DIEL REDOX CYCLING AND ITS IMPACT ON INORGANIC NITROGEN IN AN ENGINEERED WETLAND DESIGNED FOR WATER TREATMENT

by

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ABSTRACT

The Prado Wetland Basin located near Orange County, CA consists of experimental, unvegetated, wetland cells that were designed and implemented to remove incoming nitrate (NO$_3^-$) from the diverted Santa Ana River, an anthropogenically-impaired drinking water source. NO$_3^-$ is a federally-mandated compound that causes human disease and eutrophication of waterbodies. The sediment, a.k.a. biomat, within these wetland cells is instrumental in nitrogen transformations and removal and consists of photosynthetic diatoms, bacteria, and archaea. The processes by which this biological consortium removes NO$_3^-$ remain elusive.

In order to assess NO$_3^-$ removal in these experimental cells, surface water samples were collected at the inlet and outlet of a mature, open-water wetland cell with a hydraulic residence time of one day during June and September of 2018. Physical and chemical parameters, such as pH, dissolved oxygen (DO), nitrogen oxides, and dissolved metals, revealed that the wetland water chemistry changes on a day versus night basis, or by a predictable, diel pattern.

Since the biomat is a key facilitator in nitrogen transformation and removal within the wetland cell, inorganic nitrogen species within the biomat porewater were quantified at various depths during day and night conditions. Porewater sampling revealed a diel pattern in NO$_3^-$ and nitrite (NO$_2^-$) at more surficial biomat depths, as well as the presence of oxidized nitrogen species at deeper biomat depths, depths assumed to harbor extremely reduced conditions. The presence of intermediate, inorganic nitrogen species in the surface and porewater provide clues as to the fate and removal of nitrogen within the wetland.

Stable nitrogen isotope tracer experiments using $^{15}$NO$_2^-$ were performed to quantify nitrogen transformation and removal pathways on a day versus night basis. Of the measured biochemical reactions, all reactions were determined to occur faster at night than during the day. Coupled nitrification-denitrification, a metabolic process that converts aqueous nitrogen
to gaseous nitrogen, was identified as the dominant nitrogen removal pathway during both the day and night. An additional pathway that does not contribute to the net removal of aqueous nitrogen was identified. More research is needed to quantify other nitrogen cycling pathways not addressed here.

The identification and quantification of nitrogen transformation rates and documentation of diel changes in surface and porewater chemistry on a day versus night basis within these experimental, unvegetated, wetland ecosystems can better inform wetland design and operation, leading to water quality and ecosystem optimization.
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1.1 The Nitrogen Cycle

The nitrogen cycle is a series of microbially-mediated oxidative and reductive reactions of organic, inorganic, aqueous, and gaseous nitrogen species that contributes and intertwines with global nutrient cycling (Figure 1.1). The nitrogen cycle begins with nitrogen fixation, the conversion of dinitrogen gas ($N_2$) into aqueous ammonia ($NH_3$). $NH_3$ is fixed as organically-bound $NH_3$ and is liberated into free-form $NH_3$ via ammonification. Aqueous $NH_3$ is in equilibrium with aqueous ammonium ($NH_4^+$), with the pKa of $NH_4^+/NH_3$ equal to 9.3. In most freshwater environments, $NH_4^+$ is more common than $NH_3$ simply because of the pKa (Hemond & Fechner 2015). In the presence of oxygen ($O_2$), $NH_4^+$ can act as the substrate for nitrifying bacteria and archaea, thereby oxidizing $NH_4^+$ to nitrogen oxides – nitrate ($NO_3^-$) and nitrite ($NO_2^-$). $O_2$ acts as the terminal electron acceptor during nitrification. Denitrification is the anaerobic reduction of nitrogen oxides into the gaseous byproducts nitrous oxide ($N_2O$) or $N_2$ (Martens 2004). $NO_3^-$ and $NO_2^-$ act as the terminal electron acceptors in denitrification. Complete denitrification is when nitrogen oxides are reduced to $N_2$, whereas incomplete denitrification produces $N_2O$, a potent greenhouse gas.

There are several “shortcuts” in the nitrogen cycle, those reactions predominantly being anaerobic ammonium oxidation (anammox) and dissimilatory nitrate reduction to ammonium (DNRA). Anammox is the coupled, anaerobic conversion of $NO_2^-$ and $NH_4^+$ to $N_2$. $NO_2^-$ is the electron acceptor, while $NH_4^+$ is the electron donor. The conditions that select for and facilitate anammox involves the complex cohabitation and interaction of many microbes. There are several microbial community combinations that can occur to create anammox conditions. At its simplest, anammox bacteria can coexist with ammonium oxi-
dizing bacteria (AOB), thereby creating an environment where the AOB convert NH$_4^+$ to NO$_2^-$, and then NO$_2^-$ becomes the primary substrate for anammox bacteria. AOB also consumes the majority of dissolved oxygen (DO), creating an anaerobic environment for the obligate anaerobic anammox bacteria. Anammox bacteria can also coexist with nitrate reducing bacteria (NRB), which produce NO$_2^-$ as a byproduct of biosynthetic processes. NO$_2^-$ is the preferred substrate for anammox bacteria, so this combination, or a mixture of AOB, NRB, and anammox is likely to be found in an ecosystem capable of anammox (Zhu et al. 2010). DNRA is a process that converts NO$_3^-$ to NH$_4^+$ under NO$_3^-$-limiting conditions. The first step of denitrification, the reduction of NO$_3^-$ to NO$_2^-$, is a five-electron transfer reaction, whereas DNRA is an eight-electron transfer reaction (Madigan et al. 2015). DNRA has the potential to reduce N$_2$O emissions by redirecting electrons away from the denitrification pathway and into the DNRA pathway (Jones et al. 2017).

1.2 Influences on the Nitrogen Cycle and Environmental Implications

In natural systems, nitrogen fixation, the conversion of N$_2$ into NH$_3$, is the rate limiting step for the nitrogen cycle as a whole (Galloway et al. 2003). The Haber-Bosch process accelerates nitrogen fixation, making soluble nitrogen substrates readily available for agri-
cultural applications. A fraction of the soluble nitrogen produced from the Haber-Bosch process, usually \( \text{NH}_3 \) or urea, runs off agricultural lands in the form of \( \text{NO}_3^- \) and pollutes fresh, drinking water sources (Glibert et al. 2014, Smith et al. 2007).

The maximum contamination level (MCL) for \( \text{NO}_3^- \) in municipal drinking water is 10 mg/L \( \text{NO}_3^- \) as N, a value set by US Environmental Protection Agency (USEPA). MCLs are enforceable standards that are determined with the public’s health and safety in mind (USEPA 2009). High concentrations of \( \text{NO}_3^- \) in drinking water (>10 mg/L \( \text{NO}_3^- \) as N) can cause the potentially fatal Blue Baby Syndrome, a condition that decreases the blood’s ability to carry \( \text{O}_2 \) in infants (Knobeloch et al. 2000). In addition to adverse human health effects, high \( \text{NO}_3^- \) concentrations in surface water is a direct cause of eutrophication, or the overloading of nutrients into ecosystems, which leads to harmful algal blooms and massive fish kills (Mclsaac et al. 2001, Boesch et al. 2001, Glibert et al. 2005, Glibert et al. 2014).

Orange County Water District (OCWD) in Southern California, an arid and highly populated area, has embraced many new technologies for drinking water treatment. Orange County is home to the Aquifer Recharge Basin, where drinking water is treated to USEPA drinking water standards and stored in the local groundwater aquifer until its distribution through the municipal water infrastructure. Of the many progressive technologies OCWD has adopted, one of them is the utilization of engineered wetlands to treat anthropogenically impaired surface water (SAWPA 2005).

1.3 The Use of Engineered Wetlands for Nitrate-Impaired Surface Water

A wetland is defined as, “areas flooded or saturated by surface water or groundwater often long enough to support vegetation and aquatic life that are adapted to saturated soil conditions.” A plethora of research exists on vegetated wetland ecosystems because they are naturally occurring and have a high potential for nutrient storage and recycling (Hammer 1989). Wetlands have been studied and engineered for various water treatment applications, including wastewater, agricultural runoff, acid mine drainage, and landfill leachate (Stottmesiter et al. 2003, Conrad 1996, Mays & Edwards 2001, Barr & Robinson 1999).
Prado Wetlands is located in Riverside County in Southern California, USA (Figure 1.2). The primary purpose of these engineered wetlands is to remove non-point source NO$_3^-$ pollution from the Santa Ana River (SAWPA 2005). NO$_3^-$ is a prolific, water soluble nutrient that poses a risk to human and ecosystem health, as well as air and water quality. Typical NO$_3^-$ removal during peak summer months in a similar, pilot-scale system exceeded 80% (Jasper et al. 2014). Mean residence time through the entire wetland property (200 ha) is roughly 100 hours (Lin et al. 2003, Jasper et al. 2013). The Prado Wetland flow is then discharged into Chino Creek, which provides water to the Orange County Aquifer Recharge Facility (SAWPA 2005).

Prado Wetlands as a whole is a collection of smaller, constructed cells with varying physical properties, such as size, depth, and residence time. A majority of the wetland designs implemented at Prado are traditional, vegetated wetlands that remove NO$_3^-$ via plant uptake and assimilation and a smaller portion consists of new, experimental, unvegetated, shallow wetlands that remove NO$_3^-$ via diffuse bioactive sediment (a.k.a biomat). The “A” cell design at Prado Wetlands are unvegetated, shallow, open-water treatment cells that

Figure 1.2: Site location map.
minimize hydraulic shortcutting and provide more predictable hydraulic residence time. Each cell is lined with a geotextile liner and is split into three, smaller cells, thereby optimizing hydraulic flow and functional predictability (Jasper et al. 2013).

Cell 3A is a 250-meter long, unvegetated, engineered wetland that is characterized by a hydraulic residence time of one day and is colonized by a carbon-rich, microbially-active biomat (Figures 1.3 & 1.4, Bear et al. 2017). The biomat has been previously characterized as a carbon-rich, microbially active consortium of diatoms, bacteria, and archaea, while also being naturally stratified and offering microenvironments of varying redox potential and metabolic substrate (Jasper et al. 2014, Jones 2017). Biomat thickness, on average is between 2.5 cm to 15 cm; deeper biomat thickness trends towards the inlet of Cell 3A, while shallower biomat thickness trends towards the outlet. Despite observing high NO$_3^-$ removal during the peak summer months, fundamental nitrogen cycling processes and underlying mechanisms within the biomat is elusive (Jasper et al. 2014, Jones 2017). The complexity of the biomat arises from natural stratification, a gradient of redox potential, and the intertwined nature of nutrient webs.

Figure 1.3: A view of Cell 3A from the outlet in September 2018. In-situ columns were installed and sampled in June and September at the boardwalk on the left. Porewater samples were also collected just off the left boardwalk. The boardwalk was constructed out of cinder blocks, long wooden boards, and plywood for the platform. Boardwalks were necessary to construct so that personnel had easy access to the center of the wetland flow path without disturbing the biomat. The wetland inlet is ~250 meters away and the boardwalks are ~70 meters away from this vantage point.
Figure 1.4: Aerial imagery of the Prado Wetland Basin provided by Google Maps. The lateral distance from the inlet to the outlet of the A cells is roughly 250 meters. The sampling site for both June and September incubations occurred ~70 meters from the outlet of Cell 3A (square symbol). ISCO samplers were present at the inlet and outlet of Cell 3A on the June trip and only at the outlet of the September trip (star symbol). Locations are represented relative to the wetland and are not exact or to scale.
CHAPTER 2
DIEL CHANGES IN SURFACE WATER QUALITY ACROSS THE LENGTH OF AN
UNVEGETATED ENGINEERED WETLAND

2.1 Introduction

Diel changes in water chemistry are caused by changes in redox potential, a measurement for the amount of potential chemical energy that can be transferred between an oxidant and reductant species. The most energetic, naturally-occurring, redox reactions involve $O_2$ as the terminal electron acceptor, followed by $NO_3^-$, $MnO_2$, $Fe(OH)_3$, $SO_4^{2-}$, and $CO_2$. (Hemond & Fechner 2015). The solubility and speciation of contaminants, as well as the ability and efficiency of an ecosystem to transform or remove these contaminants, is dependent on the redox state. It is important to understand and characterize diel changes in water quality within natural and engineered aquatic systems and its relation to redox potential because variations in water chemistry give insight into overall ecosystem and wetland functionality.

This study aims to quantify the degree of diel changes to water quality in-situ within Cell 3A, one of the experimental, macrophyte-free, shallow, open-water treatment, engineered cells in Prado Wetlands that is hypothesized to greatly contribute to nitrogen removal via microbial nitrogen cycling processes (Jasper et al. 2014, Jones et al. 2017). Diel changes in various aqueous physical and chemical parameters were monitored in Cell 3A in both June and September of 2018, including but not limited to inorganic nitrogen species, alkalinity, pH, dissolved oxygen (DO), and many dissolved metals. The observed diel pattern in Cell 3A is especially important to understand and document because the functionality and nitrogen removal capabilities of the wetland as a whole has the potential to vary not only seasonally, but also within a 24-hour window. The background knowledge developed here will allow for deeper inquiry into nitrogen cycling processes in a later chapter and can also inform wetland management practices.
2.2 Methods

2.2.1 Field Techniques

Water column physical parameters were collected using the Eureka Water Probes Manta multiprobe, submersible sonde equipped with an optical DO sensor, a specific conductivity (SpC) probe, a temperature probe, and a glass, pH electrode. These background physical parameters were monitored on a diel basis for ≥ 60 hours with 15-minute resolution. Each probe was calibrated on site according to the manufacturer’s recommendations on the day of sonde deployment. The sondes were placed at the inlet and outlet of Cell 3A on the June and September 2018 site visits, with the sensors a few centimeters above the biomat. Both inlet and outlet sondes did not record data on the September 2018 site visit. The sondes were deployed again in October 2018 and functioned without mishaps (Note in Figure 2.1 that the comparison of sonde data is being made between June and October conditions, not June and September).

Inlet and outlet surface water chemistry samples were collected using Teledyne ISCO autosamplers. Inlet water was collected at a weir connecting the forebay to the wetland. Outlet water was collected just before the outflowing weir on the outlet side of Cell 3A. The sampling scheme for water column chemistry for each site visit can be found in Table 2.1. ISCO samples were collected in 1 L plastic bottles and stored on ice. In June, the 1 L sample collected by the ISCO autosampler was filtered using a 60 mL plastic syringe and 0.45 µm Millex syringe filter in an on-site laboratory within ≤ 6 hours of sample collection. In September, the 1 L sample was filtered through a 0.45 µm cartridge filter connected to a peristaltic pump in an on-site laboratory within ≤ 6 hours of sample collection. All samples from June and September were filtered directly into the appropriate containers and preserved accordingly. A list of samples collected on the June and September site visits along with the preservation method can be found in Appendix A (Table A1).
Table 2.1: Sampling scheme for water column chemistry for each site visit in 2018.

<table>
<thead>
<tr>
<th></th>
<th>Sample interval (hours)</th>
<th>Samples collected at every time point</th>
<th>Samples collected at random intervals</th>
<th>Samples collected manually</th>
<th>Sample location</th>
</tr>
</thead>
<tbody>
<tr>
<td>June</td>
<td>2</td>
<td>cations, anions</td>
<td>alkalinity</td>
<td>N$_2$O, CH$_4$</td>
<td>inlet, outlet</td>
</tr>
<tr>
<td>September</td>
<td>3</td>
<td>cations, anions, dissolved metals, alkalinity</td>
<td>none</td>
<td>N$_2$O, CH$_4$</td>
<td>outlet, few at inlet</td>
</tr>
</tbody>
</table>

2.2.2 Analytical Techniques

June NH$_4^+$ and major dissolved cation samples were analyzed using a Dionex ICS-5000 Ion Chromatograph (IC) system equipped with CS12A analytical column, CG12A guard column, and CERS 500 4-mm suppressor. The columns were kept at 30°C during analysis. The line voltage for the suppressor was set to 47 mA using the Chromeleon software provided by Dionex. 20 mM methanesulfonic acid was used as the eluent and was pumped through the system at a rate of 1 mL/min. The eluent reservoir was pressurized to 3 psi with ultra-high purity helium. Samples were loaded into the AS-DV autosampler prior to analysis. Cation analysis gives concentrations for dissolved sodium (Na$^+$), NH$_4^+$, potassium (K$^+$), calcium (Ca$^{2+}$), and magnesium (Mg$^{2+}$). Standards from 5 to 1000 µM for Na$^+$, K$^+$, Ca$^{2+}$, Mg$^{2+}$ were prepared using concentrated solution purchased from High Purity Standards and analyzed alongside all Prado samples. Standards from 2.5 to 500 µM for NH$_4^+$ were prepared using concentrated solution purchased from High Purity Standards and analyzed alongside all Prado samples. USGS standard reference samples (SRSs) for Na$^+$, NH$_4^+$, K$^+$, Ca$^{2+}$, Mg$^{2+}$ (high and low concentrations) were run with each sample set to ensure data accuracy. Field blanks were collected and run alongside samples to ensure no sample contamination occurred during collection. 20% of samples were rerun with each batch of samples to ensure...
the instrument was functioning reliably and consistently. SRSs, standards, and field blanks were acidified with H$_2$SO$_4$ to obtain a pH <2.

September NH$_4^+$ concentrations were colorimetrically quantified utilizing the methods described in Scheiner (1976). Methods from Scheiner (1976) were utilized on September NH$_4^+$ samples because the NH$_4^+$ concentration in the outlet surface water was lower in September resulting in greater Na$^+$ peak interference during IC analysis. A methods comparison was performed between the colorimetric NH$_4^+$ assay and IC. The two methods gave identical results for standards and SRSs, but the Prado sample matrix tended to somewhat underestimate the concentration of NH$_4^+$ for the colorimetric assay compared to the IC. Results of the internal methods comparison can be found in Figure A1, Appendix A. Scheiner (1976) determined an array of interference compounds, none of which occur in high enough concentrations in Prado samples to be of concern.

