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In Situ Plant Uptake of Excess Nutrients and Consequential Alteration of Rhizosphere Dynamics

An Honors Thesis submitted in partial fulfillment of the requirements of Honors Studies in

Environmental, Soil, and Water Science

by

Srusti Maddala

Spring 2020

Crop, Soil, and Environmental Sciences
Dale Bumpers College of Agricultural, Food, and Life Sciences
University of Arkansas

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Abstract

The use of phytoremediation in ecological remediation projects has numerous benefits including soil stabilization and nutrient uptake. Recently, microdialysis, a diffusion-based sampling technique commonly used in biomedical research, has been recognized as a candidate for monitoring chemical changes in the rhizosphere. The real-time, in situ data it provides about nutrient diffusion may improve the management and success of restoration projects. Therefore, the objective of this study was to employ the technique of microdialysis in the novel application of quantifying the diffusive flux of inorganic nitrogen compounds in the rhizosphere of native plants of Arkansas. The microdialysis technique was first optimized for the soil setting, which included flow rate determination and experimentation with soil saturation and the addition of prepared and natural spikes. Switchgrass (*Panicum virgatum*) and Reed canarygrass (*Phalaris arundinacea*) plants were planted in soil obtained from Lake Keith and maintained in a greenhouse along with a soil control. Two different watering regimes were established and microdialysis samples were collected six times during a 2-week experiment while watering with nitrate-N-rich (5-6 μg/mL) water acquired from Lake Keith. Samples were analyzed for nitrate-N and ammonium-N fluxes using colorimetric assays. Fluxes of nitrate-N in the Reed canarygrass rhizosphere increased throughout the experiment such that they were different from the Switchgrass treatment by the second sampling day and were different from both Switchgrass and the control during the second week of experimentation. Ammonium-N in all samples was below detection limits. Overall, this study revealed that in situ nitrogen sampling can be accomplished successfully using the microdialysis technique and that Switchgrass is a likely candidate for future phytoremediation applications, as shown by temporal nitrate-N distributions and total nitrogen analysis of above- and belowground biomass.

Introduction & Literature Review

Rapid urbanization is a defining characteristic of this century. With more than 60% of the world population predicted to live in cities by the year 2030, anthropogenic changes continue covering the surface of the Earth, increasing the pressure on the surrounding ecosystems (Bolund & Hunhammar, 1999; Walsh, 2000). Expansive development of impervious layers such as roadways, parking lots, and rooftops results in increased surface runoff after major precipitation events due to limited infiltration into the soil. Stormwater runoff is, according to Walsh (2000), more difficult to manage, and has been studied far less than the management of sewage, another product of urbanization that degrades receiving waters.

Excess nutrients such as nitrate-nitrogen (N) and soluble reactive phosphorus found in stormwater runoff from urban areas cause eutrophication or hypoxia in downstream receiving water (Yan et al., 2009). These excess nutrients arise from several, complex urban practices, some of which cannot be properly identified, and are labeled as nonpoint source water pollution (Walsh, 2000). One such site affected by nonpoint source water pollution is Lake Keith, located in Cave Springs, Northwest Arkansas (36.261787, -94.230138).

Lake Keith is a small body of water situated on a 30-acre plot of land managed by the Illinois River Watershed Partnership (http://irwp.org/). Water emanating from a cave is Lake Keith's source of water. From Lake Keith, water continues to flow into Osage Creek, ultimately reaching various water channels and tributaries of the Illinois River. The water in Lake Keith contains concentrations of elemental N present in nitrate (nitrate-N) ranging from 5.7-6.6 mg/L, resulting in excessive algal blooms, which often cover sections of the lake. Nuisance algal growth is exacerbated by added nutrients from guano of cave-residing bats and the regional karst topography that allows transport of nutrients into the water system.

A mode of remediating Lake Keith would be to create a streambank restoration site that functions to remove excess nutrients through phytoremediation, namely phytoextraction. Phytoremediation is an emerging study of bioremediation that employs plants for the removal of pollutants from the soil and water (Panesar, 2019). Through the phytoextraction mechanism, the plants extract the excess nutrients into harvestable biomass, which is also known as hyperaccumulation.

To monitor the impact of excess nutrient uptake, the rhizosphere, a zone of dynamic microbial activity and symbiotic relationships at the plant root-soil interface, was studied (Figure 1). Often, destructive sampling measures such as soil extractions are used to examine N availability within the rhizosphere (Mulvaney, 1996). While extraction of free and exchangeable forms of N using water and salts such as potassium chloride (KCl) and potassium sulfate (K_2SO_4) are the conventional methods of sampling nitrogenous ions such as nitrate-N and ammonium-N from the soil, this method is both destructive and requires significant periods of time between sampling and analysis. Recent studies have investigated applications of microdialysis to sample these inorganic forms of N and measure the chemical changes in the rhizosphere without removing soil or destroying the soil structure (Inselsbacher et al., 2011).

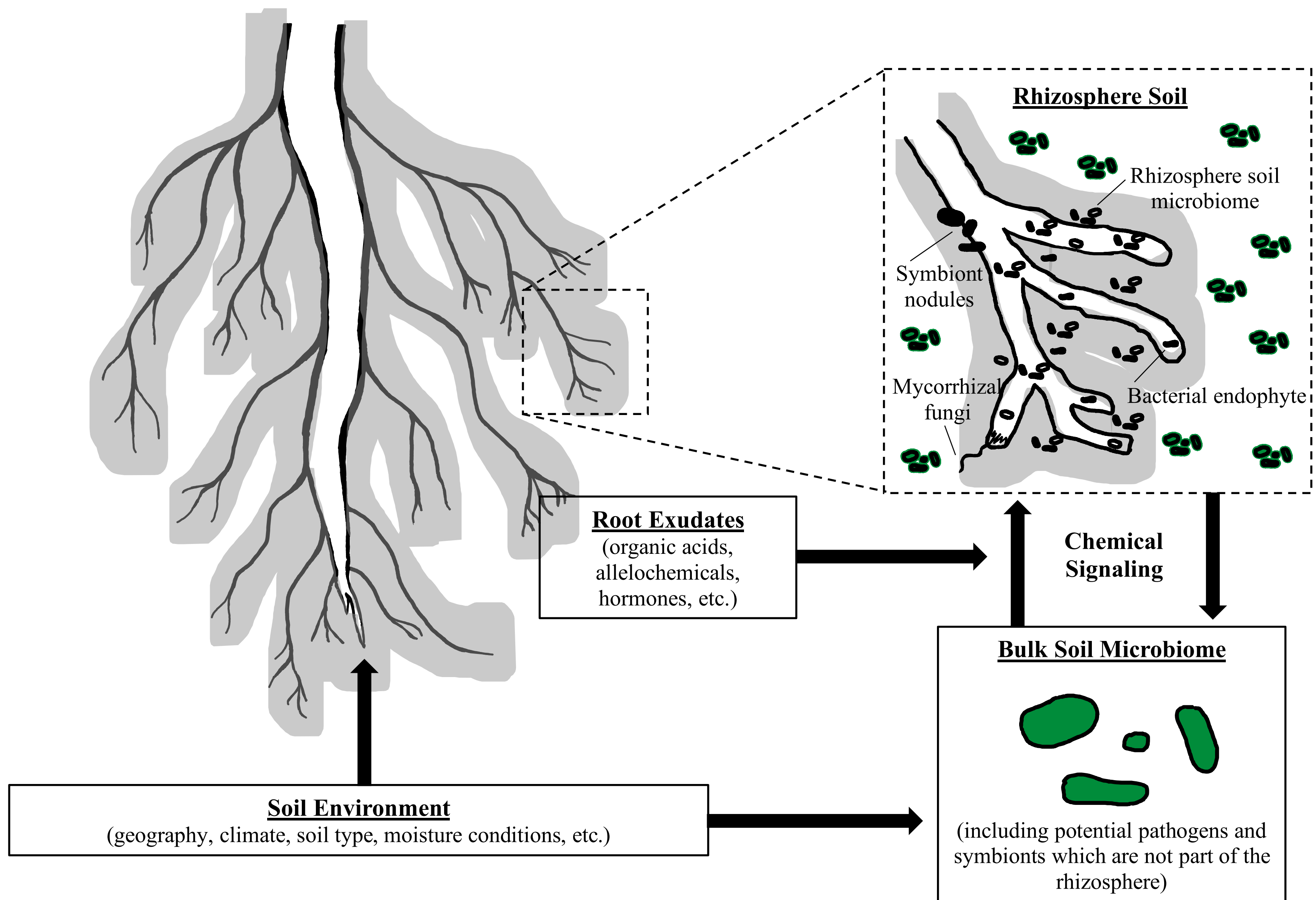

Figure 1. The rhizosphere, a zone of dynamic symbiotic relationships at the plant root-soil interface.

Microdialysis is a diffusion-based sampling method that has been used for nearly three decades in the field of medicine and has been established as one of the major research tools in experimental psychopharmacology and neuropathology (Duo et al., 2006; Kehr, 1993; Stenken, 2006). The central component of the microdialysis device is a semipermeable membrane 500 µm in diameter and 10 mm in length (Figure 2). A perfusion fluid is passed through the device at flow rates in units of µL/min, and compounds diffuse from the sample medium into the probe according to their concentration gradient. The compounds are then carried to the outlet to undergo chemical analysis (de Lange, 2013; Stenken, 2006).

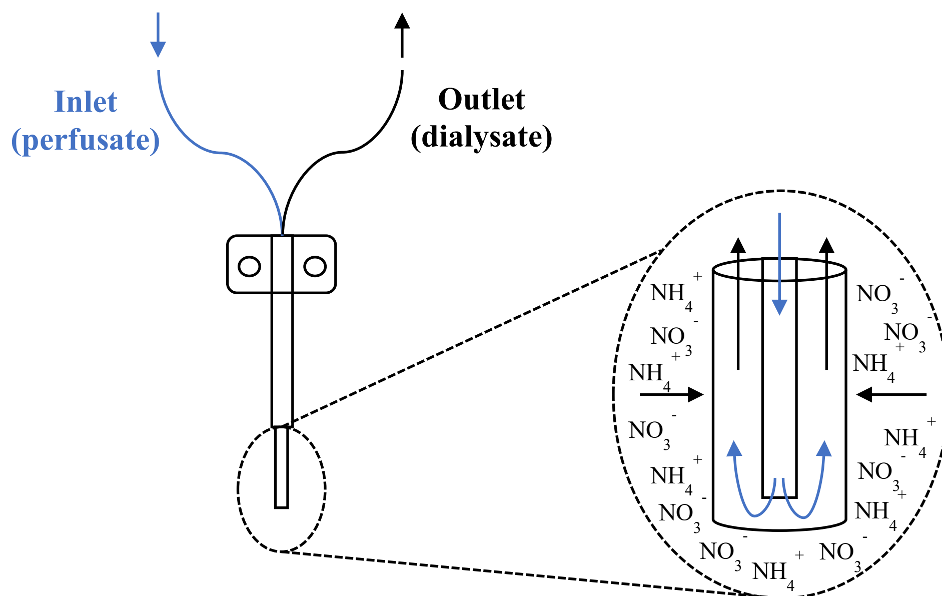

Figure 2. An overview of the microdialysis device showing the potential for diffusion of nitrate and ammonium ions from soil solution through the probe membrane as an example in the expanded inset.

In the soil, namely the rhizosphere, microdialysis has the potential for providing real-time, in situ data with greater temporal and spatial resolutions than current standard salt-solution extraction methods. The microdialysis technique allows for measuring N flux (rate of movement in space) over time, which can be utilized to achieve an enhanced understanding of the mechanisms involved in the availability of nutrients to plants, which can help facilitate managing systems by using phytoremediation.

The outcome of adapting the technique of microdialysis to the soil could be an improved, minimally invasive method of studying the fate and transport of nitrogenous compounds in dynamic microsites such as the rhizosphere. This study focuses on: 1) optimizing the microdialysis technique to sample the rhizosphere of nitrogen-hyperaccumulating plants; 2) developing a mesocosm experiment in the greenhouse with Arkansas-native plants *Panicum virgatum* and *Phalaris arundinacea*, which are capable of growing under excessive nutrient concentrations; and 3) studying the efficacy of the plants to uptake N by using microdialysis flux and plant biomass data.

The results of this study could aid in identifying effective hyperaccumulators of N, along with understanding the extent and reach of applying the microdialysis technique to the rhizosphere setting. Microdialysis flux data provides information about nutrient diffusion, which may improve the usage of fertilizers and the management and success of remediation projects.

Materials and methods

Experimental approach

This study was conducted in three phases. The first step involved laboratory experiments with microdialysis optimization performed in solutions containing nitrate-N and ammonium-N. The second step involved conducting microdialysis in soil obtained from the Lake Keith site, still

in the laboratory setting. Experimentation in this step included soil saturation to different water-filled pore space percentages, the addition of prepared spikes, the addition of Lake Keith water (natural spike), and water and KCl extractions. The third and final phase of the study was the establishment of a mesocosm experiment in the greenhouse. Switchgrass (*Panicum virgatum*) and Reed canarygrass (*Phalaris arundinacea*) plants were planted in soil obtained from Lake Keith and watered with Lake Keith water to investigate the fluxes of nitrogen in the rhizosphere of the plants during uptake over a two-week sampling period. Two different watering regimes were experimented with, and above- and belowground plant biomass production was evaluated for nitrogen concentration and total nitrogen to determine the efficacy of uptake.

Microdialysis sampling in nutrient solutions

Microdialysis set-up

Four syringe pumps (MD-1001, BASi, Lafayette, IN) were equipped with a total of 12 gas-tight syringes (MDN-0250, 2.5 mL, BASi) that delivered the perfusate HPLC-grade water (VWR, Radnor, PA) at the specified flow rates using a four-syringe drive pump controller (MD-1020, BASi). HPLC-grade water was used ubiquitously as the perfusate for all microdialysis experiments in this study, as water is the universal solvent in soil samples. HPLC-grade water was used specifically for its purity and low UV absorbance. Each syringe was connected to extra tubing (MF-5164, 1 meter, FEP (fluorinated ethylene propylene), 0.65 mm OD x 0.12 mm ID, BASi), which was connected to the inlet of a CMA 20 Elite probe (CMA8010436, 10 mm membrane length, PAES (polyarylethysulfone) membrane, and 20 kDa molecular weight cut-off, Harvard Apparatus, Holliston, MA). The equilibration time used at the beginning of every sampling was 15 minutes.

Optimization of microdialysis sampling based on relative recovery percent (RR%)

Determination of optimum flow rate. A 10-µg/mL nitrate-N solution was prepared in HPLC-grade water using sodium nitrate (Mallinckrodt, St. Louis, MO) and functioned as the sample medium. Three microdialysis probes were placed into the 10-µg/mL solution. Dialysates (180 µL) were collected in pre-weighed 0.5 mL microcentrifuge tubes at flow rates of 2.0, 3.75, and 5.0 µL/min (0.8, 1.5, and 2.0 µL/min on the pump controller). The relative recovery percent ($\boldsymbol{RR}$%) was calculated at each flow rate using equation 1:

$$RR\% = \frac{C_d}{C_{SM}} \times 100\% \qquad (1)$$

where C_d is the concentration of nitrate-N in the dialysate and C_{SM} is the concentration of nitrate-N in the sample medium. An equilibration time of 15 minutes was used between changing flow rates. The same procedure was repeated with a 10-µg/mL solution of ammonium-N prepared using ammonium chloride (Mallinckrodt, St. Louis, MO).

