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Soil Physicochemical Conditions, Denitrification Rates, and *nosZ* Abundance in North Carolina Coastal Plain Restored Wetlands

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Abstract

Over the last century, North Carolina has seen a severe reduction in the percentage of wetlands and a rise in negative environmental impacts related to this loss. To counter these effects, efforts have been enacted to mitigate wetland loss and create new wetland areas. The objective of this study was to assess the impact of hydrological restoration at several sites in the North Carolina coastal plain. Nine sites were selected for study. Hydrologically restored wetlands were compared with natural wetlands and prior converted (PC) croplands (i.e., historic wetlands under agricultural production). Each site was analyzed along a relative wetness gradient, and physicochemical properties, denitrification enzyme activity, and N_2O reductase gene (*nosZ*) abundances using real-time PCR were measured. Physicochemically, restoration resulted in significantly increased levels of total C as compared with PC cropland sites. Restored wetland sites also saw pH, soil moisture, P, and NO_2^-+NO_3^- approximate levels similar to those of natural wetlands. Denitrification enzyme activity rates varied based on relative wetness within individual sites, generally increasing with increasing soil moisture. However, denitrification tended to be lower in restored wetland sites relative to natural wetlands. Gene abundances of *nosZ* saw statistically significant decreases in restored wetland soils. In conclusion, although analysis of restored wetlands reveals clear changes in several physicochemical characteristics and significant decreases in *nosZ* gene abundances, restoration efforts appear to have not significantly affected the denitrification component of the N cycle.

Wetland biogeochemical processes play a significant role in the global cycling of nitrogen (N), sulfur (S), carbon (C), and phosphorus (P). When located in an agricultural landscape, wetlands are especially important for the recycling of N, primarily found in nitrate (NO_3^-) form. This NO_3^- is removed via reductive processes, with as much as 90% of the N that enters wetlands often removed through denitrification (Gilliam, 1994; Hunter et al., 2009; Reilly et al., 2000). If denitrification proceeds to completion, N is converted to molecular nitrogen (N_2) and released into the atmosphere. However, if the process is incomplete, N is emitted in the form of the greenhouse gas nitrous oxide (N_2O). The gene responsible for the reduction of N_2O to N_2 is *nosZ*, which encodes the enzyme N_2O reductase. In previous reports, *nosZ* abundances, as measured by quantitative real-time PCR (qPCR), have been correlated with $N_2O/(N_2 + N_2O)$ rates (Ducey et al., 2011; Philippot et al., 2009).

In addition to nutrient cycling, wetlands provide other critical ecosystem services to the watersheds they are associated with. These services include regulating water movement, filtering of suspended solids and other contaminants, and providing habitat for flora and fauna (Zedler and Kercher, 2005). Despite these important ecosystem services, it has been estimated that, over the past century, the total wetland area of the United States has been halved, with losses driven primarily by the drainage of freshwater wetlands to support agricultural, rural, and urban development (Dahl and Stedman, 2013; Tiner, 1984). To counteract these losses, efforts have been enacted to protect, enhance, and restore wetland systems. To further this goal, since 1987 the United States has fostered a "no net loss" approach, which aims to prevent or offset future wetland losses (Fretwell et al., 1996). Since the adoption of this policy, a number of federal programs have been instituted to support wetland restoration efforts. In 2003, the USDA initiated a multiagency project, termed the

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Abbreviations: CEAP, Conservation Effects Assessment Project; DEA, denitrification enzyme activity; EC, electrical conductivity; MIAR CEAP-Wetland, Mid-Atlantic Regional Conservation Effects Assessment Project-Wetland; NMS, nonmetric multidimensional scaling; PC, prior converted; qPCR, quantitative real-time PCR; RW, relative wetness; TC, total carbon.

Conservation Effects Assessment Project (CEAP), to quantify the effects and effectiveness of conservation practices, including wetland restoration. These assessments occur at the national, regional, and watershed scales and are critical for establishing a scientific basis for continued conservation efforts.

The Mid-Atlantic Regional CEAP-Wetland (MIAR CEAP-Wetland) study is one of several regional studies undertaken as part of the Wetland Component of the CEAP National Assessment. The study assesses the provision of wetland ecosystem services along a human alteration gradient from seminatural wetlands to restored wetlands to prior converted (PC) croplands within the states of North Carolina, Virginia, Maryland, Delaware, and New Jersey. Prior converted croplands are all sites that were originally wetlands that were drained or filled for agricultural purposes before 1985. Primary study areas were selected in the Delmarva Peninsula and North Carolina–Virginia coastal regions, which represent areas of concentrated wetland restoration implementation.

In a previous study, Hunt et al. (2014) looked at the effect of hydrological restoration on soil properties, denitrification enzyme activity (DEA), and *nosZ* gene abundances in depressional palustrine wetlands with mineral soils in the Delmarva Peninsula and southeastern Virginia. In that work, Hunt et al. (2014) demonstrated that restored wetlands continued to share some characteristics with wetlands converted to agricultural use (PC croplands), which was their status before restoration. However, despite these findings, the restoration efforts appeared to have resulted in lower denitrification enzyme rates and *nosZ* gene abundances as compared with the PC sites (Hunt et al., 2014). This current study addresses restoration efforts in the pocosin region of North Carolina. These palustrine wetlands were formed on low-permeability clay soil materials where water is lost predominantly via evapotranspiration rather than infiltration. These wetlands are often intermittently flooded, with the water table typically reaching its peak in the winter and spring (Phillips and Shedlock, 1993). However, unlike the mineral clay soils (Ultisols) of the Delmarva region, the pocosin soils of North Carolina are organic rich and acidic. The significant differences in wetland soil properties between these regions prompted a separate report. Our objectives were to compare the natural wetlands, PC croplands, and restored wetlands based on the following criteria: (i) physicochemical conditions, (ii) DEA, and (iii) *nosZ* gene abundances.

Materials and Methods

Site Description

Three types of sites were chosen for this study: (i) restored wetlands, (ii) natural wetlands, and (iii) PC croplands. Restored sites underwent hydrological restoration between 2004 and 2006 (Table 1) and were chosen from a list of representative wetland restorations performed by the NRCS. After permissions were granted from landowners for access, natural (forested) wetland and PC (historic wetlands under agricultural production) cropland sites were selected to have similar soils and geomorphic positions and to be within 1 to 4 km of the restored wetland.

There were nine total sites selected (three restored, three natural, and three PC croplands), all located in Hyde and Tirell Counties, North Carolina. These sites were located in the pocosin region of the northeastern North Carolina Coastal Plain and are not tidally influenced. These soils are composed mainly of high quantities of organic matter in various stages of decomposition due to historically high water levels. Two profile descriptions were made at each site, and the profile best representative of the wetland was used to measure bulk density. Additionally, hydric soil field indicators were determined based on these selected profiles (USDA–NRCS, 2010). The soils at all nine sites met hydric soil field indicators A1 (Histosol) or A2 (Histic Epipedon). Soils meeting indicator A1 were classified as Terric Haplosaprists because of the presence of 40 to 130 cm thickness of organic soil materials (Soil Survey Staff, 2014). The remaining soils with <40 cm of organic materials were Humaquepts. According to the Web Soil Survey (USDA–NRCS, 2010), the soil series used to name the map units where the sites were located were Belhaven (Loamy, mixed, dysic, thermic Terric Haplosaprists), Ponzer (Loamy, mixed, dysic, thermic Terric Haplosaprists), Roper (Fine-silty, mixed, semiactive, acid, thermic Terric Haplosaprists), and Scuppernong (Loamy, mixed, dysic, thermic Terric Haplosaprists). According to the Web Soil Survey, all sites were classified as very poorly drained (Table 1), and field soil morphological observations confirmed this. The landscape was relatively flat, with groundwater close to the surface during much of the year (Table 1). The NRCS restorations were performed by blocking ditches using dikes, with weirs as water control structures. Shallow water areas for wildlife were created by excavating a pool within each restored wetland. The natural sites were all located in the adjacent Pocosin Lakes National Wildlife Refuge. Because most of the area had been drained or logged at one time, natural sites were chosen based on least disturbance. Prior converted cropland was typically located on the same farm as the restored wetland under corn–wheat–soybean rotations. Each PC site was drained for the purpose of production agriculture using open ditches.