June NO$_3^-$ and major dissolved anion samples were analyzed using a Dionex ICS-5000 IC system equipped with AS4 analytical column, AG4 guard column, and AERS 500 4-mm suppressor. The columns were kept at 30°C during analysis. The line voltage for the suppressor was set to 27 mA using the Chromeleon software provided by Dionex. 1.8 mM sodium carbonate/1.7 mM sodium bicarbonate was used as the eluent and pumped through the system at 2 mL/min. The eluent reservoir was pressurized to 3 psi with ultra-high purity helium. Samples were loaded into the AS-DV autosampler prior to analysis. Anion analysis gives concentrations for chloride (Cl$^-$), NO$_3^-$, phosphate (PO$_4^{3-}$), and sulfate (SO$_4^{2-}$). Standards from 5 to 1000 µM for Cl$^-$ and SO$_4^{2-}$ were prepared using concentrated solutions purchased from High Purity Standards and analyzed alongside all Prado samples. Standards from 2.5 to 500 µM for NO$_3^-$ and PO$_4^{3-}$ were prepared using a concentrated solution purchased from High Purity Standards and analyzed alongside all Prado samples. USGS SRSs for Cl$^-$, NO$_3^-$, PO$_4^{3-}$, and SO$_4^{2-}$ (high and low concentrations) were run with each sample set to ensure data accuracy. Field blanks were collected and run alongside samples to ensure no sample contamination occurred during collection. 20% of samples were rerun with each batch
of samples to ensure the instrument was functioning reliably and consistently. All samples were thawed at room temperature (25°C) prior to analysis.

NO$_3^-$ concentrations were also quantified by reducing NO$_3^-$ to nitric oxide (NO) in a heated reaction chamber containing vanadium chloride (VCl$_3$) in 1N hydrochloric acid. The NO gas that was produced was carried by ultra-high purity helium to the chemiluminescent detector. Details on NO$_3^-$ quantification via chemiluminescent detector can be found in Cox (1980). Each analysis contained NO$_3^-$ standards that were prepared weekly using concentrated stock solutions purchased from High Purity Solutions. USGS SRSs with certified NO$_3^-$ concentrations were run alongside Prado samples to ensure data accuracy. Each sample was injected in duplicate with <5% difference between injections. If >5% error was obtained, the sample was analyzed until at least two injections were within <5% of each other. The detection limit for NO$_3^-$ analysis was 0.025 µmol (100 µL injection of 0.25 µM standard).

NO$_2^-$ concentrations were quantified by reducing NO$_2^-$ to NO in a reaction chamber containing acetic acid and sodium iodide (NaI). The NO gas that was produced was carried by ultra-high purity helium to the chemiluminescent detector. Details on NO$_2^-$ quantification via chemiluminescent detector can be found in Cox (1980). Each sample was injected in duplicate with <5% difference between injections. If >5% error was obtained, the sample was analyzed until at least two injections were within <5% of each other. Each analysis contained NO$_2^-$ standards that were prepared weekly using concentrated stock solutions purchased from High Purity Solutions. The detection limit for NO$_2^-$ analysis was 0.025 nmol (100 µL injection of 0.25 µM standard).

Total dissolved metal samples were submitted to the USGS Water Mission Area common services laboratory located in Boulder, Colorado. All metal samples were analyzed on the inductively coupled plasma optical emission spectrometry (ICP-OES), which quantifies the total mass of all water-soluble metal valences.

Dissolved N$_2$O and CH$_4$ were collected in the field using a plastic syringe and needle. Approximately 20 mL of wetland water was injected into a He-flushed, 37 mL glass serum
bottle with 25 µL of 12.5 N KOH (in duplicate). Dissolved gas samples were analyzed on the Shimadzu GC17A equipped with an electron capture detector (ECD) in series with a flame-ionized detector (FID) gas chromatograph. Analytical conditions for the GC17A are as follows:

- **Column:** Porapak N, 8 feet, 2 mm ID, 80/100 mesh size
- **Carrier gas and flow rate:** High purity N₂ @ 20 mL/min

Sample injections were automated using a 50 and 500 µL sample injection loop and pre-programmed software. Standard curves were established for N₂O and CH₄ using 1, 10, and 100 ppm N₂O and 10, 100, and 1000 ppm CH₄ certified standards from Scotty Gas Company. All injections, including standards, were injected at a fixed volume. N₂O was detected by the ECD but was too low to reliably quantify. The percent relative standard error for some N₂O concentrations were as high as 125%. N₂O data will not be presented or discussed here because of the poor replication due to the N₂O concentrations being at or below the detection limit of the GC. N₂O concentrations can be loosely estimated in nM concentrations.

### 2.2.3 Methods Comparison - IC versus NOA

A methodological comparison between the IC and the NOA was performed to ensure unbiased NO₃⁻ data and because both methods were used in this study to quantify NO₃⁻ concentrations. The extent of methodological bias needed to be recognized and quantified for reference in the data interpretation stage. A methods bias could alter data interpretation, so it is important to address upfront.

There are several advantages of using the NOA over IC for the analysis of NO₃⁻ concentrations. The sample volume requirement for NO₃⁻ analysis on the NOA is very low; typically, less than 500 µL is required for NO₃⁻ concentration analysis on the NOA versus 2 mL for IC analysis. The detection limit for NO₃⁻ concentrations is much lower on the NOA at 0.025 nmol NO₃⁻ versus the detection limit of NO₃⁻ on the IC being 2.5 µM NO₃⁻. Additionally,
high enough concentrations of Cl− can interfere with NO₃− concentrations analysis on the IC. 

Prado surface water in the unvegetated cells was approximately 5000 µM in both June and September.

The NOA was used to quantify NO₃− and NO₂− concentrations for the June and September in-situ stable isotope tracer test because the experimental design was a volume-limited system. In June, 20 mL of the overlying water column (≈1% of the total water column volume of the polycarbonate chamber) was removed for sample analysis. If a greater volume of water was removed during the tracer test, the act of sampling the chambers may have impacted the chemical makeup of the overlying water column enough to bias the resultant rates. In September, the total sample volume between all analyses was ≈1700 mL and the chamber water column volumes were ≈2000 mL. The 1700 mL does not account for water used to rinse the filters and sample bottles or for unintended water loss during the sampling event. For this reason, less water volume was collected for NO₃− and NO₂− concentration analysis, thereby favoring NOA analysis for these ions.

NO₃− concentrations at the inlet and outlet of the wetland were quantified using the IC because water volume was not limiting. Inlet and outlet surface water samples were used in this methodological comparison. The concentration range for this methodological comparison was 20 µM to 250 µM NO₃−. The methods comparison yielded a slope of 0.91 and an $r^2 = 0.988$ when plotting the full range of NO₃− concentrations from the IC versus the full range of NO₃− concentrations from the chemiluminescent detector (Figure A2, Appendix A). NO₃− concentrations from 0 to 150 µM yielded a slope of 0.93 and $r^2$ of 0.993, indicating slightly less bias for lower NO₃− concentrations between the IC and NOA. Overall, the two methods gave identical results for standards and SRSs, but the Prado sample matrix tended to somewhat overestimate the concentration of NO₃− for the NOA compared to the IC. Standards and SRSs follow the y=x line, indicating no method bias. Deviation from the y=x line for Prado samples indicates the matrix of Prado samples interferes with the chemical analysis of NO₃− during NOA analysis.
2.3 Results

2.3.1 Physical Parameters

The outlet of Cell 3A exhibited a diel pattern in pH, DO concentrations, SpC, and temperature on the June site visit and pH, DO concentrations, SpC, temperature, and alkalinity on the September site visit (Figures 2.1 & 2.2). The extent of diel variations in physical parameters was less pronounced at the inlet of the wetland due to the lack of contact with biomat (Figure A3, Appendix A). Outlet DO concentrations and pH varied on a diel basis between 2.5 mg/L and 15 mg/L, and pH from 7.2 and 9.0 (Figure 2.1).

The only parameter that varies significantly between June, September, and October is temperature (Figure A4, Appendix A). As expected, water column temperature at the outlet of Cell 3A was lower at night, and higher during the day (Figure 2.1). Peak outlet water column temperatures in June exceeded 30°C during the day and dipped to 20°C at night. The outlet water column temperature in June was similar to the outlet water column temperatures in September, with a maximum exceeding 30°C. The minimum water column temperature in September, however, was observed to be 14°C, 6°C lower than the minimum observed in June and 4°C higher than the minimum observed in October. The outlet water column temperature in October did not exceed 30°C as it did in June and September. The maximum observed water column temperature in October was 26°C, and the lowest outlet water column temperature observed was 10°C. June and September had more consistent outlet water column temperature fluctuations, whereas a steady increase in water column temperature was observed in October over the course of the week (Figure 2.1).

2.3.2 Nutrients

Inlet water chemistry displayed diel changes in NO$_3^-$ and NO$_2^-$, but the signal is less pronounced and does not align with the diel pattern in NO$_3^-$ and NO$_2^-$ observed at the outlet in June 2018 (Figure 2.3). NO$_2^-$ concentrations are also much lower at the inlet. NO$_2^-$ concentrations at the inlet do not exceed 2 µM, whereas the minimum observed NO$_2^-$
Figure 2.1: Diel variations in DO concentration, SpC, pH, and temperature in Cell 3A in June 2018 and October 2018. The first readings were taken on June 25, 2018 and October 19, 2018. The shaded regions represent sunset to sunrise.
Figure 2.2: Diel changes in water column alkalinity observed in September 2018. The first sample was collected September 17, 2018. The grey, shaded regions represent sunset to sunrise based on September solar time.

At the outlet was roughly 2 µM. At the inlet, NO$_2^-$ concentrations were observed to decrease during the night and increase during the day whereas the opposite was true for the outlet. Outlet NO$_2^-$ concentrations exhibited an increase at night and a decrease during the day (Figure 2.3).

Diel variations in NO$_2^-$ concentrations at the outlet of Cell 3A were more pronounced in June 2018 than in September 2018 (Figure 2.4). NO$_2^-$ concentrations in June varied from a maximum of 12 µM during the nighttime, to a minimum of 2 µM during the daytime. In September, NO$_2^-$ concentrations varied from 5 µM during the nighttime to 2.5 µM in the daytime. The same trend of higher NO$_2^-$ concentrations during the nighttime was observed in June and September (Figure 2.4). NH$_4^+$ concentrations followed the same trend of surface water NO$_2^-$ in June and September, although this relationship is more prominent in June (Figure 2.4).
NO$_3^-$ was the dominant, dissolved, inorganic nitrogen species in both the inlet and outlet surface water of Cell 3A year-round. Incoming NO$_3^-$ concentrations in June and September of 2018 averaged 310 µM NO$_3^-$ and 240 µM NO$_3^-$, respectively (Figure 2.3). Outgoing NO$_3^-$ in June varied between 160 and 220 µM NO$_3^-$ and 40 and 140 µM in September (Figure 2.4). NO$_3^-$ removal was less during the daytime than during the nighttime (Figure 2.5). Average NO$_3^-$ removal for June was observed to be 36%. Maximum NO$_3^-$ removal in June was observed during the nighttime at 50%. A similar day versus night comparison cannot be made for the September samples set because the sampling of the inlet was limited to manual, daytime sampling only. Diel changes in NO$_3^-$ exhibited different patterns between June and September (Figure 2.4). During the June site visit, NO$_3^-$ concentrations decrease at night, and increase during the day. The opposite is true for September; NO$_3^-$ concentrations increase at night and decrease during the day.

Inlet NH$_4^+$ concentrations in June were low (<5 µM on average) and displayed no strong correlation to changes in light, temperature or time of day (Figure 2.3). Outlet NH$_4^+$, however, displayed a diel pattern in both June and September (Figure 2.4). The diel pattern of NH$_4^+$ concentrations at the outlet in June were similar in pattern and concentrations of NO$_2^-$ at the outlet in June. The maximum water column NH$_4^+$ concentration in June was 14 µM and 21 µM in September.

Water column PO$_4^{3-}$ concentrations were observed to vary on a diel basis in June and September 2018. June PO$_4^{3-}$ concentrations varied between 21 to 62 µM PO$_4^{3-}$ and 3 to 50 µM PO$_4^{3-}$ during September (Figures 2.6). In June and September, peak PO$_4^{3-}$ concentrations in the water column were observed to be just after sunrise, while minimum concentrations of PO$_4^{3-}$ occurred just before sunset. The minimum PO$_4^{3-}$ water column concentration occurs in September just before sunset and is roughly ten times less than the minimum concentration of PO$_4^{3-}$ observed in the water column in June (minimum of 2.6 µM PO$_4^{3-}$ in September, versus minimum of 21 µM PO$_4^{3-}$ in June). ICP-OES P data and IC PO$_4^{3-}$ data from September were strongly correlated when plotted against each other with a
slope equal to one (Appendix A, Figure A5). Mean inlet PO$_4^{3-}$ concentrations in June and September were 38 µM and 27 µM, respectively.

Figure 2.3: Aqueous concentrations of NO$_2^-$, NO$_3^-$, and NH$_4^+$ measured at the inlet and outlet of Cell 3A in June 2018. The first sample was collected on June 26, 2018. The grey, shaded regions represent sunset to sunrise based on June solar time.
Figure 2.4: Diel changes in the concentration of aqueous NO$_3^-$ (cyan), NO$_2^-$ (red), and NH$_4^+$ (black) at the outlet of Cell 3A. The first samples were taken on June 26, 2018 and September 17, 2018. The grey, shaded regions represent sunset to sunrise based on June and September solar time.
Figure 2.5: NO$_3^-$ percent removal during June 2018. Black squares represent unadjusted NO$_3^-$ removal and red stars represent NO$_3^-$ removal when taking into account hydraulic residence time of one day. The grey, shaded regions represent sunset to sunrise based on June solar time.

### 2.3.3 Dissolved Metals

Total dissolved Ca, Br, Ba, Zn, P, Mn, Mg, and Fe concentrations exhibited the same, 24-hour, diel pattern of increasing concentrations at night and decreasing concentrations during the day (Figure 2.7). V and Cu show the opposite diel pattern from other trace metals with decreasing concentrations at night and increasing concentrations during the day. Ca$^{2+}$ and Mg$^{2+}$ were the only two metals detected in the mg/L range, whereas the other dissolved metals were measured to be in the µg/L range. Zn is the only dissolved metal that consistently showed lower concentrations at the outlet versus the inlet. The other metals showed diel variation above and below the inlet surface water concentration. Other dissolved metals were quantified, but are not shown in Figure 2.7 because they did not exhibit a diel pattern.

Figure 2.8 summarizes the primary diel concentration changes of outlet water column data for major cations and anions in June 2018. The outlet water chemistry exhibited diel changes
Figure 2.6: Diel variation in PO$_4^{3-}$ concentrations in June (red) and September (black). Inlet concentrations are represented by closed circles, outlet concentrations are represented as open circles. The grey, shaded regions represent sunset to sunrise based on June and September solar time.

in NH$_4^+$, NO$_2^-$, NO$_3^-$, PO$_4^{3-}$, Ca$^{2+}$, and Mg$^{2+}$ concentrations during June. The top panel of Figure 2.8 shows that a decrease in DO correlates with an increase in more reduced inorganic nitrogen species. The middle panel of Figure 2.8 shows that Ca$^{2+}$ and Mg$^{2+}$ exhibit a similar temporal, diel pattern, with increasing concentrations at night and increasing concentrations in the day. The bottom panel shows that pH and NO$_3^-$ follow a similar temporal pattern, a trend that relates to biological activity in the wetland. In September, NH$_4^+$, NO$_2^-$, NO$_3^-$, PO$_4^{3-}$, Ca$^{2+}$, Mg$^{2+}$, and various heavy metals concentrations were observed to vary on a diel basis (Figures 2.4 & 2.7).

2.3.4 Greenhouse Gases

Dissolved methane (CH$_4$) and N$_2$O were detected in the surface water at the outlet of Cell 3A in September. N$_2$O concentrations were too low for the current configuration of the Shimadzu GC17A to detect, but N$_2$O concentrations at the outlet are most likely in the nM region. Aqueous CH$_4$ exhibited a diel response in the water column of Cell 3A (Figure 2.9) with maximum concentrations reaching 12 µM CH$_4$ and occurring just after sunrise and
Figure 2.7: Diel changes in various concentrations of metals at the outlet (hollow circles) versus the inlet (filled in circles) of Cell 3A during the September site visit. The first sample was taken on September 17, 2018. The grey, shaded regions represent sunset to sunrise based on September solar time.
Figure 2.8: Diel variations in concentrations of various inorganic nitrogen species, metals, and physical parameters observed at the outlet of Cell 3A in June 2018. The first sample was taken on June 26, 2018. The grey, shaded regions represent sunset to sunrise based on June solar time.
decreased over the course of daylight hours to 2 µM CH₄ just before sunset.

Figure 2.9: Diel changes in dissolved CH₄ concentrations were observed at the outlet of Cell 3A in September. Error bars represent the standard deviation between duplicate samples. The first sample was taken on September 18, 2018. The shaded regions represent sunset to sunrise.

2.4 Discussion
2.4.1 Physical Parameters

The physical parameter data aligns well with previously observed ecological phenomena (Nimick et al. 2003, Hem 1989, Harrison et al. 2005). Diel changes in pH and DO concentrations were observed, as expected. The highest DO concentration occurs just after solar noon (14:00), while the lowest DO concentration occurs just before sunrise (5:00) (Figure 2.1). DO saturation reached a maximum of 255% saturation, relative to atmospheric equilibrium (19 mg/L DO at 29°C), in June and 250% saturation, relative to atmospheric equilibrium (20 mg/L DO at 26°C), in October. During the day, photosynthetic diatoms that inhabit the oxic layer of the biomat carry out metabolic processes that creates degradable organic carbon (DOC) and DO as a byproduct (Jones et al. 2017). DOC is presumed to be a driving factor in biomat reactions because the biomat is comprised of approximately 6% carbon and
carbon can act as an electron donor for biochemical reactions in the biomat (Figure 3.4). At the highest intensity of sunlight (i.e. solar noon), the DO concentration is near its highest because the diatoms are most active in carrying out photosynthetic processes. During the night cycle, diatoms stop photosynthesis because of the lack of light. Heterotrophic microorganisms consume water column DO, drawing down the concentration of DO in the water column. Heterotrophic production of carbon dioxide (CO$_2$) as a byproduct directly links microbial processes to the diel fluctuations in pH, depicted in Figure 2.1. Aqueous CO$_2$ is a weak, diprotic acid that influences the surface water pH, more dissolved CO$_2$ means a lower pH, and vice versa (Hemmond & Fechner 2015). Both pH and DO concentration show larger swings at the outlet than at the inlet. The maximum and minimum values are more pronounced because of the biogeochemical reactions occurring within the biomat, a variable that the outlet water is subjected to, but not the inlet water.

