MICROBIAL CONTROLS ON NITROGEN POLLUTION MITIGATION WITHIN STORMWATER BASINS

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When considering sustainable urban development, trade-offs between water quality and greenhouse gas emissions exist. Therefore, this study examined the nitrogen (N) cycling dynamics, water quality treatment, and greenhouse gas emissions from stormwater basins located on Cornell University's campus. Stormwater basins that were often saturated ("Wet Basins") had a greater abundance of denitrification genes, and denitrified a greater proportion of incoming N. However, overall N treatment (measured as the difference between incoming N and N leaving the basin underdrains) was greater in quick draining "Dry Basins" likely due to the large volume of infiltrated stormwater. Rain events did not create hot-moment emissions of N_2O or CH_4 from these basins. Using EPA calculations, stormwater sent to a WWTP would produce 7X more $CO_{2\text{-eq}}$ per L than if it were sent to a Dry Basin. Conversely, stormwater sent to a Wet Basin instead of the WWTP would lead to an approximately 8X increase in CO_2. These results suggest that stormwater basins with saturated conditions are likely doing a better job of completely removing N via denitrification, but are also emitting greater quantities of GHGs. Thus, the tradeoff between better N water quality treatment and greater GHG emissions must be considered when designing stormwater basins.

In an effort to understand how increased urbanization will influence the soil microbial community, we examined the soil microbiome in stormwater basins after 2-months and 2-years of exposure. Overall, the microbial communities did not shift dramatically within the stormwater sites. Microbial sub-pathways of methanogenesis and V-ATPase were increased within the Wet Basin treatment, likely due to excess Na and soil moisture. While denitrification is also an anaerobic

process like methanogenesis, the denitrification genes did not increase within the often saturated Wet Basin treatment. This indicates that denitrification may take longer than 2-years to adjust to new environments, and practices like stormwater basins that rely on denitrification to remove nitrogen and improve water quality may be initially limited.

In addition to these field studies, a lab-scale mesocolumn study done in collaboration with Monash University examined the plant-microbe interactions and their impact on N treatment within stormwater basins. We conducted N water quality, N partitioning, soil metagenomics and 16S profiling across 7 unique plant species and 1 soil control over the 2-year experiment. Plant species with greater root volume, plant and microbial assimilation, and NOx removal, had lower denitrification genes and rates. Our hypothesis that greater denitrification would lead to better NOx removal was not supported by these data, because plant species with high NOx removal depressed denitrification genes and rates, but led to a better 'treatment' rate as a larger proportion of incoming N was assimilated and did not directly exit the column drainage. This aligns with the other projects where increased denitrification did not necessarily lead to higher N treatment, unless the ultimate fate of N is considered, and then the amount denitrified is more critical. Overall, anaerobic conditions in stormwater basins promoted denitrification and complete removal of N from downstream waters. However, because of excess greenhouse gas emissions, designers should consider the tradeoffs when installing these stormwater treatment technologies.

BIOGRAPHICAL SKETCH

Natalie Morse has a B.S. degree in Biosystems Engineering from Michigan State University and a M.S. degree in Biological and Agricultural Engineering from North Carolina State University. Born and raised in a suburb of Detroit, Michigan, Natalie has always had a curiosity for urban water management. She began her PhD at Cornell University in 2014 with the hopes of improving water quality in urban areas.

Dedicated to Nicholas Morse, without your support this PhD would have been impossible.

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ABSTRACT

The nitrogen (N) cycling dynamics of four stormwater basins, two often saturated sites ("Wet Basins") and two quick draining sites ("Dry Basins"), were monitored over a ~1-year period. This study paired stormwater and greenhouse gas monitoring with microbial analyses to elucidate the mechanisms controlling N treatment. Annual dissolved inorganic N (DIN) mass reductions (inflow minus outflow) were greater in the Dry Basin than in the Wet Basin, 2.16 vs. 0.75 g N m^{-2} yr^{-1}, respectively. The Dry Basin infiltrated a much larger volume of water and thus had greater DIN mass reductions, even though incoming and outgoing DIN concentrations were statistically the same for both sites. Wet Basins had higher proportions of denitrification genes and potential denitrification rates. The Wet Basin was capable of denitrifying 58% of incoming DIN, while the Dry Basin only denitrified 1%. These results emphasize the need for more mechanistic attention to basin design because the reductions calculated by comparing inflow and outflow loads may not be relevant at watershed scales. Denitrification is the only way to fully remove DIN from the terrestrial environment and receiving waterbodies.

INTRODUCTION

By 2050 the global population is expected to increase by roughly 2.5 billion people and nearly all of that growth will be concentrated in urban areas(United Nations, 2015). Stress to local water resources is one of the biggest concerns associated with urbanization. These include increased water demand, aquatic habitat degradation, reduced water quality, harmful algae blooms, and property damage associated with flooding (Carpenter et al., 1998; Mitchell et al., 2003;

Rabalais et al., 2002; Walsh et al., 2005). Green infrastructure (GI) or low impact development (LID) is one popular tool to alleviate the pressures of development on water resources.

