ORIGINAL RESEARCH

Denitrification Potential in Geographically Isolated Wetlands of North Carolina and Florida, USA

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Abstract Denitrification dynamics in "geographically isolated wetlands" (GIWs) may provide a link between GIWs and aquatic systems by converting N to other end products (e.g., N_2, N_2O, etc.), protecting downstream waters from excessive N. We compared GIW ambient and amended denitrification rates and soil/water covariate relationships in areas of two ecoregions. The average unamended denitrification rate was 6.89 ± 5.02 (range: 1.67–18.91) µg N kg DW^{-1} (dry weight) hr^{-1}, and no ecoregional differences were found. Areal calculations were 0.010–0.356 g N m^{-2} day^{-1}. Carbon amended denitrification samples decreased -18 %, while samples amended with N or N+C averaged 2730–3675 % above background levels; N+C rates were tested and did not differ between ecoregions. DW denitrification rates were correlated with soil covariates NH_4, %N, and %C while ash-free DW samples were also correlated with soil TP and water TN. A tree-based classification grouped GIWs based on soil NH_4 values, though the results were not conclusive. The findings suggest that GIWs embedded in areas with substantial loadings of N and P and ample C (e.g., agricultural land uses) may limit exposure of other waters to N pollution.

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Introduction

"Geographically isolated wetlands" (GIWs) lack surface water connection to other waterbodies and are typically defined as wholly surrounded by uplands (Tiner 2003a), though this definition incompletely describes the ecological connectivity and functional gradient. At a finer scale, determining adjacency, connectivity, and subsequently relative isolation may be an imposing task due to intermittent surficial connectivity (e.g., Leibowitz and Vining 2003; Rains et al. 2006; Wilcox et al. 2011; Lang et al. 2012), groundwater and/or hydraulic connectivity (e.g., McLaughlin et al. 2014), biological connectivity (e.g., Subalusky et al. 2009), and biogeochemical connectivity (e.g., Creed et al. 2003). GIWs are located throughout the U.S. with higher densities found in certain areas such as near-surface karst geological areas of the southeast, playas in the southwest, and woodland vernal pools in the New England region (see Tiner 2003b). Likens et al. (2000) estimate that up to 20 % of wetland area in the contiguous U.S. may be considered GIWs, and Lane et al. (2012) reported approximately 9 %, almost 1.2 million ha, of freshwater wetlands in an eight-state area of the southeast as potential GIWs. No national map of putative GIW extent currently exists.

Most GIWs are currently not regulated under the Clean Water Act (CWA; Downing et al. 2003) due to perceived difficulties associated with quantifying the effect of GIWs on navigable waters. As a result of a 2001 U.S. Supreme Court decision (Solid Waste Agency of Northern Cook County [SWANCC] vs. US Army Corps of Engineers, 531 U.S. 159), the presence of migratory birds by itself ceased to be a sufficient basis for the U.S. Army Corps of Engineers and the U.S. Environmental Protection to exert jurisdiction over

geographically isolated, non-navigable, intrastate wetlands under the CWA. In 2006, the U.S. Supreme Court further clarified the scope of federal jurisdiction (Rapanos v. United States, US 126 S. Ct. 2006; see Leibowitz et al. 2008). However, understanding the influence of GIWs as individual systems and/or as a class of aquatic systems on the integrity of downstream traditional navigable waters may inform the determination of the jurisdictional extent of the CWA for these wetland resources.

The processing of reactive nitrogen (Nr) may provide a key to understanding the influence of GIWs on downstream waters. Reactive nitrogen includes all, "…biologically active, photochemically reactive, and radiatively active N compounds in the atmosphere and biosphere…[including] inorganic reduced forms of N (e.g., NH_3, NH_4^+), inorganic oxidized forms (e.g., NO_x, HNO_3, N_2O, NO_3^-), and organic compounds (e.g., urea, amines, proteins)" (Galloway and Cowling 2002, p. 71). Since the 1860s, anthropogenic Nr creation increased from 15 Tg N per year to over 165 Tg N by 2000 (Galloway and Cowling 2002). Approximately half of the Nr fertilizer created worldwide for agricultural purposes is incorporated into crops, while the remainder is lost to the atmosphere (e.g., NH_3, NO, N_2O, N_2) or transferred to aquatic systems, primarily as NO_3^- (Galloway et al. 2003). The Nr in aquatic ecosystems can impair system condition and function (Nixon 1995; Rabalais et al. 2002), while some forms of Nr may also affect human health through drinking water contamination (e.g., methemoglobinemia, also known as blue-baby syndrome, and certain gastric cancers; see Curşeu et al. 2011; but see also van Grinsven et al. 2006).

Due to frequent anoxic conditions, high concentrations of labile carbon, and the presence of facultative microbes and available nitrogen sources, wetlands can be a significant sink for Nr, with denitrification a major pathway for transformations that result in the release of N_2 (Reddy and DeLaune 2008) and N_2O (an important greenhouse gas; U.S. EPA 2010). In a meta-analysis, Jordan et al. (2011) reported approximately 20 % of the Nr load reaching wetlands in the contiguous U.S. was removed through plant uptake, denitrification, absorption, burial, and anaerobic ammonium oxidation. Wetland type (e.g., palustrine forested, palustrine emergent marsh) affects nitrogen removal determinants such as carbon quality and microbial composition (Boon 1991). Ullah and Faulkner (2006) found nitrate amended and control palustrine forested wetlands had significantly higher potential denitrification rates than palustrine herbaceous submergent/emergent systems, while conversely Dodla et al. (2008) reported significantly higher potential denitrification rates in non-amended freshwater palustrine marsh systems than palustrine forested wetlands or saline (i.e., estuarine) marshes, suggesting that finer wetland classification (e.g., Comer et al. 2003) may be necessary to better quantify denitrification rates at landscape scales. Similarly, wetland spatial

location vis-à-vis sources of nitrogen-laden overland flow or groundwater and/or atmospheric deposition affects the availability of Nr for denitrification or immobilization (Rheinhardt et al. 2002; Ullah and Faulkner 2006; Jordan et al. 2007; Racchetti et al. 2011). (Note that where appropriate, all literature values of N_2O have been converted to N to facilitate comparisons.)