2.4.2 Nutrients

Inlet water chemistry is hypothesized to be influenced by stationary and floating macrophytes, such as the common cattail (*Typha latifolia*) and duckweed (*Lemna spp.*) (Bear et al. 2017). Plant uptake of NO$_3^-$ and NO$_2^-$ would occur during the day and not at night, resulting in a diel response in NO$_3^-$ and NO$_2^-$ concentrations decreasing during the day and increasing at night. This pattern broke the cycle on the last day of sample collection in June where NO$_3^-$ concentrations increased during the day (Figure 2.3). The increase in inlet NO$_3^-$ concentration could be caused by varying water chemistry of the Santa Ana River, but is otherwise unexplained.

The periodicity of NO$_3^-$ concentrations entering the wetland changed from June to September, thereby changing the diel pattern of NO$_3^-$ entering the unvegetated wetland cells. In June, NO$_3^-$ concentrations decreased during the nighttime while in September, NO$_3^-$ concentrations increased during the nighttime (Figure 2.4). The upstream factors that influence incoming NO$_3^-$ periodicity is unknown. The observed increase in NO$_3^-$ during the daytime in June and during the nighttime in September is hypothesized to be the diffusive
mixing of inlet and outlet water, since water with higher NO$_3^-$ concentrations enter the wetland during the daytime in June and during the nighttime in September. The increase in surface water NO$_3^-$ in June is also hypothesized to be from nitrification. The increase in NO$_3^-$ concentrations coupled with the decrease in surface water NO$_2^-$ and NH$_4^+$ support the idea of daytime nitrification. At night in June, lower water column DO concentrations enabled heterotrophic bacteria in the upper portions of the biomat to reduce NO$_3^-$ to N$_2$O and N$_2$ via denitrification. During the day in September, NO$_3^-$ is hypothesized to decrease due to diatom NO$_3^-$ uptake. Diatoms have been shown to uptake NO$_3^-$ in storage vacuoles up to 200 mM and is used as an energy supply in the sudden absence of light and oxygen (Kamp et al. 2011).

The decrease in water column NH$_4^+$ concentrations during the daytime in both June and September is hypothesized to be caused by NH$_4^+$ diatom assimilation. NH$_4^+$ assimilation is usually followed by the intercellular conversion to organic nitrogen (Kamp et al. 2015). The increase in NH$_4^+$ at night in June and September is hypothesized to be the diffusive flux of NH$_4^+$ out of the porewater and into the water column. Diffusive flux of NH$_4^+$ is always occurring within the wetland, but the NH$_4^+$ concentration in the overlying water column is predicted to be heavily influenced by diatom assimilation. If the diatoms are not assimilating NH$_4^+$, such as during the nighttime, then NH$_4^+$ concentrations in the water column increase. If the diatoms are assimilating NH$_4^+$, the NH$_4^+$ concentration will decrease because the diatoms are actively removing NH$_4^+$ from the water column. Porewater NH$_4^+$ concentrations were measured to be up to 2 orders of magnitude higher in NH$_4^+$ concentration than in the water column (Chapter 3).

The diel pattern for water column NO$_2^-$ concentrations is the same in June and September. NO$_2^-$ concentrations increased during the nighttime and decreased during the daytime. NO$_2^-$ concentrations are hypothesized to decrease during the daytime due to diatom NO$_2^-$ uptake. NO$_2^-$ uptake was reported in cell cultures of _T. pseudonana_, a marine diatom, but only after an acclimation period of NO$_3^-$ assimilation (Waser et al. 1998). The diatoms in
Cell 3A are safely assumed to be acclimated to a high NO$_3^-$ environment, so the assimilation of NO$_3^-$ and NO$_2^-$ by these diatoms is highly plausible. At night and June and September, diatoms are rendered less active due to the absence of light and lower DO concentrations, thereby slowing their uptake of NO$_2^-$. The observed increase in NO$_2^-$ in June and September is hypothesized to be the reduction of NO$_3^-$ to NO$_2^-$, the first step of denitrification. There is a clear denitrification signal in the surface water chemistry in June but not in September, however, stable isotope tracer tests performed in September confirmed denitrification activity during that site visit.

Water column PO$_4^{3-}$ removal occurred in September on a diel basis; light conditions appeared to remove PO$_4^{3-}$, while dark conditions liberated it (Figure 2.6). PO$_4^{3-}$ could be in higher biochemical demand in June because of the longer daylight hours and therefore more time for the diatoms to photosynthesize, leading to higher, overall nutrient demand. September harbors fewer daylight hours and is the tail end of the growing season, meaning most microorganisms have well established biomass. If PO$_4^{3-}$ concentrations in the water column is correlated with nutrient demand, then one would expect to observe larger diel fluctuations in PO$_4^{3-}$ during the longer daylight hours (June), rather than during days of shorter daylight hours (September).

PO$_4^{3-}$ can be liberated from Fe and Mn complexes in the porewater due to changes in DO distribution and change in redox potential (Boulton et al. 1998). PO$_4^{3-}$ and Mn follow the same diel pattern in September, with minimum concentrations occurring just before sunset, and maximum concentrations occurring just before sunrise (Figures 2.6 & 2.7). In June, PO$_4^{3-}$ availability could be influenced by diel changes in the porewater chemistry, even though diel changes in PO$_4^{3-}$ is not apparent in the water column in June. Dahm et al. (1987) found that PO$_4^{3-}$ and total P were higher in concentration in anaerobic porewater than in the overlying, oxic water column. Porewater PO$_4^{3-}$ analysis revealed the presence of PO$_4^{3-}$ in the porewater in June, but no diel pattern was observed. PO$_4^{3-}$ concentrations were erratic in the porewater relative to the overlying surface water (Figure 3.5). June samples
analyzed for PO$_4^{3-}$ concentrations were not collected, stored, or analyzed in an optimal manner. Inquiry into seasonal factors affecting PO$_4^{3-}$ cycling and removal in Cell 3A could lead to a greater understanding of how the wetland functions on an annual basis.

2.4.3 Dissolved Metals

Water column pH influences water column metal speciation. Dissolved V and Cu concentrations follow the same diel pattern as pH, meaning V and Cu compounds become more soluble at night during lower pH conditions. The remaining dissolved metals, Ca, Br, Ba, Zn, P, Mn, Mg, and Fe, have the opposite diel pattern as pH, meaning these metal compounds become less soluble during lower pH conditions.

The adsorption/desorption model describes the change in sorption processes of trace metals under varying temperature and pH (Nimick et al. 2011). Cationic sorption increases in the presence of organic matter and clays (Sheoran & Sheoran 2006). The biomat is not carbon limited; it was estimated in June to be just under 6% carbon (Figure 3.4), so sorption processes must play an important role in the observed diel changes in dissolved metals in Cell 3A. Sorption of dissolved trace metals such as Cu, have a high affinity for sorption processes, whereas Zn has the potential to sorb to the biomat, but does so less readily than Cu (Sheoran & Sheroan 2006).

V is typically less than 10 µg/L in surface and ground water (Landergren 1974). Diel variations in groundwater V concentrations has been observed at the Hegeler Zinc Superfund Site, but otherwise V is not an element of interest at superfund sites or mine-impacted streams (Kay et al. 2011). V typically forms anionic compounds in oxic conditions, while Cu typically forms CuCO$_3$ in highly buffered systems (Hem 1989). Both Cu and V are essential to photosynthesis, so the increase in the dissolved concentrations of these elements during the nighttime could be a secondary indicator of slowing primary production in the diatoms. At night, diatoms are no longer photosynthesizing because of the lack of light, meaning no additional inputs of DO into the water column beyond atmospheric diffusion of O$_2$ (g) O$_2$ (aq), which is slow relative to heterotrophic DO consumption. Heterotrophic
bacteria draw down the DO concentrations in the water column via metabolic processes and produce CO$_2$ as a byproduct. The resultant CO$_2$ from heterotrophic bacteria metabolism decreases the water column DO concentrations, leading to a decrease in observed pH and alkalinity and the re-speciation of V and Cu compounds at night (Hems 1989).

Cu and Ba are the only metals that display diel variations and have an associated MCL. Both Cu and Ba concentrations found in the wetland are orders of magnitude below the MCL. Ba found in surface and groundwaters are often from natural sources, such as the underlying geology (Hems 1989). Several additional metals appear on the secondary MCL list, which is a non-enforceable limit that is based on aesthetics, like taste and smell, that can alter perceived water quality but do not pose any health risks (USEPA 2009). The secondary MCL for Mn is 0.05 mg/L and the maximum measured concentration for Mn in Cell 3A is 0.06 mg/L. Table 2.2 compares the maximum observed metal concentration in June to the primary and secondary MCL standards set by the US EPA.

MnO$_2$ reduction and its interaction with the nitrogen cycling in suboxic marine sediments has been shown in microcosm experiments and in-situ experiments (Mogollon et al. 2016, Lin & Taillefert 2014, Luther et al. 1997). In all instances, MnO$_2$ acts as an oxidant in sub- to anoxic conditions to oxidize NH$_4^+$ to NO$_2^-$, where NO$_2^-$ is then used as substrate for anammox bacteria and archaea. Luther et al. (1997) proposed that Mn$^{2+}$ is regenerated through contact with DO. Figure 2.7 shows that Mn$^{2+}$ accumulated in the water column during the nighttime in September 2018, a period where the DO concentration was decreasing (Figure 2.1). Mn$^{2+}$ accumulated in the water column and was not regenerated to MnO$_2$ during the nighttime, which could indicate a slow down or halt in Mn-catalyzed anammox in the depths of the biomat. Similar to MnO$_2$ reduction, ferrihydrite (Fe(OH)$_3$) has been shown to abiotically catalyze the oxidation of NH$_4^+$ to NO$_2^-$, then coupled with anammox to produce N$_2$ (Yang et al. 2012). Dissolved Fe and Mn are fairly similar in concentration in the water column in September (Figure 2.7) and follow the same diel trend. The potential for Mn and Fe mediated anammox and its tie to nutrient cycling within the biomat and
unvegetated wetlands is a crucial topic to document and understand for water quality and management purposes.

Table 2.2: Total dissolved metal concentrations in September water samples taken from Cell 3A compared to the MCL. Primary and secondary MCLs are based on a 2009 EPA publication. Dashes represent no assigned MCL value.

<table>
<thead>
<tr>
<th>Metal</th>
<th>Maximum observed concentration (mg/L)</th>
<th>Primary MCL (mg/L)</th>
<th>Secondary MCL (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>V</td>
<td>0.005</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Cu</td>
<td>0.002</td>
<td>–</td>
<td>1.3</td>
</tr>
<tr>
<td>Ba</td>
<td>0.05</td>
<td>2.0</td>
<td>–</td>
</tr>
<tr>
<td>Zn</td>
<td>0.015</td>
<td>–</td>
<td>5.0</td>
</tr>
<tr>
<td>Mn</td>
<td>0.06</td>
<td>–</td>
<td>0.05</td>
</tr>
<tr>
<td>Mg</td>
<td>0.014</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Fe</td>
<td>0.05</td>
<td>–</td>
<td>0.30</td>
</tr>
</tbody>
</table>

2.4.4 Greenhouse Gases

The observed production of CH$_4$ supports the hypothesis that there exists an extreme redox gradient within the biomat of Cell 3A. Methanogenesis is the last step in the ecological redox series, where CO$_2$ acts as the terminal electron acceptor and CH$_4$ is formed as a byproduct. Methanogenesis is also the last step in carbon reduction and can only occur if all other electron acceptors are depleted (Hemond & Fechner 2015). A methanogen genus, *Methanoregula*, has been found to be present in the deeper portions of the biomat, a trend that is to be expected since O$_2$ inhibits methanogen growth and metabolism (Jones et al. 2017). Other redox reactions, such as Mn and Fe reduction and their interactions with nutrients such as SO$_4^{2-}$ and PO$_4^{3-}$ remain to be explored in terms of nutrient attenuation and removal in the unvegetated wetland cells.
CHAPTER 3
CHARACTERIZATION OF NITROGEN SPECIES IN THE BIOMAT POREWATER

3.1 Introduction

The biomat porewater is important to characterize because the porewater is a hotspot for biochemical transformations that occur in the experimental, open-water, unvegetated, treatment wetland. The biomat has been previously characterized as a carbon-rich, microbially active consortium of diatoms, bacteria, and archaea, while also being naturally stratified and with microenvironments of varying redox potential and metabolic substrate (Jasper et al. 2014, Jones 2017). Aerobic metabolic processes can occur at the biomat-water column interface, and potentially just below the interface, as DO in the overlying water column diffuses into the biomat. Nitrification and assimilation, both aerobic processes, are likely to occur at the interface. Anaerobic processes occur within the depths of the biomat, because DO is depleted by both biotic and abiotic processes, thereby thermodynamically favoring denitrification, DNRA, ammonification, and anammox, all of which are nitrogen cycling redox reactions. Figure 3.1 summarizes the potential locations for nitrogen biochemical transformations to occur relative to the biomat. It is hypothesized that the redox zones of the biomat and biomat porewater are dictated by diel changes in nutrients and diatom activity in the upper layers of the biomat and at the biomat-water column interface. The biomat oxic layer is hypothesized to expand and contract as DO concentrations in the water column waxes and wanes, which dictates the location and contribution of different nitrogen cycling processes. The data set presented here documents shifts in inorganic nitrogen species at several depths of the biomat over a 48-hour period.
Figure 3.1: Biochemically-mediated reactions in terms of where they are likely to occur in Cell 3A relative to the biomat (not to scale). The black arrows represent biochemical transformations while the white arrows represent diffusion. NO$_3^-$ is the dominant, inorganic, aqueous, nitrogen species present in the water column and is hypothesized to be transformed or removed through diel changes in biomat redox potential.

3.2 Methods
3.2.1 Field Techniques

Porewater samples were collected in June 2018 using a Minipoint piezometer tripod connected to a low flow peristaltic pump (Duff et al. 1998, Figure 3.2). Porewater was collected at three-hour intervals for 48 hours. The biomat was sampled at 1, 3, 5, 7, 9, and 11-centimeter (cm) depth increments. The porewater was collected using small-diameter plastic tubing and was filtered through a 0.45 μm Millex filter directly into 20 mL plastic scintillation vials for NO$_3^-$ and major, dissolved anion analysis and baked, glass scintillation vials for ammonium and major, dissolved cation analysis. Each tubing line was flushed for two minutes at 1.5 mL/min flow rate prior to collecting the sample. Vials were rinsed thoroughly with porewater before the samples were collected. NO$_3^-$ and major anions were preserved by freezing the samples in a -4°C freezer. NH$_4^+$ and major cation samples were
preserved by adding 40 µL of 18N H₂SO₄ to each scintillation vial and refrigerating at 4°C.

Intact biomat cores were collected at 16:00 on June 29, 2018, 70 meters laterally from the outlet of Cell 3A, proximal to the location of the June in-situ tracer test experiment (Figure 1.3). Cores were collected using a cut-off 60 mL syringe barrel and plunger. The cores were partitioned into 0-1 cm, 1-6 cm, and 6-12 cm sections and placed directly into clean, 100 mL glass jars, baked at 450°C for 4 hours prior to arrival. Biomat cores were collected until each jar was full in order to minimize headspace and oxidation of the lower biomat sections. Samples were shipped on ice, overnight back to the laboratory in Boulder, Colorado. The biomat was then used for denitrification potential assays with added NO₃⁻ and acetylene following the methods described in Repert et al. (2014).

Figure 3.2: The Minipoint piezometer sampler. The color-coded acrylic disk in the center of the tripod is the portion of the instrument that was carefully lowered into the biomat. The tubing wrapped around the neck of the tripod was used along with the peristaltic pump to sample each depth.
3.2.2 Analytical Techniques

June NH$_4^+$ and major dissolved cation samples were analyzed using a Dionex ICS-5000 IC system equipped with CS12A analytical column, CG12A guard column, and CERS 500 4-mm suppressor. The columns were kept at 30°C during analysis. The line voltage for the suppressor was set to 47 mA using the Chromeleon software provided by Dionex. 20 mM methanesulfonic acid was used as the eluent and was pumped through the system at a rate of 1 mL/min. The eluent reservoir was pressurized to 3 psi with ultra-high purity helium. Samples were loaded into the AS-DV autosampler prior to analysis. Cation analysis gives concentrations for dissolved Na$^+$, NH$_4^+$, K$^+$, Ca$^{2+}$, and Mg$^{2+}$. Standards from 5 to 1000 µM for Na$^+$, K$^+$, Ca$^{2+}$, Mg$^{2+}$ were prepared using concentrated solution purchased from High Purity Standards and analyzed alongside all Prado samples. Standards from 2.5 to 500 µM for NH$_4^+$ were prepared using concentrated solution purchased from High Purity Standards and analyzed alongside all Prado samples. USGS SRSs for Na$^+$, NH$_4^+$, K$^+$, Ca$^{2+}$, Mg$^{2+}$ (high and low concentrations) were run with each sample set to ensure data accuracy. Field blanks were collected and run alongside samples to ensure no sample contamination occurred during collection. 20% of samples were rerun with each batch of samples to ensure the instrument was functioning reliably and consistently. All samples, including SRSs, standards, and field blanks, were acidified with H$_2$SO$_4$ to obtain a pH <2.

NO$_3^-$ concentrations were also quantified by reducing NO$_3^-$ to NO in a heated reaction chamber containing VCl$_3$ in 1N hydrochloric acid. The NO gas that was produced was carried by ultra-high purity helium to the chemiluminescent detector. Details on NO$_3^-$ quantification via chemiluminescent detector can be found in Cox (1980). Each analysis contained NO$_3^-$ standards that were prepared weekly using concentrated stock solutions purchased from High Purity Solutions. USGS SRSs with certified NO$_3^-$ concentrations were run alongside porewater samples to ensure data accuracy. Each sample was injected in duplicate with <5% difference between injections. If >5% error was obtained, the sample was analyzed until at least two injections were within <5% of each other. The detection limit for NO$_3^-$ analysis
was 0.025 µmol (100 µL injection of 0.25 µM standard).

