CHARACTERIZATION OF MICROBIAL NITRIFICATION AND DENITRIFICATION IN AN URBAN WISCONSIN MARSH

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CHARACTERIZATION OF MICROBIAL NITRIFICATION AND DENITRIFICATION IN AN URBAN WISCONSIN MARSH

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We recommend acceptance of this thesis in partial fulfillment of the candidate's requirements for the degree of Master of Science in Biology.

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ABSTRACT

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This thesis consists of an introduction and two manuscripts. The first manuscript concerns nitrification and the ammonia oxidizing communities found in ponds within an urban Wisconsin marsh. The second manuscript covers denitrification and the denitrifiers within these same ponds. Nitrogen can limit production in many ecosystems, and so this research covered two important processes of the nitrogen cycle. Little research has been done on characterizing nitrification and denitrification from freshwater urban marshes, which was the focus of this study. We measured water and sediment chemistries, process rates, ammonia-oxidizer and denitrifier abundance, and assessed diversity of ammonia-oxidizing community, seasonally, from two pond types (based on annual water cover) from Myrick Marsh, La Crosse, WI. Nitrification and denitrification process rates followed a seasonal pattern. Ammonia-oxidizer abundance correlated to nitrification rates during spring and summer months, whereas denitrifier abundance was not correlated to denitrification rates. Annual water cover did not affect denitrifier abundance or denitrification rates, but moist sample locations without overlying water had higher rates of denitrification compared to sample locations with overlying water. Our study suggests that there is a functional redundancy within the ammonia-oxidizing community maintaining a relatively consistent rate of nitrification and that denitrification thrived in moist, aerobic locations.
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CHAPTER I

INTRODUCTION
INTRODUCTION

Nitrogen is an element essential for the synthesis of proteins, nucleic acids, and amino sugars in all living organisms. Thus nitrogen can often limit organismal growth and production in terrestrial and aquatic ecosystems. In aquatic systems, environmental nitrogen is converted into an array of nitrogenous compounds via the nitrogen cycle. Two of the main microbial transformations are nitrification and denitrification (Fig. 1). Nitrification is the aerobic oxidation of NH$_3$ to NO$_3^-$ and denitrification is the anaerobic reduction of NO$_3^-$ to N$_2$. Both transformations are carried out by different groups of bacteria (Ward 1996) with microbial metabolism regulating dissolved environmental inorganic nitrogen concentrations (Bonin et al. 1998). Toxic levels of inorganic nitrogen compounds (NH$_3$, NO$_2^-$, and NO$_3^-$) can have an adverse effect on aquatic environments, possibly leading to eutrophication, anoxia, fish kills, toxic algal blooms, and loss of aquatic biodiversity (Feray et al. 1999, Kemp and Dodds 2002, Purkhold et al. 2002).

There is a need for information concerning the nitrogen cycle because of the increasing amounts of biologically usable forms of nitrogen, reactive nitrogen, used to sustain an adequate supply of food for the growing human population on Earth. Increased reactive nitrogen can result in eutrophication due to run-off of unused fertilizers into watersheds. Marshes can be effective as a bio-filter cleansing the water flowing through it of excess nutrients and toxins because of the large numbers of plants and microbes combined with the high residence time of water within the marsh. The
increased flux of reactive nitrogen makes denitrification an important process to transform reactive nitrogen to N₂ gas, which is largely unavailable biotically. The removal of water and even destruction of marshes may have an adverse affect on this removal of reactive nitrogen.

Oxygen is important in regulating nitrification and denitrification as these processes are tightly coupled to the oxic/anoxic interface in either open water or sediment (Kemp et al. 1990, Knowles 1996, Ward 1996, Zehr and Ward 2002). The diffusion of oxygen into different sediments is similar with oxygen penetrating the top 2 to 3 mm in freshwater sediments (Bodelier et al. 1996), the top 1.5 to 3.5 mm in estuary sediments (Binnerup et al. 1992), and the top 2.5 mm in coastal sediments (Nielson and Glud 1996). Temperature can affect diffusion, as oxygen does not penetrate as deeply in warm months as it does in cooler months (Kemp et al. 1990), possibly due to increased microbial activity quickly consuming the available oxygen.

**Nitrification**

Nitrification is an important component of the nitrogen cycle with NH₃ being oxidized to NO₂⁻ followed by NO₂⁻ being oxidized to NO₃⁻ (Fig. 1). The end product of nitrification, NO₃⁻, is the main source of NO₃⁻ in the majority of ecosystems (Kemp and Dodds 2001, Strauss et al. 2004). The bulk of nitrification in aquatic systems is done in oxic benthic zones because the process is obligately aerobic (Kemp and Dodds 2002, Ward 1996, Zehr and Ward 2002). Furthermore, Kemp et al. (1990) suggests that nitrification is concentrated close to the bottom of the oxic zone in sediments where there is a high concentration of NH₄⁺ from decomposition. Nitrification rates can be decreased by the stimulation of other organisms that consume the oxygen needed for the process to
occur. Nitrification has been studied in a variety of environments including soils (Degrange and Bardin 1995), wastewater (Knowles 1996), ocean water (Dore and Karl 1996, Ward 1996, Zehr and Ward 2002), freshwater lakes (Bodelier et al. 1996, Dodds and Jones 1987), salt water sediments (Henriksen et al. 1993), freshwater marshes (Robertson et al. 1995), streams (Feray et al. 1999, Kemp and Dodds 2002, Kemp and Dodds 2001), rivers (Strauss et al. 2004) and aquaria (Hovance and DeLong 1996).

Besides oxygen concentration, nitrification is also regulated by an array of other abiotic factors including temperature, C:N ratio, pH, and redox potential (Feray et al. 1999, Kemp et al 1990, Strauss and Lamberti 2000, Strauss et al. 2004). According to Henriksen et al. (1993), nitrifying bacteria in the Bering-Chukchi shelf sediments are well adapted to temperatures as low as 4º to -2ºC. Although activity and temperature are normally directly related, Kemp et al. (1990) found that temperature and nitrification were inversely related in Chesapeake Bay sediments, probably due to the inverse relationship between oxygen and temperature. The C:N ratio is also inversely related to nitrification rates with increased organic matter or decreased NH$_4^+$ likely resulting in a low nitrification rate. The low rate of nitrification is possibly due to other organisms that consume oxygen needed for nitrification or due to heterotrophic bacteria outcompeting nitrifying bacteria for available NH$_4^+$ or a combination of the two (Kemp and Dodds 2002, Strauss et al. 2002). Conversely, a decreased C:N ratio can increase the rate of nitrification due to more available nitrogen (Kemp and Dodds 2002). Henriksen et al. (1993) found that marine sediments with high organic nitrogen inputs tend to have increased nitrification rates compared to sediments with low organic nitrogen. The
organic nitrogen can undergo decomposition leading to the NH$_4^+$ needed for the nitrification process.

Nitrification rates can vary greatly from season to season. Kemp and Dodds (2002) found that in a prairie stream the lowest nitrification rate occurred in the summer months. The highest seasonal rate of nitrification occurred during fall even though there was not any significant difference in oxygen levels between the seasons. Nitrification rates in sediment from the upper Mississippi River had seasonal nitrification rate trends with the highest rates during the spring and summer months and lower rates during the autumn and winter months (Strauss et al. 2004). The seasonal pattern in the upper Mississippi River was probably driven by the river temperature (Strauss et al. 2004).

Nitrification is a process carried out by a group of bacteria called nitrifiers that are obligate chemolithoautotrophs (Ward 1996). The oxidation of NH$_4^+$ to NO$_3^-$ is a step-wise reaction split mainly into two oxidations. Each oxidation is done by a specialized group of bacteria that are either ammonia oxidizing bacteria also called nitrosifyers, (NH$_4^+$ $\rightarrow$ NO$_2^-$) or nitrite oxidizers, also called the true nitrifiers (NO$_2^-$ $\rightarrow$ NO$_3^-$). Both groups of nitrifiers are chemolithotrophic obtaining their energy from environmental sources of inorganic nitrogen compounds (NH$_4^+$ or NO$_2^-$ respectively) (Koops and Pommerening-Röser 2001, Malhautier et al. 1998, Zehr and Ward 2002).

Ammonia oxidizing bacteria are grouped into two classes of the Proteobacteria based on 16S rRNA and all have the genes for ammonia monooxygenase (*amo*), which is responsible for catalyzing the first step of ammonia oxidation (Avrahami and Conrad 2003, Rotthauwe et al. 1997). The majority of the nitrosifyers belong to the β class of the Proteobacteria, with one genus, *Nitrosococcus*, in the γ class (Kopps and Pommerening-
The ammonia-oxidizing genera in the β class of Proteobacteria are *Nitrosomonas, Nitrosolobus, Nitrosovibrio*, and *Nitrosospira* (Koops et al. 2006). The most dominant class of these in terrestrial, aquatic, and marine environments is thought to be the *Nitrosomonas/Nitrosospira* class (Hovance and DeLong 1996, Kowalchuk et al. 1997, Zehr and Ward 2002) with *Nitrosomonas europaea* being the most studied (Malhautier et al. 1998).

The four genera of nitrite oxidizing bacteria are *Nitrobacter, Nitrospira, Nitrococcus*, and *Nitrospina* (Koops et al. 2006). *Nitrobacter* was thought to be the most abundant based on culturing methods, but with the aid of molecular techniques, *Nitrospira* was found to be the most abundant nitrite oxidizing genus in the environment (Koops et al. 2006).

**Denitrification**

Denitrification is an anaerobic process of the nitrogen cycle where NO₃⁻ and NO₂⁻ are used as terminal electron acceptors for energy production (Braker et al. 2001, Knowles 1982). Denitrification begins with NO₃⁻ being reduced to NO₂⁻ and further reduced to N₂ when oxygen concentrations are below 0.2 mg/L (Knowles 1982, Scala and Kerkhof 2002, Ward 1996, Zehr and Ward 2002) (Fig. 1). The low oxygen concentrations required for denitrification are usually localized just below the oxic layer of water or sediment (Binnerup et al. 1992). The reduction of NO₃⁻ to N₂ leads to a loss of nitrogen in soils and sediments (Binnerup et al. 1992, Nijburg et al. 1997).

In coastal and large river backwater environments, denitrification rates are largely determined by the amount of available NO₃⁻ produced by nitrification (Knowles 1982, Richardson et al. 2004). Strauss et al. (2006) found that during winter months algal
growth can increase dissolved oxygen concentrations leading to increased rates of nitrification, and therefore, increased rates of denitrification in backwater sediment of the Upper Mississippi River. In the summer, lower nitrification rates lead to reduced NO$_3^-$ concentrations resulting in low rates of denitrification in marine sediments (Bonin et al. 1998, Kemp et al. 1990). However, the reduction of NO$_3^-$ to NO$_2^-$ in denitrification may stimulate NO$_2^-$ oxidizers with a source of energy for nitrification (Smorczewski and Schmidt 1991). Since the two processes are tightly coupled, together they can remove a large portion of the nitrogen inputs, therefore, acting as a buffering system against eutrophication (Kemp et al. 1990).

Denitrification can have both adverse and beneficial effects on an ecosystem. One adverse effect is that denitrification decreases the efficiency of fertilizer use by removing available nitrogen from crop lands before plant uptake. Denitrification can decrease primary productivity in nitrogen-limited systems by further decreasing the amount of nitrogen available to phytoplankton (Liu et al. 2003). Denitrification also can produce N$_2$O and NO that contribute to global warming, acid rain, and the depletion of ozone (Knowles 1996, Liu et al. 2003). Denitrification can, however, be beneficial for an ecosystem by assisting in the control of the global nitrogen budget (Knowles 1996). Since denitrification converts reactive nitrogen (Nr) to N$_2$, a form of nitrogen that is mostly unavailable biologically to organisms, it acts as a buffer to prevent eutrophication (Bonin et al. 1998). This process of removing Nr is an essential process used in tertiary wastewater treatment centers as well (Knowles 1996).

