of more complex functional attributes of wetlands (Wolf et al., 2011). Created and restored wetlands tend to show lower levels of organic C and N, higher bulk densities, and lower productivity than their natural counterparts (Craft et al., 2002; Bruland and Richardson, 2005; Fennessy et al., 2008). Wetland soils serve as sites of important biogeochemical processes that contribute to the myriad of ecosystem services for which wetlands are recognized (e.g. nutrient cycling and water quality improvements). The degree to which soil physicochemical properties mature to resemble natural soils in created wetlands is critical for both vegetation community development (Dee and Ahn, 2012) and the development of biogeochemical function (e.g., denitrification) (Wolf et al., 2011).

Still, in most cases of wetland mitigation, vegetation has been used as the sole measure of mitigation success (Breaux and Serefiddin, 1999; Spieles, 2005). Relying on vegetation alone leaves out soil physicochemical (e.g. soil moisture, pH, C content) and biological (e.g. bacterial communities) attributes that are mostly responsible for the functional development of wetlands. With increasing age and additional plant growing seasons, the soil properties of a created wetland should mature and develop. An excellent indicator of soil development and quality is SOM content (Howard and Howard, 1993; Bruland and Richardson, 2005), as it is a major source of nutrients (especially N) (Sollins et al., 1999). SOM provides both organic N, the substrate of mineralization, and organic carbon (SOC), which is a required energy source of both mineralizing and heterotrophic denitrifying microbes (Beauchamp et al., 1989; Groffman, 1994; Hill and Cardaci, 2004). Thus SOM provides the energy source and nutrients necessary for bacterial growth that can directly limit or enhance the development of ecological

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functions (Strauss and Lamberti, 2000; Wolf et al., 2011). The ability of wetlands to support diverse metabolic and catabolic processes depends on the ability to support anaerobic and aerobic environments (Ogram et al., 2006), which are directly affected by SOM and the resulting water holding capacity (D'Angelo et al., 2005; Wolf et al., 2011).

Denitrification is one of the key ecological functions of natural wetlands extensively studied (Groffman, 1994; Hunter and Faulkner, 2001; Hill and Cardaci, 2004). The loss of fixed nitrogen from the system is important to water quality functions and the release of greenhouse gases has climate change implication for wetlands. The sequential biogeochemical reactions can be carried out by one organism or each reaction may be carried out by different members of the microbial community. Denitrifying bacteria play a significant role in the denitrification function of wetlands (Groffman, 1994). It is known that denitrifiers constitute a taxonomically diverse functional guild with members belonging to all three domains, including more than 60 genera of bacteria, and they can represent up to 5% of the total soil microbial community (Fierer and Jackson, 2006; Demaneche et al., 2009). The assumption that the composition of the denitrifying community is of minor importance in controlling denitirification has been challenged by studies including those of Cavigelli and Robertson (2000), and Holtan-Hartwig et al. (2000), which suggested that denitrifier communities vary in their tolerances to environmental stresses. Therefore, denitrifying bacterial communities may act as a medium through which environmental controls on denitrification are realized. Wallenstein et al. (2006), in a literature review of environmental controls over denitrification, noted that C availability, pH, moisture and temperature are key factors in determining denitrifying community structure. Specifically, it has been suggested that increased SOC can be associated with bacterial diversity and may control the enzymatic/metabolic rates of the bacterial communities responsible for N processing (D'Angelo et al., 2005; Chen et al., 2010; Dandie et al., 2011). Peralta et al. (2010) studied denitrifying microbial community composition with the *nosZ* gene (denitrifier) in a mitigation wetland in Illinois and found microbial community assemblage was highly variable compared to a reference wetland.

We studied soil physicochemical properties and denitrifying bacterial community structure in created and natural wetlands in the Piedmont region of Virginia. Specifically we hypothesized that there would be positive association between the maturation of soil properties that are indicative of wetland development (Wolf et al., 2011) and the abundance and genetic heterogeneity of bacterial nitrite reducers. Ahn and Peralta (2012) found that the development of denitrification function in young created wetlands were fairly influenced and controlled by the degree of maturation of soil properties. Identifying soil physicochemical properties that may drive soil bacterial communities, especially denitrifying populations, will enhance our understanding of the development of denitrification function in created wetlands.

2. Materials and methods

2.1. Site descriptions

Five non-tidal freshwater wetlands located in the Piedmont physiographic region of northern Virginia were chosen for this study (mean annual precipitation 109 cm, mean temperature min 7 °C/max 18 °C). Three of the wetlands are mitigation wetlands created by Wetland Studies and Solutions Inc. (WSSI) on old farmland with a predominantly herbaceous cover. The other two are natural wetlands that were found right next to the created wetlands and include bottomland riparian forested wetlands and open herbaceous wetlands.