While some GIWs may have lost CWA protection (Leibowitz et al. 2008), they are nevertheless significant landscape elements with characteristics conducive to denitrification (Neely and Baker 1989; Forman 1995; McComb and Qiu 1998). However, we currently have limited knowledge of their contribution to landscape nutrient dynamics especially as related to N removal via denitrification (Galloway et al. 2003). Understanding how GIWs affect the integrity of lakes, rivers, and stream systems through denitrification processes can inform local and landscape-scale best management practices and land use decisions. In this study we assessed base denitrification rates and rates when carbon and nitrogen were not limited (i.e., amended rates) in select GIWs in two ecoregions to contrast between ecoregions and to identify major determinants of GIW denitrification.
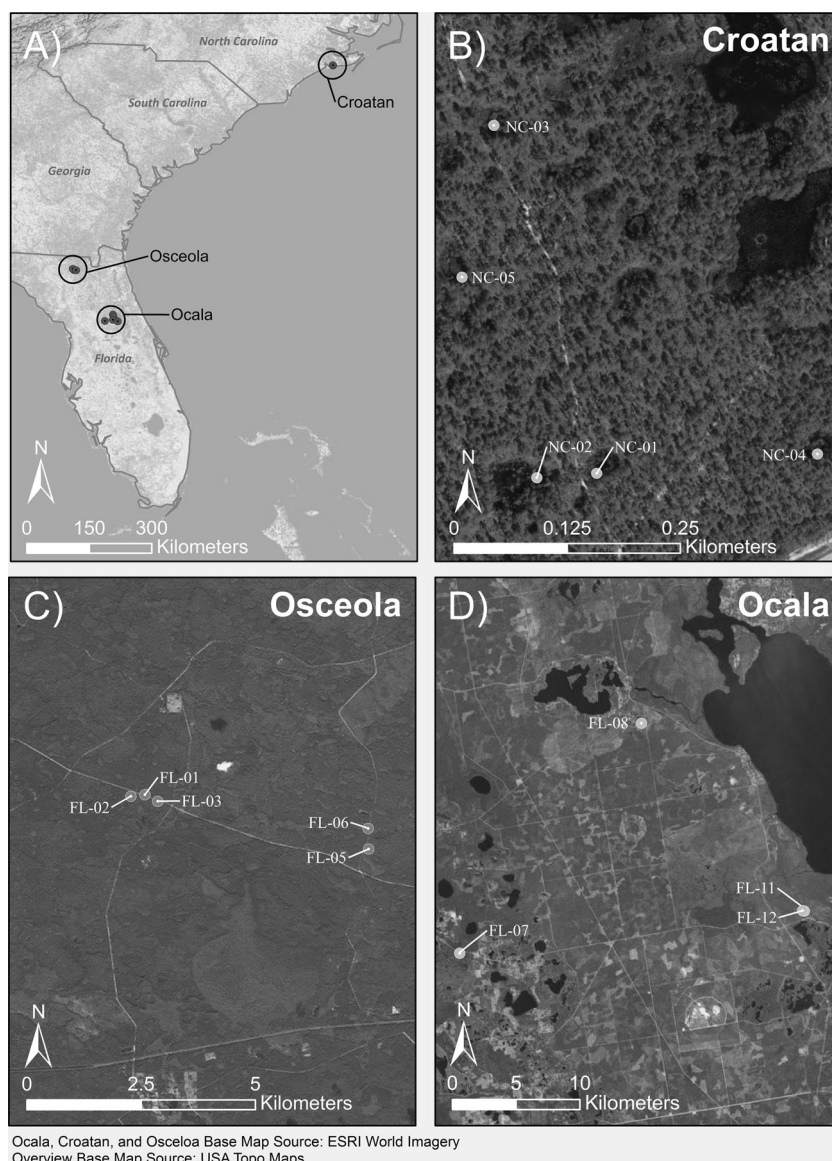
Methods

Soil and Water Collection and Processing

GIWs in the Middle Atlantic Coastal Plain of North Carolina ("North Carolina sites," $n=5$) and the Southern Coastal Plain of Florida ("Florida sites," $n=9$) were selected for this study based on site location, degree of isolation, and assumed reference condition (Fig. 1, Omernik 1987; Reiss et al. 2010). Study sites averaged 1.68 ha (±3.10 ha standard deviation (SD), range 0.03 to 12.25 ha) and were located in protected lands (i.e., Croatan National Forest in North Carolina; Osceola and Ocala National Forests in Florida) without any obvious sources of nitrogen pollution outside of unmeasured atmospheric wet and dry deposition.

A single composite soil sample at each site was collected from a series of six subsamples, three collected from within a 3.1 m^2 circle in the interior of the wetland, and three collected from a 3.1 m^2 circle in the exterior portion of the wetland, approximately half-way between the interior and exterior of the site. A stainless-steel coring device (inner diameter 7.5 cm) was inserted approximately 10 cm into the soil surface and extracted. Any surface water atop the core was gently poured off. If present, coarse plant material such as roots and leaves were removed from the subsamples, which were homogenized on-site with a clean hand trowel. Approximately 1/6 (~83 cm^3) of each homogenized sample was collected and a composite sample created. The labeled sample was double

Fig. 1 **a** to **d**. Site locations (*n*=14) across the **a** southeastern US, **b** the Croatan National Forest in North Carolina, and **c** the Osceola National Forest and **d** Ocala National Forest in Florida

Ocala, Croatan, and Osceloa Base Map Source: ESRI World Imagery
Overview Base Map Source: USA Topo Maps

bagged, placed on wet ice and shipped overnight to the EPA's National Health and Environmental Effects Research Laboratory in Duluth, MN, for analyses described below.