Effects of sample medium concentration on RR%. Standard solutions of 1, 3, 5, 8, and 10-µg/mL nitrate-N and ammonium-N were prepared in HPLC-grade water. Microdialysis sampling was performed in each solution using a 2.0 µL/min flow rate to collect 180 µL of dialysate. Equilibration times of 15 minutes were used between changing sample medium concentrations. The $RR\%$ was calculated for each sample medium concentration using equation 1.

Comparison of RR% in a combined solution of nitrate-N and ammonium-N. Standard solutions of 5-µg/mL nitrate-N and 5-µg/mL ammonium-N were prepared in HPLC water. Three microdialysis probes were placed into the 5-µg/mL nitrate-N solution and sampled for 30-minute intervals for a total of 90 minutes at 2.0 µL/min. Aliquots from the sample medium were collected before initial collection and at the end of each 30-minute interval.

Microdialysis was performed in the 5-μg/mL ammonium-N solution using the same sampling procedure. After dialysates were collected from each ion solution, 10-μg/mL nitrate-N and 10-μg/mL ammonium-N solutions were added to a 5-mL centrifuge tube in a 1:1 (v/v) ratio. The solution was vortex-mixed to ensure homogeneity, and then microdialysis was performed at 2.0 μL/min for 60 minutes. The $RR\%$ for the individual ion solutions, as well as the mixed solution was calculated using equation 1.

Field description

The field site was located at Lake Keith in Cave Springs, AR, USA (36.261787, -94.230138). The soil at this site is classified as a clayey-skeletal, mixed, semiactive, mesic Typic Paleudult (Noark series) with a 20 to 40 percent slope. The Noark series consists of deep, well-drained, moderately permeable soils that formed in colluvium and clayey residuum from cherty limestone. The site is currently managed by the Illinois River Watershed Project but has historically been a heavily disturbed site due to its use as the primary drinking water source of Cave Springs, as well as a fish hatchery, in the past.

Soil core samples and bulk soil were obtained from the upper 15 cm of the soil along a 4 m by 4 m plot on the southern bank, approximately 1 m from the edge of the lake in April 2019. Bulk soil samples were sieved using a 6-mm sieve to remove large debris and sediments. Volumetric soil moisture and soil temperature readings were collected directly from the field site at the time of soil sampling. Soil moisture was measured using a moisture sensor (SM150 Kit, Delta-T Devices, Cambridge, UK) and was 53.1-65%. Soil temperature (Traceable Products, Webster, TX) ranged from 17.1-17.5 °C. Discrete water samples (175 mL) were collected from the lake in a stratified pattern, preserved using acidification to a pH of 2.8, and stored in 175 mL HDPE containers (Nalge Nunc International Corporation, Rochester, NY) at 4 °C.

Bulk density and volumetric water content

Soil core (6-cm diameter, 5-cm height) samples were collected from the Lake Keith site, and their masses while field-moist and after they were oven-dried (VWR, Radnor, PA) at 55 °C for five days were used for determining bulk density (D_B, equation 2) and volumetric water content (θ_v, equation 3):

$$D_B = \frac{m_{OD}}{V_{core}} \tag{2}$$

$$\theta_v = \frac{\frac{m_{FM} - m_{OD}}{m_{OD}} \times D_B}{\rho_{H_2O}} \tag{3}$$

where m_{OD} is the mass of the oven-dried soil, m_{FM} is the mass of the field-moist soil, v_{core} is the volume of the soil core, and ρ_{H_2O} is the density of water.

Soil organic matter using the loss-on-ignition method

Determining the soil organic matter percentage was a necessary preliminary step for particle analysis of the soil obtained from the Lake Keith site (Gee & Bauder, 1986; "Total Carbon, Organic Carbon, and Organic Matter,"). The composite soil obtained from the Lake Keith site was first prepared by sieving through a 2-mm sieve (W.S. Tyler, Mentor, OH). It was then homogenized and set out to air-dry overnight. Upon overnight air-drying, the exact masses of 3 subsamples of 8 g aliquots of soil were measured and placed into the oven at 105 °C overnight. After approximately 24 hours, the masses of the oven-dried soils were recorded, and the 3 samples were placed into a muffle furnace (BF51800 series, 1100 °C Box Furnace, Lindberg/Blue, Riverside, MI) at 360 °C for 2 hours for combustion analysis of soil organic matter. The percentage of soil organic matter ($\% \, SOM$) was calculated using equation 4:

$$\% \, SOM = \frac{m_{OD} - m_C}{m_{OD}} \times 100 \tag{4}$$

where m_{OD} is the mass of the oven-dried soil and m_C is the mass of the soil after combustion of all organic matter.

Particle analysis

Soil obtained from the Lake Keith site was sieved using a 2-mm sieve, homogenized, and air-dried overnight. Before particle analysis, the organic matter was removed through oxidation using hydrogen peroxide. Due to the high amounts of organic matter present in the soil, six portions of 40 g soil were oxidized separately. Approximately 25 mL of HPLC-grade water was added to each portion of dry soil and mixed. A 30% hydrogen peroxide (H_2O_2) (Macron Fine Chemicals, Radnor, PA) solution was added in 5 mL increments and the portions were also heated to 90 °C to increase the rate of oxidation. This process of adding H_2O_2 and heating was repeated until the foaming reaction ceased and the soil became grey in color. Excess water was removed through evaporation, and the soil was pulverized using a mortar and pestle and homogenized.

After the removal of organic matter, particle analysis was performed on the soil using the hydrometer method (Gee & Bauder, 1986). The gravimetric water content (θ_g, equation 5) of the oxidized soil was determined by using the mass of the soil, placing in the oven overnight at 105 °C, and recording the mass of the soil of the oven-dried soil. Gravimetric water content was calculated using equation 5:

$$\theta_g = \frac{m_W - m_{OD}}{m_{OD}} \quad (5)$$

where m_W and m_{OD} are the mass of the soil before placing in the oven and the mass of oven-dried soil, respectively. The gravimetric water content of the oxidized soil was used to calculate the needed mass of soil to obtain 40 g without desiccation. Approximately 40 g of soil, 250 mL HPLC-grade water, and 100 mL of 50 g/L sodium-hexametaphosphate (EM Science, Cherry

Hill, NJ) were added to a 600 mL beaker and allowed to soak. After soaking for approximately 12 hours, the samples were transferred to a dispersing cup and mixed with an electric mixer (HMD400, Hamilton Beach, Glen Allen, VA) for 5 min. The suspension was transferred to a sedimentation cylinder, which was filled to the 1L mark after placing in the hydrometer. The contents of the cylinder were mixed, the hydrometer was lowered into the suspension, and readings were taken at 40 s, 6 hr, and 12 hr. These values were used to calculate the percentages of sand, silt, and clay in the soil samples, which were then used to determine the texture of the soil (Gee & Bauder, 1986).

Potassium chloride extraction

Traditional potassium chloride extraction of N from the soil is performed using a concentrated potassium chloride solution (Mulvaney, 1996). This extraction method was used to obtain concentrations of nitrate-N and ammonium-N in the soil obtained from the Lake Keith site. A 2 M KCl (VWR, Radnor, PA) was prepared in HPLC water. Soil (~ 1 g) was measured into a 20-mL plastic vial, and 10 mL of the prepared 2M KCl solution was pipetted into the vial, resulting in a 1:10 ratio (w/v). The vials were placed on a shaker for 30 minutes at room temperature. The contents of the vial were then filtered through an ashless, grade 42, Whatman filter paper with 2.5 µm pore size (VWR, Radnor, PA), and the resulting filtrate was stored at 4 °C.

Microdialysis in soil solution

Soil saturation

Calculations. The volume of water needed to saturate the soil to either 50% or 100% water-filled pore space (WFPS) in the laboratory was calculated using a series of equations based on bulk density, total pore space, volumetric water content, and gravimetric water content

(equations 6 – 11). These soil saturations were studied due to their relevance to the wetland setting- 50% WFPS is the theoretical optimum for aerobic processes and 100% is complete inundation of soil pores. The bulk density (D_B) of the soil was calculated by measuring the mass of soil, which filled a 30 cm^3 scoopula (equation 6):

$$D_B = \frac{m_{soil}}{30\ cm^3} \tag{6}$$

The total pore space (T_p) of the soil was calculated by using the bulk density and the particle density (D_P) of the soil, which was assumed to be 2.65 g/cm^3 (equation 7):

$$T_p = 1 - \frac{D_B}{D_P} \tag{7}$$

The volumetric water content was then calculated by:

$$\theta_v = 0.5 \times T_p \tag{8A}$$

$$\theta_v = 1 \times T_p \tag{8B}$$

where θ_v is the volumetric water content, equation 8A was used for 50% WFPS, and equation 8B was used for 100% WFPS. The gravimetric water content was calculated by using equation 9:

$$\theta_g = \frac{\theta_v \times D_W}{D_B} \tag{9}$$

where D_W is the density of water and was assumed to be 1 g/mL. Lastly, the mass of water needed for the specified percent saturation was calculated by:

$$m_{water} = \theta_g \times m_{soil} = V_{water} \tag{10}$$

where m_{soil} is the mass of soil being saturated. Since the density of water was assumed to be 1 g/mL, the mass of water needed was assumed to be equivalent to the volume of water needed to be added to the soil.

Effects of soil saturation on microdialysis sampling. Lake Keith soil (~26 g), which had been prepared by sieving using a 2-mm sieve, homogenized, and oven-dried at 55 °C, was measured

into each of two 20-mL beakers. Using the mass of soil and the bulk density of the soil (equation 6), the volume of water needed to bring the soil to 50% and 100% saturation was calculated (equations 7-11). The soil in one beaker was saturated to 50% and the other to 100% using HPLC water. After the addition of water, the beakers of soil were covered, and the soil water was allowed to equilibrate for 72 hours. After equilibration, microdialysis was performed in three different sample media: HPLC-water, 50%, and 100% saturated soils. The first was HPLC water, which served as a control. Three microdialysis probes were placed into a 20-mL beaker containing approximately 20 mL of HPLC-grade water. Dialysates (120 µL) were collected for a total of 180 minutes at a flow rate of 2 µL/min. The same sampling procedure was repeated for the 50% and 100% saturated soils. A needle was used to introduce an insertion point into the soil to aid in the implantation of probes into the soils. The dialysates were collected in 0.5-mL microcentrifuge tubes, which were placed on ice during the collection period to minimize microbial activity. Potassium chloride extracts from the soil were also obtained to compare with the concentrations of nitrate-N and ammonium-N obtained from the dialysates collected. The tubes containing dialysates and the KCl extracts were stored at 4 °C until analyzed.

The concentrations of nitrate-N and ammonium-N obtained from the dialysate samples were used to calculate the diffusive flux of the analytes across the semipermeable membrane of the probe. Diffusive flux (J) is defined as being the negative product of the diffusion coefficient, D, and the concentration gradient, $\left(\frac{dC}{dx}\right)$. In this study, the diffusive fluxes of analytes were calculated using the concentration analyte in the dialysate, C_d, the surface area of the membrane, $SA_m = 0.159$ cm^2, and the sampling time, t (equation 11):

$$J = -D\left(\frac{dC}{dx}\right) = \frac{C_d}{SA_m \times t} \tag{11}$$

Spike recovery

Spike with prepared solution. Prepared Lake Keith soil (~32 g) was measured in each of two 20-mL beakers. The volume of water needed to bring the soil to 50% and 100% saturation was calculated as previously mentioned. The soil in one beaker was saturated to 50% and the other to 100% using HPLC water. After allowing the water to equilibrate into the soil pores, three microdialysis probes were inserted into each beaker, and dialysates (120 µL) were collected for a total of 180 minutes at a flow rate of 2 µL/min. A spike solution consisting of a 1:1 (v/v) mixture of 25 µg/mL nitrate-N and 70 µg/mL ammonium-N was prepared in HPLC water. After initial microdialysis collection, the probes were removed from the beakers, 4 mL of the spike solution was added to each beaker of soil, and the soils were thoroughly mixed using a stirring rod. The microdialysis probes were re-inserted and dialysates (120 µL) were again collected for a total of 180 minutes at a flow rate of 2 µL/min. All dialysates were collected on ice for the sampling period. Aliquots of the spike solution were saved for concentration verification during chemical analysis. KCl extracts from the soil were also obtained. All dialysate samples, spike solution aliquots, and KCl extracts were stored at 4 °C.

Spike with Lake Keith water. Sieved (2 mm), homogenized, field-moist Lake Keith soil (~17 g) was measured into a 20-mL beaker. Microdialysis was performed in the field-moist soil with no addition of water. Dialysates (120 µL) were collected for a total of 60 minutes at a flow rate of 2 µL/min using 3 probes. After collection, the volume of water needed to bring the soil to 100% saturation was calculated. The soil was then saturated to 100% using water obtained from Lake Keith. After allowing the soil water to equilibrate, three microdialysis probes were inserted into the beaker, and dialysates (120 µL) were collected for a total of 180 minutes at a flow rate of

2 µL/min. All dialysates were collected on ice during sampling. Aliquots of the spike (Lake Keith water) and dialysates were stored at 4 °C until analysis.

Prepared field-moist Lake Keith soil (~17 g) was measured into each of two 20-mL beakers. The soil in one beaker was saturated to 100% using HPLC-grade water while the soil in the other beaker was saturated to 100% using Lake Keith water. After equilibration of soil water, two 1-g aliquots of soil were removed from each beaker for KCl and water extractions. Microdialysis probes (3 in each beaker) were inserted and dialysates (120 µL) were collected for a total of 180 minutes at a flow rate of 2 µL/min. Aliquots from the HPLC water and Lake Keith water were stored for nitrate-N and ammonium-N concentration verification. All dialysates, aliquots of Lake Keith water, and extracts were stored at 4 °C.

Microdialysis sampling in the greenhouse

Greenhouse study preparation

Composite soil was obtained from the Lake Keith site, sieved using a 6-mm sieve to remove large rocks and debris, and transported to a greenhouse at the Altheimer Lab (University of Arkansas Agricultural Extension Center), for a mesocosm experiment. In July 2019, three Northern switchgrass (*Panicum virgatum*, Daylily Nursery, Rock Island, TN), and three Reed canarygrass (*Phalaris arundinacea*, Daylily Nursery, Rock Island, TN) were planted in 8.5-inch diameter pots (Misco Enterprises, Dunellen, NJ) using approximately 3.05 kg of soil obtained from the site and pots were placed in the greenhouse. The pots containing the plants, along with three control pots of only soil, were watered on alternating days using 265 mL of Lake Keith water starting one month prior to microdialysis sampling. This greenhouse study took place in the months of July and August (2019) under natural light conditions, where temperatures ranged from 31 °C - 50 °C.

Microdialysis probe test

The twelve microdialysis probes needed for the greenhouse study were placed in a standard nitrate-N solution (10 μg/mL) with stirring. Dialysates (80 μL) were collected for three 40-min intervals for a total of 120 minutes at a flow rate of 2 μL/min. The $RR\%$ of each probe was calculated using equation 1.

Probe implantation and general schematic of set-up in the greenhouse

All twelve of the microdialysis probes were randomly assigned and implanted into the rhizosphere of the plants being grown in the greenhouse. A series of tubings with differing diameters were used to insert the probes to a depth of approximately 15 cm. One probe was placed into each pot in groups 1 and 2, while two probes were placed in each pot in group 3. The microdialysis syringe pumps and syringes were assigned randomly to each probe and were placed in the center of the pot set-up (Figures 3 and 4).