All created wetlands contain at least a 0.3 m low permeability subsoil layer covered with the same site topsoil that was supplemented with commercially available topsoil to a depth of 0.2 m. This design creates a perched, precipitation-driven water table close to the soil surface and limits groundwater exchange in the wetland. Loudoun County Mitigation Bank (LC) (4-yr old) is a 12.9 ha wetland and upland buffer complex, constructed in the summer of 2006 in Loudoun County, Virginia (39°1′ N, 77°36′ W). LC receives surface water runoff from an upland housing development and forested buffer, as well as minor groundwater inputs from toe-slope intercept seepage. LC consists of two wetland basins (LCs 1 and 2). LCs 1 and 2 are two contiguous sites separated by a berm and connected by a drainage channel with LC1 approximately 0.4 m higher in elevation than LC2. This design causes LC1 to drain more quickly leaving it inundated for shorter periods after precipitation than LC2, while LC2 can remain under standing water (e.g., <∼12 cm) for longer periods. Bull Run Mitigation Bank (BR) (8-yr old) is a 20.2 ha wetland and upland buffer complex, constructed in 2002 in Prince William County, Virginia (38°51′ N, 77°32′ W). The site may receive water from Bull Run from a culvert structure that routes water via a central ditch through the wetland, as well as overbank flow from Bull Run, which sharply bends around the corner of the site. The wetland receives limited surface water runoff from wetlands and negligible groundwater. North Fork Mitigation Bank (NF) (11-yr old) is a 50.6 ha wetland, constructed in 1999 in Prince William County, Virginia (38°49′ N, 77°40′ W). With the exception of minor contributions from toe-slope intercept seepage, the site is disconnected from the groundwater by an underlying clay liner. Study plots were located in two created hydrologic regimes: main pod area-fed by upland surface water runoff and a tributary of the North Fork of Broad Run that is controlled by an artificial dam; and vernal pool area – located in the south west quadrant of the wetland and fed solely by precipitation. All vegetation in LC1, LC2, BR and NF were planted by WSSI with the same seed mix applied at the time of construction, and was mostly herbaceous, interspersed with young tree saplings and shrubs at the time of the study.

Manassas National Battlefield Park (BP), is a 2000 ha site with areas of natural wetland coverage located in Prince William County, Virginia (38°49′ N, 77°30′ W). Study plots were located in an area of herbaceous wetland within a matrix of forested floodplain to be comparable with the study areas of created wetlands. The site is connected to Bull Run by a culvert on its eastern end and also receives groundwater and upland surface water runoff. Vegetation is mostly herbaceous with a few mature trees interspersed throughout. Banshee Reeks Nature Preserve (BN) is a 290 ha site with areas of seep and riparian wetlands located in Loudoun County, Virginia (39°1′ N, 77°35′ W). These floodplain riparian wetlands receive water from groundwater springs, surface water runoff, and occasional overbank flooding from Goose Creek. Vegetation is a mixture of herbaceous plants dominated with mature wet bottomland forest.

2.2. Soil sampling

Soil samples were collected on four dates: October and December 2010 and April and June 2011. A total of 20 study plots (i.e., 2 plots for LC1, 3 plots for LC2, 5 plots for BR, 6 plots for NF, 2 plots for BP, and 2 plots for BN) were established in the study wetlands. Each plot was 100 m^2 (e.g. 10 m × 10 m) and was divided into four (e.g. 5 m × 5 m) quadrants. Within each quadrant, three soil samples were taken at the depth of 10 cm from the top by use of an auger (1¼ in. diameter) at random and combined in a polyethylene bag. A total of 320 (e.g. 20 plots × 4 quadrants × 1 soil depth × 4 sampling periods) samples were processed. All samples

were kept in a cooler with ice packs to slow bacterial activity until further processing in the laboratory. At the laboratory, each bag was homogenized manually to mix all three samples for each quadrant. Any visible root or plant material was manually removed prior to homogenization.

2.3. Soil physicochemical analyses

Oven dried soils were macerated using a mortar and pestle and any large constituents (e.g. rocks and large organic debris) were removed by passing the soil through a 2 mm sieve and analyzed for percent TOC (∼TC) and percent TN by use of a Perkin-Elmer 2400 Series II CHNS/O Analyzer (PerkinElmer Corporation, Norwalk, CT, USA) Sub-samples (2–3 g of air dried soil) were separated for SOM, loss on ignition (LOI) method (Wilson and Sander, 1996), and oven dried at 105 °C for 24 h, weighed and placed in 405 °C for 16 h. SOM was calculated as the difference between the dry soil mass and the mass of the soil after oxidation of organic matter [(dry mass − ovened at 405 °C mass)/(dry mass) × 100]. For gravimetric soil moisture (GSM), field-wet mass was measured and samples dried at 105 °C for 48 h. GSM was calculated by: [(wet mass − dry mass)/(dry mass) × 100] (Gardner, 1986). For pH determination, 10 g dried soil samples were combined with 10 mL of deionized water, swirled and left to stabilize for 10 min prior to measurement with pH meter (Thomas, 1986). Bulk density (D_b) (Blake and Hartge, 1986) was measured once during the study period in November 2010. D_b was determined by collecting 5 cm by 10.2 cm cores, weighing the entire field-moist core, converting to dry weight based on GSM percentage, and dividing by the total volume of the soil in the core (200.2 cm^3). Soil temperatures were measured using ibuttons (Embedded Data Systems Inc.) for each sampling periods, including a week before and after the period. The ibuttons are computer chips that contain temperature sensors and are encased in portable button sized capsules. All ibuttons were buried at each plot at a soil depth of 5–10 cm.

