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ORIGINAL RESEARCH

Greenhouse Gas Emissions and Denitrification within Depressional Wetlands of the Southeastern US Coastal Plain in an Agricultural Landscape

Jarrod O. Miller[1] · **Thomas F. Ducey**[1] · **P. William Brigman**[1] · **Charlie O. Ogg**[1] · **Patrick G. Hunt**[1]

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Abstract Carolina Bays are depressional wetlands on the Coastal Plain of the southeastern United States. These wetlands are often the recipient of nutrient runoff from adjacent agricultural lands and there is potential for production of greenhouse gases during nitrification and denitrification processes occurring in the wetland sediments. Because of their saturated conditions, Carolina Bays may improve regional water quality through denitrification of soil nitrate. Three small bays in South Carolina were selected for denitrification and greenhouse gas analysis. A transect of four points was sampled within each Carolina Bay in May, July, September, and November over a two year period. Gas emissions were measured in-situ using a photoacoustic gas analyzer and soil samples were brought back to the lab for denitrification enzyme activity and microbial analysis. Emissions of nitrous oxide (N_2O) averaged 1.8 mg m^{-2} d^{-1}, with a median of 0.47 (with a range of below detectable limits to 9.414 mg m^{-2} d^{-1}). Many measurement events of N_2O were below detection and did not vary within the bays. The carbon dioxide emissions from Carolina Bays averaged 15.8 g m^{-2} d^{-1} and were largely controlled by temperature. Denitrification enzyme activity had a larger response to nitrate additions further into the bays. Gram + bacteria were also greater deeper into the bays, while Gram- and fungal populations were greater at the field/wetland interface. Manure application had some minor effects on DEA within the bays, but did not appear to increase gas emissions over the period measured.

✉ Thomas F. Ducey
Thomas.Ducey@ars.usda.gov

[1] ARS-USDA, Coastal Plains Soil, Water, and Plant Research Center, 2611 W. Lucas St, Florence, SC 29501, USA

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Introduction

Carolina Bays are depressional wetlands on the South Atlantic Coastal Plain of the United States, with a water cycle largely dependent on precipitation (Newman and Schalles 1990; Lide et al. 1995; Bruland et al. 2003; Sharitz 2003). While rainfall and evaporation dominate inputs into these wetlands, ground and surface water from adjacent uplands also flows through Carolina Bays (Sun et al. 2006). Water inputs from uplands are governed by topography as well as the presence of restrictive layers beneath the landscape (Sun et al. 2006). Because of the variability in water inputs, these wetlands may have different hydrologic regimes, with some being consistently drier than others (Lide et al. 1995; Sharitz 2003).

Carolina Bays also have a large range in size, with some bays being greater than 3000 ha, but most others less than 1 ha (Sharitz 2003). Many of the smaller bays have been drained for agricultural use (Kirkman et al. 1996; Bruland et al. 2003; Sharitz 2003), but have demonstrated the ability to return to a forested state (Kirkman et al. 1996; Bruland et al. 2003). Soils within Carolina Bays can be composed of organic or mineral materials, and are often underlain by sandy or clay layers (Sharitz 2003; Caldwell et al. 2007). Mineral soils are more likely to be found inland, while those bays near the coast are more likely to consist of peat materials (Newman and Schalles 1990; Sharitz 2003). Soil characteristics of surface horizons will vary as they move towards the center of the bays. Reese and Moorehead (1996) have reported thicker A horizons at the rim, while pH, bases (Ca, Mg, K), and sand content typically increase towards the center of the bays.

Carolina Bays may be connected to drainage systems, but most are considered to be isolated from local streams and rivers (Sharitz 2003; Whigham and Jordan 2003; Mushet et al. 2015). Due to their perceived isolated nature, in 2001 Carolina Bays lost most federal protections under the Clean Water Act, leaving their protection up to the individual states (Downing et al. 2003; Sharitz 2003). However, these wetlands can affect the quality of downstream waters, particularly when considered on a regional basis. Water quality of depressional wetlands are dependent on the source of the water, soil properties, and landscape use (Whigham and Jordan 2003). Those Carolina Bays which receive water inputs through rainfall will have in turn lower nutrient contents (Ralston and Richter 1980), while others supplied with surface or groundwater can be nutrient sinks (Clément et al. 2002; Bruland et al. 2003; Whigham and Jordan 2003). Upon draining for agriculture, these bays can be a source of nutrients back into surface and groundwater (Bruland et al. 2003; Whigham and Jordan 2003). Greater concentrations of P, nitrite (NO_2), and nitrate (NO_3) in surface runoff from drained wetlands has been observed, compared to restored and reference Carolina Bays (Bruland et al. 2003).

Carolina Bays are widespread on the coastal plain, often adjacent to agricultural fields, and therefore potential recipients of nutrient runoff (Phillips et al. 1993; Sharitz 2003). Although they are known to be potential nutrient sinks (Bruland et al. 2003; Whigham and Jordan 2003), the actual N-cycling processes occurring within Carolina Bays has limited study. Riparian buffers have been studied extensively, and are known to decrease groundwater NO_3 content (Peterjohn and Correll 1984; Spruill 2000; Weller and Baker 2014). For riparian buffers, topography has a greater effect on denitrification than vegetation (Clément et al. 2002). Across an agroecosystem, local geometry of a slope can significantly impact soil moisture as well as soil organic matter deposition; in turn impacting denitrifiers and N_2O emissions (Florinsky et al. 2004). Greater populations of denitrifying bacteria occur at the wetland/upland interface, and are a function of the entire soil profile (Clément et al. 2002). While a paucity of research exists on denitrification in forested Carolina Bays, they are known to reduce NO_3 contents relative to unsaturated uplands (Phillips et al. 1993; Bruland et al. 2003).