Green infrastructure, which modifies natural hydrologic features to manage water and provide ecosystem and community benefits, became popular in the 1990's and is now an institutionalized and accepted global practice(U.S. Environmental Protection Agency (EPA), 2014). Initially, GI practices were installed to detain water and reduce nuisance flooding(Mitchell, 2006). Recently, designers and regulators seek to obtain water quality as well as quantity benefits from GI(Dietz, 2007; Hunt et al., 2012) by leveraging physical mechanisms such as settling and filtration and biological processes such as plant uptake and microbial cycling to remove nutrients, pathogens, petroleum hydrocarbons, and sediment from stormwater(Hatt et al., 2009; LeFevre et al., 2015).

While GI has grown in popularity, the nutrient removal performance of these systems is often inconsistent. Of particular concern is nitrogen (N), which can lead to waterbody eutrophication, harmful algae blooms, and decreased biodiversity, especially in coastal areas (Howarth, 2008; McKinney, 2008); N is also increasingly recognized as playing an important role in harmful freshwater algal blooms (Paerl and Scott, 2010). Urban areas receive 2-4 times higher N deposition from fossil fuel combustion than nearby rural areas(Bettez and Groffman, 2013; Lovett et al., 2000; Redling et al., 2013). Fertilizer applications, septic systems, and leaky sanitary sewers also contribute to N pollution near urban areas(Law et al., 2004; Sercu et al., 2008). With future land use change and global climate change, there is a strong need to mitigate N pollution from urban areas(Kaushal et al., 2008).

Mitigating dissolved N pollution is notoriously difficult because the filtering and settling mechanisms employed in GI are ineffective. Two mechanisms of dissolved N removal in GI are

plant uptake and denitrification (Payne et al., 2014a). Dissolved N is only completely removed from the system via denitrification, a microbially-mediated process which transforms nitrate (NO_3^-) to nitrous oxide (N_2O) and N_2 gas and thus permanently removes NO_3^- from the GI feature (Seitzinger et al., 2006). Relying on vegetation and microbial cycling for N removal has proved inconsistent (Payne et al., 2014a), with variable N reductions and even N leaching in field monitoring trials (Stanley, 1996; Dietz and Clausen, 2005; Hunt et al., 2006; Hatt et al., 2009).

The pollutant removal performance of GI practices is traditionally treated as a black-box input minus output (input-output) difference and generally neglects internal processes or fluxes that bypass the system. However, several recent studies have begun moving towards a more mechanistic understanding of N cycling in urban GI. Bettez and Groffman (2012) found that potential denitrification rates were higher in GI practices than nearby riparian zones, landscape features considered to be denitrification hotspots. Zhu et al.(Zhu et al., 2005) found much higher denitrification rates in detention basins than the surrounding desert ecosystem. Payne et al. (2014b) used isotopic methods in bioretention mesocosms and noted that plant uptake is a large sink of N but that denitrification is particularly key where NO_3^- levels are higher. Chen et al. (2013) has been the only study of which we are aware that has directly examined the microbial community related to N cycling in stormwater GI features. In a bioretention cell, they found that the abundance of denitrification genes quantified with qPCR in the soils was correlated with inundation time.

Consequently, this research builds upon this body of work by connecting traditional field observations of N in basin inflows and outflows along with quantification of microbial denitrification capacity using multiple methods. One thing that distinguishes this study from other studies that have considered denitrification gene abundance in a stormwater basin is the use of metagenomics in lieu of qPCR, which is particularly advantageous for characterizing very

phylogenetically diverse communities such as denitrifiers(Wallenstein et al., 2006). We aimed to determine if microbial function associated with denitrification led to better N treatment within stormwater detention basins at the field scale. By connecting microbial function and ecosystem processes to stormwater basin design, we can better design basins that cultivate microbial communities to promote certain desirable ecosystem services such as denitrification.

METHODS

We used a three-pronged approach: (1) soil DNA metagenomics, (2) coupled *in situ* N_2O flux monitoring and potential denitrification measurements, and (3) typical stormwater monitoring techniques.

Study Sites

This study utilized four grassed stormwater detention (or retention) basins (two slow draining Wet Basins and two fast draining Dry Basins) on Cornell University's campus in Ithaca, New York (Figures S1 and S2, Table 1). All sites receive runoff from individual contributing drainage areas, and operate independently of each other. These sites were previously monitored in 2013 for N_2O and CH_4 fluxes and soil volumetric water content (McPhillips and Walter, 2015). Additionally, soil from an adjacent 'reference' site not receiving stormwater located near Dry Basin2 were sampled for comparison.