2.4. Microbial community analyses

2.4.1. Extraction of DNA

DNA was extracted from approximately 0.5–1 g of soil per sample using the UltraClean® Soil DNA Isolation Kit (MoBio Laboratories, Solana Beach, CA, USA) and following manufacturer's instructions. Extractions were quantified using the NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, Willmington, DE, USA). Nanodrop is an equipment of measuring DNA content of relatively pure samples. There are uncertainties with this method to quantify DNA extracted from soil samples with phenolics and humic acids. We have analyzed the same soils from same sites in another study (Ahn and Peralta, 2009) and not found any compounds that interfered with DNA extraction of the soils. We also confirmed relative yields by running samples through gels and a size standard.

2.4.2. PCR amplification of nirK fragments

Bacterial *nirK* gene fragments were amplified using the primer pair F1aCu–R3Cu (approximately 470 bp) developed by Hallin and Lindgren (1999). The forward primers (F1aCu) were 5′-end FAM labeled (Operon Inc.). We used 10 ng DNA for PCR reaction. PCR amplification was done with 50-μL reaction mixtures in 0.5 mL Eppendorf tubes. Each reaction contained 1 μL of extracted DNA, 1.25 U of GoTaq® polymerase (Promega, Madison, WI, USA), manufacturer's reaction buffer containing 25 mM $MgCl_2$, 2.5 mM of each deoxynucleotide triphosphate, 1.2 μg/μL non-acetylated BSA, and 20 uM of each primer. The PCR was run in a Mastercycler® gradient cycler (Eppendorf, Hamburg, Germany) with an initial denaturing step of 4 min at 94 °C; 35 cycles of denaturation at 94 °C for 30 s, primer annealing at 59 °C for 1 min, and extension at 72 °C for 1 min; then a final extension at 72 °C for 7 min. Products were confirmed by electrophoreses of 5 μL of each reaction on 1% agarose gel. Negative and positive controls were included in each experimental run.

Amplification of *nirS* gene fragments was attempted by PCR using primer pairs F1acd-R4 cd (Hallin and Lindgren, 1999), and also primer pairs *nir*S1F-*nir*S6R developed by Braker et al. (1998). These amplifications yielded strong products for the positive control (*Pseudomonas stutzeri*), but faint if any amplification products in environmental samples. Since *nirS* gene detections were rare and faint, therefore we focused on *nirK* gene amplification products for the study.

2.4.3. Terminal restriction fragment length polymorphism analysis

Screening of the nitrite reducing bacterial communities was done by terminal restriction fragment length polymorphism (TRFLP) analysis. Amplified *nirK* fragments were digested with *HaeIII* (New England BioLabs, Beverly, Mass., USA) restriction endonuclease enzyme for at least 4 h at 37 °C. Aliquots (2–4 μL) of each digest were mixed with 12 μL deionized formamide and 0.5 μL of GeneScan-ROX500 (Applied Biosystems Instruments, Foster City, CA, USA) size standard. Mixtures were denatured for 3–5 min at 93 °C in and snap cooled on ice for 2 min. Fragment lengths were determined by using an automated DNA sequencer, model ABI 310 (Applied Biosystems Instruments, Foster City, CA, USA). The fluorescently labeled fragments were detected and analyzed by the GeneMapper® v4.1 (Applied Biosystems Instruments, Foster City, CA, USA) software. Terminal restriction fragment (T-RF) peaks from all samples were aligned using the interactive binner script (Ramette, 2009) for R statistical software environment. T-RFs were only considered if sized between 50 and 400 bp with relative abundances greater than 1%. Usually T-RFs < 50 bp are error and spurious bands, and only fragments >400 bp are uncut products. Most samples yielded detectable amounts of *nirK* gene fragments, except for samples collected in June 2010 for LC1 and BP.

