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FACTORS CONTROLLING MICROBIAL NITROGEN REMOVAL EFFICACY IN
CONSTRUCTED STORMWATER WETLANDS

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Abstract

Agricultural runoff and precipitation have contributed to increased nitrogen loading in freshwater ecosystems. Stormwater wetlands have been constructed and used to reduce excess nitrogen loading to rivers and estuaries. However, the microbial processes, denitrification and anammox, involved in N removal have not been fully examined in constructed wetlands. In order to assess the efficacy and factors influencing microbial nitrogen removal in stormwater wetlands, molecular and stable isotope analyses of anammox and denitrifying communities were conducted with the sediment samples collected from the large regional JEL Wade wetland in Wilmington, NC. Water quality parameters were measured with surface water samples, while the abundance and activity of ANAMMOX and denitrifying communities were measured using quantitative PCR and ^{15}N tracer incubation experiments, respectively. ANAMMOX and denitrifying communities in bare and rhizospheric sediments were characterized from the samples collected in the summer and fall of 2011 as well as winter of 2012. Denitrification was found to be the dominant N removal pathway of this system, contributing up to 71% of the N_2 production in the bare sediments and 78% in the rhizosphere. The activity and abundance of both ANAMMOX and denitrification were found to be higher in the rhizosphere compared to bare sediments. Rhizospheric denitrification and ANAMMOX activities were much higher in the summer than in the fall. These results demonstrate (and add to previous insights) that wetland vegetation plays a major role in N-removal from incoming stormwater. Furthermore, following wetland construction specific wetland species can be emphasized in plantings in order to maximize N-removal, with *Pontederia cordata* a clear choice. Additionally, this research shows that locally invasive species such as cattail *Typha angustifolia*, alligatorweed *Alternanthera philoxeroides* and soft rush *Juncus effuses* can play a significant role in N-removal as well. This research also indicates that constructed stormwater wetlands in warm climates (with a long growing season) may be particularly effective in enhancing denitrification.

Acknowledgements

1.0. Introduction

1.1. Constructed Wetlands and Nutrient Removal

Nitrogen (N) is considered the principal nutrient of concern in estuarine systems (NRC 2000) as well as in the abundant blackwater fluvial systems that characterize much of the U.S. southeastern Coastal Plain (Mallin et al. 2004a). Some of North Carolina's largest fluvial ecosystems (Neuse, Pamlico, and New Rivers) are considered nutrient-sensitive waters with N loading as a primary concern. Eutrophication in response to N loading is recognized nationally as the single largest threat to the integrity of coastal ecosystems, and stormwater runoff is considered the primary source of impairment to 32% of all U.S. estuaries (NRC 2009). One major source of N loading to streams, rivers, lakes and estuaries is stormwater runoff. Stormwater runoff is generally considered to be non-point source runoff draining urban and suburban areas.

In order to reduce runoff and remove pollutants such as suspended solids, nutrients and fecal bacteria from stormwater runoff, constructed stormwater wetlands (CSWs) are gaining popularity as cost effective passive attenuation systems (Lee et al., 2009). A constructed wetland is an engineered system designed for natural pollutant attenuation by wetland vegetation, sediments, and microbial processes. Within wetlands, various physical, chemical and biological processes are involved in the removal of pollutants and nutrients such as N.   Although volatilization, sedimentation, chemical absorption and plant uptake are involved in N removal in wetlands (Weisner et al. 1994; Woltemade 2000), sedimentary microbial processes are major pathways of removing N in the ecosystems (Lee et al., 2009; Vymazal, 2007).   Organic N is decomposed by microbes to NH_4^+ (ammonification), which can be taken up by primary producers, or converted to NO_2^- and then NO_3^- by nitrification. Nitrate can be assimilated by plants and bacteria, or dissimilated in denitrification, anaerobic ammonium oxidation (ANAMMOX) and dissimilatory NO_3^- reduction to NH_4^+ (DNRA) depending on the prevailing conditions. Among these processes, only denitrification and ANAMMOX remove fixed N from the environment. Denitrification typically removes fixed N by reducing NO_3^- to N_2 or N_2O while consuming available organic carbon. The N gases produced during denitrification are emitted to the atmosphere. In most wetland sediments, sufficient reducing power exists to result in the production of N_2 as the dominant terminal denitrification end product.   Denitrification rates are influenced by labile organic carbon and nitrate availability, both of which can vary spatially through the sediment profile (Tobias et al. 2001; Cornwell et al. 1999). Direct denitrification (fueled by water column NO_3^-) and 'coupled denitrification' (fueled by NO_3^- produced by nitrification) constitute major pathways for N removal. High rates of coupled denitrification often accompany high mineralization rates, if sufficient oxygen is available for nitrification (Seitzinger and Giblin 1996; Seitzinger 1994). Increased NO_3^- loading to aquatic ecosystems (i.e. increased NO_3^- concentration) influences total denitrification (direct plus coupled) rates, and the partitioning between direct and coupled pathways respond primarily to changes in water column NO_3^- (Nedwell et al. 1999; Seitzinger et al., 2006). It has been estimated that denitrification removed fixed N up to 60 to 95% while the uptake by algae and wetland plants ranged from 1 to 34% in constructed wetlands (Lee et al., 2009). High concentrations of nitrate in the inflow are thought to lead to higher denitrification within the inlet

sediments (Sirivedhin and Gray, 2006). In addition, emergent plant species have important roles of providing labile organic carbon to support denitrification in wetland sediments (Bastviken et al., 2005).