Denitrification within wetlands and riparian buffers has become an important environmental filter of agricultural runoff, but the consequential production of nitrous oxide (N_2O), a greenhouse gas, has become a separate concern (Bouwman 1990; Hunt et al. 2007). N_2O emissions from soils are the results of nitrification and denitrification due to microbial processes (Mosier et al. 1998). In agricultural systems, nitrification and denitrification are often coupled with fertilizer and manure applications (Velthof et al. 1997; Goossens et al. 2001; Hunt et al. 2007). The largest N_2O emissions are observed immediately following fertilizer or manure applications (Lowrance et al. 1995; Velthof et al. 1997), with N_2O flux dropping within a few weeks after application (Velthof et al. 1997). Across fertilizer types, nitrates typically cause a larger N_2O flux from the soil compared to ammonia fertilizers or animal manures (Velthof et al. 1997; Flechard et al. 2005).

The water table is another important factor in N_2O emissions, as higher groundwater tables and saturated soils will increase incomplete denitrification (Velthof et al. 1996, 1997; Clément et al. 2002; Klemedtsson et al. 2005; del Prado et al. 2006). Poorly drained sandy soils, such as those found at the rim of Carolina Bays (Reese and Moorhead 1996), have shown a greater potential to produce N_2O when compared to clay soils (Velthof et al. 1997). It is also known that C:N ratios less than 25 are important in N_2O production (Hunt et al. 2007), where up to 88 % of N_2O emissions can come from soils with lower C:N ratios (Ernfors et al. 2008). Although the C:N ratios of the soil and plant inputs are important (Klemedtsson et al. 2005; Hunt et al. 2007; Ernfors et al. 2008), land use (Goossens et al. 2001), and topography also play important roles (Velthof et al. 2000; Clément et al. 2002).

The high carbon content, saturated conditions and possible influx of runoff from manure-impacted agricultural fields will all affect nitrogen cycling in Carolina Bays. For this study our goals were to observe some of the processes of denitrification by season in Carolina Bays by (1) measuring N_2O emissions, (2) measure potential denitrification through denitrification enzyme assays (DEA), and (3) compare N_2O emissions and DEA to microbial populations within the bays.

Materials and Methods

Site Selection

Three forested Carolina Bays (Bay1, Bay2, Bay3) were selected on a farm in Lee County, SC. This farmer used poultry litter as a fertilizer on fields adjacent to the forested bays. A four point transect was sampled from the field edge to the middle of each bay, representing 0, 10, 20 and 30 m into the center for twelve total sample sites. These distances were chosen in an attempt to capture a sufficient spatial gradient while capturing the variability of wetland activity and functions. A majority of NO_3 removal in riparian buffers typically occurs within the first 19 m (Peterjohn and Correll 1984); therefore, 30 m was considered to be a sufficient sampling distance into each bay. Tree species were dominated by loblolly pine (*Pinus taeda*), red maple (*Acer rubrum*), sweet gum (*Liquidambar styraciflua*) and southern red oak (*Quercus falcata*). The adjacent crop land was in a three year cotton-corn-soybean rotation. The soil of each bay was classified as Rains loamy sand (Fine-loamy, siliceous, semiactive, thermic Typic Paleaquults). The Rains series is the poorly drained component of the

Norfolk loamy sand (well drained), Goldsboro loamy sand (moderately well drained), Lynchburg loamy fine sand (somewhat poorly drained) catena. Most of the Carolina Bays in the surrounding region were classified as Rains loamy sand. Depth to water was measured by an adjacent Natural Resource Conservation Service well in one of the bays. Over the sampling period, water table depths ranged from 0 to >200 cm (Table 1). The water table did not rise above 80 cm below the surface in 2011 due to dry weather.

Soil and Gas Emission Sampling

Gas emissions (N_2O and CO_2) were measured with an Innova (LumaSense Technologies, Santa Clara, California) 1412 Photoacoustic gas analyzer (PAGA) at each sampling point. Gas emissions were measured every other month from May through November, during 2010 and 2011, for a total of eight measurement events per transect point. No sampling was performed in the winter because previous observations at this site noted no microbial activity in cold weather. For each sampling point the PAGA measured gas emissions twice (duplicate runs), for a minimum time of 10 min each run. A static chamber was inserted 5 cm into the forest soil, leaving a 1200 cm^3 headspace in the chamber. The PAGA sampled the headspace gas once a minute, measuring N_2O, CO_2, SF_6, and water vapor. The water vapor was used to correct N_2O concentrations by the PAGA, while manually injected SF_6 was measured to test the system for leaks.

For quality control, 5 mL gas subsamples were taken at the beginning and end of selected runs from the measurement chamber using a syringe at the end of the second run. These QA/QC samples were injected into 2.5 mL crimp cap GC vials and analyzed on a Model 3800 gas chromatograph (Varian, Palo Alto, CA) equipped with a 15-mCi^{63}Ni electron capture detector operating at 350 °C to test the accuracy of the PAGA.

Each time gas emissions were sampled we also measured electrical conductivity (EC), moisture content, and temperature of the in situ soil. Electrical conductivity and soil temperature were measured using a ECTestr11+ meter (Spectrum Technologies, East Plainfield, IL) while soil moisture was measured using a Delta-T HH2 Moisture Meter (Dynamax, Houston, TX). Soil samples for the measurement of denitrification enzyme activity (DEA) and microbial analysis were taken from the upper 15 cm of each lysimeter and placed on ice.