Abiotic factors such as pH, organic carbon, and contributions of nitrogen oxides (NO$_3^-$, NO$_2^-$, and N$_2$O) can influence the rate of denitrification. A low pH decreases the
rate of denitrification with the optimum pH range between 7.0 and 8.0 (Knowles 1982). Organic carbon levels also influence denitrification rates with higher concentrations of organic carbon usually resulting in higher rates. Dodds and Jones (1987) noticed that in freshwater sediment cores with high organic carbon concentrations, the rate of denitrification was 30 times higher than in cores with low levels of organic carbon. Nitrate concentration can also regulate denitrification since it is the substrate for the process and must be present in either the water column or the upper layers of sediment (Knowles et al. 1982). For instance, a study showed that an under saturation of N$_2$O and NO$_2^-$ is usually correlated with NO$_3^-$ deficits and were indicators of denitrification (Ward 1996). In a study done by Nijburg et al. (1997), the addition of NO$_3^-$ greatly increased the number of denitrifying bacteria in freshwater sediments. Nitrate concentration is inversely related to sediment depth, typically limiting denitrification to the upper portions of sediments. The various sediment depths where denitrification occurs range from the top 2-3 cm of freshwater sediments (Chan and Knowles 1979) to the top 6 cm in marine sediments (Sorensen 1978).

Denitrification is done by a diverse array of anaerobic heterotrophic opportunists (Knowles 1982, Ward 1996, Zehr and Ward 2002). Organisms able to denitrify are spread throughout the Bacteria and Archaea with the majority belonging to the domain Bacteria (Knowles 1996). The denitrifier community composition changes as distances between communities increase, with the most similar communities located within centimeters from each other (Scala and Kerkhof 1999).
Objectives

The goal of this study was to determine if nitrification and denitrification rates and the sediment communities carrying out these processes differed based on annual water presence and seasonal variations in Myrick Marsh, La Crosse, Wisconsin. We determined process rates in sediment samples collected from perennial and intermittent pond types throughout the different seasons. Intermittent water cover can be an inconsistent environment for these two processes because there is a high chance of variable oxygen concentrations and water availability as the sediments go from underwater to moist to dry. The variable environmental conditions can also affect nutrient transport and availability to the pertinent microbial communities. On the other hand, perennial ponds may provide stable environments because of the constant water cover. These differences may be exacerbated or attenuated by environmental, seasonal, and temporal variations in nitrification and denitrification processes and communities. We hypothesized that:

- Perennial pond types will have higher process rates, higher microbial abundance, and more stable community structure compared to intermittent ponds due to the more consistent water cover likely resulting in a more stable environment.
- Nitrification will increase as temperature increases due to high metabolic activity to a point where oxygen is no longer abundant and then nitrification rates will decrease.
- Denitrification rates and nitrification rates will follow the same trend since the two are tightly linked. Optimal process rates should occur where
temperatures are moderate. As temperatures approach freezing, both process rates will dramatically decline.

The urban marsh location of our study makes this research project unique in that this will be the first study of this kind and magnitude conducted in a non-tidal freshwater urban marsh. Our study of nitrification process rates, and the ammonia-oxidizing population size and diversity is found in Chapter 2 and will be submitted to Applied and Environmental Microbiology for publication. The portion of our study covering denitrification process rates and denitrifier population size is found in Chapter 3 and will be submitted to Limnology and Oceanography for publication.
REFERENCES


Figure 1. Major Processes of the Nitrogen Cycle.
CHAPTER II

NITRIFICATION
Running Head: Characterization of microbial nitrification in an urban Wisconsin marsh.

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ABSTRACT

Nitrification is an aerobic process of the nitrogen cycle where NH$_4^+$ is oxidized to NO$_3^-$ which is the main source of NO$_3^-$ in many ecosystems. Excess NO$_3^-$ in ecosystems can lead to eutrophication that can have detrimental effects. Nitrification is typically split into two steps with each done by a specialized group of bacteria. The first step is done by ammonia-oxidizing bacteria (AOB) that oxidize NH$_4^+$ to NO$_2^-$. There has been little research done on AOB communities from non-tidal freshwater urban marshes, which is the focus of this study. We measured water column and sediment chemistries, nitrification process rates, AOB abundance, and AOB community diversity from perennial and intermittent pond types (based on annual water cover) from Myrick Marsh in La Crosse, WI over four seasons. The highest measured nitrification rates were in sediment samples collected during summer and the lowest during winter. AOB abundance did not vary by pond type but did vary seasonally and was directly related to spring and summer nitrification rates. AOB community diversity, as determined by denaturing gradient gel fingerprints, ranged from one dominant community member to 14 community members. Cluster analysis indicated there was 55% similarity between the sites examined; however, there was no obvious grouping by pond type or season. It appears that annual water cover doesn’t directly affect the nitrification rates or abundance and diversity of the AOB communities. Functional redundancy within the AOB
community may be responsible for the stability of the AOB population size and activity levels.
INTRODUCTION

Nitrification is the aerobic process where NH$_4^+$ is oxidized stepwise to NO$_3^-$.

Nitrification, therefore, is the main source of NO$_3^-$ in many ecosystems (12, 27). In aquatic systems, nitrification occurs in oxic benthic zones since the process is obligately aerobic (13, 21, 30, 32). Furthermore, Kemp et al. (11) suggests that nitrification is concentrated close to the bottom of the oxic zone in sediments where there is a high concentration of NH$_4^+$ from decomposition.

Nitrification rates can vary greatly from season to season with influencing factors being temperature, NH$_4^+$, organic matter, and dissolved oxygen. Kemp and Dodds (13) found that in a prairie stream the lowest and highest measured rates of nitrification occurred during the summer and fall months respectively, although there was no difference in oxygen concentration. There was, however, a relationship between low C:N and high nitrification rates. Strauss et al. (27) found a different seasonal trend with nitrification rates highest during the spring and summer months and lower rates during the fall and winter in sediment from the upper Mississippi River. The nitrification rates in the upper Mississippi River also varied depending on sediment type. Strauss et al. (27), however, suggested that the seasonal pattern was more likely driven by the river temperature rather than sediment type.

Nitrification is mainly carried out by a group of bacteria, called nitrifiers, that are obligate chemolithoautotrophs (30). The oxidation of NH$_4^+$ to NO$_3^-$ is a step-wise
reaction split into two oxidations. Each oxidation is done by a specialized group of bacteria; the initial step by ammonia oxidizers \((\text{NH}_4^+ \rightarrow \text{NO}_2^-)\) and the next step by nitrite oxidizers \((\text{NO}_2^- \rightarrow \text{NO}_3^-)\). Both groups of nitrifiers are typically chemolithotrophic obtaining their energy from environmental sources of inorganic nitrogen compounds \((\text{NH}_4^+ \text{ or } \text{NO}_2^- \text{ respectively})\) (14, 16, 32).

Aerobic ammonia oxidizing bacteria (AOB) are grouped into two classes of the Proteobacteria based on 16S rRNA and all have the genes for ammonia monooxygenase \((\text{amo})\), which is responsible for catalyzing the first step of ammonia oxidation (3, 22). Molecular techniques based on both the 16S rRNA and \(\text{amoA}\) genes have been used to study AOB (15, 22). Rotthauwe et al. (22) found that the \(\text{amoA}\)-based approach, compared to the 16S rRNA approach, had less false-positives and therefore was more specific for only AOB. Besides AOB, recent discoveries have found that some members of the archaeal group \textit{Crenarchaeota} have \(\text{amoA}\) genes. These ammonia-oxidizing archaea (AOA) can outnumber AOB in some natural environments and may have an important role in nitrification (10).

Little research has been conducted on nitrification in non-tidal freshwater urban marshes. Wetlands are an important natural ecosystem. Wetlands filter out toxins, pollutants, and nutrients; provide habitat for many different species, and are used for recreation and aesthetic purposes (7). The filtering function of wetlands is essential to help lessen the effects that nutrient rich runoff have when directly drained into waterways. Without this filtering, there is an increased risk of nutrient loading that may lead to aquatic eutrophication. Aquatic eutrophication has a negative impact on the aquatic ecosystem in that it can lead to toxic algal blooms, anoxic water conditions, and
even lead to a loss of aquatic biodiversity. Even though urban marshes are crucial to
maintaining ecosystem health, many are being drained due to urban expansion (7).
Approximately half have been drained in the continental United States with some regions
having over 80% wetland loss since European settlement (7).

Our study took place in Myrick Marsh, a non-tidal freshwater urban marsh in La
Crosse, Wisconsin, USA. The objectives of our study were to measure nitrification
process rates, AOB abundance, and AOB community diversity in the sediment of a non-
tidal freshwater urban marsh. There are two pond types within Myrick Marsh,
intermittent and perennial, that were sampled for this study. Intermittent pond types are
ponds where annual water cover is not continuous. This irregularity may lead to a range
of environmental conditions that affect oxygen concentration, water availability, nutrient
transport and nutrient availability. Oxygen conditions can range from anoxic in stagnant
water to aerobic in dry sediments. Nutrient transportation and availability can also vary
considerably due to environmental changes including redox potential, temperature, and
various levels of water availability. Perennial ponds are ponds where annual water cover
is more continuous and, therefore, may have less variability in environmental conditions
compared to intermittent pond conditions. In our study, we measured AOB community
abundance, AOB community diversity and nitrification rates in sediment samples
collected seasonally and by pond type. We hypothesized that nitrification rates in our
samples would follow the seasonal patterns seen in the nearby Mississippi River (27).
We predicted that AOB community abundance and diversity would be different between
pond types with communities from perennial ponds being more stable compared to
intermittent ponds due to regular water cover. We believed that there would also be a seasonal AOB community abundance and diversity trend mirroring nitrification rates.
MATERIALS AND METHODS

Site Description and Sample Collection

Myrick Marsh is a non-tidal freshwater urban marsh located in La Crosse, Wisconsin, USA, that makes up the southern part of the 2,500 acre La Crosse River Marsh (31) (Fig. 1). Myrick Marsh is approximately 2.4 km to the east of the Mississippi River and splits the city of La Crosse, WI into north and south with bluffs to the east. There are two golf courses, approximately 0.3 km and 4.2 km to the southeast and northeast respectively, that are likely having adverse affects on Myrick Marsh due to chemical treatments. There are between 24 and 29 different ponds within the marsh which are categorized as having either perennial or intermittent annual water cover (31). We selected five perennial and five intermittent ponds to be sampled twice a season for one year totaling eight sample sessions. Seasonal sample sessions were as follows: summer was collected 29 July and 31 July 2004 and 16 July and 18 July 2005, fall was collected 2 October and 4 October 2004 and 14 November and 16 November 2004, winter was collected 27 December and 30 December 2004, and 26 February and 28 February 2005, and spring was collected 23 April and 25 April 2005 and 11 June and 13 June 2005. Three separate random sampling locations predetermined with the aid of the ArcView extension from the Minnesota Department of Natural Resources (17) were sampled from each pond during each sampling session.
Site locations were determined using a handheld GPS unit (Magellan SporTrak Map). Water depth was measured with a PVC depth pole. Water temperature and dissolved oxygen were determined just above the sediment at sites with overlying water using a precalibrated oxygen meter (YSI, Yellow Springs, Ohio). Unfiltered site surface water was collected at each site in sterile, one liter and 100 mL Nalgene® bottles. In addition, approximately 50 mL of site surface water was filtered in the field through a glass fiber filter (Whatman, Piscataway, New Jersey USA) and acidified with hydrochloric acid to a pH of approximately 2 for later analysis of NO₃⁻/NO₂⁻. All site water collected was stored on ice while in the field and then stored at 0-4°C until processed. Triplicate sediment cores were collected at each of the three sites within a pond by manually advancing a PVC sediment corer (i.d. of 7 cm) into the sediment to a depth of at least five centimeters. Sediment was extracted by inserting a plunger at the bottom of the sediment corer and pushing it up through the clear polycarbonate tube until all overlying water was expelled. Approximately the top five centimeters of sediment was extruded and placed in a sterile plastic bag and stored on ice until processed. The triplicate sediment cores for a given site were combined into one sterile plastic bag, homogenized by hand and stored on ice until analysis.