ANAMMOX is a recently identified microbial process involved in N removal by producing N_2 while oxidizing NH_4^+ coupled to NO_2^- reduction under anoxic conditions. ANAMMOX has shown to be a key N removal pathway in marine sediments (e.g., Thamdrup and Dalsgaard 2002; Risgaard-Petersen et al. 2004a; Engström et al. 2005; Hietanen and Kuparinen 2008; Rich et al., 2008; Dale et al., 2009) and suboxic or anoxic marine water columns (e.g., Kuypers et al. 2003; Kuypers et al. 2005; Thamdrup et al. 2006; Hamersley et al. 2007; Galán et al., 2009). ANAMMOX contributions to total N_2 production can be highly significant, varying in different ecosystems and accounting for 24-67% of the total N_2 production in coastal and continental shelf sediments (Thamdrup and Dalsgaard, 2002). Erler et al (2008) reported that ANAMMOX accounted for 24% of N removal in a surface flow constructed wetland and proposed oxygen as a key regulator for ANAMMOX rates. Sediment mineralization showed a positive correlation to ANAMMOX significance by supplying NO_2^- from heterotrophic denitrification (Trimmer et al., 2003). Meyer et al (2005) also reported strong correlations between ANAMMOX rates and the production of NO_2^- by nitrification and denitrification in the Logan and Albert River sediments. The availability of $NO_3^- + NO_2^-$ (NO_x^-) in the suboxic zone of sediments may be a key controlling factor for ANAMMOX in constructed wetlands. In addition, wetland vegetation has been suggested to have an important role of ANAMMOX as shown in a mesocosm study of Tao and Wang (2009). Thus, ANAMMOX and denitrifying bacteria in constructed wetlands appear to have either a mutualistic or competitive relationship for available NO_x^- substrates, as well as being supported by different wetland vegetation types/species. In addition, wetland vegetation may enhance ANAMMOX and denitrification by supplying substrates and niches to both bacterial communities. Particle size of sediment could also influence microbial N removal processes since higher denitrification has been measured with fine textured soils with high contents of silt and clay (Pinay et al., 2000). With the crucial importance of N as a pollutant and major component of stormwater runoff, determining the environmental parameters controlling ANAMMOX and denitrification in constructed wetland sediments is imperative to enhancing its removal. Such information can lead to optimizing the performance of N removal from constructed wetlands and enhancing protection of the waterways receiving outflows from the engineered ecosystems, in the most cost-effective manner.

1.2. Objectives

1) Quantify the seasonal N removal rates via denitrification and ANAMMOX in a large CSW system using ^{15}N stable isotope techniques. 2) Determine spatial variation of N removal capacity within the test CSW system and define areas of optimal N removal. 3) Examine shifts in denitrification and ANAMMOX rates in response to observed changes in meteorological, physical, chemical and microbial parameters as well as vegetation type and coverage.

1.3. Hypotheses Tested

1) Increased water temperatures will lead to increased denitrification rates within CSWs, while lower temperatures may favor ANAMMOX enhancement; 2) Plant rhizospheric material will enhance denitrification and ANAMMOX rates relative to unvegetated sediments; 3) Sediments comprised of smaller grain size will enhance both denitrification and ANAMMOX rates 4) Areas containing emergent macrophyte vegetation will produce greater denitrification and ANAMMOX rates than areas containing submersed, loosely attached or free-floating macrophytes; 5) Abundance and community structures of ANAMMOX and denitrifying bacteria will influence spatial and temporal variation of their rates in SCW sediments.

1.4. Site Description

The study site was the large regional JEL Wade wetland (Mallin et al. 2012), located in New Hanover County, southeastern North Carolina (Fig. 1). This wetland was constructed in 2007 after excavating soils. Since the construction, the wetland has treated about 9% of the stormwater runoff entering estuarine Hewletts Creek. This tidal creek has been determined experimentally to be a principally N-limited system that hosts significant algal blooms (Mallin et al. 2004b). Hewletts Creek is classified by the North Carolina Department of Environment and Natural Resources as SA, HQW (high-quality waters) yet is impaired and closed to shellfishing. This constructed wetland drains and treats runoff from a watershed of approximately 589 acres (238 ha) consisting primarily of suburban development. The wetland facility covers an area of approximately 11.5 acres (4.7 ha) consisting of 5.7 acres (2.3 ha) of wetland, 1.9 acres (0.77 ha) of open water and 3.4 acres (1.4 ha) of uplands (Fig. 2). The wetland was designed to treat the first inch of rainfall from the drainage basin (Dewberry & Davis, Inc. 2008).

Figure 1. Sediment and water sampling sites at the JEL Wade constructed stormwater wetland in Wilmington, N.C. The GPS coordinates for the inflow area (FB1) are N34.1781, W77.8805.

Stormwater runoff enters the facility at two points, designated within as Forebay1 (FB1) and Forebay 2 (FB2 - Fig. 1). FB1 is located at the southwest corner of the site and consists of a double concrete box culvert that accepts 100% of the inflowing water from its drainage through to the wetland. The FB2 provides inflow to the wetland through a lateral inflow diversion structure. This structure diverts low flows into the wetland through a pair of 24 inch pipes, which are equipped with backflow prevention devices. Water and suspended soils entering the wetland from both channels is directed into forebays; as it passes through the wetland there are two concrete weirs designed to drop the water level 6 inches (15 cm). Flashboard risers within the weirs are designed to slowly draw down the water surface into the next segment (Dewberry & Davis, Inc. 2008). The wetland is designed to hold water continuously, with two main channels meandering through the system at low flow rising to extensive water coverage at elevated flows (Fig. 1). The wetland is designed to contain and convey events up to the 100 years, 24 hr flood without overtopping the embankment surrounding the system. Water exits the wetland through a primary riser outlet structure and enters an outflow channel that leads to Hewletts Creek. The inflowing suspended soils are accumulated in the wetland system. A broad variety of herbaceous and woody wetland plants were planted in 2007, including emergent, submersed, and shoreline species.

A storm-event study found that the JEL Wade wetland was very effective in reducing pollutant concentrations and loads in the stormwater entering the CSW (Mallin et al. 2012). In terms of N-removal, average nitrate concentrations and loads were reduced by 46-59% and 83%, respectively, average ammonium concentrations and load by 70-81% and 92% respectively, and average total nitrogen concentrations and loads by 39-56% and 85%, respectively (all load reductions were statistically significant at $p < 0.05$). An intriguing pattern in nitrate removal emerged in which nitrate load reduction was strongly correlated ($r^2 = 0.71$, $p < 0.001$) with increasing water temperature for the January-June period. Thus, seasonality and/or temperature were expected to be a major factor controlling denitrification and ANAMMOX rates in the wetland.