Table 1. Study site characteristics. Conducted monitoring highlights the monitoring employed at each site, "DNA" = soil metagenomic DNA, "SW" = stormwater, "pot. denit." = potential denitrification, "soil" = soil characterization

		Wet Basin1	Wet Basin2	Dry Basin1	Dry Basin2	Control
Drainage Area	m^2	11,049	4,000	3,000	4,249	NA
Basin Area	m^2	1,500	550	400	480	NA
Drainage Area: Basin Area	ratio	7.37	7.27	7.50	8.85	NA
Year Constructed	Year	2004	2002	2007	2006	NA
Impervious Drainage Area	%	95	100	95	100	NA
Contributing Drainage Area		Parking Lot	Parking Lot	Roof	Parking Lot	Grass
Conducted Monitoring		DNA, SW, pot. denit., N_2O, soil	pot. denit., soil	pot. denit., soil	DNA, SW, pot. denit., N_2O, soil	DNA, soil

All basins were designed as dry infiltration basins following New York Department of Environment and Natural Resources (NYDENR) guidelines, but either due to construction error, clogging, or ecosystem succession, some basins developed into a wetland like system with various saturated areas throughout the basin, *i.e.*, the Wet Basins. The Dry Basins function as dry infiltration basins that quickly infiltrate stormwater runoff and standing water is seldom observed between storm events. The basins were all originally planted with turfgrass (primarily perennial ryegrass- *Lolium perenne*) and have 10-15 cm topsoil which is underlain by native silt loam, and then a layer of sand. Below the sand is an underdrain (perforated pipe) that connects to the storm sewer system. Consequently, this study exploited these differing wetness regimes to explore how alternative designs may influence stormwater treatment performance and functional microbial gene abundance. Inclusion of wet and dry sites were intended to highlight how similar systems with different hydrology may alter the N treatment from urban areas. This is important because of the variability in basin design, construction, and site succession which leads to differing hydrology

and subsequent treatment in real-world conditions. Only 2 sites, Wet Basin1 and Dry Basin2, were monitored for stormwater quality and N_2O emissions (Table 1). All four basins were monitored for soil metagenomics and potential denitrification during 2015.

Soil Metagenomics

High-throughput next-generation sequencing was used to analyze the soil DNA genome (metagenomics) at each stormwater basin and the reference site. Soils were collected on June 29, 2015. At each site three soil cores were collected and pooled from each of the 3 sample points, for DNA extraction and soil characterization (5 sites x 3 sample points = 15 total samples). Soils were collected with a steel push probe (2.5 cm diameter) from the top 5 cm of soil. Soils were kept on ice during sampling, and stored at 4°C until DNA extraction within one week. Soil DNA was extracted with Mo-Bio PowerSoil® DNA Isolation Kit. For each of the 15 samples, DNA was extracted in triplicate and then pooled to reduce variability within the soil sample and provide ample DNA for sequencing. Concentration of extracted DNA was assessed using a Qubit fluorometer with dsDNA BR and HS assay kits. Extractant was frozen at -20ºC until sequencing.

Approximately 18M 100 base single-strand reads were sequenced for each sample (15 samples x 18M reads = 270M reads total) by the Columbia University Genome Center on an Illumina HiSeq 2500. Next, DIAMOND (double index alignment of next generation sequencing data), a high throughput alignment program compared sample DNA sequence reads against a custom, manually curated database of reference proteins critical for N cycling. DIAMOND is analogous, but faster, than BLASTx (Buchfink et al., 2015). DIAMOND returned a matrix of matches for each sequence, within each sample; results were filtered where percent sequence identity (pid) >50, and assigned read length >25 base pairs.

This study focused on reads corresponding to functional genes, as opposed to phylogenetic classifications common with 16S rRNA amplicon sequencing. Focusing on functional genes within the soil microbial metagenomes allows us to better understand microbial nutrient cycling functions and drivers in this diverse soil ecosystem(Lombard et al., 2011). Once functional gene count reads were obtained, they were normalized by total reads per sample to compensate for the slight variation in sample to sample reads (14M to 21M per sample), and multiplied by one million to obtain reads per million reads (rpm).

As denitrification is the process of interest for complete N treatment, our analyses focused on these functional genes: NO_3^- to NO_2 reduction via nitrate reductase (*nap* and *nar*); NO_2 to NO reduction via nitrite reductase (*nirK* and *nirS*); NO to N_2O via nitric oxide reductase (*cnor* and *qnor*); and N_2O to N_2 via nitrous oxide reductase (*nosZ*). The normalized sum of sequencing reads that matched these proteins is herein referred to as 'normalized total denitrification reads' (rpm).

Soil Characteristics

Each soil core collected for metagenomics was analyzed for bulk density, total carbon (C), metals, pH, and NO_3^- and NH_4^+. The cores were oven-dried at 105°C for 24-hours and weighed to determine bulk density (Blake and Hartge, 1986). A subsample was ground to less than 250 µm and analyzed for % total C (g C g^{-1} dry soil), which was measured at the Cornell University Stable Isotope Laboratory (Ithaca, NY) through dry combustion on a Conflo III Elemental Analyzer. Soil available NO_3^- and NH_4^+ was measured after a 1 M KCl extraction (Robertson et al., 1999) and the extractant was analyzed on a Lachat QuikChem 8000 Flow Injection Analyzer using the NO_3^- low flow method and the salicylate method. Nitric acid and HCl extractions were used to extract total metals (US EPA, 1996), which were quantified via ICP at Cornell University USDA lab. Soil pH was determined with a 1:1 soil to water ratio.

