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Impact of Salt on Denitrification Potential in Roadside Environments

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Impact of Salt on Denitrification Potential in Roadside Environments

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Impact of Salt on Denitrification Potential in Roadside Environments

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Abstract

Urban systems are impacted by changes in hydrology and water chemistry, altering their ability to mitigate detrimental impacts to coastal water bodies, particularly those that result from high volumes of stormwater runoff. The elevated level of impervious cover increases not only runoff but also contaminant loading of nutrients, metals, and road salt used for deicing in cold weather climates. Current stormwater management strategies, while focused on managing the changes in hydrology associated with urbanization, also are designed to mitigate the pollutant loading. Here we investigate the impact that road salt will have on nitrogen removal in roadside environments. Sediments were collected from a series of forested and roadside ponds and treated with a range of Cl\(^-\) concentrations from 0-5000 mg/L for 96 hrs. Denitrification rates were measured by the isotope pairing technique using \(^{15}\)N-NO\(_3^\). Our results suggest that road salt inhibits denitrification in both roadside and forested ponds. The decrease in denitrification rates is lower for the roadside ponds which previously have been exposed to elevated concentrations of Cl\(^-\) due to their proximity to roadways (<10 m). Forested ponds demonstrated a greater decrease in denitrification rates suggesting that ponds that historically have not been exposed to Cl\(^-\) are not able to adapt as quickly to increases in Cl\(^-\). At 5000 mg/L, denitrification rates in forested ponds decreased by three orders of magnitude from the baseline compared with a decrease of only two orders of magnitude in the roadside ponds. As development increases the impact to natural systems, the effectiveness of roadside ponds and stormwater detention ponds for mitigating nitrogen loading is critical to protect local and downstream water resources. Included is the necessity, as in the current investigation, to evaluate the synergistic impacts among pollutants in roadside environments.
Introduction

Anthropogenic activities have increased the nitrogen (N) loading in rural, suburban, urban, and mixed land use watersheds. One of the most important consequences of higher N loading is the increase in N exports from urban watersheds to coastal ecosystems. Excessive nitrogen exports to receiving water bodies can result in eutrophication, hypoxic conditions, and/or loss of biodiversity (Paerl, 1997). The input rate of N has doubled since the 1940’s, mostly due to agriculture and the burning of fossil fuels (Vitousek et al., 1997). As N inputs increase, the ability of freshwater systems to act as buffer between the terrestrial landscape and coastal ecosystems remains essential. In forested streams, for example, 95% of N inputs are retained in the stream (Groffman et al., 2004). However, retention of N in suburban/urban watersheds can range from 25% to 95%, with 40% being the expected value (Groffman et al., 2004).

Nitrogen removal in urban streams is impacted by hydrologic, geomorphological, and biogeochemical changes as well as elevated N loading. Changes in hydrology are driven by the increase in impervious cover in urban areas which generates high volumes of stormwater runoff. Stormwater runoff creates higher peak flows leading to channel incision and a lower hydraulic retention time, decreasing the interaction of water with stream sediments and hyporheic zones where removal and uptake occur (Paul and Meyer, 2001; Groffman et al., 2002; Sweeney et al., 2004). Interaction with riparian zones is reduced by erosive runoff and the loss of hydrologic connectivity that is associated with decrease in groundwater infiltration (Groffman et al., 2002). In addition to these physical changes, stormwater runoff increases the concentration of nutrients and other pollutants. High concentrations of N in particular can lead to biological saturation where the available N exceeds the processing ability of organisms (O’Brian et al., 2007). N
processing can further be impacted by increased pollutants associated with stormwater including metals (Sakadevan et al., 1999) and chloride from road salt (Hale and Groffman, 2006, Groffman et al., 1995).

Road salt usage is high in urban areas contributing to high concentrations of Cl⁻ in surrounding water bodies, the negative environmental impacts of which have been documented (e.g., Findlay and Kelly, 2011). As Cl⁻ concentrations increase in urban and rural environments (Kaushal et al., 2005), determining the impacts that the combined increase in N and Cl⁻ will have on N exports from urban watersheds is critical. In urban streams, Cl⁻ concentrations have reached nearly 4,600 mg/L (Kaushal et al., 2005) and have been measured as high as 25,000 mg/L in snowmelt (Makepeace et al., 1995). Elevated Cl⁻ concentrations have been shown to have negative impacts on aquatic plants and animals (Kaushal et al., 2005) and cause changes to microbial community structure and respiration rates (Baldwin et al. 2006). The impact of Cl⁻ on N cycling has also been investigated; however, the results vary. In forest soils treated with Cl⁻, nitrification and N mineralization rates have been shown to decrease, but denitrification was not inhibited (Groffman et al., 1995). Studies in freshwater sediments have measured decreases in denitrification potential with higher concentrations of Cl⁻ in sediments without prolonged exposure to salinity (Hale and Groffman, 2006). Conversely, in sediments with prolonged exposure to salinity, denitrification rates remain unchanged (Hale and Groffman, 2006; Laverman et al., 2007). In freshwater forested wetlands subjected to coastal flooding, denitrification rates increased with salinity in sediments collected along one river but remained unchanged in sediments from two similar rivers (Marton et al., 2012). However, the long-term salinity exposure at these study sites was uncertain due to potential exposure of the coastal flood plain in the past. These studies suggest that microbial communities that are exposed to elevated
concentrations of Cl$^-$ for prolonged periods of time may adapt to the physiochemical stress associated with an increase in salinity. However, Cl$^-$ concentrations used in these experiments were below the maximums observed for urban settings during winter months. No studies have yet to determine the impacts of Cl$^-$ concentrations in freshwater systems over a broad range of Cl$^-$ values. Determining the response of denitrification rates over a broad range of Cl$^-$ concentrations provides insight into the threshold at which microbial communities can adapt based on prior exposure.