**Water and Sediment Chemistries**

Surface water and sediment characteristics and chemistries were measured at each of the three sites within each pond sampled. Nitrate, NH₄⁺ and organic matter concentrations along with pH were determined for each site. Porewater and water column NO₃⁻/NO₂⁻ were analyzed on a Dionex ICS-90 Ion Chromatography System using standard procedures (1). An external standard reference material sample (Ultra
Scientific: QCI-740 Ampule 2) was measured in each run for quality assurance. Water column, porewater and exchangeable NH$_4^+$ concentrations were determined. Porewater was collected by centrifugation of a known amount of sediment at 1000 x g for 8 minutes (24, 27). Exchangeable NH$_4^+$ was obtained by adding a volume of 2 N KCl to an equal amount of sediment. The KCl displaced the NH$_4^+$ bound to soil particles thus exchanging NH$_4^+$ with K$^+$. After the addition of KCl, the sediment was incubated for one hour with regular periods of mixing prior to centrifugation as was done for porewater. The water collected after centrifugation was filtered with a Seraclear™ filter (Technicon Instruments Corporation, Tarrytown, New York), acidified with 10% sulfuric acid (final concentration 0.2%) and stored at 4ºC until quantification. The water column NH$_4^+$ concentrations were determined from site water filtered through a glass fiber filter (Whatman, Bound Brook, New Jersey). All NH$_4^+$ determinations were done spectrophotometrically using the phenol-hypochlorite method with appropriate controls and reference material (24). Organic content was determined by drying a known mass of sediment at 105ºC for 24 h or until a constant mass was achieved, followed by ashing at 550ºC for 4 h. The weight of the remaining mass was the ash weight (6) and the difference in mass between dry weight and ash weight represented the ash-free organic dry weight of each sample. Sediment pH was determined with an Oakton Waterproof pHTestr 3 pen-type meter.

**Nitrification Rate Determination**

The rate of nitrification in sediment was determined using the nitrapyrin method as described (27). For each site, two 125 mL flasks were set up with 25 mL of sediment and 81 mL of site water each to make a sediment slurry. If the sample site lacked
overlying water, distilled water was used (2). One flask was amended with 10 mg/L nitrpyrin dissolved in dimethyl sulfoxide (DMSO) for a final concentration of 1.9 µg/L nitrpyrin and the other flask with only an equal volume of DMSO. The addition of nitrpyrin inhibited the ammonia oxidation step of nitrification. At time point zero, a sub-sample of 6 mL of the sediment slurry was taken from each flask. The flasks were then covered loosely with foil, incubated in the dark at the average site water temperature for the sampling session and shaken at 175 rpm to maintain aerobic conditions. After a 72 h incubation, a second 6 mL slurry sub-sample was taken. Approximately 15% of all samples were done in duplicate for quality control. In addition, approximately 15% of all samples were spiked with a known concentration of NH$_4^+$ for quality control. All sub-samples were analyzed for exchangeable NH$_4^+$ as described previously for sediment chemistry. The rate of nitrification was determined from the difference in NH$_4^+$ concentration between the flask containing the nitrpyrin in DMSO (where nitrification was inhibited) and the one with DMSO only as described by Strauss et al. (27). Dry sites sampled during the winter that were frozen were not tested for activity.

**AOB Community Diversity**

DNA was extracted from sediment using a MoBio UltraClean Soil DNA Kit as described by the manufacturer (MoBio Laboratories, Inc., Carlsbad, CA). The approximate concentration of extracted DNA was determined by measuring absorbance at 260 nm and 280 nm. The extracted DNA was stored at -20°C until analysis.

The *amoA* gene was amplified with PCR using primers from Okano et al. (19) (forward primer A337-short-clamp: 5’ GC clamp-TTC-TAC-TGG-TGG-CRC-ACT-ACC-CCA-TCA-ACT 3’ and reverse primer *amoA*-2R-TG: 5’ CCC-CTC-TGG-AAA-
GCC-TTC-TTC 3’). Each PCR reaction consisted of A337-short-clamp (1.2 µM), \textit{amoA}-
2R-TG-modified (3.2 µM), 1X GoTaq Green Master Mix (Promega, Madison WI),
sample DNA (1 to 100 ng), and PCR grade sterile water. PCR reactions were carried out
on a GeneAmp PCR System 9700 (PE Applied Biosystems, Faster City, CA) thermal
cycler with an initial denaturation of 3 min at 95°C, followed by 35 amplification cycles
consisting of 1 min at 95°C, 1 min at 55°C, and 45 s at 75°C, with a final 5 min extension
at 72°C as described by Okano et al. (19). Amplification and proper fragment size were
verified on a 1.5% agarose gel. Amplifications were repeated up to four times with
various DNA concentrations for DNA samples that did not amplify initially.

Amplicons of \textit{amoA} were separated using denaturing gradient gel electrophoresis
(DGGE) to determine the microbial diversity of the AOB community. At least one
positive, \textit{amoA} amplicons from \textit{N. europea} ATCC® 19718D™, and one negative
control, a sample from a PCR reaction containing no added DNA, was run with each gel.
The DGGE gel gradient used was 25% to 60% as determined from a perpendicular
DGGE gel (18). Electrophoresis was performed using the DCode System (Bio-Rad
Laboratories, Hercules, CA) at 130V for approximately 6.5 hours at 60°C (18). The gel
was stained with SYBR Gold (Molecular Probes Inc., Eugene, OR) with the resulting
bands likely indicative of different species (18).

\textbf{AOB Abundance}

A standard was created by amplifying the \textit{amoA} gene from \textit{N. europea} ATCC®
19718D™ with primers A189 (19) (5’ GGH-GAC-TGG-GAY-TTC-TGG 3’) and A337R
(modified from 19) (5’ AGT-GYG-ACC-ACC-AGT-AGA-A 3’). Primer A337R was
modified from Okano et al. (19) by removing 12 nucleotides from the 5’ end for better
coverage as revealed by analyzing 36 different known AOB amoA DNA sequences obtained from Genbank. The PCR reaction was carried out as described above except with primers A189 (3.6 µM) and A337-modified (3.4 µM), and *N. europea* ATCC® 19718D™ DNA. The resulting amoA fragments were cloned into plasmids using the pGEM®-T Vector System I as described by the manufacturer (Promega, Madison, WI). Plasmid DNA was extracted and purified from an overnight culture using a Plasmid Maxi Prep Kit as described by the manufacturer (Qiagen, Valencia, CA). Plasmid concentrations were measured spectrophotometrically and copy number of the amoA gene was determined to be 3.8 x 10^{11} copies/µL. The amoA containing plasmid DNA and was used to create real-time PCR standard curves.

Real-time PCR was performed in duplicate with a reaction mixture consisting of 1X SYBR-Green I Master Mix (Roche Applied Science, Indianapolis, IN), primer A189 (900 nM) and primer A337R-modified (850 nM), sediment DNA (1 to 50 ng), and PCR grade sterile water. The following amoA quantification program was used: initial denaturation of 2 min at 50ºC and 10 min at 95ºC, 40 cycles of 95ºC for 45 s, 55ºC for 60 s, and 72ºC for 45 s (19). An external standard curve was generated with DNA from the clone created from *N. europea* ATCC® 19718D™ DNA. amoA copy number was quantified by comparing averaged sample Cp values to the amoA quantification standard curve. AOB abundance was estimated by dividing our calculated amoA copy number by 2.5 since typical AOB chromosomal DNA contains either two or three copies of the amoA gene (19).
Analysis of Data

Data were analyzed using SAS v.9.1 (Cary, NC). A general linear model two-way ANOVA procedure was used to determine significant interactions between multiple factors. If there was a significant interaction between two main factors (i.e. season with pond type), then type III sum of squares was used. If the paired main factors did not have a significant relation, then type II sum of squares was used. Once significance was determined, a bivariate correlation test was done using the Spearman correlation coefficient due to missing values in some of the analyzed factors. A $p$-value of less than or equal to 0.05 was considered significant. AOB diversity was assessed by performing a cluster analysis from a binary diversity matrix (23) with the aid of the PRIMER 6 statistical software (PRIMER-E Ltd, Ivybridge, United Kingdom).
RESULTS

Water and Sediment Chemistries

Differences were found in water and sediment chemistries within and between pond types, water depths, and seasons (Table 1). The water column of perennial ponds were deeper (mean ± SE = 64.6 ± 2.6 cm) than intermittent ponds (32.4 ± 2.3 cm). Surface water temperature, dissolved oxygen concentration, and organic matter all did not vary significantly between pond types but did vary seasonally.

Water column NH$_4^+$ concentrations were also not significantly different by pond type but were by season. Mean water column NH$_4^+$ concentration was highest during the fall for perennial ponds (0.24 ± 0.10 mg/L) and winter for intermittent ponds (0.17 ± 0.04 mg/L) while it was lowest during the summer for perennial ponds (0.01 ± 0.00 mg/L) and lowest in spring (0.030 ± 0.01 mg/L) for intermittent ponds (Fig. 2A).

Likewise, sediment porewater NH$_4^+$ concentrations were not significantly different between pond types ($p = 0.22$) but were between seasons ($p < 0.0001$) (Fig. 2B). Mean porewater values were highest in the summer for perennial pond types (2.91 ± 0.50 mg/L) and spring for intermittent pond types (2.27 ± 0.29 mg/L) and lowest during fall for both pond types with 0.05 ± 0.01 mg/L in perennial pond types and 0.06 ± 0.01 mg/L in intermittent pond types.

Concentrations of exchangeable NH$_4^+$, however, were significantly different between pond types and season (Fig. 2C). The mean perennial pond exchangeable NH$_4^+$
concentration (15.7 ± 1.1 mg/L) was significantly higher than that for intermittent ponds (12.1 ± 1.1 mg/L). Seasonally fall was significantly higher than spring and summer for both pond types (Fig. 2C).

Water column NO$_3$\textsuperscript{-}/NO$_2$\textsuperscript{-} concentrations were not significantly different by pond type, but were significantly different seasonally. In both perennial and intermittent ponds, mean water column NO$_3$\textsuperscript{-}/NO$_2$\textsuperscript{-} concentration was highest during winter (0.39 ± 0.013 mg/L and 0.19 ± 0.07 mg/L, respectively) and lowest in the spring (0.01 ± 0.00 mg/L and 0.03 ± 0.01 mg/L, respectively) (Fig. 3A).

Likewise, sediment porewater NO$_3$\textsuperscript{-}/NO$_2$\textsuperscript{-} concentrations were not significantly different by pond type but were by season. Mean porewater NO$_3$\textsuperscript{-}/NO$_2$\textsuperscript{-} concentrations for perennial and intermittent ponds were highest during summer at 2.43 ± 1.44 mg/L and 2.45 ± 1.48 mg/L respectively and lowest during spring at 0.02 ± 0.00 mg/L in both pond types (Fig. 3B).