Potential Denitrification Monitoring

We employed the denitrification enzyme assay (DEA), a long-standing method used to characterize a site's ability to denitrify under optimal conditions using lab incubations(Groffman et al., 1999). This method is well suited to compare site-to-site differences, but does not quantify actual denitrification rates *in situ* (Attard et al., 2011; Groffman et al., 1999). Monitoring was conducted at three locations at each basin site in 2016 (Figure S1 and S2). At each sampling event, two soil samples were collected from each location with a steel push-probe (0-5 cm depth) and pooled. The assay was done in duplicate on each pooled sample (4 sites x 3 locations/site x 2 duplicates = 24 samples/event). A total of four monitoring events were conducted approximately monthly from June to September 2016.

The DEA method is described by Groffman et al.(1999). Briefly, collected fresh soil was hand sieved to remove coarse debris and then 5 grams were transferred to 125-mL glass serum bottles amended with 25-ml solution media (100 mg L^{-1} NO_3^- and 500 mg L^{-1} glucose). The bottles were sealed, evacuated, and flushed with N_2 twice. Then 10-ml of acetylene (C_2H_2) was added to inhibit N_2O to N_2 reduction. Headspace samples were collected at t=0, 20, 40, and 60 minutes and analyzed for N_2O concentration via gas chromatograph (GC; Agilent 6890N). Potential denitrification rates were calculated as the linear rate of change in N_2O over time normalized to soil dry mass (ug N_2O-N kg $soil^{-1}$ hr^{-1}).

Stormwater Monitoring

Stormwater quantity and quality monitoring was completed at each site from July – November 2015, and from May 2016 to November 2016. A total of 20 and 17 storms were monitored at Dry Basin2 and Wet Basin1, respectively. Inflow volume was determined based on precipitation measured on site via rain gauges and the U.S. Natural Resource Conservation Service

(NRCS) Curve Number method, which estimates runoff based on land-use terms (Ponce and Hawkins, 1996; Wilson et al., 2014). Because the site contributing drainage areas are nearly 100% impervious, little ambiguity exists surrounding initial abstraction or infiltration: almost all of the rainfall is converted to runoff directed toward the basins. A v-notch weir-box on the underdrain was fitted with HOBO pressure-transducer water depth loggers recording at 1-minute intervals to quantify outflow volume. ISCO automated samplers (ISCO 6712) collected stormwater samples over the storm duration. Individual samples were flow-weight composited for an event mean concentration (EMC), prior to analysis: one inflow and one outflow sample characterized each storm at each site.

Water quality analyses included total suspended solids, nitrite, nitrate, and ammonium following general water quality guidance(American Public Health Association, American Water Works Association, and Water Environment Federation (APHA, AWWA, and WEF), 1998; USEPA, 2007). A subset of each sample was immediately filtered using 0.45μm Pall mixed cellulose ester filters and filtrate was stored at 4°C until analysis of dissolved nutrients. NO_3^- and NH_4^+ were quantified colorimetrically (as described above) in 2015 via Lachat QuikChem 8000 Flow Injection Analyzer and in 2016 via microplate reader.

Greenhouse gas monitoring

Nitrous oxide monitoring was also conducted at Wet Basin1 and Dry Basin2 during 2016. Three in-situ static chambers were deployed during each sampling period following methods of McPhillips and Walter (2015). Gas samples were collected at 10 minute intervals over 30 minutes and injected into pre-evacuated 10-mL glass serum vials. A total of 17 sample events were conducted to capture a range of environmental conditions. Gas samples were analyzed as described

above and flux rates were calculated based the concentration rate of change over time. Fluxes were converted from volumetric to mass-based units (μg gas m^{-2} hr^{-1}) using the ideal gas law.

Nitrogen Budget

An annual N budget was calculated to determine the fate of N within these systems. First, a water budget (Equation 1) was calculated which scaled observational data by the ratio of total rainfall over the monitoring period (NRCC, 2016) to the sum of rainfall measured over the monitored ~20 storms(Wilson et al., 2014). This yields annual volumes (m^3 yr^{-1}) instead of the partial volumes observed over the individually monitored storm events.