Soil Sampling

Sites were stratified based on potential relative wetness using topography as a primary indicator. The gradient and sampling points were determined before field sampling using ArcGIS (ESRI). Digital elevation models were used to define boundaries of each study site, and four evenly proportioned topographic class variables were defined at each site using ArcGIS. These digital elevation models were further refined based on field observation. Each topographic class served as one of four sampling locations; topographic classes are referred to throughout this manuscript as "relative wetness" (RW) class variables 0 (wettest) through 3 (driest). For each class variable, sampling points were selected randomly, with geographic coordinates uploaded into a global positioning system (Trimble Navigation Limited).

A total of three samples from the upper 10 cm of soil were collected with a soil probe (AMS, Inc.) from within a 0.5-m radius at each sampling point. These soil samples were composited and then placed on ice to be returned to the lab for processing. Soils were kept under refrigeration (4°C) at their initial field moisture content before DEA or were stored at −80°C until DNA extraction. Sampling was performed a total of three times in a spring-fall-spring pattern in the years of 2010 and 2011, resulting in collection of restored wetland samples between 4 and 7 yr after restoration. A total of 108 samples (nine

sites total, including three of each treatment type, three sampling dates per site, and four relative wetness class variables per site) were included in this study.

For bulk density samples, the profile best representative of the wetland was selected, and duplicate samples were collected from each horizon to a depth of 100 cm using the core method (Blake and Hartage, 1986).

Soil Chemical Properties

Soil temperature, electrical conductivity (EC), and moisture were measured in situ at the same time samples were collected for DEA analysis. Soil temperature and EC were measured using a ECTestr11+ meter (Spectrum Technologies), and moisture was measured using a Delta-T HH2 moisture meter

Table 1. Descriptions and characteristics of sites chosen for analysis.

Name	Soil series used to name soil map unit	Vegetation†	BD‡	SDC§	HSI‡	DWT§	Land use	County
Site 1	Ponzer	primarily woody shrubs [*Clethra alnifolia* L., *Morella cerifera* (L.) Small, *Vaccinium fuscatum* Aiton], trees (*Acer rubrum*, *Ilex opaca* Aiton, *Magnolia virginiana* L., *Nyssa biflora*), and vines (*Parthenocissus quinquefolia, Smilax glauca* Walter, *Smilax rotundifolia* L., *Toxicodendron radicans* (L.) Kuntze, *Vitis rotundifolia*); also includes ferns (*Dicranales* sp., *Sphagnum* sp.) and mosses.	0.24	very poorly drained	A1	15 cm	natural	Tyrrell
Site 2	Scuppernong	primarily woody shrubs [*Clethra alnifolia* L., *Morella cerifera* (L.) Small], trees (*Acer rubrum*, *Liquidambar styraciflua* L., *Magnolia virginiana* L., *Salix* sp.), and vines (*Parthenocissus quinquefolia*, *Smilax glauca* Walter, *Smilax rotundifolia* L.); also includes ferns and mosses	0.23	very poorly drained	A2	15 cm	natural	Hyde
Site 3	Belhaven	primarily woody shrubs [*Clethra alnifolia* L., *Lyonia ligustrina*, *Morella cerifera* (L.) Small, *Vaccinium fuscatum*], trees (*Acer rubrum*, *Liquidambar styraciflua* L., *Magnolia virginiana* L.) and vines (*Parthenocissus quinquefolia*, *Smilax glauca* Walter, *Smilax rotundifolia* L., *Vitis rotundifolia* Michx.); also ferns, mosses, and herbaceous (*Boehmeria cylindrica*) plants	0.20	very poorly drained	A1	15 cm	natural	Hyde
Site 4	Ponzer	corn, wheat, soybean rotation	0.86	very poorly drained	A2	15 cm	prior converted	Tyrrell
Site 5	Belhaven	corn, wheat, soybean rotation	0.73	very poorly drained	A2	15 cm	prior converted	Tyrrell
Site 6	Roper	corn, wheat, soybean rotation	0.86	very poorly drained	A1	15 cm	prior converted	Tyrrell
Site 7	Belhaven	primarily herbaceous plants [*Boehmeria cylindrical* (L.) Sw., *Cicuta maculate* L., *Ludwigia alternifolia* L., *Ludwigia palustris*, *Sesbania herbacea, Vernea bonariensis* L.] and grasses [*Echinochloa crus-galli* (L.) P. Beauv., *Juncus effusus* L., *Phragmites australis* (Cav.) Trin. Ex Steud., *Schoenoplectus* sp., *Scirpus cyperinus* (L.) Kunth]; also containing woody shrubs [*Morella cerifera* (L.) Small] and trees (*Magnolia virginiana* L., *Salix caroliniana* Michx.)	0.57	very poorly drained	A1	15 cm	restored, 2004	Tyrrell
Site 8	Belhaven	primarily herbaceous plants [*Compositae* sp., *Euthamia caroliniana* (L.) Green ex Porter & Britton, *Hypericum denticalatum* Walter] and grasses (*Juncus* sp.), also containing woody shrubs [*Ilex glabra* (L.) A. Gray, *Morella cerifera* (L.) Small]	0.37	very poorly drained	A2	15 cm	restored, 2005	Tyrrell
Site 9	Scuppernong	primarily herbaceous plants [*Cyperaceae* sp., *Eupatorium capillifolium* (Lam.) Small] and grasses [*Dichanthelium aciculare* (Desv. ex Poir.) Gould & C.A. Clark), *Scirpus cyperinus* (L.) Kunth]; also containing woody shrubs [*Baccharis halimifolia* L., *Morella ceriferia* (L.) Small, *Rhexia mariana* L., *Rubus occidentalis* L.] and trees (*Acer rubrum*).	0.29	very poorly drained	A1	15 cm	restored, 2006	Tyrrell

† Plants are listed alphabetically (Yepsen et al., 2014).

‡ Two profiles taken from each site. BD, bulk density. Hydric soils indicator (HSI) and depth to water table (DWT) are listed in profile from which bulk density was measured (Fenstermacher, 2012).

§ As reported for this map unit by Soil Survey Staff (2015). SDC, soils drainage class.

(Dynamax). Soil C and N were measured using a TruSpec CN analyzer (LECO Corp.). Soil pH was measured using a 1:1 (w/w) mixture of soil and water. Soil samples were further air dried and extracted using Mehlich 1 solution to determine plant-available nutrients (Mehlich, 1953). Extracts were analyzed for Al^{3+}, Ca^{2+}, Cu^{2+}, Fe^{3+}, K^{+}, Mg^{2+}, Na^{+}, P, and Zn^{2+} with a Vista Pro inductively coupled plasma–atomic emission spectrometer (Varian Inc.). Soil samples were further extracted using a 5:1 ratio of water:extract and filtered through a 0.2-μm filter. This water extraction was used to determine the concentration of Cl^{-}, SO_4^{2-}, NO_2^{-} + NO_3^{-}, and PO_4^{3-} using a Dionex 2000 Ion Chromatograph (Thermo Scientific) according to ASTM standard D4327–11 (Roades, 1996).

