Denitrification Enzyme Activity as an Indicator of Nitrate Movement through a Diversion Wetland

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The Davis Pond freshwater diversion is intended to help restore Louisiana's coastal wetlands by reintroducing Mississippi River water to Barataria Basin. We hypothesized that the high NO_3^- concentration (2.0 mg NO_3–N L^{-1}) of the Mississippi River water would control the rate of denitrification in the receiving marsh given that the soils are saturated, anaerobic, and contain high C. Therefore, areas of high denitrification enzyme activity (DEA) in the marsh would represent soils exposed to river NO_3^- and actively involved in denitrification. Data from 88 soil samples (0–10 cm) collected throughout the marsh revealed significantly higher rates of DEA in a 715-ha area adjacent to the diversion inflow. This area of generally high DEA contained >80% of all DEA observed while representing only 19% of the total marsh area at the low discharge rate of 39.5 m^3 s^{-1}. The area of high DEA coincided with the highest surface water NO_3^- and indicated that the marsh has a greater aerial capacity for NO_3^- removal than is utilized. A laboratory experiment suggested that soils loaded with external NO_3^- typically had higher DEA rates than soils receiving no added NO_3^-. The DEA was strongly dependent on soil depth (92% of DEA occurred at 0–5 cm) and internal N cycling was substantial in this wetland soils. This study demonstrates the applicability of using soil DEA to map where denitrification activity is greatest, the aerial extent of soils involved in denitrification, and the general flow path of introduced nutrients in large wetlands where NO_3^- is the limiting factor for denitrification.

Abbreviations: DEA, denitrification enzyme activity; MBC, microbial biomass carbon.

The Mississippi River delta plain is experiencing the highest rate of land loss in the United States, with approximately 88 km^2 of marsh converting to open water every year (USGS, 2003). Natural processes (soil compaction and eustatic sea level rise) and anthropogenic disturbances (artificial levees, dams, and canal dredging) have resulted in subsidence of the Louisiana coast (Day et al., 1995; Penland and Ramsey, 1990; Day et al., 2009). Economically important fishing grounds, oil and gas infrastructure, shipping channels, and wildlife habitat in the Louisiana coastal zone are threatened by this wetland loss (National Research Council, 2000; Turner and Cahoon, 1987). The construction of large-scale freshwater diversions along the lower Mississippi River is part of a coastal restoration plan being implemented by the U.S. Army Corps of Engineers and the Louisiana Department of Natural Resources to mitigate wetland loss.

Freshwater diversions are intended to mimic the natural flooding regime of the Mississippi River by the controlled release of freshwater, nutrients, and sediments into the riparian and coastal wetlands of the delta plain (Green, 2006; White et al., 2009). The Davis Pond freshwater diversion is the largest diversion constructed to date, with the capacity to redirect up to 302 m^3 s^{-1} of Mississippi River flow into the upper Barataria Basin, a historic distributary west of the present Mississippi River (U.S. Army Corps of Engineers, 2006). The goals of the Davis Pond diversion are to counteract saltwater intrusion and help offset marsh subsidence in Barataria Basin (Green, 2006).

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Davis Pond marsh is a 3760-ha freshwater wetland that serves as the receiving marsh for reintroduced river water; the marsh has not received any river inputs since 1904 when a dam was constructed upstream (Evers et al., 1992; U.S. Army Corps of Engineers, 2006). Since the hydraulic isolation of upper Barataria Basin and Davis Pond marsh, the mean annual NO_3^- concentrations in the Mississippi River have doubled (Goolsby et al., 2001). In the Lower Mississippi River, the mean annual NO_3^- is between 1.0 and 1.2 mg NO_3–N L^{-1}, with spring concentrations reaching 2.0 mg NO_3–N L^{-1} (Antweiler et al., 1995; DeLaune et al., 2005). The high nutrient concentrations of the Mississippi River are linked to algal blooms, hypoxia, and fish kills in the northern Gulf of Mexico (Anderson et al., 2002; Rabalais et al., 2002). Barataria Basin is an N-limited estuary (Patrick and DeLaune, 1976) and could be negatively impacted by the reintroduced nutrient-rich Mississippi River water if the excess N is not removed within the receiving marsh.

The Caernarvon freshwater diversion demonstrated high efficiency (88–97%) for removing and transforming NO_3 and NO_2 in the receiving marsh (Lane et al., 1999). A pilot study in Davis Pond marsh indicated near-complete removal of NO_3^- at low discharge rates (35 m^3 s^{-1}), but <40% removal efficiency at moderate discharge rates (100 m^3 s^{-1}). Denitrification was the primary mechanism through which NO_3^- was removed in Davis Pond (DeLaune et al., 2005).

Denitrification is occurring in Davis Pond marsh (DeLaune et al., 2005), but the size of the marsh (3760 ha) and the dearth of information on topography and hydrology make it difficult to determine the spatial distribution and rates of denitrification activity. Dissolved NO_3^- in the water column has been quantified using a mass balance approach and indicates a decrease in NO_3^- concentration between the marsh inflow and outflow (DeLaune et al., 2005). This "black box" method provides little information, however, on the mechanisms and site conditions that control N cycling. This knowledge is critical for planning future diversion projects and protecting the Gulf of Mexico from NO_3–induced eutrophication.

In upland soils, denitrification is often limited by C availability and a paucity of anaerobic soil sites. In contrast, wetland soils are defined by flooded conditions that favor the accumulation of organic matter (White and Reddy, 2001). In wetlands, NO_3^- supply is typically the limiting factor for denitrification (Cooper, 1990). The major sources of NO_3^- in Davis Pond marsh are from the internal cycling (mineralization and nitrification) of organic N, and NO_3^- dissolved in the Mississippi River water being reintroduced through the diversion. The contribution of internally cycled N to the Davis Pond marsh (measured as the in situ concentration when the river diversion is off) is between 0.0 and 0.2 mg NO_3–N L^{-1}, whereas the NO_3^- concentration in the diverted Mississippi River water is >10 times greater (2.0 mg NO_3–N L^{-1}) (DeLaune et al., 2005). Therefore, because C is not limiting and soils are saturated in Davis Pond marsh, we can conclude that NO_3^- is the limiting factor for denitrification, the reintroduced Mississippi River water is the overwhelming source of NO_3^- in the system and DEA should provide a spatial record of where the NO_3^- (and water) is moving in the marsh.

The limited supply of electron acceptors in wetland soils drives the diffusion of NO_3^- into the soil, where it is quickly denitrified, leaving little or no detectable NO_3^- in the soil pore water (Reddy et al., 1978). The enzymes produced by denitrifiers to catalyze the reduction of NO_3^- remain in the soil, however, as evidence that the sites are primed for denitrification. Laboratory studies have shown that denitrifiers grown in a C-rich medium under anaerobic conditions produce enzymes at a rate proportional to the concentration of NO_3^- in the substrate (Downey, 1966). Denitrification enzyme activity quantifies the activity of these enzymes and is a reflection of the quantity of NO_3^- being denitrified (Schipper et al., 1993). Denitrification enzyme activity differs from other denitrification assays because a short incubation time and the addition of an enzyme inhibitor ensure that all N_2O is produced by the active pool of denitrifiers in situ, and not from new enzymes synthesized following the introduction of idealized laboratory conditions (Smith and Tiedje, 1979). Therefore, DEA can be considered a reflection of the environmental history of the site (Tiedje et al., 1989). After the cessation of NO_3^- loading, it has been demonstrated in the Florida Everglades that DEA values return to baseline values within several months (White and Reddy, 1999), further illustrating the ability of DEA to serve as a snapshot of the current site conditions (Luo et al., 1996).