$$I = O + ET + I_f \quad (1)$$

Where, I = total inflow water volume (m^3 yr^{-1}), O = total outflow water volume (m^3 yr^{-1}), ET= evapotranspiration (m^3 yr^{-1}), and I_f = infiltrated water volume (m^3 yr^{-1}). The annual ET rate (cm) was obtained from Sanford et al.(Sanford and Selnick, 2013) and multiplied by the basin area (m^2) to obtain ET volume (m^3 yr^{-1}). Next, the N budget used the water budget, average measured incoming and outgoing DIN concentrations (NO_3^- + NH_4^+), and average N_2O gas flux measurements to determine fluxes of N within these systems (Equation 2):

$$I_N = O_N + I_{fN} + D_N \quad (2)$$

where, I_N = incoming N mass (mg N yr^{-1}) calculated via I × average incoming concentration (mg N m^{-3}), O_N = outgoing N mass (mg N yr^{-1}) calculated via I × average outgoing concentration (mg N m^{-3}), I_{fN} = infiltration N mass (mg N yr^{-1}), and D_N = denitrification N mass (mg N yr^{-1}) as calculated in Equation 3. As infiltration through the basin was not measured, I_{fN} was calculated via subtraction to close the N budget. Mass N denitrified was based on average N_2O fluxes measured

in 2016 and modeled $N_2O/(N_2O+N_2)$ ratio (N_2R), and scaled by correction factors to account for daily fluctuations (Equation 3):

$$D_N = N_E \; x \;\; A \;\; x \, K \; (3)$$

where, N_E = average N_2+N_2O emissions (mg N m^{-2} $year^{-1}$), A = basin surface area (m^2), and K = correction factor reducing emissions during night 0.77 (Maljanen et al., 2002). This assumes plant uptake is at steady state where any N accumulated within the plant is recycled back to the soil within the year. Since N_2R was not monitored at each site, we used Monte Carlo simulations to estimate the likely mean and standard error (se) of N_2+N_2O emissions. At each sample time, the 3 N_2O measurements per site were averaged and then divided by a randomly selected N_2R value from a uniform distribution with the domain of (0,1). We conducted 1,000 simulations using random sampling and then calculated the site mean and se for N_E at each site. This created a robust approximation of N_E, and allowed us to estimate the likely variability of D_N.

In addition to the above calculations, we explored two alternative calculations to better bound this budget. First, we used N_2R values from previous publications that were based on average soil volumetric water content (Schlesinger, 2009) multiplied with the average measured N_2O emissions. This method again relied on subtraction to calculate I_{fN} to close the budget. Second, we assumed the infiltrated water had a comparable DIN concentration to the incoming runoff, and back-calculated the % denitrified.

Statistical Analyses

The statistical analysis was conducted using R software (version 3.1.1; R Development Core Team, 2014). A criteria of 95% confidence (α=0.05) was selected for all analyses herein. Due to evidence of heteroscedasticity, non-parametric tests were used for stormwater quality

assessments. Comparisons between the two sites used the Mann-Whitney test, and comparisons of paired data used the Wilcoxon signed rank test. Analysis of variance (ANOVA) with Tukey HSD was used to test differences of metagenomics gene reads and denitrification potential between the sites. Due to evidence of heteroscedasticity, denitrification values were log transformed prior to statistical analysis. Mixed effects models, where site was the random effect, were used to test which site variables (*i.e.*, soil moisture, carbon, and NO_3^-) significantly affected metagenomic read abundances.

RESULTS AND DISCUSSION

Soil Characteristics

Average soil characteristics were fairly consistent among the basins (Table 2). All sites were classified as sandy loam texture based on the U.S. Department of Agriculture (USDA) soil classification (Soil Survey Staff, 1999). All of the basins had near neutral soil pH, and negative impacts on denitrification associated with acidic soils are not expected at these sites (Liu et al., 2010; Šimek and Cooper, 2002).

Table 2. Average basin soil characteristics and dimensions.

		Wet Basin1	Wet Basin2	Dry Basin1	Dry Basin2	Control
pH		7.3	7.3	7.7	7.5	7.7
Carbon	%	10.7	5.0	7.0	5.3	4.2
Cu	mg kg^{-1}	12.8	51.6	31.6	11.9	11.0
Pb	mg kg^{-1}	8.4	16.4	16.4	23.3	18.7
Zn	mg kg^{-1}	76.0	133.3	140.8	87.7	71.9
sand	%	83.7	79.2	88.3	76.4	78.5
silt	%	3.0	15.0	5.9	6.4	6.1
clay	%	13.3	5.9	5.9	17.2	15.4
soil moisture	g water g dry $soil^{-1}$	0.56	0.60	0.28	0.35	0.30
soil NO_3^-	mg kg^{-1}	1.01	2.00	6.96	3.82	2.86

Stormwater Monitoring

Incoming concentrations of NO_3^- were not statistically different for both basins: average EMCs were 0.21 and 0.18 mg L^{-1} for Wet Basin1 and Dry Basin2, respectively. This is slightly lower than other studies, e.g., 0.5 mg L^{-1} (Dietz and Clausen, 2005) and 0.5-0.7 mg L^{-1} (US EPA, 1983). Outflow NO_3^- concentrations were similar to inflow concentrations and did not differ between the sites (Figure 1). Neither basin significantly reduced NO_3^- outgoing concentrations relative to incoming concentrations (paired Wilcox test p=0.25 and 0.58 for Wet Basin1 and Dry Basin2, respectively). Average incoming NH_4^+ concentrations (0.20 and 0.17 mg L^{-1} for Wet Basin1 and Dry Basin2, respectively) and outgoing NH_4^+ concentrations (0.10 and 0.09 mg L^{-1} for Wet Basin1 and Dry Basin2, respectively) were similarly low for both basins and no significant differences were observed between the sites (Figure 1). However, Wet Basin1 had significant differences between inflow and outflow NH_4^+ concentrations (paired Wilcox test $p<0.01$), while Dry Basin2 did not (p=0.85). These results are similar to other field monitoring reports with little to no DIN reductions (Dietz and Clausen, 2005; Hatt et al., 2009; Hunt et al., 2006; Stanley, 1996).