Denitrification Enzyme Analysis

Denitrification enzyme analysis using the acetylene inhibition method was performed as previously described (Hunt et al., 2014; Tiedje, 1994). Although the scope of this study did not allow exhaustive measures of DEA, only a NO_3^{-} amendment treatment was chosen because NO_3^{-}—not carbon—is typically the limiting factor for DEA in C-rich soils (Davidsson and Stahl, 2000; Hunt et al., 2002). Briefly, a total of 10 to 15 g of field-wet soil from each sample was placed in a 60-mL serum bottle. A 5-mL solution of chloramphenicol (0.1 g L^{-1}) was then added to each bottle to suppress additional protein synthesis during the course of the microbial assay. Before capping the bottles with rubber septa, NO_3^{-} was added to bottles used for the measurement of incomplete and potential incomplete DEA. The bottles were then attached to a vacuum manifold to induce an anaerobic environment, at which point acetylene—for complete and potential complete DEA measures—was injected. A total of 12 bottles were prepared from each soil sample and split into triplicates for a total of four different DEA treatments. These treatments measured different aspects of DEA and were as follows:

- Complete denitrification: Acetylene (15×10^{-3} L) was used to block denitrification at the N_2O reduction step, resulting in an increased accumulation of N_2O, a portion of which would typically be reduced to nitrogen gas.
- Incomplete denitrification: Denitrification was allowed to occur unimpeded, with N_2O accumulating at natural rates.
- Potential complete denitrification: Nitrates (200 mg L^{-1} NO_3–N) in nonlimited quantities and acetylene (15×10^{-3} L) were added to measure maximal enzyme activity rates with blockage at the N_2O reduction step; similar to complete denitrification.
- Potential incomplete denitrification: Nitrates (200 mg L^{-1} NO_3–N) in nonlimited quantities were added to measure maximal enzyme activity rates; similar to incomplete denitrification.

DNA Extraction

A total of 0.2 g from each soil sample was used for DNA extractions using a PowerSoil DNA Extraction Kit (MO BIO Laboratories Inc.) according to manufacturer directions. Both DNA quality and quantity were determined using a NanoDrop 2000c spectrophotometer (Thermo Scientific) and by electrophoresis on a 0.8% agarose gel stained with SYBR Safe (Life Technologies).

Quantitative Real-Time Polymerase Chain Reaction

All qPCR assays were performed using a LightCycler 480 Real-Time PCR Detection System (Roche Diagnostics). The primer pair, nosZF (5′-CGYTGTTCMTCGACAGCCAG-3′) and nosZ-1622R (5′-CGSACCTTSTTGCCSTYGCG-3′), was used to produce a ~450–base pair amplification product. Primers were synthesized and HPLC purified by Integrated DNA Technologies. Assays were performed using SYBR GreenER qPCR SuperMix (Invitrogen) in a total volume of 25 μL. Final reaction concentrations were as follows: 1X SYBR GreenER qPCR SuperMix, 200 nmol L^{-1} of each primer, and 10 ng of DNA template. The qPCR reaction conditions were as follows: an initial denaturation at 95°C for 5 min; 50 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and elongation at 72°C for 30 s; and a final melting curve analysis to confirm amplification product specificity. Fluorescent measurements were taken during the annealing phase of each cycle. Data were collected and processed using the LightCycler 480 software package. All qPCR assays included control reactions without template and were performed in triplicate. A *nosZ* DNA standard, derived from the linearized plasmid pCPDnosZ1 (Ducey et al., 2011), was used to develop a standard curve from between 10^1 and 10^8 copies per reaction; this standard was also used to calculate an amplification efficiency of 1.92 according to the equation $E = 1 + 10^{[-1/\text{slope}]}$ (Pfaffl, 2001).

Statistics

All data were statistically analyzed using SAS v. 9.3 (SAS Institute). Denitrification enzyme activity rates were analyzed using the GLIMMIX (General Linearized Mixed Models) procedure, with sites, sampling dates, and laboratory replicates pooled and considered random. Land use and the relative wetness class variable were considered fixed. Denitrification enzyme activity treatments were $\log_{10}$ transformed to meet normalization criteria and analyzed using the least squares mean method; treatment differences of analyzed variables were compared using the pdiff option. T value grouping for treatment least squares mean was $P \le 0.05$. Soil physicochemical measures and *nosZ* gene abundances were analyzed using the general linear model procedure, and Duncan's multiple range test ($P \le 0.05$) was used to detect statistical differences. Relationships between DEA rates and *nosZ* gene abundances with environmental variables were performed using Pearson correlation coefficients under the CORR (Correlation) procedure. For visualization of physicochemical characteristic differences between land use, nonmetric multidimensional scaling (NMS) plots, using a Sorensen distance measure, were produced in PCORD v. 6.0 (MjM Software Design).

Results

Wetland Soil Physicochemical Properties

All selected sites consisted of organic-rich soils, with a shallow water table (Table 1). The sites had flat relief, making demarcation of RW class variables more difficult than in the Delmarva study (Hunt et al., 2014). However, collection

of samples in relation to the relative wetness class variable is supported by soil moisture values (r^2 = 0.42; P < 0.001). When comparing soil moisture for each land use by the relative wetness, the three wettest (RW0, RW1, and RW2) were significantly wetter than RW3 (P < 0.05) (Table 2). Given this delineation, further discussion of statistics will focus on comparisons between the wettest (RW0) and driest (RW3) areas among each land use.

Bulk densities were determined (Table 1), with higher bulk densities collected from PC cropland sites. Bulk density values for restored wetland sites were not as low as bulk densities measured in natural wetland soils but have decreased in the short period of time (4–7 yr) after restoration. As part of a larger vegetation survey that included all sites analyzed in this study, Yepsen et al. (2014) reported that woody species accounted for >70% of cover in natural wetlands, whereas in restored wetland sites the cover from woody species was <10%. Restored wetland sites showed the highest species richness but were primarily herbaceous in their plant community composition.

Wetland soil physicochemical properties were compared based on land use and relative wetness; all values and statistical comparisons can be found in Tables 2, 3, and 4. For visualization of the physicochemical properties of these wetlands soils in relation to land use, ordination by NMS of data summarized in Tables 2, 3, and 4 were used to construct a two-axis plot (Fig. 1). The NMS plot reveals distinct separation of soils under PC cropland and natural wetland land use and overlap of both with restored wetland soils. The relationship of individual soil properties to specific land use can also be determined in Fig. 1 and in the statistical groupings provided in Tables 2, 3, and 4. Regarding differences among land use, some of the more pronounced physicochemical differences between PC cropland and natural wetlands were as follows: pH, EC, soil temperature, Ca^{2+}, K^+, Mg^{2+}, P, and $NO_2^- + NO_3^-$ were all significantly greater in PC cropland soils; conversely, soil moisture, Al^{3+},

Table 2. Wetland soil physicochemical properties for different land use and relative wetness class variables.