The purpose of this study is to determine how Cl$^-$ addition impacts denitrification rates in ponds that are exposed to Cl$^-$ over a long period of time relative to ponds that have not been impacted by Cl$^-$. The study site includes three roadside and three forested ponds; the roadside ponds are exposed to elevated concentrations of Cl$^-$ throughout the year due to road salt application while forested ponds are not. Specific objectives were to measure denitrification rates in roadside and forested ponds, to monitor changes in water chemistry with Cl$^-$ exposure, and to assess potential differences in microbial community structure in roadside and forested pond sediments. In urban environments, where there is an increasing interest in managing stormwater, the synergistic impacts of N loading and Cl$^-$ are important to determine effective N management strategies. Detention ponds are considered a best management practice for managing stormwater in urban environments to prevent stream degradation and remove nutrients (EPA, 2012). Here, we evaluate the impact of Cl$^-$ on N removal via denitrification and water quality to demonstrate the impact that road salt use in urban environments will have on N removal and retention in wet detention ponds/wetlands.
Background

Nitrogen cycling and loading

Anthropogenic inputs of nitrogen in the urban environment include point sources such as wastewater treatment plants and sanitary and combined sewer overflows as well as non-point sources (NPS) such as impervious surface runoff, agricultural runoff, and atmospheric deposition (Vitousek et al., 1997). The amount of ecosystem bioavailable nitrogen has increased dramatically with human activity due to fossil fuel combustion, fertilizer use, and the mobilization of nitrogen through land use change (i.e. land clearing and loss of wetlands; Vitousek et al., 1997). As receiving waters become increasingly stressed by nutrient inputs, efforts have been directed towards limiting N loading, particularly from point sources. For example, Connecticut has established a N permit system to limit inputs from water pollution control facilities (WPCF; DEEP, 2012). However, NPS pollution is more difficult to address due to the diffuse nature of the source. As the incremental return on N controls for WPCF decreases, NPS pollution and the ability of streams to alleviate the elevated loads is becoming increasingly important.

Excessive nitrogen inputs into the environment lead to water quality degradation such as eutrophication and hypoxia. The N loading in urban environments often exceeds the ability of the ecosystem to attenuate N leading to high exports of N into coastal ecosystems. Eutrophication is the biologic response to excessive nutrients such as nitrogen and phosphorus (Pearl, 1997). High concentrations of nitrates and phosphates stimulate algal growth leading to large blooms of phytoplankton. When the phytoplankton die and sink, they decompose which results in the consumption of oxygen by microorganisms in the bottom waters. When the water
column stratifies, oxygen is consumed more quickly near the sediments than the rate of
replenishment via vertical mixing in the water column. Fish and other organisms that depend on oxygen for survival cannot live in these environments. In addition to disruption of natural ecosystem cycles, large algal blooms decrease the aesthetic and recreational value of surface waters and fisheries, each of which impacts the economic welfare of coastal communities. To help prevent eutrophication, water quality managers seek to maintain a balance between nitrogen inputs and the ability of ecosystems to attenuate or remove nitrogen.

**Nitrogen in the urban environment**

Urban streams have high nutrient inputs, altered hydrology and geomorphology, and increased concentrations of other pollutants that can create poor water quality. “Urban stream syndrome” is a well-described phenomenon that results in the loss of ecosystem function (Walsh et al., 2005). As the percent of impervious cover increases, runoff increases and infiltration decreases. During precipitation events the enhanced runoff results in a flashier hydrograph characterized by a higher peak flow and shorter hydraulic retention time in the stream channel. The higher flow velocity contributes to scouring of the stream bed, sediment erosion, incision of the stream channel, and loss of riparian zones. The decreased interaction of the water column with the stream sediments and riparian zone reduce the uptake, retention, and removal of N which typically occurs in the hyporheic zone (McClain et al., 2003).

In addition to the hydrology and geomorphology changes, water chemistry changes with increased loading of nutrients, pesticides, metals, petroleum byproducts, and pharmaceuticals which also can impact ecosystem functioning (Gobel et al., 2007). Freshwater ecosystems in colder regions also experience an increase in salinity due to the application of road salt and
deicers (Marsalek, 2003). These chemicals can directly impact biogeochemical cycling or can act synergistically. For example, road salt increases conductivity and can mobilize metals from sediments. Lofgren (2001) documented increased H\textsuperscript{+}, Zn\textsuperscript{2+} and Cd\textsuperscript{2+} mobilization, demonstrating the increase in metals mobilization with acidification and increase in ionic strength.

**Impacts of salinity on nitrogen cycling**

Salinization of freshwater is an increasingly widespread concern (Kaushal et al., 2005). In suburban and urban streams, impervious cover is linked to high usage of road salt and concentrations of Cl\textsuperscript{-} in the streams and shallow groundwater (Likens and Buso, 2009). Concentrations of Cl\textsuperscript{-} in snowmelt have been measured as high as 25,000 mg/L (Makepeace et al. 1995). Baseline concentrations of Cl\textsuperscript{-} in streams are increasing and remaining elevated beyond the seasonal spikes associated with road salt application. (Kaushal et al, 2005; Kelly et al., 2008). The extended increase in Cl\textsuperscript{-} concentration suggests that there is retention of Cl\textsuperscript{-} within the watershed and that road salts applied during the winter are not immediately flushed out of the system (Findlay and Kelly, 2011). During the winter, concentrations greater than 4,600 mg/L have been reported in an urban stream in Baltimore, MD (Kaushal et al., 2005).