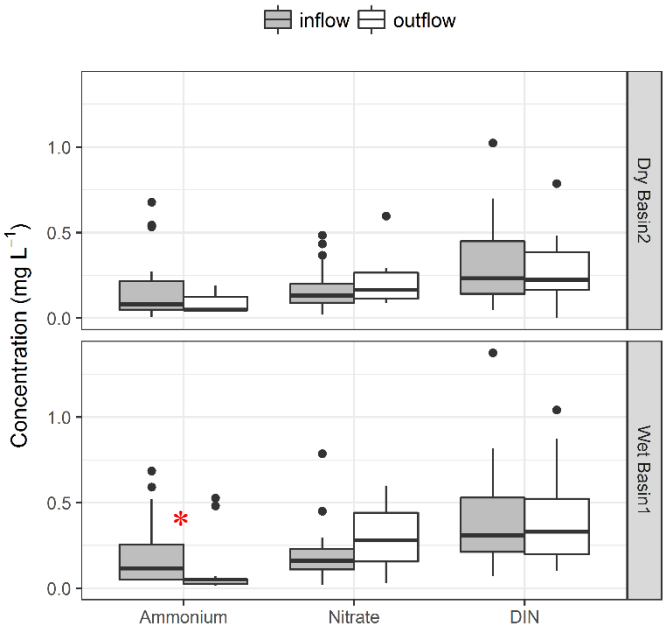

Figure 1. Stormwater inflow and outflow concentrations. * indicates a significant difference between inflow and outflow concentration at that site.

The hydrology between the wet and dry basin was markedly different. Outflow (drainage pipe outlet) from Dry Basin2 was only observed for 8 of the 20 storm events, while Wet Basin1 produced outflow for 16 of the 17 events. Dry Basin2 was able to remove a much higher percentage of runoff volume (averaging ~99% per storm) than Wet Basin1 (~38%). Almost no runoff exited Dry Basin2 through the underdrains and no stormwater was observed backing up onto the parking lot. These basins are unlined, which indicates the majority of the stormwater infiltrated to underlying soils. This change in volume reduction is the only significant difference between the two basins ($p<0.001$), as no concentration differences were observed. Consequently, mass load reductions were much easier to achieve within Dry Basin2 simply due to stormwater volume reductions. Table 3 displays the annual mass loads and % reductions at each site.

Table 3. Annual mass loads of nitrate, ammonium, and dissolved inorganic N. Dissolved inorganic N is the sum of NO_3^- + NH_4^+. % reductions are the difference between inflow (In) and outflow (Out) mass loads. A total of 17 and 20 storms were monitored for Wet Basin1 and Dry Basin2, respectively.

Site	Nitrate			Ammonium			Dissolved Inorganic N		
	In	Out	Reduction	In	Out	Reduction	In	Out	Reduction
	g N yr^{-1}		%	g N yr^{-1}		%	g N yr^{-1}		%
Wet Basin1	1,425	1,252	12	1,384	437	68	2,809	1,689	40
Dry Basin2	529	9	98	521	4	99	1,050	13	99

Soil Metagenomics

Wet Basin 1 and 2 had significantly greater normalized total denitrification reads compared to the Dry Basins and control ($p<0.001$; Figure 2). Denitrifiers are facultative aerobes, and the wet basins, which are often more saturated than the dry basins, may have more anaerobic areas to promote denitrification. Available carbon and nitrogen are also important drivers of denitrification. Carbon content was comparable across the sites (Table 2), but soil NO_3^- was much lower in the wet basins. The lower NO_3^- levels in the wet basins could be due to depletion via denitrification, although we did not investigate NO_3^- utilization. Results of the mixed effects models indicated that soil gravimetric water content (g water g dry $soil^{-1}$) was a good predictor of normalized total denitrification reads ($p=0.057$, Figure S3), while % carbon ($p=0.69$) and NO_3^- ($p=0.98$) were not. Consequently, the higher normalized total denitrification reads within the wet basins is likely due to greater soil water content.

Because denitrification is a multi-step process, upstream reductions may affect downstream capacities, *e.g.*, if *nap* and *nar* levels are low, subsequent reduction steps may also decrease. Generally, the Wet Basins had higher proportions of denitrifying reads in all categories, except *nap, nirK, and qnor*, which did not significantly differ between the sites (Figure S4). Table

4 displays the ANOVA results for each individual gene, along with the Tukey-HSD differences (if ANOVA was significant) between the sites. Importantly, the *nosZ* enzyme, which reduces N_2O to N_2, was higher within the Wet Basins. Many denitrifiers do not possess all genes in the denitrification pathway(Shapleigh, 2013). Thus, it is difficult to discern how effective a microbial community is simply from the mix of denitrification genes. However, the observation that wet basins had proportionally higher denitrification genes could signal these systems are primed for this ecosystem service.