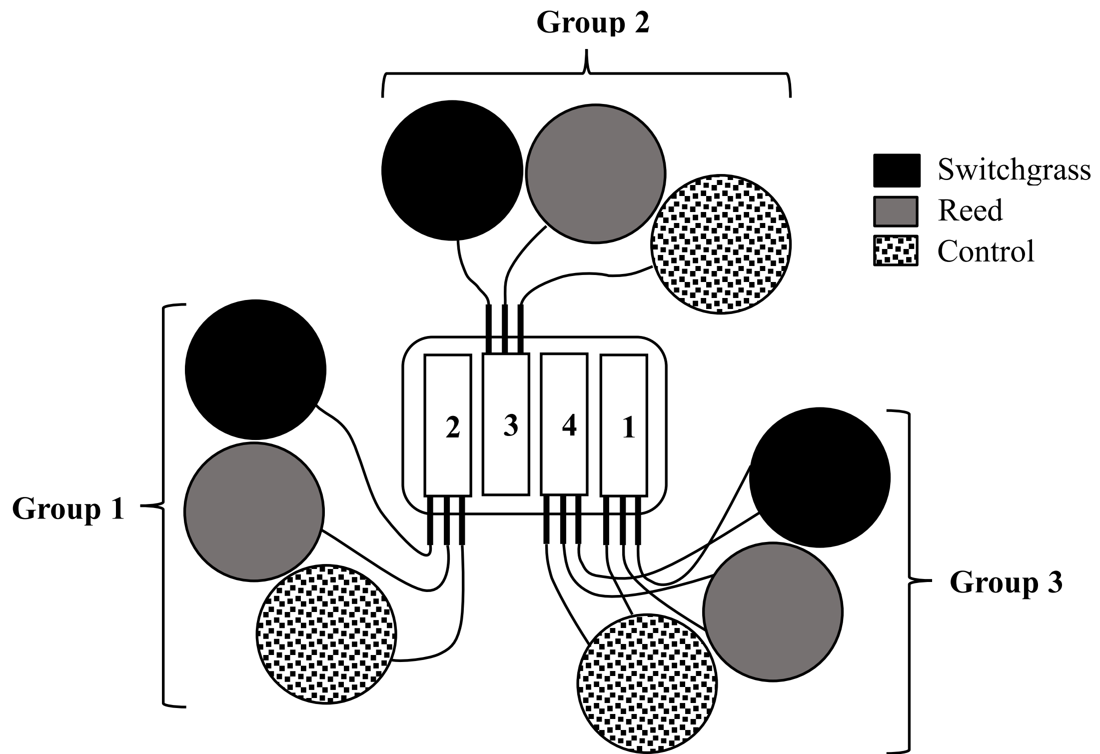

Figure 3. Schematic representation of the set-up of pots in the greenhouse.

Figure 4. Actual set-up of treatment pots in the greenhouse.

Establishment of watering regimes and times of sampling after watering

The initial watering regime consisted of watering all pots with 265 mL of water obtained from the Lake Keith site. Aliquots of the Lake Keith water (500 µL) were saved for verification of nitrate-N and ammonium-N concentrations. In order to establish the optimum time of sampling after watering, dialysates (120 µL) were collected for 60 minutes at four time points: 1.5, 2.5, 4, and 6 hours after watering. Soil moisture, soil temperature, and air temperature readings were recorded at each time point. All dialysates were collected and stored on ice during the duration of the experiment, and then stored at 4 °C.

After obtaining N fluxes from the watering regime with equal volumes of water, a different watering regime was established- differing volumes of water were used to bring the soil in the pots to field capacity, which resulted in volumetric water contents in the range 33-40%. To obtain this range of volumetric water content, 265, 565, and 365 mL of Lake Keith water were added to the control, Switchgrass, and Reed canarygrass pots, respectively. These volumes were determined by measuring soil moisture approximately 10-15 minutes after watering. Dialysates (120 µL), soil moisture, soil temperature, and air temperature readings were recorded at each of four time points: 1.5, 2.5, 4, and 6 hours after watering to determine the optimum time of sampling after employing the second watering regime.

Nitrogen flux data collection

After determining the optimum time after watering for each of the watering regimes, all of the pots were watered with 265 mL of Lake Keith water and dialysate samples from each treatment (120 µL) were collected for 60 minutes four hours after watering. Soil moisture before and during collection, and soil and air temperatures during collection were recorded. All dialysate samples were collected on ice, and an aliquot (500 µL) of the Lake Keith water used

for watering, were stored on ice during the duration of the experiment, and then stored at 4 °C until analysis. Three such samplings were performed in total, with sampling occurring every other day. The same sampling scheme was repeated for the second watering regime, but the volumes of Lake Keith water added were 265, 565, and 365 mL for the control, Switchgrass, and Reed canarygrass pots, respectively.

Plant biomass and soil

After microdialysis experimentation in the greenhouse, each treatment pot was dismantled. The aboveground and belowground biomasses of the plants were separated at the soil surface and placed to dry in an oven at 55 °C for five days. After drying, portions of the biomasses of the plants were ground into a fine powder using a Wiley Mini Mill (3383-L10, Thomas Scientific, Swedesboro, NJ). The soil from each pot was homogenized, and aliquots were oven-dried at 55 °C for five days, and large aggregates were broken up using a mortar and pestle. The soil was then sieved through a 2-mm sieve. Samples were analyzed using combustion analysis (Elementar VarioMax CN Cube, Elementar, Langenselbold, Germany) for total nitrogen of the aboveground and belowground biomasses of the plants and total nitrogen and carbon for the soil samples.

Chemical assays and UV-Vis spectrophotometry

Nitrate-N (Griess reaction)

The Griess reaction occurs in the presence of nitrite, so the nitrate (NO_3^-) in the samples was first reduced to nitrite (NO_2^-) using vanadium chloride (VCl_3). A 0.05M solution of VCl_3 (Strem Chemicals, Newburyport, MA) was prepared in 1M HCl. The solution was mixed using a stir-bar until homogeneous and was slowly passed through a 0.2-µm Whatman syringe filter (GE Healthcare, Chicago, IL) (Miranda et al., 2001; Mulvaney, 1996).

Once the VCl_3 had converted the nitrate in the sample to nitrite, the Griess reaction could be employed for colorimetric analysis. The Griess reaction consisted of two reagents: Reagent 1 consisted of 1% (w/v) sulfanilamide (Tokyo Chemical Industry, Tokyo, Japan) which was prepared in HPLC water, added to 5% (v/v) phosphoric acid; also prepared in HPLC-grade water. Reagent 2 consisted of 0.1% (w/v) N-naphthylethylenediamine (Sigma-Aldrich, St. Louis, MO) in HPLC water. Both Griess reagent 1 and Griess reagent 2 were stored at 4 °C for further use. The Griess reagents were stable for only two weeks and were re-made as necessary. The assay for nitrate in a 96-well plate (3590, Corning Inc., Corning, NY) was as follows: 50 μL of samples or standards, 50 μL of 0.05M VCl_3, 50 μL of Griess reagent 1, and 50 μL of Griess reagent 2. The plate was then covered in aluminum foil and placed on a plate shaker at 200 RPM for 2.5 hours at room temperature.

Ammonium-N (Indophenol Berthelot reaction)

The Indophenol Berthelot reaction requires two solutions: Reagent 1 and Reagent 2. Reagent 1 was prepared by dissolving 3.4 g of sodium salicylate (Alfa Aesar, Haverhill, MA), 2.5 g of trisodium citrate (BeanTown Chemical, Hudson, NH), 2.5 g of sodium tartrate (Alfa Aesar, Haverhill, MA), and 0.0125 g of sodium nitroprusside (MilliporeSigma, Burlington, MA) in 50 mL of HPLC-grade water. The second solution, Reagent 2, contained NaOH (Sigma-Aldrich, St. Louis, MO) and hypochlorite (Clorox, Oakland, CA). A solution of 60 g/L NaOH was prepared in HPLC-grade water, and Reagent 2 was prepared by mixing 10% hypochlorite solution and the prepared NaOH in a 0.2:10 ratio (v/v). Both Reagents 1 and 2 were stored at 4 °C for further use (Mulvaney, 1996; Willis et al., 1996). The assay for ammonium in the microplate was as follows: 40 μL of samples or standards, 80 μL of Reagent 1, and 80 μL of

Reagent 2. The plate was covered in aluminum foil and placed on a plate shaker at 200 RPM for 1.5 hours at room temperature.

UV-Vis spectrophotometry

Absorbance values from the azo dye produced by the Griess reaction were measured at 540 nm using a Tecan infinite m200 plate reader (Tecan, Männedorf, Switzerland). The absorbance values of the colored indophenol dye produced by the Indophenol Berthelot reaction were measured at 650 nm.

Statistical analysis

Statistical analyses were performed using one-way ANOVA followed by Tukey's as a post-hoc test, Student's t-test, two-way repeated-measures ANOVA followed by Bonferroni tests, and ANOVA on Ranks for non-parametric data using SigmaPlot 14.0 (Systat Software, Inc). All statistical analyses were performed using a 95% confidence interval; therefore, differences were considered statistically different at $p \leq 0.05$.

Results

Microdialysis sampling in nutrient solutions

Optimization of microdialysis sampling based on RR%

Optimum flow rate determination. Flow rates of 2.0, 3.75, and 5.0 µL/min were studied, and the resulting relative recovery percentages ($RR\%$) were compared to establish an optimum flow rate for all subsequent experiments. The $RR\%$ of nitrate-N (denoted as mean $\pm$ SE) were 42.7 $\pm$ 1.3 %, 20.4 $\pm$ 0.5 %, and 14.4 $\pm$ 0.4 % for flow rates of 2.0, 3.75, and 5.0 µL/min, respectively (Figure 5; n = 3). The recoveries yielded by the flow rates were all statistically different from each other, with 2.0 µL/min yielding the greatest $RR\%$ ($p < 0.001$).

The $RR\%$ of ammonium-N (denoted as mean $\pm$ SE) were 51.0 $\pm$ 1.2 %, 23.2 $\pm$ 0.5 %, and 15.6 $\pm$ 1.0 % for flow rates of 2.0, 3.75, and 5.0 µL/min, respectively (Figure 5; n = 3). The recoveries yielded by the three different flow rates were all statistically different from each other, with 2.0 µL/min yielding the greatest $RR\%$ ($p < 0.001$).

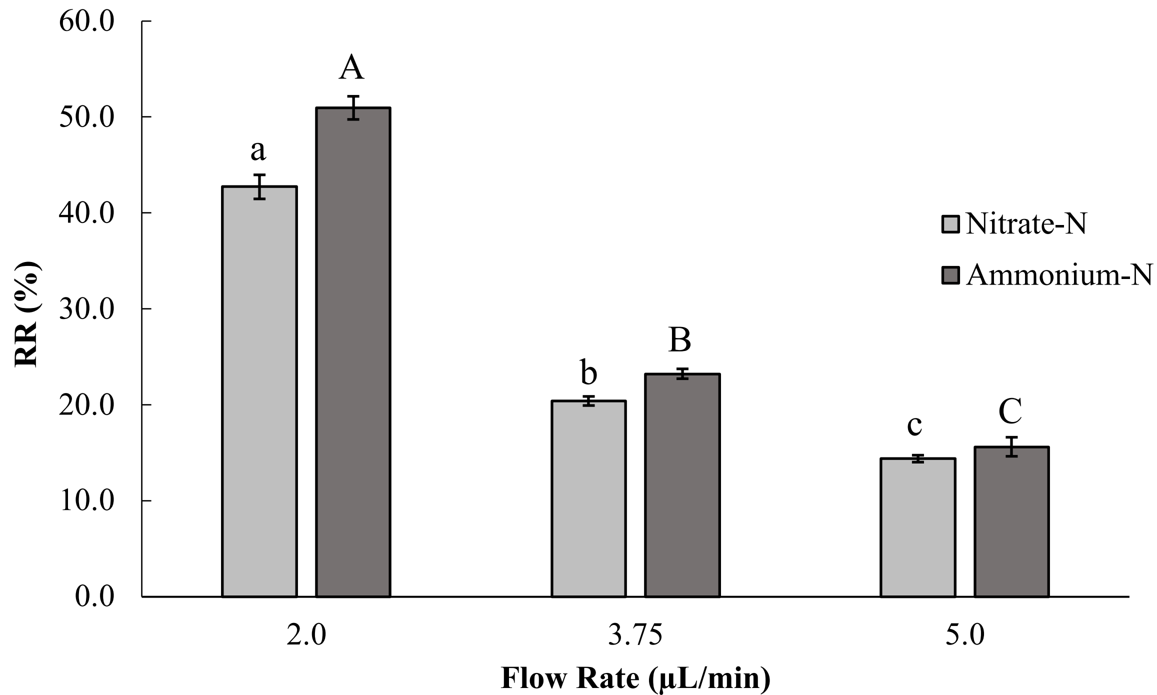

Figure 5. The relative recovery percent ($RR\%$) of nitrate-N and ammonium-N obtained from microdialysis sampling in solutions of 10-µg/mL at flow rates of 2.0, 3.75, and 5.0 µL/min. Nitrate-N and ammonium-N $RR\%$ were analyzed separately. Bars represent means $\pm$ SE (n = 3). Different lowercase letters indicate differences between RR% of nitrate-N ($p < 0.001$) and different uppercase letters indicate differences between RR% of ammonium-N ($p < 0.001$).

Effect of differing sample medium concentrations. Microdialysis was performed in solutions of 1, 3, 5, 8, and 10-µg/mL nitrate-N and ammonium-N, respectively, and the relative recoveries of the analytes from each sample medium were calculated. The relative recoveries of nitrate-N (denoted as mean ± SE) were 37.0 ± 3.1 %, 31.0 ± 1.1 %, 29.3 ± 1.7 %, 38.2 ± 3.7 %, and 29.9 ± 0.3 % in 1, 3, 5, 8, and 10-µg/mL solutions, respectively (Figure 6; n = 3). Varying the concentration of the sample medium did not yield statistically different relative recovery percentages for nitrate-N ($p = 0.093$).