2.4.4. Quantification of nirK gene copies

Quantitative PCR (qPCR) assays were used to quantify the abundance of *nirK* gene copies within soil bacterial communities, using the primer pair F1aCu–R3Cu (Hallin and Lindgren, 1999). QuantiTect® SYBR® green PCR kit (Qiagen Inc., La Jolla, CA) was used in 25 μL reactions containing 0.6 μM of each primer, 1X quantitect SYBR green PCR master mix, and 1 μL of DNA template in a Stratagene MX3000P thermal cycler (Agilent Technologies, La Jolla, CA, USA). Run conditions included an initial denaturing step of 15 min at 95 °C; 45 cycles of denaturation at 95 °C for 30 s, primer annealing at 55 °C for 30 s, and extension at 72 °C for 1 min; then a final cycle of 95 °C for 30 s, 55 °C for 30 s and 95 °C for 30 s. We tested for PCR inhibition in every sample by doing a spike of nirK DNA in parallel qPCR reactions for each sample to ensure it was amplifying correctly in qPCR reactions and that was not a problem. The efficiency of the nirK qPCR reaction was generally in the 86–88% range, therefore the 10 copy standard would not amplify in 40 cycles. We needed 41 cycles or so for that sensitivity. This is why we added 5 cycles to the typical 40 cycle qPCR reaction. Non-template controls were included in each experimental run, and melting curves were also performed to assess product integrity. Standard curves were obtained with serial plasmid dilutions of a known amount of plasmid DNA containing a fragment of the *nirK* gene, and runs with amplification efficiency >90% were deemed acceptable. Assay sensitivity was 10 copies. This is the detection limit of the nirK qPCR reaction. A standard curve is prepared for each qPCR run using serial dilutions of nirK containing plasmid, from 10^6 copies to 1 copy, and the sensitivity of the assay was 10 copies. That is, the 10 copy standard amplified in the qPCR reaction. The 1 copy standard

Table 1
Soil properties by wetland site in the study (mean ± standard error).

	Created wetlands				Natural wetlands		*F*	*p*
Site	LC1	LC2	BR	NF	BP	BN		
Age (years)	4	4	8	11				
SOM (%)	4.8 ± 0.2[a]	3.1 ± 0.2[b]	3.2 ± 0.2[b]	4.3 ± 0.2[a]	3.3 ± 0.3[b]	5.2 ± 0.4[a]	19.4	**
pH	5.3 ± 0.1[b]	5.1 ± 0.1[b]	5.3 ± 0.1[b]	6.4 ± 0.1[a]	4.3 ± 0.1[c]	5.5 ± 0.1[b]	71.5	**
TOC (%)	1.9 ± 0.1[a]	1.0 ± 0.1[b]	1.1 ± 0.1[b]	1.1 ± 0.0[b]	1.2 ± 0.1[b]	2.0 ± 0.2[a]	19.4	**
TN (%)	0.34 ± .10[a]	0.28 ± .08[b]	0.25 ± .06[b]	0.37 ± .10[a]	0.25 ± 0.10[b]	0.38 ± 0.10[a]	15.3	**
GSM (%)	34 ± 3[b]	33 ± 1[b]	32 ± 1[b]	36 ± 2[b]	31 ± 2[b]	43 ± 1[a]	6.4	**
D_b (g/cm3)	1.3 ± .08 [ab]	1.4 ± .03 [a]	1.3 ± .08 [ab]	1.2 ± .02 [ab]	1.4 ± .04 [ab]	1.1 ± .06 [b]	3.96	**

SOM (soil organic matter), TOC (total organic carbon), TN (total nitrogen), GSM (gravimetric soil moisture), pH (soil pH), LC (Loudoun County Mitigation Wetland), BR (Bull Run Mitigation Wetland), NF (North Fork Mitigation Wetland), BP (Battlefield Park Natural Wetland), BN (Benshee Reeks Natural Wetland).
Different letters between sites indicated significance at $\alpha < 0.05$ after Dunnett's T3 post hoc tests.
** $p < 0.05$.

did not. When inhibitory effects of coextracted substances were seen we diluted the DNA to relieve the inhibition and reran the qPCR, but there were almost no issues with the samples in this study.

2.5. Data analyses

We composited the data from the four sampling periods since there were not meaningful differences by season with no definitive trends. Soil condition (SC) groups were determined by cluster analysis at 75% similarity of TOC, TN, GSM, D_b, and pH, the soil properties known to indicate soil maturation in wetlands (Wolf et al., 2011; Dee and Ahn, 2012). Statistical significance of the SC groups was verified by applying a similarity profile test (SIMPROF) which performs permutation tests at each node of the cluster analysis dendogram. SIMPROF thus determines whether each cluster set has significant ($\alpha = 0.05$) evidence of a multivariate pattern different from the rest (Clarke and Gorley, 2006). We compared physicochemical and nitrite reducer community assemblages using multivariate analysis of similarities (ANOSIM) (Clarke and Gorley, 2006; Ahn and Peralta, 2009). Additionally, principal component analysis (PCA) was used to visualize 'best fit' of plots along soil physicochemical properties. All test described thus far were performed using PRIMER 6, version 6.1.5 (Primer-E Ltd., Plymouth, United Kingdom). PCA-generated principal coordinates were used for further analysis in bivariate correlation and/or regressions. Shannon–Weiner's diversity index (H') (Hill et al., 2003) was calculated based on the observed fragment peak areas generated by T-RFLPs of the wetland soils.