2.0. Methods

2.1. Wetland Surveys

The wetland was first surveyed to obtain depth estimates (under non-rain conditions). Visual estimates were made of areas containing vegetation coverage, with species comprising > 20% of coverage targeted for sampling. Bare areas were likewise mapped for potential sampling sites and the GPS coordinates of sampling sites were recorded. Fourteen sites representing the entire wetland were chosen for a preliminary nutrient survey that was performed in May 2011. Based on the nitrate concentrations obtained, denitrification and ANAMMOX sampling was concentrated within the two forebays (FB1 and FB2) of the wetland (Fig. 1). Each forebay was divided into four transects labeled sequentially from the inflow points as A, B. C and D. Based on coverage, seven rooted and submersed macrophytes were chosen for rhizospheric sampling (Fig. 2). These species were cattail *Typha angustifolia* L., giant cutgrass *Zizaniopsis miliacea*

(Michx.) Doell & Aschers., Eastern bur-reed *Sparganium americanum* Nutt., pickerelweed *Pontederia cordata* L., alligatorweed *Alternanthera philoxeroides* Griseb., soft rush *Juncus effuses* L. and parrot feather *Myriophyllum aquaticum* (Vell.) Verdc.

2.2. Field Sampling

Surface sediment core samples (0-3 cm) were collected and split into two parts. The samples for DNA extraction were collected from the core sampler and stored in liquid nitrogen until transferred to the laboratory, and then stored at -80 ℃ until DNA was extracted. The sediments for ^{15}N incubation experiments were stored in sterile glass jars at 4 ℃ until transferred to the laboratory. ANAMMOX and denitrification activities were measured immediately as described below. Plant rhizospheric material was sampled by hand. Samples were obtained from monospecific stands of selected species by pulling up the material by the roots (Fig. 2), and cutting away the rhizospheric material from the plant body. Samples were stored in labeled plastic bags and stored on ice until returned to the laboratory. Sediments attached to root materials were suspended in site water and the sediments were collected by centrifugation.

Figure 2. Left: Plants in Forebay 1 including *Sparganium* (back center), *Typha* (back right), *Juncus* (left), *Pontederia* (front center) and *Alternanthera* (submersed). Right: Sampling macrophyte rhizosphere material.

Plant rhizospheric material was sampled in June, August and October 2011. Unvegetated sediments were sampled in August and October 2011, and limited sediment samples were collected in February 2012. In August, samples for sediment grain size analysis were also collected (surface cores, 3 cm). On each sampling occasion water temperature, conductivity, pH, dissolved oxygen (DO), and turbidity were measured in the overlying water column using a YSI 6920 Multiparameter Water Quality Probe (sonde) linked to a YSI 650 MDS display unit.

2.3. Measuring ANAMMOX and Denitrification Rates

Sediment slurry incubation experiments with ^{15}N tracer were conducted to measure potential rates of denitrification and ANAMMOX using a modified method of Dale et al. (2009). Eight sediment slurries containing one gram of homogenized sediment and porewater were pre-incubated in helium purged Exetainer tubes (Labco Limited, High Wycombe, Buckinghamshire, England) in the dark overnight to remove residual NO_x^-. After pre-incubation, two of the

Exetainers were sacrificed to measure residual NO_x^- in sediment porewater via Vanadium reduction and chemiluminescence detection (Braman and Hendrix1989). The residual concentration of NO_x^- was used to correct the mole fraction ^{15}N enrichment of the added $^{15}NO_3^-$ (Song and Tobias, 2011) in subsequent rate calculations for ANAMMOX and denitrification. Remaining Exetainer tubes with sediment slurries were again purged with helium, amended with 200 nmoles $^{15}NO_3^-$ and 200 nmoles $^{14}NH_4^+$ and placed in the dark during incubations. Time series incubations (0, 1 and 2 hr) were carried out in duplicates and the activities stopped by the addition of saturated $ZnCl_2$. Production of $^{29}N_2$, and $^{30}N_2$ was measured on an Isotopic Ratio Mass Spectrometer (Delta V Plus, Thermo Fisher Scientific, Waltham, MA) and used to calculate the rate of ANAMMOX and denitrification following the method of Thamdrup and Dalsgaard (2002) as modified by Song and Tobias (2011). Percent ANAMMOX was estimated based on the rates of ANAMMOX and total N_2 production in each sample.

2.4. Molecular Characterization of Denitrifying Communities

Sediment DNA was extracted using PowerSoil DNA Kit (Mo-Bio Laboratories, Inc., Carlsbad, CA) following the manufacture's protocol with two modifications, 1) the amount of wet sediment was increased to 0.6 g and 2) Thermo Savant Fast Prep FP 120 Cell Disrupter (Qbiogene Inc. Carlsbad, CA) was used for cell disruption. Denitrifying bacterial communities in the sediment samples were examined by targeting the nitrous oxide reductase (*nosZ*) genes. Terminal Restriction Fragment Length Polymorphism (T-RFLP) of *nosZ* genes was conducted to compare denitrifying community structures in different sediment samples using the primers of Henrey et al (2006). The amplicons were purified using the Wizard SV Gel and PCR Clean-Up System (Promega, Fitchburg WI). DNA concentration was measured using the Quant-iT ds DNA Assay Kit (Life Technologies, Carlsbad, CA). A total of 20 ng of PCR products was digested overnight at 37 ° C with 5 units of *Cfo1* restriction endonuclease (Promega, Fitchburg WI). The digested products were precipitated with isopropenol and run on a 3130x/ Genetic Analyzer (Life Technologies, Carlsbad CA). Fragment analysis was conducted using the Gene Mapper 4.0 (Life Technologies, Carlsbad CA) and T-RFLP Analysis Expedited (T-REX) programs (Culman et al., 2009). Variations in *nosZ* gene fingerprints were assessed using a Bray-Curtis similarity matrix and cluster analysis in the Primer-5 software package (Primer-E Ltd, Lutton, UK). Q-PCR of *nosZ* genes was also conducted to measure the abundance of denitrifying bacteria following the method of Henry et al (2006).

2.5. Water Column Nutrient Analysis

Surface water samples were collected on site for nutrient analysis. A selection of sites throughout the wetland was sampled in May 2011 for background information. On subsequent visits nutrient samples were collected at the locations where sediment and plant material were collected, primarily in the two forebays. Nutrients (Total nitrogen, nitrate+nitrite, total phosphorus, and orthophosphate were measured using a Bran-Luebbe continuous Flow Autoanalyzer III using EPA techniques. Ammonium was measured using an Ammonia Selective Electrode, Thermo Scientific Electrode on an Orion 4 Star Benchtop meter.