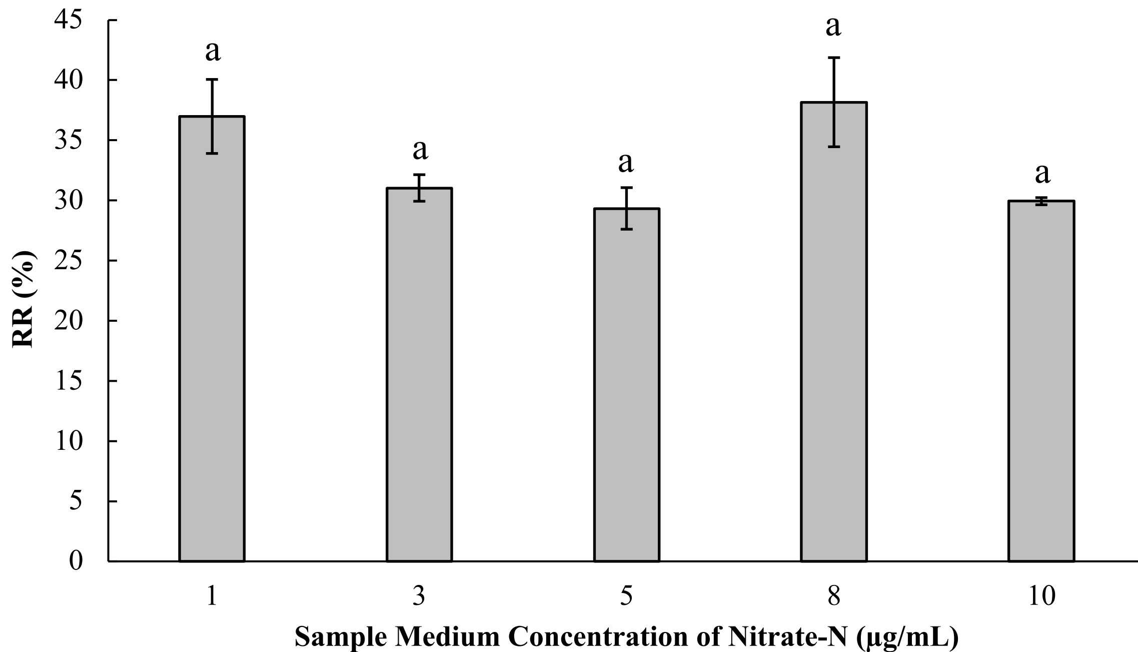

Figure 6. The relative recovery percent ($RR\%$) of nitrate-N obtained from microdialysis sampling in 1, 3, 5, 8, and 10-µg/mL solutions at 2.0 µL/min. Bars represent means ± SE (n = 3).

The relative recoveries of ammonium-N were 46.9 ± 1.3 %, 40.5 ± 3.3 %, 37.2 ± 1.9 %, 43.3 ± 2.8 %, and 45.1 ± 2.8 % in 1, 3, 5, 8, and 10-µg/mL solutions, respectively (Figure 7; n = 3). Varying the concentration of the sample medium did not yield statistically different relative recovery percentages for ammonium-N (p = 0.130).

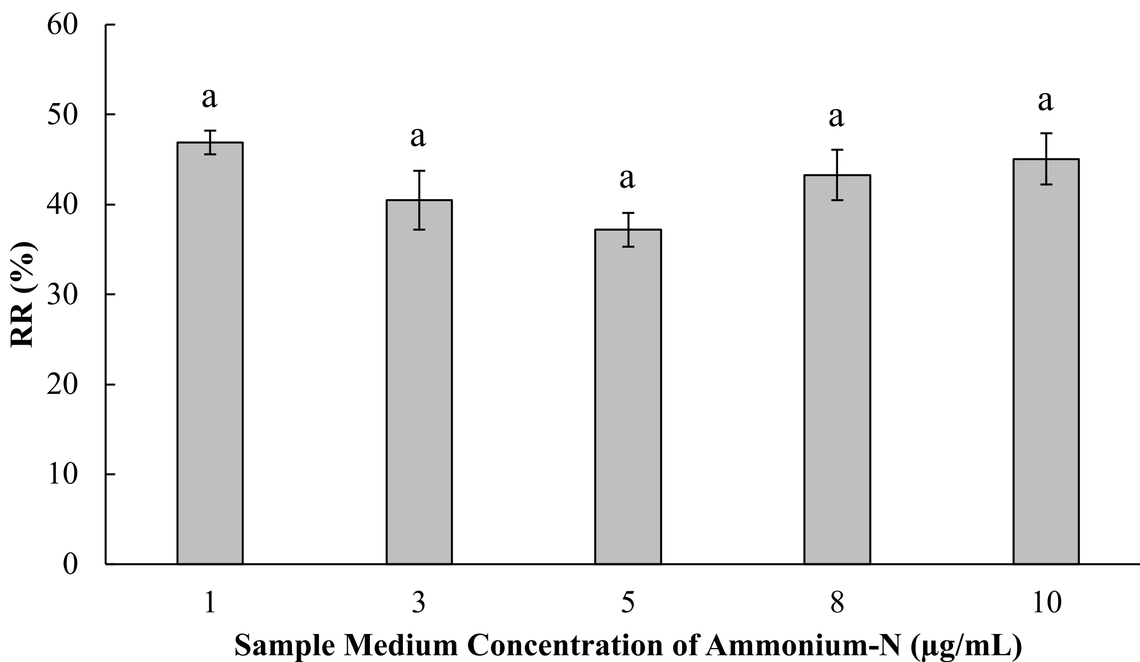

Figure 7. The relative recovery percent (*RR*%) of ammonium-N obtained from microdialysis sampling in 1, 3, 5, 8, and 10-µg/mL solutions at 2.0 µL/min. Bars represent means ± SE (n = 3).

Comparison of RR% of individual analytes with RR% in combined solution. Relative recoveries of the analytes were measured from individual solutions and were compared to the recoveries when the analytes were present in the same solution. In the 5 µg/mL nitrate-N solution, the average *RR*% (denoted as mean ± SE) was 39.9 ± 0.7 % (n = 3), while in the

nitrate-N and ammonium-N combined solution the average recovery was 41.2 ± 0.6 % (n = 3). The $RR\%$ obtained from the individual nitrate-N solution and $RR\%$ obtained from the combined solution were not statistically different (p = 0.199).

The average $RR\%$ in the 5-µg/mL ammonium-N solution and the combined nitrate-N and ammonium-N solution were 43.7 ± 0.8 % (n = 3) and 43.9 ± 1.1 %, respectively (n = 3). The recoveries yielded by the individual ammonium-N solution and the combined solution were not statistically different (p = 0.863).

Lake Keith soil experimental analysis

Soil organic matter (SOM) was 9.31 ± 0.07 % (n = 3), and based on the measured fractions of sand, silt, and clay, the soil at the Lake Keith site was deemed to be a loam (Table 1). Total soil carbon and nitrogen were 5.29 and 0.38 %, respectively. The pH and electric conductivity (EC) were 7.8 ± 0.0 (n = 3) and 251 ± 2 µmhos/cm (n = 3).

Table 1. Summary of Lake Keith soil data reported as mean ± SE (n = 3).

Bulk Density (g/cm^3)	Volumetric Water Content	% Sand	% Silt	% Clay	% SOM	% C	% N	pH	EC (μmhos/cm)
0.84 ± 0.07	0.56 ± 0.03	38.2 ± 1.1	36.6 ± 1.0	25.2 ± 0.3	9.31 ± 0.05	5.29	0.38	7.8 ± 0.0	251 ± 2

Mehlich III Extraction (μg/g soil)										
P	K	Ca	Mg	S	Na	Fe	Mn	Zn	Cu	B
54 ± 1	129 ± 1	5738 ± 28	67 ± 0	27 ± 0	8.2 ± 0.5	197 ± 2	257 ± 3	39 ± 0	3.3 ± 0.0	2.6 ± 0.1

Microdialysis sampling in soil solutions

The effects of soil saturation

Fluxes of nitrate-N (denoted as means ± SE) were 0.05 ± 0.01, 0.08 ± 0.02, and 0.03 ± 0.00 μg mL^{-1} cm^{-2} h^{-1} in HPLC water, 50% saturated soil, and 100% saturated soil, respectively (Figure 8; n = 3). The fluxes in all sample media were not statistically different ($p = 0.076$). The concentration of nitrate-N yielded by KCl extraction of the soil was 1.74 ± 0.24 μg N/g soil (n = 3).

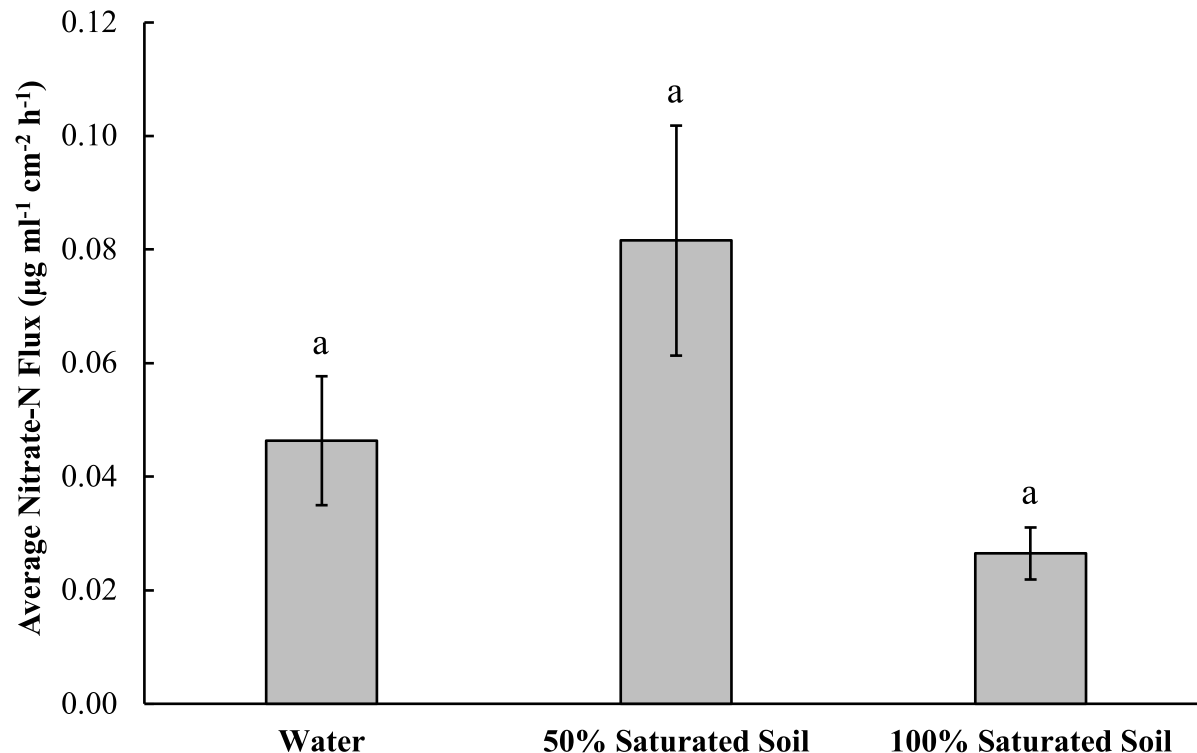

Figure 8. The fluxes (μg mL^{-1} cm^{-2} h^{-1}) of nitrate-N obtained from microdialysis sampling in HPLC water, 50% saturated soil, and 100% saturated soil. Dialysate samples were collected at 2.0 μL/min. Bars represent means ± SE (n = 3).

The fluxes of ammonium-N were 0.28 ± 0.04, 11.54 ± 1.33, and 30.06 ± 0.46 μg mL^{-1} cm^{-2} h^{-1} in HPLC water, 50% saturated soil, and 100% saturated soil, respectively (Figure 9; n = 3). The fluxes in all sample media were statistically different ($p < 0.001$), with ammonium-N fluxes being greatest in the 100% saturated soil. The concentration of ammonium-N yielded by KCl extraction of the soil was 0.96 ± 0.02 μg N/g soil (n = 3).

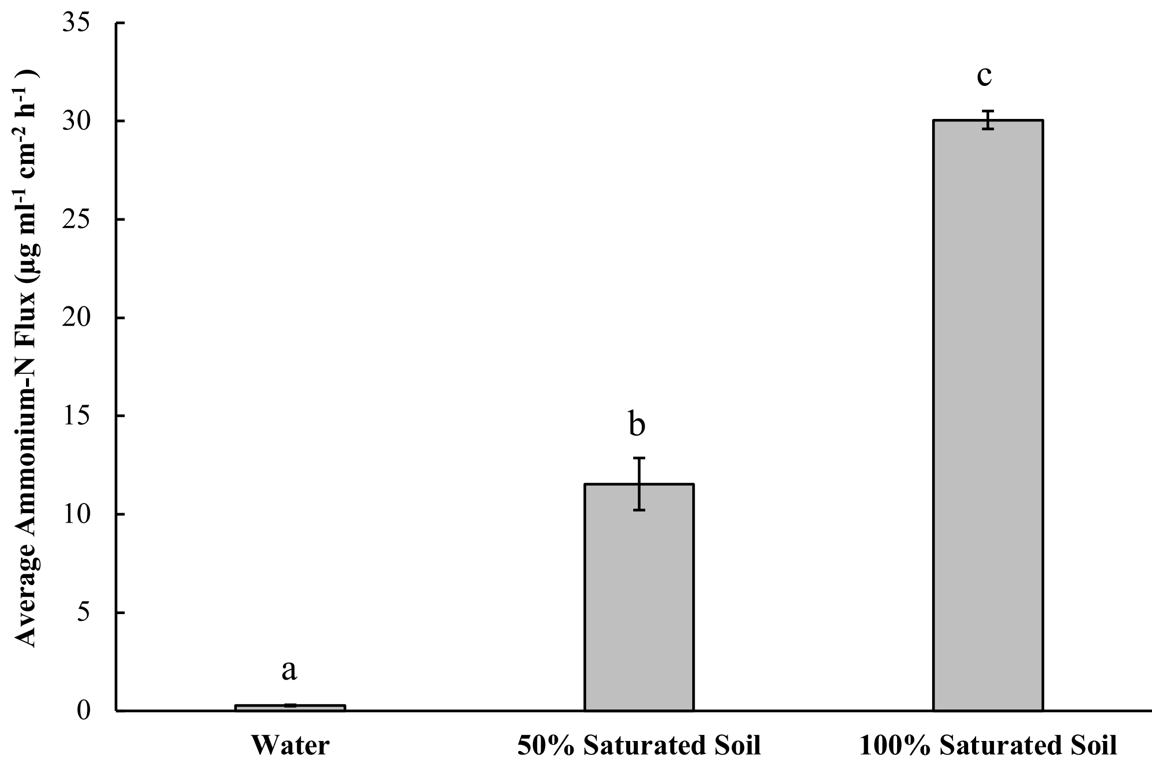

Figure 9. The fluxes (μg mL^{-1} cm^{-2} h^{-1}) of ammonium-N obtained from microdialysis sampling in HPLC water, 50% saturated soil, and 100% saturated soil. Dialysate samples were collected at 2.0 μL/min. Bars represent means ± SE (n = 3). Different letters indicate treatments with statistically different fluxes ($p < 0.001$).

Comparison of spike recoveries

Experimentation with prepared spike. In the 50% saturated soil, the background flux of nitrate-N was not within detection limits and after the spike solution was added, nitrate-N flux was 1.97 ± 1.01 µg mL^{-1} cm^{-2} h^{-1} (Figure 10; n = 3). In the 100% saturated soil, the background flux of nitrate-N was below detection limits and was 0.25 ± 0.25 µg mL^{-1} cm^{-2} h^{-1} (n = 3) after the spike was added. The spiked fluxes obtained from the 50% and 100% saturated soils were not statistically different (p = 0.172). The concentration of nitrate-N yielded by KCl extraction of the soil was 0.85 ± 0.02 µg N/g soil (n = 3).