Analysis of variance (ANOVA) was used to compare soil physicochemical variables, T-RF diversity and *nirK* gene copies abundance, all between SC groups. Dunnett's post hoc tests for uneven variances were carried out for each ANOVA to determine between-group differences. In addition, correlation tests were conducted between DEA rates from a companion study (Ahn and Peralta, 2012) and *nirK* gene copy numbers (i.e., abundance). ANOVAs, correlations, and regressions were all conducted using SYSTAT 12 (Cranes Software International Ltd).

Redundancy analysis was performed on denitrifying bacteria community composition based on *nirK* gene T-RFLP to test if there is any relationship between dominant members of the bacterial community and soil properties. Soil physicochemical attributes used for redundancy analysis (RDA) included pH, SOM, TOC, TN, GSM and temperature (°C). RDAs were carried out using CANOCO, version 4.5 (Biometrics-Plant Research International, Wageningen, Netherlands). The significance of the relationships between the soil physicochemical attributes and the T-RFs were calculated by use of Monte Carlo permutations and $p < 0.05$ were considered non random.

3. Results and discussion

3.1. Soil properties by wetland site

The outcome of the study showed no relationship between site age and soil maturation reflected in the measurements of SOM, TOC, TN, D_b, and GSM (Table 1). Wetland soil matures over several growing seasons since they were created. Generally, greater SOM (or TOC), lower D_b, and higher GSM are all indicative of soil maturation or development in wetlands (Bruland and Richardson, 2005; Dee and Ahn, 2012). SOM and TN were significantly higher in the forested natural wetland (BN), the oldest created wetland (NF) and one of the youngest created wetlands (LC1) with SOM contents of 4.1% up to 5.6% and TN from 0.24% to 0.48% (Table 1). TOC content was highest in LC1 and BN with values averaging 1.9% and 2.0%, respectively. LC1 showed a significantly higher TOC content than the same-aged LC2, indicating that soil properties varied both within and among sites (Table 1). Soil pH was significantly higher in NF (6.4) and lower (4.3) in BP (Table 1). Soil pH in the other wetlands (e.g. LC1, LC2, BR and BN) ranged from 5.0 to 5.6 with no significant differences between these sites (Table 1), showing a typical, acidic characteristic of the soils of Virginia Piedmont (Farrel and Ware, 1991). GSM was highest in BN soils where the average was 43%, but there was no significant difference of GSM between the other sites (Table 1).

3.2. Soil properties by SC groups across the sites

Soil properties develop through the accumulation of SOM which is usually closely associated with age related factors such as seasonal plant senescence (Ballantine and Schneider, 2009). However, age-based soil development trajectories have been found to be highly variable and not predictive of plant community development (Wolf et al., 2011; Dee and Ahn, 2012). SOM accumulation can vary due to variables that may facilitate or impede autochthonous (e.g. seasonal plant senescence) and allochthonous or allogenic (e.g. sediment brought by flooding or runoff) sources of organic matter. The construction process itself can also compact soils, increasing D_b and decreasing microtopography and leading to a loss in water holding capacity and SOM (Moser et al., 2009). Therefore comparison of soil development within and between wetland sites may be better achieved by identifying soil attributes that specifically contribute to soil development. Accumulation of SOM, TOC, and TN along with the resulting lower D_b and increased GSM have been identified as structural attributes of wetland soil development or maturation (Craft et al., 2002; Bruland and Richardson, 2005; Hossler and Bouchard, 2010; Wolf et al., 2011) and significantly correlated with plant community establishment (Dee and Ahn, 2012) and the development of nitrogen cycling (Wolf et al., 2011).

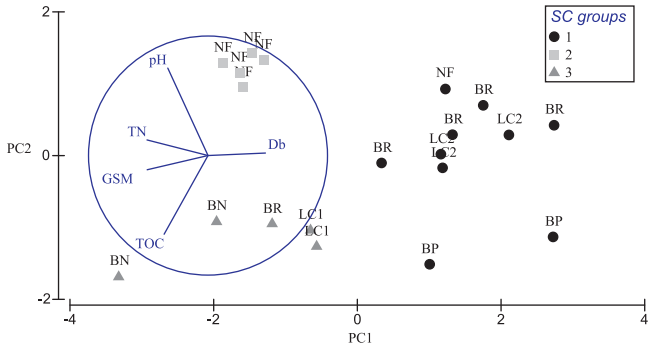

Fig. 1. Principal component analysis (PCA) of physicochemical attributes of soils collected in the study during all four sampling periods. Symbols represent the plots at the indicated wetland soil condition. TOC (total organic carbon), TN (total nitrogen), GSM (gravimetric soil moisture), and D_b (bulk density). Sixty two percent of all data variability was explained by PC1 and 21 percent by PC2. SC groups discriminated plots according to progressive soil development/maturation (e.g., SC1 < SC2 < SC3), irrespective of site.