In July and September of 2011 we were not able to sample Bay2 with the PAGA due to the adjacent field being planted over the access path. Instead static chambers were sampled three times over 15 min using 2.5 mL GC crimp cap vials as described above. Lab analysis of the PAGA vs static chamber GC samples had been previously performed to ensure they would lead to similar results. Soil samples from a Norfolk loamy sand were treated with 100 mg NO_3 L^{-1} to ensure emissions would be measured. Emissions were measured in triplicate for a total of three pots. The static chamber was placed in each pot and headspace gas was sampled by the PAGA for 10 min runs. Flux of N_2O was measured at 0, 4, 24, 28, and 48 h, and there were duplicate runs for each time sampled. Gas samples (5 mL) were taken from the static chamber and injected into 2.5 mL crimp cap vials to be analyzed by a Varian CP-3800 GC. Strong relationships between GC and PAGA N_2O concentrations were observed ($r^2 = 0.96$), as well as no observable differences in N_2O flux between the GC and PAGA ($r^2 = 0.99$; data not shown). The minimal detection limit for the PAGA was determined to be 0.1468 mg N_2O m^{-2} d^{-1}; when detection fell below this threshold, this value was used for statistical analysis, including the production of non-metric multidimensional scaling plots.

Flux Analysis

Analysis of the flux was done using Microsoft Excel 2007 © and Graphpad Prism 5. Raw PAGA data was imported into Excel and converted from ppmv to mg m^{-3} units. In Graphpad Prism, gas concentrations (mg m^{-3}) were regressed against time using a linear function:

$$C = C_o + ((J_c{*}A_d)/V){*}t.$$

Where A_d = surface are at the saturation depth (m), J_c = instantaneous flux, C = bulk headspace concentration (mg m^{-3}), C_o = initial headspace concentration, t = time (min), and V = headspace volume (m^3). Flux (Jc) was calculated by Graphad using the time and gas concentration data collected by the PAGA.

DEA Analyses

The DEA was measured by the acetylene inhibition method (Tiedje 1994). For this analysis, soil (10-15 g) was placed in 60 mL serum bottles (three bottles per sample per replication). The treatments were as follows: (deaA) 5 ml of a solution containing chloramphenicol (1 g L^{-1}) to inhibit protein

Table 1 Water table depth (cm) over the sampling period in 2010 and 2011

	Water Table Depth (cm)	
Month	2010	2011
May	97	148
July	16	>200
September	175	>200
November	>200	>200

synthesis and to measure actual incomplete denitrification; (deaB) 5 ml of a solution containing chloramphenicol (1 g L^{-1}) and 15×10^{-3} L of acetylene (produced from calcium carbide) to block denitrification at the nitrous oxide phase for measuring actual complete denitrification; (deaC) 5 ml of a solution containing chloramphenicol (1 g L^{-1}) and nitrates (200 mg L^{-1} NO_3-N) to measure potential incomplete denitrification; and (deaD) 5 ml of a solution containing chloramphenicol (1 g L^{-1}) and nitrates (200 mg L^{-1} NO_3-N), and 15×10^{-3} L of acetylene to measure potential complete denitrification.

Phospholipid Fatty Acid (PLFA) Analysis

A total of 15 g of soil was taken from fresh field samples, and then stored and shipped at −20 °C. Phospholipid fatty acid (PLFA) extraction and analysis was then performed by Microbial ID, Inc. (Newark, DE) as previously described (Buyer and Sasser 2012). Select markers were analyzed to determine the relative abundance (mole percent of total PLFA) of specific microbial groups (Moore-Kucera and Dick 2008). Fungal population relative abundance was determined from 18:2ω6, while bacterial population relative abundances were measured as follows: a) actinomycetes from 10Me16:0, 10Me17:0, and 10Me18:0; b) Gram-positive from *a*15:0, i15:0, i16:0, a17:0, and i17:0; and c) Gram-negative from cy17:0, 16:1ω7, and 18:1ω7.

DNA Extraction

Microbial DNA was extracted from all soil samples using a PowerLyzer Powersoil DNA isolation kit (MO BIO Laboratories Inc., Carlsbad, CA), according to manufacturer specifications. Final DNA quantity was determined via Biophotometer (Eppendorf, Hamburg, Germany), while DNA quality was assessed by electrophoresis on a 1 % agarose gel prepared with SYBR Safe (Thermo Fisher Scientific, Waltham, MA) as previously described (Ducey et al. 2011).

Quantitative Real-Time PCR Assays

All qPCR assays were performed using the LightCycler 480 Real-Time PCR Detection System (Roche Diagnostics, Indianapolis, IN). All primers used in these assays were obtained from Integrated DNA Technologies (Coralville, IA) and are listed in Table 2. Assays were carried out using SYBR GreenER qPCR SuperMix (Invitrogen, Carlsbad, CA) in a total volume of 25 μL. The final reaction concentration of reagents was as follows: 1X SYBR GreenER qPCR SuperMix; 200 nM each of forward and reverse primers; and 10 ng of DNA template. The qPCR reaction conditions were as follows: (1) an initial denaturation at 95 °C for 5 min; (2) 50 cycles of denaturation at 95 °C for 30 s, the appropriate annealing temperature (see Table 5) for 30 s, and elongation at 72 °C for 30 s; (3) melting curve analysis to confirm amplification product specificity. Fluorescent measurements were taken during the annealing phase of each cycle. Data was collected and processed using the LightCycler 480 software package. All qPCR assays included control reactions without template. Each assay also contained appropriate standard DNA reactions with concentrations between 10^1 and 10^9 copies per reaction, and were used to calculate amplification efficiencies according to the equation: $E = 1 + 10^{[-1/slope]}$ (Pfaffl 2001). DNA standards consisted of plasmids carrying the appropriate target gene, which were sequenced to confirm their identity and primer binding site. Each assay was performed in triplicate, with duplicate measurements for each sample.