The effect of chloride on nitrogen dynamics in forested and urban debris dams has been studied in streams in the Baltimore Long Term Experimental Research site (BES LTER). Debris dams are hot spots for denitrification since they are rich in organic matter, have high hydraulic retention times, and reducing conditions are present (McClain et al., 2003). Sediments were collected from debris dams in the summer from all streams and brought back to the lab to measure denitrification enzyme activity. Results showed that Glyndon, an older suburban watershed in the headwaters of the BES LTER, which has the highest Cl\textsuperscript{-} concentrations of the
three sites, exhibited similar denitrification enzyme activity (DEA) before and after incubation with 2500 mg/L Cl\(^-\) (Hale and Groffman, 2006). Conversely, Pond Branch, the forested reference site for the BES LTER, showed a decrease in DEA following incubation with 2500 mg/L of Cl\(^-\). The discrepancy in DEA response between the two sites suggests that forested sites exposed to high chloride concentration will have a more drastic decrease in denitrification relative to developed sites with previous exposure to elevated Cl\(^-\) levels.

Studies have demonstrated a notable shift in microbial community structure as a function of Cl\(^-\) exposure (Baldwin et al., 2006). Estuaries provide a natural salinity gradient and are the most common location for researchers interested in the impact of salinity on microbial community structure to conduct their field investigations. Smith et al. (2007) demonstrated that there was a decrease in denitrifier diversity with increasing salinity. In a similar study, Santoro et al. (2006) showed that the highest diversity occurred in the mid range of salinity concentrations in an estuary. These studies suggest that diverse microbial communities can exist in saline environments and that salinity alone is not an accurate indicator of microbial community structure. While these field studies provided an opportunity to assess the microbial diversity in the environment, identifying a specific cause of the differences in community composition is difficult due to the system biogeochemical and hydrologic complexity.

Laboratory experiments conducted by Laverman et al. (2007) and Baldwin et al. (2006) were designed to investigate the impact of salinity on anaerobic metabolic processes and nutrient cycling. Laverman et al. (2007) were unable to determine if there was a significant decrease in denitrification over a three-day period using flow-through-reactors amended with 10% NaCl. Results did show that some processes in the nitrogen cycle had been impacted by the increase in salinity. Specifically, nitrate reduction and nitrite production increased. Release rates for DOC
and the production of ammonium were also stimulated by the NaCl amendment. Baldwin et al. (2006) did not specifically measure denitrification rates but demonstrated that net anaerobic respiration decreased with increasing salinity and was correlated with a shift in microbial community structure.

Prior studies have demonstrated that microbial community structure changes along a salinity gradient and that there may potentially be a change in denitrification rates in response to increasing Cl$^-$ concentrations. However, the impact of these observed changes in microbial community structure along a salinity gradient on denitrification rates has not been determined. Moreover, the majority of the prior studies have occurred in salt-water environments, notably estuaries, where recruitment readily occurs. Studies in freshwater ecosystems are more limited.

**Denitrifiers**

One of the most critical pathways in the nitrogen cycle is denitrification because bioavailable nitrogen is permanently removed from the system as N$_2$(g). Denitrification is carried out under anaerobic conditions by microorganisms. These denitrifiers use nitrate as a terminal electron acceptor in their respiratory process. Microbial community structure, including that for denitrification, has been shown to be influenced by distal controls which act on the community over the long-term and include factors such as carbon substrate, temperature, moisture and oxygen availability, pH, predation, and physical disturbances (Wallenstein et al., 2006). The actual rate at which these communities carry out denitrification is influenced instantaneously by proximal controls, or surrounding environmental conditions. More specifically, oxygen and carbon availability, pH, and nitrate supply are considered the primary drivers for these rates (Wallenstein et al., 2006). Although proximal and distal controls are well-defined, determining if
there is a relationship between the structure and function of microbial communities remains unclear (Philippot and Hallin, 2005).

The link between structure and function has been studied using microbial techniques that target functional genes that are expressed by denitrifying microorganisms (Philippot and Hallin, 2005). Genes that code for nitrite reductase (nir) and nitrous oxide reductase (nos) are the most commonly used (Wallenstein et al., 2006). Denitrifiers are composed of a phylogenetically diverse group of microorganisms. Using the functional genes associated with enzymes that are responsible for denitrification allows researchers to examine the microbial communities that are actually participating in denitrification apart from the diverse microbes that are present in the environment. Community composition and diversity can be analyzed using microbial techniques such as Terminal Restriction Fragment Length Polymorphism (T-RFLP) and Denaturing Gradient Gel Electrophoresis (DGGE; Wallenstein et al., 2006). These represent fingerprinting techniques that provide limited information about the actual identity of microorganisms that are present but allow for observations about structure of community. Both techniques require PCR amplification using primers designed to target the functional genes. Once the DNA is amplified, T-RFLP involves cutting fragments using a restriction enzyme and determining the length of those fragments using electrophoresis. The profile that is generated provides an approximation of genetic variation that is present as well as the relative abundance of those variants. Fingerprints from DGGE are obtained by running the PCR product through a denaturing gel and to generate fragments. Fragments of different base pair length show up as distinct bands on the gel. This provides an additional benefit in that the bands that are observed on the gel can be cut and sequenced to find more detailed information about the phylogenetic structure.
Denitrifiers provide an opportunity to study the relationship between community composition and function since there are specific genes that are expressed in the denitrification pathway and there are measurable products from the respiration process (Wallenstein et al., 2006). Cavagelli and Roberts (2001) determined that the microbial community structure at two sites were significantly different. Soils samples were exposed to varying concentrations of oxygen while other environmental conditions were held constant. Under the same oxygen concentration, the two different soils had different rates of denitrification. This shows that distal controls that shape the community over the long-term are important for determining the response of the community to proximal controls over the short-term. Therefore, knowledge of microbial community structure may play a role in predicting the ability of that community to function under a myriad of environmental stressors.