We compared study plots across the wetlands by grouping them along a SC gradient. SC groups were identified by cluster analysis of all plots discriminated by five easily measurable soil physicochemical properties; TOC, TN, pH, GSM, and D_b. SC groups effectively discriminated plots according to progressive soil development/maturation (e.g., SC1 < SC2 < SC3), irrespective of site (Table 2). Fig. 1 shows the PCA of physicochemical attributes which clearly demonstrated SC groupings. Sixty two percent of all data variability was explained by PC1 and 21 percent by PC2 (Fig. 1). PC1 was found correlated with TOC, TN, D_b, and GSM, and PC2 with soil pH (Fig. 1). SOM ranged on average from 3.2% in SC1 to 5.0% in SC3 and was significantly different for each group ($p < 0.01$). While TOC and TN are closely related to SOM, there was no difference in TOC and TN contents between SC1 and SC2 ($p > 0.05$). TOC and TN were highest in SC3 ($p < 0.05$) which on average had contents of 2.0% and 0.2%, respectively (Table 2). D_b and GSM followed a similar pattern indicating that SC1 was the least mature group having higher D_b ($p < 0.01$) and lower GSM ($p < 0.01$; Table 2). These values are comparable to young (e.g., <20-yr old) created wetlands in Virginia (TOC 0.3–4.0%) (Moser et al., 2009), North Carolina (SOM 0.6–4.03, D_b 0.99–1.64 g^{-1} cm^3) (Bruland and Richardson, 2005), and New York (SOM 6.2%, D_b 1.1 g^{-1} cm^3) (Ballantine and Schneider, 2009). Natural wetlands were also included in the SC groups and while SOM and TOC contents are lower than reported in Pennsylvania (SOM mean 11.5%) (Campbell et al., 2002) and Maryland (TOC mean 5.7%) (Hogan et al., 2004) they were comparable to other Virginia natural sites (0.7–7.7%) (Moser et al., 2009). In conclusion, all soil attributes showed a trend from more to less developed as you move from SC3 to SC1, except soil pH (Table 2). Soil pH was generally acidic in all samples, showing a typical acidic characteristic of the Virginia piedmont soils (Moser et al., 2009; Wolf et al., 2011). However, the soil pH of SC2 was significantly higher (6.6) than both SC1 and SC3 (5.2 and 5.4, respectively).

3.3. Abundance of nitrite reducers by qPCR

Denitrifying bacterial community abundances were assessed by quantifying the number of *nirK* functional gene copies per sample. *Nir*K gene copies ranged between 3.8×10^4 and 3.4×10^7 copies g^{-1} dry soil weight among all soil samples. To account for any differences in biomass between samples, we also calculated the abundance of copies normalized to the amount of extracted DNA, which ranged from 1.2×10^2 to 4.4×10^3 copies ng^{-1} extracted DNA. The gene copy numbers by SC groups ranged between 2.2×10^4 and 1.1×10^7 copies g^{-1} dry soil weight, and from 1.1×10^2 to 2.3×10^3 copies ng^{-1} extracted DNA (Table 2).

While it is difficult to find comparable studies in created wetland soils quantifying *nirK* genes, our gene copy abundances were lower than those reported in one study of denitrifying community abundances in free water surface constructed wetlands, which had a range between 1.6×10^9 and 2.1×10^{11} *nir*K copies g^{-1} soil (Garcia-Lledo et al., 2011). However, these wetland soils were significantly different from those in this study in that they were from a treatment wetland for water quality improvement, and the study consisted of sites with continuously waterlogged sediments planted with *Phragmites* and *Typha*. Other more comparable values were found in studies looking at *nir*K gene copy abundances in various soils (9.7×10^4 to 3.9×10^6 copies g^{-1}) (Henry et al., 2004) and organic humus in south Bohemia (2.7×10^4 to 1.2×10^6 copies g^{-1}) (Barta et al., 2010).