We hypothesized that DEA rates in the organic soils of Davis Pond marsh will indicate the spatial distribution of the introduced NO_3^-–rich Mississippi River water by demonstrating where the NO_3^- limitation has been met and denitrification is occurring. To investigate this hypothesis, we first confirmed our assumption that the NO_3^- supply was the limiting factor for denitrification in the marsh soils by (i) analyzing the spatial distribution of soil moisture, organic matter, and total C within the marsh, and (ii) measuring potential denitrification in the laboratory. We then conducted a laboratory experiment using intact soil cores from the marsh that were exposed to various levels of NO_3^- loading for a 45-d period to determine the relationships (i) between surface water NO_3–N concentration and DEA, and (ii) among DEA, soil depth, and general soil properties in a controlled setting. Finally, we quantified DEA in 88 soil samples collected throughout Davis Pond marsh. The field samples were used to (i) create a map of the spatial distribution of DEA in the marsh, and (ii) determine the relationship between DEA and field site characteristics (soil properties, vegetation type, and a limited number of surface water NO_3–N samples).

MATERIALS AND METHODS

Site Description

The Davis Pond freshwater diversion was constructed in 2002 by the U.S. Army Corps of Engineers and is operated by the Louisiana Department of Natural Resources. It consists of four 18-m^2 box culverts with manually operated gates built into the west bank levee of the Mississippi River in St. Charles Parish, Louisiana, approximately 19 km

upstream from New Orleans. Using gravity flow, the structure can divert up to 302 $m^3 s^{-1}$ (10,650 cfs) of river water down a 3-km inflow channel leading to Davis Pond marsh, a 3760-ha receiving wetland (Fig. 1) (Louisiana Department of Natural Resources, 2004; U.S. Army Corps of Engineers, 2006). When the marsh reaches holding capacity, water is intended to sheet flow over an outflow weir into Lake Cataouatche, Lake Salvador, and eventually Barataria Bay (Fig. 1). Since construction, several design modifications have been required along the outflow weir to permit greater water conveyance, thus limiting the operation of the diversion until recently (Letter, 2005; Louisiana Department of Wildlife and Fisheries, 2005).

The Davis Pond marsh is underlain by fluvial sediments deposited by the historic Mississippi River 200 to 3500 yr before present (Turner and Cahoon, 1987). A crevasse in the levee in 1884 assisted in the formation of the current marsh (Ensminger and Simon, 1993). The western portion of the marsh contains a series of splay ridges running east to west that support declining stands of *Taxodium distichum* (L.) Rich. The majority of the marsh consists of emergent herbaceous plants, predominately *Sagittaria lancifolia* L., *Eichhornia crassipes* (Mart.) Solms, *Alternanthera philoxeroides* (Mart.) Griseb., *Bidens* spp., and *Typha* spp. (Ensminger and Simon, 1993). A natural channel has developed through the center of the marsh that connects the inflow and outflow. This central channel is the major conduit for the diverted water, primarily when the diversion is operating under low-flow conditions, leading to minimal sheet flow across the marsh.

Confirmation of Assumptions

To confirm that the NO_3^- concentration is limiting denitrification in Davis Pond, we collected soil samples from 88 sites throughout the marsh and measured the soil moisture, organic matter, and total C contents. An ANOVA of soil properties for 20 soil samples clustered nearest to the inflow, and another 20 located near the outflow, was performed to ensure that soils near the inflow were not favored for denitrification due to unique soil characteristics or greater C availability.

Additionally, a denitrification potential study was conducted in the laboratory using eight soil samples (four collected near the inflow and four collected near the outflow) to determine if all soils were equally capable of supporting denitrification given the appropriate conditions. The denitrification potential represents the denitrification rate of both existing and newly synthesized denitrifying enzymes given non-limiting growth conditions (no enzyme inhibitor) during a 24-h period. The denitrification potential differs from DEA in the longer length of time N_2O-N production is measured and the lack of chloramphenicol (an enzyme production inhibitor) addition. All eight soils were characterized by organic matter contents between 47 and 62%. Incubations were prepared in glass serum bottles (described below), purged with N_2 gas, and 15% of the headspace was replaced with C_2H_2. Dextrose C (288 mg L^{-1}) and 1.0 mg KNO_3–N L^{-1} were added to the slurry. Samples were continuously agitated on a longitudinal shaker in the dark at 23°C. The headspace was sampled for N_2O-N every 2 to 6 h, analyzed on a Shimadzu GC-8A ECD (Shimadzu Scientific Instruments, Columbia, MD), and plotted against time.

Laboratory Experiment

Thirty-six field-replicate intact soil cores were collected within a 20- by 20-m area in the southwest quadrant of Davis Pond marsh on 25 June 2007. These replicate cores were collected in a monoculture of *S. lancifolia* to minimize soil and microbial differences that might be related to the vegetation community. The intact cores were flooded with site water, transported to the laboratory at 4°C, and wrapped in foil to exclude light. A 1-cm-diameter hole was drilled in each core tube exactly 10 cm above the soil surface (this hole served as a drain and ensured identical water column depths among the cores). Each core was randomly assigned to one of four NO_3^- treatment groups—0.0, 0.5, 1.0, or 2.0 mg NO_3–N L^{-1}—for a total of nine cores in each treatment. Nitrate concentrations were chosen to represent NO_3–N levels typically observed in the Mississippi River (Antweiler et al., 1995). The cores were incubated in a water bath in the dark at 23°C while NO_3^- solution (in the form of KNO_3 and deionized water) was continuously pumped into each core at equal rates using a peristaltic pump. In situ surface water NO_3–N, dissolved O_2, temperature, and redox potential were regularly monitored during the incubation. Three cores from each NO_3^- treatment were randomly chosen and destructively sampled