Soil samples were analyzed for physical and chemical parameters using standard methods including: soil moisture (dry weight (g)), NO_3 (mg/kg), NH_4 (mg/kg), %N, %C, C:N, total phosphorus (soil TP, mg/kg), pH, and conductivity (μS/cm). All nutrient samples were analyzed using a Lachat flow-injection analyzer with appropriate listed QuikChem methods (Lachat Instruments, Loveland, CO, USA). Field moist sub-samples were extracted with 2 M potassium chloride (KCl) for available NO_3^- and NH_4^+ according to Keeney and Nelson (1986); extracts were analyzed using the cadmium reduction and phenolate method (APHA 1998), respectively. Soil water content was determined by gravimetric methods using a drying oven. The percent solids were used to calculate available

nutrient content on a dry weight basis. Soil samples were dried and ground for TC, TN, and TP analyses. The TC and TN were analyzed by combustion using a 1112 EA Carlo Erba elemental analyzer; for TP determinations, samples were first digested in reagent grade concentrated nitric acid (HNO_3) using a CEM Corporation microwave, then neutralized with sodium hydroxide (NaOH) and analyzed by the molybdate-ascorbic acid method (APHA 1998). The pH and conductivity were measured by direct soil probes (ph meter by IQ Scientific Instruments, California, USA; HI92331 Soil Conductivity and Temperature meter by Hanna Instruments, Rhode Island, USA). We collected an additional single 30 cm core from each wetland and measured the average bulk density (g/cm^3) in 2 cm increments over the top 10 cm of the core. These values were collected by cutting the frozen samples, drying the two cm slices 48–72 h and weighing the air-dried samples.

Four L of site water (>10 cm in depth) were collected for use in denitrification analyses and placed on ice. In addition, a composite 1 L of site water was collected from ten 100 ml subsamples taken from throughout the wetted portions (>10 cm in depth) of each wetland and similarly stored on ice. The water samples were shipped along with the soils overnight to the laboratory for analyses that included: total (TOC, mg/L) and dissolved organic carbon (DOC in mg/L), color (PTU), pH, alkalinity (mg/L CaCO$_3$), total phosphorus (TP, µg/L), total nitrogen (TN, µg/L), orthophosphate (µg/L), NH$_4^-$ (µg/L), NO$_x$ (µg/L), and dissolved inorganic nitrogen (DIN, µg/L). TOC and DOC were preserved with H$_2$PO$_4$ and measured by UV-sodium persulfate oxidation and non-dispersive infrared (NDIR) detection with the Dohrmann Phenix 8000 TOC analyzer (5310-C; APHA 1998). Color was measured with a Perkin Elmer UV–vis Model 20S spectrometer (Perkin Elmer, Waltham, MA, USA) and alkalinity following EPA Method 130.2 (US EPA 1978). Unfiltered and filtered (0.45 µm pore membrane) samples for N- and P-species were preserved frozen. Nutrient samples were analyzed using a Lachat flow-injection analyzer 8000 with QuickChem methods (Lachat Instruments, Loveland, CO, USA). The unfiltered subsamples were digested using the persulfate method for TN and TP (APHA 1998). TP was determined by the molybdate-ascorbic acid method (10-115-01-1-B). TN and dissolved NOx-N were analyzed using the cadmium reduction method (10-107-04-1O), and NH$_4$-N was analyzed with the phenolate method (10-107-06-1-F).

Denitrification Measurements

Denitrification potential was measured using the chloramphenicol-amended acetylene block method (Smith and Tiedje 1979; Arango et al. 2007). We measured denitrification by dry weight (DW, g N kg DW^{-1} h^{-1}) and as ash-free dry weight (AFDW, g N kg AFDW^{-1} h^{-1}) to account for the influence of organic matter on denitrification kinetics. We added 30 mL of soil to 50 mL of unfiltered site water with chloramphenicol at a final concentration of 0.3 mM in a 75 mL slurry. The sample bottles were sealed with septum caps for headspace sampling and purged with ultrahigh-purity N$_2$ for 5 min, shaking periodically to induce anoxia. After purging, the bottles were returned to ambient atmospheric pressure and 10 mL of C$_2$H$_2$ were added to achieve a 1:5 atmosphere of C$_2$H$_2$ in the assay bottle. The bottles were shaken for several seconds to equilibrate dissolved gases with the headspace before collecting gas samples. A 10 mL headspace subsample was then removed after 15 min and injected into a 10 mL evacuated vial for N$_2$O analysis. Constant pressure was maintained in the assay bottles by replacing each headspace subsample with 10 mL of 1:5 C$_2$H$_2$. Additional headspace gas samples were collected after 1, 2, 3, and 4 h. Headspace N$_2$O concentrations were injected into an Agilent

HP 5890A gas chromatograph with a Porapak Q column and electron capture detector with the following settings: inlet temperature 55 C^o, oven temperature 65 C^o, detector temperature 270 C^o, with a 5 % CH$_4$/95 % Ar carrier gas at 30 mL min^{-1}. The headspace autosampler settings were: oven temperature 40 C^o, sample loop temperature 45 C^o, and transfer line temperature 50 C^o. Bunsen coefficients were used to calculate total N$_2$O produced in the bottle to plot N$_2$O production against time. The N$_2$O production rates were calculated as the slope of the regression line. Denitrification rates were determined by dividing the N$_2$O production rate by the mass of sediment in the assay bottle and assay duration and converted to nitrogen. Incubations were conducted at room temperature to minimize variability not associated with soil characteristics. As we were interested in both ambient and amended denitrification measures, we amended Florida samples with N (NO$_3$-N as KNO$_3^-$ at 6 mg N L^{-1} above ambient levels), C (as glucose at 30 mg C L^{-1} above background), and both N+C (Inwood et al. 2005; Beaulieu et al. 2009) and analyzed these for denitrification as noted above. Due to sample volume limitations we were only able to amend North Carolina samples with N+C. Duplicates of seven assays, including ambient and amended measures, were assessed for sample accuracy.