Land use	Relative wetness	Total C	Total N	C/N ratio	pH	EC†	Moisture	Soil temperature
		%				µS cm^{-1}	%	°C
Natural	0 (wettest)	29.1 (4.9)‡bc§	1.1 (0.1)ab	26.6 (3.2)bcd	4.6 (0.2)de	82.7 (7.1)cd	68.9 (1.6)a	19.3 (0.9)e
	1	32.0 (5.8)bc	1.0 (0.1)ab	30.7 (4.1)bcd	4.5 (0.2)de	84.3 (6.1)cd	63.8 (4.1)ab	19.3 (0.8)e
	2	47.0 (4.3)a	1.3 (0.1)a	37.0 (3.0)abc	3.9 (0.2)e	106.5 (16.0)bcd	57.5 (4.2)b	20.0 (0.9)de
	3 (driest)	15.5 (5.5)de	0.6 (0.2)cd	24.6 (2.7)cd	4.3 (0.2)e	51.0 (16.5)d	25.7 (4.9)de	20.4 (0.9)de
Restored	0 (wettest)	40.7 (5.7)ab	1.0 (0.2)ab	45.7 (9.7)a	4.5 (0.2)de	140.4 (20.6)abc	57.0 (5.0)b	22.9 (1.1)cde
	1	41.5 (6.3)ab	1.0 (0.1)ab	40.6 (5.9)ab	4.4 (0.2)de	159.1 (17.0)abc	56.4 (3.5)b	22.7 (1.3)cde
	2	40.3 (6.6)ab	1.0 (0.1)ab	40.4 (5.7)ab	4.5 (0.3)de	176.3 (11.7)ab	56.8 (3.1)b	23.5 (1.0)bcd
	3 (driest)	20.4 (5.5)cd	0.6 (0.1)cd	31.0 (3.6)bcd	5.2 (0.4)cd	87.5 (14.5)bcd	31.8 (4.7)cd	23.4 (1.5)bcd
Prior converted	0 (wettest)	15.1 (1.5)de	0.6 (0.1)cd	26.8 (2.1)bcd	6.0 (0.3)ab	210.6 (45.5)a	37.0 (3.3)cd	26.5 (1.2)abc
	1	19.2 (1.7)cde	0.7 (0.1)bc	28.8 (2.2)bcd	5.6 (0.2)bc	165.7 (34.5)abc	39.3 (3.4)c	26.1 (1.5)abc
	2	19.7 (2.5)cde	0.8 (0.1)bc	26.8 (2.2)bcd	6.1 (0.3)ab	215.7 (59.8)a	31.6 (3.3)cd	26.7 (1.4)ab
	3 (driest)	5.2 (1.3)e	0.3 (0.1)d	17.7 (1.8)e	6.7 (0.1)a	101.5 (25.8)bcd	18.1 (2.3)e	28.1 (1.4)a

† Electrical conductivity.

‡ Values are mean and SE.

§ Columns are statistically grouped according to Duncan's multiple range test based on a P < 0.05 level. Those with the same letter are not significantly different.

Table 3. Plant-available nutrients (Mehlich I) for different land use and relative wetness class variables.

Land use	Relative wetness	Al^{3+}	Ca^{2+}	Cu^{2+}	Fe^{3+}	K^+	Na^+	Mg^{2+}	P	Zn^{2+}
		mg kg^{-1}								
Natural	0	1304 (236)†a‡	507 (115)f	0.15 (0.03)ab	56 (11)ab	66 (15)d	45 (9)abc	64 (14)c	26 (5)c	1.6 (0.3)b
	1	969 (235)ab	558 (193)f	0.13 (0.04)ab	54 (12)b	63 (13)d	44 (9)abc	77 (25)c	15 (4)c	1.8 (0.6)b
	2	755 (177)bc	496 (119)f	0.09 (0.02)ab	57 (6)ab	103 (21)bcd	54 (9)a	91 (45)c	24 (4)c	2.0 (0.3)b
	3	514 (75)bcd	394 (162)f	0.21 (0.05)a	80 (10)a	58 (18)d	30 (9)abc	76 (24)c	22 (4)c	2.5 (0.9)ab
Restored	0	469 (165)cd	2267 (592)de	0.11 (0.03)ab	19 (5)cd	107 (27)bcd	53 (10)a	499 (132)b	24 (7)c	5.1 (1.5)ab
	1	449 (148)cd	2404 (548)de	0.12 (0.04)ab	20 (4)cd	100 (19)bcd	57 (8)a	523 (113)b	23 (6)c	4.8 (1.4)ab
	2	429 (159)cd	2285 (533)de	0.13 (0.05)ab	18 (4)cd	123 (33)bc	49 (7)ab	506 (112)b	19 (5)c	5.0 (1.6)ab
	3	582 (104)bcd	1840 (307)e	0.14 (0.06)ab	40 (16)bc	57 (11)d	41 (13)abc	427 (79)b	17 (4)c	2.6 (0.4)ab
Prior converted	0	439 (108)cd	4618 (503)ab	0.11 (0.04)ab	14 (4)d	154 (18)abc	26 (3)bc	486 (61)b	78 (27)b	5.2 (1.7)ab
	1	593 (117)bcd	3834 (318)bc	0.09 (0.02)ab	13 (4)d	136 (25)abc	32 (4)abc	534 (87)b	43 (14)bc	6.0 (1.6)a
	2	161 (36)d	5273 (289)a	0.05 (0.01)b	5 (1)d	222 (52)a	35 (6)abc	757 (58)a	52 (15)bc	4.6 (1.2)ab
	3	308 (50)cd	3269 (536)cd	0.20 (0.05)a	24 (5)cd	167 (57)ab	21 (3)c	427 (74)b	135 (40)a	4.0 (1.2)ab

† Values are mean and SE.

‡ Columns are statistically grouped according to Duncan's multiple range test based on a P < 0.05 level. Those with the same letter are not significantly different.

Table 4. Water soluble anions for different land use and relative wetness class variables.

Land use	Relative wetness	Cl^-	SO_4^{2-}	PO_4^{3-}	$NO_2^- + NO_3^-$
		mg kg^{-1}			
Natural	0	28.6 (5.3)†b‡	29.6 (6.8)abc	1.6 (0.6)c	4.4 (2.1)c
	1	26.5 (2.5)b	22.8 (4.0)abc	1.5 (0.5)c	2.6 (0.6)c
	2	42.7 (5.5)ab	42.6 (5.5)a	7.9 (0.8)bc	4.4 (1.3)c
	3	20.6 (17.2)b	22.2 (7.3)abc	3.3 (1.2)c	3.8 (1.3)c
Restored	0	30.4 (3.5)b	30.4 (9.0)abc	18.0 (7.8)a	4.8 (2.0)c
	1	31.4 (4.7)b	34.1 (8.9)ab	16.3 (6.4)ab	4.9 (2.6)c
	2	32.6 (6.3)b	29.9 (6.6)abc	9.1 (2.4)abc	11.2 (5.0)c
	3	26.7 (6.9)b	15.7 (3.3)bc	2.6 (0.9)c	9.8 (4.7)c
Prior converted	0	29.8 (10.0)b	15.7 (3.0)bc	2.5 (0.6)c	87.1 (52.7)ab
	1	35.3 (5.2)b	20.5 (5.1)bc	2.4 (0.6)c	60.2 (34.0)b
	2	63.0 (19.9)a	25.5 (6.9)abc	5.1 (1.7)c	168.5 (89.6)a
	3	16.8 (5.7)b	10.9 (4.6)c	5.7 (2.3)c	50.9 (30.7)b

† Values are mean and SE.

‡ Columns are statistically grouped according to Duncan's multiple range test based on a $P < 0.05$ level. Those with the same letter are not significantly different.

and Fe^{3+} were significantly greater in natural wetland soils. When focusing on these 11 soil properties, restored wetland soils showed mixed results. Restored soils were more similar to natural wetland soils in relation to pH, soil moisture, P, and $NO_2^- + NO_3^-$ levels while being more similar to PC soils in relation to EC, Al^{3+}, Fe^{3+}, and Mg^{2+}. Soil temperature, K^+, and Ca^{2+} values for restored wetlands were in between those values recorded for PC and natural wetland soils. Soluble inorganic phosphorus (PO_4^{3-}) at RW0 in restored sites was significantly greater than in natural and PC cropland sites.