Table 2 Primers used in this study for the qPCR analysis

Primers	Sequence (5′ to 3′)	Target	Tm†	Product Length	Reaction Tm	Efficiency
amoA-1F	GGGGTTTCTACTGGTGGT	*amoA*	54.1 °C	491 bp	54 °C	1.92
amoAr NEW	CCCCTCBGSAAAVCCTTCTTC		58.8 °C			
cd3aF_nirS	GTSAACGTSAAGGARACSGG	*nirS*	57.1 °C	425 bp	55 °C	1.97
R3cd_nirS	GASTTCGGRTGSGTCTTGA		55.8 °C			
1F_nirK	GGMATGGTKCCSTGGCA	*nirK*	58.0 °C	516 bp	53 °C	1.91
nirK5R	GCCTCGATCAGRTTRTGG		52.8 °C			
nosZF	CGYTGTTCMTCGACAGCCAG	*nosZ*	58.6 °C	453 bp	55 °C	1.90
nosZ-1622R	CGSACCTTSTTGCCSTYGCG		63.1 °C			
PolF	TGCGATCCSAATGCBGACTC	*nifH*	55.9 °C	360 bp	55 °C	1.99
PolR	ATSGCCATCCTYTCRCCGGA		57.9 °C			
67F	TGAAAACTGAACGAAACAAAC	16S v1-v3 region	50.0 °C	480 bp	50 °C	1.95
519R	GWATTACCGCGGCKGCTG		58.1 °C			

† Tm = Melting temperature

Statistics

Three bays, all within 200 ft of each other, were chosen as replicates for statistical analysis and averaged by SAS. This sampling approach was selected as opposed to having several transects within one bay, in order to capture landscape variability. Due to the length of time required to sample gas emissions with the PAGA, only one sampling approach could be utilized. The statistical design was setup as a factorial with one factor being distance into the bay and the second factor being time (month). As mentioned previously, when PAGA results were determined to be below detectable limits, the minimal detection limit of 0.1468 mg N_2O m^{-2} d^{-1} was substituted for statistical analysis purposes. Comparisons of soil properties, gas emissions, DEA, microbial gene abundances, and microbial PLFA were done using SAS v. 9.3 (SAS Institute Inc., Cary, NC). The PROC GLIMMIX procedure was used to compare soil properties, DEA, and gas emissions. Denitrification enzyme activity treatments were log10 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 $P \leq 0.05$. Microbial gene abundances were log transformed and analyzed using the general linear model procedure (PROC GLM), and Duncan's multiple range test ($P \leq 0.05$) was used to detect statistical differences. The PROC CORR procedure was used to correlate DEA, gas emissions, gene abundances, microbial PLFA, and soil properties to each other. To ensure statistical certainty, only correlations above 0.35 ($p < 0.01$) are indicated. For visualization of temporal shifts in PLFA profiles, non-metric multidimensional scaling (NMS) plots, using a Sorenson distance measure, were produced in PCORD v. 6.0 (MjM Software Design, Gleneden Beach, OR). The environmental variable second matrix in the NMS ordination was comprised of all physicochemical and gas emission data, as well as log10 transformed DEA values.

Results and Discussion

Emissions of N_2O were highly variable during the observed time period, ranging from below detection to 9.4 mg N_2O m^{-2} d^{-1}. Flux of N_2O from these Carolina Bays had an average of 1.8, a median of 0.47, and a standard deviation (sd) of 2.1 mg N_2O m^{-2} d^{-1}. Our range of N_2O flux is greater than the 0.09 to 0.62 mg N_2O m^{-2} d^{-1} reported from undrained forest soils (Mojeremane et al. 2012), but similar to the 3.04 to 9.12 mg N_2O m^{-2} d^{-1} flux range observed in tropical forest soils (Kiese and Butterbach-Bahl 2002).

Carbon dioxide ranged from 3.7 to 44.3 g CO_2 m^{-2} d^{-1}, with an average of 15.8 g CO_2 m^{-2} d^{-1} (6.9 sd), and median of 14.6 g CO_2 m^{-2} d^{-1}. Average CO_2 flux was greater than from Brazilian wetlands (Belger et al. 2011) and undrained forests soils (Mojeremane et al. 2012), which were 8.1 and 4.9 g CO_2 m^{-2} d^{-1}, respectively. Additionally, CO_2 flux was also lower than the 24 h average of 18.7 g CO_2 m^{-2} d^{-1} from tallgrass prairies, (Mielnick and Dugas 2000), and greater than the reported range of 8.1 to 12.1 g CO_2 m^{-2} d^{-1} from tropical forest sites (Kiese and Butterbach-Bahl 2002).

There were no observed differences in N_2O or CO_2 emissions by distance into the bays (Fig. 1a). Any spatial differences in gas emissions may have been reduced by temporal variation (Groffman and Tiedje 1989b; Rochette et al. 1991). Measured N_2O emissions were greatest in July (2.73 mg N_2O m^{-2} d^{-1}) and lowest in November (0.36 mg N_2O m^{-2} d^{-1}). This may be related to the wet conditions observed in July 2010 (Table 1, Fig. 1b) as well as the warmer soil temperatures (Table 3). Carbon dioxide emissions were greatest in July (21.2 g CO_2 m^{-2} d^{-1}) and September (18.4 CO_2 m^{-2} d^{-1}), compared to May (14.4 CO_2 m^{-2} d^{-1}) and November (11.5 CO_2 m^{-2} d^{-1}). The highest average soil temperatures were also in July (25.6 °C, Table 3) and September (23.0 °C). Temperature has a strong affect on microbial activity (Groffman and Tiedje 1989b; Fang and Moncrieff 2001), and in turn on greenhouse gas emissions. Although N_2O emissions could have come from deeper within the profile, soil