Statistical Analyses

We calculated the non-parametric Wilcoxon Mann–Whitney statistic to test for ecoregional differences between ambient and amended denitrification rates and soil and water parameter concentrations. Bivariate linear correlations amongst all measured variables were tested to look for redundant variables and to explore factors affecting denitrification rates. We removed redundant variables from subsequent multivariate analysis to limit the emergence of spurious relationships with our small data set. Duplicate assays were assessed using student's t-test. The above analyses were conducted using SAS (SAS Institute, Cary, NC, version 9.2).

Conditional inference (CI) trees were grown to explore the relationships between denitrification rates and measured soil and water variables using the "ctree" function in the package "party" in R (R version 3.0.1, "party" version 1.0-9; Hothorn et al. 2006). Much like classification and regression trees (e.g., "CART" [Breiman et al. 1984]), CI trees are tree-based non-parametric regression algorithms that recursively partition the dataset exploring all bifurcation permutations to identify covariates that optimize the best split between nodes (see Hothorn et al. 2006 for additional information). As our study was limited to a small number of sites, we relaxed the minimum group membership number and terminal node weight to zero and established a minimum split criterion of 0.20.

Results

Measured denitrification rates for each composite averaged 6.89 ± 5.02 μg N kg dry weight^{-1} h^{-1} (DW denitrification range: 1.67–18.91 μg N kg dry weight^{-1} h^{-1}; Table 1). The AFDW rates averaged 23.60 ± 52.72 μg N kg AFDW^{-1} h^{-1}, ranging from 2.00 to 204.15 μg N kg AFDW^{-1} h^{-1}. There were no significant differences in denitrification rates between duplicate samples ($p=0.6742$). Average base DW denitrification rates increased when amended with N and N+C, though they decreased when amended only with C. This held for both DW and AFDW measures, though the greatest change in AFDW results was when samples were only amended with N. Differences in soil measures ($n=14$, Table 2) were found for conductivity ($p=0.0092$) and bulk density ($p=0.0136$), both greater in North Carolina sites, while significantly greater values for water variables (Table 3) were found in Florida sites for pH ($p=0.0268$) and alkalinity ($p=0.0388$). Wilcoxon Mann–Whitney U-tests indicated no differences between ecoregions for neither ambient DW ($p=0.3861$) or AFDW ($p=0.4634$) nor C+N amended DW ($p=0.1252$) or AFDW ($p=0.3173$).

Many soil (Table 4) and water variables (Table 5) were strongly correlated with each other. DW and AFDW denitrification measures were significantly correlated with soil NH$_4$ (as well as %N and %C, while AFDW rates were also correlated with TN in the water column and TP in the soil matrix. For the conditional inference tree analyses we maintained water DIN (not NH$_4^-$), pH (not alkalinity), DOC (not color or TOC), and soil C:N (not %N or %C) as variables, selecting amongst correlated variables expected to impact denitrification rates, those more commonly measured, and the more inclusive variables (e.g., DIN).

The conditional inference bifurcation to classify GIW denitrification rates was not significant for either DW ($p=0.07$) or AFDW ($p=0.19$). We present the results for exploratory purposes since the statistical significance of the DW conditional inference tree was marginal and the AFDW tree contained the same sites and bifurcation (data not shown), despite a relatively small final sample size ($n=14$). A conditional inference split occurred for the DW and AFDW measures depending on the availability of soil NH$_4$, with bifurcation occurring at measured soil NH$_4 \leq 14.329$ mg/kg (figure not shown). Low denitrification sites ($n=12$, average DW denitrification rate 5.49 ± 3.51 μg N kg DW^{-1} h^{-1}) predominated, with denitrification ranging from 1.97 to 13.72 μg N kg DW^{-1} h^{-1}. Only two sites were identified in the relatively high-denitrification branch (average 15.31 μg N kg DW^{-1} h^{-1}), and the denitrification range overlapped that of the first group (11.71–18.91 μg N kg DW^{-1} h^{-1}). (Note that conditional inference trees were also grown using all variables, including highly correlated variables, with no difference with the DW tree grown using the parsimonious suite of variables and no successful tree grown using the AFDW data [not shown]).

Discussion

Denitrification Rates

In this study we assessed the ambient (i.e., unamended) and amended denitrification rates in a small number of select palustrine GIWs from two ecoregions and explored major determinants of denitrification rates. Though a small study, ambient denitrification rates reported herein (6.89 μg N kg DW^{-1} h^{-1}) fall within the range of studies reported for other wetland systems, though the range varied one to two orders of magnitude across the 14 sites analyzed (see Table 1). For instance, in a critical review Johnson (1991, p. 539) reported a denitrification range for unamended wetland samples from multiple studies and wetland types from 4.17 to 667 μg N kg DW^{-1} h^{-1}. Dodla et al. (2008) assessed DW denitrification in 25 cm deep sediment cores in freshwater forested and emergent marsh wetlands in Louisiana, reporting average ambient rates of 3.75 μg N kg DW hr^{-1} and 19.58 μg N kg DW hr^{-1}, respectively. Marton et al. (2014) found Indiana depressional wetlands sampled to depths of 5 cm had ambient denitrification rates of 88.8 μg N kg DW^{-1} h^{-1}. These results suggest that in addition to a host of habitat and hydrologic functions performed by GIWs (e.g., Gibbons et al. 2006; Pomeroy et al. 2014), denitrification services provide a pathway to improved water quality and downstream system integrity, assuming the maintenance of conditions conducive to nutrient assimilation.