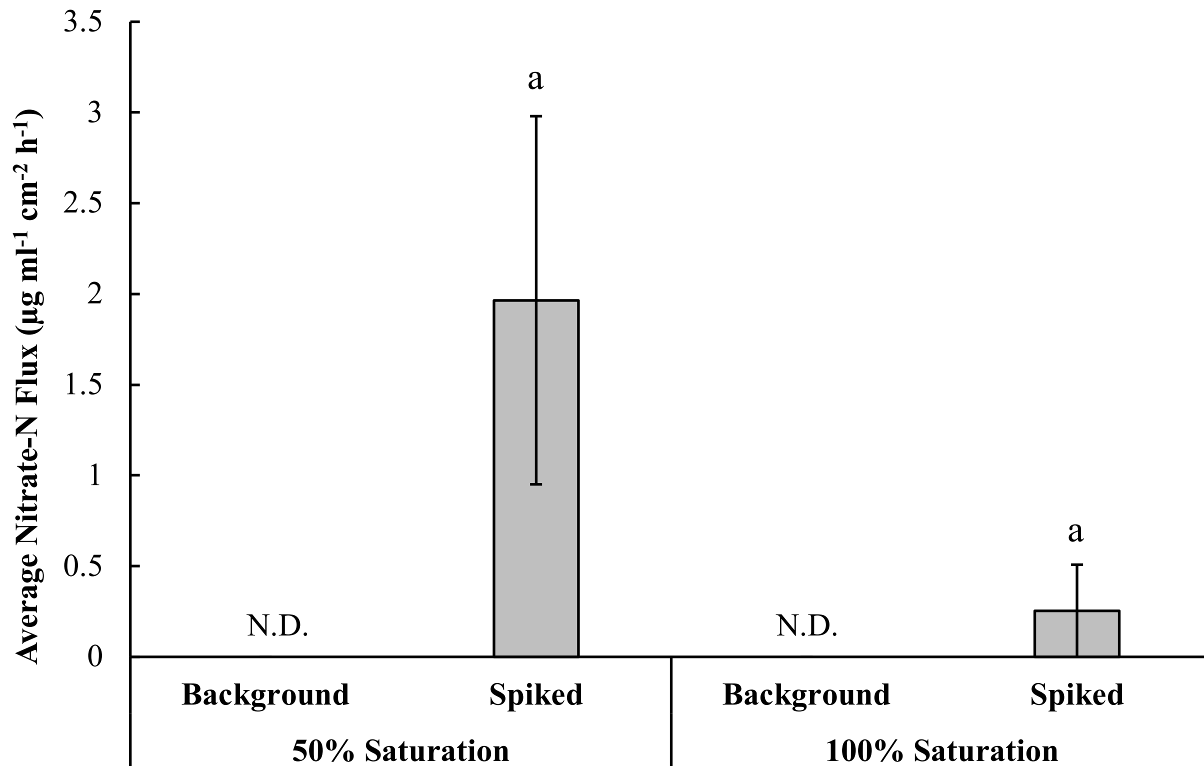

Figure 10. The fluxes (µg mL^{-1} cm^{-2} h^{-1}) of nitrate-N obtained from microdialysis sampling in 50% and 100% saturated soils. Dialysate samples were collected at 2.0 µL/min. Bars represent means ± SE (n = 3).

The background and spiked fluxes of ammonium-N (denoted as means ± SE) in the 50% saturated soil were 24.10 ± 1.85 and 56.98 ± 12.62 μg mL^{-1} cm^{-2} h^{-1}, respectively (Figure 11; n = 3). The background and spiked fluxes in the 100% saturated soil were 119.77 ± 2.50 and 126.34 ± 1.41 μg mL^{-1} cm^{-2} h^{-1}, respectively. When compared across treatments, the fluxes obtained were statistically different from each other ($p < 0.001$). The background and spiked samples obtained from the 100% saturated soil were not statistically different ($p = 0.890$) and exhibited greater fluxes than the 50% saturated soil. Conventional potassium chloride extraction of the soil yielded an ammonium-N concentration of 10.22 ± 0.21 μg N/g soil (n = 3).

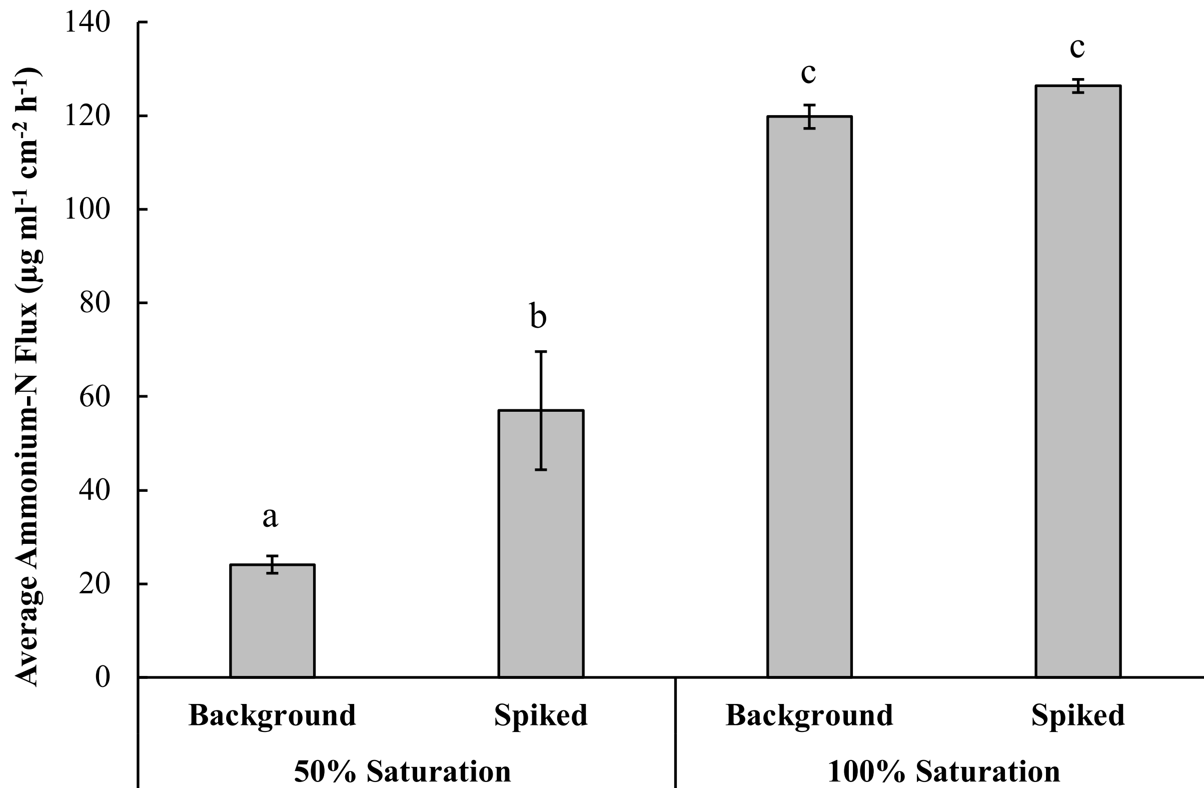

Figure 11. The fluxes (µg mL^{-1} cm^{-2} h^{-1}) of ammonium-N obtained from microdialysis sampling in 50% and 100% saturated soils. Dialysate samples were collected at 2.0 µL/min. Bars represent means ± SE (n = 3). Different letters indicate treatments with statistically different fluxes ($p < 0.001$).

Experimentation with Lake Keith water as a spike. Water obtained from the Lake Keith study site was used as a spike as it is rich in nitrogen. The fluxes of nitrate-N (denoted as means ± SE) were 0.19 ± 0.19 and 72.49 ± 1.58 μg mL^{-1} cm^{-2} h^{-1} (n = 3) in the field-moist and spiked soil dialysate samples, respectively, and were statistically different (Figure 12; $p < 0.001$). The spiked soil exhibited greater fluxes in nitrate-N than the field-moist soil. The water obtained from Lake Keith contained 6.42 ± 0.13 μg/mL of nitrate-N.

Ammonium-N fluxes were below detection limits in the field-moist soil, the spiked soil, and the Lake Keith water.

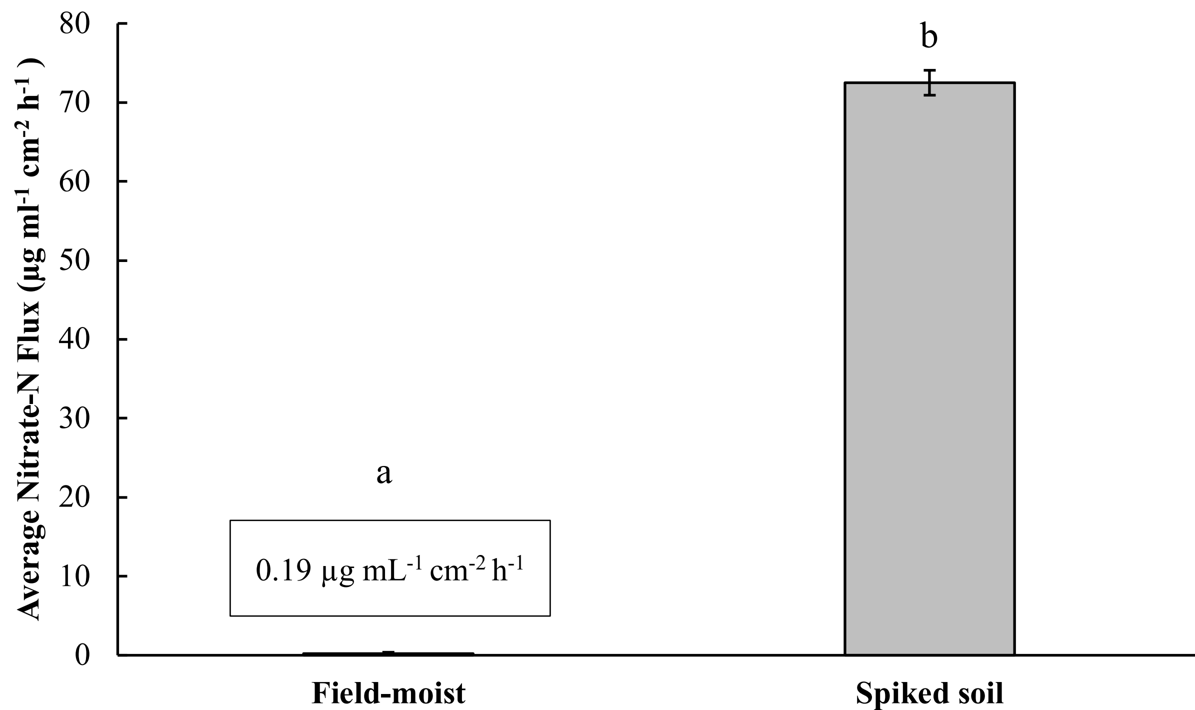

Figure 12. The fluxes (μg mL^{-1} cm^{-2} h^{-1}) of nitrate-N obtained from microdialysis sampling in field-moist and spiked soil. Dialysate samples were collected at 2.0 µL/min. Bars represent means ± SE (n = 3). Different letters indicate treatments with statistically different fluxes ($p < 0.001$).

Fluxes of nitrate-N (denoted as means ± SE) were 63.69 ± 4.67 µg mL^{-1} cm^{-2} h^{-1} and 69.35 ± 0.54 µg mL^{-1} cm^{-2} h^{-1} in the soil spiked with HPLC water and the spiked with Lake Keith water, respectively (Figure 13; n = 3). The fluxes obtained from the two treatments were not statistically different (p = 0.295). The HPLC water contained nitrate-N below detection limits, and the Lake Keith water contained 5.80 ± 0.02 µg/mL nitrate-N.

Fluxes of ammonium-N could not be quantified due to concentrations obtained in the dialysate samples being below detection limits.

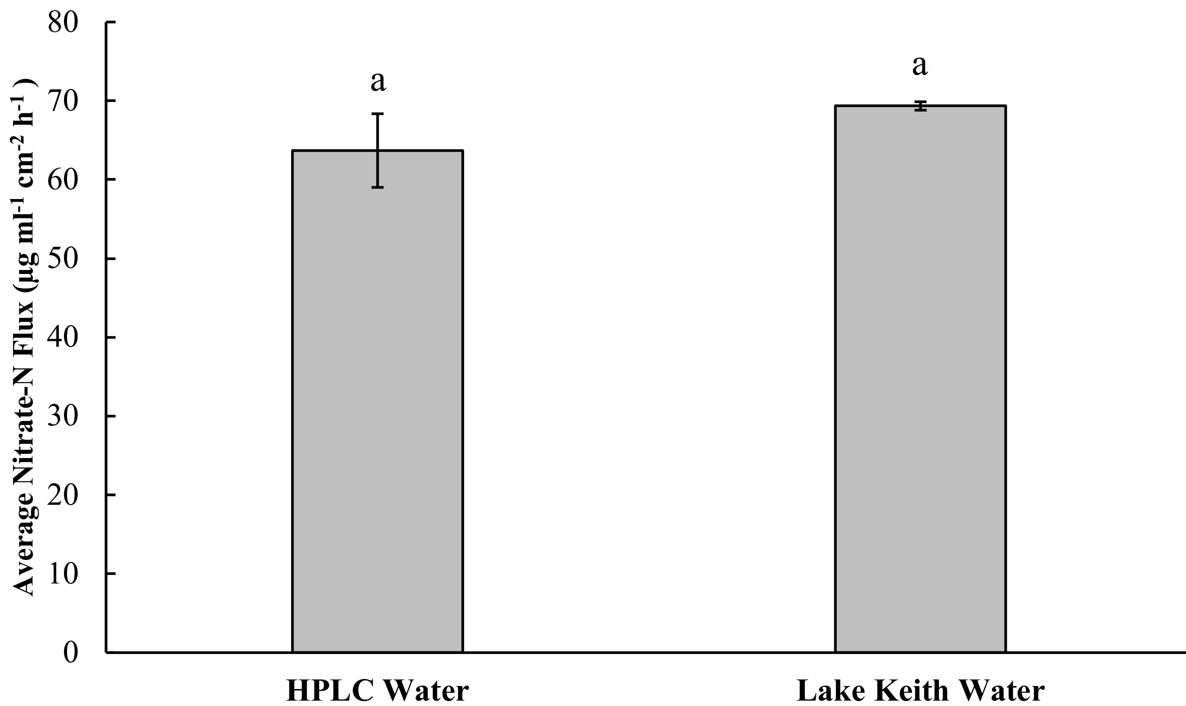

Figure 13. The fluxes (µg mL^{-1} cm^{-2} h^{-1}) of nitrate-N obtained from microdialysis sampling in soils spiked with HPLC water and Lake Keith water. Dialysate samples were collected at 2.0 µL/min. Bars represent means ± SE (n = 3).

The concentrations of free and exchangeable nitrate-N from the soil were obtained from KCl and water extractions. In the soil spiked with HPLC water, nitrate-N concentrations (denoted as means ± SE) were 0.79 ± 0.04 and 1.10 ± 0.03 μg N/g soil from the KCl and water extractions, respectively (Figure 14; n = 3). In the soil spiked with Lake Keith water, concentrations were 0.84 ± 0.02 and 1.45 ± 0.01 μg N/g soil from the KCl and water extractions (n = 3), respectively. Across the treatments, the concentrations of nitrate-N were statistically different ($p < 0.001$), although the concentrations obtained from the KCl extractions were not ($p = 0.984$).