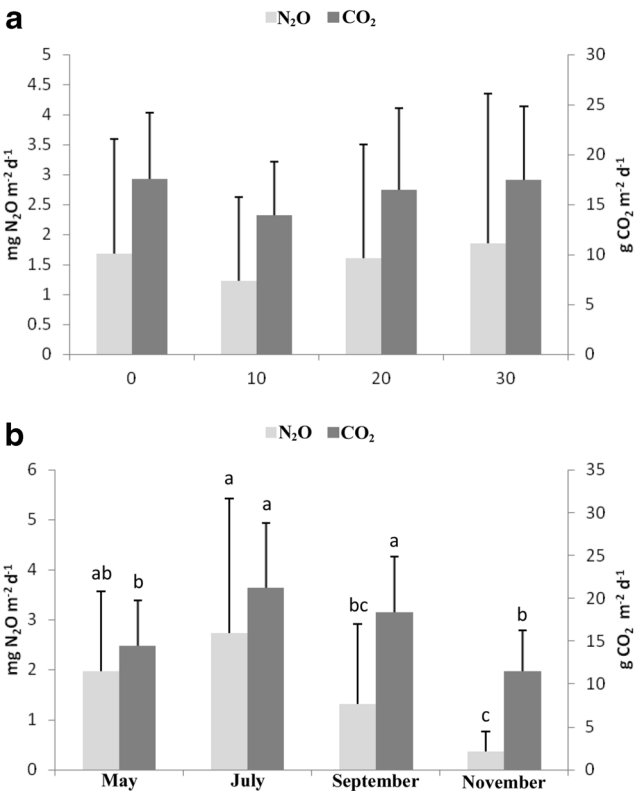

Fig. 1 Gas flux (N_2O, CO_2) from Carolina Bays by **a** distance into the bay and **b** month sampled. Statistical differences are for each gas across months by LSD ($\alpha = 0.05$), and statistically different samples ($P < 0.05$) are indicated by letter

Table 3 Soil properties in the Carolina Bays by distance into the bays and the month sampled

Distance (m)	pH	Moisture (%)	Temperature (°C)	Carbon (%)	Nitrogen (%)	C/N†
0	4.6 (0.7)	15.6 (8.7) b§	21.7 (4.0)	5.1 (2.2) b	0.26 (0.1) b	18.9 (4.7) c
10	4.5 (0.3)	23.1 (8.1) a	20.9 (3.4)	6.3 (1.5) b	0.29 (0.1) b	21.5 (3.1) b
20	4.4 (0.3)	25.6 (9.9) a	20.9 (3.5)	8.9 (3.3) a	0.37 (0.1) a	23.5 (3.0) ab
30	4.4 (0.3)	27.6 (10.8) a	21.1 (3.8)	9.7 (3.6) a	0.38 (0.1) a	24.7 (4.1) a
		****‡		****	****	****

Month	pH	Moisture (%)	Temperature (°C)	Carbon (%)	Nitrogen (%)	C/N
May	4.2 (0.2) b	30.1 (10.2) a	18.9 (1.4) c	7.5 (3.1)	0.34 (0.1)	21.4 (4.0)
July	4.6 (0.7) a	22.6 (9.3) b	25.6 (1.8) a	7.8 (3.4)	0.34 (0.1)	22.4 (4.3)
Sept.	4.5 (0.3) a	18.7 (9.1) b	23.0 (1.0) b	6.7 (2.7)	0.3 (0.1)	21.9 (4.3)
Nov.	4.5 (0.3) a	20.4 (9.6) b	17.1 (2.1) d	7.9 (4.0)	0.34 (0.1)	22.9 (4.9)
	***	****	****			

† Carbon to Nitrogen ratio

‡ – significance indicated by * = 0.1, ** = 0.05, *** = 0.01, **** = 0.0001, ns = not significant

§ statistically different samples ($P < 0.05$) are indicated by letter

data does not indicate that N content in the surface was higher in July. If this is the case, greater N_2O emissions could have occurred in July due to leaching into the subsurface from the adjacent manured fields. In addition, it cannot be determined from field measurements whether N_2O is from nitrification or denitrification.

Emissions of N_2O did not have any significant correlations with soil properties (Table 4). Carbon dioxide positively correlated with temperature ($r = 0.55$, Table 4), which is due to temperatures effect on microbial respiration (Groffman and Tiedje 1989a, b; Fang and Moncrieff 2001). That soil moisture did not have an effect on N_2O is interesting, as soil moisture has been previously shown to correlate with denitrification (Sexstone et al. 1985). For this study however, soil moisture was highest in May, where N_2O was lower. It should also be noted that the Rains soil series is poorly-drained, and Groffman and Tiedje (1989a) have previously demonstrated that poorly-drained soils can hamper predictors of denitrification (such as DEA) given their variable moisture regimes, and therefore, influence on microbial populations. Additionally, soil N, soil C, and the C/N ratio were both greater with distance into the bays (Table 3), but did not have a concurrent effect on N_2O emissions.

While there was no correlation with moisture, it can be observed that greater N_2O emissions (Fig. 1b) did coincide with higher moisture in May and July (Table 3). The drier conditions in 2011 may have limited a stronger correlation with moisture, potentially by limiting N movement to the wetland due to a lower water table (Table 1). Therefore, temperature is probably a more important factor within these wetlands during the period sampled.

DEA Analysis (July and November)

It was hypothesized that the field edge would have greater DEA due to nutrient inputs from agriculture, but this was

Table 4 Correlations of DEA treatments and Greenhouse Gas fluxes to soil chemical and physical properties

	Month	Year	Bay	Point	Moisture	Temp	EC	pH	%C	%N	CN	N_2O	CO_2
deA†	ns‡	ns	ns	ns	0.35 ***	ns	0.49 ****	ns	ns	ns	ns	ns	ns
deB	ns	ns	−0.37 ***	ns	0.35 ***	ns	0.52 ****	ns	ns	0.35 ***	ns	ns	ns
deC	ns	ns	ns	ns	0.47 ****	ns	0.37 ***	ns	0.41 ***	0.45 ****	ns	ns	ns
deD	ns	ns	ns	0.35 ***	0.40 ***	ns	0.42 ****	ns	ns	ns	ns	ns	ns
N_2O	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
CO_2	ns	ns	ns	ns	ns	0.55 ****	ns	ns	ns	ns	ns	ns	ns

† – deaA = incomplete DEA, deaB = complete DEA, deaC = incomplete DEA plus NO_3, deaD = complete DEA plus NO_3