As with many other wetland studies, our study demonstrated much greater potential denitrification rates when N limitations were removed. Amended samples varied greatly, up to three orders of magnitude in change, depending on whether the sample was amended with N, C, or N+C. N amendments increased denitrification rates on average>2730 %, while samples amended with both N and C increased>3675 %, suggesting carbon limitations may occur at excessive N loadings. Carbon amendments alone decreased denitrification rates by −18 %. Amended values (typically NO$_3$, but the amendment varied by study) reported by Johnson (1991) ranged from 0.417 to 22,208 μg N kg DW^{-1} h^{-1}; the mean denitrification rate in two different restored wetland systems in North Carolina amended with N and C were 195.97 and 541.75 μg N kg DW^{-1} h^{-1} (Sutton-Grier et al. 2010). A riparian wetland amended with both C and N was reported to have potential denitrification rates of 779.17–845.83 μg N kg DW^{-1} h^{-1} (Muñoz-Leoz et al. 2011). Wetland cores were amended by Dodla et al. (2008) and average denitrification rates were reported as 64.58 μg N kg soil hr^{-1} (amended with 2 mg L^{-1} NO$_3^-$ - N) and 307.92 μg N kg soil hr^{-1} (amended with 10 mg L^{-1} NO$_3^-$ - N) for forested systems and

Table 1 Ambient and amended dry weight (DW) and ash-free dry weight (AFDW) denitrification rates for sampled sites

Site name	Wetland type	Ambient		N Amended		C Amended		N+C Amended	
		μg N kg DW⁻¹ h⁻¹	μg N kg AFDW⁻¹ h⁻¹	μg N kg DW⁻¹ h⁻¹	μg N kg AFDW⁻¹ h⁻¹	μg N kg DW⁻¹ h⁻¹	μg N kg AFDW⁻¹ h⁻¹	μg N kg DW⁻¹ h⁻¹	μg N kg AFDW⁻¹ h⁻¹
FL-01	Forested	5.26	6.18	16.25	19.09	12.01	14.11	36.45	42.82
FL-02	Forested	2.54	3.63	147.65	211.14	3.86	5.52	178.15	254.75
FL-03	Forested	1.67	2.00	73.65	88.28	2.29	2.74	103.08	123.56
FL-05	Forested	4.11	4.72	74.36	85.36	3.39	3.89	79.97	91.79
FL-06	Forested	1.97	2.41	102.85	126.35	1.91	2.35	124.84	153.36
FL-07	Emergent marsh	11.71	36.51	691.29	2156.24	9.51	29.66	770.86	2404.44
FL-08	Emergent marsh	18.91	204.15	417.19	4503.16	11.93	128.81	142.05	1533.31
FL-11	Emergent marsh	5.09	5.99	115.17	135.62	3.81	4.49	208.37	245.37
FL-12	Emergent marsh	9.60	11.55	54.91	66.04	2.44	2.94	127.17	152.93
NC-01	Emergent marsh	2.64	3.42	nd	nd	nd	nd	186.94	242.66
NC-02	Emergent marsh	13.72	16.09	nd	nd	nd	nd	141.15	165.59
NC-03	Emergent marsh	5.27	10.73	nd	nd	nd	nd	964.16	1961.62
NC-04	Emergent marsh	7.51	13.88	nd	nd	nd	nd	351.84	649.79
NC-05	Emergent marsh	6.47	9.13	nd	nd	nd	nd	130.32	183.89
	Average	6.89	23.60	188.15	821.25	5.68	21.61	253.24	586.13
	Standard deviation	5.02	52.72	221.70	1539.34	4.216	41.16	272.75	780.06

Notes: *nd*, no data collected

Table 2 Measured soil characteristics for each site sampled

Site name	NO$_3$ (mg kg^{-1})	NH$_4$ (mg kg^{-1})	%N	%C	C:N	TP (mg kg^{-1})	pH	Specific conductivity (μS cm^{-1})	Bulk density (g cm^{-3})
FL-01	1.440	4.357	0.156	6.073	38.850	101.216	3.17	0.060	0.825
FL-02	1.852	5.622	0.489	13.314	27.231	265.455	3.31	0.050	0.812
FL-03	1.402	4.345	0.246	7.095	28.876	122.786	4.24	0.030	0.490
FL-05	0.495	3.327	0.204	8.463	41.486	100.180	3.39	0.040	0.467
FL-06	1.048	5.371	0.319	12.035	37.682	125.168	3.37	0.060	0.479
FL-07	1.011	54.296	1.895	40.371	21.310	521.773	3.47	0.110	0.196
FL-08	2.836	73.003	3.628	54.928	15.141	413.410	4.00	0.045	0.181
FL-11	0.588	14.329	0.188	4.154	22.106	71.870	4.38	0.030	0.292
FL-12	0.657	5.904	0.099	3.503	35.512	88.183	4.08	0.015	0.123
NC-01	0.918	7.361	0.210	8.384	39.939	85.859	3.65	0.107	1.183
NC-02	1.567	2.357	0.152	4.680	30.760	67.965	3.67	0.112	0.923
NC-03	2.152	5.904	1.585	30.190	19.049	310.490	3.72	0.100	0.879
NC-04	8.273	9.768	0.918	17.857	19.444	184.205	4.00	0.118	0.724
NC-05	1.030	8.218	0.389	7.596	19.501	95.250	4.11	0.090	0.616
Average	1.805	14.583	0.748	15.617	28.349	182.415	3.75	0.069	0.585
Standard deviation	1.971	21.317	1.000	15.499	9.072	142.271	0.38	0.036	0.320

770 μg N kg soil hr^{-1} (2 mg L^{-1}) and 1005 μg N kg soil hr^{-1} (10 mg L^{-1}) for freshwater marshes. Marton et al. (2014) reported amended samples from Indiana depressions were four times higher (329.3 μg N kg DW^{-1} h^{-1}) than unamended samples. Ullah and Faulkner (2006) showed lower Mississippi River floodplain wetland soils amended with

nitrate had 55–67 % greater potential denitrification rates than unamended soils. The repeated wet/dry cycling in GIWs facilitates carbon breakdown rates and may create a carbon-rich environment beneficial to denitrifying microbes such that when presented with abundant nitrogen sources excessive denitrification may occur (Winter and LaBaugh 2003; Reddy

