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

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.
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$_2$O 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$_2$O-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$_6$H$_5$ Dextrose C (288 mg L$^{-1}$) and 1.0 mg KNO$_3$–N L$^{-1}$—for a total of nine cores in each treatment. Nitrate concentrations were chosen to represent NO$_3^-$ 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$_3^-$, 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 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$_3^-$ 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$_3^-$, 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
by separating into three depth segments (0–5, 5–10, and 10–20 cm) after 7, 20, or 45 d of \( \text{NO}_3^- \) loading. The moisture content, DEA, dry weight bulk density, total C, and total N were quantified on all samples. Extractable \( \text{NO}_3^- \) and \( \text{NH}_4^- \) were measured on soil extracts (25 mL of 0.5 mol L\(^{-1}\) \( \text{K}_2\text{SO}_4 \)) 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\(^{-1}\) \( \text{K}_2\text{SO}_4 \), 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 \( k_{\text{EC}} \) = 0.37 was applied (Sparling et al., 1990).

Microbial biomass N was measured using the chloroform-fumigation method developed by Brookes et al. (1989b), 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 \( k_{\text{EN}} \) = 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.

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 \( \text{NO}_3^- \). 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 \( \text{NO}_3^- \) 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 \( \text{O}_2 \)-free \( \text{N}_2 \) gas for 1 min. Eight milliliters of \( \text{N}_2 \)-purged deionized water was added to create a slurry, and approximately 15% of the headspace was replaced with acetylene gas (\( \text{C}_2\text{H}_2 \)) 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 K\( \text{NO}_3 \)-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 \( \text{N}_2\text{O} \) 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 \( \text{N}_2\text{O} \)-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 (α = 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, and 10–20 cm) in the laboratory experiment. Significance differences were identified using Fisher’s LSD post-hoc test. One-way ANOVA models (α = 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 infl ow vs. near the outfl ow) in the fl eld experiment to confrm 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 fl eld parameters of soil moisture, bulk density, pH, organic matter content, total N, total C, DEA, and vegetation community. The georeferenced spatial data from the fl eld experiment were mapped using ArcGIS 9.0 (ESRI, Redlands, CA) and the delineation of the region of high DEA was done by hand.

RESULTS

Confrmation of Assumptions

The assumptions of normality and homogenous 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 signifcant 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 infl ow (81 ± 10%) and sites located near the outfl ow (88 ± 9%). In general, soils located near the infl ow tended to contain more mineral matter, as seen by the slightly higher bulk density and signifcantly lower organic matter content and total C (P < 0.01) compared with soils located farthest from the infl ow (Table 1). Denitrifcation 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 infl ow and near the outfl ow showed the same potential to support denitrifcation. The denitrifcation potential for soils collected near the infl ow had a mean N$_2$O-N production rate of 0.83 mg N$_2$O-N kg$^{-1}$ h$^{-1}$ (n = 4) during the flrst 2 h (the period representing DEA) and reached the maximum potential denitrifcation rate (2.3 mg N$_2$O-N kg$^{-1}$ h$^{-1}$ or 44.2 mg N$_2$O-N m$^{-2}$ d$^{-1}$) within 4 h of incubation under idealized conditions. In contrast, soils located near the outfl ow had a mean denitrifcation rate of 0.13 mg N$_2$O-N kg$^{-1}$ h$^{-1}$ (n = 4) in the flrst 2 h and required 10 h to reach the maximum potential denitrifcation rate (Fig. 3). The N$_2$O-N production rate for all samples, regardless of spatial location, stabilized at 2.3 mg N$_2$O-N kg$^{-1}$, representing the maximum denitrifcation rate achievable with the provided NO$_3^-$- addition (Fig. 3). These results demonstrate that river NO$_3^-$-reaching parts of the marsh at low fl ow rates can very quickly (<1 d) stimulate denitrifcation and hence enzyme activity in the soil.