Additional soil DNA temporal sampling was not feasible. Lauber et al. (2013) noted site-to-site differences of soil 16S results remained relatively consistent over time, although the exact numbers could fluctuate slightly. Consequently, the greater normalized total denitrification reads associated with the wet basins are likely indicative of a greater denitrifier community throughout the year monitoring period.

Table 4. Analysis of variance results for individual and total denitrification gene reads among the basin and control sites. Significant ANOVA p-values are in bold. Significant ($p<0.05$) Tukey-HSD differences are given by differing letters; letters are in ascending order where sites with the lowest normalized reads are denoted with "a".

	p-value	**Control**	**Dry Basin1**	**Dry Basin2**	**Wet Basin1**	**Wet Basin2**
nap	0.16	----------NA----------				
nar	**<0.001**	a	a	a	b	b
nirK	0.51	----------NA----------				
nirS	**<0.001**	a	a	a	b	b
cnor	**0.003**	a	a	a	b	ab
qnor	0.06	----------NA----------				
nosZ	**<0.001**	ab	a	abc	bc	c
Total Denit.	**<0.001**	a	a	a	b	b

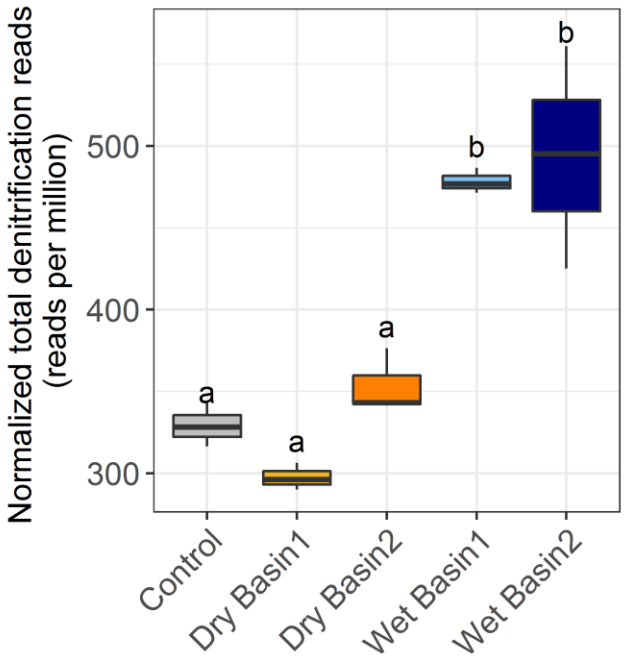

Figure 2. Total denitrification reads (*nap*, *nar*, *nirK*, *nirS*, *cnorB*, *qnorB*, and *nosZ*) normalized by total reads per sample. Significant ($p<0.05$) Tukey HSD differences are denoted by different letters.

Potential Denitrification

In addition to the DNA metagenomics, Wet Basin1 had higher overall potential denitrification rates than both dry basins ($p <0.05$). While not significant, Wet Basin2 also had slightly higher denitrification rates than the dry basins (Figure 3). These results are in accordance with the metagenomics results, where the wet basins had a greater proportion of denitrification genes.

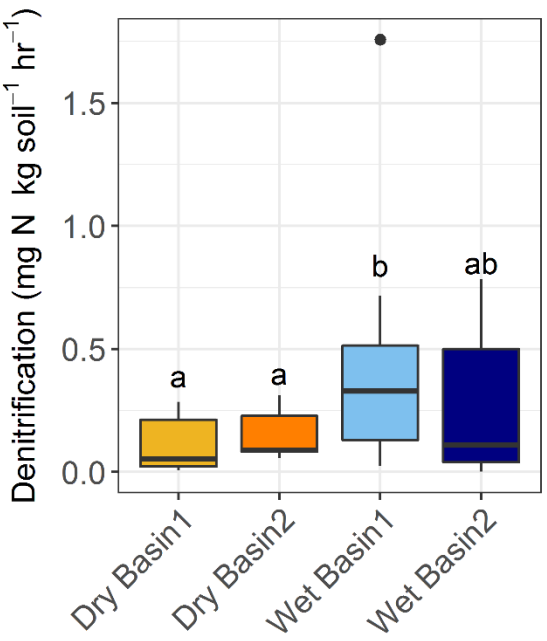

Figure 3. Potential denitrification enzyme assay (DEA) rates (n=16). Tukey HSD differences ($p<0.05$) are shown by different letters.

Water and nitrogen budget

On an annual basis, a much larger proportion of incoming water exits the underdrains as outflow in Wet Basin1 (62%) than Dry Basin2 (1%). Consequently, a much smaller proportion of water is infiltrated within Wet Basin1 (6%) than Dry Basin2 (80%). Evapotranspiration percentages were similar at the sites, where Wet Basin1 and Dry Basin2 average annual percentages were approximately 32% and 19%, respectively.