Table 3 Measured site water characteristics

Site name	NH$_4$ (μg L^{-1})	NOx (μg L^{-1})	DIN (μg L^{-1})	PO$_4$ (μg L^{-1})	TP (μg L^{-1})	TN (μg L^{-1})	ALK (mg L^{-1} CaCO$_3$)	pH	CLR (PTU)	DOC (mg L^{-1})	TOC (mg L^{-1})
FL-01	21.9	15.7	37.6	8.5	17.2	1349.8	0.5	4.1	818.8	34.8	38.2
FL-02	33.5	4.3	37.8	9.5	43.5	1010.0	bdl	4.0	759.0	32.9	35.4
FL-03	30.8	16.3	47.1	11.6	22.0	1313.8	19.6	5.9	1075.6	46.3	48.7
FL-05	26.9	3.5	30.3	19.8	38.5	1261.0	1.4	4.2	746.0	28.6	31.4
FL-06	16.6	bdl	16.6	8.1	32.2	851.9	bdl	4.0	1029.6	38.1	41.6
FL-07	135.3	5.7	141.0	4.5	57.0	1567.5	bdl	3.9	758.2	41.3	43.8
FL-08	60.2	3.8	63.9	8.1	5.6	2016.0	1.0	4.5	255.7	16.7	17.2
FL-11	44.0	3.3	47.3	8.5	35.1	767.5	0.9	4.9	265.7	12.1	12.3
FL-12	54.1	2.7	56.8	9.6	10.1	878.3	1.0	4.6	262.8	15.6	17.7
NC-01	29.1	2.4	31.5	9.4	12.7	1768.0	bdl	3.6	649.7	34.7	36.5
NC-02	24.8	4.3	29.1	8.2	49.2	1092.8	bdl	3.9	686.9	32.2	34.3
NC-03	31.9	7.4	39.3	9.0	15.7	1172.0	bdl	3.9	349.4	16.6	18.7
NC-04	18.1	4.6	22.8	8.5	10.8	1223.0	bdl	4.1	362.8	16.7	17.6
NC-05	14.9	6.1	21.0	7.5	6.0	1059.0	bdl	4.0	185.5	10.1	11.7
Average	38.7	5.7	44.4	9.3	25.4	1237.9	1.7	4.3	586.1	26.9	28.9
Standard deviation	30.9	4.7	30.9	3.4	17.0	353.3	5.2	0.6	300.3	11.9	12.6

Note: *bdl*, below detection limit (of 1.26 μg L^{-1} for NOx and 0.01 mg L^{-1} for alkalinity)

Table 4 Pearson correlation matrix for soil variables sampled at each site

Soil Chemistry Variable	NH$_4$ (mg kg^{-1})	%N	%C	C:N	TP (mg kg^{-1})	pH	Specific conductivity (µS cm^{-1})	Bulk density (g cm^{-3})	µg N kg DW^{-1} h^{-1}	µg N kg AFDW^{-1} h^{-1}
NO$_3$ (mg kg^{-1})	0.09	0.28	0.26	−0.44	0.18	0.15	0.42	0.16	0.18	0.17
NH$_4$ (mg kg^{-1})		0.90	0.89	−0.55	0.80	0.11	0.03	−0.54	0.73	0.87
%N			0.98	−0.66	0.84	0.08	0.14	−0.35	0.69	0.88
%C				−0.62	0.92	−0.04	0.21	−0.33	0.62	0.80
C:N					−0.59	−0.51	−0.25	0.26	−0.47	−0.48
TP (mg kg^{-1})						−0.17	0.24	−0.32	0.48	0.57
pH							−0.23	−0.36	0.18	0.17
Specific conductivity (µS cm^{-1})								0.57	0.16	−0.11
Bulk density (g cm^{-3})									−0.39	−0.41
µg N kg DW^{-1} h^{-1}										0.78

Note: Underlined correlations are significant at $p<0.05$

and DeLaune 2008). That the N and N+C amended values increased to such extent strongly suggests that non-sampled GIWs in similar biogeophysical settings (e.g., with sufficient labile carbon and anoxic conditions) may provide substantial denitrification when exposed to ample reactive nitrogen. These conditions are frequently found in agricultural settings, encouraging the maintenance of GIWs in these land use modalities to prevent nutrient loading of other aquatic systems.

Controls on Denitrification Rates

Denitrification is typically mediated by pH, temperature, anoxic conditions, carbon quality, microbial biomass, and available nitrogen. While temperature and oxygen levels were controlled in the laboratory, we anticipated pH to have a controlling effect on denitrification rates in this study, as pH was typically below 4.0 and studies have suggested low pH affects microbial denitrification (Morse et al. 2012). However, despite the acidic average soil pH (3.75±0.38) and water pH (4.24±0.58), pH was neither correlated nor a factor in the conditional inference tree, perhaps due to the narrow range of values across our limited number of sites. We further expected but did not find DOC to be correlated with denitrification, as Buford and Bremner (1975) (as noted by Reddy and DeLaune 2008) suggested a strong linear relationship between denitrification capacity and organic C. Our study did find DW and AFDW denitrification rates to be correlated with soil ammonium (discussed below), percent carbon (suggesting

Table 5 Pearson correlation matrix for water chemistry variables sampled at each site

Water Chemistry Variable	NOx (µg L^{-1})	DIN (µg L^{-1})	PO$_4$ (µg L^{-1})	TP (µg L^{-1})	TN (µg L^{-1})	ALK (mg L^{-1} CaCO$_3$)	pH	CLR PTU	DOC (mg L^{-1})	TOC (mg L^{-1})	µg N kg DW^{-1} h^{-1}	µg N kg AFDW^{-1} h^{-1}
NH$_4$ (µg L^{-1})	−0.07	0.99	−0.38	0.42	0.35	−0.07	−0.02	−0.03	0.18	0.16	0.47	0.33
NOx (µg L^{-1})		0.08	0.02	−0.15	0.16	0.64	0.46	0.33	0.35	0.35	−0.19	−0.13
DIN (µg L^{-1})			−0.38	0.39	0.37	0.03	0.05	0.02	0.23	0.22	0.44	0.31
PO$_4$ (µg L^{-1})				0.02	−0.06	0.25	0.24	0.21	0.06	0.08	−0.36	−0.19
TP (µg L^{-1})					−0.22	−0.07	−0.14	0.50	0.52	0.52	−0.03	−0.26
TN (µg L^{-1})						0.07	−0.11	0.02	0.21	0.19	0.43	0.66
ALK (mg L^{-1} CaCO$_3$)							0.85	0.45	0.44	0.42	−0.28	−0.09
pH								0.13	0.09	0.07	−0.12	0.08
CLR (PTU)									0.95	0.96	−0.46	−0.34
DOC (mg L^{-1})										1.00	−0.31	−0.24
TOC (mg L^{-1})											−0.32	−0.26
µg N kg DW^{-1} h^{-1}												0.78