Total C (TC) is high in all land use and relative wetness classes, but it is significantly greater in restored wetland

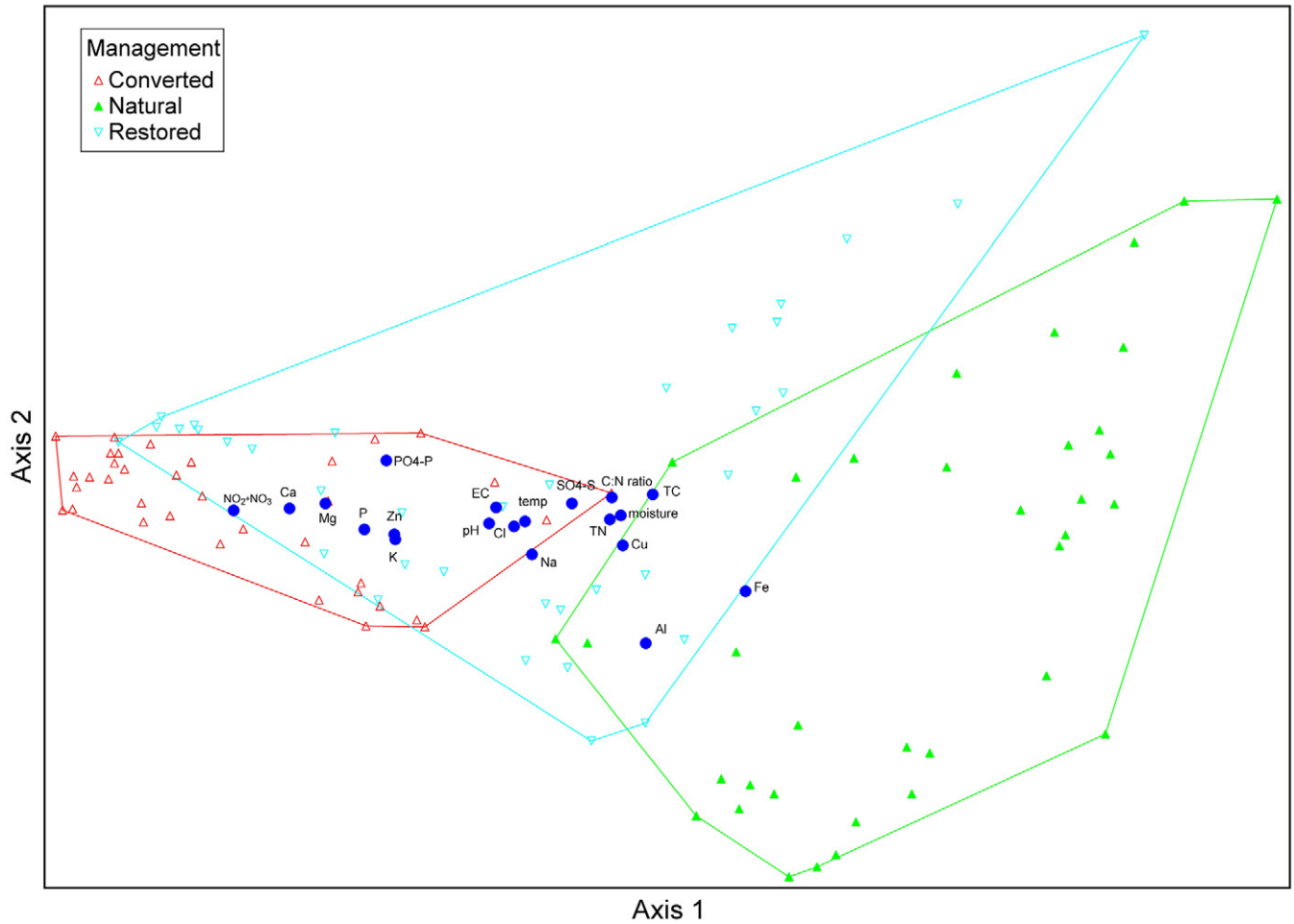

Fig. 1. Two dimensional ordination using nonmetric multidimensional scaling (NMS) showing relative differences between prior converted cropland, restored, and natural wetland soil samples. Individual soil physicochemical properties are displayed as labeled dots. EC, electrical conductivity.

sites as compared with PC cropland sites, with natural sites having values between the other two. Total N is statistically greater in RW0 for natural and restored sites compared with PC cropland, although there is no statistical difference at RW3 between the three land uses. Restored wetland sites, as a consequence of higher TC values, also display higher C/N ratios than natural wetland and PC cropland sites. This difference is greatest at RW0, with C/N ratios of 45.7, 26.6, and 26.8 for restored wetland, natural wetland, and PC cropland sites, respectively.

Denitrification Enzyme Activity

Denitrification enzyme activity rates, sorted by land use and relative wetness, are listed in Table 5. As a general trend, DEA rates decreased as relative wetness increased, with a majority of DEA rates significantly higher ($P < 0.05$) in wetter areas (RW 0) as compared with the drier areas (RW3). Additionally, when looking at land use, natural wetlands typically had higher DEA rates as compared with restored wetlands and PC croplands.

Complete and Potential Complete Denitrification

Complete and potential complete DEA rates were determined using an acetylene block. As mentioned previously, this block prevents the activity of N_2O reductase, disrupting the formation of N_2 gas, resulting in higher accumulations of N_2O. For complete denitrification, within the wettest areas (RW0) of the restored wetlands, DEA rates were in between the natural and PC cropland DEA rates, with rates of 89.7 μg N_2O–N kg^{-1} soil h^{-1}. In the driest areas (RW3) of the restored wetlands, however, complete (9.9 μg N_2O–N kg^{-1} soil h^{-1}) DEA rates were the lowest among the three land uses, although none of the measured rates across the different relative wetness class was statistically different at the $P < 0.05$ level (Table 5).

Additional comparisons of complete DEA by land use revealed that at RW0, natural wetlands (145.3 μg N_2O–N kg^{-1} soil h^{-1}) had significantly higher rates than PC croplands (49.7 μg N_2O–N kg^{-1} soil h^{-1}); with lower soil moisture levels at RW3, rates between the natural wetlands and PC croplands were not statistically different. Potential complete denitrification rates at RW0 were significantly greater in natural wetlands (330.5 μg N_2O–N kg^{-1} soil h^{-1}) than in PC croplands (41.2 μg N_2O–N kg^{-1} soil h^{-1}), although, once again, rates were not statistically different at the driest areas of each site (RW3).

With NO_3^- as a nonlimiting factor, potential complete DEA rates in restored wetlands sites showed a trend similar to the complete DEA rates. At RW0, restored wetland (143.9 μg N_2O–N kg^{-1} soil h^{-1}) rates bridged the natural (330.5 μg N_2O–N kg^{-1} soil h^{-1}) and PC cropland (41.2 μg N_2O–N kg^{-1} soil h^{-1}) rates, with natural and PC cropland rates being statistically different ($P < 0.05$). At RW3, restored wetland (13.8 μg N_2O–N kg^{-1} soil h^{-1}) rates were significantly lower than natural wetlands (81.6 μg N_2O–N kg^{-1} soil h^{-1}) only at the $P < 0.10$ level and were not significantly different from PC cropland (41.2 μg N_2O–N kg^{-1} soil h^{-1}) rates.