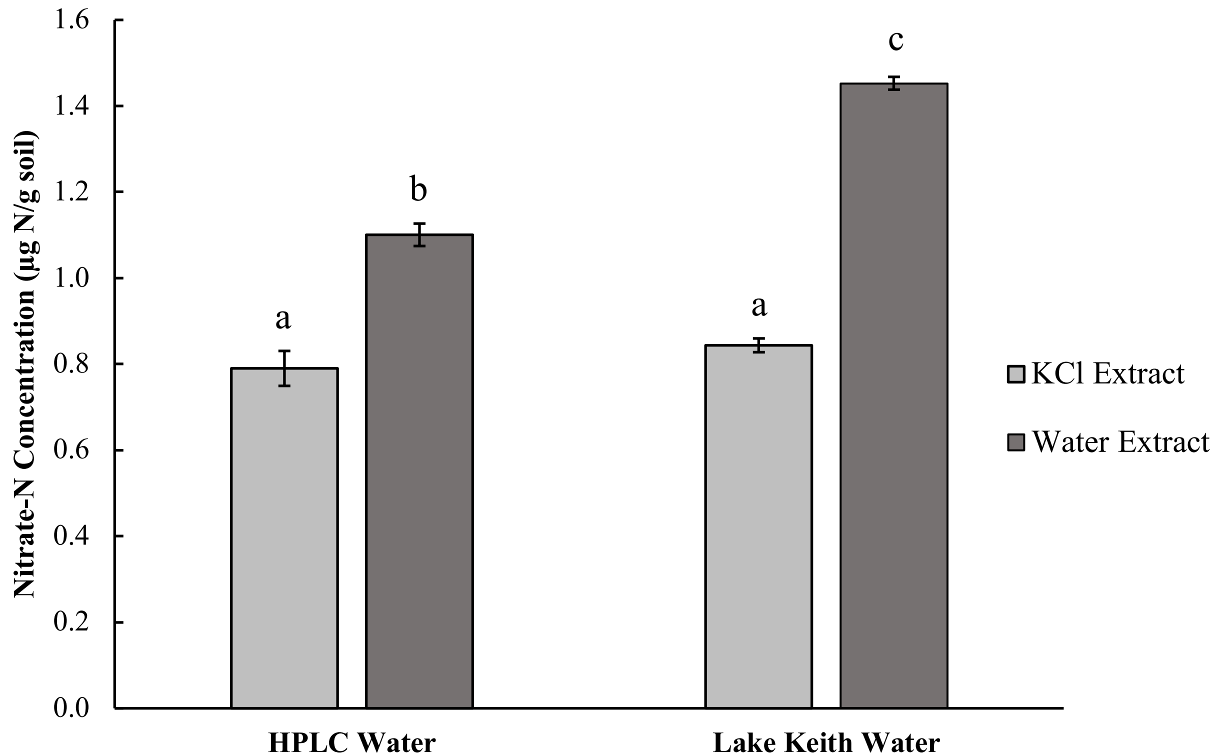

Figure 14. The concentrations (μg N/g soil) of nitrate-N obtained from KCl and water extractions. Bars represent means soil ± SE (n = 3). Different letters indicate treatments with statistically different fluxes ($p < 0.001$).

The concentrations of ammonium-N from the soil were also obtained from KCl and water extractions. In the soil spiked with HPLC water, ammonium-N concentrations were 1.26 ± 0.01 and 1.79 ± 0.02 µg/mL from the KCl and water extractions, respectively (Figure 15; n = 3). In the soil spiked with Lake Keith water, concentrations were 1.04 ± 0.00 and 1.99 ± 0.03 µg N/g soil (n = 3) from the KCl and water extractions, respectively. The concentrations of ammonium-N obtained from all soil and extraction combinations were statistically different ($p < 0.001$).

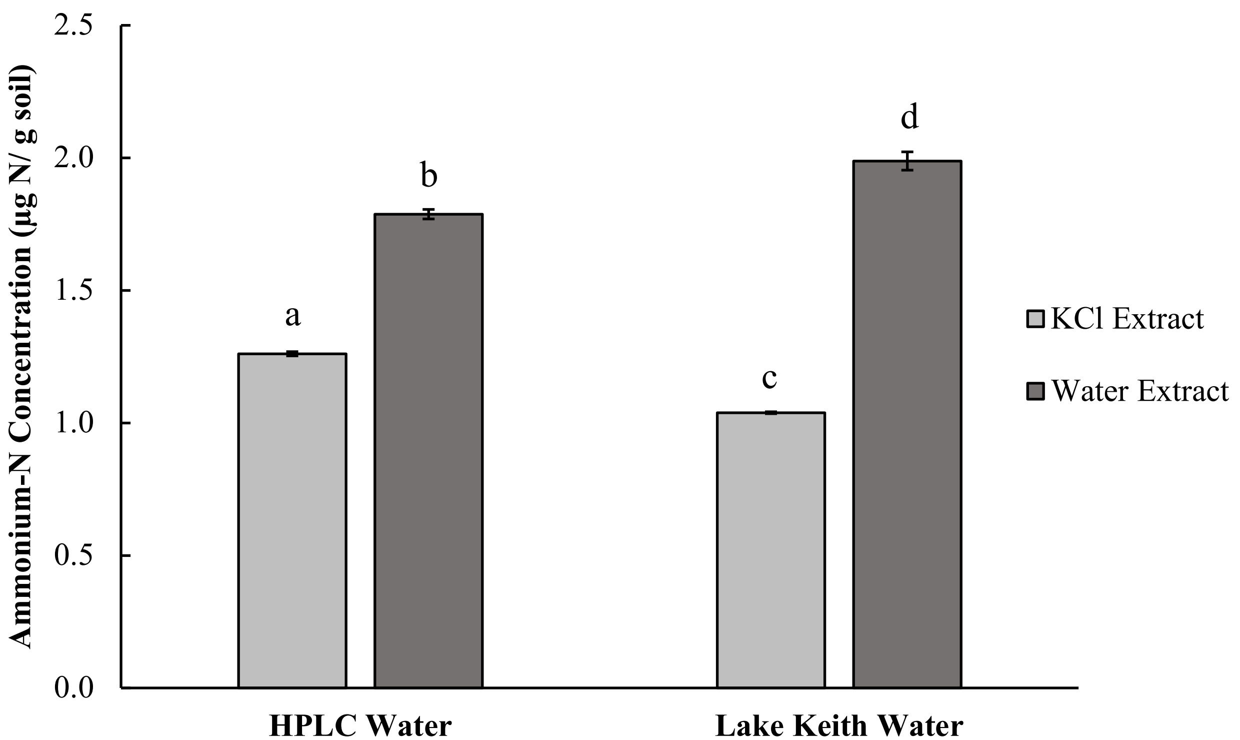

Figure 15. The concentrations (µg N/g soil) of ammonium-N obtained from KCl and water extractions. Bars represent means ± SE (n = 3). Different letters indicate treatments with statistically different fluxes ($p < 0.001$).

Microdialysis sampling in the greenhouse

Twelve-probe comparison

The $RR\%$ of each probe was calculated and compared to each other in order to determine the presence of differences in probe performance. The relative recovery percentages ranged from 48.8% to 79.3% and the probes statistically differed in performance. In order to overcome these differences and to minimize the role of probe variability in the fluxes obtained from the differing treatments, the probes were categorized into groups based on performance and the replicate pots of each treatment were randomly assigned probes from each group. This procedure was followed to avoid complete random assignment, which could result in the assignment of all high- or low-performing probes to all the replicates within one treatment, putting that treatment at a severe advantage or disadvantage in comparison to the other treatments.

Establishment of watering regimes

Constant volumes of water and effects on soil moisture and nitrate-N concentration. The moisture contents within the control treatment (denoted as means $\pm$ SE) were 40.74 $\pm$ 1.16, 38.58 $\pm$ 1.91, 35.19 $\pm$ 1.68, and 33.24 $\pm$ 1.91 % at 1.5, 2.5, 4, and 6 hours after watering, respectively (Figure 16A; $n = 9$). These moisture contents were statistically different from each other ($p < 0.001$); however, consecutive time points did not display statistically different moisture contents. The moisture content at 1.5 hours was not different from 2.5 hours, 2.5 hours was not different from 4 hours, and 4 hours was not different from 6 hours. Nitrate-N fluxes within the control treatment (denoted as means $\pm$ SE) were 237.2 $\pm$ 49.2, 232.6 $\pm$ 40.4, 233.5 $\pm$ 38.8, and 224.7 $\pm$ 38.3 $\mu g\ mL^{-1}\ cm^{-2}\ h^{-1}$ at 1.5, 2.5, 4, and 6 hours after watering, respectively (Figure 16C; $n = 4$). The fluxes of nitrate-N were not statistically different between any of the

sampling times ($p = 0.910$). Ammonium-N fluxes within the control treatment were below detection limits (Figure 16D; $n = 4$).

The moisture contents within the Switchgrass treatment (denoted as means $\pm$ SE) were 31.34 ± 2.12, 30.81 ± 1.54, 27.87 ± 2.02, and 28.31 ± 1.66 % at 1.5, 2.5, 4, and 6 hours, respectively (Figure 16A; $n = 9$). These moisture contents were not statistically different ($p = 0.064$). Nitrate-N fluxes within the Switchgrass treatment (denoted as means $\pm$ SE) were 21.6 ± 18.0, 28.6 ± 25.1, 32.9 ± 26.1, 20.5 ± 17.7 µg mL^{-1} cm^{-2} h^{-1} at 1.5, 2.5, 4, and 6 hours after watering, respectively (Figure 16C; $n = 4$). The fluxes of nitrate-N were not statistically different any sampling time ($p = 0.836$). Ammonium-N fluxes within the treatment were 1.2 ± 1.2, 2.4 ± 2.4, 2.8 ± 2.8, and 3.4 ± 3.4 µg mL^{-1} cm^{-2} h^{-1} at 1.5, 2.5, 4, and 6 hours after watering, respectively (Figure 16D; $n = 4$). The fluxes of ammonium-N were not statistically different between any of the sampling times ($p = 0.981$).

The moisture contents within the Reed canarygrass treatment (denoted as means $\pm$ SE) were 40.89 ± 1.99, 39.96 ± 1.79, 36.71 ± 2.06, and 34.98 ± 1.56 % at 1.5, 2.5, 4, and 6 hours, respectively (Figure 16A; $n = 9$). The moisture contents were statistically different from each other ($p = 0.001$) but were not different between consecutive time points. Nitrate-N fluxes within the Reed canarygrass treatment (denoted as means $\pm$ SE) were 411.0 ± 111.0, 428.3 ± 90.8, 448.3 ± 75.6, and 404.3 ± 49.7 µg mL^{-1} cm^{-2} h^{-1} at 1.5, 2.5, 4, and 6 hours after watering, respectively (Figure 16C; $n = 4$). The fluxes of nitrate-N were not statistically different at any sampling time ($p = 0.982$). Ammonium-N fluxes within the control treatment were below detection limits (Figure 16D; $n = 4$).

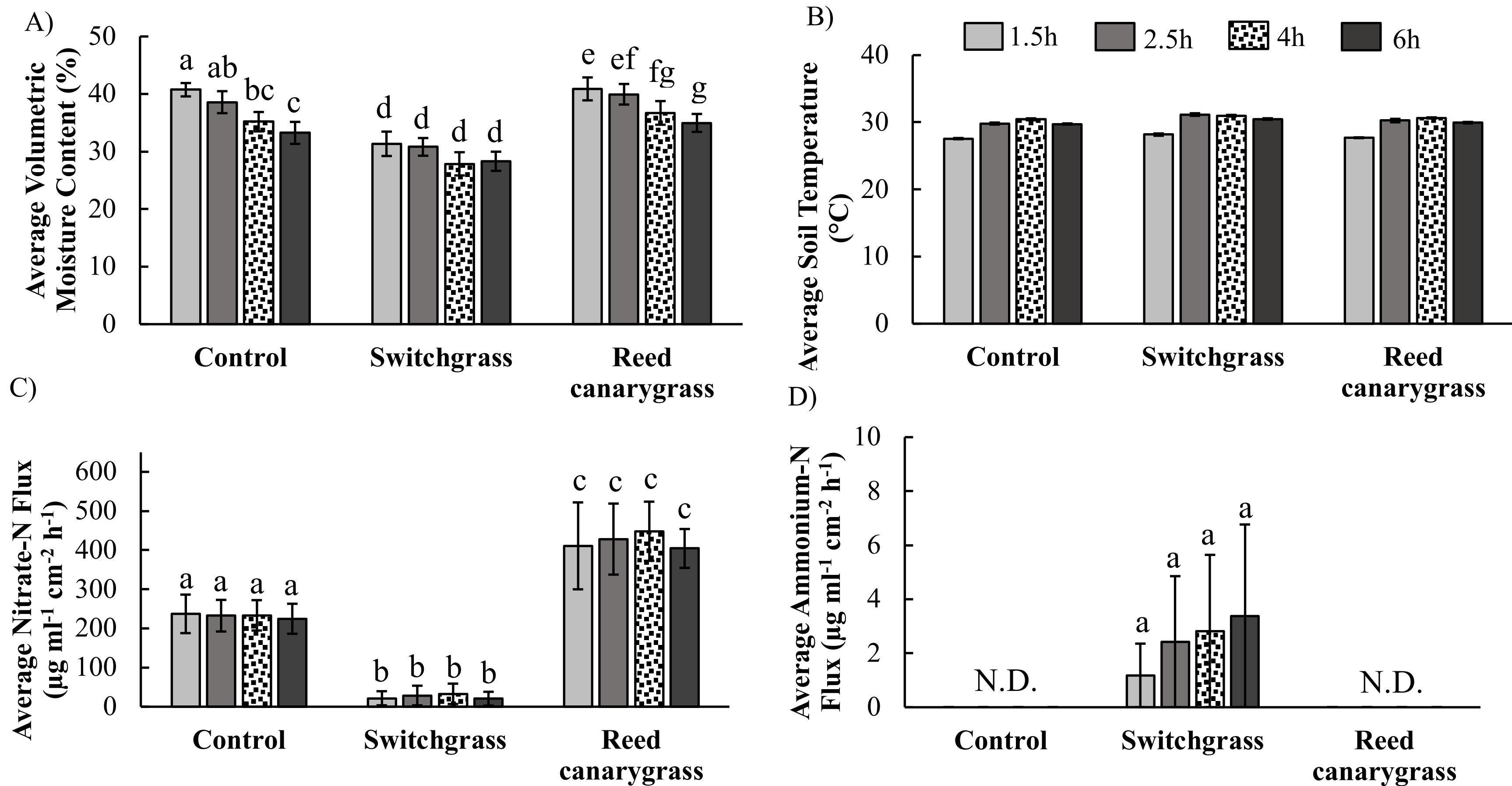

Figure 16. (A) The moisture contents (percentage) (B) soil temperature (°C) (C) nitrate-N fluxes (µg mL^{-1} cm^{-2} h^{-1}) and (D) ammonium-N fluxes (µg mL^{-1} cm^{-2} h^{-1}) in the soil 1.5, 2.5, 4, and 6 hours after watering. Bars represent mean ± SE (n = 9 for moisture and temperature and n = 3 for fluxes). Different letters indicate treatments with statistically different moisture contents and fluxes ($p < 0.001$).

Field capacity and effects on soil moisture and nitrogen fluxes. The treatment pots were watered with different volumes of water to bring volumetric water content of each pot into the 33-40% range, which is approximated to be field capacity in loamy soil (Gholami Bidkhani & Mobasheri, 2018).