‡ = significance indicated by *** = 0.01, **** = 0.0001, ns = not significant

not the case. While the C/N ratio is lower at the field edge (18.9, Table 3) and within range to promote denitrification (Hunt et al. 2007), incomplete denitrification (deaA) was lower at the field edge (although not significantly). The addition of NO_3 (deaC), and NO_3 plus acetylene (deaD) to the soil slurry did reveal in an increase in denitrification potential towards the bay interior. Both deaC and deaD were the greatest at 30 m into the bays, while they were lowest at the field edge (Table 5). The higher C/N towards the center of the bays may promote this, as the addition of NO_3 may stimulate denitrifying bacteria in high carbon environments (Miller et al. 2012). In addition, moisture was greater deeper into the bays (Table 3), which may have supported greater microbial activity. In this case, moisture may be a stronger driver than C/N for the soils and conditions studied. Another consideration would be presence of oxic conditions closer to the field edge; such conditions would prove unfavorable for denitrification despite the potential availability of NO_3 (Seitzinger et al. 2006). Overall, DEA was greater in July than November (Table 5), this is largely due to the greater temperature observed in those months (Table 2).

All four DEA treatments had a strong positive correlation with soil moisture (Table 4), which is due to the microbial nature of DEA. Soil C and N also had positive correlations, but neither were greater than 0.45 with any of the DEA treatments (Table 4). Because DEA is driven by C and N contents, this positive correlation is not surprising (Hunt et al. 2007). The correlation of deaC and deaD to temperature may be due to lower NO_3 contents in July, although there were no differences in total N by month (Table 3). Although none of the correlations were above 0.35 (Table 4), distance into the bay also had a positive correlation with deaB, C, and D. Control DEA (deaA), was unaffected by distance into the bay, which is similar to the lack of difference seen with N_2O emissions (Fig. 1a). Because deaA represents potential incomplete denitrification, it should be similar to observed N_2O field emissions. No direct correlation between DEA treatments and field N_2O emissions was observed.

Microbial Communities and their Relationship to Emissions and DEA

Carolina bay soil bacterial and fungal community composition based on PLFA analysis is detailed in Table 6. With the exception of the actinomycetes, all measured groups saw statistically significant differences as a function of distance. Overall higher percentages (42.6 %) of microbially-related PLFAs – in particular Gram- (10.9 %) and fungal PLFAs (3.9 %) – were found at the field edge. Only Gram + PLFAs had a higher percentage internal to the bays. Non-metric multidimensional scale (NMS) analysis of the data revealed strong relationships between the microbial community and several physicochemical variables, with the first two axes explaining 85.5 % of the variation (Fig. 2). Changes in microbial community composition could most easily be seen when grouped by sampling month. This change strongly correlated with EC, pH, temperature, and nitrous oxide emission rates (Fig. 2). Gram + PLFA markers i17:0 (r = 0.72) and a17:0 (r = 0.68) most strongly correlated with axis 1, while i16:0 (r = 0.50) most strongly correlated with axis 2. Gram- PLFA markers, 16:1ω7 (r = −0.77), and 18:1ω7 (r = −0.54) most strongly, and negatively correlated with axis 1, while cy17:0 (r = −0.46) negatively correlated with axis 2. Fungal PLFA marker 18:2ω6 positively correlated (r = 0.87) with axis 1.

Comparison of microbial PLFAs with CO_2 and N_2O emissions and denitrification enzyme activity revealed several significant relationships. While no individual PLFA markers correlated with CO_2 emissions, N_2O emissions negatively correlated with actinomycete markers 10Me17:0 (r = −0.46) and 10Me18:0 (r = −0.37). In relation to DEA, the actinomycete markers 10Me17:0 (r = −0.38) and 10Me18:0 (r = −0.38) negatively correlated with deaB, while only 10Me18:0 negatively correlated with deaC (r = −0.37) and deaD (r = −0.36). The

Table 5 Differences in the four DEA treatments by distance into the Bay and the month sampled

Distance (m)	deaA[†]	deaB	deaC	deaD
0	1.57 (1.2)	2.4 (1.4)	3.89 (3.3) c[§]	5.66 (5.2) c
10	1.87 (2.0)	2.68 (2.9)	5.8 (5.3) bc	9.17 (8.8) bc
20	14.8 (37.5)	26.95 (67.9)	24.9 (46.8) ab	30.03 (51.6) ab
30	8.06 (16.3)	13.77 (27.3)	20.5 (16.7) a ***[‡]	25.11 (17.5) a ***
Month	deaA	deaB	deaC	deaD
July	9.18 (27.7)	16.46 (49.9)	19.7 (34.5) a	24.00 (37.4) a
Nov.	3.97 (9.0)	6.43 (15.5)	8.38 (11.1) b **	10.98 (14.2) b **

[†] – deaA = incomplete DEA, deaB = complete DEA, deaC = incomplete DEA plus NO_3, deaD = complete DEA plus NO_3

[‡] – significance indicated by ** = 0.05, *** = 0.01, ns = not significant

[§] statistically different samples ($P < 0.05$) are indicated by letter

Table 6 Phospholipid fatty acid (PLFA) groupings by distance into Carolina Bays

Distance (m)	Gram -	Gram +	Fungi	Actinomycetes	Total
0	10.88 a[§]	18.52 b	3.94 a	9.22	42.55 a
10	9.53 a	19.45 ab	2.00 b	9.88	40.86 ac
20	7.77 b	19.85 a	2.28 b	9.44	39.34 b
30	7.86 b	19.82 ab	2.03 b	9.81	39.52 bc
p-value[†]	****	*	***	ns	***

[†] – significance indicated by * = 0.1, ** = 0.05, *** = 0.01, **** = 0.0001, ns = not significant

[§] statistically different samples ($P < 0.05$) are indicated by letter

relationships between nitrous oxide and DEA rate with actinomycete markers 10Me17:0 and 10Me18:0 are of particular interest. Studies have demonstrated that the actinomycetes are capable of nitrous oxide production (Kumon et al. 2002), and form a sizeable portion of the soil microbiota (Janssen 2006). In this study these two actinomycete markers account for an average of 30 % of the total actinomycete PLFA. It is possible that in this system, these markers are indicative of a subset of actinomycetes responding to environmental factors which likewise result in depressed nitrous oxide emissions. Future efforts to identify the actinomycete species associated with these PLFA markers, through deep 16S rDNA sequencing of environmental samples, could be undertaken with the understanding that these organisms may serve as biomarkers to indicate soils with the potential for lower nitrous oxide emissions.