Note: Underlined correlations are significant at $p<0.05$

carbon forms other than dissolved are important mediators of denitrification), and percent nitrogen. The AFDW rates were also significantly correlated with water TN and soil TP. It was not surprising that TN, percent carbon, and percent nitrogen would be correlated with denitrification. However it was unexpected that TP was a correlated variable with AFDW denitrification. White and Reddy (2003) found TP to be a significant predictor of potential denitrification rates in the detrital and 0–10 cm soil profiles of Everglades' soil samples, but not in the lower profiles, concluding that P was a limiting variable in areas of high Nr concentrations. The GIWs in this study had no known direct nitrogen inputs outside of atmospheric deposition, the decomposition of organic matter, and surface water and ground water inputs (which can be a significant N source; Whitmire and Hamilton 2008), and amendments suggested strong N limitation. As P has an effect on the microbial biomass of the system, the correlation may reflect the abundance of microbial denitrifiers (Atkinson et al. 2011) or that GIWs are typically P (or N and P) limited (e.g., Craft and Casey 2000). That P was correlated with denitrification rates in this study further suggests that GIWs with additional P loading (e.g., those in agricultural landscapes, including cattle or crop land use modalities; Dunne et al. 2007) may serve as hot spots for nitrogen conversion to N_2 or as a source for N_2O, (e.g., Kerrn-Jespersen and Henze 1993; Kuba et al. 1993; but see White and Reddy 1999).

The conditional inference tree results were not significant (at $p < 0.05$) but the relative importance of soil ammonium in classifying site denitrification rates could be informative and perhaps useful in future modeling efforts. Organic matter decomposition is likely the main source of Nr in the study's GIWs located in relatively pristine settings, though atmospheric, groundwater, and occasional overland flows (which may bring substantial amounts of OM and N to the system) all contribute to the mass balance of N in the system. The organic N is used by soil microbes and mineralized to ammonium, which can be further, and rapidly, utilized by the microbial community for respiration as well as by macrophytes and periphyton as an N source. Internal cycling of nitrogen in GIWs is facilitated by the frequent wetting/drying of most GIWs, which can also provide soil conditions conducive to nitrification and increase the nitrate-nitrogen concentration. The two wetlands classified in the CI tree occurring with soil $NH_4 > 14.329$ were the largest and third largest wetlands sampled in this study, at 12.2 ha (FL-08) and 1.2 ha (FL-07). Though variable and also prone to completely drying (C. Lane, personal observation), these systems likely had more stable water levels than the smaller wetland systems sampled in this study and hence greater available OM for both mineralization and nitrification; these two wetlands had the highest %C rates of any site in this study (see Table 2). These sites were also amongst the deepest (maximum depths > 2 m, especially in potential alligator holes) and the most flocculent sites

sampled (C. Lane, personal observation). The abundant mucky soils in the larger and deeper wetlands – when compared with mixtures of coarse organic matter, sand, and abundant roots frequently observed throughout shallower and smaller sites – likely harbored abundant microbes and anoxic conditions conducive to microbial denitrification and harbingered the relatively high denitrification rates. These hypotheses suggest further understanding soil types within GIWs and in different subecoregions could improve our understanding of denitrification rates in wetland soils and further our ability to accurately model denitrification within wetland systems at the landscape scale.

Scaling Results to Areal Measures and Potential Effects

Determining areal denitrification rates with sufficient temporal and spatial resolution to capture intrasite as well as intersite variation is a goal to understanding nutrient processing in wetland systems. This information can furthermore be utilized to more accurately model wetland nutrient processing capacities and fluxes at multiple spatial scales. Using the average DW denitrification data from this study and the average site bulk density measure, we determined *ex post facto* to explore the hypothetical impact of GIWs at larger scales on landscape-level denitrification processing. To do so, we calculated areal denitrification capacity estimates using the following equation (modified from Belmont et al. 2009):

$$deN_{areal-cap} = (deN_{ambient})(0.10\ m)(BD) \tag{1}$$

where $deN_{areal-cap}$ is the denitrification capacity of GIWs (g m^{-2} day^{-1}), $deN_{ambient}$ is the average ambient denitrification rate (g N kg DW^{-1} day^{-1}), 0.10 m is the sampled top 10 cm of soil from the study sites and an area of high denitrification rates (White and Reddy 2003; Muñoz-Leoz et al. 2011), and BD is the average bulk density (kg m^{-3}). Based on the data from this study (average $deN_{ambient} = 0.000165$ g N kg DW^{-1} day^{-1}, average BD = 584.9 kg m^{-3}), the ambient areal capacity ($deN_{areal-cap}$) of GIWs was calculated to be 0.010 g N m^{-2} day^{-1}. This calculated result is within the range of reported areal values. For example, Olde Venterink et al. (2006) measured a range of ambient denitrification in floodplains of 0.008–0.015 g N m^{-2} day^{-1}, and in a review paper Johnson (1991) reported denitrification rate ranges from 0.0001 to 0.016 g m^{-2} day^{-1} for unamended soil samples (and < 0.0001–0.5330 g m^{-2} day^{-1} for amended samples, see below). This measure of areal capacity multiplied by the average area (15,900 m^{-2}) of the sites sampled resulted in the average denitrification capacity of 153.8 g N per GIW per day. Using the same equation, but the N+C amended denitrification rate from Table 1 converted to g N per kg DW^{-1} day^{-1}