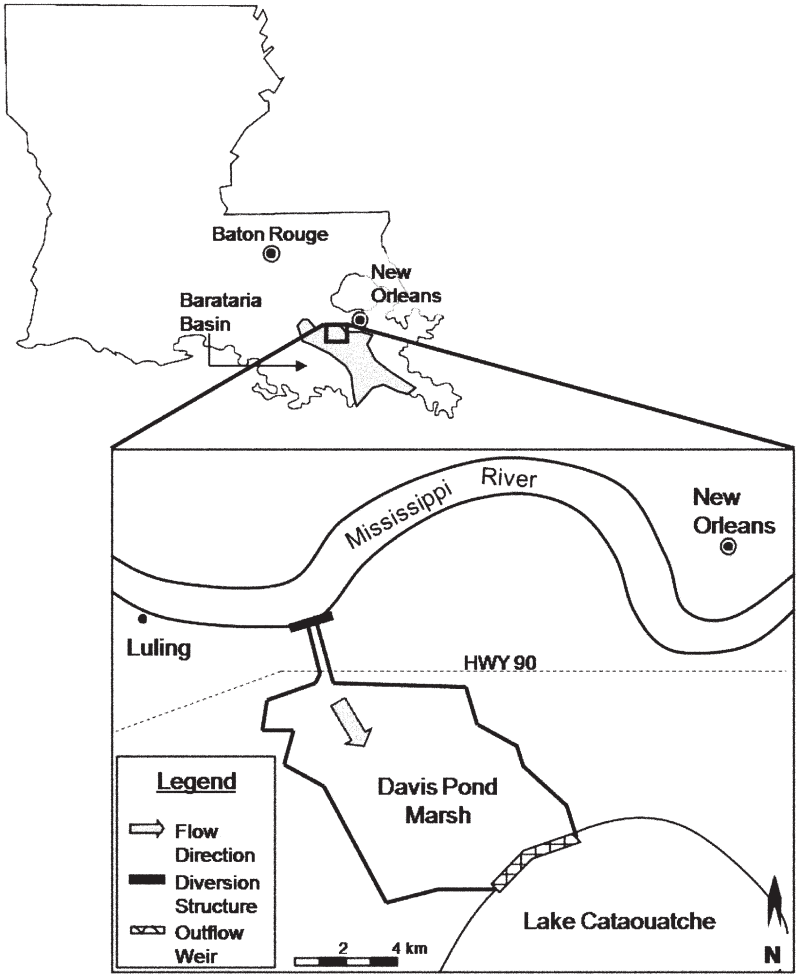

Fig. 1. Location map and schematic of Davis Pond diversion and marsh.

by separating into three depth segments (0–5, 5–10, and 10–20 cm) after 7, 20, or 45 d of NO_3^- loading. The moisture content, DEA, dry weight bulk density, total C, and total N were quantified on all samples. Extractable NO_3–N and NH_4–N were measured on soil extracts (25 mL of 0.5 mol L^{-1} K_2SO_4) and analyzed on a SEAL AQ2 Automated Discrete Analyzer (SEAL Analytical, West Sussex, UK; USEPA Methods 353.2 and 350.2, USEPA, 1983).

Microbial biomass C (MBC) and N were also determined for each of the three depth segments of each core using the fumigation-extraction method after Vance et al. (1987). Duplicate 5-g wet weight samples were prepared in 25-mL centrifuge tubes. One set was fumigated for 24 h and the other set served as the unfumigated control. Following the chloroform treatment, both fumigated and unfumigated samples were extracted with 25 mL of 0.5 mol L^{-1} K_2SO_4, agitated for 30 min on a longitudinal shaker, and centrifuged at 5000 rpm for 10 min (Malecki-Brown et al., 2007). The supernatant was vacuum filtered through Whatman no. 42 filter paper and stored at 4°C until analyzed for total organic C (TOC) (Shimadzu Scientific Instrument TOC-VCSN, Columbia, MD). Microbial biomass C was determined by subtracting the TOC of the unfumigated samples from the corresponding fumigated sample (White and Reddy, 2000). An extraction efficiency coefficient of k_{EC} = 0.37 was applied (Sparling et al., 1990).

Microbial biomass N was measured using the chloroform-fumigation method developed by Brookes et al. (1985b), followed by an acid total Kjeldahl N (TKN) digestion (Bremner and Mulvaney, 1982). The TKN was quantified on the SEAL AQ2 Automated Discrete Analyzer (SEAL Analytical, West Sussex, UK) using USEPA Method 351.2 (USEPA, 1983). An extraction efficiency coefficient of k_{EN} = 0.54 was applied (Brookes et al., 1985a).

Field Experiment

The upper 20 cm of the soil profile was collected in a 7-cm-diameter Plexiglas tube and global positioning system coordinates recorded at 88 sites chosen with a sampling grid designed to optimize geospatial interpolation. All field samples were collected between 13 May and 10 July 2007. Because the soils consisted of moderately decomposed organic matter, coring often involved cutting the organic mat with a serrated knife as the core tube was pushed down. While some surface compaction was unavoidable, samples compacted >5 cm were discarded and recollected. Soils were extruded in the field and divided into 0- to 10- and 10- to 20-cm increments, placed on ice, and transported back to the laboratory for storage at 4°C. Bulk density, DEA, total C, total N, and organic matter contents were quantified for all samples within 50 d of collection (Luo et al., 1996). All sampling sites had either standing water or saturated soils at the time of sample collection. During soil collection, the vegetation community was categorized at each sampling site as submerged aquatic, emergent macrophyte, or woody dominated.

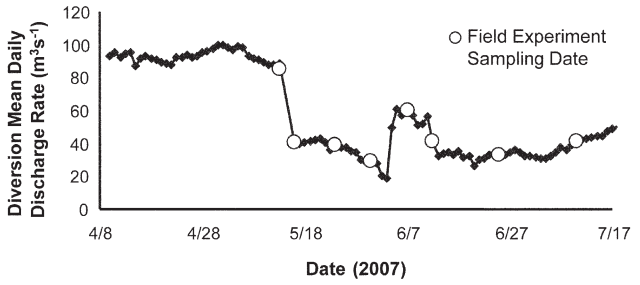

Fig. 2. Mean daily discharge rate of the Davis Pond freshwater diversion, April to August 2007. Sampling dates indicate when the 88 soil samples were collected for the field study.

Eleven surface water samples were collected on 10 July 2007 in the central channel that has become the preferential flow path connecting the marsh inflow and outflow. At the time water samples were collected, the discharge rate had been 38.0 ± 11.3 m^3 s^{-1} (±1 standard deviation) for 57 continuous days (Fig. 2). Samples were field filtered, placed on ice, and transported back to the laboratory for storage at 4°C. The surface water was analyzed within 2 wk of collection for NO_3–N. Water samples were not collected at all 88 sites because, despite all soils being saturated, not all had standing surface water. Surface water samples were analyzed for NO_3–N on a SEAL AQ2 Automated Discrete Analyzer (SEAL Analytical, West Sussex, UK) using USEPA Method 353.2 (USEPA, 1983).

Soil Analysis

All soils were analyzed for bulk density, total C, total N, organic matter content, and DEA. The bulk density was determined after drying a subsample at 70°C to constant weight. Total C and N were measured on the dried, ground subsample using an Elemental Combustion System (Costech Analytical Technologies, Valencia, CA). The organic matter content was estimated by mass loss-on-ignition, where dry soils were combusted at 550°C for 5 h and the final weight was subtracted from the initial weight.