While it is possible to find relationships between broad microbial groups, N_2O emissions, and DEA rates, only a portion of the overall microbial community carries genes responsible for each step in the N-cycle. Therefore we used quantitative Real-Time PCR (qPCR) to better understand the effect that the abundance of these genes might have on nitrous oxide production. Accordingly, we looked at the following genes involved in N-cycling: 1) the nitrogenase encoding *nifH*; 2) the ammonia monooxygenase structural gene *amoA*; 3) the nitrite reductase genes *nirK* and *nirS*; and 4) the nitrous oxide reductase gene *nosZ*.

Examination of the temporal and spatial patterns of the microbial 16S rDNA and N-cycling genes paints a complex picture. From a temporal standpoint, significant differences based on month were found for both the 16S rDNA gene ($p = 0.05$) and *amoA* ($p = 0.006$), which had higher abundances in November as opposed to July. Likewise, with the exception of *nifH*, all genes showed a statistically significant yearly difference ($p < 0.03$) with 16S and *nosZ* significantly higher in the studies first year and *amoA*, *nirK*, and *nirS* all significantly higher in the second year of the study. However spatially, *nifH* gene abundances were significantly lower at the edge of the field ($p = 0.01$) as compared to within the bays (Fig. 3).

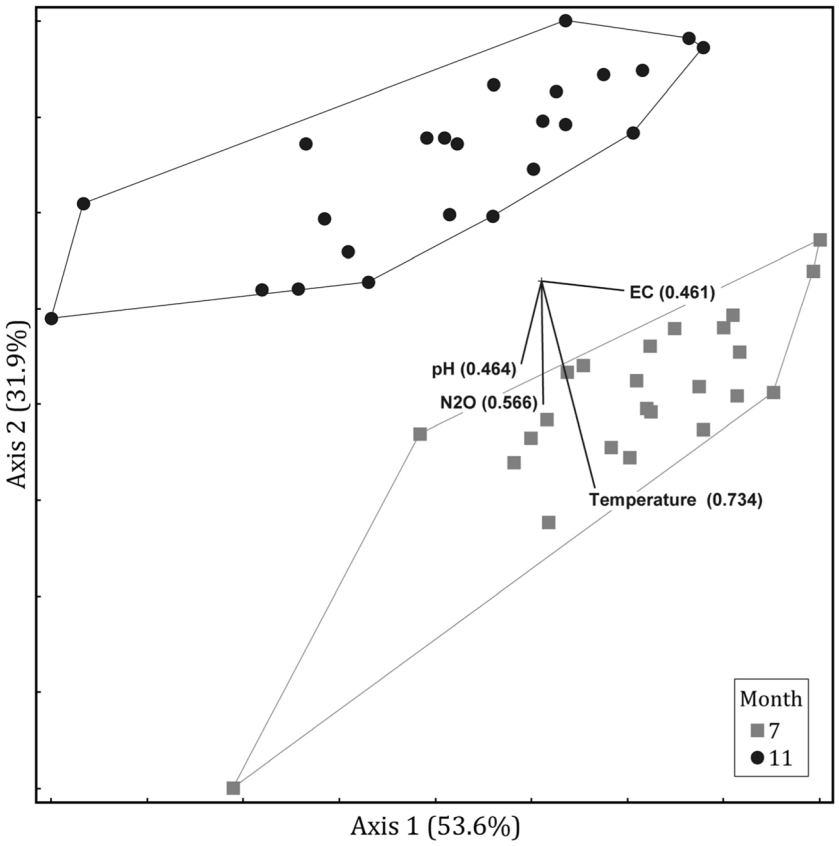

Fig. 2 NMS ordination of soil samples based on % PLFA, grouped by sampling month. Significant factors for variation extracted from environmental data are given as vectors, with correlation to ordination axes given in brackets