**Implications for stormwater management**

Stream restoration efforts in urban watersheds are increasingly focused on managing the downstream flux of pollutants. Many of these efforts focus on reconnecting the stream channel with the floodplain and managing stormwater runoff through the use of best management practices (BMPs). Filoso and Palmer (2011) evaluated the effectiveness of stream restoration alternatives on controlling N flux under low and high flow conditions over a three year period. Results from this study and others suggest that restoration efforts that increase hydraulic retention times are best for controlling N flux (Filoso and Palmer, 2011; Kaushal et al., 2008). Stream-wetland complexes are one way to limit spill-over and increase the hydraulic retention time, allowing N to be removed through denitrification. However, simply increasing the hydrologic retention time does not guarantee that N removal will occur. The focus of restoration
efforts has been on improving hydrologic condition of the stream to remove N. However, further studies are necessary to investigate the impact that other pollutants, such as road salt, will have on N removal. Both hydrologic and biogeochemical conditions are important in maintaining an ecosystem's ability to reduce N loading.

**Materials and Methods**

**Study Site**

Sediment slurries were collected from six ponds within the Yale Myers Experimental Forest (YEF) located in Northeastern Connecticut (Figure 1). YEF covers 7,840 acres and is mostly surrounded by large private and state forest. It is mostly characterized as a remote area of the state; however, some of the surrounding area is suburban. The soil in YEF and the surrounding area is classified as glacial till and the region is forested by a mix of hardwoods (YEF, 2009).

The ponds selected for sampling are based on proximity to roadways with three of the ponds located adjacent to a roadway and three ponds located within the forest. Roadside ponds are located within 10 m of the roadways and forested ponds are more than 200 m from the roadway. Each roadside pond is paired with a forested pond so that canopy cover, size, and geography were similar. Water quality data was collected for the ponds in 2008 as part of a previous study (Aragon-Jose et al., 2012).

**Sample Collection**
Sediment and pond water chemical samples were collected on November 3rd, 2011 and January 27th, 2012. The November sampling event occurred the week after a heavy snowstorm during which road salt was applied. During the month of January, all of the ponds were covered with a thick layer of ice necessitating the use of an axe for sample collection. Sediment slurries were collected using a sediment corer to penetrate the layer of roots and leaves on top of the sediment. Three cores were collected from each pond and homogenized in a zip lock back to form a composite sample. Each core contained approximately 2 in of leaf litter and 4 in of sediment. The leaf litter was scraped off and only the sediment was used to form the slurry. Grab samples of the surface water was collected into acid-cleaned (3% HCl v/v) HDPE bottles. The sediment slurries for microbial analysis were frozen at -80°C in the dark within 24 hr of collection until analysis. Slurries for denitrification analysis were collected 96 hr prior to analysis and stored at 4°C in the dark. Grab water samples were filtered (0.45 um) and frozen (-20°C) in the dark until analysis of dissolved organic carbon (DOC), Cl\(^-\), pH, alkalinity and N speciation.

**Mesocosms**

Sediments from all ponds were acclimated for 96 hr across a range of Cl\(^-\) concentrations. Salt solutions for the mesocosms were prepared using NaCl in DI water to Cl\(^-\) concentrations of 0, 500, and 1000 mg/L (January 2012) and 0, 2500, and 5000 mg/L (November 2011). In addition to mesocosms with salt water, a control mesocosms for each pond was prepared with Milli-Q water. Each mesocosm was prepared with 20 g of wet sediment and 40 mL of salt solution. After acclimating for 96 hours, sediment subsamples from each slurry were divided into seven exetainers (~2 g wet wt each), sealed with caps containing rubber septa and flushed with He\(_{(g)}\) for 3 min to purge oxygen. The vials were stored overnight at room temperature to allow
existing nitrate in the sample to denitrify prior to laboratory assessment of the denitrification rates.

Additional mesocosms were performed in parallel to measure changes in water chemistry as a function of Cl\textsuperscript{−}. Sediment and water chemical samples were collected on February 24\textsuperscript{th}, 2012, consistent with prior sampling methods. Pond conditions between the January and February samples are expected to be consistent as the unusually dry winter resulted in no additional precipitation events during this period. Mesocosms were prepared in HDPE bottles by mixing 150 g of wet sediment with 300 mL of filtered pond water. NaCl was added for Cl\textsuperscript{−} concentrations of 0, 500, 1000, 2500, and 5000 mg/L and samples were placed on a shaker table rotating at 60 rpm for 96 hours. The slurries were centrifuged with the supernatant filtered (0.45 um) and analyzed for DOC, Cl\textsuperscript{−}, pH, alkalinity, and N speciation.

**Denitrification Analysis**

Denitrification rates were determined using \textsuperscript{15}N-labeled NO\textsubscript{3}\textsuperscript{−} and measuring the production of \textsuperscript{30}N\textsubscript{2}(g). After sitting overnight to remove existing nitrate, the exetainers were flushed with He\textsubscript{(g)} for 5 min and spiked with 0.1 mL of 550 µM \textsuperscript{15}N-NO\textsubscript{3}\textsuperscript{−} and \textsuperscript{14}N-NH\textsubscript{4}\textsuperscript{+} using a He-flushed syringe. After spiking, sediments were vortexed and allowed to equilibrate for 30 minutes. A continuous flow isotope ratio mass spectrometer (IRMS) was programmed to collect gas samples from the vials at 15 minute intervals. Helium blanks were included at the beginning and end of each run to detect any potential leaks from the vials during the experiment. Anammox and denitrification were determined by the production of \textsuperscript{29}N\textsubscript{2}(g) and \textsuperscript{30}N\textsubscript{2}(g), respectively. The peak area measurements were converted to mass production using air calibration standards. The
moles per gram of sediment were graphed against time and rates were determined from the slope of the linear regression.