2.6. Sediment Grain Size Analysis

Sediment grain size analysis was performed for the August 2011 sampling. Sediments (top 3 cm) collected in cores on-site were digested to remove organics using 30% Hydrogen Peroxide (H_2O_2). Analyses were performed using a Beckman Coulter Counter.

2.7. Statistical Analysis

Data for denitrification and ANAMMOX rates, nutrient concentrations, water temperature, pH, dissolved oxygen, turbidity and sediment grain size were tested for normality using the Shapiro-Wilk test. Non-parametric data were log transformed. These data were explored using correlation analyses to assess environmental factors influencing N loss rates. Denitrification and ANAMMOX rates, for both plant rhizospheric material and bare sediments, were compared by month using analysis of variance (ANOVA); differences between sampling stations were also tested. Denitrification and ANAMMOX rates comparing individual plant species were also tested using ANOVA. Denitrification and ANAMMOX rates for rhizospheric material were tested against bare sediment rates using t-tests. Statistical analyses were performed using SAS (Schlotzhauer and Littell 1997) with the significance level set at $\alpha = 0.05$.

3.0. Results

3.1. Nitrate measurements in different parts of the JEL Wade wetland

Nitrate concentrations in surface water of different parts of the wetland system (Fig. 3) were measured in in May of 2011. Sedimentary Forebay 1 (FB1) received much lower levels of nitrate/nitrite than Forebay 2 (FB2) (Fig. 3A and 3B), possibly due to the long vegetated ditch delivering stormwater to the wetland at FB1. Nitrate/nitrite removal efficiency was found to be 70% in FB1 while FB2 removed 46% of nitrate that entered. Less than 3 ppb of nitrate/nitrite was released as effluent from this wetland system (Fig. 3C).
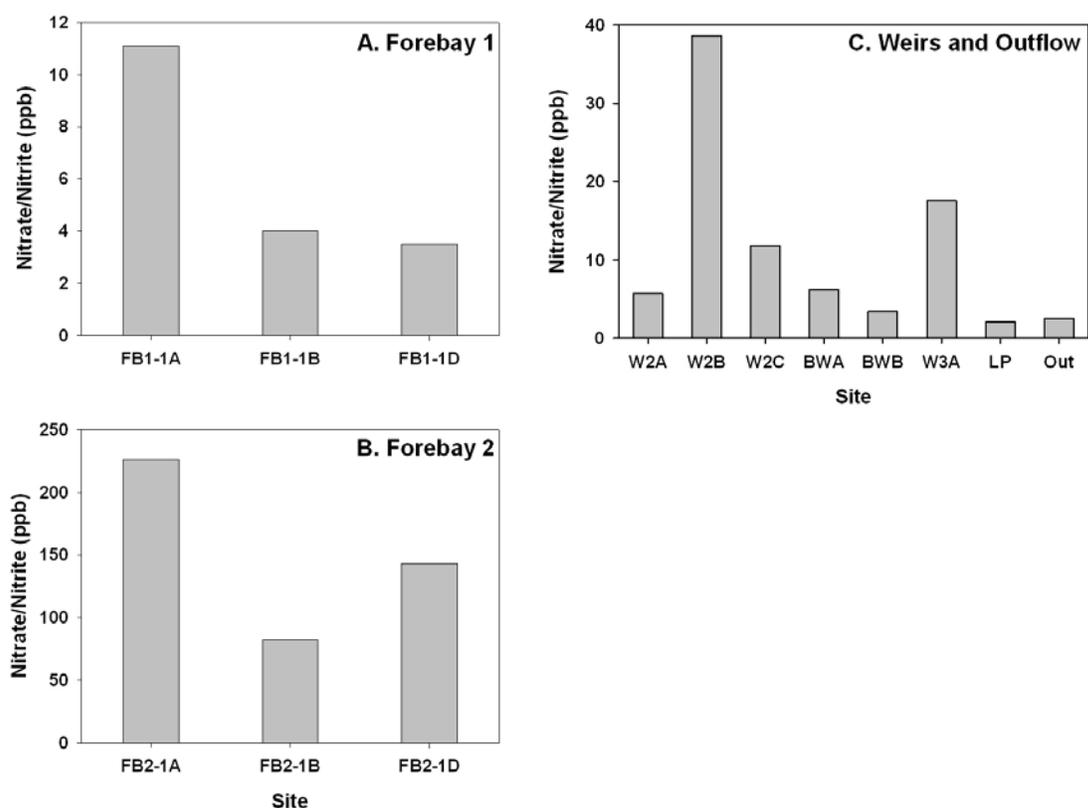
Figure 3. May 2011 Nitrate reduction through Forebay 1 (A), Forebay 2 (B) and beyond the forebays through the weirs and at the outfall (C).

3.2. Seasonal comparison of ANAMMOX and denitrification activities in wetland sediments

ANAMMOX and denitrification rates were measured in the unvegetated sediments and rhizospheric materials collected in summer of 2011 (June and August), fall (October), and winter of 2012 (February). Denitrification was found to be the dominant N_2 producing pathway in all of the samples examined (Fig. 4). ANAMMOX contributed up to 29% and 26 % of N_2 production in wetland sediments and rhizopheric sediments, respectively. Both denitrification and ANAMMOX were much more active in rhizospheric sediments than the bare sediments (Fig. 4). Seasonal variation in both microbial processes was also observed in rhizospheric and wetland sediments. Higher activities of ANAMMOX and denitrification in wetland sediments were measured in winter while rhizospheric sediments were more active in summer (Table 1).
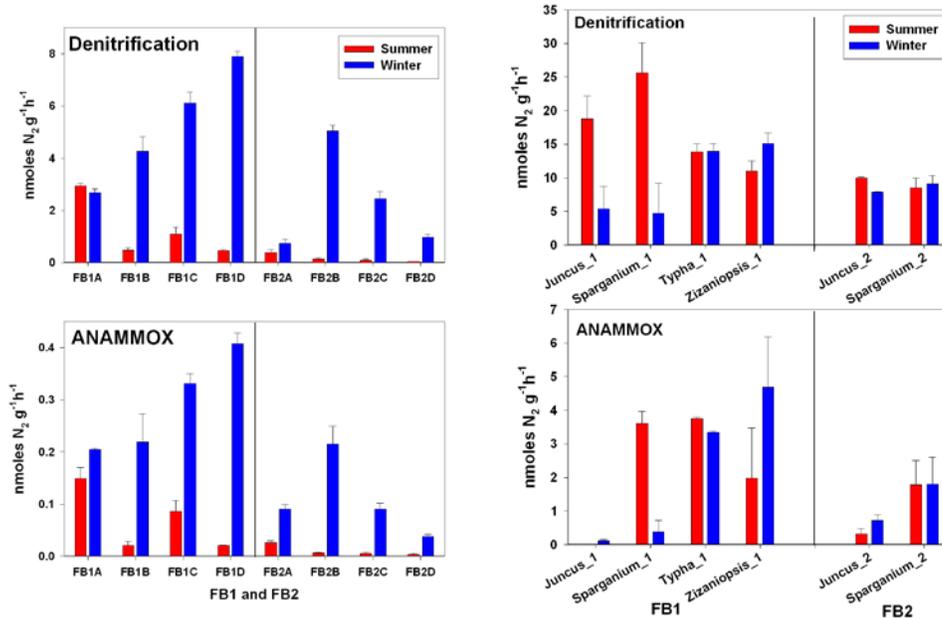
Fig. 4. Seasonal comparison of ANAMMOX and denitrification measured in bare sediments (left) and macrophyte rhizospheres (right).