$\text{NO}_2^-$ concentrations were quantified by reducing $\text{NO}_2^-$ to NO in a reaction chamber containing acetic acid and NaI. The NO gas that was produced was carried by ultra-high purity helium to the chemiluminescent detector (Cox 1980). Each sample was injected in duplicate with <5% difference between injections. If >5% error was obtained, the sample was analyzed until at least two injections were within <5% of each other. Each analysis contained $\text{NO}_2^-$ standards that were prepared weekly using concentrated stock solutions purchased from High Purity Solutions. The detection limit for $\text{NO}_2^-$ analysis was 0.025 nmol (100 µL injection of 0.25 µM standard).

$\text{PO}_4^{3-}$, and $\text{SO}_4^{2-}$ anion samples were analyzed using a Dionex ICS-5000 IC system equipped with AS4 analytical column, AG4 guard column, and AERS 500 4-mm suppressor. The columns were kept at 30°C during analysis. The line voltage for the suppressor was set to 27 mA using the Chromeleon software provided by Dionex. 1.8 mM sodium carbonate/1.7 mM sodium bicarbonate was used as the eluent and pumped through the system at 2 mL/min. The eluent reservoir was pressurized to 3 psi with ultra-high purity helium. Samples were loaded into the AS-DV autosampler prior to analysis. Standards from 2.5 to 500 µM for $\text{PO}_4^{3-}$ and 5 to 1000 µM for $\text{SO}_4^{2-}$ were prepared using concentrated solutions purchased from High Purity Standards and analyzed alongside all Prado samples. USGS SRSs for $\text{PO}_4^{3-}$ and $\text{SO}_4^{2-}$ (high and low concentrations) were run with each sample set to ensure data accuracy. Field blanks were collected and run alongside samples to ensure no sample contamination occurred during collection. 20% of samples were rerun with each batch of samples to ensure the instrument was functioning reliably and consistently. All samples were thawed at room temperature (25°C) prior to analysis.

Denitrification potential rates were measured using the acetylene block method (Repert et al. 2014). Incubation bottles were prepped in anaerobic glovebox under a 95% CO$_2$, 5% H$_2$ atmosphere, with ~0.5 grams biomat and ~30 grams NO$_2^-$ and NO$_3^-$—free, anoxic, artificial wetland water (Appendix B, Tables B1 & B2). Bottles were flushed for 20 minutes
at room temperature with ultra-high purity helium before the start of the incubation. An anoxic NO$_3^-$ stock was added to a final concentration of 100 µM to each replicate. Incubations were performed at room temperature (25°C) and were turned end-over-end using a rotary mixer throughout the experiment to maintain a biomat-water slurry inside the bottles. N$_2$O concentrations in the headspace were quantified on an HNU electron capture gas chromatograph with a radioactive $^{63}$Ni detector and backflush valve to prevent acetylene from entering the detector. The carrier gas consisted of 95% argon and 5% methane and was set to a flowrate of 40 cm$^3$/min during analysis. The oven temperature was set to 90°C and the detector temperature was set to 240°C.

The remaining biomat not used in the denitrification assay was dried at 50°C for one week. Each dried biomat depth interval (0-1 cm, 1-6 cm, 6-12 cm) was ground and homogenized by hand using a mortar and pestle. The ground and homogenized biomat was weighed into aluminum capsules using a Cahn C-33 microbalance. The microbalance was calibrated with a 200 mg weight daily. Aluminum capsules were crimped and placed in a nickel sleeve and run on the Exeter Analytical Inc. CE-440 Elemental Analyzer. The combustion oven was set to 980°C and the reduction oven was set to 700°C. The particulate sample was combusted with pure O$_2$, which produced CO$_2$, H$_2$O and N$_2$. The resultant gases were analyzed via thermal conductivity (EPA Method 440.0, Zimmerman et al. 1997).

3.3 Results

3.3.1 Porewater Overview

Several interesting trends in inorganic nitrogen species were observed in the porewater from depths 1 to 11 cm below the biomat surface. NH$_4^+$ was the most prevalent dissolved, inorganic nitrogen species in the porewater, with concentrations between approximately 150 µM to 2000 µM for all observed depths and time points (Figure 3.3). The maximum NH$_4^+$ concentration was observed at the 7 cm depth at a concentration of 2100 µM NH$_4^+$, closely followed by 1600 µM observed at the 11 cm depth. The lowest observed NH$_4^+$ concentrations occurred at the 1 and 7 cm depths. NO$_3^-$ concentrations throughout the full depth of the
biomat ranged from 0 to 180 µM NO$_3^-$, NO$_3^-$ was detected at all depths whereas NO$_2^-$ was detected at all depths except 11 cm. NO$_2^-$ was the least prominent nitrogen species in the porewater with concentrations ranging from 0 to 5 µM NO$_2^-$. The maximum NO$_2^-$ porewater concentration was 5.2 µM at the 7 cm depth. The 5 cm biomat depth exhibited an increase in NH$_4^+$ porewater concentration while NO$_3^-$ and NO$_2^-$ porewater concentrations exhibited a decrease (Figure 3.3). The 7 cm biomat depth showed the inverse trend, there was an observed decrease in NH$_4^+$ porewater concentrations and increased NO$_3^-$ and NO$_2^-$ concentrations (Figure 3.3). The 7 and 11 cm biomat depths had low NO$_3^-$ concentrations and high NH$_4^+$ concentrations. These depths are the mostly likely location for methanogenesis to occur. CH$_4$ was detected in the outlet water column and was observed to vary on a diel basis (Figure 2.9). Percent carbon (%C), percent nitrogen (%N), and carbon to nitrogen ratio (C:N) for the biomat at various depths were determined on average to be 5.8%C, 2.2%N, and 2.6:1 (Figure 3.4). %C, %N, and C:N ratios were not significantly different with changing depth.

The vertical profiles of other porewater constituents are shown in Figures 3.5 and 3.6. Porewater SO$_4^{2-}$ concentrations were lower at every biomat depth when compared to water column SO$_4^{2-}$ concentrations (Figure 3.5). SO$_4^{2-}$ concentrations at the 7 cm depth were closest to surface water SO$_4^{2-}$ concentrations with an average SO$_4^{2-}$ concentration of 870 µM (+/- 54 µM) (excluding the first two, outlier time points, with measured SO$_4^{2-}$ concentrations of 94 µM and 99 µM). The average water column SO$_4^{2-}$ concentration during the porewater sampling event was 1000 µM (+/- 20 µM). SO$_4^{2-}$ exhibited a diel trend at 1 cm biomat depth, with higher SO$_4^{2-}$ concentrations during the day and lower SO$_4^{2-}$ concentrations during the night (Figure B1, Appendix B). Porewater PO$_4^{3-}$ concentrations were erratic, show no discernible pattern, and range from 24 µM to 140 µM (Figure 3.5). K$^+$, Ca$^{2+}$, Mg$^{2+}$, and Na$^+$ concentrations display fairly consistent vertical profile trends (Figure 3.6). Porewater concentrations of these cations are similar to surface water concentrations.
3.3.2 Nitrogen porewater chemistry at 1 cm biomat depth

A steep increase in NH$_4^+$ concentration occurred within the first 1 cm of biomat depth. Porewater NH$_4^+$ concentrations were between 17 to 50 times more concentrated than concentrations found in the water column. At 5:00, a maximum of 13 µM NH$_4^+$ was observed in the water column and 220 µM in the porewater at the 1 cm depth (Figure 3.7). At 16:00, a minimum water column NH$_4^+$ concentration of 3.2 µM was observed and 160 µM NH$_4^+$ was observed in the porewater at the next closest corresponding time point taken at 18:00. The average NH$_4^+$ concentration at the 1 cm depth was 210 µM (+/- 40 µM) (Figure 3.3). Some evidence exists for diel changes in NH$_4^+$ in the porewater; the lowest NH$_4^+$ porewater concentration occurred at the 15:00 time points. Diel changes in NH$_4^+$ concentrations were observed in the overlying water column (Figure 2.4).

NO$_3^-$ porewater concentrations ranged from 2.6 µM to 140 µM. A noticeable decrease in NO$_3^-$ concentration was observed between the water column and 1 cm biomat depth. The maximum concentration of 140 µM in the porewater at 1 cm was observed at 15:30 on the first day of sampling (Figure 3.7). In comparison, the water column at 14:00 was observed to be 220 µM NO$_3^-$. NO$_2^-$ porewater concentrations increase during the first dark cycle then steadily decreased over the course of the next light and dark cycles. Forty-eight hours after the first increase in NO$_3^-$ concentration was observed in the porewater, NO$_3^-$ concentrations appear to increase again heading into the dark cycle (Figure 3.7).

NO$_2^-$ porewater concentrations ranged from 0.16 µM to 5.30 µM at 1 cm biomat depth. The minimum NO$_2^-$ porewater concentration occurred at 21:30 and was nearly 40-times less concentrated than the overlying water column at that same time. NO$_2^-$ porewater concentrations follow a very similar temporal pattern to NO$_3^-$ porewater concentrations (Figure 3.7).
3.3.3 Nitrogen porewater chemistry at 3 cm biomat depth

At 3 cm biomat depth, the average porewater NH$_4^+$ concentration increased from 210 µM (+/- 40 µM) at the 1 cm depth to 620 µM (+/- 260 µM) NH$_4^+$ (Figure 3.3). There is more variation in NH$_4^+$ porewater concentrations at the 3 cm depth when compared to the 1 cm depth. NH$_4^+$ did not exhibit a diel pattern at the 3 cm depth (Figure 3.7).

NO$_3^-$ porewater concentrations exhibited a diel pattern, with concentrations ranging from 1 µM to 62 µM (Figure 3.7). The first maximum porewater NO$_3^-$ concentration occurred at 1:30. The second maximum occurred at 6:10, almost 29 hours after the first NO$_3^-$ concentration maximum (Figure 3.7). The diel pattern of NO$_3^-$ in the porewater does not align with the 24-hour diel pattern observed in the water column in June. Water column NO$_3^-$ concentrations in June were observed to decrease during the nighttime and increase during the daytime at the outlet of Cell 3A between 160 to 220 µM NO$_3^-$ (Figure 2.4). Porewater NO$_3^-$ concentrations were observed to increase during the nighttime and decrease during the daytime (Figure 3.7).

NO$_2^-$ porewater concentrations at the 3 cm depth also exhibited a diel pattern, with concentrations ranging from 0 µM to 2.4 µM NO$_2^-$ (Figure 3.7). The NO$_2^-$ maxima occurred on consecutive days at the same time as the NO$_3^-$ porewater maxima. The diel pattern of NO$_2^-$ in the porewater roughly aligns with the 24-hour diel pattern observed in the water column in June. Water column NO$_2^-$ concentrations in June were observed to increase during the nighttime and decrease during the daytime (Figure 2.4). The maximum NO$_2^-$ concentrations in the water column occurred at 5:00, whereas the maximum porewater NO$_2^-$ concentration occurred at 1:30 and 6:10.
3.3.4 Nitrogen porewater chemistry at 5 cm biomat depth

The 5 cm depth yielded the second highest, average, porewater NH$_4^+$ concentration at 1100 µM (+/- 130 µM). Porewater NH$_4^+$ concentration nearly doubled from the 3 cm to the 5 cm biomat depth (Figure 3.3). There is less variation in NH$_4^+$ porewater concentration at the 5 cm depth when compared to the 3 cm depth. NH$_4^+$ did not exhibit a diel pattern at the 5 cm depth.

NO$_3^-$ porewater concentrations ranged from 0 µM to 10 µM (Figure 3.7). The maximum porewater NO$_3^-$ concentration at the 5 cm depth is nearly 6-times lower than the maximum NO$_3^-$ porewater concentration at the 3 cm biomat depth. One NO$_3^-$ porewater concentration maximum was observed at 1:30 on the first day of sampling. The minimum for NO$_3^-$ porewater concentrations occurred at 18:00 and 0:00 (Figure 3.7). Porewater sampling of the 5 cm depth ended at 15:10, which corresponded with an increase in NO$_3^-$ porewater concentrations.

NO$_2^-$ porewater concentrations ranged from 0 to 0.66 µM NO$_2^-$ (Figure 3.7). The maximum porewater NO$_2^-$ concentration at the 5 cm depth is more than 15-times lower than the maximum NO$_2^-$ porewater concentration at the 3 cm depth. The maximum NO$_2^-$ porewater concentration of 0.65 µM occurred between 1:30 to 6:10. Porewater sampling of the 5 cm depth ended at 15:10, which corresponded with an increase in NO$_2^-$ porewater concentrations.

3.3.5 Nitrogen porewater chemistry at 7 cm biomat depth

At 7 cm biomat depth, the average porewater NH$_4^+$ concentration decreased from 1100 µM (+/- 130 µM) at the 5 cm depth to 610 µM (+/- 560 µM) NH$_4^+$ at the 7 cm depth (Figure 3.3). There is more variation in NH$_4^+$ porewater concentrations at the 7 cm depth when compared to the 5 cm depth. The minimum NH$_4^+$ concentration was 200 µM and occurred at 23:00. The maximum NH$_4^+$ concentration was 2100 µM and occurred at 15:30. NH$_4^+$ did not exhibit a diel pattern at the 7 cm depth.
The 7 cm depth yielded the highest, average, porewater NO$_3^-$ concentration of 44 µM (+/- 30 µM). The maximum porewater NO$_3^-$ concentration at the 7 cm depth is 7-times higher than the maximum NO$_3^-$ porewater concentration at the 5 cm depth (Figure 3.3). The maximum NO$_3^-$ concentration at the 7 cm depth occurs at 23:00 and the minimum occurred at 18:00 with 0 µM NO$_3^-$. NO$_3^-$ concentrations did not exhibit diel changes at the 7 cm depth (Figure 3.7).

The 7 cm depth yielded the highest, average, porewater NO$_2^-$ concentration of 1.8 µM (+/- 1.5 µM). The maximum porewater NO$_2^-$ concentration at the 7 cm depth is just over 7-times higher than the maximum NO$_2^-$ porewater concentration at the 5 cm depth. The maximum NO$_2^-$ concentration at the 7 cm depth occurred at the same time the maximum NO$_3^-$ concentration occurred at 23:00. NO$_2^-$ concentrations did not exhibit diel changes at the 7 cm depth, but the shape of the NO$_2^-$ concentration curve versus time is the same as the NO$_3^-$ concentrations curve versus time (Figure 3.7).

3.3.6 Nitrogen porewater chemistry at 9 cm biomat depth

At 9 cm biomat depth, the average porewater NH$_4^+$ concentration increased from 390 µM (+/- 110 µM) at the 7 cm depth to 820 µM (+/- 230 µM) NH$_4^+$ (Figure 3.3). There is more variation in NH$_4^+$ porewater concentrations at the 9 cm depth when compared to the 7 cm depth. NH$_4^+$ did not exhibit a diel pattern at the 7 cm depth. A decrease in porewater NH$_4^+$ concentration coincided with an increase in porewater NO$_3^-$ and NO$_2^-$ concentration at the 9 cm depth at 0:05 and again at 18:00 (Figure 3.7). The decrease in NH$_4^+$ was more pronounced at 0:05 than the decrease that occurs at 18:00.

NO$_3^-$ concentrations did not exhibit diel changes at the 9 cm depth (Figure 3.7). The variation in porewater NO$_3^-$ concentrations was very erratic at the 9 cm depth (average NO$_3^-$ concentration = 35 µM +/- 45 µM).
NO$_2^-$ concentrations did not exhibit diel changes at the 9 cm depth, but the shape of the NO$_2^-$ concentration curve versus time is the same as the NO$_2^-$ concentrations curve versus time (Figure 3.7). The variation in porewater NO$_2^-$ concentrations was very erratic at the 9 cm depth (average NO$_2^-$ concentration = 0.85 µM +/- 1.0 µM).

3.3.7 Nitrogen porewater chemistry at 11 cm biomat depth

The 11 cm depth yielded the highest, average, porewater NH$_4^+$ concentration at 1200 µM (+/- 230 µM). The average porewater NH$_4^+$ concentration increased from 820 µM (+/- 230 µM) at the 9 cm depth to 1200 µM (+/- 230 µM) (Figure 3.3). NH$_4^+$ did not exhibit a diel pattern at the 11 cm depth (Figure 3.7).

NO$_3^-$ concentrations did not exhibit diel changes at the 11 cm depth (Figure 3.7). The variation in porewater NO$_3^-$ concentrations is very erratic at the 11 cm depth (average NO$_3^-$ concentration = 0.90 µM +/- 1 µM).

There is no detectable NO$_2^-$ at the 11 cm depth (Figure 3.7).