The moisture contents within the control treatment (denoted as means $\pm$ SE) were 37.60 $\pm$ 1.78, 36.58 $\pm$ 1.62, 35.57 $\pm$ 1.20, and 33.97 $\pm$ 1.39 % at 1.5, 2.5, 4, and 6 hours after watering, respectively (Figure 17A; $n = 9$). These moisture contents, namely at 1.5 and 6 hours, were statistically different ($p = 0.039$). Nitrate-N fluxes within the control treatment were 131.3 $\pm$ 16.9, 139.9 $\pm$ 10.1, 136.7 $\pm$ 10.2, and 145.5 $\pm$ 10.5 µg mL^{-1} cm^{-2} h^{-1} at 1.5, 2.5, 4, and 6 hours after watering, respectively (Figure 17C; $n = 4$). The fluxes of nitrate-N were not statistically different between any of the sampling times ($p = 0.622$). Ammonium-N fluxes ($n = 4$) within the control treatment were below detection limits.

The moisture contents within the Switchgrass treatment (denoted as means $\pm$ SE) were 27.69 $\pm$ 1.15, 26.89 $\pm$ 0.39, 24.50 $\pm$ 1.58, and 21.04 $\pm$ 1.43 % at 1.5, 2.5, 4, and 6 hours after watering, respectively. These moisture contents were statistically different ($p < 0.001$). Nitrate-N fluxes within the Switchgrass treatment were 2.3 $\pm$ 1.4, 1.4 $\pm$ 1.0, 1.0 $\pm$ 1.0, and 34.2 $\pm$ 34.2 µg mL^{-1} cm^{-2} h^{-1} at 1.5, 2.5, 4, and 6 hours after watering, respectively (Figure 17C). The fluxes of nitrate-N were not statistically different at any sampling time ($p = 0.883$). Ammonium-N fluxes ($n = 4$) within the control treatment were below detection limits.

The moisture contents within the Reed canarygrass treatment were 29.11 $\pm$ 2.17, 27.41 $\pm$ 1.53, 27.22 $\pm$ 1.92, and 24.79 $\pm$ 2.17 % at 1.5, 2.5, 4, and 6 hours after watering, respectively. These moisture contents were not statistically different ($p = 0.081$). Nitrate-N fluxes within the Reed canarygrass treatment were 266.8 $\pm$ 74.2, 296.8 $\pm$ 60.2, 315.5 $\pm$ 63.2, and 255.1 $\pm$ 33.7

μg mL^{-1} cm^{-2} h^{-1} at 1.5, 2.5, 4, and 6 hours after watering, respectively (Figure 17C). The fluxes of nitrate-N were not statistically different at any sampling time ($p = 0.884$). Ammonium-N fluxes ($n = 4$) within the control treatment were below detection limits.

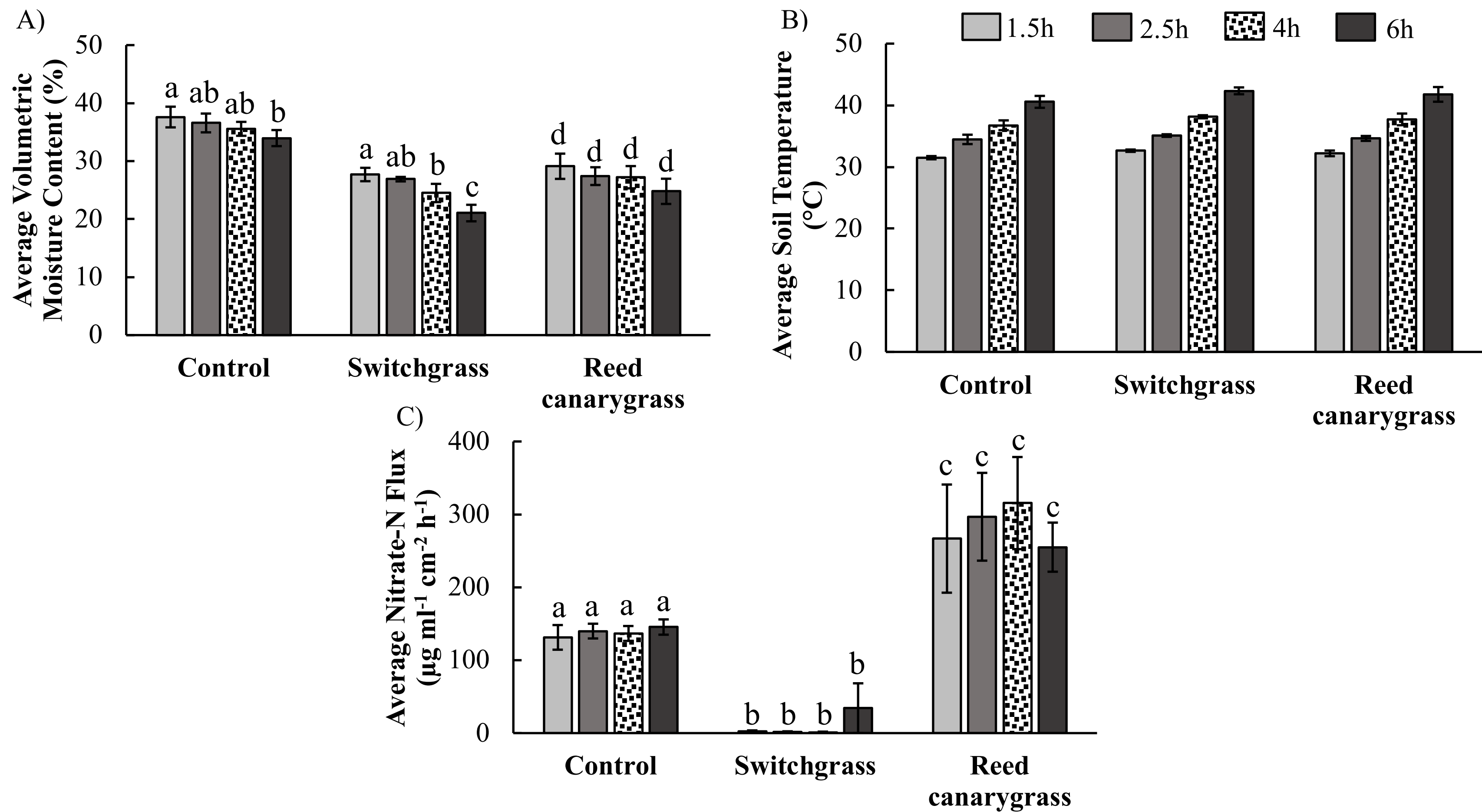

Figure 17. (A) The moisture contents (percentage) (B) soil temperature (°C) and (C) nitrate-N fluxes (µg mL^{-1} cm^{-2} h^{-1}) in the soil 1.5, 2.5, 4, and 6 hours after watering. Bars represent mean ± SE (n = 9 for moisture and temperature and n = 3 for fluxes). Different letters indicate treatments with statistically different moisture contents and fluxes ($p < 0.001$).

Quantifying nitrogen fluxes

Watering regime: constant volumes of water. Nitrate-N fluxes in the control treatment (denoted as means ± SE) were 151.82 ± 76.18, 174.61 ± 18.88, and 152.70 ± 19.60 $\mu g\ mL^{-1}\ cm^{-2}\ h^{-1}$ during sampling days 1, 2, and 3, respectively (Figure 18; n = 4). Fluxes of nitrate-N in the Switchgrass treatment were 8.36 ± 8.36 $\mu g\ mL^{-1}\ cm^{-2}\ h^{-1}$ during sampling day 1 and were below detection levels during days 2 and 3. The fluxes of nitrate-N in the Reed canarygrass treatment were 139.79 ± 64.06, 299.52 ± 55.11, and 352.61 ± 104.55 $\mu g\ mL^{-1}\ cm^{-2}\ h^{-1}$ during days 1, 2, and 3, respectively.

Reed canarygrass exhibited a greater flux in nitrate-N than the Switchgrass treatment during the second and third sampling days. However, neither the Switchgrass nor Reed canarygrass treatments were statistically different from the control during all three sampling days. Fluxes of ammonium-N were below detection limits in all treatments during all sampling days.

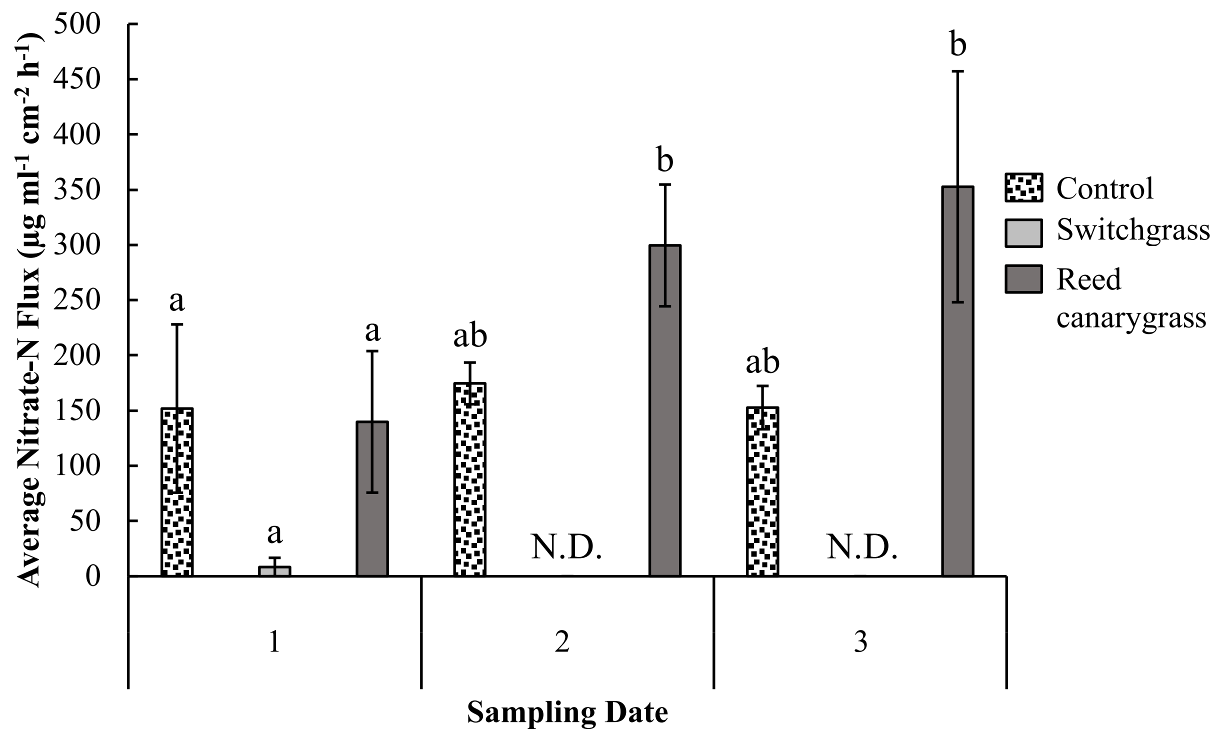

Figure 18. The fluxes (µg mL^{-1} cm^{-2} h^{-1}) of nitrate-N obtained from microdialysis sampling in the control, Switchgrass, and Reed canarygrass treatments over three sampling days. The same volumes of water were added to each treatment. Dialysate samples were collected at 2.0 µL/min. Bars represent means ± SE (n = 4). Different letters indicate treatments with statistically different fluxes.

Watering regime: field capacity. Nitrate-N fluxes in the control treatment were 133.71 ± 35.32, 128.48 ± 6.85, and 100.83 ± 20.11 μg mL^{-1} cm^{-2} h^{-1} during sampling days 4, 5, and 6, respectively (Figure 19; n = 4). Fluxes of nitrate-N in the Switchgrass treatment were 2.25 ± 1.22, 0.64 ± 0.52, and 1.07 ± 0.87 μg mL^{-1} cm^{-2} h^{-1} during sampling days 4, 5, and 6, respectively. The fluxes of nitrate-N in the Reed canarygrass treatment were 478.33 ± 140.79, 505.53 ± 126.80, and 516.17 ± 180.01 μg mL^{-1} cm^{-2} h^{-1} during days 4, 5, and 6, respectively.

During these three sampling days, the control and Switchgrass treatments were not statistically different and the Reed canarygrass treatment exhibited greater nitrate-N fluxes than both the control and Switchgrass treatments.

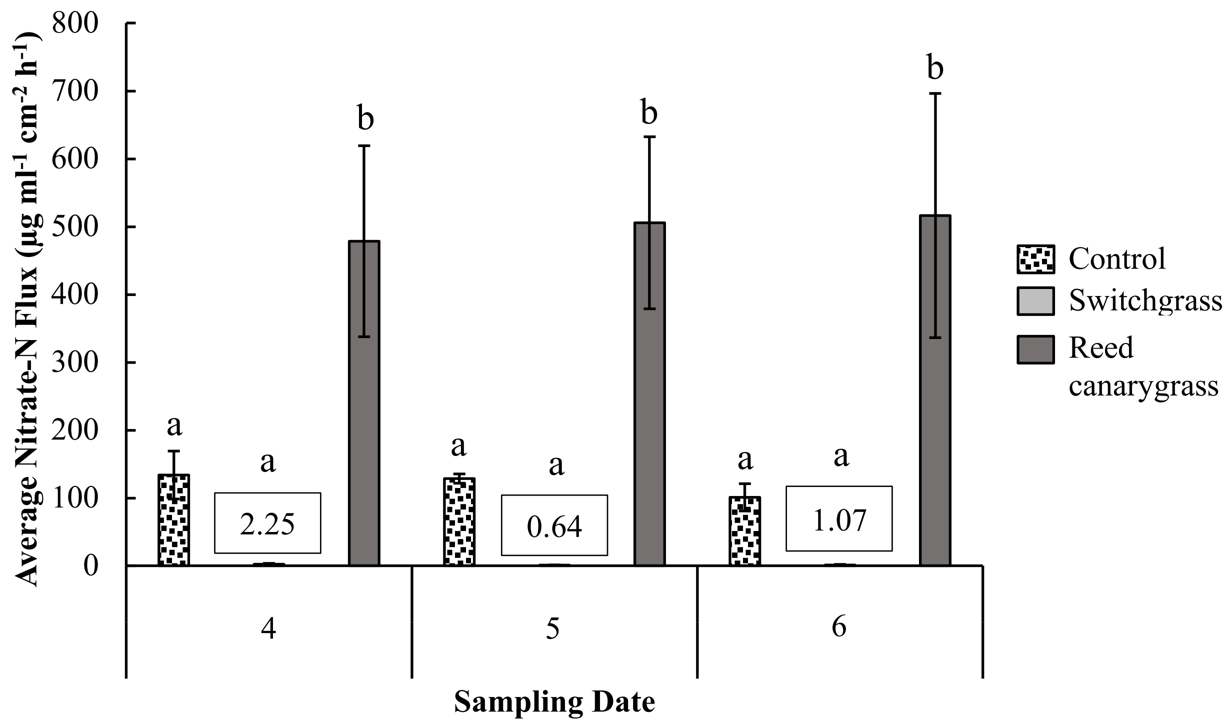

Figure 19. The fluxes (µg mL^{-1} cm^{-2} h^{-1}) of nitrate-N obtained from microdialysis sampling in the control, Switchgrass, and Reed canarygrass treatments over three sampling days. Each treatment was watered to bring the moisture content of the soil to field capacity. Dialysate samples were collected at 2.0 µL/min. Bars represent means ± SE (n = 4). Different letters indicate treatments with statistically different fluxes.