Denitrification enzyme activity was determined in accordance with the methods outlined in Tiedje (1982), with adaptations by White and Reddy (1999). The 0- to 10-cm soil sample was homogenized and a 5-g wet weight subsample was placed in a glass serum bottle. The bottle was sealed with a rubber septa and aluminum crimp cap; the headspace was evacuated from the bottle to −75 kPa, then purged with O_2–free N_2 gas for 1 min. Eight milliliters of N_2–purged deionized water was added to create a slurry, and approximately 15% of the headspace was replaced with acetylene gas (C_2H_2) while maintaining atmospheric pressure within the bottle (Yoshinari and Knowles, 1976). The bottles were agitated on a longitudinal shaker for 30 min to distribute the acetylene. Eight milliliters of a solution of 56 mg KNO_3–N L^{-1}, 288 mg dextrose C L^{-1}, and 2 mg chloramphenicol L^{-1} was added, creating a slight overpressure. Chloramphenicol is an enzyme inhibitor used to prevent de novo synthesis of enzymes during incubation under idealized conditions (Smith and Tiedje, 1979). Samples were continuously agitated in the dark at 23°C and the headspace was sampled at approximately 30, 60, 90, and 120 min. Gas samples were analyzed on a Shimadzu GC-8A ECD (Shimadzu Scientific Instruments, Columbia, MD) and N_2O-N production was calculated, with consideration for product in the aqueous phase using the Bunsen absorption coefficient (Tiedje, 1982). The rate was calculated as the slope of the line when milligrams N_2O-N per kilogram soil was plotted against time.

Data Analysis

Statistical analysis was performed using SAS 9.1 software (SAS Institute, Cary, NC). All data sets were first tested to determine if the

assumptions of homogeneity and normality were met using Levene's test and the Shapiro–Wilk test, respectively. Where these assumptions were not met, the raw data were logarithmically transformed and further statistical analysis was conducted using the data set that fulfilled the assumptions of homogeneity and normality. A three-way ANOVA model ($\alpha = 0.05$) was used to determine the interaction between NO_3^- treatment (0.0, 0.5, 1.0, or 2.0 mg NO_3–N L^{-1}), time (7, 20, or 45 d), and soil depth (0–5, 5–10, or 10–20 cm) in the laboratory experiment. Significance differences were identified using Fisher's LSD post-hoc test. One-way ANOVA models ($\alpha = 0.05$) were also used to identify significant differences between soil core properties (DEA, moisture content, bulk density, total C, total N, extractable NO_3–N, and extractable NH_4–N) and NO_3^- treatment (0.0, 0.5, 1.0, or 2.0 mg NO_3–N L^{-1}), time (7, 20, or 45 d), and soil depth (0–5, 5–10, and 10–20 cm) in the laboratory experiment. A one-way ANOVA was also performed to determine if significant differences occurred between soil parameters (soil moisture, bulk density, organic matter content, total C, total N, and pH) and spatial distribution (near the diversion inflow vs. near the outflow) in the field experiment to confirm our assumptions of non-limiting C and anaerobic conditions throughout the marsh. Pearson's product moment correlations were used to determine if correlations existed among the various field parameters of soil moisture, bulk density, pH, organic matter content, total N, total C, DEA, and vegetation community. The georeferenced spatial data from the field experiment were mapped using ArcGIS 9.0 (ESRI, Redlands, CA) and the delineation of the region of high DEA was done by hand.

Table 1. Select properties of field soils used to verify our assumptions about the factors limiting denitrification in the marsh. Inflow values are means ± standard deviations of 20 sample sites located near the inflow (northern portion of the marsh). Outflow values are from the 20 sampling sites located near the outflow (southern portion of the marsh). Overall values are for all 88 sites.

Soil parameter	Inflow sites (n = 20)	Outflow sites (n = 20)	Wetland mean (n = 88)
Soil moisture, %	81 ± 10	88 ± 9	89 ± 8
Bulk density, Mg m^{-3}	0.22 ± 0.14	0.12 ± 0.10	0.12 ± 0.10
Organic matter, %	24 ± 14 a†	63 ± 21 b	58 ± 26
Total C, g kg^{-1}	110 ± 80 a	316 ± 112 b	291 ± 137
Total N, g kg^{-1}	8.2 ± 5.2 a	22.2 ± 7.8 b	19.5 ± 9.0
pH	7.3 ± 0.3	6.9 ± 0.2	7.0 ± 0.3

† Means followed by different letters are significantly different between inflow and outflow ($P < 0.01$).

RESULTS

Confirmation of Assumptions

The assumptions of normality and homogeneous variance were met for most of the data sets (soil moisture, bulk density, organic matter content, total C, total N, and pH). Extractable NO_3–N and extractable NH_4–N were logarithmically transformed to meet these assumptions and DEA was logarithmically transformed and a constant was added to correct for the significant number of measurements below the detection limit. Once these transformations were made, the assumptions of normality and homogenous variance were verified.

The surface soils (0–10 cm) from 88 sites distributed throughout the marsh had mean soil moisture of 89.2 ± 8.1% (mean ± standard deviation). The mean organic matter content was 58 ± 26%, bulk density was 0.12 ± 0.10 Mg m^{-3}, and total C was 291 ± 137 g kg^{-1} (Table 1). The soil moisture was not significantly different between sites located near the diversion inflow (81 ± 10%) and sites located near the outflow (88 ± 9%). In general, soils located near the inflow tended to contain more mineral matter, as seen by the slightly higher bulk density and significantly lower organic matter content and total C ($P < 0.01$) compared with soils located farthest from the inflow (Table 1). Denitrification enzyme activity was inversely correlated ($r = -0.42$) with total C and organic matter content ($P < 0.01$), indicating that there was no C limitation in the marsh soils (Table 2).

Soils collected near the inflow and near the outflow showed the same potential to support denitrification. The denitrification potential for soils collected near the inflow had a mean N_2O-N production rate of 0.83 mg N_2O-N kg^{-1} h^{-1} ($n = 4$) during the first 2 h (the period representing DEA) and reached the maximum potential denitrification rate (2.3 mg N_2O-N kg^{-1} h^{-1} or 44.2 mg N_2O-N m^{-2} d^{-1}) within 4 h of incubation under idealized conditions. In contrast, soils located near the outflow had a mean denitrification rate of 0.13 mg N_2O-N kg^{-1} h^{-1} ($n = 4$) in the first 2 h and required 10 h to reach the maximum potential denitrification rate (Fig. 3). The N_2O-N production rate for all samples, regardless of spatial location, stabilized at 2.3 mg N_2O-N kg^{-1}, representing the maximum denitrification rate achievable with the provided NO_3^- addition (Fig. 3). These results demonstrate that river NO_3^- reaching parts of the marsh at low flow rates can very quickly (<1 d) stimulate denitrification and hence enzyme activity in the soil.

Table 2. Product-moment correlation coefficients (*r*) for soil and site characteristics measured during the field experiment (for $n = 88$, at $P = 0.05$, $r = 0.22$, and at $P = 0.01$, $r = 0.28$). Correlations that are significant at $P < 0.05$ are indicated in bold. The analysis was performed using raw data, except for denitrification enzyme activity (DEA), which was transformed to meet the assumptions of normality and homogeneity of variance.