N-loss rates were compared by month to assess seasonal influence, for both rhizospheric material and unvegetated sediments. There were monthly differences, but these followed different patterns for plant rhizosphere material and unvegetated sediments (Table 1). For plant rhizospheric material, August denitrification rates were significantly greater than October rates, while August rates were not different from June rates. There were no significant monthly differences in ANAMMOX rates between months for rhizospheric material, however.

An opposite pattern was displayed by N-loss rates from unvegetated sediments. For denitrification, both cool months (February and October) showed significantly greater rates than August. Monthly ANAMMOX rates, while far lower than denitrification rates, also showed significantly greater rates in February and October compared to August (Table 1). This indicates that wetland plants support both denitrification and ANAMMOX in rhizospheric sediments.

Table 1. Comparison of N-loss rates by month (data as nmol N/g sed. wet wt./hr)

Plant Rhizospheric Material

Denitrification		ANAMMOX	
August	16.68 + 8.37	June	2.37 + 1.76
June	16.01 + 8.28	August	1.93 + 1.90
October	8.88 + 4.16	October	1.67 + 1.73
August > October (p = 0.0001)		no significant difference	

Unvegetated Sediments

Denitrification		ANAMMOX	
February	$4.57 + 0.33$	February	$0.65 + 0.63$
October	$3.77 + 2.52$	October	$0.20 + 0.12$
August	$0.67 + 1.08$	August	$0.04 + 0.07$
February, October > August ($p < 0.0001$)		February, October > August ($p = 0.0004$)	

3.3. Comparison of Vegetated versus Unvegetated Sediments

N-loss rates from rhizospheric material were tested against unvegetated sediments for all data pooled. Mean denitrification rates for rhizospheric material (14.41 ± 7.95 nmol N/g sed. wet wt./hr) were approximately seven-fold greater than those of bare sediments (2.15 ± 2.35 nmol N/g sed. wet wt./hr); this difference was highly significant ($p < 0.0001$). Mean ANAMMOX rates for rhizospheric material (2.03 ± 1.76 nmol N/g sed. wet wt./hr) were likewise much greater than rates from unvegetated sediments (0.15 ± 0.24 nmol N/g sed. wet wt./hr); again this difference was highly significant ($p < 0.0001$).

Overall potential N removal capacity in FB1 and FB2 sediments was estimated based on the rate measurements in summer and winter (Fig. 4). Rhizospheric sediments in both FB1 and FB2 are capable of producing 2.1 to 4.5 mmoles N_2 m^{-2} d^{-1} while unvegetated wetland sediments had potential activities of 0.04 to 1.3 mmoles N_2 $m^{-2}d^{-1}$. Higher N removal capacity in both FB1 and FB2 was measured in summer when wetland plants were actively growing (Table 1; Fig. 5).
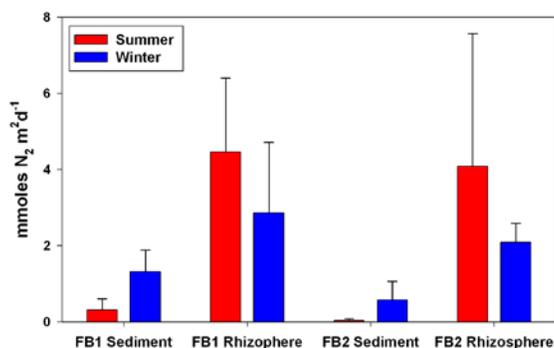
Figure 5. Seasonal comparison of daily N-removal between bare sediments and rhizospheres.

3.4. Comparison of abundance and structure of denitrifying microbial communities

Molecular analysis supports the results of the rate measurements. Higher abundance of denitrifying bacteria was also measured in rhizospheric sediments based on quantitative PCR of *nosZ* genes (Fig. 6). We also conducted denitrifying community analysis in rhizospheric and wetland sediments using T-RFLP of *nosZ* genes.
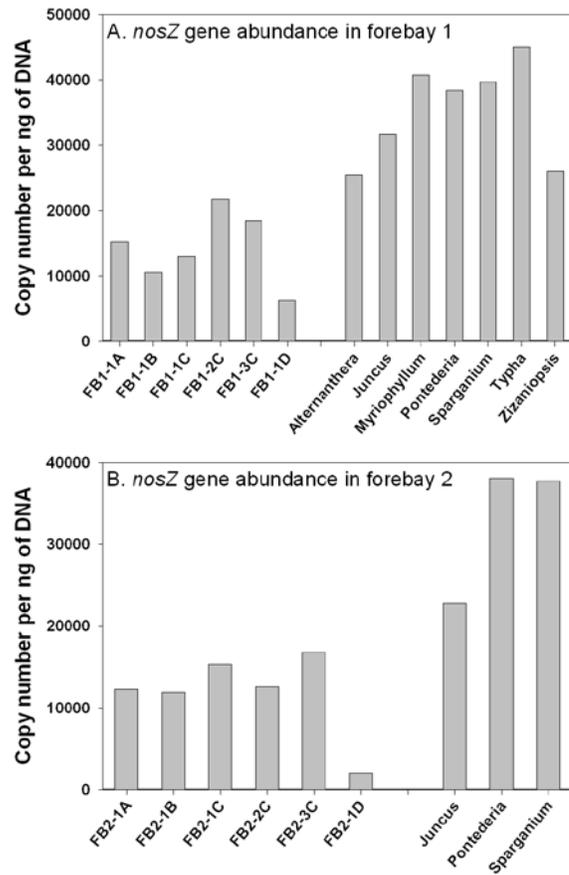
Figure 6. Abundance of microbial denitrifying communities in bare sediments and rhizospheric sediments measured by quantitative PCR of *nosZ* genes.