3.3.8 Denitrification Potential Assay

The potential for denitrification activity was observed in the 0-1 cm and 1-6 cm biomat depths, but not in the 6-12 cm biomat depth (Figure 3.8). The 1-6 cm biomat depth exhibited the fastest denitrification potential rate with a rate of 32 µmol-N g$^{-1}$ hr$^{-1}$. The 0-1 cm depth exhibited a denitrification rate of 24 µmol-N g$^{-1}$ hr$^{-1}$. The 1-6 cm biomat depth produced 22 µM N$_2$O-N over the course of the assay, more N$_2$O than the 0-1 cm biomat depth which produced 17 µM-N$_2$O-N. There was greater variability between triplicate measurements for the 0-1 cm biomat depth when compared to the 1-6 cm and 6-12 cm depths. Standard error of the slope was higher for the 1-6 cm biomat depth than for the 0-1 cm biomat depth.
Figure 3.3: Porewater chemistry profiles of the biomat, located 70 meters laterally from the outlet. Red, dashed lines represent nighttime sampling events (21:00 to 3:00). The black, solid lines represent the daytime sampling events (6:00 to 18:00). The experiment spanned 48 hours and every depth was sampled at every time point.
3.4 Discussion

3.4.1 Predicted nitrogen cycling activity from 0 to 5 cm biomat depth

In June, there was a clear concentration gradient of high NO$_3^-$ concentrations in the water column and lower NO$_3^-$ concentrations in the porewater. Assuming diffusive NO$_3^-$ flux, depletion of NO$_3^-$ between the water column and the 1 cm depth can be attributed to the presence of the oxic-anoxic interface (Figure 3.3, refer to Chapter 2 for water column measurements). An oxidized species, like NO$_3^-$ would be rapidly denitrified and reduced under anoxic conditions in non NO$_3^-$-limiting environments. This type of reactivity has been
Figure 3.5: The vertical profiles of $\text{SO}_4^{2-}$ and $\text{PO}_4^{3-}$ in the biomat including the $\text{SO}_4^{2-}$ and $\text{PO}_4^{3-}$ concentrations in the overlying water column during the June 2018 site visit. Red, dashed lines represent dark sampling events (21:00 to 3:00). The black, solid lines represent the light sampling events (6:00 to 18:00). The experiment spanned 48 hours and every depth was sampled at every time point. The difference in $\text{SO}_4^{2-}$ concentrations between the biomat and surface water supports the idea that the piezometers did not cause leakage of surface water into the depths of the biomat.
Figure 3.6: The vertical profiles of cation species (K$^+$, Ca$^{2+}$, Mg$^{2+}$, and Na$^+$) in the biomat during the June 2018 site visit. Red, dashed lines represent dark sampling events (21:00 to 3:00). The black, solid lines represent the light sampling events (6:00 to 18:00). The experiment spanned 48 hours and every depth was sampled at every time point.
Figure 3.7: Horizontal profiles of \( \text{NH}_4^+ \) (black), \( \text{NO}_3^- \) (blue), and \( \text{NO}_2^- \) (orange) at each sampled biomat depth during the June 2018 site visit. The first sample was taken at 15:30 on June 26, 2018. The shaded regions represent sunset to sunrise based on June solar time.
Figure 3.8: Denitrification potential experiment using the acetylene block technique with added NO$_3^-$ described in Repert et al. (2014). The biomat was partitioned by depth in the field and shipped overnight on ice. Error bars represent the standard deviation of triplicate samples. Wet biomat weights were used to calculate denitrification rates.
shown to occur within the porewater of stream sediments (Nimick et al. 2011). Denitrification potential in the first centimeter of the biomat was estimated at 24 µmol-N g⁻¹ hr⁻¹ for biomat tested in June 2018 (Figure 3.8).

Diel changes in NO₃⁻ and NO₂⁻ at the 3 cm depth followed the same diel pattern of Mn and Fe in the water column in September. NO₃⁻, NO₂⁻ and dissolved Mn and Fe increase during the night and decrease during the day (Figure 2.7). MnO₂ and Fe(OH)₃ reduction coupled with NH₄⁺ oxidation to nitrogen oxides (NO₃⁻, NO₂⁻) could explain the observed diel pattern of nitrogen oxides at the 3 cm biomat depth. A diel pattern is not noticeable in the NH₄⁺ pool because the background NH₄⁺ concentration is very high at the 3 cm depth. MnO₂ and Fe(OH)₃ regeneration via diffusion of DO into the biomat at the 3 cm depth would be critical to catalyze these reactions.

At the 5 cm depth, the observed increase in NH₄⁺ concentrations along with the observed decrease in NO₃⁻ and NO₂⁻ concentrations suggest DNRA. The conversion of NO₃⁻ to NH₄⁺ via DNRA is not evident in the NH₄⁺ pool because of the high NH₄⁺ background concentration. NO₂⁻ is an intermediate species in the DNRA pathway and NO₂⁻ concentration versus time follows a similar pattern to NO₃⁻ concentration over time at the 5 cm depth, indicating that the NO₂⁻ signal is from the first step of DNRA, the reduction of NO₃⁻ to NO₂⁻. Sulfide-induced DNRA is also a proposed mechanism for NO₃⁻ removal within the biomat (Jones et al. 2017). There is a considerable decrease in SO₄²⁻ concentration at the 5 cm depth of the biomat, which potentially indicates that SO₄²⁻ had been reduced to hydrogen sulfide (HS⁻) (Figure 3.5). More directed, future inquiry into the presence of HS⁻ at the 5 cm is needed to confirm the presence of HS⁻ in-situ.

### 3.4.2 Predicted nitrogen cycling activity at the 7 cm biomat depth

Anammox is a likely biochemical nitrogen transformation process that is responsible for the observed decrease in NO₃⁻ and NO₂⁻ at the 7 cm depth. NO₂⁻ is the preferred substrate for anammox, and average NO₂⁻ concentration is highest at the 7 cm depth. Genetic sequencing analysis of the biomat has found the family Brocadiaceae to be present in deeper sections of
the biomat, a family of microorganisms associated with anammox activity (Jones et al. 2017). No denitrification activity was detected at this depth using the acetylene block technique (Figure 3.7), further supporting alternative biotic activity at the deeper biomat depths, such as DNRA and anammox. Anammox incubations following the guidance of Thamdrup and Dalsgaard (2002) are needed to confirm potential anammox activity in the biomat.

It is uncertain if the piezometer at the 7 cm biomat depth was leaking surface water. Surface water leakage was suspected at the 7 cm depth because of the presence of nitrogen oxides, an environment assumed to harbor extremely reduced conditions. (Figure 3.7). \( \text{SO}_4^{2-} \) was detected at levels similar to surface water concentrations at the 7 cm biomat depth further suggesting surface water leakage (Figure 3.5). However, low levels of \( \text{NO}_3^- \) were detected during the first two porewater samples taken at 15:30 and 18:00, 0.13 µM and 0.21 µM respectively. At 23:00, porewater \( \text{NO}_3^- \) concentration jumped to 110 \( \text{NO}_3^- \) µM. \( \text{NO}_3^- \) concentrations decreased to 12 µM across the span of 6 time points, then increased again to roughly 40 µM over the course of 5 time points (Figure B1, Appendix B). If surface water leakage was occurring at the 7 cm depth, one would expect high \( \text{NO}_3^- \) concentrations at the first two time points, not at the third and fourth time points. Additionally, there is another \( \text{NO}_3^- \) concentration increase roughly 24 hours into the sampling, indicating a pattern caused by unknown factors, not random surface water leakage.

### 3.4.3 Methanogenesis at the 9 and 11 cm biomat depth

The production of \( \text{CH}_4 \) is hypothesized to occur at the 9 and 11 cm biomat depths. Methanogenesis is the last step in the ecological redox sequence, meaning the substrate for other, more energetic reactions must be depleted before methanogenesis occurs (Hemond & Fechner 2015). \( \text{NO}_3^- \) and \( \text{NO}_2^- \), on average are found in very low concentrations at the 9 and 11 cm biomat depths. Dissolved \( \text{CH}_4 \) was observed to vary on a diel basis in the water column in September (Figure 2.9). Diffusive flux predicts that \( \text{CH}_4 \) is present in higher concentrations in the biomat and low concentrations in the overlying water column, hence a net flux of \( \text{CH}_4 \) out of the biomat. Re-oxidation of \( \text{CH}_4 \) can occur via methanotrophic
bacteria and archaea, but the degree of biotic CH$_4$ oxidation in the biomat is unknown.

### 3.4.4 Spatial availability of nitrate within the biomat

There are many hypothesized factors that influence the spatial availability of NO$_3^-$ within the biomat, including the rate of diffusive flux, the ability for diatoms to act as NO$_3^-$ reservoirs, and subsurface, lateral hydrologic flows.

Diffusive flux is the molecular movement of solutes from area of high concentration to low concentration in order to achieve equilibrium (Hemond & Fechner 2015). DO and NO$_3^-$ are in higher concentrations in the water column than in the biomat, thereby directing DO and NO$_3^-$ into the biomat via diffusive flux. NH$_4^+$ is found in high concentrations in the biomat versus the water column, so diffusive fluxes would predict the movement of porewater NH$_4^+$ into the water column. Diffusive flux of NO$_3^-$ has been shown to contribute significantly less to nitrogen biotransformation in sediment when compared to internally-derived benthic fluxes (Horak et al. 2013, Devol & Christensen 1993).

Jones (2017) classified the diatoms within the biomat of Cell 3A to be *Staurosira construens* var. *venter*. These photosynthetic diatoms have the potential to influence both spatial DO and NO$_3^-$ availability within the biomat, which has huge implications for wetland redox potential, functionality, and ultimately NO$_3^-$ removal. The distribution of DO within the biomat dictates the thermodynamic favorability of biotic nitrogen transformations. The relation between the diatoms and DO availability was discussed in more detail in Chapter 2. The mobility and distribution of the diatoms throughout the full depth of the biomat is unknown. Diatoms have been shown to store NO$_3^-$ in concentrations far above ambient porewater NO$_3^-$ concentrations, an evolutionary commonality among many organisms as NO$_3^-$ can act as the terminal electron acceptor in the sudden absence of oxygen (Kamp et al. 2011, Kamp et al. 2015). Additionally, Kamp et al. (2011) showed that under anoxic conditions, diatoms are capable of metabolizing stored NO$_3^-$ via DNRA to enter a long-term resting phase. If diatoms are found in deeper, anoxic zones of the biomat, diatoms undergoing DNRA could act as a source for the high NH$_4^+$ concentrations observed in the biomat. Ul-
timately, diatoms possess the capability to redirect NO$_3^-$ away from heterotrophic microbes that undergo denitrification and anammox, two pathways that result in NO$_3^-$ removal and towards DNRA, an ineffective NO$_3^-$ removal pathway.

Lateral flow within the biomat could explain the presence of nitrogen oxides at the 7 cm depth. Lateral flow within the biomat could be caused by the waxing and waning of liner bubbles. Liner bubbles are caused by gaseous build up below the geotextile liner of the wetland. Since gases cannot penetrate the geotextile liner, it is also assumed that water cannot penetrate it either. As a result, porewater is diverted around the bubble, causing lateral flow within the biomat and vertical redistribution of nitrogen oxides. This hydrologic phenomenon could explain the presence of nitrogen oxides at the 7 cm depth.
CHAPTER 4
QUANTIFYING NITROGEN TRANSFORMATION PROCESSES AT THE BIOMAT-WATER INTERFACE USING STABLE NITROGEN ISOTOPES

4.1 Introduction

A tracer test is a commonly used method in hydrology to monitor the change in concentration of a tracer over time and/or space. The type of information a tracer test can yield spans from hydraulic residence time to establishing biogeochemical rates, all in-situ. Stable isotope tracer tests are unique because they provide reactive tracer that can be followed from substrate to product. Biogeochemical rates can be established by tracking this isotope tracer into various N-containing analytes following its addition in the desired tracer form. $^{15}$N is a good tracer for environmental reactions because the majority of all naturally occurring N is $^{14}$N and small, excess abundances of $^{15}$N can be measured with high sensitivity. Here, $^{15}$N was utilized as a reactive tracer in the form of $^{15}$NO$_2^-$. NO$_2^-$ serves as a unifying and reactive intermediate and substrate in many biochemical pathways, including nitrification, denitrification, anammox, and DNRA (Figure 4.1). This study aims to quantify NO$_2^-$ transformation within the unvegetated, experimental wetland cells and also assess the influence of light and dark conditions on biomat activity in terms of inorganic nitrogen speciation rates. Observing NO$_2^-$ turnover and transformation can give insight into the relative contributions and potential rates of many biochemical pathways, which translates into the ability to optimize the engineered wetland system for a variety of downstream water quality applications (Lee et al. 2009, Jasper et al. 2013).
Figure 4.1: A depiction of the nitrogen cycle with an emphasis on NO$_2^-$, a reactive intermediate in many biochemical nitrogen transformation processes. AMO = ammonium oxidation, DNRA = dissimilatory nitrate reduction to ammonium.

4.2 Methods

Two, in-situ tracer experiments were performed in June and September of 2018. Both experiments were conducted in the unvegetated wetland Cell 3A, characterized by a hydraulic residence time of approximately one day. Placement of the chambers is depicted in Figure 1.3.

4.2.1 June in-situ tracer test experimental design

The June tracer test was designed to quantify gross NO$_2^-$ production and turnover. In-situ chambers were 15 cm diameter, 38 cm tall, clear, hollow, polycarbonate columns (Figures 4.2 & 4.3). Each chamber was gently lowered into the biomat to minimize disturbance just before the start of the incubation and the top was sealed with a clear cap equipped with an electronic stir motor to ensure a homogenous, overlying water column. The bottom of the chamber was open and rested on the geotextile liner of the wetland, below the biomat. Biomat thickness in each chamber ranged from 14 to 18 cm, with the water column comprising the remainder.
of the column volume (20 to 24 cm water depth). Since the chamber height exceeded the water depth of the wetland, wetland water was used to fill the remainder of the chamber volume. Each chamber was capped, without headspace or bubbles, the stir motor initiated, and remained undisturbed and in-place for the duration of the experiment. Two incubations were performed, one in the light and one in the dark. For each incubation, there was a biomat + water column treatment and a water column only treatment. Next, both NaCl and $^{15}$NO$_2^-$ were added to each chamber, the conservative and reactive tracers, respectively. The target concentration for the conservative and reactive tracers were 20 mM and 20 µM, respectively. $^{15}$NO$_2^-$ enrichment was ~30 atom percent (AT% as $^{15}$N). The dark incubation took place from 20:00 to 11:00 the day following the light incubation. Time points were taken 0, ½, 1, 3, 5, 10, and 15 hours after the initial start of the experiment. At the completion of the light incubations, the chambers were removed from the biomat and repositioned in new locations in the wetland for the light incubations. The light incubation spanned from 13:00 to 17:45 with time points at 0 ½, 1, 2, and 4 hours after the initial start of the experiment. A sampling event in June consisted of accessing a sample port from the chamber cap, removing 20 mL of incubated water from the overlying water column with a syringe, and partitioning into 10 mL sample aliquots (one for bulk cation analysis, one for bulk anion and $^{15}$NO$_2^-$ analysis). 20 mL of wetland water was used to replace the lost volume in the column due to the sampling event. A wetland water sample was taken at each time point to factor in the water chemistry that was added to the columns at each time point. A Millex 0.45 µm syringe filter was used to filter the samples directly into their designated scintillation vials. Dark incubation anion and $^{15}$NO$_2^-$ samples were collected in 37 mL serum bottles, flushed with ultra-high purity helium and stoppered prior to arrival. Samples collected in the serum bottles were not filtered, but rather preserved with 20 µL of concentrated potassium hydroxide, to achieve a final pH of ~12.
4.2.2 September in-situ tracer test experimental design

In-situ stable nitrogen isotope tracer experiments were performed in batch mode assays described in Steingruber et al. (2001) for the purpose of quantifying the rates of direct denitrification and coupled nitrification-denitrification. The columns were the same specifications as described for the June incubations (Figure 4.2 & 4.3). Biomat cores were collected ~70 meters (+/-2.0 meters) from the outlet of Cell 3A, then the bottoms of the chambers were capped with a rubber seal. The chambers were placed in the wetland for the remainder of the incubation. For each incubation, there was a biomat + water column treatment and a water column only treatment. Biomat thickness in the chambers ranged from 8 to 20 cm, with the water column comprising the remainder of the column volume (18 to 30 cm water depth). All chambers were assumed to be chemically and physically similar at the start of the incubations. The chambers were pre-incubated as a closed system for approximately 12
Figure 4.3: Depiction of the column setup for the June and September in-situ tracer test experiments. More details on experimental design can be found in Table 4.1.
hours during the nighttime before the addition of the reactive and conservative tracers. More information regarding the pre-incubation can be found in Appendix C. The target concentration for NaCl and $^{15}$NO$_2^-$ tracers were 20 mM and 20 µM, respectively. $^{15}$NO$_2^-$ enrichment was 80 AT%. The entirety of both the light and dark incubations occurred in-situ, as a closed system, and during the daytime. DO concentration and temperature of the incubated water column was measured periodically. Treatments consisted of light and dark conditions. Light conditions allowed for light penetration through the clear, polycarbonate walls of the chamber, whereas the dark conditions consisted of wrapping the outside wall of the chambers in heavy duty aluminum foil, thereby blocking light penetration to the inside of the chamber. Chambers were sacrificially-sampled every two hours for approximately eight hours. The top 5 cm of biomat was disturbed and homogenized with the overlying water column in order to optimize $^{15}$N tracer recovery, including the porewater of the denitrification reaction zone. All samples were filtered through a 0.7 µm GF/F filter, followed by a 0.22 µm polyethersulfone membrane filter and collected into the designated sample bottle. Samples were preserved according to USGS NWQL and the Reston Stable Isotope Laboratory standards (Appendix A, Table A1). A summary of experimental design for June and September varied slightly and can be found in Table 4.1.