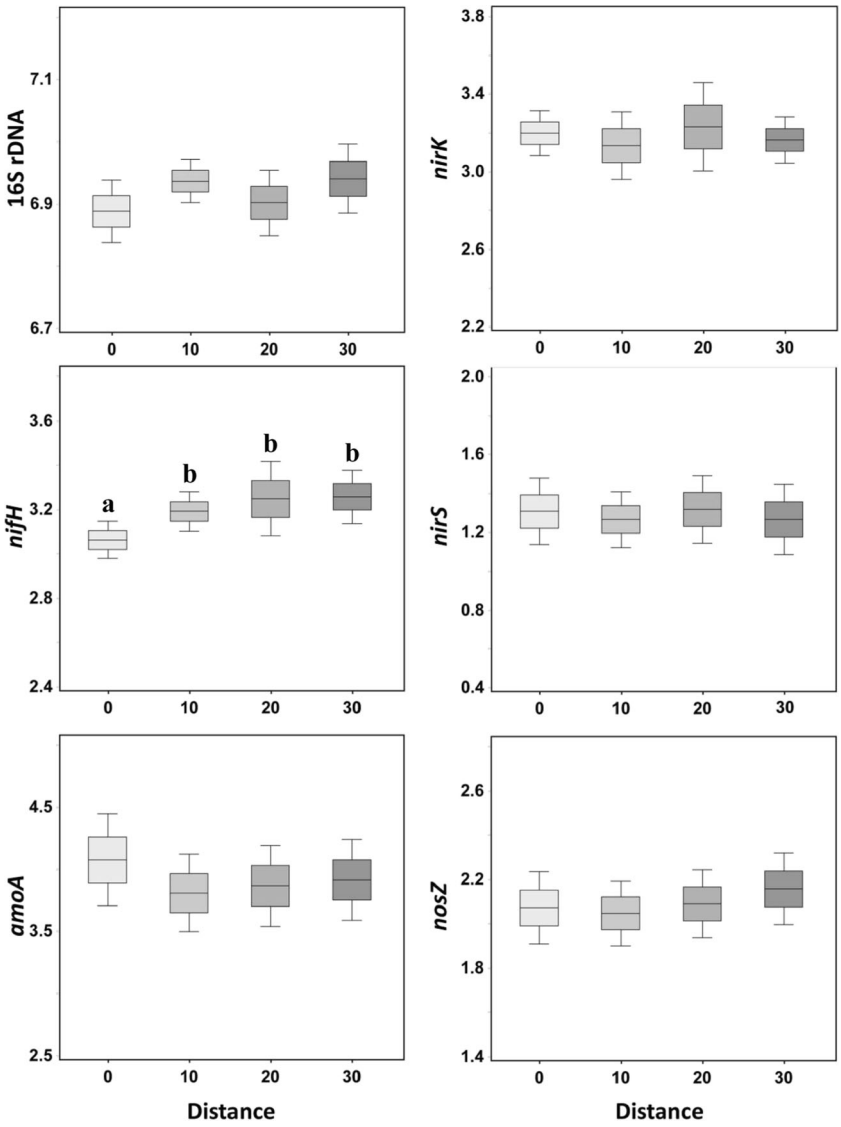

Fig. 3 Spatial representation based on distance, of gene abundances as determined by qPCR. X axis represents log transformed gene copy numbers per μg DNA, while Y axis represents distance from agricultural field in meters. Only *nifH* demonstrated significant differences upon spatial analysis, and statistically different samples ($P < 0.05$) are indicated by letter

This may be indicative of a reduced need for microbial nitrogen fixation in the agricultural fields due to N fertilization (Romero et al. 2012). Comparisons of the relative abundances of all N-cycling genes, with environmental variables, nitrous oxide emissions, and DEA rates show several significant relationships (Table 7). Negative correlations between the *amoA*, *nirS,* and *nosZ* genes with C, N, and C/N ratio were identified. Since the organisms which carry bacterial *amoA* are obligate autotrophs, this negative relationship may be indicative of the inability of these organisms to compete with heterotrophs in high carbon environments (Nogueira et al. 2002). It is unlikely that microbial consumption can be implicated in the negative

Table 7 Correlations between gene abundances, environmental factors, and nitrous oxide production

	C	N	C:N	Moisture	Temp	EC	pH	deaA	deaB	deaC	deaD	CO_2	N_2O
16S	ns[†]	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
amoA	−0.42 ***	−0.46 ***	−0.49 ***	−0.41 ***	ns	ns	0.40 ***	ns	ns	ns	ns	ns	ns
nifH	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
nirS	−0.43 ***	−0.46 ****	−0.48 ***	−0.40 ***	ns	ns	0.49 ***	ns	ns	ns	ns	ns	ns
nirK	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
nosZ	−0.35 **	−0.38 ***	−0.45 ***	ns	ns	ns	0.53 ****	ns	ns	ns	ns	ns	ns

[†] = significance indicated by * = 0.1, ** = 0.05, *** = 0.01, **** = 0.0001, ns = not significant

relationship between C and N with *nirS* and *nosZ*. In fact, two recent studies, performed from soil collected in agriculture-purposed fields, have documented either steady state or increased denitrifier gene abundances with C (Miller et al. 2009; Henderson et al. 2010). It should be noted however that both amount and type of organic carbon play a role in this relationship, making it possible that the available organic carbon found in the forested Carolina Bays was not as conducive to denitrifier growth. Additionally, while no genes demonstrated a correlation between their relative abundance and DEA rates or greenhouse gas emissions, *nosZ* relative abundances did correlate ($r = 0.49$; $p = 0.0003$) with the ratio of N_2O / N_2 + N_2O. Similar measures have been reported previously in other studies (Philippot et al. 2009; Ducey et al. 2011).

Conclusions

This study was performed to examine if Bays adjacent to agricultural production served as denitrification sites for agricultural runoff. However, over the 2 year period of this study there were no differences in N_2O or CO_2 emissions by distance into Carolina Bays, possibly due to dry conditions. In a normal year, water table levels should have been closer to the surface in May and November.

As expected, N_2O and CO_2 emissions were greater in warmer summer months than in the fall. Compared to soil physical and chemical properties, temperature had a much greater effect on gas emissions. Although conditions in 2011 were drier than normal, it still appears that moisture content is a dominant factor at these sites. Even with lower C/N ratios at the field edge, DEA potential was greater deeper into the Bays, where greater soil moisture was also observed.

Distance from the field edge also had an effect on bacterial and fungal populations, where Gram+, Gram- and fungal population PLFAs were greater at the field/Bay boundary. Only actinomycetes correlated with N_2O emissions, and these populations may be an indicator of lower emissions in some soils. Nitrogen fixation was also lower at the bay/field boundary, where the *nifH* gene was observed to be significantly lower. This is potentially due to the more abundant N from manure application in these soils.

There appears to be very little effect on N_2O emissions from Carolina Bays adjacent to agricultural fields receiving manure applications. This may indicate that little runoff is occurring at the field edge, although subsurface drainage may pose more of a factor. Subsurface drainage can bypass typical denitrification pathways (Denver et al. 2014). The greater DEA response to NO_3 additions towards the middle of the Bay does indicate their ability to filter runoff from high rainfall events.

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