**Water chemical analysis**

Filtered water samples from the ponds and parallel Cl⁻ mesocosms were analyzed for DOC, Cl⁻, pH, alkalinity and N speciation (TN, NO₃, ammonia, Organic N) via *Standard Methods* (APHA/AWWA/WEF, 2005). DOC was analyzed via thermal oxidation (Teledyne Tekmar Company, Mason, OH; ASTM, 1994). Cl⁻ and pH were analyzed via ion-selective electrodes (Fisher Scientific accruement probe). Alkalinity was measured via titration to an endpoint of pH 4.3 (Method 2320 B). N speciation was analyzed via Lachat QuikChem 8500 Flow Injection Ion Auto Analyzer by the University of Connecticut Center for Environmental Sciences and Engineering, a state-certified laboratory. Quality assurance was assessed via continuous calibration verification (±10%) and a 10% rate for lab duplicates, field duplicates and matrix spikes. Recovery of spikes and replicate error were within 10%.

**Microbial Analysis:**

**DNA Extraction**

DNA was extracted from 0.25 g of wet sediment using a PowerSoil DNA Isolation Kit (MO BIO Laboratories, Inc.). The kit contains a patented Inhibitor Removal Technology containing a salt reagent for the removal of humic substances, cell debris, and proteins that may inhibit amplification of DNA as well as downstream processes.

**PCR Amplification**
NosZ genes were amplified using the 1F (WCSYTGTTCMTCGACAGCCAG) and 2R (CAKRTGCAKSGCRTGGCAGAA) primers (Henry et al., 2006). The PCR reactions contained 1 uL dNTPs, 2.2 uL of each primer, 0.25 uL Fisher Taq polymerase, and 10 uL template DNA. The amplification conditions were 1 cycle at 94°C for 5 minutes, 35 cycles of 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 1.5 minutes, with a final extension step of 72°C for 10 minutes.

**T-RFLP Analysis**

After verifying successful amplification of the nosZ gene using PCR gel, the products were purified using the Promega Wizard SV Gel and PCR Clean-Up System. DNA concentration was measured using a fluorometer and 20 ng of DNA was used for enzyme digestion using the CfoI restriction enzyme. Following overnight digestions at 36°C, DNA was precipitated out of solution by adding 75% isopropanol and vortexing for 15 minutes at maximum speed. Hi-Di with 500 ROX size standard was added and then 10 uL of the sample was put onto a 96 well plate. The plate was run an Applied Biosystems 3130xl Genetic Analyzer for fragment analysis. Four profiles were generated and did not provide sufficient data to conduct further analysis.

**Results and Discussion**

**Water Chemistry**

Samples collected in November 2011 were higher in DOC than samples collected in January 2012 at all but one sampling site (Table 1). Forested ponds had, on average, a concentration of 10 mg/L in the fall and 5.8 mg/L in winter. One of the roadside ponds, CH, had a higher DOC concentration during the winter sampling event (6.1 mg/L) compared with the fall
sample (8.7 mg/L). The other two roadside ponds had an average DOC concentration of 2.7 mg/L in the fall and 1.2 mg/L in the winter. Higher concentrations are expected during summer and fall months due to increases in primary productivity and allochthonous inputs (Mann and Wetzel, 1995). With the exception of CH, the results presented here are consistent with the expected seasonal trend. CH was the only pond that was not covered with ice during the winter sampling event and the higher DOC concentration might be influenced by runoff, snowmelt, or the warmer pond temperature (Westerhoff and Anning, 2000).

The concentrations of Cl⁻ measured in the roadside ponds were higher than concentrations measured in forested ponds (Table 1). On average the roadside and forest ponds had concentrations of 3608 mg/L and 113 mg/L in November and 4842 mg/L and 159 mg/L in January, respectively. The high concentrations of Cl⁻ measured in November can be explained by the unexpected snowstorm that delivered more than a foot of snow in most parts of Connecticut during the week prior to sampling. The trends are consistent with previous data (Aragon-Jose et al., 2012) which showed concentrations averaging 80 to 300 mg/L in the roadside ponds and 2 to 6 mg/L in forested ponds during spring and summer months. Concentrations are lower in the spring and summer due to an increase in rainfall and termination of road salt application. The roadside ponds have elevated concentrations throughout the entire year. Cl⁻ concentrations can remain elevated during the year due to storage in the watershed and aquifers (Findlay and Kelley, 2011).

NOₓ varied seasonally in both forested and roadside ponds. The concentration of NOₓ in both sets of ponds was higher during the winter sampling event, however; there was not a difference between the roadside and forested ponds. Aragon-Jose et al. (2012) measured higher concentrations of NOₓ in all of the ponds during spring and summer. Despite seasonal variability,
NOx concentrations are consistent between roadside and forested ponds. Typically urban environments have higher concentrations of NOx compared to forested environments (Hale and Groffman, 2006). However, N pollution in urban environments is from point sources such as WWTPs, fertilizer use, atmospheric deposition, or stormwater runoff. In the ponds that were selected for this study, N pollution is limited to atmospheric deposition and stormwater runoff from impervious surfaces. Atmospheric deposition will likely influence the forested ponds in the study area to the same extent that it impacts the roadside ponds since ponds were selected based on similar canopy cover. Runoff from impervious surfaces is limited to the adjacent roadway and does appear to carry high concentrations of NOx into the ponds.