4.2.3 Analytical Techniques

Analytical constraints for isotopic enrichment necessitated the two incubations with differing isotopic enrichments between June and September. The target for June $^{15}$N isotopic enrichment was approximately 30 AT% and September was approximately 80 AT%. The experimental design between the two incubations allowed for $^{15}$NO$_2^-$ disappearance and $^{14}$NO$_2^-$ production to be observed in June and for noticeable enrichment to occur in other nitrogen species in September, all while operating on the natural abundance isotope mass ratio mass spectrometer (IRMS) configuration. A summary of analytical techniques for the determination of $^{15}$N isotopic enrichment can be found in Table 4.2.
Table 4.1: Experimental design for the June and September in-situ tracer test incubations. $^{15}\text{NO}_2^-$ AT% is the atom percent of the stock concentrations. Actual AT% for June can be found in Table C1, Appendix C.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>biomat</th>
<th>water</th>
<th>$^{15}\text{NO}_2^-$</th>
<th>NaCl</th>
<th>Replication</th>
<th>$^{15}\text{NO}_2^-$ AT%</th>
</tr>
</thead>
<tbody>
<tr>
<td>June</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
<td>triplicate</td>
<td>30%</td>
</tr>
<tr>
<td>light &amp; dark</td>
<td>✔</td>
<td>✔</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>light &amp; dark</td>
<td>✔</td>
<td>✔</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>light &amp; dark</td>
<td>✔</td>
<td>✔</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>September</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
<td>6 columns</td>
<td>80%</td>
</tr>
<tr>
<td>light &amp; dark</td>
<td>✔</td>
<td>✔</td>
<td></td>
<td></td>
<td>sample</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>sacrificially</td>
<td></td>
</tr>
<tr>
<td>light &amp; dark</td>
<td>✔</td>
<td>✔</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
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<td></td>
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</tr>
</tbody>
</table>

Table 4.2: Summary of analytical techniques for the determination of nitrogen analyte concentration and $^{15}\text{N}$ isotopic enrichment.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Analysis</th>
<th>Method</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>June</td>
<td>$^{14}\text{NO}_2^-+^{15}\text{NO}_2^-$</td>
<td>Concentration</td>
<td>NOA</td>
</tr>
<tr>
<td>$^{14}\text{NO}_3^-+^{15}\text{NO}_3^-$</td>
<td>Concentration</td>
<td>NOA</td>
<td>Böhlke et al. (2007)</td>
</tr>
<tr>
<td>$^{15}\text{NO}_2^-$</td>
<td>Enrichment</td>
<td>GC-IRMS</td>
<td></td>
</tr>
<tr>
<td>September</td>
<td>$^{14}\text{NH}_4^++^{15}\text{NH}_4^+$</td>
<td>Concentration</td>
<td>Colorimetric</td>
</tr>
<tr>
<td>$^{14}\text{NO}_2^-+^{15}\text{NO}_2^-$</td>
<td>Concentration</td>
<td>NOA</td>
<td>Cox (1980)</td>
</tr>
<tr>
<td>$^{14}\text{NO}_3^-+^{15}\text{NO}_3^-$</td>
<td>Concentration</td>
<td>NOA</td>
<td>Hannon &amp; Böhlke (2008)</td>
</tr>
<tr>
<td>$^{15}\text{NH}_4^+$</td>
<td>Enrichment</td>
<td>EA-IRMS</td>
<td></td>
</tr>
<tr>
<td>$^{29}\text{N}_2, ^{30}\text{N}_2$</td>
<td>Enrichment</td>
<td>GC-IRMS</td>
<td>Böhlke et al. (2004)</td>
</tr>
</tbody>
</table>
\(^{15}\text{NO}_2^-\) was bacterially reduced to N\(_2\)O by \textit{Stenotrophomonas nitritireducens} (Böhlke et al. 2007). The resultant headspace was analyzed on a gas chromatograph in tandem with a continuous-flow isotope-ratio mass spectrometry (GC-IRMS) for isotope enrichment values (\(\partial^{15}\text{N}\)), which is defined as:

Equation 4.1.

\[
\partial^{15}\text{N}_{\text{sample}} = \left[ \frac{\left( \frac{^{15}\text{N}}{^{14}\text{N}} \right)_{\text{sample}}}{\left( \frac{^{15}\text{N}}{^{14}\text{N}} \right)_{\text{reference}} - 1} \right] \times 1000 \tag{4.1}
\]

Air standards were used as the reference for calculating isotope enrichment values. Isotope enrichment values were converted to mole fraction excess (\(X^{15}\text{N}\)), which is defined as:

Equation 4.2.

\[
X^{15}\text{N}_{\text{sample}} = \left[ \frac{\left( \frac{^{15}\text{N}}{1000} + 1 \right) \left( \frac{^{15}\text{N}}{^{14}\text{N}} \right)}{1 + \left( \frac{^{15}\text{N}}{1000} \right) \left( \frac{^{15}\text{N}}{^{14}\text{N}} \right)} \right] - 0.004 \tag{4.2}
\]

Where 0.004 is the mole fraction of naturally abundant \(^{15}\text{N}\). Conceptually, the mole fraction excess is the amount of \(^{15}\text{N}\) in a system beyond the naturally abundant \(^{15}\text{N}\).

\(^{15}\text{NH}_4^+\) enrichment was quantified by converting aqueous \(^{15}\text{NH}_4^+\) to gaseous \(^{15}\text{NH}_3\) in a closed container by raising the pH of the sample to \(>9\) with magnesium oxide. The \(^{15}\text{NH}_3\) gas was trapped as ammonium sulfate onto a glass fiber filter supplemented with sodium bisulfate monohydrate. The filter was tightly wrapped in aluminum foil, combusted, and the resultant gas stream was analyzed for isotope enrichment values on an elemental analyzer in series with isotope ratio mass spectrometer (EA-IRMS) (Table 4.2). \(^{15}\text{NH}_4^+\) standards of the same target mass were diffused and analyzed alongside Prado samples to ensure high diffusion efficiencies. Blank injections were run between each Prado sample on the EA-IRMS to ensure that any residual, highly enriched tracer gas was flushed out of the system before the next sample injection. USGS Reston Stable Isotope Glutamic Acid Reference Materials U40 and U41 were run in sequence with Prado samples to ensure data accuracy and that the instrument was functioning reliably and consistently. \(^{15}\text{NH}_4^+\) enrichment values were used to calculate the rate of conversion of \(^{15}\text{NO}_2^-\) to \(^{15}\text{NH}_4^+\) (Table 4.3).
$^{29}\text{N}_2$ and $^{30}\text{N}_2$ enrichment were quantified by GC-IRMS. Air standards were used to calculate the isotope enrichment values (Equation 4.1) and Equation 4.2 was used to calculate $X^{15}\text{N}$. All $\text{N}_2$ present in the aqueous and gaseous forms are assumed to be flushed out of the serum bottle by sparging the aqueous fraction with helium and flushing the headspace directly into the GC-IRMS. The measured enrichments of mass $^{29}\text{N}_2$ and mass $^{30}\text{N}_2$ isotopologues were used to calculate coupled and direct denitrification rates described in Steingruber et al. (2001) (Table 4.3). The production of $^{29}\text{N}_2$ and $^{30}\text{N}_2$ and the associated slopes can be found in Figure 4.9 and Table 4.4, respectively.

Table 4.3: Analytes of interest and their application in various calculations.

<table>
<thead>
<tr>
<th></th>
<th>Analyte</th>
<th>Rate calculation</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>June</td>
<td>$^{15}\text{NO}_2^-$</td>
<td>Gross $^{15}\text{NO}_2^-$ regeneration, $^{15}\text{NO}_2^-$ turnover</td>
<td>Tobias et al. (2003)</td>
</tr>
<tr>
<td></td>
<td>$^{15}\text{NH}_4^+$</td>
<td>$^{15}\text{NO}_2^-$ $\rightarrow^{15}\text{NH}_4^+$</td>
<td>Plummer et al. (2015)</td>
</tr>
<tr>
<td></td>
<td>$^{29}\text{N}_2$, $^{30}\text{N}_2$</td>
<td>Isotope Pairing Technique</td>
<td>Steingruber et al. (2001)</td>
</tr>
</tbody>
</table>

$\text{NO}_2^-$ concentrations were quantified by reducing $\text{NO}_2^-$ to NO in a reaction chamber containing acetic acid and NaI. The NO gas produced was carried by ultra-high purity helium to the chemiluminescent detector (Table 4.3, Cox 1980). Each sample was injected in duplicate with <5% difference between injections. If >5% error was obtained, the sample was analyzed until at least two injections were within <5% of each other. Each analysis contained $\text{NO}_2^-$ standards that were prepared weekly using concentrated stock solutions purchased from High Purity Solutions. The detection limit for $\text{NO}_2^-$ analysis was 0.025 nmol (100 µL injection of 0.25 µM standard).

$\text{NO}_3^-$ concentrations were quantified by reducing $\text{NO}_3^-$ to NO in a heated reaction chamber containing heated $\text{VCl}_3$ in 1N hydrochloric acid. The NO gas stream was carried by ultra-high purity helium to the chemiluminescent detector (Table 4.3, Cox 1980). Each analysis contained $\text{NO}_3^-$ standards that were prepared weekly using concentrated stock solu-
tions purchased from High Purity Solutions. USGS SRSs with certified NO$_3^-$ concentrations were run alongside Prado samples to ensure data accuracy. Each sample was injected in duplicate with $<5\%$ difference between injections. If $>5\%$ error was obtained, the sample was analyzed until at least two injections were within $<5\%$ of each other. The detection limit for NO$_3^-$ analysis was 0.025 nmol (100 µL injection of 0.25 µM standard).

September NH$_4^+$ concentrations were colorimetrically quantified utilizing the methods described in Scheiner (1976) (Table 4.2). Methods from Scheiner (1976) were utilized on September NH$_4^+$ samples because the NH$_4^+$ concentration in the outlet surface water was lower in September resulting in greater Na$^+$ peak interference during IC analysis. A methods comparison was performed between the colorimetric NH$_4^+$ assay and IC; results of the internal methods comparison can be found in Appendix A. Scheiner (1976) determined an array of interference compounds, none of which occur in high enough concentrations in Prado samples to be of concern.

The mass of NO$_2^-$, NO$_3^-$, and NH$_4^+$ (in µmol) was determined by multiplying the measured concentrations (µM) by the water column volume (L) for each column. Water column volume was calculated by multiplying the inner column area by the water column height. Water column height was individually determined for each column by taking a biomat height measurement and subtracting it from the total height of the column. The volumes for the water column only treatments were calculated by multiplying the inner column area by the height of the column. Measuring the change in mass per column in µmol allowed for a degree of normalization between each time point, since water volume varied between each column in September. Figure 4.5 depicts both µM and µmol values for the reader’s interpretation. Table C2 in Appendix C gives water volumes for each column during the September incubation.
4.3 Results

4.3.1 June light incubations

For the biomat + water column treatment, the net NO$_3^-$ mass decreased more dramatically than the decrease in mass of NO$_2^-$ over the course of the 4-hour incubation (Figures 4.4 & 4.5a). The mass of NO$_3^-$ decreased from 360 µmol to 290 µmol (20% decrease in NO$_3^-$ mass). The net mass of NO$_2^-$ was observed to decrease from 39 µmol to 34 µmol (13% decrease in NO$_2^-$ mass). The mass of $^{15}$NO$_2^-$ decreased by 18% over the course of the incubation from 6.0 µmol to 4.9 µmol (Figure 4.6c). The mole fraction excess of $^{15}$NO$_2^-$ decreased slightly from 0.15 to 0.14 (Figure 4.6d). The amount of net NO$_2^-$ and NO$_3^-$ disappearance in the light conditions was much less than the disappearance of these same analytes in the dark conditions, despite the light conditions having more NO$_2^-$ mass at the beginning of the incubation.

Very little net change in mass was observed for NO$_2^-$ and NO$_3^-$ in the water column only treatment (Figure 4.5a). No change in the mass of NO$_3^-$ was observed. Net NO$_2^-$ mass was observed to increase from 52 µmol to 55 µmol (6.2% increase in NO$_2^-$ mass). $^{15}$NO$_2^-$ mass increased from 7.9 µmol to 8.5 µmol (7% increase in $^{15}$NO$_2^-$) (Figure 4.6c). No change in NO$_2^-$ mole fraction excess was observed (Figure 4.6d).

4.3.2 June dark incubations

For the biomat + water column treatment, the net mass of NO$_2^-$ and NO$_3^-$ were observed to decrease of the course of the 15-hour incubation (Figures 4.4 & 4.5b). A net decrease of 33% in NO$_2^-$ mass was observed, with a beginning mass of 33 µmol and a final mass of 22 µmol. The mass of $^{15}$NO$_2^-$ decreased by 57% over the course of the incubation from 7.0 µmol to 3.0 µmol (Figure 4.6a). $^{15}$NO$_2^-$ mole fraction excess was observed to decrease from 0.21 to 0.13 (Figure 4.6b). A 55% decrease in net NO$_3^-$ mass was observed. The starting mass of NO$_3^-$ was 410 µmol and the final mass was 180 µmol (Figure 4.5b).
For the water column only treatment, minimal changes in NO\textsubscript{2}\textsuperscript{-} and NO\textsubscript{3}\textsuperscript{-} mass were observed. The net mass of NO\textsubscript{2}\textsuperscript{-} decreased from 26 µmol to 23 µmol (13% decrease in NO\textsubscript{2}\textsuperscript{-} mass) (Figure 4.5b). The mass of \textsuperscript{15}NO\textsubscript{2}\textsuperscript{-} decreased from 5.9 µmol to 4.4 µmol (25% decrease in \textsuperscript{15}NO\textsubscript{2}\textsuperscript{-}) (Figure 4.6a). \textsuperscript{15}NO\textsubscript{2}\textsuperscript{-} mole fraction excess for the water column did not change (Figure 4.6b). NO\textsubscript{3}\textsuperscript{-} mass decreased from 660 µmol to 610 µmol (7% decrease in NO\textsubscript{3}\textsuperscript{-} mass).

4.3.3 September light incubations

For the biomat + water treatment, the net mass of NO\textsubscript{2}\textsuperscript{-}, NO\textsubscript{3}\textsuperscript{-}, and NH\textsubscript{4}\textsuperscript{+} was observed to decrease over the course of the 8-hour incubation (Figure 4.7a). In the water column, net NO\textsubscript{2}\textsuperscript{-} was observed to decrease from 38 µmol to 25 µmol (34% decrease), net NO\textsubscript{3}\textsuperscript{-} decreased from 41 µmol to 23 µmol (44% decrease), and net NH\textsubscript{4}\textsuperscript{+} decreased from 130 µmol to 71 µmol (35% decrease). \textsuperscript{15}NH\textsubscript{4}\textsuperscript{+} mass increased slightly from 0.47 µmol to 0.93 µmol and the NH\textsubscript{4}\textsuperscript{+} mole fraction excess increased by 0.009 (0.004 to 0.013) (Figure 4.8). The production of \textsuperscript{29}N\textsubscript{2} and \textsuperscript{30}N\textsubscript{2} was minimal during the light incubation (Figure 4.9).

For the water column only treatment, no significant change in net NO\textsubscript{2}\textsuperscript{-}, NO\textsubscript{3}\textsuperscript{-}, or NH\textsubscript{4}\textsuperscript{+} concentration or mass was observed (Figure 4.7a). Net NO\textsubscript{2}\textsuperscript{-} mass was observed to be 32 µmol at the beginning of the incubation and 34 µmol at the end of the incubation. Net NO\textsubscript{3}\textsuperscript{-} mass was observed to decrease from 230 µmol to 210 µmol across the course of the incubation. Net NH\textsubscript{4}\textsuperscript{+} mass was observed to decrease slightly from 8.8 µmol to 8.0. (Figure 4.8). The mole fraction excess for the water column only treatment was not determined due to the very low NH\textsubscript{4}\textsuperscript{+} concentration. There was no observed change in \textsuperscript{29}N\textsubscript{2} and \textsuperscript{30}N\textsubscript{2} mass over the course of the incubation (Figure 4.9).

4.3.4 September dark incubations

For the biomat + water column treatment, the net mass of NO\textsubscript{2}\textsuperscript{-} and NO\textsubscript{3}\textsuperscript{-} was observed to decrease over the course of the 9-hour incubation, while the mass of the water column NH\textsubscript{4}\textsuperscript{+} was observed to increase (Figures 4.7b). The net mass of NO\textsubscript{2}\textsuperscript{-} at T\textsubscript{0} was 35 µmol and decreased to 13 µmol by the end of the incubation (37% decreased in NO\textsubscript{2}\textsuperscript{-}). The net
mass of NO$_3^-$ at T$_0$ was 27 µmol and 16 µmol at the end of the incubation. NH$_4^+$ exhibited a significant increase in both concentration and isotopic enrichment. The net mass of NH$_4^+$ increased approximately 100 µmol (97 µmol to 200 µmol) (Figure 4.7b). The mole fraction excess and the mass of $^{15}$NH$_4^+$ increased from 0.63 µmol to 3.3 µmol (Figures 4.8). During the dark incubation, 5.2 µmol $^{29}$N$_2$ was produced and 0.89 µmol $^{30}$N$_2$ (Figure 4.9).

For the water column only treatment, changes in water column NO$_2^-$, NO$_3^-$, and NH$_4^+$ mass and enrichment were observed (Figure 4.7b). Net NO$_2^-$ mass was observed to be 30 µmol at T$_0$ and 35 µmol by the end of the incubation. Net NO$_3^-$ mass was observed to decrease from 170 µmol to 120 µmol and net NH$_4^+$ mass was observed to decrease from 2.9 µmol to 0.50 µmol. The mole fraction excess for the water column only treatment was not determined due to the very low NH$_4^+$ concentration. There was no observed change in $^{29}$N$_2$ and $^{30}$N$_2$ mass over the course of the incubation (Figure 4.9).

![Figure 4.4: Change in NO$_2^-$ mass in June and September. Dark incubations are represented with dashed lines and light incubations are represented by solid lines. June data points are represented as the average of triplicate measurements. September data points are single measurements. Error bars represent the standard deviation of triplicate samples.](image-url)
Figure 4.5: Changes in NO$_2^-$, NO$_3^-$, and NH$_4^+$ concentration and mass in the overlying water column of the biomat + water column treatment during the June light (left) and dark (right) in-situ incubations. N/Cl ratios depict the change in reactive tracer relative to the conservative tracer. All data is represented as an average of triplicate samples. Error bars are present on concentration measurements and represent the standard deviation between triplicate samples.
Figure 4.6: Change in mass of \( ^{15}\text{NO}_2^- \) and their associated mole fraction excess values \( (X^{15}\text{N}) \) at \( T_0 \) (white) and \( T_f \) (shaded). \( \text{NO}_2^- \) samples were collected and analyzed from the June in-situ incubation. Mass measurements are represented by the average of triplicate measurements. Mole fraction excess measurements are represented by the average of triplicate measurements. Error bars represent standard deviation of triplicate measurements. (*) Measurement and error bar represented by the average of duplicate measurements.
Figure 4.7: Changes in NO$_2^-$, NO$_3^-$, and NH$_4^+$ concentration and mass in the overlying water column of the biomat + water column treatment during the September light (A) and dark (B) in-situ incubations. N/Cl ratios depict the change in reactive tracer relative to the conservative tracer. Each data point for the September incubations represents a unique, in-situ incubation.
Figure 4.8: Change in mass of $^{15}\text{NH}_4^+$ (black) and the mole fraction excess of $^{15}\text{N}$ (red). The biomat + water column treatment is shown. $\text{NH}_4^+$ enrichment data was collected and analyzed from the September in-situ incubation. Mass measurements are represented by singular measurements. Enrichment measurements are represented by the average of duplicate measurements. Error bars represent the standard deviation between duplicate samples. (*) Represents a single measurement, not an average of duplicate measurements.
Figure 4.9: Change in mass of $^{29}\text{N}_2$ (black) and $^{30}\text{N}_2$ (red). The biomat + water column treatment is represented as a time series (hollow points connected by lines) and the water column only treatment is represented by triangle points at the first and last time points. $\text{N}_2$ enrichment data was collected and analyzed from the September in-situ incubation. Each time point represents the average of duplicate measurements. Error bars represent the standard deviation of duplicate samples.