Cluster analysis based on the *nosZ* gene T-RFLP fingerprints showed that denitrifying communities in rhizospheric sediments were quite different from those in bare sediments (Fig. 7). Thus, both molecular and stable isotope analyses of sediment communities support that denitrifiers in rhizospheric sediments are more efficient in removing fixed N from the JEL wetland ecosystem.
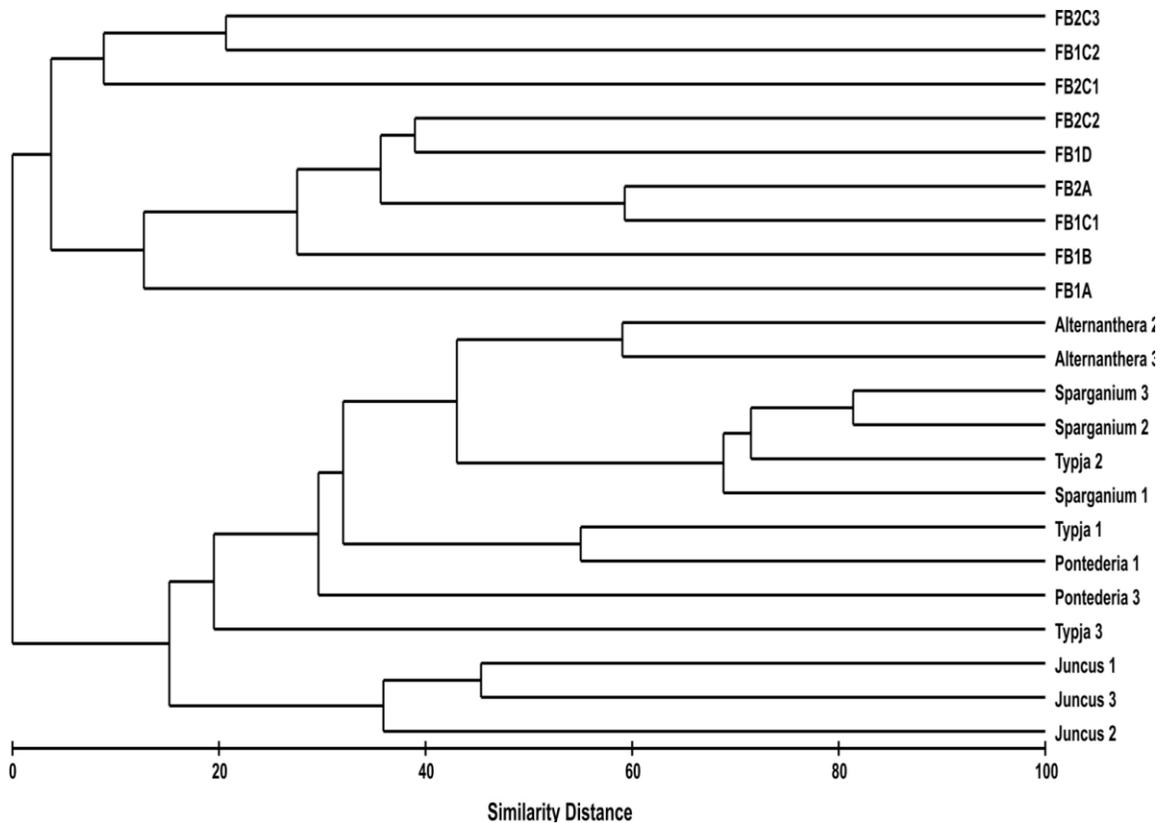
Figure 7. Cluster analysis of denitrifying communities based on *nosZ* gene T-RFLP fingerprints.

3.5. Environmental Factors Influencing N-Loss Rates

Correlation analyses were performed to help assess what environmental factors influenced denitrification and ANAMMOX rates. For all data combined (Table 2) water temperature was positively correlated with denitrification, whereas there was no significant relationship between temperature and ANAMMOX. Dissolved oxygen was negatively correlated with denitrification; however dissolved oxygen was highly negatively correlated with water temperature (i.e. DO was lower in summer than winter). Thus, the DO influence on denitrification may have been a seasonal, temperature-driven effect instead. Water-column nitrate and orthophosphate were both negatively correlated with denitrification and ANAMMOX rates. Nitrate was negatively correlated with water temperature, but orthophosphate was positively correlated with temperature.

Table 2. Results of correlation analysis of physical and chemical factors potentially influencing N-loss rates for all data combined, presented as Pearson correlation coefficient (r) / probability (p).

	Denitrification	ANAMMOX	water temperature
Water temperature	0.33 0.019	ns	1.000
Dissolved oxygen	-0.29 0.043	ns	-0.934 <0.001
Nitrate	-0.35 0.013	-0.335 0.021	-0.397 0.005
Orthophosphate	-0.29 0.041	-0.424 0.003	ns
Total phosphorus	ns	-0.397 0.006	0.398 0.005

When considering the plant rhizospheric samples alone, denitrification was positively correlated with water temperature and negatively correlated with DO (Table 3). Again, DO was strongly negatively correlated with water temperature. ANAMMOX rates were not correlated with any physical factors. Water-column nutrient concentrations were not significantly correlated with either N-loss factor for the rhizospheric samples.

Table 3. Results of correlation analysis of physical and chemical factors potentially influencing N-loss rates for plant rhizospheric samples, presented as Pearson correlation coefficient (r) / probability (p).

	Denitrification	ANAMMOX	water temperature
Water temperature	0.399 0.040	ns	1.000
Dissolved oxygen	-0.357 0.068	ns	-0.978 <0.001

The unvegetated sediment samples showed a very different pattern than plant rhizospheric material in terms of factors influencing N-loss rates (Table 4). Both denitrification and

ANAMMOX were strongly negatively correlated with water temperature and positively correlated with DO. Several nutrient species (TN, TP and orthophosphate) were negatively related to both denitrification and ANAMMOX. Orthophosphate and TP were both positively correlated with water temperature.