(i.e., $deN_{N+C\ amended} = 0.006077$ g N kg DW^{-1} day^{-1}) resulted in an average denitrification capacity measurement of 5652.2 g N per day with an areal measurement of 0.356 g N m^{-2} day^{-1}. Further assuming that the ambient areal measures approximate the lower range and N+C amended areal denitrification rates approximate the higher range it is possible to extrapolate total denitrification – albeit from a small sample size – to a large area using Eq. 2:

$$deN_{cap} = (deN_{areal-cap})(\text{total area of potential GIWs}) \quad (2)$$

where deN_{cap} is the estimated total g N removed by GIWs, $deN_{areal-cap}$ is given above in Eq. 1, and (total area of potential GIWs) is in m^{-2}. Using the National Wetlands Inventory data and a 10-m buffer on National Hydrography Dataset features, Lane et al. (2012) calculated the abundance of potentially geographically isolated wetlands in an eight state region of the southeastern US (Alabama, Delaware, Florida, Georgia, Maryland, North Carolina, South Carolina, and Virginia) to be approximately 1.2 million ha. Applying Eq. 2 to the total extent from Lane et al. (2012) suggests that in total, potential GIWs of the southeastern US may remove between 0.1 and 4.2 million kg N day^{-1} (or between 0.04 and 1.5 million metric tons N yr^{-1}); potential GIWs in Florida alone (~535,000 ha) could remove between 0.05 and 1.9 million kg N day^{-1}, up to 694 million kg N yr^{-1} (or between 0.02 and 0.7 million metric tons N yr^{-1}). As noted, these conclusions are based on extrapolating from a small sample size and certainly warrant further exploration.

Wetland Type

While generalizations on wetland typology such as those found in Cowardin et al. (1979) may be useful in quantifying denitrification rates at larger scales, a given wetland classified as a palustrine forested system may have a substantial herbaceous layer or substantial vegetative heterogeneity. We attempted to minimize local effects by creating a composite sample of typical vegetation/habitats found in the center and intermediate portions of the wetland. We did not randomly select our sampling locations and it is possible that we inadvertently chose areas favorable to collecting a suitable core. The vegetative structures typifying classification regimes likely reflect, or are affected by, the determinant factors affecting microbial denitrification and will almost certainly vary by soil composition, latitude and ecoregion, prevailing wind conditions and atmospheric deposition of N, soil moisture and a host of additional site-specific characteristics (Cronk and Fennessy 2001; McClain et al. 2003). Approximately half of the wetlands sampled in Florida consisted of palustrine forested wetlands, while the other half and all of the North Carolina sites were palustrine emergent marsh systems (Cowardin et al.

1979; see Table 1). While not a goal of this study, we found that while ecoregion (i.e., state in the immediate study) did not affect the average denitrification rates, wetland type did affect rates (Wilcoxon Mann–Whitney test $Z = -2.6000$, $p = 0.0093$ for DW; $Z = -2.4667$, $p = 0.0136$ for AFDW). Ullah and Faulkner (2006) found significantly higher potential denitrification rates in forested wetlands, while Dodla et al. (2008) reported emergent marsh systems had five times the ambient denitrification rate of forested systems. In our study, DW denitrification rates in palustrine emergent marshes (8.99 ± 5.08 μg N kg DW^{-1} h^{-1}) were almost three times those in forested wetland systems (3.11 ± 1.53 μg N kg DW^{-1} h^{-1}), while average AFDW rates increased from 3.79 ± 1.71 μg N kg AFDW^{-1} h^{-1} in forested wetlands to 34.605 ± 64.289 μg N kg AFDW^{-1} h^{-1} in emergent marshes – perhaps because of increased carbon lability in emergent marsh macrophytes. The influence of wetland structure and its concomitant covariates, too, remains an active area for nitrogen processing research in all wetlands, not just in GIWs.

Limitations and Conclusions

This research provides supporting data on ecosystem N processing in GIWs, though with caveats. Questions exist on the utility and precision of the acetylene block method (e.g., Groffman et al. 2006) for measuring denitrification, though it is frequently used due to high throughput and relative ease of field and laboratory sampling efforts. Our study focused on two ecoregions, but the breadth of our sampling locations was limited by access and site availability to a single study region in North Carolina, with a wider distribution in Florida. Additional analyses conducted over a larger area would decrease autocorrelation and increase relative precision. In our study, we created a composite sample to account for intra-site variability, but discriminating between heterogeneous vegetation zones and subsampling within those zones could help to identify important denitrifying areas within a wetland site and provide additional data on average denitrification rates across different wetland types, thereby helping to quantify denitrification rate covariates. Lastly, a composite sample also diminishes the importance of zonation within a wetland, especially within GIWs, typified by a transitional zone between the upland and wetland composed of a combination of obligate, facultative, and facultative-upland plants. Differences in denitrification zones between shallow and deep habitats would be expected and should be further explored.

We conclude that GIWs contribute to nitrogen removal and that by intercepting Nr species, geographically isolated wetlands may thereby limit the exposure of downgradient aquatic systems and ground water to N. Results further suggest that GIWs with ample N and P supplies may have substantially elevated denitrification rates which suggests a potentially important role in agricultural landscapes or urban settings with

exposures to both N and elevated P. These wetlands are frequently considered degraded or impaired (Brown and Vivas 2005; Lane and Brown 2006; Reiss et al. 2010) yet they still perform a multitude of wetland functions (e.g., Johansson et al. 2005; Dunne et al. 2007; Atkinson et al. 2011; Marton et al. 2014). Belying their name, "geographically isolated wetlands" have been shown in many cases to be hydrologically connected to near-surface groundwater (Sun et al. 2000; McLaughlin and Cohen 2013) as well as linked intermittently through overland flow (Leibowitz and Vining 2003; Forbes et al. 2012). The movement of nitrogen-laden overland or ground water into wetlands with favorable conditions for denitrification prevents these pollutants from reaching other aquatic systems and can affect traditional navigable waters. The presence on the landscape and performance of GIWs in capturing N from various sources (Sobota et al. 2013) suggests that extant GIWs can help maintain the integrity of other aquatic systems.

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