Comparison of complete DEA rates to potential complete DEA rates provides insight as to the NO_3^- limitation of the soil microbial communities capable of denitrification. Examination of natural wetlands revealed that at RW0 the addition of NO_3^- resulted in a significant ($P < 0.05$) DEA rate increase (145.3–330.5 μg N_2O–N kg^{-1} soil h^{-1}). For restored wetland sites at RW0, NO_3^- addition resulted in a 60% mean increase (89.7–143.9 μg N_2O–N kg^{-1} soil h^{-1}) between complete and potential complete DEA rates, although this increase was not statistically significant. Additionally, although DEA rates did increase slightly in both natural and restored sites at RW3, the increases were not significant. Similarly, for PC croplands the difference between complete and potential complete DEA rates was negligible, with no significant increases at RW0 or RW3.

Incomplete and Potential Incomplete Denitrification

Incomplete and potential incomplete denitrification rates were determined without the use of acetylene, allowing for N_2O reductase activity and production of N_2 gas production. For these DEA treatments, all remaining N_2O is considered an endpoint product for the N cycle in those soils.

Comparisons of incomplete DEA rates between the three land uses revealed a different pattern than the one for complete DEA. At the wettest (RW0) and driest (RW3) points, natural wetlands had significantly higher incomplete DEA rates than

Table 5. Denitrification enzyme activity rates in North Carolina Conservation Effects Assessment Project designated wetlands.

Land use	Relative wetness	Incomplete (no additions)	Complete (+ acetylene)	Potential incomplete (+ NO_3^-)	Potential complete (+ NO_3^- + acetylene)
		μg N_2O–N kg^{-1} soil h^{-1}			
Natural	0	70.1 (32.4)†a‡	145.3 (64.4)a	231.1 (157.6)a	330.5 (192.7)a
	1	29.1 (11.7)a	97.4 (49.6)ab	139.8 (50.9)a	210.2 (67.6)a
	2	11.8 (4.1)ab	21.3 (9.8)cd	18.8 (5.9)bcd	28.1 (9.4)bc
	3	28.4 (15.4)b	59.7 (34.6)cd	48.2 (29.3)bcde	81.6 (46.2)bc
Restored	0	23.3 (12.9)bc	89.7 (53.1)bc	67.3 (30.6)abcd	143.9 (58.3)ab
	1	40.6 (18.5)ab	77.0 (32.3)abc	89.9 (39.4)ab	132.2 (44.3)ab
	2	19.2 (12.7)bc	35.3 (22.4)cd	34.1 (18.3)cde	68.7 (32.4)bc
	3	4.5 (2.0)c	9.9 (3.3)c	7.8 (3.4)e	13.8 (5.4)c
Prior converted	0	18.5 (7.8)bc	49.7 (13.1)ab	23.6 (9.3)bcd	41.2 (10.5)b
	1	35.9 (12.9)ab	88.2 (28.8)a	42.5 (15.4)bcd	102.3 (27.5)ab
	2	24.3 (9.3)ab	58.1 (14.6)ab	46.5 (15.9)abc	75.3 (24.6)b
	3	4.5 (1.8)c	33.0 (15.5)c	22.0 (12.8)de	55.2 (21.8)bc

† Values are mean and SE.

‡ Columns are statistically grouped according to Duncan's multiple range test using least significant mean differences based on a $P < 0.05$ level. Those with the same letter are not significantly different.

restored wetlands and PC cropland sites ($P < 0.05$) (Table 5). At RW0, natural wetland sites had incomplete DEA rates of 70.1 μg N_2O–N kg^{-1} soil h^{-1}, with DEA rates dropping to 28.4 μg N_2O–N kg^{-1} soil h^{-1} for soils collected at the driest points of the natural wetlands. However, unlike complete DEA rates of restored wetlands trending between natural and PC cropland sites, restored wetland and PC cropland incomplete DEA rates were similar across all relative wetness class.

For potential incomplete DEA, natural wetlands had significantly higher DEA rates at RW0 (231.1 μg N_2O–N kg^{-1} soil h^{-1}) compared with restored wetland and PC cropland sites ($P < 0.05$). Like the potential complete DEA treatment, restored wetlands trended between natural wetlands and PC croplands. For restored wetland sites, the potential incomplete DEA rate at RW0 was 67.3 μg N_2O–N kg^{-1} soil h^{-1}, compared with 23.6 μg N_2O–N kg^{-1} soil h^{-1} for PC croplands at RW0. At RW3, although natural wetland sites had higher DEA rates than both restored wetland and PC cropland sites, there were no significant differences between all three land uses.

NO_3^- Limitation

Comparison of incomplete/complete DEA to potential incomplete/complete DEA treatments provides insight into the NO_3^- limitation of the soil microbial communities capable of denitrification. For complete DEA treatments, examination of natural wetlands revealed that at RW0 the addition of NO_3^- resulted in a significant ($P < 0.05$) 2.3-fold increase of DEA rate (145.3–330.5 μg N_2O–N kg^{-1} soil h^{-1}). For restored wetland sites at RW0, NO_3^- addition resulted in 1.7-fold increase in mean rates (89.7–143.9 μg N_2O–N kg^{-1} soil h^{-1}) between complete and potential complete DEA, although this increase was not statistically significant. Additionally, although DEA rates did increase slightly in both natural and restored sites at RW3, the increases were not significant. Similarly, for PC croplands the difference between complete and potential complete DEA rates was negligible, with no significant increases at RW0 or RW3.

For incomplete DEA treatments, natural wetlands at RW0 saw a significant increase in DEA rates with the addition of NO_3^- ($P < 0.05$; 70.1–231.1 μg N_2O–N kg^{-1} soil h^{-1}). Restored wetlands at RW0 also saw an increase (23.3–67.3 μg N_2O–N kg^{-1} soil h^{-1}), although it was significant only at the $P < 0.10$ level. Natural and restored wetlands at the driest points (RW3) and PC croplands at both RW0 and RW3 did not see significant increases in DEA rate with the addition of NO_3^-.

Quantification of the N_2O Reductase Gene

Abundances of the gene encoding the enzyme N_2O reductase (*nosZ*), as determined by qPCR, are shown by land use (Fig. 2A) and by relative wetness (Fig. 2B). Based on land use, comparison of log-transformed mean gene copy numbers (± SE) per gram of soil shows that PC cropland sites had the highest abundance of *nosZ* (6.64 ± 0.12), followed by natural (6.49 ± 0.12) and restored wetlands (6.09 ± 0.10); restored wetland sites had significantly ($P < 0.001$) lower *nosZ* abundances compared with PC cropland and natural wetland sites (Fig. 2A). Measurement of *nosZ* copy numbers along the relative wetness gradient (Fig. 2B) revealed several differences. Gene abundance patterns between the three land uses varied, with PC cropland and restored wetland sites having lower mean gene abundance values at RW0 (6.46 and

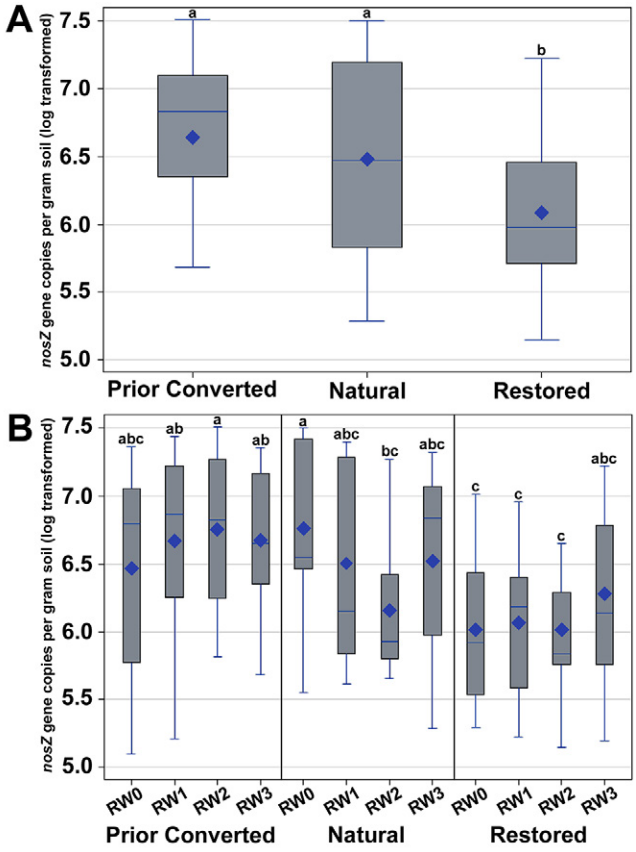

Fig. 2. Box and whisker plots of *nosZ* gene abundances per gram of soil by land use (A) and by relative wetness (B). All values have been log transformed. *Statistical significance is based on Duncan's multiple range test ($P < 0.05$). **Those with the same letter are not significantly different.**

6.01 log-transformed gene copies per gram soil, respectively), whereas mean gene abundance values were greatest (6.76 log-transformed gene copies per gram soil) in RW0 for natural wetlands.