Table 4. Results of correlation analysis of physical and chemical factors potentially influencing N-loss rates for unvegetated sediment samples, presented as Pearson correlation coefficient (r) / probability (p).

	Denitrification	ANAMMOX	Water temperature
Water temperature	-0.704 <0.001	-0.739 <0.001	1.000
Dissolved oxygen	0.538 0.001	0.519 0.013	-0.903 <0.001
Total nitrogen	-0.497 0.019	-0.554 0.007	ns
Orthophosphate	-0.507 0.016	-0.680 0.001	0.557 0.007
Total phosphorus	-0.790 <0.001	-0.770 <0.001	0.640 0.001

3.6. Sediment Grain Size and N-Loss Rates

Denitrification and ANAMMOX rates were tested against sediment grain size for the August 2011 sample period. There was no significant ($p < 0.05$) relationship between either denitrification or ANAMMOX rates and grain size. However, we note that the size range of the wetland sediments was limited. Median grain sizes by station ranged from 135-400 µm, and mean grain sizes by station ranged from 222-458 µm. Based on the Wentworth (1922) classification scheme, grain sizes were primarily within the sand category, which ranges from 63-2,000 µm. Based on this classification < 10% of the wetland sediment material sampled was in the clay or silt range, and < 2% of the material sampled was gravel-sized or greater. Thus, in this wetland grain size range may have been too limited to detect a significant influence on N-loss rates.

3.7. Comparison of N-Loss Rates by Individual Macrophyte Species

Denitrification and ANAMMOX rates were compared among individual macrophyte species using analyses of variance. Significant differences among species were seen for both processes (Table 5). *Pontederia* (Fig. 8) rhizospheric denitrification rates were significantly greater than any of the other plants with the exception of *Alternanthera* (Fig. 8), which in turn yielded higher rates than *Myriophyllum*. Several species (*Sparganium*, *Zizaniopsis*, *Typha* and *Juncus*) yielded denitrification rates that were very similar (Table 5). We note that only three of these species were actually planted while the other species entered the wetland and spread opportunistically. ANAMMOX rates also differed significantly among species (Table 5), with *Pontederia*, *Typha*, *Zizaniopsis* and *Sparganium* similar and yielding greater ANAMMOX rates than the other species.

Table 5. Comparison of N-loss rates among individual macrophyte species (rates as nmol N/g sed. wet wt./hr). Note: *Pontederia*, *Sparganium*, and *Zizaniopsis* (Fig. 8) were planted in the wetland following construction; the other species were opportunistic invaders into the wetland.

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Denitrification
Pontederia cordata	27.39 ± 2.68
Alternanthera philoxeroides	16.90 ± 3.64
Sparganium americanum	13.65 ± 9.33
Zizaniopsis miliacea	12.37 ± 2.40
Typha angustifolia	11.92 ± 3.90
Juncus effuses	11.66 ± 5.49
Myriophyllum aquaticum	4.59 ± 0.14

Pontederia > *Sparganium*, *Zizaniopsis*, *Typha*, *Juncus*, *Myriophyllum*
Alternanthera > *Myriophyllum* (p for the model = 0.0037)
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ANAMMOX
Pontederia cordata	3.72 ± 2.34
Typha angustifolia	2.91 ± 1.49
Zizaniopsis miliacea	2.90 ± 1.55
Sparganium americanum	2.22 ± 1.33
Alternanthera philoxeroides	1.50 ± 2.13
Myriophyllum aquaticum	0.61 ± 0.13
Juncus effuses	0.33 ± 0.26

Pontederia, *Typha*, *Zizaniopsis*, *Sparganium* > *Alternanthera*, *Juncus* (p for the model = 0.048)
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Figure 8. Forebay 2 of the JEL Wade constructed stormwater wetland. Macrophyte species include *Pontederia cordata* (purple flowers), *Sparganium americanum* (center emergent), *Juncus effuses* (flowering emergent in foreground), *Typha angustifolia* (tall emergent in back), *Zizaniopsis miliacea* (flowering emergent in front of *Typha* stand) and *Alternanthera philoxeroides* (submersed between *Sparganium* and *Pontederia*, right side of photo).

4.0. Discussion

4.1. Denitrification vs. ANAMMOX as N Loss Processes

FB1 has higher N removal capacity than FB2 as higher rates of ANAMMOX and denitrification were measured in unvegetated sediments and rhizospheric materials. Potential N_2 production rates in FB1 sediments were estimated to be 0.316 to 1.326 mmoles N_2 m^{-2} d^{-1}. The rates measured in unvegetated sediments were in the low range of N_2 production rates reported from different wetland soils (Davidsson and Stahl 2000).

4.2. Vegetated vs. Unvegetated Sediments

Macrophyte rhizospheric material in our study produced far greater N-loss through denitrification and ANAMMOX than did unvegetated sediments. Potential N_2 production rates ranged from 2.096 to 4.462 mmoles N_2 m^{-2} d^{-1}. Wetland soils with higher organic contents were shown to have similar rates as measured in rhizospheric materials (Davidsson and Stahl 2000).

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Some reasons that have been hypothesized for this include the production of detritus (a carbon source) through plant decay, extracellular release of dissolved organic carbon from leaves and roots, and providing surfaces for attachment of bacteria (Weisner et al. 1994). Additionally, the presence of photosynthesizing plants adjacent to decaying organic material can provide porewater oxic/suboxic gradients to support coupled nitrification/denitrification (Woltemade 2000).

4.3. Environmental Factors Influencing N Loss Processes

Statistically, water temperature was a significant driver influencing denitrification, with higher rates within macrophyte rhizospheres associated with higher water temperatures. This was also reflected in the strong seasonal pattern of June and August rates approximately doubling those in October. This provides a mechanism as to why our previous research (Mallin et al. 2012) found nitrate loss in the wetland strongly related to increasing water temperatures. However, ANAMMOX rates in vegetated sediments showed no relation to temperature, and monthly rates did not differ.