**Nitrification Rate Determination**

Nitrification rates were not significantly different between sediment samples collected from the different pond types (Fig. 4) with sediment samples from perennial pond types averaging 0.37 ± 0.04 µg N·cm$^{-2}$·h$^{-1}$ and samples from intermittent pond types averaging 0.28 ± 0.03 µg N·cm$^{-2}$·h$^{-1}$. Even though there was not a significant difference between pond types, nitrification rates in sediment samples collected during the different seasons varied significantly. Summer sediment samples had the highest mean rate (0.66 ± 0.07 µg N·cm$^{-2}$·h$^{-1}$) while winter had the lowest mean rate (0.06 ± 0.01 µg N·cm$^{-2}$·h$^{-1}$) (Fig. 4). Nitrification rates were not determined for samples from winter locations that did not have overlying water (six sample locations) since the sediment was frozen and not
amenable to the technique used for measuring activity; therefore the winter nitrification rate average may be an underestimate if there was indeed some minimal activity occurring. In addition to seasonal relationships, nitrification rates in the sediment samples were positively correlated to temperature and porewater NH$_4^+$ concentration. Nitrification rates, however, were not significantly related to exchangeable NH$_4^+$ or organic matter concentrations.

**AOB Abundance**

AOB abundance, based on *amoA* gene copy number, did not vary significantly by pond type. Perennial ponds average AOB abundance was slightly higher (1.2 x 10$^7$ ± 1.3 x 10$^6$ AOB per gram of sediment wet weight) than intermittent ponds (9.8 x 10$^6$ ± 2.1 x 10$^6$ AOB per gram of sediment wet weight). Water depth was positively correlated with AOB abundance during spring and summer while temperature was negatively correlated with AOB abundance during the fall and summer. AOB abundance was seasonally different with fall and summer both having the highest (1.7 x 10$^7$ ± 2.2 x 10$^6$ AOB per gram of sediment wet weight and 1.7 x 10$^7$ ± 4.0 x 10$^6$ AOB per gram of sediment wet weight). The lowest AOB abundance was during the spring and winter seasons (6.1 x 10$^6$ ± 9.1 x 10$^5$ AOB per gram of sediment wet weight and 2.5 x 10$^6$ ± 3.3 x 10$^5$ AOB per gram of sediment wet weight, respectively) (Table 1). Nitrification rates were positively correlated with AOB abundance only during spring and summer.

**AOB Community Diversity**

Amplification of the *amoA* gene with A337-short-clamp and *amoA*-2R-TG was detected for approximately 80% (193 of 240) of the DNA samples extracted from the sediment from the sample sites. Only 51% of the amplicons resulted in visible bands on
the DGGE polyacrylamide gel (42% from perennial ponds and 58% from intermittent ponds) (Fig. 5). Fall sample sites had the highest percentage of sites (33%) producing detectable DGGE bands, followed by winter (28%), spring (20%), and summer with the lowest (18%). Of the samples with detectable DGGE bands, the most diverse community was from an intermittent pond sampled during early winter that had 14 different bands (Fig. 5 – Lane 4). The majority of samples with detectable DGGE bands only had two or three different bands. Cluster analysis revealed that all sites were approximately 55% similar (Fig. 6). The dendrogram revealed that there were not defined groupings of sediment AOB communities based on pond type or season.
DISCUSSION

The mean nitrification rates determined from Myrick Marsh sediment samples followed a seasonal cycle with the highest rates during summer then lower in fall and the lowest rates during winter. There was a direct relationship between nitrification rates measured in the samples and surface water temperature from the site sampled; both also had a seasonal trend. Therefore, the seasonal trend in the rates of nitrification was likely due to temperature differences. There was, however, no significant difference in the measured nitrification rates between pond types regardless of the season. This lack of difference in measured nitrification rates between pond types may indicate that water cover consistency does not affect nitrification rates in Myrick Marsh sediment.

Strauss et al. (27) found the same seasonal trend for nitrification rates approximately one mile away in the Upper Mississippi River, but had nitrification rates approximately three times higher than ours although measured in similar sediment types with the same nitrification assay procedure. The Upper Mississippi River nitrification rates were also high compared to other aquatic ecosystems (27). Although nitrification rates in Myrick Marsh were lower than those in the Mississippi River, they were approximately four times higher than mean nitrification rates measured from 42 streams in the northern United States using the same nitrapyrin method that was used in our study (26). The water column NH$_4^+$ concentrations that Strauss et al. (27) found are similar to ours with spring having the lowest concentration and winter having the highest. Water
column NO$_3^-$/NO$_2^-$ concentrations were upwards of 10 times higher in the Mississippi River (27) compared to our sample locations. The high NO$_3^-$/NO$_2^-$ concentrations reported by Strauss et al. (27) may be due to the higher rates of nitrification compared to Myrick Marsh.

In Myrick Marsh, AOB numbers were also directly related to temperature, which is common (9) and may explain the high nitrification rates in sediment collected during the summer. Organic matter concentrations can be important in nitrification regulation (25), however, we did not find a significance relationship between nitrification rates and organic matter concentration in the Myrick Marsh sediment samples likely because there is an abundant supply from the dense macrophytes cover. We did, however, determine that porewater NH$_4^+$ was positively correlated to nitrification rates which may indicate that this is what is limiting the rate of nitrification in Myrick Marsh.

AOB abundance in Myrick Marsh ranged from 1.7 x 10$^5$ to 6.9 x 10$^7$ AOB per g sediment wet weight (sed ww) and was similar to numbers found in other sediments; salt marsh sediments ranged from 5.6 x 10$^4$ to 1.3 x 10$^6$ AOB per g sed ww (8) and Michigan soils ranged from 10$^4$ to 10$^6$ cells per g as determined using 16S rRNA gene-based competitive PCR (20). Recent studies have indicated that ammonia-oxidizing Archaea (AOA) may be more abundant than AOB in estuarine sediments (4), oceans (10), and salinity gradients (5). Another study, however, discovered that β-AOB abundance was higher than AOA in estuary sediments (29). Some Crenarchaeota are known to thrive in colder environments and so if they are present in these sediments it may explain the lack of correlation between AOB abundance and rates during the cooler fall and winter seasons in Myrick Marsh. In soil samples from a pristine forest, AOB abundance
increased with soil temperature while AOA abundance remained approximately the same 
(28). The abundance of AOB bacteria may be strongly influencing the higher 
nitrification rates in Myrick Marsh during the warmer spring and summer seasons where 
AOB may be higher than AOA.

We determined that the AOB community in Myrick Marsh ranged from low 
diversity (two to three bands) to relatively high diversity (14 bands), however, the 
communities did not form defined groups by season or pond type. We may have seen 
seasonal or pond type groups if we had been able to obtain DGGE fingerprints from all of 
our samples. DNA extracted from sample sites was amplified at least four times more if 
no amoA amplicons were visualized initially. Likewise, samples with amoA amplicons 
were run on the denaturing gradient gels at least twice more if no DGGE bands were 
visualized on the first gel. We know that all sample sites had AOB and that the DNA 
sample did not contain compounds that inhibited amplification since AOB abundance 
was determined using real-time PCR for all sample sites. Real-time PCR used a slightly 
different primer set and was more sensitive and so this may be why we were unable to 
detect amplification in all of the samples prepared for DGGE. Additionally, it is possible 
that some of the PCR reactions did not have sufficient amplicon copies per community 
member to visualize bands on the gel. The majority of sample locations that resulted in 
DGGE bands contained N. europea like DGGE fingerprints and could indicate that a N. 
europea like species may be dominant in Myrick Marsh. This was not confirmed, 
however, by sequencing DNA from the DGGE bands. Our cluster analysis indicated that 
the amoA community in Myrick Marsh is fairly similar (55% similar) but, as previously 
mentioned, there were no obvious groupings based on season or pond type. The lack of
groupings may indicate that the total AOB community was the same throughout Myrick Marsh, but that the dominant AOB community at different sample sites that was detected by denaturing gradient gels differed, likely due to different environmental and nutritional conditions that varied from pond to pond and season to season.

In summary, nitrification rates measured in the sediment samples from our non-tidal freshwater urban Wisconsin marsh varied seasonally, but not by pond type indicating that the consistency of overlying water and therefore, nutrient availability and dissolved oxygen may not be major regulating factors. Nitrification rates followed a seasonal trend with the highest rates during the summer then declining through to the winter months. The seasonal trend in this study was also followed by AOB abundance indicating that the number of AOB may be directly linked to nitrification rates. Although the structure of the ammonia-oxidizing community was not stable, our study did not reveal changes in AOB community diversity that varied with respect to season or pond type. One or two groups of AOB may have been responsible for the majority of nitrification that occurred depending on a given environmental condition or conditions within the ponds that we did not determine in this study. The changes seen in the AOB then could have been in response to a change in this unknown environmental factor(s). These community shifts appear to have had minimal impact on the abundance of AOB and were not correlated to changes in nitrification rates. The relatively consistent activity although there were shifts in the community, was likely due to functional redundancy within the ammonia-oxidizing community in Myrick Marsh.
REFERENCES


17. Minnesota Department of Natural Resources. 


TABLE 1. Water and sediment chemistries and estimated ammonia oxidizer population size for perennial and intermittent ponds sampled seasonally from Myrick Marsh located in La Crosse, Wisconsin.

<table>
<thead>
<tr>
<th>Pond type</th>
<th>Season</th>
<th>Depth (cm)</th>
<th>Water column temp (°C)</th>
<th>Dissolved oxygen (mg/L)</th>
<th>AOB estimation (bacteria/g sed ww)(^a)</th>
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</thead>
<tbody>
<tr>
<td>Perennial</td>
<td>summer</td>
<td>Mean 58.8</td>
<td>23.0</td>
<td>0.47</td>
<td>1.6x10^7</td>
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<td></td>
<td>Range</td>
<td>(0.0 - 116)</td>
<td>(19.5 - 35.8)</td>
<td>(0.14 - 1.94)</td>
<td>(3.4x10^5 - 6.9x10^7)</td>
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<tr>
<td></td>
<td>Fall</td>
<td>Mean 67.4</td>
<td>8.2</td>
<td>4.77</td>
<td>2.0x10^7</td>
</tr>
<tr>
<td></td>
<td>Range</td>
<td>(2.5 - 120)</td>
<td>(3.0 - 19.0)</td>
<td>(0.80 - 16.5)</td>
<td>(1.3x10^6 - 9.1x10^7)</td>
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<td></td>
<td>Winter</td>
<td>Mean 64.8</td>
<td>2.2</td>
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<td>2.9x10^6</td>
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<td>Range</td>
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<td>(0.1 - 5.6)</td>
<td>(0.15 - 9.2)</td>
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<td></td>
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<td>17.1</td>
<td>2.99</td>
<td>7.7x10^6</td>
</tr>
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<td>Range</td>
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<td>(7.1 - 28.4)</td>
<td>(0.84 - 8.5)</td>
<td>(1.1x10^6 - 4.9x10^7)</td>
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<td>Intermittent</td>
<td>summer</td>
<td>Mean 31.0</td>
<td>21.6</td>
<td>0.96</td>
<td>1.9x10^7</td>
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<td>Range</td>
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<td>(4.3x10^5 - 2.3x10^6)</td>
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<tr>
<td></td>
<td>Fall</td>
<td>Mean 28.4</td>
<td>9.2</td>
<td>3.31</td>
<td>1.9x10^7</td>
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<td>Range</td>
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<td>(4.1 - 20.5)</td>
<td>(0.39 - 18.0)</td>
<td>(7.4x10^5 - 5.9x10^7)</td>
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<tr>
<td></td>
<td>Winter</td>
<td>Mean 31.7</td>
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<td>1.58</td>
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<tr>
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<td>(12.6 - 26.2)</td>
<td>(0.25 - 12.1)</td>
<td>(6.9x10^5 - 1.2x10^7)</td>
</tr>
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</table>