Table 1. Select properties of fl eld soils used to verify our assumptions about the factors limiting denitrifcation in the marsh. Infl ow values are means ± standard deviations of 20 sample sites located near the infl ow (northern portion of the marsh). Outfl ow values are from the 20 sampling sites located near the outfl ow (southern portion of the marsh). Overall values are for all 88 sites.

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

† Means followed by different letters are signifcantly different between infl ow and outfl ow (P < 0.01).

Table 2. Product-moment correlation coeffcients (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 signifcant at P < 0.05 are indicated in bold. The analysis was performed using raw data, except for denitrifcation enzyme activity (DEA), which was transformed to meet the assumptions of normality and homogeneity of variance.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Sampling date</th>
<th>Soil moisture</th>
<th>Bulk density</th>
<th>pH</th>
<th>Organic matter</th>
<th>Total N</th>
<th>Total C</th>
<th>DEA</th>
<th>Vegetation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soil moisture</td>
<td>0.06</td>
<td>-0.99</td>
<td>Bulk density</td>
<td>-0.05</td>
<td>-0.36</td>
<td>0.37</td>
<td>Organic matter</td>
<td>0.01</td>
<td>0.80</td>
</tr>
</tbody>
</table>
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\(_3\)-N loading), the Day 20 sampling event showed a significant increase in DEA with NO\(_3\)-N concentration at both the 0- to 5-cm (one-way ANOVA, \( P < 0.05 \)) and 5- to 10-cm depths (one-way ANOVA, \( P < 0.01 \)) soil depths (Fig. 4). This trend was not significant on Days 7 or 45.

Intertreatment 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\(_2\)O-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\(_3\)-N 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).

![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, \( n = 4 \).](image-url)
Denitrification enzyme activity rates ranged from below detection (0.006 mg N$_2$O-N kg$^{-1}$ h$^{-1}$) to 2.10 mg N$_2$O-N kg$^{-1}$ h$^{-1}$ (0.08–92.4 mg N$_2$O-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$_2$O-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$_2$O-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, $P < 0.0001$). The area of generally high DEA radiated from the marsh inflow point in a southeaster 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...
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$_2$O-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$_2$O 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$_2$O-N kg$^{-1}$ h$^{-1}$ (21.9–92.4 mg N$_2$O-N m$^{-2}$ d$^{-1}$) within the 715-ha area. Outside this area, DEA ranged from below detection to 0.30 mg N$_2$O-N kg$^{-1}$ h$^{-1}$ (3.90 mg N$_2$O-N m$^{-2}$ d$^{-1}$).

Surface water NO$_3^-$ 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$_3^-$ 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).

**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$_3^-$.

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 O₂, DEA signals where the NO₃⁻ 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 NO₃⁻ 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 NO₃⁻ loading because of the uniformly high C availability, high soil moisture, and the external source of NO₃⁻ that far exceeds ambient NO₃⁻ concentrations. While DEA cannot be used to predict the exact NO₃⁻ concentration at a specific site, we found that NO₃⁻ 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 ~39.5 m³ 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 NO₃⁻ availability is the limiting factor for denitrification. With a single sampling, DEA can provide information on the spatial impact of NO₃⁻, the capacity for denitrification, and the general hydrologic flow path of NO₃⁻-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 NO₃⁻ 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.

ACKNOWLEDGMENTS

We would like to acknowledge the efforts of Maverick LeBlanc, Ben LeBlanc, Brett Marks, and Jeremy Conkle for assistance in sample collection. In addition, we would like to acknowledge Prof. Murray Lark and Dr. Ben Marchant from Rothamsted Research, UK, for their contribution in identifying sampling locations. Funding was provided by the Louisiana Board of Regents Research Competitive Subprogram Grant LEQSF (2005-08)-RD-A-14 and a Louisiana Board of Regents Fellowship to Lisa Gardner.

REFERENCES


U.S. Army Corps of Engineers. 2006. Freshwater diversions. USACE, New Orleans, LA.