Plant biomass and soil nitrogen

The nitrogen in the Switchgrass plants (denoted as mean ± SE) was 0.33 ± 0.03 and 0.60 ± 0.02 g N/g biomass for aboveground and belowground biomasses, respectively (Table 2; n = 3). Nitrogen in the biomass of the Reed canarygrass plants was 0.16 ± 0.01 and 0.08 ± 0.02 g N/g biomass for the aboveground and belowground biomasses, respectively. Nitrogen in the

aboveground biomasses and belowground biomasses were statistically different ($p = 0.004$ and $p < 0.001$, respectively) between the plant species.

The total N in the Switchgrass plants (denoted as mean $\pm$ SE) was 10.43 ± 1.39 and 22.24 ± 0.92 for aboveground and belowground biomasses, respectively ($n = 3$). Total N in the Reed canarygrass plants was 0.98 ± 0.08 and 0.47 ± 0.18 for aboveground and belowground biomasses, respectively ($n = 3$). When comparing the two plant species, the Switchgrass plants contained greater amounts of N in the aboveground ($p = 0.002$) and belowground biomasses ($p < 0.001$) than the Reed canarygrass plants.

Total soil N percentages (denoted as means $\pm$ SE) were 0.36 ± 0.01, 0.30 ± 0.00, and 0.35 ± 0.00 % in the control, Switchgrass, and Reed canarygrass treatments, respectively ($n = 3$). Total carbon percentages were 5.38 ± 0.23, 5.04 ± 0.01, and 5.27 ± 0.01 % in the control, Switchgrass, and Reed canarygrass treatments, respectively ($n = 3$). When comparing among the three treatments, the soil in the Switchgrass pots contained a decreased quantity of nitrogen in comparison to the control and Reed canarygrass treatments ($p < 0.05$).

Table 2. Nitrogen concentration and total nitrogen in *Panicum virgatum* and *Phalaris arundinacea* biomass reported as mean ± SE (n = 3).

	Switchgrass		**Reed canarygrass**	
	N Concentration (g N/g plant)	**Total N (g N)**	**N Concentration (g N/g plant)**	**Total N (g N)**
Aboveground	0.33 ± 0.03	10.43 ± 1.39	0.16 ± 0.01	0.98 ± 0.08
Belowground	0.60 ± 0.02	22.24 ± 0.92	0.08 ± 0.02	0.47 ± 0.18

Discussion

The objectives of this study were to: 1) optimize the microdialysis technique to the rhizosphere setting in order to quantify fluxes of N during plant uptake, 2) develop a mesocosm experiment to study the fluxes of N in the rhizospheres of *Panicum virgatum* (Switchgrass) and *Phalaris arundinacea* (Reed canarygrass), and 3) study the efficacy of these plants to hyperaccumulate N by analyzing results obtained from microdialysis flux and plant biomass data.

Optimization of microdialysis sampling for rhizosphere setting

The efficiency of microdialysis sampling can be measured in two ways: relative recovery and mass recovery. Relative recovery is the ratio between the concentration of analyte collected in the dialysate to the analyte concentration in a sample medium. Mass recovery is the amount of analyte extracted from the sample per unit time (de Lange, 2013; Kehr, 1993). Relative recovery can be readily calculated when microdialysis is performed in solutions of nitrate-N and ammonium-N, as the solutions can be sampled to calculate the ratio between the dialysates and the sample medium. Accomplishing this task becomes difficult when sampling in soil, an incredibly complex matrix where analytes can be affected by microbial degradation and oxidation-reduction reactions, adsorption to soil particles, availability of soil water, and other physico-chemical processes (Inselsbacher et al., 2011). Therefore, while initial optimization of the microdialysis technique was based on relative recovery percentage ($RR\%$), mass recoveries are used for soil sampling.

The effect of differing flow rate on the recovery of nitrate-N and ammonium-N was studied in order to determine the optimal flow rate for microdialysis sampling. Flow rate affects both recovery and sampling times: slower flow rates yield greater relative recoveries but also

result in longer sampling times, which could be problematic to the microdialysis equipment such as the syringe pumps as they are exposed to heat, moisture, and a non-sterile environment for longer periods of time. Prior studies employ a flow rate of 5.0 μL/min and report low recoveries of target molecules from the soil (Buckley et al., 2017; Inselsbacher et al., 2014; Inselsbacher et al., 2011; Shaw et al., 2014). Therefore, flow rates of 2.0, 3.75, and 5.0 μL/min were studied, and a flow rate of 2.0 μL/min was determined to be an optimum rate for subsequent experiments using CMA 20 microdialysis probes (Figure 5). Relative recoveries at 2.0 μL/min were significantly greater than the other two flow rates and would result in sampling times of 60 minutes in order to sample the needed volumes for nitrate-N and ammonium-N chemical analysis.

The effect of compound concentration on the relative recoveries was studied in order to determine if the concentrations of analytes obtained in the dialysate samples were still proportional to the concentrations in the sample medium regardless of the magnitude of the actual concentration of the sample medium. Determining the presence of sample medium concentration effect on the recoveries of nitrate-N and ammonium-N was important to the overall study of quantifying N fluxes in the soil. The concentration of N in the soil is a dynamic property and is constantly changing depending on microbial activity, precipitation, temperature, and other abiotic and biotic factors. If the recoveries of the analytes were contingent on the concentrations present, precise quantification of the diffusive fluxes occurring the rhizosphere would not be possible. However, according to this set of experiments, the $RR\%$ for both nitrate-N and ammonium-N did not display a statistically significant dependence on the concentration of analyte present in the sample medium (Figures 6 and 7).

The presence of nitrate-N and ammonium-N in the same solution was studied in order to study the differences in microdialysis performance in the laboratory setting. Both forms of N are present in the soil environment and are sampled simultaneously during microdialysis sampling. Since the analytes are of opposite charges and different molecular weights, this study was performed to determine if the presence of both analytes impose interferences or confound measurement. Interferences were not present, and the presence of both nitrate-N and ammonium-N in the same solution did not exhibit any influence on the $RR\%$ of either analyte.

Development of a mesocosm experiment

The development of a mesocosm experiment in the greenhouse required preliminary experimentation with microdialysis sampling in soil solution. Microdialysis is a diffusion-based sampling technique, and therefore is dependent on the presence of analytes in the aqueous phase. The volumetric water content of a soil is an extremely dynamic property and changes throughout the day depending on various environmental factors such as temperature, season, humidity, precipitation, geological conditions, and biological processes. The overarching objective of this study was to investigate the efficacy of wetland plant uptake of N for the construction of a riparian buffer zone along the southern bank of Lake Keith. Therefore, the volumetric water contents studied were 50% and 100%, where 50% saturation is the theoretical optimum soil saturation for aerobic processes and 100% is complete inundation of soil pores. While soil moisture contents vary widely in the environment, these two saturations were studied due to their relevance to the wetland setting.

Mass recoveries were also used in soil solution instead of relative recoveries. These mass recoveries were used to calculate the diffusive flux of the analytes in the samples. Diffusive flux, the amount of analyte which passes across the semipermeable membrane of the microdialysis

probe, can provide substantial real-time information about root uptake kinetics of nitrate-N and ammonium-N in microsites such as the rhizosphere (Buckley et al., 2017). Therefore, all following soil microdialysis data were reported as fluxes.

The percentage of water-filled pore space (WFPS) did not have a significant effect on the fluxes of nitrate-N; 50% and 100% saturated soils had statistically similar fluxes (Figures 9 and 11). The mobility and free movement of the nitrate ion results in a mostly homogeneous distribution of nitrate-N in the soil, thus nitrate-N fluxes do not show a significant dependence on soil saturation (Inselsbacher et al., 2011). However, according to Figure 9, field-moist soil (9.9% volumetric water content) yielded significantly less diffusive flux in nitrate-N, which may be attributed to the absence of adequate moisture present in the soil for concentration gradient establishment and for movement of analyte across the microdialysis probe to occur. The absence of adequate moisture was not expected to be problematic during mesocosm experimentation; the appropriate watering regime would be adopted to prevent moisture contents below 28%.

On the other hand, WFPS had a significant effect on the fluxes of ammonium-N. Ammonium-N, unlike nitrate-N, is highly retained in the soil due to adsorption to negatively charged clay particles and organic matter. The increase in saturation from 50% to 100% yielded greater fluxes in the 100% saturated soil (Figures 10 and 12).

According to Inselsbacher et al., conventional soil extractions with KCl yielded greater concentrations of all nitrogenous compounds (inorganic and organic) in comparison to the water extractions. Our study shows strikingly different results, with the water extractions yielding greater concentrations of both nitrate-N and ammonium-N (Figures 14 and 15). This difference might be attributed to differences in the chemical composition of the soils used in the individual studies.

Efficacy of wetland plants

The plants used in this study belong to differing wetland indicator statuses: Switchgrass is a facultative (FAC) plant whereas Reed canarygrass is a facultative-wet (FACW) plant. Therefore, these plants are adapted to differing growing conditions and availability of water. The dynamic nature of soil water and other differences in physiological and biochemical processes such as magnitude of root and shoot growth, rate of hyperaccumulation, and rhizosphere microbial composition regulate fluxes of nitrogen. One of the objectives of this study was to determine the efficacy of hyperaccumulation by the chosen plants, which is governed by the aforementioned physiological and biochemical differences. In order to account for the possible effect of differing bioavailability of soil water on the studied N fluxes, two watering regimes were established: 1) constant volumes were added to each of the treatments and 2) differing volumes of water were added to bring the overall moisture content to 33-40%, which was estimated to be approximately field capacity in a loamy soil.

In order to determine the time of sampling after watering for each watering regime, dialysates were collected at 1.5, 2.5, 4, and 6 hours after watering for flux quantification. Soil moisture and soil temperature data were also collected. A time point at which there was still adequate moisture present for diffusive fluxes, along with quantifiable nitrate-N and ammonium-N diffusive fluxes in all treatments was chosen.

According to the data presented in Figure 16, 4 hours after watering was chosen because water content was not statistically different compared to 2.5 hours or 6 hours in the control or Reed canarygrass treatments, and because water content was not statistically different from all other time points in the Switchgrass treatment. The soil water in the pores needed to equilibrate upon watering, so 1.5 hours was not preferred, and since the moisture contents at 4 hours were

similar to the moisture contents at 2.5 hours, it was decided to allocate more time for equilibration. The diffusive fluxes in nitrate-N were not statistically different among time points within each treatment, and the quantifiable fluxes of ammonium-N in the Switchgrass treatments were also not different between time points. Therefore, the optimum time of sampling 4 hours after watering was selected based on the soil moisture contents.

A second watering regime was adopted in order to determine if the differences in fluxes of nitrate-N across the treatments were due to the actual root dynamics during hyperaccumulation or if it was due to the availability of water in the soil (Figure 17). While the same volumes of water were added to each treatment, the plants used were of different indicator statuses and adapted for different bioavailabilities of soil water, which could impact the quantified diffusive fluxes. Therefore, the moisture content in each treatment was brought to field capacity to ensure that the same amount of water was available for the plants and microdialysis probes. The same procedure was followed for determining the sampling time after watering. One striking difference between the data from the first watering regime and second was that the mass recovery of ammonium-N was not above detection limits for any of the treatments (Figure 17). The diffusive fluxes of nitrate-N were not statistically different among all time points within each treatment, so only the differences in soil moisture content was studied for determination of sampling time. The 4-hour time point was deemed appropriate as it was not statistically different to 2.5 hours for all treatments.

The major objective of this study was to quantify the fluxes of N in the forms of nitrate-N and ammonium-N in the rhizospheres of Switchgrass and Reed canarygrass plants during hyperaccumulation of N. The use of the first watering regime yielded significantly different nitrate-N fluxes between the two plants. The Reed canarygrass treatment exhibited greater fluxes

of nitrate-N in soil than the Switchgrass treatment, which did not exhibit fluxes above detection limits (Figure 18). However, neither of the plant treatments yielded statistically different fluxes than the control. During the time period of the second watering regime, the Reed canarygrass exhibited soil fluxes of nitrate-N greater than the control and Switchgrass treatment (Figure 19). Although the fluxes in the control treatment were not statistically greater than the fluxes in the Switchgrass treatment, fluxes of nitrate-N were very low in the Switchgrass treatment in comparison to the other two treatments.

The fluxes of nitrate-N in the Switchgrass treatment were either significantly decreased or were below detection limits across both watering regimes. This could be attributed to the Switchgrass plants readily hyperaccumulating the nitrate-N being added. On the contrary, the Reed canarygrass treatment consistently exhibited the greatest fluxes of nitrate-N, which may be attributed to microbial nitrification activity within the rhizosphere. Evidently, the Reed canarygrass rhizosphere was accommodating of nitrification either through bacterial or archaeal ammonia and nitrite oxidizers, resulting in the production of nitrate, despite inputs of water with nitrate-N present. Nitrate-N was not measured in the dialysate of either the control or Switchgrass treatments, and thus the difference in the fluxes of nitrate-N exhibited can be ascribed to the presence of the Reed canarygrass rhizosphere, as all treatments contained soil from the same source, all received Lake Keith water, and all pots were subject to the same conditions. If the source of increased nitrate-N was the Lake Keith water itself, the control treatment would exhibit similar fluxes to the Reed canarygrass treatment, which was not the case. Therefore, the increased fluxes of nitrate-N in the Reed canarygrass was attributed to the presence of exudates and metabolites exclusive to the rhizosphere of Reed canarygrass, which functioned to sustain the production of nitrate.

Fluxes of ammonium-N across both watering regimes and within in all treatments were below detection limits. A possible reason may be the chemicals used in the colorimetric assay to detect ammonium, and a solution to increase detection may be to substitute salicylate with 2-pheylphenol sodium salt tetrahydrate to lower the detection limit (i.e. increase the intensity) of the ammonium-N chemical analysis method (Hood-Nowotny et al., 2010).

During the phytoremediation technique of phytoextraction, contaminants, in this case excess nutrients, are extracted from the soil and into harvestable biomass (Panesar, 2019). Therefore, to determine efficacy of hyperaccumulation aboveground and belowground biomasses of the Switchgrass and Reed canarygrass plants were analyzed for biomass N (Table 2). Of the two plants, Switchgrass displayed the greatest concentrations and amounts of total N within its above- and belowground biomasses, with the greater accumulation in its roots. Switchgrass's increased N bioaccumulation, along with its greater biomass formation makes it a physiologically adept plant species for future phytoremediation efforts at the Lake Keith study site.

Conclusion & Future Work

This study revealed that in situ N sampling can be accomplished successfully using the microdialysis technique. Although the technique and equipment are still yet to be completely adapted to environmental studies, microdialysis has shown to be a viable alternative to measure movement of soil nutrients rather than use of conventional methods of soil extractions for soil concentrations of extractable nutrients. The results from this study also indicated that in the soil obtained from surrounding Lake Keith, a mesic Typic Paleudult, nitrate-N flux was not limited by soil moisture content in the ranges studied. In conclusion, Switchgrass displayed a high

efficacy of N hyperaccumulation as displayed by temporal nitrate-N distribution and total N analysis of above- and belowground biomass.

Future directions for this study would be to optimize the microdialysis method for successfully sampling and analyzing ammonium-N. The reagents used for the colorimetric reaction, along with a change in perfusion fluid are two possible ways to increase the concentrations/fluxes of ammonium-N to detectable levels. Studying the microbial communities of the rhizosphere during N uptake would also provide valuable insight into the roles of microorganisms in nutrient cycling and could be accomplished by PCR techniques and DNA sequencing.

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