Correlations between gene abundances and soil physicochemical properties revealed significant positive relationships of *nosZ* with pH ($r = 0.57$; $P < 0.05$) and NO_2^- + NO_3^- ($r = 0.57$; $P < 0.05$), whereas significant negative relationships were identified between *nosZ* and TC ($r = -0.73$; $P < 0.005$), C/N ratio ($r = -0.88$; $P < 0.001$), Na^+ ($r = -0.71$; $P < 0.009$), and PO_4^{3-} ($r = -0.75$; $P < 0.005$). No significant relationship between *nosZ* gene abundances and DEA rates was identified. Likewise, a relationship between *nosZ* gene abundances and the ratio between incomplete and complete DEA [$N_2O/(N_2 + N_2O)$] was not elucidated.

Discussion

It has been estimated that development and industrial activity have led to a significant reduction in North Carolina's pocosin regions (Richardson, 2003). Loss of wetland function has been associated with a number of environmental impacts, such as (i) degradation of surface water quality, (ii) decreased flood mitigation and storm abatement, and (iii) conversion of lost wetland areas from a net sink to a net source of C in the atmosphere (Hirano et al., 2012; Zedler and Kercher, 2005). To this end, the depletion of wetlands in North Carolina has

long been recognized as a threat of significant economic and environmental costs (Mitsch and Gosselink, 2000; Richardson, 1983). To counter these effects, public policy has been enacted to mitigate wetland loss, create new wetland areas, and promote hydrological restoration of wetlands previously converted for other use (Whigham, 1999). There are many parameters for estimating wetland restoration success, physicochemical parameters being of primary interest. Indeed, a number of studies have assessed these parameters in the process of creation and restoration of wetlands in North Carolina (Bruland et al., 2003; Bruland and Richardson, 2005; Bruland and Richardson, 2006; Dimick et al., 2010). Two additional parameters for measuring effects of wetland restoration are analysis of microbial communities and microbial enzymatic activity (Bossio et al., 2006; Bruland et al., 2006; Hunter and Faulkner, 2001; Morse et al., 2012). Therefore, in this study, our objectives were to measure the effects of wetland restoration in the pocosin region of North Carolina based on the following parameters: (i) soil physicochemical properties, (ii) DEA rates, and (iii) N_2O reductase (*nosZ*) gene abundances.

As evidenced by a battery of physicochemical parameters, the restored wetland sites continue to exhibit an agricultural legacy, as demonstrated by the similar levels of a number of soil chemical properties with the PC sites. This is illustrated in Fig. 1, with an overlap of a number of restored wetland sites with the PC sites. In the restored wetlands, the mean values for the chemical properties of Al^{3+}, Fe^{3+}, and Mg^{2+} are similar to levels found in the PC sites. Still other properties appear to be in transition between their previous PC status and natural wetlands, such as levels of Ca^{2+} and the physical properties of pH, EC, moisture, soil temperature, and bulk density. The observed decrease in restored wetland bulk densities is a result of organic matter buildup and increased pore space from plant roots and earthworms in restored sites (Joschko et al., 1989). The PC sites may undergo tillage practices that destroy these macropores and result in soil compaction (Osunbitan et al., 2005). Still other soil properties have come to approximate natural wetland conditions, such as Na^+, P, Zn^{2+}, total N, and NO_2^- + NO_3^-. Immediate agricultural inputs, which can be measured by NO_2^- + NO_3^- and plant-available P, were significantly lower in restored wetlands as compared with their agriculturally used PC counterparts. This is a clear indication that these nutrients are removed from the soil quickly after restoration (Ardón et al., 2010). Although plant-available P (measured by Mehlich I extraction) in restored wetlands had dropped to levels similar to those found in natural wetlands, soluble inorganic PO_4^{3-} saw significant increases over both PC cropland and natural wetland sites. This is not unexpected because hydrological restoration leads to anaerobic conditions with eventual Fe reduction and solubilization of Fe oxides, conditions conducive to soluble inorganic PO_4^{3-} release associated with Fe oxides (Moorberg et al., 2015; Szogi et al., 2004). Ardón et al. (2010) predicted that restored wetlands would most likely release agricultural P for 3 to 16 yr after initiation of restoration efforts. These values support the results of this study, with P values being measured between 4 and 7 yr after restoration. Although wetlands are typically considered a sink for P, pocosin soils have been demonstrated to have low P retention capacity as compared with other wetland types in part due to the low levels of extractable Al^{3+} from these soils (Richardson, 1985); the restored wetland sites would have an even lower capacity for P retention given their significantly lower levels of soil Al^{3+} (Table 3). However, other nutrients used as liming agents to increase soil pH, such as Ca^{2+} and Mg^{2+}, persist in the restored soils and are reflected in the restored wetlands sites with higher soil EC values. Levels of Mg^{2+} were essentially unchanged when compared with the PC sites, and, although Ca^{2+} was approximately half of what could be found in PC sites, it was still several times higher than the levels found in natural wetland sites.

One effect of the restoration efforts was a significant increase in TC at the restored wetland sites. At the three wettest points of restored wetland sites (RW0, RW1, and RW2), restored wetland sites have significantly higher TC levels as compared with PC sites; these values are on par with their natural wetland counterparts. These results indicate a sizeable increase in accumulated TC pools at restored wetland sites over the short period of time after restoration. This accumulated TC has also resulted in high C/N ratios, and restored wetland sites at RW0 have significantly higher C/N ratios than natural and PC cropland sites. High C/N ratios (>25) are commonly associated with complete, rather than incomplete, denitrification (Hunt et al., 2007). This is supported by the data in Table 5, where incomplete to complete DEA rate ratios were often below 50% (range, 13.7–55.2%); a majority of NO_3^- is converted to N_2, as opposed to N_2O during denitrification. Gene abundances of *nosZ* showed a strong negative relationships to TC (r = −0.73) and, as a consequence, to C/N ratios (r = −0.88). A negative relationship between *nosZ* and soil C has been previously documented in dairy-grazed pasture soils and was associated with a concomitant positive relationship to NO_3 (Jha et al., 2012). These findings, similar to those reported in this study, potentially indicate that NO_3^- availability is a stronger influence over denitrifier abundance than C availability.