Table 4.4: Summary of slope (m), r-squared ($r^2$), and standard error of the slope (SE) for the biomat + water column treatment used in the IPT calculations.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Isotopologue</th>
<th>m (µmol hr$^{-1}$)</th>
<th>$r^2$</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dark</td>
<td>$^{29}\text{N}_2$</td>
<td>0.57</td>
<td>0.94</td>
<td>$8.3 \times 10^{-2}$</td>
</tr>
<tr>
<td></td>
<td>$^{30}\text{N}_2$</td>
<td>0.10</td>
<td>0.84</td>
<td>$1.1 \times 10^{-2}$</td>
</tr>
<tr>
<td>Light</td>
<td>$^{29}\text{N}_2$</td>
<td>$3.3 \times 10^{-2}$</td>
<td>1.6 $\times 10^{-2}$</td>
<td>$5.4 \times 10^{-2}$</td>
</tr>
<tr>
<td></td>
<td>$^{30}\text{N}_2$</td>
<td>$6.8 \times 10^{-3}$</td>
<td>3.9 $\times 10^{-2}$</td>
<td>$1.7 \times 10^{-2}$</td>
</tr>
</tbody>
</table>
4.4 Discussion

4.4.1 Gross nitrite regeneration

Conceptually, gross NO$_2^-$ regeneration is the rate at which NO$_2^-$ is generated and/or fluxed from the biomat into the water column (Figure 4.10). Since it is predicted there is a small inventory of NO$_2^-$ in the porewater, the gross NO$_2^-$ regeneration is equivalent to NO$_2^-$ production rate in the sediments at steady state with the overlying water column. NO$_2^-$ production can come from either NH$_4^+$ oxidation (AMO) or the reduction of NO$_3^-$ from the overlying water column (Figure 4.10). Gross NO$_2^-$ regeneration was estimated to be 5.5 mmol-N m$^{-2}$ d$^{-1}$ for the light conditions and 8.7 mmol-N m$^{-2}$ d$^{-1}$ for the dark conditions (Table 4.5), meaning more NO$_2^-$ was generated in dark incubation versus the light incubation. Average NO$_2^-$ turnover time, or the theoretical length of time to completely replace the NO$_2^-$ pool to naturally abundant nitrogen ratios, was estimated to be 12 minutes in the light and dark conditions under low, porewater NO$_2^-$ concentrations (Table 4.6). Short NO$_2^-$ turnover time indicates NO$_2^-$ is being readily consumed and produced in biochemical reactions. NO$_2^-$ turnover was estimated to be 8.6 hours and 7.5 hours in the dark and light conditions, respectively, under higher NO$_2^-$ porewater concentrations, however, these differences between light and dark are minimal.

<table>
<thead>
<tr>
<th></th>
<th>Total denitrification ($^{15}$NO$_2^-\rightarrow$ $^{29}$N$_2$, $^{30}$N$_2$)</th>
<th>$^{15}$NO$_2^-\rightarrow$ $^{15}$NH$_4^+$</th>
<th>Gross NO$_2^-$ regeneration</th>
<th>Net NO$_2^-$ loss</th>
</tr>
</thead>
<tbody>
<tr>
<td>unit</td>
<td>mmol-N m$^{-2}$ d$^{-1}$</td>
<td>mmol-N m$^{-2}$ d$^{-1}$</td>
<td>mmol-N m$^{-2}$ d$^{-1}$</td>
<td>mmol-N m$^{-2}$ d$^{-1}$</td>
</tr>
<tr>
<td>Dark</td>
<td>7.3</td>
<td>1.8</td>
<td>8.7</td>
<td>-5.8</td>
</tr>
<tr>
<td>Light</td>
<td>0.39</td>
<td>0.58</td>
<td>5.5</td>
<td>-2.1</td>
</tr>
</tbody>
</table>
Table 4.6: NO$_2^-$ regeneration times in the light and dark conditions in June. NO$_2^-$ concentrations are the minimum and maximum observed concentrations at the 1 cm biomat depth in June.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Average NO$_2^-$ regeneration (µmol L$^{-1}$ hr$^{-1}$)</th>
<th>Porewater NO$_2^-$ concentration (µmol L$^{-1}$)</th>
<th>Regeneration time (hour)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dark</td>
<td>0.61 (+/- 0.16)</td>
<td>0.11</td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5.3</td>
<td>8.6</td>
</tr>
<tr>
<td>Light</td>
<td>0.70 (+/- 0.19)</td>
<td>0.11</td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5.3</td>
<td>7.5</td>
</tr>
</tbody>
</table>

4.4.2 The transformation of $^{15}$NO$_2^-$ to $^{15}$NH$_4^+$

Stable nitrogen isotope tracer experiments revealed that the reduction rate of $^{15}$NO$_2^-$ to $^{15}$NH$_4^+$ is faster at night than during the daytime (Table 4.5). $^{15}$NO$_2^-$ reduction to $^{15}$NH$_4^+$ was estimated to be approximately 3-times faster in the dark than in the light (1.8 mmol-N m$^{-2}$ d$^{-1}$ versus 0.58 mmol-N m$^{-2}$ d$^{-1}$). NO$_3^-$ is typically the least preferred form of nitrogen for heterotrophic bacteria, but in aqueous environments with high NO$_3^-$ loads, it has been shown that higher NO$_3^-$ uptake can occur (Kirchman & Wheeler 1998, Middelburg & Nieuwenhuize 2000). Diatoms have been shown to store NO$_3^-$ intercellularly and utilize NO$_3^-$ reserves in the sudden absence of light and oxygen (Kamp et al. 2011, Lomas & Glibert 1999). At night, photosynthetic processes within the diatoms cease and alternate energy production methods ramp up. Water column NH$_4^+$ concentrations were observed to increase during the nighttime in both June and September (Figure 2.4). The $^{15}$NO$_2^-$ tracer is not specific to the DNRA pathway, but it is predicted that the majority of the nighttime $^{15}$NO$_2^-$ to $^{15}$NH$_4^+$ conversion is due to DRNA activity for several reasons. DRNA activity is predicted in the porewater at the 5 cm depth because of the observed decrease in NO$_3^-$ and NO$_2^-$ concentrations relative to the 3 and 7 cm biomat depths. DNRA is also the most efficient pathway to reduce NO$_2^-$ to NH$_4^+$, something that is more likely to occur during a short incubation (Giblin et al. 2013).
Estimated daytime $^{15}$NO$_2^-$ to $^{15}$NH$_4^+$ rates were similar for the biomat + water column treatment and the water column only treatment. The daytime, water column only treatment had an estimated conversion rate of 0.74 mmol-N m$^{-2}$ d$^{-1}$ versus the daytime, biomat + water column treatment rate of 0.58 mmol-N m$^{-2}$ d$^{-1}$. The reason for this similarity is unknown. It is assumed that the biomat consists of an microbially active community of diatoms, bacteria, and archaea that catalyze nitrogen transformation processes. The absence of the biomat in the water column only treatment and the similarity of $^{15}$NO$_2^-$ to $^{15}$NH$_4^+$ rates suggests that nitrogen transformation occurs in the water column during the daytime, potentially as nitrogen assimilation.

The reduction of NO$_3^-$ and NO$_2^-$ to NH$_4^+$ is a highly diverse, biochemical pathway that is generally associated with the nrfA gene (Giblin et al. 2013). Analytical difficulties prevented the amplification and identification of nrfA within the biomat, a gene associated with DNRA activity (Jones 2017). The nrfA gene has not been found or quantified in diatoms, but the functional capacity of DNRA has been shown to be closely linked with diatoms communities (Kamp et al. 2011, Stief et al. 2013). The capacity for the diatoms in the biomat to utilize the DNRA pathway would greatly alter the interpretation of NO$_3^-$ removal with the unvegetated, experimental cells. If the diatoms in Cell 3A possess the capability of DNRA, that would signify the capability of redirecting NO$_3^-$ away from denitrification and anammox, two pathways that result in NO$_3^-$ removal and towards DNRA, an ineffective NO$_3^-$ removal pathway. The origin of high NH$_4^+$ concentrations is unknown within the biomat, but diatom DNRA is a logical contributing factor.

More research is needed into the role and impacts of diatoms on NO$_3^-$ transformation and removal within the biomat of the unvegetated wetland cells. Diatoms have been shown to move NO$_3^-$ into intercellular pools against a NO$_3^-$ concentration gradient and store up to 274 mM NO$_3^-$ in intercellular vacuoles (Kamp et al. 2011, Stief et al 2013). This type of biotic activity effects the spatial availability of NO$_3^-$ within the biomat, a reactive zone that has shown dentification activity and the potential for anammox (Figure 3.8, Jones et
Diatoms could be hosting a massive reservoir of NO$_3^-$ within the biomat, thereby overestimating NO$_3^-$ removal by just quantifying the inlet versus outlet NO$_3^-$ concentrations.

### 4.4.3 The transformation of $^{15}$NO$_2^-$ to $^{29}$N$_2$ and $^{30}$N$_2$

Typically, $^{15}$NO$_3^-$ is used as the tracer for the IPT calculations. In this application, $^{15}$NO$_2^-$ was used as the tracer, which altered the interpretation of these calculations. Direct denitrification rates describe denitrification of NO$_2^-$ in the overlying water column while coupled nitrification-denitrification includes denitrification of NO$_2^-$ produced in the biomat following mineralization and oxidation plus NO$_2^-$ produced from the reduction of water column NO$_3^-$ following NO$_3^-$ diffusion into the biomat. A summary of calculated IPT rates can be found in Table 4.7.

Table 4.7: Summary of rates derived from the IPT calculations for the biomat + water column treatment from $^{29}$N$_2$ and $^{30}$N$_2$ samples collected in September 2018.

<table>
<thead>
<tr>
<th></th>
<th>Total denitrification ($^{15}$NO$_2^-$→$^{29}$N$_2$, $^{30}$N$_2$)</th>
<th>Direct denitrification</th>
<th>Coupled nitrification-denitrification</th>
</tr>
</thead>
<tbody>
<tr>
<td>unit</td>
<td>mmol-N m$^{-2}$ d$^{-1}$</td>
<td>mmol-N m$^{-2}$ d$^{-1}$</td>
<td>mmol-N m$^{-2}$ d$^{-1}$</td>
</tr>
<tr>
<td>Dark</td>
<td>7.3</td>
<td>0.51</td>
<td>6.7</td>
</tr>
<tr>
<td>Light</td>
<td>0.39</td>
<td>0.03</td>
<td>0.36</td>
</tr>
</tbody>
</table>

Coupled nitrification-denitrification during the light and dark conditions as the dominant denitrification pathway aligns well with high NH$_4^+$ concentrations measured in the biomat porewater, since NH$_4^+$ is not limiting within the system. If NH$_4^+$ was a limiting reagent, one would expect higher contributions of direct denitrification. Total denitrification (dark + light rates) is equal to 7.7 mmol-N m$^{-2}$ d$^{-1}$, a rate that is above the mean denitrification rate, according to Fennel et al. (2009), but is still within a range to consider as an overall, reasonable environmental rate estimate. Denitrification occurred at a faster rate during the nighttime than during the day time (7.3 mmol-N m$^{-2}$ d$^{-1}$ versus 0.39 mmol-N m$^{-2}$ d$^{-1}$, respectively). Denitrifying bacteria require anoxic conditions and high NO$_2^-$ and/or NO$_3^-$
concentrations, two conditions that are met in the biomat during the nighttime. During the daytime, denitrification slows down because the autotrophs outcompete the denitrifying heterotrophs for NO$_3^-$.

The autotrophs additionally create and oxic water column and biomat-water column interface which further inhibits denitrification activity. Aerobic processes such as assimilation and nitrification become the dominant pathways during the daytime.

### 4.4.4 Mass balance

A mass balance was performed to account for NO$_2^-$ sink(s) that were not directly quantified during this tracer experiment (Table 4.8 & Figure 4.10). It is predicted that anammox is a large contributor to NO$_2^-$ loss within the biomat. Anammox activity was predicted to occur at the 7 cm biomat depth because of the observed increase in NO$_3^-$ and NO$_2^-$ concentrations coupled with a decrease in NH$_4^+$ concentrations. Genetic sequencing of the biomat has also shown the anammox gene hzsA to be present in larger quantities with depth (Jones et al. 2017). Anammox rates can be calculated using the methods described in Thamdrup & Dalsgaard (2002). Anammox is a biochemical mechanism that contributes to overall NO$_3^-$ removal. NO$_3^-$ removal was observed to occur during the nighttime in Cell 3A in June (Figure 2.5). It is hypothesized that denitrification (7.3 mmol-N m$^{-2}$ d$^{-1}$) and anammox (potentially 5.4 mmol-N m$^{-2}$ d$^{-1}$) are the primary, contributing biochemical pathways for observed NO$_3^-$ removal.

It is hypothesized that NO$_2^-$ assimilation is unaccounted for in the light incubations (Figure 4.10 & Table 4.8). NO$_3^-$ assimilation in diatoms is a well-documented phenomenon (Lomas & Glibert 1999, Kamp et al. 2011). NO$_2^-$ uptake was reported in cell cultures of *T. pseudonana*, a marine diatom, but only after an acclimation period of NO$_3^-$ uptake (Waser et al. 1998). The diatoms in Cell 3A are safely assumed to be acclimated to a high NO$_3^-$environment, so the uptake of NO$_3^-$ and NO$_2^-$ by these diatoms is highly plausible. Nitrification is hypothesized to contribute less to NO$_2^-$ removal during the daytime because of the autotrophs' ability to outcompete the heterotrophs in the light.
Figure 4.10: Mass balance of nitrogen cycling processes measured at the biomat-water interface using stable nitrogen isotopes.

Table 4.8: The predicted NO$_2^-$ sinks for light and dark conditions in the biomat and their associated rate estimates.

<table>
<thead>
<tr>
<th></th>
<th>Net NO$_2^-$ loss</th>
<th>Sum of calculated rates</th>
<th>Remainder to satisfy mass balance</th>
<th>Predicted sink</th>
</tr>
</thead>
<tbody>
<tr>
<td>unit</td>
<td>mmol-N m$^{-2}$ d$^{-1}$</td>
<td>mmol-N m$^{-2}$ d$^{-1}$</td>
<td>mmol-N m$^{-2}$ d$^{-1}$</td>
<td></td>
</tr>
<tr>
<td>Dark</td>
<td>-5.8</td>
<td>-0.4</td>
<td>5.4</td>
<td>Anammox</td>
</tr>
<tr>
<td>Light</td>
<td>-2.1</td>
<td>4.5</td>
<td>6.6</td>
<td>Diatom nitrate uptake</td>
</tr>
</tbody>
</table>
CHAPTER 5
CONCLUSION, BROADER IMPLICATIONS, AND FUTURE RESEARCH

This study was the first to quantify in-situ denitrification in the experimental, unvegetated cells at Prado Wetlands. Denitrification potential has been shown to occur ex-situ on several occasions, but the identification and quantification of denitrification in-situ shows that the unvegetated wetlands are removing NO$_3^-$, not just transforming it. Higher NO$_3^-$ removal occurred during the nighttime due to denitrification and potentially due to anammox (Chapters 1 & 4). Additional inquiry into anammox functionality beyond gene abundance needs to be explored. The methods of Thamdrup and Dalsgaard (2002) can give the relative rates of anammox within the biomat ex-situ. Similar, in-situ incubations could be performed with some protocol modifications.

Diatoms and their influence on the spatial availability of NO$_3^-$ within the biomat should be a topic for future research. Intercellular NO$_3^-$ pools have been quantified by boiling and using freeze-thaw cycles to extract intercellular NO$_3^-$ from environmental samples. Several studies looking at intercellular NO$_3^-$ pools have found that porewater NO$_3^-$ on average accounts for 10% of the total NO$_3^-$ present when quantifying both porewater and intercellular NO$_3^-$ concentrations (Kamp et al. 2015). If we scale that to the upper one centimeter of the biomat porewater, we could have a total NO$_3^-$ pool of up to 270 µM just within the first centimeter. Additional probing for the $nrfA$ gene should be a priority to demonstrate the functional capacity for DNRA in the biomat. Beginning to understand the role of diatoms in these wetlands may help fill in the wetland nitrogen mass balance (Chapter 4) and add to our understanding of how these wetland function and whether NO$_3^-$ removal has been accurately estimated.

The same research approach used in the mature wetland cell can translate to the newer and smaller B cells just downstream of the mature A cells. The B cells also contain biomat
and were designed and implemented to improve NO$_3^-$ attenuation and removal through various hydrologic manipulations called biobarriers. The biobarriers are hypothesized to increase surface water contact with the lower biomat depths, and thereby, increase NO$_3^-$ removal via denitrification and anammox. Nitrogen transformation rates were more feasible to quantify in Cell 3A because of the singular flow regime. The rates established in Cell 3A can act as a baseline for comparison for each hydrologic alternation, coupled with carbon in the smaller B cells. Similar inlet/outlet surface water studies, porewater sampling, and potentially stable $^{15}$N isotope tracer experiments in the smaller cells could be performed in the future to quantify NO$_3^-$ removal efficiency of the smaller cells. There are many variables at play apart from the biobarriers in the B cells, such as shorter residence time, a shallower water column, and carbon availability. These factors must be considered when comparing the functionality of the wetland with a simpler flow regime (A cells) to the smaller, B cells.

The data presented here can also be used as a comparison between vegetated and unvegetated wetlands designed for NO$_3^-$ removal. A plethora of research exists on vegetated wetlands because they are naturally occurring and have high potential for nutrient storage and recycling (Hammer 1989). Vegetated wetlands remove NO$_3^-$ through the complex interaction of microorganisms located in the rhizosphere, including the assimilation of NO$_3^-$ in high NO$_3^-$ environments, nitrification and denitrification (Vymazal 2007). Reilly et al. (2000) reported that macrophyte and algal NO$_3^-$ uptake in Prado Wetlands accounts for 15 mmol-N m$^{-2}$ d$^{-1}$ (~17% of total NO$_3^-$ removal) versus benthic NO$_3^-$ removal accounting for 71 mmol-N m$^{-2}$ d$^{-1}$ (83% of total NO$_3^-$ removal). The rates established by Reilly et al. (2000) are much higher than rates established in this study and is most likely an artifact of methodological differences.