\(^a\)Bacteria/g sed ww = bacteria per gram of sediment wet weight
Figure 1. Five intermittent (I-1 to I-5) and five perennial (P-1 to P-5) ponds sampled in Myrick Marsh, La Crosse, Wisconsin (31). Pond identifications were given to distinguish between pond type and replicate.
Figure 2. Seasonal water column $\text{NH}_4^+$ (A) porewater $\text{NH}_4^+$ (B) and exchangeable $\text{NH}_4^+$ (C) concentrations from five perennial and five intermittent ponds in Myrick Marsh, La Crosse, Wisconsin. Error bars represent standard error from the mean.
Figure 3. Seasonal water column NO$_3^-$/NO$_2^-$ (A) and porewater column NO$_3^-$/NO$_2^-$ (B) concentrations from five perennial and five intermittent ponds in Myrick Marsh, La Crosse, Wisconsin. Error bars represent standard error from the mean.
Figure 4. Seasonal nitrification rates measured in sediment collected from five perennial and five intermittent ponds in Myrick Marsh, La Crosse, Wisconsin. Error bars represent standard error from the mean.
Figure 5. DGGE analysis of AOB community diversity based on the *amoA* gene. Bacterial 16S rDNA PCR fragments from intermittent pond samples were loaded in lanes 1, 2, 4-6, 9, 10, 13, and 14. Bacterial 16S rDNA PCR fragments from perennial pond samples were loaded in lanes 3, 7, 8, 11, and 12. Samples were collected either during fall (lanes 1, 9-11), winter (lanes 4-8, 13, and 14), spring (lanes 2 and 12), or summer (lane 3). Lane 15 contains 16S rDNA fragments amplified from *N. europea* ATCC® 19718D™ DNA and served as a positive control. Lane 16 was loaded with a sample from a PCR reaction containing no added DNA and served as a negative control.
Figure 6. Cluster analysis of *amoA* DGGE banding patterns for the *amoA* gene from DNA from the ammonia-oxidizing communities sampled seasonally from five perennial and five intermittent ponds located in Myrick Marsh, La Crosse, Wisconsin. Cluster analysis was performed using PRIMER 6 statistical software.
CHAPTER III
DENITRIFICATION
Running Head: Characterization of microbial denitrification in an urban Wisconsin marsh.

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Denitrification is an anaerobic dissimilatory process of the nitrogen cycle that is responsible for removing biologically available nitrogen, in the form of NO₃⁻, from the environment by converting it to N₂. There has been little research done on the denitrifier communities from non-tidal freshwater urban marshes, which was the focus of our study. We measured sediment and water column chemistries, denitrification process rates, denitrification enzyme activity, and abundance of the denitrifying communities from perennial and intermittent pond types (based on annual water cover) from a non-tidal freshwater urban marsh. In addition, individual sample sites were also categorized being either covered or uncovered based on water cover at the time of sampling. Our results show both denitrification rates and denitrification enzyme activity measured in the sediment samples were directly related to organic matter concentrations. Porewater NO₃⁻/NO₂⁻ varied seasonally and was directly related to denitrification rates but not with denitrifier abundance. Denitrification rates varied seasonally. Denitrifier abundance ranged over at least four orders of magnitude, but was not correlated to pond type or season indicating that the type of denitrifier is likely more important than abundance. To our surprise, uncovered sites had the highest concentration of porewater NO₃⁻/NO₂⁻ and the highest rates of denitrification. Our results indicated that porewater, not water column, NO₃⁻/NO₂⁻ was more influential for increased denitrification rates. Our study suggests that moist, aerobic locations high in porewater NO₃⁻/NO₂⁻ promote increased rates of denitrification in a non-tidal freshwater urban marsh.
INTRODUCTION

Denitrification is an anaerobic process where NO$_3^-$ is reduced to N$_2$. The reduction of NO$_3^-$ to N$_2$ consists of many intermediate reductions with some bacteria able to do all of the reductions while others can only do a few. The end product of denitrification, N$_2$, is fundamentally biotically unavailable to organisms due to its strong chemical bonds (Binnerup et al.; 1992; Nijburg et al. 1997). Denitrification occurs when oxygen concentrations are below 0.2 mg/L and is usually localized to just below the oxic/anoxic layer of the water column or the sediment where NO$_3^-$ is located (Knowles 1982; Seitzinger et al. 2006). In these low oxygen environments, the reduction of NO$_3^-$ to N$_2$ leads to a loss of nitrogen from soils and sediments, with the majority of N$_2$ formed being lost to the atmosphere (Nijburg et al. 1997).

Denitrification can be either beneficial or detrimental to an ecosystem depending on the nutritional state of the system. It can be beneficial assisting in the control of the global nitrogen cycle by maintaining atmospheric N$_2$ in balance (Knowles, 1996). Also, denitrification removes nitrogen from ecosystems, thus acting as a buffer helping to prevent eutrophication (Seitzinger 1988; Bonin et al. 1998; Seitzinger et al. 2006). Denitrification, however, can be detrimental by lessening the amount of nitrogen available to phytoplankton and, therefore, decreasing primary productivity in nitrogen-limited systems (Liu et al. 2003). In addition, denitrification can decrease the efficiency of nitrogen fertilizer applied to agricultural ecosystems thereby lowering the growth rate of targeted plants.
Wetlands play a key role in the natural processes of environments. Wetlands act as a sink and filter for pollutants and toxicants that otherwise may have adverse effects on an aquatic ecosystem (Mitsch et al. 2001; Poe et al. 2003). Pollutant runoff from rural and urban areas often drain into streams that can lead to a wetland; this runoff may contribute to an increased rate of nutrient loading (Boyer and Polasky 2004) that can lead to aquatic eutrophication. Aquatic eutrophication can lead to toxic algal blooms and anoxic water conditions that can cause a loss of aquatic biodiversity (Helmut and Sommer 2000). These detrimental effects can be lessened by an urban marsh’s potential to enhance the ability of the watershed to adequately control flooding, filter nutrients and pollutants, and maintain environmental stability. Many wetlands, however, are being drained to accommodate the expansion of cities thus reducing the number of urban marshes (Boyer and Polasky 2004). Approximately half of all wetlands in the continental United States of America have been drained since European occupation (Boyer and Polasky 2004).

The majority of denitrification research has focused on streams (Kemp and Dodds 2002; Böhlke et al. 2004; Mulholland et al. 2004, Bernot and Dodds 2005), rivers (Richardson et al. 2004), estuary sediments (Binnerup et al. 1992), marine sediments (Scala and Kerkhof 2000; Braker et al. 2001; Liu et al. 2003; Davis et al. 2004), agricultural soils (Dandie et al. 2008; Seitzinger 1994) and freshwater sediments (Chan and Knowles 1979; Dodds and Jones 1987). Little research, however, has been conducted on denitrification in non-tidal, natural freshwater urban marshes.

This study took place in intermittent and perennial pond types in Myrick Marsh, an urban marsh located in La Crosse, Wisconsin. The microbial characteristics of denitrification were the focus of this study with pond type and season being the major
variables. Intermittent ponds have irregular annual water cover. This irregularity could potentially lead to a harsh environment for denitrifiers due to the extreme changes in physical conditions, nutrient transport, and nutrient availability. Physical conditions include dissolved oxygen concentrations, pH, temperature, and hydrology which can vary greatly from stagnant water to dry sediment. Nutrient transportation and availability can also vary considerably due to environmental changes including reduction and oxidation potential, temperature, pH, and various levels of moisture. These variable conditions can promote coupling between nitrification and denitrification because the end product of nitrification, NO$_3^-$, is the substrate needed for the first set of denitrification. Perennial ponds are ponds where water cover is generally year round. We hypothesized that perennial ponds would have a higher rate of denitrification and higher denitrifier populations compared to intermittent ponds since the more consistent water cover would provide a more stable anaerobic environment and efficient nutrient transport.

The goals of the study were to compare denitrification rates and denitrifier abundance between perennial and intermittent pond types in Myrick Marsh over an entire year. The variables studied were annual water presence (i.e., pond type) seasonal variations, and denitrifier abundance and rates, making this research project unique in that, to the best of our knowledge, this is the first study of this kind and magnitude done in a non-tidal freshwater urban marsh.
MATERIALS AND METHODS

Site Description

Myrick Marsh is a non-tidal freshwater urban marsh located in La Crosse, Wisconsin. The marsh makes up the southern part of the 2,500 acre La Crosse River Marsh (Wisconsin Department of Natural Resources WebView 2005) (Fig. 1). Myrick Marsh is approximately 2.4 km to the east of the Mississippi River and divides the city of La Crosse WI into north and south and there are bluffs directly to the east. Besides urban runoff into Myrick Marsh, the two golf courses close to Myrick Marsh, approximately 0.3 km and 4.2 km to the southeast and northeast respectively, are likely contributing to the runoff that enters the marsh. There are 24 to 29 different ponds within the marsh that are categorized as having either perennial or intermittent annual water cover (Wisconsin Department of Natural Resources WebView 2005). Five perennial and five intermittent ponds were selected to be sampled twice a season for one year totaling eight sampling sessions. Seasonal sample sessions were as follows: summer was collected 29 July and 31 July 2004 and 16 July and 18 July 2005, fall was collected 2 October and 4 October 2004 and 14 November and 16 November 2004, winter was collected 27 December and 30 December 2004, and 26 February and 28 February 2005, and spring was collected 23 April and 25 April 2005 and 11 June and 13 June 2005. With the aid of the ArcView extension from the Minnesota Department of Natural Resources Sampling Generator Extension (Minnesota Department of Natural Resources 2005), three random predetermined sampling locations were selected for each pond for each sampling session.
Surface water and sediment characteristics and chemistries were measured at each of the three sites within each pond sampled. Sampling sites were located with a handheld GPS unit (Magellan SporTrak Map). Water depth was measured with a PVC depth pole. Water temperature and dissolved oxygen were measured in the water column just above the sediment with a precalibrated oxygen meter (YSI, Yellow Springs, Ohio). Unfiltered surface water was collected at each site in sterile, one liter and 100 mL Nalgene® bottles. In addition, approximately 50 mL of site surface water was filtered in the field through a Whatman glass fiber filter (Whatman, Piscataway, New Jersey) and acidified with concentrated hydrochloric acid to a pH of approximately 2 for later analysis of NO$_3^-$/NO$_2^-$. All site water collected was stored on ice in the field and then stored at 0-4°C until processed. Triplicate sediment cores were collected at each of the three sites within a pond by manually advancing a PVC sediment corer (i.d. of 7 cm) into the sediment to a depth of at least five centimeters. Sediment was extracted by inserting a plunger at the bottom of the sediment core and pushing it up through the clear polycarbonate tube until all overlying water was expelled. Approximately the top five centimeters of sediment, which is where denitrification likely occurs (Sorensen 1978), was removed and placed in a sterile plastic bag and stored on ice until returned to the lab. The triplicate sediment cores for a given site were combined into one sterile plastic bag, homogenized by hand and stored on ice until assayed.