Parameter	Sampling date	Soil moisture	Bulk density	pH	Organic matter	Total N	Total C	DEA
Soil moisture	0.06							
Bulk density	−0.05	**−0.99**						
pH	0.10	**−0.36**	**0.37**					
Organic matter	0.01	**0.80**	**−0.78**	**−0.53**				
Total N	0.02	**0.81**	**−0.80**	**−0.51**	**0.98**			
Total C	0.01	**0.79**	**−0.77**	**−0.51**	1.00	**0.98**		
DEA	0.04	−0.06	0.06	**0.37**	**−0.42**	**−0.39**	**−0.42**	
Vegetation	−0.19	**0.32**	**−0.32**	**−0.29**	**0.33**	**0.26**	**0.31**	0.20

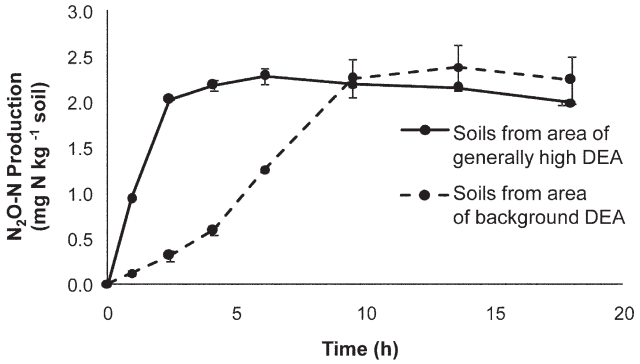

Fig. 3. Denitrification potential of soils collected in the area of high denitrification enzyme activity (DEA) (near the inflow) compared with soils collected in the area of low DEA (near the outflow), indicating the ability of all soils to synthesize denitrifying enzymes given the appropriate conditions. Error bars indicate standard error, *n* = 4.

Laboratory Experiment

Soil properties of the 36 field replicate intact cores are presented in Table 3. A significant increase in total C, total N, and MBC with soil depth was observed, as well as a significant decrease in bulk density with soil depth (one-way ANOVA, $P < 0.01$) (Table 3). None of the soil properties measured showed a significant difference between treatments. Nitrate solution was added at a rate of 55.4 ± 2.3 mL h^{-1}, which resulted in the replacement of the 10-cm water column approximately four times a day (Table 4). The surface water temperature within the cores was 23 ± 1.1°C. The redox potential indicated that the soils were poised for denitrification and that redox conditions between treatments did not differ significantly (Table 4). In situ surface water NO_3–N concentration increased significantly (one-way ANOVA, $P < 0.01$) with treatment concentration but was, on average, 20 to 27% below the concentration of the added solution. Calculating the denitrification rate as the difference between the NO_3–N added and that observed in situ, the denitrification rate (mg NO_3–N lost m^{-2} d^{-1}) increased significantly with treatment concentration (one-way ANOVA, $P < 0.01$) (Table 4).

During the 45-d lab experiment, soil cores continuously loaded with 1.0 mg NO_3–N L^{-1} had significantly higher DEA rates than cores receiving no external N (three-way ANOVA, $P < 0.05$). When DEA was investigated with each time step (duration of NO_3^- loading), the Day 20 sampling event showed a significant increase in DEA with NO_3^- concentration at both the 0- to 5-cm (one-way ANOVA, $P < 0.05$) and 5- to 10-cm (one-way ANOVA, $P < 0.01$) soil depths (Fig. 4). This trend was not significant on Days 7 or 45.

Intratreatment variability of DEA rates was high (Fig. 4). Overall, the mean rates of DEA for the 0.0, 0.5, 1.0, and 2.0 mg NO_3–N L^{-1} treatments at the 0- to 5-cm soil depth were 0.47 ± 0.4, 0.93 ± 0.7, 1.67 ± 1.0, and 1.55 ± 0.8 mg N_2O-N kg^{-1} h^{-1}, respectively. The greatest variability and overall highest rates of DEA were observed on Day 7. The mean rates of DEA appeared to decrease with time, but this trend was not significant (Fig. 4).

Denitrification enzyme activity decreased significantly with increasing soil depth ($r = -0.65$). Averaged across time, approximately 92% of all DEA activity occurred in the top 0 to 5 cm of soil, 7% at the 5- to 10-cm depth, and <1% at 10 to 20 cm. The proportion of DEA occurring below 5 cm increased as the length of time the soils were loaded with NO_3–N increased (one-way ANOVA, $P < 0.001$). On Day 7, <1% of the DEA activity occurred below 5 cm; on Day 20, 7% of DEA was observed below 5 cm; 16% of DEA activity occurred below 5 cm after 45 d. Several soil properties (i.e., extractable NO_3^-, total C, and total N) that differed significantly with soil depth also showed a significant relationship with DEA.

Field Study

The discharge rate of the Davis Pond diversion was 93.1 ± 3.6 m^3 s^{-1} for about 1 mo before field sampling. Soil collection began on 13 May 2007 when the discharge rate was 88 m^3 s^{-1}. The following day, the discharge rate dropped to 39.5 ± 10.4 m^3 s^{-1} and remained at a similar rate from 14 May to 10 July 2007 (Fig. 2). On field sampling days, the minimum air temperature ranged from 19 to 26°C and the maximum air temperature was between 27 and 33°C (data not shown). The water temperature was influenced by many variables (e.g., proximity to the diversion inflow, the discharge rate, river water temperature, air temperature, etc.) but tended to be lower closer to the inflow due to the mixing of cold Mississippi River water. Because standing water was not present at all sites, water temperature, NO_3^- concentration, and other surface water parameters were not collected. All soils were saturated, however, as evidenced by the high moisture content and therefore ideal environment for denitrification to occur once NO_3^- is introduced.

The soil properties (0–10 cm) for the entire marsh are presented in Table 1. The aerial coverage of the dominant vegetation communities in the marsh consisted of 64% emergent macrophyte, 22% submerged aquatic, and 14% woody plant dominated. Denitrification enzyme activity was not correlated with plant community, soil moisture, or bulk density (Table 2); however, DEA was negatively correlated with organic matter content, total N, and total C and positively correlated with pH (one-way ANOVAs, all $P < 0.01$) (Table 2).

Table 3. Select soil properties of intact cores (laboratory experiment) from three depths quantified after destructive sampling. Data are means ± standard deviation (*n* = 35 unless indicated).

Soil parameter	0–5 cm	5–10 cm	10–20 cm
Total C, g C kg^{-1}	119 ± 37 a†	323 ± 67 b	453 ± 27 c
Total N, g N kg^{-1}	15.7 ± 2.7 a	25.2 ± 4.9 b	33.8 ± 2.0 c
Bulk density, Mg m^{-3}	0.095 ± 0.005 a	0.073 ± 0.003 b	0.059 ± 0.004 c
pH	6.9 ± 0.2	6.9 ± 0.3	7.0 ± 0.2
Microbial biomass C, g C kg^{-1}‡	23.8 ± 6.0	25.4 ± 5.0	30.9 ± 6.6
Microbial biomass N, g N kg^{-1}‡	0.29 ± 0.18	0.39 ± 0.22	0.37 ± 0.22
Extractable NO_3^-, mg N kg^{-1}	0.03 ± 0.06	0.24 ± 0.18	0.11 ± 0.14
Extractable NH_4^+, mg N kg^{-1}	1.73 ± 0.54	1.81 ± 0.97	1.13 ± 0.59

† Means followed by different letters are significantly different with soil depth ($P < 0.01$) for each parameter.
‡ $n = 9$.