No relationship between *nosZ* gene abundances and DEA rates was confirmed in this study. This was not unexpected because several studies have failed to identify a relationship between *nosZ* gene abundances to N_2O emission or DEA rates (Ducey et al., 2011; Morales et al., 2010; Philippot et al., 2009). Those studies, however, did report a correlation between *nosZ* gene abundance levels and the ratio between incomplete and complete DEA [$N_2O/(N_2O + N_2)$], a relationship not identified in this study. It is possible that other genes, such as *nirS*—which encodes for nitric oxide reductase—would serve greater utility in future studies (Morales et al., 2010). Another possibility is that a recently identified clade (clade II) of the *nosZ* gene may play a significant role in N_2O reduction in North Carolina coastal plain soils (Jones et al., 2013). This previously undetected clade is not covered by the qPCR primers used in this study and may have potentially hampered attempts to elucidate the relationship between *nosZ* and DEA rates.

The fate of NO_2^- + NO_3^- (primarily NO_3^-) is of particular concern because it is a key water contaminant. Prior converted croplands had statistically higher levels of NO_2^- + NO_3^-. This is a consequence of the application of fertilizer for row crop production, as evidenced by a similarly statistical increase in P in

PC croplands. The presence of NO_2 + NO_3 in PC cropland soils is also the most likely explanation for a lack of response in PC cropland soils to NO_3 addition of in the DEA assay (as evidenced by potential DEA rates). For the wettest areas (RW0) in PC croplands, the increase in DEA activity between incomplete and potential incomplete DEA was only 5.1 μg N_2O–N kg^{-1} soil h^{-1}, and, when comparing complete to potential incomplete DEA, rates dropped on addition of NO_3. When comparing the same area (RW0) in natural wetland soils, increases of 161.0 and 44.0 μg N_2O–N kg^{-1} soil h^{-1} were observed for incomplete versus potential incomplete and complete versus potential incomplete DEA rates, respectively. Likewise, sizeable increases were observed for restored wetlands, with values of 185.2 and 54.2 μg N_2O–N kg^{-1} soil h^{-1} for incomplete versus potential incomplete and complete versus potential incomplete DEA rates, respectively. By comparison, these values are a clear indication that PC cropland soils are not NO_3^- limited. Additionally, the low DEA of the PC cropland soils is most likely affected by a variety of additional environmental factors, including soil moisture (wetter soils are more anaerobic and hence more capable of denitrification) and soil C (which serves as an energy source for heterotrophic denitrifiers). In the PC cropland sites, such conditions do not exist to support high DEA rates. Although unlikely, the role of acidic conditions in the PC cropland soils can also not be discounted as playing a role in the decreased DEA rates in these soils (Simek and Cooper, 2002).

Variability of sites within a particular land use can also be observed in Fig. 1, with less variation in the PC cropland sites as compared with the restored and natural wetlands. Additionally, there is separation of PC cropland and natural wetland sites, with restored wetland sites overlapping the two other land uses. Lower variability among PC sites may be attributable to the increased management activity (i.e., liming and fertilization) associated with these sites that was implemented with the intention of maintaining defined nutrient levels for agricultural purposes. However, shortly on removal of agricultural inputs and implementation of hydrological restoration, variability increases among the restored wetland sites, which approximates the variability observed in natural wetland sites.

Analysis of DEA rates (Table 5) reveals that, when compared with PC and natural wetland sites, restored sites exhibit a trend somewhere between the other two land uses. These results indicate that wetland restoration efforts have not greatly affected this component of the N cycle. Only at RW2, for complete and potential complete denitrification, did restored site DEA rates exceed the rates found in natural wetland sites. These results differed from DEA rates analyzed in the Delmarva region, in which restored sites demonstrated a general trend of higher DEA rates than natural wetland sites (Hunt et al., 2014). A similar result was observed in *nosZ* gene abundance rates at the Delmarva sites, with restored wetland site *nosZ* gene abundances significantly greater than the natural wetland sites but with significantly less than abundances found in the PC sites (Hunt et al., 2014). In contrast to the Delmarva result, the restored wetland sites of North Carolina had significantly lower levels of *nosZ* gene copies per gram of soil than PC and natural wetland sites.

A previous report demonstrated significant differences between the physicochemical properties of the soil in North Carolina sites and the soils of the Delmarva region (Kluber et al., 2014). The differences between the soil properties found in these two distinct areas could potentially explain different responses in DEA and *nosZ* patterns with restoration. In the Delmarva and North Carolina coastal plain soils, DEA and *nosZ* gene abundances were observed as responding to restoration efforts but not approaching natural wetland site–like levels. This potentially indicates that restoration efforts have not successfully restored microbial communities capable of functioning in these sites. These results are similar to Peralta et al. (2010), who demonstrated that restored wetland sites did not successfully restore denitrifier communities. Additionally, a report by Bruland et al. (2006) reported that two of three restored wetlands studied displayed lower DEA rates than adjacent natural wetland sites. These results led them to determine that the restored wetlands did not possess microbial communities capable of the increased denitrification rates demonstrated in the natural wetlands. Another possibility is that microbial populations have reached a different paradigm given the new environmental conditions. This paradigm is consistent with the restored wetland sites vegetation survey, which revealed that in the restored sites, a large diversity of hydrophytic herbaceous plants was present without the trees native to North Carolina coastal plain wetlands. This indicates that restoration efforts resulted in supporting wetland plant communities, albeit in a manner distinct from the neighboring natural wetlands.

Regardless of the reasons for the shifts in microbial populations, a potential explanation can be found in a report by Bossio et al. (2006), which demonstrated that C quality, rather than C quantity, had significant effects on restored wetland microbial populations; these results were similar to a report by Bååth et al. (1995), which indicated that differences in soil composition have a direct, limiting effect on microbial structure and function in restored wetland sites. In the current study, the decrease of both denitrification activity and *nosZ* in restored wetland sites supports the hypothesis of a negative feedback process on the microbial populations associated with denitrification. This is potentially supported by a report from McCarty et al. (2007) that demonstrated the influence of plant residue C on denitrification potential. In that report, wetland soil microbial communities, despite being found in soils with higher C/N ratios, produced lower DEA rates, which was a result of stimulation of soil microbial communities by vegetation with a lower C/N ratio (McCarty et al., 2007). It is possible that these higher C/N ratios in restored wetlands, in addition to potentially suppressing DEA rates, could result in reduced *nosZ* gene abundances. This would be significant because *nosZ* is responsible for converting the greenhouse gas N_2O into a more environmentally friendly form as molecular N, and a decrease in *nosZ* levels could potentially result in exacerbating N_2O emissions in the restored wetland soils (Philippot et al., 2009). In fact, the opposite has occurred, with hydrological-restored soils generally demonstrating lower DEA rates than their natural wetland soils counterparts. Therefore, it is difficult to predict whether the microbial populations in these soils will reach DEA levels equal to populations in natural wetlands.

Although a number of physicochemical factors suggest that restoration efforts are resulting in conditions analogous to natural

wetlands, the continued agricultural legacy of these restored sites suggests that wetland reclamation is potentially an ongoing process that is contingent on more than hydrological restoration. It is possible that these restored sites will never achieve a state that equals their wetland status before their conversion for agricultural purposes (Zedler and Callaway, 1999). What this study and others demonstrate is that current restoration efforts in the MIAR-CEAP region have not led to serious, unintended consequences, such as an increase in greenhouse gas emissions. This is supported by Morse et al. (2012), who reported similar findings. Likewise, although not having returned to a natural state, many of the restored wetland functions are more similar to natural wetlands than their converted cropland counterparts. Although this may not be considered an ideal outcome, this is an improvement relative to the total wetland ecosystem service of improving water quality, a result consistent with original conservation program goals. Therefore, although restoration efforts continue to exhibit an agricultural legacy after almost a decade after restoration, a number of physicochemical predictors (i.e., C sequestration, nutrient reduction, and plant community richness) indicate progress toward a state capable of significant wetland ecosystem services (Gleason et al., 2008).

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