A different pattern emerged with N-loss processes in unvegetated sediments. Both denitrification and ANAMMOX rates were inversely correlated with water temperature. We found lower denitrification activities in summer than winter, which is opposite to the trends reported in the correlation between temperature and rhizospheric denitrification activities. This might be due to the presence of plant debris accumulated in the sediments during winter. Higher organic contents in sediments were shown to positive correlation to denitrification (Sirivedhin and Gray 2006).

There were some significant inverse correlations between N-loss rates and nutrient concentrations, all of which were negative and primarily concerned unvegetated sediments. In contrast, denitrification has often been considered to increase along with increasing nitrate (Seitzinger et al. 2006) since there is a greater supply available to be processed. We note that nutrient samples were collected from the upper water column while N-loss processes were measured from sediments and rhizospheres.

Dissolved oxygen was negatively correlated with denitrification in rhizospheric samples, but positively correlated with denitrification and ANAMMOX in unvegetated samples. Such processes are known to be associated with anaerobic porewater conditions (Seitzinger et al. 2006). However, our DO measurements were confined to the water column, rather than the sediments where the processes actually occurred. Thus, the correlations may have been indirect as a result of higher DO in winter as opposed to lower DO in summer due to temperature-based changes in gas solubility.

As to grain size, we noted above that during our sediment collection (confined to one month), there was a rather narrow range among samples. Thus, it may require a wider grain size variability to determine optimal sediment sizes for N-loss in wetlands.

4.4. Differences Among Macrophyte Species

Macrophytes in wetlands play an important role of providing organic carbon to support heterotrophic microbial activities such as denitrification. Ingersoll and Baker (1998) demonstrated increased nitrate removal with the addition of debris of *Typha* spp. The effect of vegetation can be dependent on species and total biomass of the macrophytes. We found that *Pontederia* rhizospheric denitrification rates were significantly greater than any of the other plants (Table 5).

5.0. Summary

- A seasonal study was conducted to assess rates of denitrification and ANAMMOX in a large regional stormwater wetland, and the impact of physical, chemical and biological factors on these processes.
- Both processes occurred in the wetland, with denitrification proving to be the dominant N-loss pathway, with ANAMMOX contributing up to 29% and 21% of N_2 production in wetland unvegetated sediments and plant rhizospheres, respectively.
- Overall, N-loss activity was approximately 4X higher in plant rhizospheres compared to unvegetated sediments.
- In plant rhizospheres, summer (June and August) denitrification rates were approximately double those of October, while there were no significant differences among June, August and October samples for ANAMMOX rates.
- In unvegetated sediments, winter and fall denitrification and ANAMMOX rates were significantly higher than those of August.
- Molecular analyses demonstrated higher abundance of denitrifying bacteria in rhizospheres compared with unvegetated sediments, while cluster analyses based on the nosZ gene T-RFLP showed that denitrifying communities were considerably different from those in unvegetated sediments.
- Water temperature was significantly correlated with denitrification in rhizospheric samples, but negatively correlated with denitrification and ANAMMOX rates in unvegetated sediments.
- Among wetland plant species, pickerelweed *Pontederia cordata* demonstrated significantly greater denitrification than than a variety of other species, while parrot feather *Myriophyllum aquaticum* showed lowest denitrification rates.
- *Pontederia*, cattail *Typha angustifolia*, giant cutgrass *Zizaniopsis miliaceae*, bur-reed *Sparganium americanum*, and alligatorweed *Alternanthera philoxeroides* showed significantly higher ANAMMOX rates than *Myriophyllum* and soft rush *Juncus effuses*.
- These results demonstrate (and add to previous insights) that wetland vegetation plays a major role in N-removal from incoming stormwater.
- A clear recommendation is that, in order to maximize N-removal, emergent and submersed wetland plant species should be planted with *Pontederia cordata* a clear choice. Additionally, this research shows that locally invasive species such as cattail *Typha angustifolia*, alligatorweed *Alternanthera philoxeroides* and soft rush *Juncus effuses* can play a significant role in N-removal as well and removal may not be advisable.

- This research also indicates that constructed stormwater wetlands in warm climates (with a long growing season) may be particularly effective in enhancing denitrification, and should thus be emphasized as N-control techniques provided sufficient space is available.

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Appendix 1. List of abbreviations and symbols, with definitions.

ANAMMOX (anaerobic ammonia oxidation): this is a recently identified microbial process involved in N removal by producing N_2 while oxidizing NH_4^+ coupled to NO_2^- reduction under anoxic conditions

CSW (constructed stormwater wetland): this is an engineered system designed for natural pollutant attenuation by wetland vegetation, sediments, and microbial processes.

Denitrification: this is a microbially-facilitated process in which fixed N is converted to an inert form by reducing NO_3^- to N_2 or N_2O while consuming available organic carbon.

DNRA (dissimilatory nitrate reduction to ammonium): this is a microbial dissimilatory reduction of nitrate and nitrate to ammonium while consuming available organic carbon.

FB (forebay): this is a man made pond in front of a larger water body.

nosZ gene (nitrous oxide reductase gene): this is a gene encoding for nitrous oxide reductase enzyme, which converts N_2O to N_2 in denitrifying bacteria.

PCR (polymerase chain reaction): this is an *in vitro* reaction of DNA polymerase to amplify a specific target gene in genomic DNA using specific primers.

T-RFLP (terminal restriction fragment length polymorphism): this is a molecular method to generate DNA fingerprint after PCR amplication of specific gene and restriction enzyme digestion.

Appendix 2. List of Presentations and Publications

Mallin, M.A., B. Song, A. Long and M.R. McIver. 2013. "High Pollutant Removal in a Large Constructed Wetland Improves a Tidal Creek." Tidal Creeks Summit, Wilmington, N.C.

Mallin, M.A., B. Song, A. Long and M.R. McIver. 2013. "Biotic, physical and meteorological factors maximizing N loss in a constructed wetland." Meeting of the Southeastern Estuarine Research Society, Charleston, S.C.

Song, B., A. Long, M.A. Mallin and M.R. McIver. 2012. "Anaerobic ammonium oxidation and denitrification in a constructed stormwater wetland." NCSU-SRP Biennial Stream Restoration Conference, Wilmington, N.C.

Long, A., M.A. Mallin, M.R. McIver and B. Song. 2012. "Anaerobic ammonium oxidation and denitrification in a constructed stormwater wetland." Water Resources Research Institute Annual Conference, Raleigh, N.C.