Denitrification enzyme activity rates ranged from below detection (0.006 mg N_2O-N kg^{-1} h^{-1}) to 2.10 mg N_2O-N kg^{-1} h^{-1} (0.08–92.4 mg N_2O-N m^{-2} d^{-1}) in the upper 0 to 10 cm of the soils. The highest rates were concentrated near the inflow channel where river water first enters the marsh. The spatial distribution of DEA rates indicated that soils with DEA rates ≥0.41 mg N_2O-N kg^{-1} h^{-1} were concentrated within a 715-ha area adjacent to the inflow (Fig. 5a). Outside this region of generally high DEA, rates were either undetectable or ≤0.30 mg N_2O-N kg^{-1} h^{-1}. The 715-ha area of generally high DEA rates adjacent to the inflow contained >80% of all observed DEA in the 0- to 10-cm soil horizon while representing only 19% of the total marsh area (Fig. 5a). The mean DEA rates in the 715-ha area were 15 times higher than the mean DEA rates of sites outside this area (one-way ANOVA, $P < 0.001$). The area of generally high DEA radiated from the marsh inflow point in a southeasterly orientation. Denitrification enzyme activity below 10 cm was negligible in all field samples.

In much of the marsh, standing surface water was confined to the central channel during the sampling period because of the low discharge rate of the diversion. Within the channel, NO_3^- concentrations varied from 2.0 mg NO_3–N L^{-1} at the Mississippi River inflow to 0.5 mg NO_3–N L^{-1} at the outflow weir (Fig. 5b). Nitrate concentrations steadily decreased as water flowed through the marsh area; however, a direct comparison cannot be made between DEA and surface water NO_3^- concentration because NO_3^- was not quantified at all sites where DEA was measured. The highest NO_3^- concentrations were observed within the area of the highest DEA rates (Fig. 5b).

DISCUSSION

Confirmation of Assumptions

In order for DEA to accurately represent the marsh area impacted by NO_3^- loading and actively involved in denitrification, the soils must be saturated and C availability must be non-limiting. While DEA rates were highest near the inflow, soil organic matter and total C showed the opposite spatial pattern, with higher concentrations near the outflow (Fig. 5a; Tables 1 and 2). This indicates that denitrifiers were not controlled by C content. Other work has demonstrated that the high soil C content and primary productivity of Davis Pond marsh make it a source of dissolved organic C (DOC) to the downstream basin (DeLaune et al., 2008). The soil moisture content (89 ± 8%) indicates that soils were saturated at the time of sampling (Table 1). Further confirmation of our assumption that these soils are NO_3^- limited was supplied by the denitrification potential study, which found that soils near to and far from the diversion inflow had a similar capacity to support denitrification given adequate time and added NO_3^- (Fig. 3).

Laboratory Experiment

Previous field studies have suggested a spatial correlation between DEA and N loading in wetland soils (Schipper et al., 1993; White and Reddy, 1999; Wigand et al., 2004); however, a controlled laboratory core study where the effect of NO_3^- could be isolated had not been performed before this study. Using intact soil cores, we found that DEA in the 0- to 5- and 5- to 10-cm soil horizons increased significantly with NO_3^- concentration after 20 d of continuous loading (Fig. 4b); however, this relationship was not significant on the Day 7 or Day 45 sampling events (Fig. 4a and 4c). On Day 7, the overall DEA rates appeared higher than on Days 20 and 45, as well as significantly higher than rates observed in the field study. This may be an artifact of soluble C released during field collection of the cores, which required root structures to be sheared. Such a spike in labile C could have accelerated denitrification at the beginning of the laboratory experiment to rates higher than those observed in the field.

Toward the end of the incubation period (Day 45) it appears that the artificial conditions may have caused DOC to replace NO_3^- as the limiting factor, presumably because the inflow solution consisted of only NO_3^- and deionized water. In the field, these soils experience a continuous influx of litter and DOC that was not effectively emulated in the laboratory (DeLaune et al., 2008). Research on denitrification in stream sediment found that a NO_3^- threshold exists below which denitrification is limited by NO_3^- concentration, but above which other factors become the controlling variable for the denitrification rate (Inwood et al., 2005). Such a threshold could account for the lower than expected DEA rates in the 2.0 mg NO_3–N L^{-1} treatment. The inability to produce a robust correlation between NO_3^- and DEA with time in the laboratory suggests the complexity of factors influencing DEA (e.g., diffusion rate, soluble organic C, and soil heterogeneity) and the possible limitations for the use of intact soil cores to simulate field conditions during extended incubations (Ambus, 1993; Bruland et al., 2006; Casey et al., 2004; Seitzinger et al., 2006). The laboratory experiment may have been improved by using freshwater that mimicked the composition of the field water (e.g., including the naturally occurring DOC and micronutrients) with various levels of NO_3–N to prevent C and other constituents from being depleted with time. A field comparison study of Coastal Plain wetlands (North Carolina) found that NO_3–N was a significant predictor of DEA at six of the eight wetland sites measured, while soluble

Table 4. Characteristics of intact cores (laboratory experiment) measured during the experiment and presented according to NO_3^- treatment concentrations of 0.0, 0.5, 1.0, and 2.0 mg NO_3–N L^{-1}. Data are means ± standard deviations (n = 12).

Parameter	0.0 mg L^{-1}	0.5 mg L^{-1}	1.0 mg L^{-1}	2.0 mg L^{-1}
Surface water NO_3^-, mg N L^{-1}	0.003 ± 0.001 a†	0.37 ± 0.03 b	0.73 ± 0.08 c	1.60 ± 0.07 d
NO_3^- removed, %	NA	26 ± 6	27 ± 8	20 ± 3
Denitrification rate, mg N m^{-2} d^{-1}	NA	44 ± 11 a	95 ± 29 b	137 ± 24 c
Surface water dissolved O_2, mg L^{-1}	2.3 ± 0.3	2.8 ± 0.7	2.7 ± 0.6	3.2 ± 0.7
Redox potential (3-cm depth), mV	−148 ± 47	−57 ± 22	−115 ± 93	−81 ± 57
Redox potential (7-cm depth), mV	−89 ± 97	−115 ± 74	−103 ± 80	−120 ± 80

† Means followed by different letters are significantly different between NO_3^- treatments using a one-way ANOVA model ($P < 0.01$).