Table 2
Summary of the soil chemical and bacterial community attributes (mean ± SE) by wetland soil condition (SC) groups.*

	SC1 $n = 10$	SC2 $n = 5$	SC3 $n = 5$	F	p
LC1			2		
LC2	3				
BR	4		1		
NF	1	5			
BP	2				
BN			2		
SOM (%)	3.2 ± 0.1^c	4.4 ± 0.1^b	5.0 ± 0.2^a	49.9	**
pH	5.2 ± 0.6^b	$6.6 \pm .05^a$	$5.4 \pm .04^b$	137	**
TOC (%)	$1.1 \pm .03^b$	$1.2 \pm .03^b$	$2.0 \pm .13^a$	53.5	**
TN (%)	0.1 ± 0.0^b	0.1 ± 0.0^b	$0.2 \pm .01^a$	60.2	**
GSM (%)	32 ± 1^b	37 ± 1^a	39 ± 1^a	20.8	**
D_b (g/cm^3)	$1.4 \pm .03^a$	$1.2 \pm .02^b$	$1.2 \pm .06^b$	9.6	**
nirK copies per g of soil ($\times 10^4$)	260 ± 170^b	29 ± 6.7^c	950 ± 120^a	3.8	**
nirK copies ng-1 DNA ($\times 10^2$)	9.8 ± 4.3^b	$1.4 \pm .31^c$	20 ± 2.5^a	2.6	**
Diversity (H')	2.8 ± 0.1^a	2.9 ± 0.2^a	3.1 ± 0.1^a	0.7	0.4
DEA rate ($\mu g\ N–N_2O/kg\ soil/h$)***	119 ± 16^b	32 ± 6^c	177 ± 25^a	17.4	**

D_b (bulk density), GSM (gravimetric soil moisture), DEA (denitrification enzyme activity), H' (Shannon-Weiner's diversity index), pH (soil pH).

* Different letters between SC groups indicated significance at a p of <0.05 after Dunnett's T3 post hoc test. Letters were omitted if there were no significant differences among SCs.

** $p < 0.05$.

*** From Ahn and Peralta (2012).

Abundances of *nir*K gene copies, both in per gram soil or in per ng of DNA, were greatest in the plots with greater SOM, TOC, TN, GSM and lower D_b, in SC3 that are indicative of soil development in created wetlands (Table 2). Denitrification is a facultative process that requires anaerobic conditions, such as those observed in inundated soils. The more developed SC plots have higher levels of SOM which not only provide an energy source but also can contribute to lower D_b. The lower D_b in turn increases pore space that allows for greater water retention, possibly leading to higher GSM. The resulting soil matrix is better able to maintain anoxic conditions, that may be able to give an advantage to those bacteria that are able to use an alternate (i.e. NO_2^-) terminal electron acceptor than O_2.

The lowest *nir*K gene abundances, however, occurred in SC2 unexpectedly rather than in SC1 (least developed or matured soils). SC2 had the pH of 6.6, being significantly higher than those in the other SC groups (i.e., SC1 and SC3), and contained an order of magnitude fewer gene copies than either SC1 or SC3 (Table 2). The negative relationship between soil pH and gene copy numbers was not limited to SC2 plots; a bivariate regression of soil pH and *nir*K abundances in all our soil samples revealed that the gene copy numbers have a negative correlation with pH ($R = 0.27$, $p = 0.03$). Among the few studies that have specifically linked *nir*K communities to pH, Barta et al. (2010) found that *nir*K gene abundances had a positive relationship with pH, which was opposite to what was found in this study. However, Barta et al. (2010) cautioned that the relationship between gene copy numbers and soil pH is not consistent in all soil types and that in some cases the denitrifying community may be adapted to low pH (e.g., <pH 6) (Parkin et al., 1985; Wallenstein et al., 2006). That might be relevant to the case of our soils studied where more than an order of magnitude higher pH (6.6 on average) in SC2 may have led to the reduction in the biomass of bacterial communities adapted to the soil pH below 6. On a companion study (Ahn and Peralta, 2012) we also found an order of magnitude reduction in DEA rates in SC2 compared to SCs 1 and 3 (Table 2; Ahn and Peralta, 2012). Thus, SC2 revealed the lowest DEA rates among the SC groups (Table 2). The DEA rates clearly showed a negative correlation ($R = -0.26$, $p < 0.05$) with soil pH (Ahn and Peralta, 2012) and was found positively correlated to ($R = 0.54$, $p < 0.001$) *nir*K gene copy numbers. Effect of soil pH, however, may not necessarily be a direct one on the denitrification processes, and may be more related to other environmental and biological factors (Simek and Cooper, 2002) that were not included in the study. Therefore further investigation is needed to test the effects of a range of soil pH in the acidic Piedmont soils on the structure of denitrifying bacterial communities.

PC1 (Fig. 1) was also found correlated with *nir*K gene abundances (*adjusted* $R = 0.32$, $p = 0.03$), indicating the positive association between soil maturation properties (i.e., the increase of TOC, TN, and GSM values) and the bacterial biomass. PC2 was not correlated to *nir*K gene abundances ($p = 0.15$).