**Denitrification Analysis**

The initial denitrification rates (without Cl− amendment) for the roadside ponds are, on average, lower than the rates for the forested pond (Figure 2). Still, the rates observed for both sets of ponds are similar to those typically observed in freshwater sediments (28 and 24 µmol N/m²/hr in the fall and 53 and 14 µmol N/m²/hr in the winter for forested and roadside ponds, respectively, following conversion to a per area basis assuming an estimated wet bulk density of 2 g/cm³ and 0.5 cm for the thickness of the denitrification zone). Denitrification rates in freshwater lake sediments range from 1 µmol N/m²/hr up to nearly 600 µmol N/m²/hr (Piña-Ochoa and Álvarez-Cobelas, 2005). The lower baseline rate in the roadside ponds relative to the forested ponds (1.38E-03 and 5.15E-03 µmol N/g/hr, respectively) may be due to the changes in water chemistry, specifically, the higher concentrations of Cl− and lower concentrations of DOC. Cl− has been demonstrated to negatively impact denitrification rates in an estuary, with rates measured at the more saline end of a salinity gradient lower relative to those at the freshwater
end (Fear et al., 2005). DOC availability can also limit denitrification (Wallenstein et al., 2006). Fear et al. (2005) also measured lower organic matter at the more saline end of the salinity gradient making a determination of the relative significance on the denitrification rate of Cl\(^-\) and DOC difficult.

Overall, the denitrification rates were more sensitive to increases in Cl\(^-\) concentration for the forested sites decreasing by 69% from 0 to 500 mg/L Cl\(^-\). Conversely, denitrification rates actually increased by 38% in the roadside ponds with exposure to 500 mg/L Cl\(^-\). This stimulatory effect has been observed in a previous study (Marton et al., 2012) where the denitrification rate increased with increasing salinity in one of three tidal freshwater forested wetlands. There was no change in the denitrification rates from the other two tidal freshwater forested wetlands.

In the forested ponds, the rate of denitrification decreased by 83% from baseline to 1000 mg/L Cl\(^-\), yet, increased by 28% in the roadside ponds relative to the baseline conditions. The two highest concentrations of Cl\(^-\) demonstrated a similar trend with lower rates of denitrification in the forested ponds compared to the roadside ponds. At 2500 mg/L of Cl\(^-\) the rate of denitrification dropped by 99% relative to the baseline in the forested ponds. In the roadside ponds, the rate decreased by 90% at 2500 mg/L. At 5000 mg/L the rates decreased by 99% and 94% for the forested and roadside ponds, respectively. Hale and Groffman (2006) showed a similar trend measuring DEA in sediments that were impacted by high Cl\(^-\) concentrations prior to the laboratory incubations and sediments that had not been previously exposed to high Cl\(^-\) concentrations. In sediments that were not exposed to Cl\(^-\) there was a 38% decrease in DEA which is consistent with impacts observed in the current study. However, the findings from Hale and Groffman (2006) showed no change in DEA in sediments incubated with 2500 mg/L of Cl\(^-\) that had been previously exposed to Cl\(^-\) (i.e. the urban/suburban stream). Cl\(^-\) concentrations at the
Hale and Groffman (2006) site have been recorded as high as 4600 mg/L in the winter but were closer to 100 mg/L at the time the sediments were collected in the summer of 2004. Marton et al. (2012) reported no change in denitrification potential after incubating sediments with 2000 and 5000 mg/L salinity, except at one location where the denitrification rates increased. At the time of the Marton et al. (2012) sample collection, river water and pore water were freshwater but there was no historic data to indicate if the sediments had been exposed to Cl$^{-}$ previously. Cl$^{-}$ concentrations were at the observed maximum for the year at the time of sample collection in our study. However, the pore water concentration of Cl$^{-}$ was not measured or accounted for when adding salt water for the mesocosms. Therefore, actual concentrations of Cl$^{-}$ likely were slightly higher than reported. The likely higher Cl$^{-}$ concentrations might explain why the sediments collected from the roadside ponds in this study exhibit a tolerance to Cl$^{-}$ at 500 mg/L and 1000 mg/L but the rates of denitrification decrease in all ponds at 2500 mg/L and 5000 mg/L. The two higher concentrations used here likely exceed the highest values used in previous studies (Marton et al., 2012; Hale and Groffman, 2006) which might explain the discrepancy in the findings. If there is an adaptation due to long-term Cl$^{-}$ exposure in these study sites, perhaps there is also a threshold that was exceeded in the current study.

**Changes in water chemistry**

Changes in water chemistry were determined using separate mesocosms from those used to determine denitrification rate. DOC concentrations were reduced by approximately 50% with the addition of Cl$^{-}$ in both forested and roadside ponds during the 96-hr acclimation. Values for DOC and Cl$^{-}$ concentration in the mesocosms were averaged for each set of three ponds. Initial concentrations of DOC were higher, 14 mg/L, in the forested ponds compared to roadside ponds,
5 mg/L, reflecting the higher background Cl\(^-\) in the roadside ponds. A significant litter mat existed on the bottom of each pond with the majority of the pond edge comprised of forested buffer similar to the forested ponds, suggesting that biotic material inputs were similar for the two sets of ponds. With the addition of 500 mg/L Cl\(^-\), DOC concentrations decreased to 7 mg/L and 3 mg/L in forested and roadside ponds, respectively. Continued increases in Cl\(^-\) concentrations did not further impact DOC concentration (Figure 2), with concentrations approaching 5 and 3 mg/L, respectively, for the forested and roadside ponds.

Alkalinity and pH both decreased as Cl\(^-\) concentration increased. Similar to the pattern observed in DOC concentrations, the largest drop in pH occurred between 0 and 500 mg/L Cl\(^-\) addition. The pH continued to decrease in smaller increments as the Cl\(^-\) concentration increased. Initial pH values for both sets of ponds were 5.5 and decreased to 4.3. Alkalinity was lower in the roadside ponds with a base level of 0.80 meq/L decreasing to 0.19 meq/L with Cl\(^-\) addition. The change in alkalinity was greater in the forested ponds with a base level of 1.17 meq/L and decreased to 0.17 meq/L with Cl\(^-\) addition. The decrease in pH and alkalinity results from the increase in NH\(_4^+\) in the mesocosms as Cl\(^-\) concentrations increase (Figure 3). The addition of Na\(^+\) likely replaces NH\(_4^+\) on the sediments, a result, that is consistent with other studies (Laverman et al., 2007).