**Water and Sediment Chemistries**

Nitrate and organic matter concentrations, along with pH, were determined for each site. Pore-water and water column NO$_3^-$/NO$_2^-$ were analyzed on a Dionex ICS-90 Ion Chromatography System using standard procedures (American Public Health Association, 1995). An external standard reference material sample (Ultra Scientific:
QCI-740 Ampule 2) was measured in each run for quality assurance. Organic content was determined by drying a known mass of sediment at 105°C for 24 h or until a constant mass was achieved (dry weight), followed by ashing at 550°C for 4 h. The weight of the remaining material was the ash weight (Bodelier et. al 1996) and the difference in mass between dry weight and ash weight represented the ash-free organic dry weight of each sample. pH determinations were done with an Oakton Waterproof pHTestr 3 pen-type meter (Vernon Hills, IL).

**Denitrification Rate Determination**

Denitrification rates were determined with the acetylene inhibition method (Sorensen 1978; Chan and Knowles 1979; Dodds and Jones 1987; Kemp and Dodds 2002; Richardson et. al 2004). For each sample, 25 mL of the combined site sediment and 20 mL of site water were placed into an air-tight 120 mL canning jar modified with a septum in the lid. If the sample site did not have overlying water, distilled water was used in place of site water (Austin and Strauss 2011). A total of two jars were prepared for each sample site. One jar was amended with 5 mL of chloramphenicol (final concentration of 100 mg/L) and the second (for measuring denitrification enzyme activity, DEA) was amended with 5 mL of DEA mixture (final concentrations of 100 mg chloramphenicol·L⁻¹, 12 mg glucose C·L⁻¹, and 14 mg potassium nitrate N·L⁻¹) (Richardson et al. 2004). Chloramphenicol is a broad-spectrum antibiotic that inhibits the de novo synthesis of proteins, thus the chloramphenicol only amendment served to determine an approximate rate of denitrification under the in situ conditions of available enzyme and substrate concentration. The chloramphenicol, carbon, and nitrogen mixture amendments were used to estimate denitrification enzyme activity (DEA), potential denitrification rates without carbon or nitrogen limitation (Richardson et al. 2004). After
the amendment addition, the jars were sealed and the head space was evacuated and replaced with helium since it will not interact with the denitrification process. The assay began when 20 mL of acetylene gas was injected into each jar to inhibit the reduction of N₂O to N₂. The jars were then incubated in the dark with continuous shaking at 175 rpm at the average surface water temperature measured from the site during sampling. Headspace gas samples of 5 mL were taken from each jar at 30, 60, 90, 120, and 240 minutes and injected into a pre-evacuated 2 mL gas storage vial. During the incubation, N₂O gas standards were aliquoted into identical 2 mL gas storage vials from stock N₂O gas concentrations of 1, 10, 100, and 1,000 ppm. The headspace gas samples and N₂O standards were analyzed for N₂O within 30 days on a Hewlett-Packard model 5870 gas chromatograph equipped with an electron capture detector (ECD ⁶³Ni). The change of N₂O during incubation times was used to estimate the rate of denitrification for the sediment sample size (Groffman et al. 1999).

**Denitrification Nutrient Limitation**

During the first sample session, additional denitrification assays were prepared to determine whether carbon and/or NO₃⁻ was limiting denitrification in Myrick Marsh sediments. Sediment was placed in 120 mL canning jars with site water as stated previously. One jar was amended with carbon (glucose) plus chloramphenicol for a final concentration of 12 mg/L carbon and 100 mg/L chloramphenicol and the other jar was amended with nitrogen (potassium nitrate) plus chloramphenicol for a final concentration of 14 mg/L nitrogen and 100 mg/L chloramphenicol. Both jars were then setup for denitrification using acetylene block inhibition as described previously.
Culture Based Most Probable Number

The abundance of the denitrifying community was determined by a culture-based Most Probable Number method (MPN) (Banward 1981). Triplicate sediment samples (1.0 gram wet weight each) were each serially diluted using phosphate buffer with dilutions ranging from $10^{-2}$ to $10^{-4}$ for sandy sites and $10^{-5}$ to $10^{-7}$ for sites with high organic content. One milliliter from each dilution was added to 9.0 mL of sterile nitrate broth (Difco, Detroit, MI) containing an inverted Durham tube to collect gas produced during incubation. The inoculated nitrate broth tubes were incubated at room temperature in the dark for seven days. After the seven day incubation, microbial growth and denitrification were assessed using three parameters: tube turbidity, presence of gas in the Durham tube, and removal of NO$_3^-$ by nitrate reduction and denitrification (Smibert and Krieg, 1981). By using these three analyses for each tube (Focht and Joseph 1973), the MPN of denitrifiers/g wet weight of sediment was estimated from a MPN table (Banward 1981). Due to insufficient dilution schemes for some of the samples, their results could only be estimated as greater than $10^6$ MPN/g. Sites were therefore categorized as having either a low (less than $5.5 \times 10^3$ MPN/g), medium (between $5.5 \times 10^3$ MPN/g and $5.5 \times 10^4$ MPN/g), or high (greater than $5.5 \times 10^4$ MPN/g) population of denitrifiers for statistical purposes.

Analysis of Data

Data were analyzed using SAS v. 9.1 (SAS Institute Inc., Cary, North Carolina, USA). A general linear model two-way ANOVA procedure was used to determine significant interactions between multiple factors. If there was a significant interaction between two main factors (i.e. season with pond type), then type III sum of squares was used to more closely delineate interactions. If the paired main factors did not have a
significant relation, then type II sum of squares was used. Once significance was
determined, a bivariate correlation test was done using the Spearman correlation
coefficient due to missing values in some of the analyzed factors. A $p$-value of less than
or equal to 0.05 was considered significant.
RESULTS

Water and Sediment Chemistries

Differences were found in water and sediment chemistries within and between pond types and seasons (Table 1). Perennial ponds (mean ± SE, 65.6 ± 5.6 cm) were deeper than intermittent ponds (32.4 ± 2.3 cm). Surface water temperatures did not vary significantly between pond types, but did vary seasonally as expected. Similarly, dissolved oxygen concentrations measured just above the sediment were not significantly different between pond types, but did vary seasonally (Table 1). No dissolved oxygen measurements were taken at locations without overlying water. Sediment organic matter, likewise, did not vary by pond type but did vary by season. The pH of the sample sites varied significantly by both pond type and season.

Porewater NO$_3^-$/NO$_2^-$ concentrations were significantly different by season ($p = 0.003$). Mean porewater NO$_3^-$/NO$_2^-$ concentration was highest during summer in both perennial and intermittent ponds (2.43 ± 1.44 mg/L, 2.45 ± 1.48 mg/L, respectively) (Fig. 2A). It was significantly higher compared to fall, winter, and spring. The lowest concentration of porewater NO$_3^-$/NO$_2^-$ was during spring in perennial and intermittent ponds (0.02 ± 0.01 mg/L, 0.02 ± 0.00 mg/L, respectively) (Fig. 2A). Porewater NO$_3^-$/NO$_2^-$ concentrations were not significantly different when comparing the interaction between pond type with season.

Water column NO$_3^-$/NO$_2^-$ concentrations were not significantly different by pond type, but were significantly different seasonally. Mean water column NO$_3^-$/NO$_2^-$
concentration was highest during winter in both perennial and intermittent ponds (0.39 ± 0.13 mg/L and 0.19 ± 0.08 mg/L respectively) (Fig. 2B). The lowest means for water column NO$_3^-$/NO$_2^-$ concentrations from both perennial and intermittent ponds occurred during the spring (0.01 ± 0.00 mg/L, and 0.026 ± 0.3 mg/L, respectively) (Fig. 2B). Water column NO$_3^-$/NO$_2^-$ concentrations were not significantly different when comparing pond type with season.

**Denitrification Rate Determination**

Variations in denitrification rates from sediment samples collected from the different seasons were significant but not different between pond types. Nor was there a significant interaction between denitrification rates with pond type and season. Denitrification rates were the lowest in the spring samples for both perennial and intermittent pond types (0.07 ± 0.03 µg N·cm$^{-2}$·h$^{-1}$, 0.09 ± 0.05 µg N·cm$^{-2}$·h$^{-1}$, respectively). The highest rates of denitrification occurred in the summer samples for both pond types. Summer intermittent pond denitrification rates (19.3 ± 6.9 µg·cm$^{-2}$·h$^{-1}$) were approximately double the rates for sediment from summer perennial ponds (11.0 ± 6.3 µg·cm$^{-2}$·h$^{-1}$), however, this was not a significant difference (Fig. 3). The mean fall denitrification rate in sediment from intermittent ponds (17.7 ± 11.7 µg·cm$^{-2}$·h$^{-1}$) was significantly higher than from fall perennial ponds (0.20 ± 0.07 µg·cm$^{-2}$·h$^{-1}$).

Variations in denitrification enzyme activity rates (potential rates without C and N limitation) for the sediment samples were also significant seasonally but not different between pond type. Unlike denitrification rates, however, denitrification enzyme activity rates for fall from samples from intermittent ponds were significantly higher than all other pond types and seasons. The range of DEA rates in sediment from perennial ponds was not as broad as those determined for intermittent ponds. Mean perennial pond DEA
rates ranged from 56.7 ± 6.5 µg N·cm⁻²·h⁻¹ in spring to 84.6 ± 9.6 µg N·cm⁻²·h⁻¹ for winter while intermittent pond mean rates ranged from 29.3 ± 7.5 µg N·cm⁻²·h⁻¹ for winter to 144 ± 31.2 µg N·cm⁻²·h⁻¹ for fall (Fig. 4).

The interaction of DEA rates with pond type and season was significant. Fall sediment sample DEA rates were significantly higher from intermittent ponds than from perennial ponds. Like summer denitrification rates, fall DEA rates for intermittent ponds were approximately twice that of perennial ponds (144 ± 31.2 µg N·cm⁻²·h⁻¹, 72.1 ± 8.2 µg N·cm⁻²·h⁻¹, respectively) (Fig. 4). An opposite trend was present for winter sediment samples with mean DEA rates for perennial ponds (84.6 ± 9.6 µg N·cm⁻²·h⁻¹) being almost three times higher than those recorded for intermittent ponds (29.3 ± 7.5 µg N·cm⁻²·h⁻¹) (Fig. 4).

Denitrification and DEA rates were not significantly related to dissolved oxygen concentrations. Denitrification and DEA rates were both positively correlated with concentrations of porewater NO₃⁻/NO₂⁻ and organic matter. Denitrification rates were positively correlated with water column NO₃⁻/NO₂⁻ concentrations, however, DEA rates were not. DEA rates were positively correlated to water depth and surface water temperature while denitrification rates were not.

**Denitrification Nutrient Limitation**

Denitrification rates were the highest in perennial and intermittent pond sediments with NO₃⁻ amendments (72.8 ± 6.88 µg N·cm⁻²·h⁻¹ and 46.6 ± 4.22 µg N·cm⁻²·h⁻¹, respectively) and with carbon and NO₃⁻ amendments (70.7 ± 7.89 µg N·cm⁻²·h⁻¹ and 54.8 ± 5.29 µg N·cm⁻²·h⁻¹, respectively). Unamended sediment samples and glucose amended samples were considerably lower than samples amended with NO₃⁻ (Fig. 5). Glucose amendments did not affect the overall denitrification rate.
Culture Based Most Probable Number

Denitrifying bacteria abundance ranged from $10^2$ to greater than $10^6$ denitrifiers/g sediment (Table 1 and Table 2). Denitrifying bacteria abundance varied by over four orders of magnitude, however, it was not significant by either pond type or season. Although not significant, fall sample locations had the most sample sites categorized with a high abundance of denitrifiers (40%) while spring had the most sample sites categorized with a low abundance of denitrifiers (68%). Both perennial and intermittent pond sample locations (38% and 37% respectively) were mainly categorized as having low denitrifier abundance. Sample locations with overlying water (covered) were also generally categorized as low in denitrifier abundance (38%) while sample locations that did not have overlying water, hereafter called uncovered sites, were split equally between low and high denitrifier abundances (36%) each. Denitrifier abundance was negatively correlated with surface water temperature. There was no significant relation between MPN and denitrification rates, DEA rates, dissolved oxygen concentration, porewater or water column NO$_3^-$/NO$_2^-$ concentration, or ash-free organic dry weight.