3.4. Denitrifying community diversity (T-RFLP)

The denitrifying bacterial communities from plots classified by three SC groups were evaluated by T-RFLP analysis of the amplified *nir*K gene fragments. While it is important to keep in mind potential biases from differential PCR amplification in using peak areas for T-RFLP community structure analysis, it has been found to be a useful tool in delineating structure between samples (Peralta et al., 2010). We detected a total of 146 different T-RFs in all our samples. The average numbers of fragments observed in each group were 131, 99 and 110 for SC1, SC2 and SC3, respectively. *Nir*K gene based community structures were not explained by SC groupings (ANOSIM *Global* $R = 0.046$, $p = 0.1$). Diversity values (H')

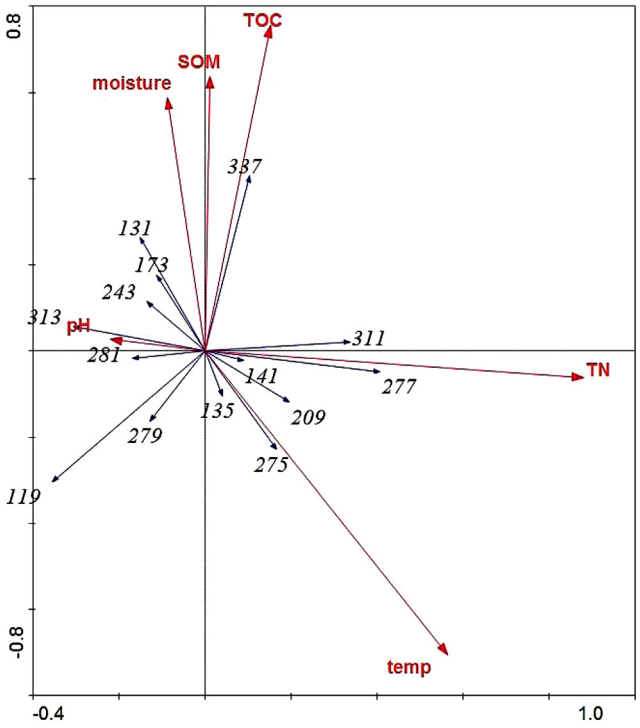

Fig. 2. Redundancy analysis diagram of *nirK* community major T-RFs and soil property variables defined by the first and second axes. Arrows represent the relationship between soil physicochemical attributes and the major denitrifiers in the communities.

ranged from 2.7 to 3.2, and did not differ by SC group (Table 1). The diversity values found in our study are a bit lower than a comparable study using T-RFLPs to assess *nirK* gene diversity in marsh ($H' = 3.6$) and upland ($H' = 4.4$) soils in Michigan (Prieme et al., 2002). The comparison was somewhat limited due to the range of primers and methods to be matched to study denitrifying bacteria communities.

Considering the effect of rarer species on diversity values we identified fourteen dominant T-RFs, from the 146 detected, which together made up more than 65% of all detected T-RFs. Dominant T-RFs did correspond to four main factors; (1) pH, (2) TN, (3) temperature and (4) GSM, SOM, and TOC (Fig. 2). Specifically we found three fragments (e.g., T-RFs-311, 277 and 141) were positively correlated with TN and negatively with pH (Fig. 2). While two (e.g., T-RFs 281 and 313) increased in relative abundance with decreasing pH and lower TN (Fig. 2). Four fragments (e.g., T-RFs 131, 173, 243 and 337) increased along with soil maturity reflected in increased GSM, SOM and TOC (Fig. 2). The other four fragments (e.g., T-RFs 135, 141, 209 and 275) showed a positive correlation with soil temperature (Fig. 2). The importance of dominant T-RFs in *nirK* soil communities was explored by Wertz et al. (2009) in Canadian agricultural soils, which compared whole and active (using mRNA transcripts from entire community) *nirK* community composition, concluding that the active portion was relatively stable and more abundant in all sampled soils. This kind of variation in *nirK* community structure has also been observed in stream sediments along an urbanization gradient (Wang et al., 2011). The study revealed *nir*K (and *nos*Z) community shifts in stream sediments along a gradient of soil conditions (measured by TN, TOC, and pH) that changed with urbanization. Another study by Peralta et al. (2010) revealed that soil moisture and soil fertility were associated with differences in denitrifying microbial community structure.

4. Conclusion

We investigated a subset of soil denitrifying communities and soil properties in created and natural wetland soils in the piedmont region of Virginia. SC groups effectively managed to organize study plots across sites according to soil development and/or maturation using five easily measured soil properties; TOC, TN, D_b, pH and GSM. The majority of soil properties related to soil maturation in wetlands shows association with the structure of *nir*K bearing denitrifiers. It seems that soil pH influences the structure of *nir*K bearing denitrifier communities within the characteristic ranges of acidic soils in the Virginia Piedmont. A further study is necessary to investigate the impacts of soil pH in acidic wetland soils on the abundance of *nir*K bearing denitrifier communities. While SC groups did not relate to whole *nir*K community structure, soil attributes that identified SC groups significantly correlated with a number of dominant members of the community. The study was limited to a single gene (i.e., *nir*K community) in the denitrification pathway and therefore represents a partial picture of the potential denitrification communities in the system.

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