The concentration of NO\(_x\) increases from 2 hr to 96 hr at 0 mg/L in all ponds likely due to nitrification. Nitrification could be stimulated by the release of NH\(_4^+\) from the sediments which explains why higher NO\(_x\) concentrations were observed with the addition of 500 mg/L in some sites, corresponding to higher concentrations of NH\(_3\). At 5000 mg/L, the concentration of NH\(_3\) increases from 0.5 mg/L at baseline to 2.5 mg/L in forested ponds. In roadside ponds at 5000 mg/L, the initial concentration is 0.4 mg/L and increases to 1.3 mg/L. However, at 5000 mg/L
there is not an increase in NO$_3$ in the forested ponds but there is an increase in the roadside ponds. Despite the higher concentration of NH$_3$ in the forested ponds, it appears that the rate of nitrification is lower compared to the forested ponds. The results from the N speciation analysis are consistent with the trends observed in the denitrification analysis in that metabolic activity (i.e. nitrification and denitrification) is inhibited to a greater extent in the forested ponds compared to the roadside ponds at elevated concentrations of Cl$^-$.

**Microbial Analysis**

T-RFLP profiles were generated for two of the roadside ponds and two of the forested ponds (Figure 5). T-RFLP profiles could not be generated for the other two ponds as PCR amplification was unsuccessful. There were a greater number of peaks present for the roadside ponds compared with the forested ponds, suggesting that there are more fragments with different numbers of base pairs, likely correlated to different organisms. A greater number of peaks, therefore, suggests that there is more genetic variability in the sample, or more diversity (Blackwood et al., 2002). Generally, microbial diversity is lower under stress since adaptation to the physiochemical stress is the highest priority (Atlas and Bartha, 1998). Since the roadside ponds in this study are exposed to numerous stressors simultaneously, it is likely that adaptations were not limited to a single stressor. Therefore, the community structure may require greater diversity to function in the presence of multiple stressors.

**Factors affecting denitrification**

Our data suggest that Cl$^-$ is the primary mechanism for inhibiting denitrification in pond sediments based on the parameters measured. Correlation matrices (Table 2) for each set of
ponds demonstrates a stronger correlation between denitrification rates and Cl\(^-\) concentration than the other measured parameters. Denitrification rates decreased in all ponds as Cl\(^-\) concentrations increased. Other water chemistry factors such as DOC, pH, and trace metals have been demonstrated to affect denitrification. However, the concentration levels in the two sets of ponds and mesocosms contradict the trends that would be expected if these additional water chemical shifts were impacting denitrification.

DOC concentration decreased by nearly half in both sets of ponds after the addition of 500 mg/L but remained unchanged as higher doses of Cl\(^-\) were added. However, denitrification rates continued to decline in both sets of ponds as the concentration of Cl\(^-\) increased suggesting that the decrease was a function of Cl\(^-\) concentration and not DOC. Additionally, despite the decrease in DOC concentration with the addition of 500 mg/L Cl\(^-\), denitrification rates did not decrease at the majority of the ponds. However, as the Cl\(^-\) concentrations continued to increase, the denitrification rates were increasingly reduced even though DOC concentrations did not decrease further. This continued decrease in denitrification uncorrelated with DOC suggests that while the level of DOC may be impacting denitrification, the rates are more influenced by changes in Cl\(^-\) concentration.

Decreases in pH have been shown to decrease denitrification rates (Baeseman et al., 2006). The optimal pH range for denitrification to occur is between 6 and 8, with inhibition observed below pH 5. In the current study, initial pH values were approximately 5.4 and 5.2 and decreased to around 4.3 and 4.6 for the forested and roadside ponds, respectively, following the 96-hr acclimation period. The pH values decreased in a similar pattern for both sets of ponds, with the largest decrease in pH occurring from 0 to 500 mg/L. These decreases in pH are likely due to the release of NH\(_4^+\) with the addition of Na\(^+\). Since the denitrification rates exhibit
contrasting patterns, especially at the lower concentrations of Cl\(^-\), it appears that the decrease in pH was not as influential as the increase in Cl\(^-\), a fact supported by the continued decrease in denitrification rate above 500 mg/L addition without further decreases in pH.

Historical trace metal concentrations are higher in roadside ponds, potentially due to increased loading or release from sediment with elevated salinity (unpublished results). Increasing concentrations of NaCl further in the mesocosms would result in additional trace metal release from the sediments (Logren, 2001). If metals were responsible for the inhibition of denitrification, there would have been a more significant decrease in denitrification with the addition of Cl\(^-\) to roadside ponds compared to forested ponds where the concentrations of metals are lower. The fact that denitrification rates in roadside ponds do not decrease as much as those in the forested ponds at higher Cl\(^-\) additions again supports Cl\(^-\) as the primary factor impacting N processing.

**Implications for pond N processing**

Microbial analysis suggests that the microbial community structure differs between the roadside and forested ponds and that the community is actually more diverse in the roadside ponds compared with forested ponds. Since greater diversity is associated with greater stability (Atlas and Bartha, 1998), the microbial community structure offers some insight into why the communities responded differently to the Cl\(^-\) treatments. The long-term stressors from roadside disturbance acting on the communities may have created a need for a more diverse community structure. The higher rates in the roadside ponds with Cl\(^-\) addition suggest that microbial communities in the roadside ponds are able to handle the stress of additional Cl\(^-\) better than the forested ponds. Given that the roadside ponds historically have been exposed to elevated
concentrations of Cl\(^-\), we hypothesize that the microbial community may have adapted to the presence of Cl\(^-\).