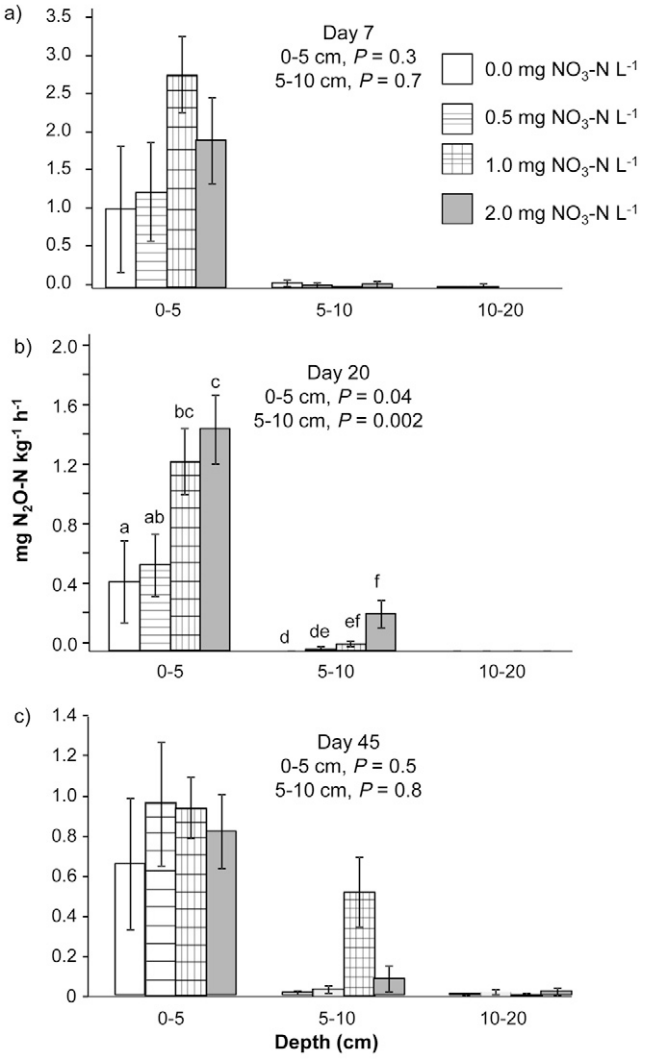

Fig. 4. Denitrification enzyme activity (DEA) from the laboratory experiment at various soil depths following the destructive sampling of intact soil cores loaded with different NO_3 treatment concentrations after (a) 7 d, (b) 20 d, and (c) 45 d. Error bars indicate standard error, *n* = 3; different lowercase letters above the error bars in (b) indicate a significant difference at $\alpha = 0.05$.

organic C was as a predictor or copredictor of DEA at three of the eight sites (Bruland et al., 2006), suggesting an interplay of factors controlling DEA. Finally, utilizing a larger volume core tube may have allowed a greater percentage of the surface area to remain unaltered by the collection process, such as root shearing and profile disturbance during coring.

A significant inverse relationship between DEA and soil depth was observed in the laboratory experiment. In the Florida Everglades, DEA in the surface 0 to 10 cm of soil averaged four times greater than rates observed at the 10- to 30-cm soil depth (White and Reddy, 1999). The present study refines the relationship between DEA and soil depth by showing that approximately 92% of all DEA was occurring in the top 0 to 5 cm of soil, 7% at the 5- to 10-cm depth, and <1% at 10 to 20 cm. The proportion of DEA occurring at lower depths increased significantly as the duration of NO_3^- loading increased, indicating that NO_3^- diffusion may have been limiting activity at greater soil depths (Reddy et al., 1978). These results suggest that a detailed quantification of the vertical distribution of DEA in the field may provide valuable information regarding the duration of flooding with river water in a specific site because higher DEA found at depth is correlated with longer NO_3^- exposure.

The laboratory study demonstrated the significant contribution of internal biogeochemical N cycling to the DEA rate (and hence denitrification) with measurable amounts of gaseous N produced from soils receiving no NO_3^- loading. Denitrification enzyme activity was observed in the soil cores receiving 0.0 mg NO_3–N L^{-1} on all sampling days and averaged 0.47 ± 0.4 mg N_2O-N kg^{-1} h^{-1} (approximately 5.2 mg N m^{-2} d^{-1}). Sediments collected in the vicinity of Davis Pond from a previous study also demonstrated significant denitrification (3.3 mg N m^{-2} d^{-1}) in laboratory control treatments (no added N) (Miao et al., 2006). Internal N cycling, including mineralization and coupled nitrification–denitrification processes, could account for N_2O production in the control treatment (White and Reddy, 2003). The influence of internal N cycling has been quantified in riverine (Malecki et al., 2004), estuarine (Burdige and Zheng, 1998), and lake (D'Angelo and Reddy, 1993) sediments and wetland soils (this study).

Field Study

The field study revealed that DEA rates in Davis Pond marsh were significantly higher in a 715-ha area adjacent to the diversion inflow channel than the rest of the marsh area (Fig. 5a). Denitrification enzyme activity ranged from 0.41 to 2.10 mg N_2O-N kg^{-1} h^{-1} (21.9–92.4 mg N_2O-N m^{-2} d^{-1}) within the 715-ha area. Outside this area, DEA ranged from below detection to 0.30 mg N_2O-N kg^{-1} h^{-1} (3.90 mg N_2O-N m^{-2} d^{-1}).

Surface water NO_3–N could not be quantified at every sampling site due to differences in flood status; however, several sites characterized by standing water containing undetectable NO_3^- yielded low rates of DEA, suggesting that the presence of floodwater alone was not controlling the rate of DEA. Sites with the highest surface water NO_3–N were located within the area of high rates of DEA found near the diversion inflow (Fig. 5b).

We assumed that denitrification was the major pathway for surface water NO_3^- disappearance, which is supported by several studies in flooded organic soils (e.g., DeLaune et al., 2005; DeBusk et al., 2001: Seitzinger, 1988). The low redox potential, high soil moisture content, and high total C also suggest that denitrification is the main pathway for NO_3^- removal in this system (Tables 1 and 4); however, other processes (e.g., assimilation, immobilization, dissimilatory reduction to NH_4, dilution, and mixing) can also result in NO_3^- loss. It is important to note that DEA only reflects enzyme activity associated with the denitrification pathway and does not provide insight on the importance of other pathways in this system. The further decline in surface water NO_3^- outside the area of high DEA indicates the existence of other mechanisms for NO_3^- loss (Fig. 5b).

The spatial distribution of DEA rates provides additional information about NO_3^- removal and hydrology in Davis Pond

that a mass-balance approach could not provide. For instance, DEA can be measured at sites where standing water is not present, a common occurrence in wetlands with fluctuating water levels or subsurface flow. Because denitrifying enzymes are able to persist in the soil for several days when conditions are no longer ideal (Smith and Parsons, 1985), DEA functions as a time-averaged indicator of N exposure (White and Reddy, 1999). In comparison, surface water NO_3^- only provides instantaneous information, varying greatly with time.