Sample Location Surface Water Cover

As expected, more intermittent pond sampling locations lacked water cover compared to perennial pond sample locations ($p < 0.001$) (Table 2). pH was lower at uncovered sites ($p < 0.001$) whereas surface temperature, organic content, and porewater NO$_3^-$/NO$_2^-$ concentrations were all higher at uncovered sites (Fig. 6). Denitrification and DEA rates were also higher at uncovered sites (both $p < 0.001$). Denitrification and DEA rates were not determined for samples from winter locations that did not have overlying water (six sample locations) since the sediment was frozen and not amenable to the technique used for measuring activity. Therefore, the winter denitrification and DEA
rates may be an underestimation if there actually was minimal activity, since the rate for these sites was set to zero (Fig. 7). Denitrifier abundance did not differ significantly between uncovered sites and sites with surface water.
DISCUSSION

We hypothesized that perennial ponds would have higher rates of denitrification compared to intermittent ponds due in part to the more stable anaerobic conditions. Our results were actually the opposite with intermittent pond sediments typically having higher rates of denitrification and potential denitrification. In fact, sediment samples from uncovered sites, which had a greater exposure to oxygen, had higher denitrification and potential denitrification rates. These higher rates appear to be due to high porewater NO$_3^-$/NO$_2^-$ and not water column NO$_3^-$/NO$_2^-$ concentrations as seen by others (Kemp and Dodds, 2002; Richardson et al. 2004). During the summer and fall seasons when porewater NO$_3^-$/NO$_2^-$ was highest, denitrification rates in the sediment samples were also the highest. Our porewater NO$_3^-$/NO$_2^-$ concentrations correlated with denitrification rates and were on average much higher than water column NO$_3^-$/NO$_2^-$ concentrations; therefore porewater NO$_3^-$/NO$_2^-$ concentrations may be a better indication of denitrification. The periodic lack of overlying water, based on our results, may actually promote increased rates of denitrification by, in essence, accumulating porewater NO$_3^-$/NO$_2^-$ since there is no water column to diffuse into. Even with aerobic conditions, denitrification is likely occurring in anaerobic microhabitats in close proximity to where the aerobic nitrification process is occurring. These uncovered sites likely enable a better coupling between nitrification and denitrification (Seitzinger 1988; Richardson et al. 2004).

The average denitrification rate calculated in our study was 6.2 µg N·cm$^{-2}$·h$^{-1}$, ranging from some nondetectable rates in all seasons to a high of 280 µg N·cm$^{-2}$·h$^{-1}$ in
fall. Our average denitrification rate was higher than the literature averages. Our
denitrification rates were approximately twenty times higher during the summer and fall
seasons than those in a study using the same method from the nearby Mississippi River
by Richardson et al. (2004), depending on the season and pond type. Richardson et al.
(2004) did not have data for the winter season but had spring denitrification rates
approximately 5 times higher than our rates. This difference was unexpected as the La
Crosse River flows through Myrick Marsh and drains directly into the Mississippi River
approximately two kilometers away. Richardson et al. (2004) found that denitrification
rates in comparable sample categories differed considerably both spatially and temporally
like the sites in our study. The backwater locations sampled by Richardson et al. ranged
from approximately 0.026 µg N·cm⁻²·h⁻¹ in fall to 0.40 µg N·cm⁻²·h⁻¹ in spring and the
impounded locations ranged from approximately 0.07 µg N·cm⁻²·h⁻¹ in fall to 0.3 µg
N·cm⁻²·h⁻¹ in spring. Both sample categories had lower denitrification rates compared to
sample locations in Myrick Marsh even though they were similar in that they both
contained high concentrations of organic carbon and primarily stagnant water. Water
column NO₃⁻/NO₂⁻ concentrations in the Mississippi River stagnant water locations were
approximately five times higher than those found in this study, further supporting our
contention that water column NO₃⁻/NO₂⁻ may not be a good indicator of denitrification
rates. Davis et al. (2004) determined denitrification rates from sediment samples from a
Rhode Island salt marsh averaged 0.59 µg N₂O-N·cm⁻²·h⁻¹ with high variability between
sample sites. This Rhode Island salt marsh average was on the higher end of the
denitrification range for a salt marsh (Davis et al. 2004). Myrick Marsh sediment
denitrification rates also had high variability between sample sites, but were on average
approximately ten times higher than those determined for the salt marsh. This difference
may be due to a difference in measuring denitrification rates. Our samples were stored on ice and processed within approximately 16 hours, whereas the Davis et al. samples were stored at ambient temperature with a 12/12 hour light/dark cycle and processed within two days. The longer time from sampling to processing may contribute to the lower denitrification rates. Other aquatic ecosystems have had similar reported denitrification rates. Seitzinger (1988) reported denitrification rates as high as 0.48 µg N₂O-N·cm⁻²·h⁻¹ in sediments from rivers. Other comparable studies have also used the acetylene inhibition technique and have found similar results. In agriculturally influenced streams, Kemp and Dodds (2002) reported a high of 0.002 µg N₂O-N·cm⁻²·h⁻¹ in samples from the streams, and Schaller et al. (2004) reported a high of approximately 0.07 µg N₂O-N·cm⁻²·h⁻¹, all of which are considerably lower than our average of 6.2 µg N·cm⁻²·h⁻¹.

Denitrification rates in our study were lowest for samples collected during the spring for both pond types most likely due to melting snow and ice from the surrounding watershed that may have diluted NO₃⁻/NO₂⁻ concentrations as both water column and porewater NO₃⁻/NO₂⁻ concentrations were the lowest during spring. The area surrounding Myrick Marsh is mostly residential and snow accumulates throughout the winter resulting in annual spring flooding of Myrick Marsh. The highest average rates of denitrification were in samples collected during the summer for both pond types, which is common (Poe et al. 2003; Richardson et al. 2004; Seitzinger et al. 2006), and is likely due to a combination of increased temperature, organic matter, and porewater NO₃⁻/NO₂⁻ concentration. Another possible reason for the increased denitrification rates during the summer could have been from nonpoint sources from the neighboring urban watershed. The surrounding watershed consists of residential properties, industrial properties, and
commercial properties including two nearby golf courses, which could conceivable contribute \( \text{NO}_3^- \) to watershed runoff. However, the water column \( \text{NO}_3^-/\text{NO}_2^- \) was not elevated during the summer months and so these nonpoint sources of \( \text{NO}_3^- \) was likely not contributing to the increased denitrification rates.

Denitrification enzyme activity rates did not vary significantly by pond type but did seasonally indicating that pond type is not a major factor in determining denitrification potential. DEA served as an indicator for potential denitrification rates where carbon and nitrogen were in excess at anaerobic conditions and is a useful tool for sediment comparisons because it corresponds well to nutrient changes that can affect denitrification rates (Groffman et al. 1999). Studies have indicated that carbon and/or nitrogen were limiting factors of denitrification (Kemp and Dodds 2002, Groffman et al. 1999, Richardson et al. 2004, and Solomon et al. 2009). Our DEA and unamended denitrification results did not correlate to each other which is not uncommon (Groffman et al. 1999). The denitrification nutrient limitation results, even though they were for just one sample session, indicated that nitrogen, not carbon, was likely limiting denitrification in Myrick Marsh sediments. With the dense aquatic macrophytes found throughout Myrick Marsh, it is not surprising that carbon was not limiting.

As expected, intermittent ponds had more samples collected from locations that were lacked overlying water compared to perennial ponds. Even though our sample size of uncovered sites was lower than covered sites, some surprising trends could be discerned. Sediment samples from uncovered sites had considerably higher rates of denitrification and potential denitrification. In addition, uncovered sites had higher surface temperatures, organic matter concentrations, and porewater \( \text{NO}_3^-/\text{NO}_2^- \) concentrations, which were likely the reason for the increased denitrification rates.
Austin and Strauss (2011), however, found that DEA rates drastically declined in an experimental stream when drying the stream sediment to below 5% moisture. Our uncovered sites did not have overlying water cover, but were moist to the touch and may have been greater than 5% moisture which could explain the opposing DEA trends. Even though our rates were higher for samples from uncovered sites, denitrifier abundance was not significantly different between covered and uncovered sites suggesting that the type of denitrifiers present, not the abundance of denitrifiers, had more influence on denitrification rates (Čuhel et al. 2010).

Overall, denitrification in sediment samples collected from the urban Myrick Marsh varied seasonally as expected, but not by pond type. With the exception of fall and winter intermittent ponds, potential denitrification rates (DEA) were very similar regardless of season or pond type indicating that nitrogen was likely limiting. Our study showed that regardless of pond type, porewater NO$_3^-$/NO$_2^-$ concentration was more critical than water column NO$_3^-$/NO$_2^-$ for increased denitrification rates in sediment from this urban Wisconsin marsh. We did not find a correlation between denitrification rates and denitrifier abundance indicating that the type of denitrifiers may have been more important than the actual number of denitrifiers at our sample sites. Myrick Park’s perennial ponds may not be the ideal environment for the denitrifying community; instead our results indicate that moist marsh environments lacking surface water with a high concentration of porewater NO$_3^-$/NO$_2^-$ may promote higher denitrification rates.
REFERENCES


Table 1. Water and sediment chemistries and most probable number of denitrifiers for perennial and intermittent ponds sampled seasonally from Myrick Marsh located in La Crosse, Wisconsin.