Understanding diel redox changes in aqueous water chemistry and nitrogen transformation and removal on a day versus night basis within unvegetated, engineered, wetland ecosystems can better inform wetland design and operation. The current design of Cell 3A allows for the surface water to experience a full day and night cycle. The diatoms produce
DOC during the daytime, which is used as an electron donor for the anaerobic, nighttime reactions. A shorter hydraulic residence time would not allow for some of the surface water to experience a day or night cycle, thereby decreasing NO$_3^-$ removal efficiency in the A cell design. The new, smaller B cells were designed to shortcut the need for a longer hydraulic residence time through the addition of biobarriers. The biobarriers provide allochthonous carbon to anaerobic environments, shortcutting the need for liable DOC synthesized by the diatoms. In theory, denitrifying bacteria can denitrify in light and dark conditions in anaerobic portions of the biomat. The ability to remove NO$_3^-$ in a shorter period of time within a smaller area with equal efficiency is an overall design improvement. A smaller wetland is more financially feasible and implementation is potentially more practical. The functionality and NO$_3^-$ removal efficiency of the smaller cells is under investigation but shows great potential.
REFERENCES CITED


Smith, D. R., Owens, P. R., Leytem, A. B., & Warnemuende, E. A. (2007). Nutrient losses from manure and fertilizer applications as impacted by time to first runoff event. Environmental Pollution, 147(1), 131-137.


Figure A.1: A method comparison for the analysis of NH$_4^+$ concentration between ion chromatography and colorimetric ammonium assay described by Scheiner (1976). SRS is an acronym for standard reference sample, a QA/QC program managed by the USGS Water Mission Area and National Water Quality Laboratory. Prado samples deviate from the y=x line, indicating the background water chemistry matrix of Prado effects the determination of NH$_4^+$ via the colorimetric analysis.
Figure A.2: A method comparison for the analysis of NO$_3^-$ concentration between IC and NOA. SRS is an acronym for standard reference sample, a QA/QC program managed by the USGS Water Mission Area and National Water Quality Laboratory. Prado samples deviate from the y=x line, indicating the background water chemistry matrix of Prado effects NOA analysis relative to IC analysis.
Table A.1: A description of preservation methods used for sample collection in June and September.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Container</th>
<th>Preservation</th>
<th>Field QA/QC</th>
</tr>
</thead>
<tbody>
<tr>
<td>June cations (Na(^+), NH(_4^+), K(^+), Ca(^{2+}), Mg(^{2+}))</td>
<td>glass scintillation vial</td>
<td>40 µL 18N H(_2)SO(_4) per 20 mL sample</td>
<td>–</td>
</tr>
<tr>
<td>June anions (Cl(^-), NO(_3^-), PO(_4^{3-}), SO(_4^{2-}))</td>
<td>plastic scintillation vial</td>
<td>frozen</td>
<td>–</td>
</tr>
<tr>
<td>June alkalinity</td>
<td>125 brown polyproylene bottle</td>
<td>chilled</td>
<td>–</td>
</tr>
<tr>
<td>September N(_2)O/CH(_4)</td>
<td>37 mL glass serum bottle</td>
<td>25 µL 12.5N KOH per 20 mL sample</td>
<td>collected in duplicate</td>
</tr>
<tr>
<td>September cations (Na(^+), NH(_4^+), K(^+), Ca(^{2+}), Mg(^{2+}))</td>
<td>glass scintillation vial</td>
<td>40 µL 18N H(_2)SO(_4) per 20 mL sample</td>
<td>–</td>
</tr>
<tr>
<td>September anions (Cl(^-), NO(_3^-), PO(_4^{3-}), SO(_4^{2-}))</td>
<td>plastic scintillation vial</td>
<td>frozen</td>
<td>–</td>
</tr>
<tr>
<td>September N(_2)O/CH(_4)</td>
<td>37 mL glass serum bottle</td>
<td>25 µL 12.5N KOH per 20 mL sample</td>
<td>collected in duplicate</td>
</tr>
<tr>
<td>September NH(_4^+)/NO(_2^-) isotopes</td>
<td>60 mL plastic bottle</td>
<td>frozen</td>
<td>collected in duplicate</td>
</tr>
</tbody>
</table>
Figure A.3: A comparison of June (red) and October (black) water column parameters collected by the multiprobe sonde on each visit. It is assumed here that the only parameter that varied considerably between site visits was temperature. The same trend of larger pH and DO concentration fluctuations occur at the outlet relative to the inlet on both the June and October site visits. The shaded regions represent sunset to sunrise based on June solar time.
Figure A.4: A comparison of water column temperature fluctuations of Cell 3A in June, September, and October. Temperature readings from June and October were collected by the multiprobe sonde described in Chapter 2. Temperature readings for September were collected using a HOBO Light/Temperature Pendant (±0.5°C between 0 and 50°C). The shaded regions represent sunset to sunrise.
Figure A.5: A method comparison for the analysis of PO$_4^{3-}$ and total P on the IC (y-axis) and ICP-OES (x-axis). Black squares represent Prado samples. A strong, 1:1 correlation between PO$_4^{3-}$ and total P indicates that nearly all dissolved P present in the surface water of Cell 3A is in the form of PO$_4^{3-}$.  

\[ y = 1.0x - 2.4 \]
\[ r^2 = 0.994 \]
Figure B.1: Horizontal profiles of $\text{SO}_4^{2-}$ at each sampled biomat depth interval during the June 2018 site visit. The first sample was taken at 15:30 on June 26, 2018. The shaded regions represent sunset to sunrise based on June solar time.
Table B.1: NO$_2^-$ and NO$_3^-$-free artificial wetland water recipe based on wetland water collected at the inlet of Cell 3A during the daytime. Major ion concentrations were obtained through ion chromatography analysis, bicarbonate ion concentration was obtained via ThermoFischer autotitrator and raw voltage readouts.

<table>
<thead>
<tr>
<th>Ion</th>
<th>Target concentration (µM)</th>
<th>Actual concentration (µM)</th>
<th>Salt</th>
<th>mg/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cl$^-$</td>
<td>4900</td>
<td>4600</td>
<td>MgSO$_4$ • 7H$_2$O</td>
<td>123</td>
</tr>
<tr>
<td>PO$_4^{3-}$</td>
<td>70</td>
<td>70</td>
<td>CaCl$_2$</td>
<td>235</td>
</tr>
<tr>
<td>SO$_4^{2-}$</td>
<td>850</td>
<td>850</td>
<td>KH$_2$PO$_4$</td>
<td>12.2</td>
</tr>
<tr>
<td>Na$^+$</td>
<td>5400</td>
<td>5400</td>
<td>NaHCO$_3$</td>
<td>302</td>
</tr>
<tr>
<td>K$^+$</td>
<td>400</td>
<td>470</td>
<td>Na$_2$SO$_4$</td>
<td>49.6</td>
</tr>
<tr>
<td>Mg$^{2+}$</td>
<td>500</td>
<td>500</td>
<td>KCl</td>
<td>24.6</td>
</tr>
<tr>
<td>Ca$^{2+}$</td>
<td>1600</td>
<td>1600</td>
<td>NaCl</td>
<td>64.2</td>
</tr>
<tr>
<td>HCO$_3^-$</td>
<td>3600</td>
<td>3600</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table B.2: Empirical mass balance of April wetland water. Wetland water was collected at the inlet of Cell 3A, not filtered, and shipped overnight on ice with minimal headspace. The wetland water was immediately filtered through a 0.7 µm GF/F filter and a vacuum manifold, then aliquoted and preserved accordingly upon arrival to the analytical laboratories.

<table>
<thead>
<tr>
<th>Ion</th>
<th>Concentration (µM)</th>
<th>Charge contribution (µeq/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cl⁻</td>
<td>4900</td>
<td>-4900</td>
</tr>
<tr>
<td>NO₃⁻</td>
<td>300</td>
<td>-300</td>
</tr>
<tr>
<td>PO₄³⁻</td>
<td>73</td>
<td>-220</td>
</tr>
<tr>
<td>SO₄²⁻</td>
<td>850</td>
<td>-1700</td>
</tr>
<tr>
<td>HCO₃⁻</td>
<td>3600</td>
<td>-3600</td>
</tr>
<tr>
<td>OH⁻</td>
<td>10⁻⁶.6 M = 0.25 µM</td>
<td>-0.25</td>
</tr>
<tr>
<td>Na⁺</td>
<td>5400</td>
<td>+5400</td>
</tr>
<tr>
<td>NH₄⁺</td>
<td>4.8</td>
<td>+4.8</td>
</tr>
<tr>
<td>K⁺</td>
<td>410</td>
<td>+410</td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>530</td>
<td>+1060</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>1700</td>
<td>+3400</td>
</tr>
<tr>
<td>H⁺</td>
<td>10⁻⁷.7 M = 0.04 µM</td>
<td>+0.04</td>
</tr>
<tr>
<td>Charge balance</td>
<td>–</td>
<td>-410</td>
</tr>
</tbody>
</table>
APPENDIX C
SUPPLEMENTAL INFORMATION FOR CHAPTER 4

Table C.1: Actual atom percent (AT%) values for the June 2018 in-situ, stable nitrogen isotope tracer tests. Values were calculated using equation C1.

<table>
<thead>
<tr>
<th></th>
<th>Biomat + water + tracers</th>
<th>Water + tracers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dark</td>
<td>15%</td>
<td>27%</td>
</tr>
<tr>
<td>Light</td>
<td>7%</td>
<td>11%</td>
</tr>
</tbody>
</table>

Note: Actual AT% values could not be accurately calculated for September because the mixed background + stock solution concentration is unknown.

Equation C1. Mixing calculation to determine actual AT%. AT% stock = 30%, AT% for natural abundance = 99.6%.

\[
AT\%_{\text{actual}} = \left[ \frac{(\mu\text{mol}_{\text{stock}})(AT\%_{\text{stock}})(\mu\text{mol}_{\text{background}})(99.6)}{(\mu\text{mol}_{\text{mix}})} \right] 
\]  

(C.1)

C.1 September dark pre-incubations

During the 12-hour pre-incubation period, changes in water column NO$_3^-$ and NH$_4^+$ concentrations were observed in both the biomat + water column treatment and water column only treatment. Biomat cores were collected in the late afternoon of September 18, 2018, which corresponded to an outlet water column concentration of 91 µM NO$_3^-$ at 15:00. The initial NO$_3^-$ concentration in the T$_0$ biomat + water column treatment was measured to be 17 µM, indicating a 74 µM loss of water column NO$_3^-$ during the pre-incubation. The water column only treatment exhibited a 27 µM decrease in NO$_3^-$. Water column NH$_4^+$ concentration increased during the pre-incubation in the biomat + water column treatment, but not in the water column only treatment. The outlet surface water NH$_4^+$ concentration
was 3.8 µM, while the T₀ biomat + water column NH₄⁺ concentration was measured to be 62 µM, indicating the production and flux of 58 µM of NH₄⁺ in the pre-incubation period into the water column. The water column only treatment exhibited a 0.90 µM increase in NH₄⁺.

C.2 September light pre-incubations

During the 12-hour pre-incubation period, water column NO₃⁻ was consumed and water column NH₄⁺ was produced. Biomat cores were collected in the late afternoon of September 17, 2018, which corresponded to an outlet water column NO₃⁻ concentration of 72 µM at 18:00. The initial NO₃⁻ concentration in the T₀ column was measured to be 21 µM, indicating 51 µM of lost NO₃⁻ during the pre-incubation phase. The water column only treatment exhibited an 8.0 µM decrease in NO₃⁻. Water column NH₄⁺ concentration increased in the biomat + water column treatment and consumed in the water column only treatment during the 12-hour pre-incubations phase. The outlet surface water concentration of NH₄⁺ at the time the biomat cores were collected was measured to be 5.4 µM, while the T₀ water column NH₄⁺ concentration was measured to be 62 µM, indicating the production of 57 µM NH₄⁺ in the pre-incubation period. The water column only treatment exhibited a 2.5 µM increase in NH₄⁺.

Table C.2: Water columns volumes for the September incubations.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Time point</th>
<th>water column volume (L)</th>
<th>Treatment</th>
<th>Time point</th>
<th>water column volume (L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Light</td>
<td>0</td>
<td>2.0</td>
<td>Dark</td>
<td>0</td>
<td>1.6</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>2.1</td>
<td></td>
<td>1</td>
<td>1.7</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1.2</td>
<td></td>
<td>2</td>
<td>1.7</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1.7</td>
<td></td>
<td>3</td>
<td>1.4</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>1.7</td>
<td></td>
<td>4</td>
<td>1.7</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>1.6</td>
<td></td>
<td>5</td>
<td>1.7</td>
</tr>
</tbody>
</table>
C.3 IPT calculations

The following IPT equations were used to calculate denitrification rates and are from Steingruber et al. (2001). Calculations were performed in the order listed.

A rearranged form of Steingruber et al. Equation (9).

where \( p_{N_2} \) is equal to the partial pressure of \( N_2 \) in the atmosphere (0.78 atm) and \( K_H(T) \) is the temperature-dependent Henry’s constant of \( N_2 \) (1513 atm L mol\(^{-1}\) at 25°C). This equation accounts for the conversion from M to µM. The resultant units are µM.

\[
\left[ ^{28}N_2 \right] = \frac{p_{N_2}}{K_H(T)} \times 1 \times 10^6 \tag{C.2}
\]

Equation (8) in Steingruber et al. (2001).

Where the ratio of \( [^{29}N_2]/[^{28}N_2] \) is equal to the mass 29 peak area divided by the mass 28 peak area obtained from GC-IRMS analysis. The resultant units are µM.

\[
[^{29}N_2] = \frac{[^{29}N_2]}{[^{28}N_2]} \times [^{28}N_2] \tag{C.3}
\]

Equation (8) in Steingruber et al. (2001).

Where the ratio of \( [^{30}N_2]/[^{28}N_2] \) is equal to the mass 30 peak area divided by the mass 28 peak area obtained from GC-IRMS analysis. The resultant units are µM.

\[
[^{30}N_2] = \frac{[^{30}N_2]}{[^{28}N_2]} \times [^{28}N_2] \tag{C.4}
\]

Equation (10) from Steingruber et al. (2001), slightly modified.

\( r_{29} \) is the production rate of \( ^{29}N_2 \) (µmol hr\(^{-1}\)), \( m_{29} \) is the slope of the regression line of \( [^{29}N_2] \) versus time, \( A \) is the surface area of the incubated sediment. \( V_{\text{water}} \) is the volume of water in each chamber. The resultant units are µmol m\(^{-2}\) hr\(^{-1}\).

\[
r_{29} = \frac{m_{29}}{A} (V_{\text{water}}) \tag{C.5}
\]

Equation (10) from Steingruber et al. (2001), slightly modified.

\( r_{30} \) is the production rate of \( ^{30}N_2 \) (µmol hr\(^{-1}\)), \( m_{30} \) is the slope of the regression line of \( [^{30}N_2] \) versus time, \( A \) is the surface area of the incubated sediment. \( V_{\text{water}} \) is the volume of water in each chamber. The resultant units are µmol m\(^{-2}\) hr\(^{-1}\).
\[ r_{30} = \frac{m_{30}}{A} (V_{\text{water}}) \]  

Equation (1) from Steingruber et al. (2001). \( D_{15} \) is the denitrification rate of \( ^{15}\text{NO}_2^- \). The resultant units are \( \mu\text{mol m}^{-2} \text{ hr}^{-1} \).

\[ D_{15} = r_{29} + 2r_{30} \]  

Equation (2) from Steingruber et al. (2001). \( D_{14} \) is the denitrification rate of \( ^{14}\text{NO}_2^- \). The resultant units are \( \mu\text{mol m}^{-2} \text{ hr}^{-1} \).

\[ D_{14} = D_{15} \times \frac{r_{29}}{2r_{30}} \]  

Equation (3) from Steingruber et al. (2001). \( D^{\text{tot}} \) is the total denitrification rate. The resultant units are \( \mu\text{mol m}^{-2} \text{ hr}^{-1} \).

\[ D^{\text{tot}} = D_{14} + D_{15} \]  

Equation (4) from Steingruber et al. (2001). \( D_w^{\text{tot}} \) represents the denitrification rate due to \( ^{15}\text{NO}_2^- \) diffusion from the water into the sediment. \( \varepsilon \) represents the isotopic enrichment of \( ^{15}\text{NO}_2^- \) during the September incubations and is described mathematically by Equation (5) in Steingruber et al. (2001).

\[ D_w^{\text{tot}} = \frac{D_{15}}{\varepsilon} \]  

Equation (5) from Steingruber et al. (2001). The subscript \( a \) is an abbreviation for the concentration of \( \text{NO}_2^- \) after the addition of the \( \text{NO}_2^- \) tracer. The subscript \( b \) is an abbreviation for the \( \text{NO}_2^- \) concentration before the addition of the \( \text{NO}_2^- \) tracer.

\[ \varepsilon = \frac{[\text{NO}_2^-]_a - [\text{NO}_2^-]_b}{[\text{NO}_2^-]_a} \]  

Equation (6) from Steingruber et al. (2001). \( D_a \) represents the coupled nitrification-denitrification rate of \( \text{NO}_2^- \) produced in the biomat following mineralization and oxidation plus \( \text{NO}_2^- \) produced from the reduction of water column \( \text{NO}_3^- \) following \( \text{NO}_3^- \) diffusion into the sediment.
\[ D_n = D^{tot} + D^{tot}_w \]  \hspace{1cm} (C.12)

Equation (7) from Steingruber et al. (2001). \( D_w \) represents the direct denitrification rate and describes the rate of denitrification of \( NO_2^- \) in the overlying water column.

\[ D_w = D^{tot}_w \times (1 - \varepsilon) \]  \hspace{1cm} (C.13)

The sum of \( D_n \) and \( D_w \) gives the total denitrification rate of the system.

\[ D_{tot} = D_n + D_w \]  \hspace{1cm} (C.14)