The ability of the Davis Pond marsh to remove NO_3^- from the surface water was of greater interest to us than the surface water NO_3^- concentration itself, which has already been shown to decrease with distance from the inflow (DeLaune et al., 2005). Soil DEA identified a "hot spot" of denitrification activity adjacent to the inflow that could not be explained by soil organic matter, total C, vegetation community, or soil moisture, but was positively related to the spatial distribution of surface water NO_3^-. The area of generally high DEA represents only 19% of the total marsh area, suggesting that Davis Pond marsh has the aerial capacity to remove a greater NO_3^- load. The rate of NO_3^- loading (i.e., the diversion discharge rate) at the time of this study was approximately 10% of the total diversion capacity. When the diversion is conveying more water, we expect to see enhanced sheet flow across the marsh, increasing the surface area available for denitrification (Blahnik and Day, 2000; Kjellin et al., 2007). The efficiency of NO_3^- removal in the marsh may resemble a normalized distribution with time, however, because denitrification may become limited by water residence time at very high discharge rates (Seitzinger et al., 2006). To accurately establish the denitrification capacity of the marsh, additional DEA measurements at higher discharge rates are needed to determine how the spatial conveyance of river water and the water residence time interact to alter the denitrification efficiency.

The spatial distribution of DEA at low discharge indicates that NO_3^- was quickly denitrified on entering the marsh, but the spatial pattern also shows that the highest rates of DEA occurred along a southeasterly flow path emanating from the inflow (Fig. 5a). This pattern suggests that river water is being deflected eastward along a preferential flow path, probably resulting from the historic splay ridges on the western side of the marsh. Managers may want to investigate how elevation differences within the marsh are contributing to channelization and short-circuiting of water during low-discharge events, which can reduce the areal capacity for denitrification. The utility of DEA in delineating surface water flow paths is another advantage of this methodology that may save time and cost during future hydraulic studies in wetlands of a similar size containing microtopographical complexity (Wang et al., 2006).

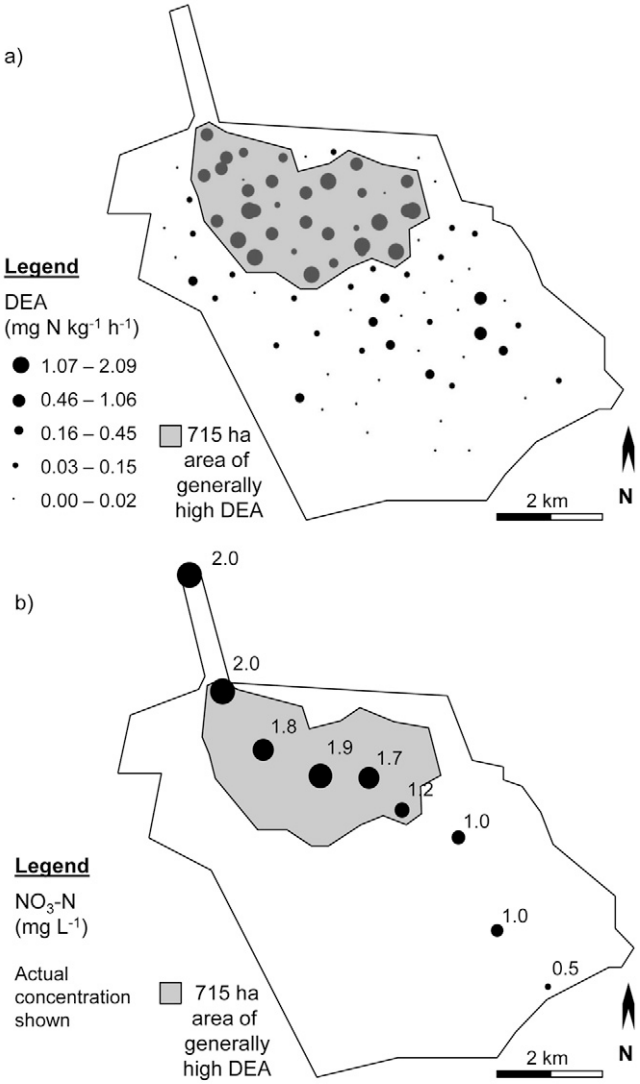

Fig. 5. Outline of Davis Pond marsh with (a) denitrification enzyme activity (DEA) concentrations and (b) surface water NO_3–N concentrations mapped with global positioning system coordinates. For (a), the size of the circle corresponds with the rate of DEA (mg N_2O-N kg^{-1} h^{-1}) indicated in the legend. Likewise for (b), the size of the circle corresponds with the concentration of NO_3^- (NO_3–N mg L^{-1}) in the water column indicated on the map. The gray area indicates the 715-ha area (19% of the marsh area) that contained >80% of all observed DEA in the 0- to 10-cm horizon.

CONCLUSIONS

Denitrification enzyme activity can be a potentially powerful tool for monitoring a wetland system receiving an N-rich input from an anthropogenic point source. Monitoring techniques comprised of calculating the mass balance of NO_3^- at the inflow and outflow provide little information on the spatial distribution of the internal N-removal processes. Denitrification enzyme activity indicates where denitrification activity is greatest, provides an estimate of the wetland area involved in denitrification, and suggests the general flow path of the introduced NO_3^-. Denitrification enzyme activity differs from the standard potential denitrification assay because is measures enzymes that were synthesized in situ in response to current field conditions.

Previous work has indicated that DEA can provide an estimation of the NO_3^- load to a system (Groffman and Tiedje, 1989; Schipper et al., 1993; White and Reddy, 1999). The utility of DEA may not be as a direct surrogate for NO_3^- concentration, however, but as a method for determining the spatial variations in the magnitude of NO_3^- loading and denitrification activity

across a large area. In organic wetland soils characterized by high C content and low O_2, DEA signals where the NO_3^- limitation has been met by triggering the synthesis of denitrifying enzymes. Future work should focus on determining the length of time that enzymes remain active in the soil after NO_3^- loading has ceased.

The Davis Pond wetland is an ideal case for testing the applicability of using soil DEA to determine the spatial extent of NO_3^- loading because of the uniformly high C availability, high soil moisture, and the external source of NO_3^- that far exceeds ambient NO_3^- concentrations. While DEA cannot be used to predict the exact NO_3^- concentration at a specific site, we found that NO_3^- concentrations were high enough to trigger significant enzyme synthesis in only 715 ha of Davis Pond, or approximately 19% of the marsh surface area, when the diversion was discharging at a rate of $\sim$39.5 $m^3\ s^{-1}$. We also noted that river inputs are flowing to the southeast on entering the marsh, suggesting that elevation differences may be contributing to short-circuiting during low discharge rates. To obtain a better understanding of the denitrification capacity of Davis Pond and the effect of the discharge rate on hydrology, future work should use additional DEA determinations during moderate and high river water discharge rates.

With an ever-increasing number of wetlands being impacted by nutrients, DEA could be considered a useful tool to monitor, map, and manage large wetland areas where NO_3^- availability is the limiting factor for denitrification. With a single sampling, DEA can provide information on the spatial impact of NO_3^-, the capacity for denitrification, and the general hydrologic flow path of NO_3^-–enriched surface water. This method is an improvement over more standard methods of using water level indicators and autosamplers to provide spatial information from large systems such as Davis Pond. The equipment, personnel, and travel costs of monitoring NO_3^- levels in water with time and at so many stations would be prohibitive and would fail to provide a definitive answer on the importance of the denitrification process over other N loss mechanisms.

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