<table>
<thead>
<tr>
<th>Pond type</th>
<th>Season</th>
<th>Depth (cm)</th>
<th>Surface water temp (°C)</th>
<th>Dissolved oxygen (mg/L)</th>
<th>pH</th>
<th>Ash-free organic dry weight (%)</th>
<th>Denitrifier abundance (MPN/g sediment)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Perennial</td>
<td>summer</td>
<td>Mean 58.8</td>
<td>23.0</td>
<td>0.47</td>
<td>6.6</td>
<td>5.17</td>
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<td>Range</td>
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<td>(19.5 - 35.8)</td>
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<td>(4.9 - 7.1)</td>
<td>(1.60 - 8.69)</td>
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<td>Fall</td>
<td>Mean 67.4</td>
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<td>4.77</td>
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<td>4.81</td>
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<td>Winter</td>
<td>Mean 64.8</td>
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<td>2.00</td>
<td>6.9</td>
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<td>Spring</td>
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<td>17.1</td>
<td>2.99</td>
<td>6.7</td>
<td>4.88</td>
<td>Low (1.1x10^5 - &gt;1.0x10^6)</td>
</tr>
<tr>
<td></td>
<td>Range</td>
<td>(19.0 - 105)</td>
<td>(7.1 - 28.4)</td>
<td>(0.84 - 8.5)</td>
<td>(6.0 - 7.1)</td>
<td>(2.07 - 6.93)</td>
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</tr>
<tr>
<td>Intermittent</td>
<td>summer</td>
<td>Mean 31.0</td>
<td>21.6</td>
<td>0.96</td>
<td>6.0</td>
<td>5.95</td>
<td>Low (3.6x10^5 - &gt;1.0x10^6)</td>
</tr>
<tr>
<td></td>
<td>Range</td>
<td>(0.0 - 74.5)</td>
<td>(12.7 - 26.8)</td>
<td>(0.20 - 5.20)</td>
<td>(4.9 - 7.0)</td>
<td>(2.69 - 14.9)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Fall</td>
<td>Mean 28.4</td>
<td>9.2</td>
<td>3.31</td>
<td>6.9</td>
<td>5.83</td>
<td>Low (7.3x10^2 - &gt;1.0x10^6)</td>
</tr>
<tr>
<td></td>
<td>Range</td>
<td>(0.0 - 67.5)</td>
<td>(4.1 - 20.5)</td>
<td>(0.39 - 18.0)</td>
<td>(6.2 - 7.4)</td>
<td>(3.30 - 12.6)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Winter</td>
<td>Mean 31.7</td>
<td>1.1</td>
<td>1.58</td>
<td>6.9</td>
<td>4.20</td>
<td>High (9.4x10^2 - &gt;1.0x10^6)</td>
</tr>
<tr>
<td></td>
<td>Range</td>
<td>(0.0 - 75.0)</td>
<td>(0.0 - 5.0)</td>
<td>(0.24 - 11.0)</td>
<td>(6.3 - 7.4)</td>
<td>(0.76 - 8.50)</td>
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</tr>
<tr>
<td></td>
<td>Spring</td>
<td>Mean 38.3</td>
<td>22.1</td>
<td>3.37</td>
<td>6.8</td>
<td>4.50</td>
<td>Low/High (7.2x10^2 - &gt;1.0x10^6)</td>
</tr>
<tr>
<td></td>
<td>Range</td>
<td>(0.0 - 85.0)</td>
<td>(12.6 - 26.2)</td>
<td>(0.25 - 12.1)</td>
<td>(5.7 - 7.2)</td>
<td>(1.87 - 8.28)</td>
<td></td>
</tr>
</tbody>
</table>

a Surface sediment temperature was taken at uncovered sites.

b Mean most probable number categories were given as some numbers could only be determined as >10^6 MPN/g sediment and do not allow for a mathematical mean. The mean category designated is the predominant category with split categories indicating the same percentage of sample locations within a designated category. Categories were defined as follows: Low < 5.5x10^2, Medium >5.5x10^2 to < 5.5x10^4, and High > 5.5x10^4 MPN/g sediment.
Table 2. Water and sediment chemistries and most probable number of denitrifiers results for covered and uncovered sample sites sampled from Myrick Marsh located in La Crosse, Wisconsin.

<table>
<thead>
<tr>
<th>Moisture classification</th>
<th>Season</th>
<th>Surface water temp (°C)</th>
<th>Dissolved oxygen (mg/L)</th>
<th>pH</th>
<th>Ash-free organic dry weight (%)</th>
<th>Denitrifier abundance (MPN/g sediment)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Covered</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Low (&lt;10^6)</td>
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<tr>
<td></td>
<td>summer</td>
<td>21.5</td>
<td>0.68</td>
<td>6.9</td>
<td>4.81</td>
<td>(2.0x10^5 - &gt;1.0x10^6)</td>
</tr>
<tr>
<td></td>
<td>Range</td>
<td>(12.7 - 28.2)</td>
<td>(0.1 - 5.2)</td>
<td>(6.4 - 7.1)</td>
<td>(1.60 - 7.32)</td>
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<tr>
<td></td>
<td>fall</td>
<td>7.9</td>
<td>4.17</td>
<td>7.1</td>
<td>4.77</td>
<td>High (&gt;1.0x10^6)</td>
</tr>
<tr>
<td></td>
<td>Range</td>
<td>(3.0 - 19.0)</td>
<td>(0.39 - 18.0)</td>
<td>(6.6 - 7.5)</td>
<td>(1.20 - 11.0)</td>
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<tr>
<td></td>
<td>winter</td>
<td>1.7</td>
<td>1.82</td>
<td>6.8</td>
<td>4.72</td>
<td>High (&gt;1.0x10^6)</td>
</tr>
<tr>
<td></td>
<td>Range</td>
<td>(0.0 - 5.6)</td>
<td>(0.15 - 11)</td>
<td>(6.4 - 7.4)</td>
<td>(2.10 - 8.77)</td>
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<td>spring</td>
<td>18.6</td>
<td>3.17</td>
<td>6.7</td>
<td>4.57</td>
<td>Low (7.2x10^2 - 1.0x10^6)</td>
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<tr>
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<td>Range</td>
<td>(7.1 - 28.4)</td>
<td>(0.25 - 12)</td>
<td>(6.0 - 7.2)</td>
<td>(1.87 - 6.93)</td>
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<tr>
<td>Uncovered</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Low/Medium/High</td>
</tr>
<tr>
<td></td>
<td>summer</td>
<td>24.5</td>
<td>NA^a</td>
<td>5.5</td>
<td>7.81</td>
<td>(2.0x10^5 - 2.7x10^5)</td>
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<tr>
<td></td>
<td>Range</td>
<td>(19.1 - 35.8)</td>
<td>(4.9 - 6.3)</td>
<td>(2.69 - 14.9)</td>
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<tr>
<td></td>
<td>fall</td>
<td>13.3</td>
<td>NA^a</td>
<td>6.6</td>
<td>8.44</td>
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<td>(4.42 - 12.6)</td>
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<tr>
<td></td>
<td>winter</td>
<td>1.3</td>
<td>NA^a</td>
<td>6.8</td>
<td>4.14</td>
<td>Low (1.2x10^3 - &gt;1.0x10^6)</td>
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<td>Range</td>
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<td>(6.3 - 7.3)</td>
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<tr>
<td></td>
<td>spring</td>
<td>25.2</td>
<td>NA^a</td>
<td>6.1</td>
<td>8.03</td>
<td>Low/High (1.5x10^5 - &gt;1.0x10^6)</td>
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<td>Range</td>
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<td>(5.7 - 6.5)</td>
<td>(7.79 - 8.28)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

^a Surface sediment temperature was taken at uncovered sites.

^b Mean most probable number categories were given as some numbers are only determined as >10^6 MPN/g sediment and do not allow for a mathematical mean. The mean category designated is the predominant category with split categories indicating the same percentage of sample locations with designated category. Categories are defined as follows: Low < 5.5 x 10^3, Medium >5.5 x 10^3 to < 5.5 x 10^4, and High > 5.5 x 10^4 MPN/g.
Fig. 1. Five intermittent (I-1 to I-5) and five perennial (P-1 to P-5) ponds sampled in Myrick Marsh, La Crosse, Wisconsin (Wisconsin Department of Natural Resources Webview). Pond identifications were given to distinguish between pond type and replicate.

Fig. 2. Seasonal porewater NO₃⁻/NO₂⁻ (A) and water column NO₃⁻/NO₂⁻ (B) concentrations from five perennial and five intermittent ponds in Myrick Marsh, La Crosse, Wisconsin. Error bars represent standard error from the mean.

Fig. 3. Seasonal denitrification rates from sediment samples collected from five perennial and five intermittent ponds in Myrick Marsh, La Crosse, Wisconsin. Error bars represent standard error of the mean.

Fig. 4. Seasonal denitrification potential rates from sediment samples collected from five perennial and five intermittent ponds in Myrick Marsh, La Crosse, Wisconsin. Error bars represent standard error of the mean.

Fig. 5. Potential denitrification enzyme activity resulting from the addition of carbon, NO₃⁻, or carbon and NO₃⁻ to Myrick Marsh sediments. Error bars represent standard error of the mean.

Fig. 6. Seasonal porewater NO₃⁻/NO₂⁻ concentrations from uncovered and covered sampling sites within ten ponds in Myrick Marsh, La Crosse, Wisconsin. Error bars represent standard error from the mean.

Fig. 7. Seasonal denitrification rate (A) and denitrification enzyme activity (B) from sediment samples collected from covered and uncovered sampling sites in Myrick Park, La Crosse, Wisconsin. Uncovered sample sites from the winter were frozen and reported as zero. Error bars represent the standard error of the mean.
Fig. 1.
Fig. 2.

**A**

Comparison of water column $\text{NO}_3^-/\text{NO}_2^-$ concentrations between perennial and intermittent sites across different seasons.

**B**

Comparison of porewater $\text{NO}_3^-/\text{NO}_2^-$ concentrations across different seasons.
Fig. 3.

Denitrification Rate (µg N·cm⁻²·h⁻¹)

Season

Perennial
Intermittent

Summer Fall Winter Spring
Fig. 4. Denitrification Enzyme Activity Rate (µg N·cm$^{-2}$·hr$^{-1}$) by Season and Perennial vs. Intermittent
Fig. 5. Denitrification enzyme activity (µg N·cm\(^{-2}·h^{-1}\))

Perennial

Intermittent

Control +C +N +C+N

Amendments
Fig. 6.
Fig. 7.

A

Denitrification Rate (µg N·cm⁻²·h⁻¹)

Covered Sample Sites

Uncovered Sample Sites

B

Denitrification Enzyme Activity (µg N·cm⁻²·h⁻¹)

Season

Summer Fall Winter Spring
CHAPTER IV

SUMMARY
SUMMARY

Nitrification and denitrification rates were not significantly related to each other by pond type or season from Myrick Marsh sediment samples, which is unlike the trend found in other studies. Both nitrification and denitrification rates followed seasonal trends. Nitrification rates were the highest during the warmest season, summer, and lowest during the coldest season, winter. Like nitrification rates, denitrification rates were also the highest during summer, but were lowest during the spring. Denitrification enzyme activity rates were similar throughout the seasons with sediment samples from fall intermittent pond sample locations having the highest rates and samples from winter intermittent ponds having the lowest rates. These results indicate that the warmer seasons promote increased process rates.

There was not a significant difference in AOB or denitrifier abundance regardless of pond type. We did, however, see a seasonal difference in AOB abundances with summer and fall having the highest number of AOB followed by spring having approximately half the AOB, and winter having the lowest number of AOB at approximately one log factor lower than summer and fall. There was a positive relationship between nitrification rates and spring and summer AOB abundance. We did not, however, find a correlation between denitrification rates and denitrifier abundance indicating that the type of denitrifiers may be more critical than the actual number of denitrifiers in Myrick Marsh sediment.
AOB community diversity was determined for only approximately half of our total sample sites because even though all sites had detectable \textit{amoA} as seen by AOB abundance results we were unable to generate \textit{amoA} DGGE fingerprints with DNA from many of our sites. The majority of sites analyzed for AOB community diversity resulted in only two or three different bands, although the fingerprint from the most diverse site consisted of 14 different bands. Based on cluster analysis, all sites were approximately 55\% similar indicating that Myrick Marsh sediments had a core group of AOB with additional members changing depending on environmental conditions. The same analysis did not reveal any pond type or seasonal groupings of sediment AOB communities. Although the communities are only 55\% similar, the relatively stable rates of nitrification may indicate a functional redundancy within the ammonia-oxidizing community in Myrick Marsh.

Sample locations without surface water, uncovered sites, had higher concentrations of porewater NO$_3^-$/NO$_2^-$ and higher rates of denitrification and DEA compared to sample sites with surface water, covered sites. These results were surprising since uncovered sites had greater exposure to oxygen and denitrification is an anoxic process. Other studies state that water column NO$_3^-$/NO$_2^-$ concentrations are good indicators of denitrification rates, whereas this study shows that porewater NO$_3^-$/NO$_2^-$ concentration may be a better indicator of denitrification. Therefore, our results indicate that moist, aerobic locations, high in porewater NO$_3^-$ supported increased rates of denitrification in Myrick Marsh sediment.