In comparison to other studies (Hale and Groffman, 2006; Marton et al., 2012), the results generated here support the hypothesis that historic exposure results in microbial communities adapted to Cl\(^-\). The denitrification rates measured in the roadside ponds decreased at the highest concentrations of Cl\(^-\) added (2500 and 5000 mg/L), suggesting that even with historic exposure to Cl\(^-\), a threshold exists beyond which denitrification rates are severely impacted. Given that Cl\(^-\) concentrations have been measured as high as 25,000 mg/L in snow melt, it is likely that there are periods of time when the threshold for developed sites is exceeded. Determining the impacts of road salt on N removal is important for successful stream restoration efforts that are focused on reducing the flux of pollutants downstream. Detention ponds, in particular, can provide a hot spot for N removal. However, their effectiveness should be evaluated in the presence of other pollutants, especially given the likelihood of elevated Cl\(^-\) accumulation due to the hydrologic retention characteristics.

**Summary and Conclusions**

Denitrification rates were inhibited in all six ponds evaluated. At concentrations of Cl\(^-\) up to 1000 mg/L, roadside ponds exhibit a tolerance to the increase in salinity. Denitrification rates decreased at different rates in the roadside and forested ponds. While the roadside ponds exhibited a tolerance to Cl\(^-\) concentrations up to 1000 mg/L, the rates in forested ponds decreased continuously. Beyond 1000 mg/L Cl\(^-\), both sets of ponds demonstrated decreases in denitrification rates. However, the decrease was not as severe for the roadside ponds. Water chemistry trends were similar for the forested and roadside ponds, with decreasing DOC and pH with Cl\(^-\) addition. However, the differences in water chemistry between the ponds suggests that
Cl⁻ is the primary influence on the alteration of N processing. The higher rates in the roadside ponds and higher microbial diversity suggest that the microbial communities have adapted to historical Cl⁻ exposure. However, the decrease in denitrification rate of two orders of magnitude, even for the roadside ponds, suggest that a threshold exists where ecosystem functioning is impacted despite long-term exposure.

The enhanced hydraulic residence time in wetlands allows for N removal through denitrification which reduces the export of N to coastal ecosystems. However, current research suggests that road salt disrupts the ability of natural ecosystems to mitigate N pollution. Urban environments are impacted by changes in water chemistry and hydrology that inhibit ecosystem functioning. The runoff that is generated from impervious surfaces contains pollutants such as metals, nutrients, and road salt. We rely on our natural and engineered wetland areas to assist in the mitigation of such pollutant loading, particularly for nutrients such as N. Our study suggests that there is a need for additional studies to determine the influence of road salt on N removal in urban settings. Questions remain regarding the ability of microbial communities to adapt to increases in salinity. While previously exposed sediments demonstrated a lower impact, significant decreases in denitrification rates still were observed. Determining the threshold at which microbial communities can no longer function and the timing required for adaptation to occur is essential for setting water quality standards that will ensure adequate N removal and protect coastal ecosystems from the detrimental impacts of excess N loading. Engineering stormwater designs must account for the alteration in rates. Additionally, the impacts of road salt loading to N processing may negatively impact roadside wetland areas, particularly as development pressure increases.
References


Table 1 Average water quality parameters measured for roadside and forested ponds in November and January. These concentrations show general trends in water quality between the two sets of ponds and between seasons. All samples were filtered prior to analysis. DOC – dissolved organic carbon; Cl\textsuperscript{−} - chloride; TN – total nitrogen (N); NO\textsubscript{x} – total nitrate plus nitrite; NH\textsubscript{3} – total ammonia species; Org N – organic N (calculated by difference). All concentrations are mg/L.

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Table 2 Correlation matrices for roadside and forested ponds testing the correlation of denitrification rates against the different parameters measured. For denitrification rates, Cl-concentration has the highest correlation

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<th>DOC</th>
<th>Denitrification</th>
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<th>Denitrification</th>
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**Figures Captions**

**Figure 1:** Map of Yale Meyers Experimental Forest showing the approximate geographic region in which the six ponds used in this study are located. The study utilized three of the five pairs from a longer-term study.

**Figure 2:** Average denitrification rates for forested and roadside ponds in Fall and Winter as a function of Cl$^-\$ concentration. Denitrification rates were more stable in roadside ponds at lower concentrations of Cl$^-\$. At high concentrations of Cl$^-\$, denitrification rates are severely inhibited for all ponds. Denitrification rates measured using the isotopic labeling method followed by measurement of $^{30}$N$_2(g)$ during the subsequent treatment.

**Figure 3:** Nitrogen (N) speciation in filtered samples for roadside and forest ponds after 96-hr acclimation. Sediments and pond water used for the mesocosms were collected in February 2012. Total NH$_3$ increases as Cl$^-\$ increases which initially stimulates the production of NO$_x$ through nitrification. However, the microbial process becomes limited at 5000 mg/L.

**Figure 4:** Dissolved organic carbon (DOC) concentration from filtered 96-hr incubations relative to Cl$^-\$ concentration. DOC decreases after the first addition of Cl$^-\$, 500 mg/L, but then reaches an asymptotic value for both ponds.

**Figure 5:** T-RFLP Profiles for roadside and ponds showing the difference in microbial community structure between the roadside ponds and forested ponds. Roadside ponds: A.) Cavar B.) ECem. Forested ponds: C.) BS D.) DON
Figure 2

-5.00
-4.50
-4.00
-3.50
-3.00
-2.50
-2.00
-1.50
-1.00
-0.50
0 1000 2000 3000 4000 5000 6000

Added Cl⁻ [mg/L]

-5.00
-4.50
-4.00
-3.50
-3.00
-2.50
-2.00
-1.50
-1.00
-0.50
0 1000 2000 3000 4000 5000 6000

Denitrification rate [log₁₀(µM N/g/hr)]

- Forest (Winter)
- Forest (Fall)
- Roadside (Winter)
- Roadside (Fall)
Figure 3
Figure 4