The annual DIN mass load budget follows a similar trend with the water budget. Approximately 60% of incoming DIN exits Wet Basin1 via outflow, whereas only 1% exits Dry Basin2 as outflow (Table 5). The average N_2O fluxes were higher in Wet Basin1, 0.30 g N_2O-N m^{-2} yr^{-1} compared to Dry Basin2, 0.01 g N_2O-N m^{-2} yr^{-1} (Supplementary Materials Table S1). According to the denitrification calculations using measured N_2O emissions and simulated N_2R, Wet Basin1 is capable of denitrifying approximately 1.09 g N m^{-2} yr^{-1}, or 58% of incoming DIN

on average (± 2%, se), while Dry Basin2 denitrifies only 0.03 g N m^{-2} yr^{-1}, or roughly 1% (±2%) of incoming DIN (Table 5). As this calculation relied on the monitored N_2O emissions and simulated N_2R, we explored alternative calculations to better bound this calculation. Using N_2R literature values (Schlesinger, 2009) (0.082 [0.024 se] Wet Basin1, and 0.492 [0.066] Dry Basin2) these denitrification rates were approximately 133% and 1% of incoming DIN for Wet Basin1 and Dry Basin2, respectively. It is unlikely Wet Basin1 could denitrify 100% of incoming DIN, expectedly some N quickly escapes the system through the underdrains as outflow, and some may also be microbially unavailable. Assuming the infiltrated water had a comparable DIN concentration to the incoming runoff, a worst case scenario where no internal processing occurred, the calculated denitrification percentages for Wet Basin1 and Dry Basin2, were 34% and 19%, respectively (Table 5). In all three calculations Wet Basin1 had considerably more denitrification than Dry Basin2, and these alternative calculations support the conclusion that more denitrification occurred within Wet Basin1. Constructed wetlands have been reported to denitrify approximately 3.5% and 25% of incoming N(Batson et al., 2012; Reinhardt et al., 2006). Venterink et al.(2002) estimated denitrification to be around 5-10% in natural wetlands. Clearly denitrification is highly variable in natural and constructed environments. Our results highlight how differing hydrology can drive denitrification rates to be relatively high in the wetland like Wet Basin1, and relatively low in the quick draining Dry Basin2.

Table 5. Annual DIN mass budget; mean (se). Nitrogen loads were calculated based on measured N_2O emissions, N_2O ratio literature values (Schlesinger, 2009) and measured N_2O emissions, and using the average inflow concentration as the concentration infiltrating underlying soils. Values in italics were calculated via subtraction to close the N budget.

Calculation		Inflow		Outflow				Denitrified				Infiltrated			
	Site	g N yr^{-1}		g N yr^{-1}		% of Inflow		g N yr^{-1}		% of Inflow		g N yr^{-1}		% of Inflow	
Measured N_2O emissions on-site	Dry Basin2	1,050	(174)	13	(4)	1	(2)	13	(25)	1	(2)	*1,025*	*NA*	*98*	*NA*
	Wet Basin1	2,808	(527)	1,689	(304)	60	(87)	1,635*	(67)	58*	(2)	*-516*	*NA*	*-18*	*NA*
Schlesinger 2009	Dry Basin2	1,050	(174)	13	(4)	1	(2)	8	(1)	1	(0.1)	*1,030*	*NA*	*98*	*NA*
	Wet Basin1	2,808	(527)	1,689	(304)	60	(87)	3,748*	(849)	133*	(30)	*-2,628*	*NA*	*-94*	*NA*
Infiltrated water = inflow conc.	Dry Basin2	1,050	(174)	13	(4)	1	(2)	*197*	*NA*	*19*	*NA*	840	(139)	80	(13)
	Wet Basin1	2,808	(527)	1,689	(304)	60	(87)	*955*	*NA*	*34*	*NA*	164	(31)	6	(1)

*Using the methodology described in the text, the calculated mass denitrified caused the budget to exceed 100%. Therefore, infiltrated values were 'negative' to balance the budget. In reality, the amount denitrified would be constrained and mass N infiltrated would be greater than zero.

Based upon the measured N_2O emissions, the calculated DIN infiltrated in Dry Basin2 was approximately 98% of incoming DIN, while Wet Basin1 was capable of denitrifying all excess N and no DIN would infiltrate underlying soils (values shown as negative infiltration in Table 5 would be constrained). These calculations likely overestimate the effectiveness of Wet Basin1 to denitrify all excess DIN, as heterogeneity throughout the basin likely limited microbial interaction with DIN. It is important to note that the infiltrated water, unless further microbially processed, will reach receiving waters and lead to N pollution. Consequently, if treatment is defined as inflow-outflow Dry Basin2 appears to be out-performing Wet Basin1, where Dry Basin2 "removed" 99% of inflow (2.16 g N m^{-2} yr^{-1}), and Wet Basin1 "removed" 40% of inflow (0.75 g N m^{-2} yr^{-1}). However, the water and N budget show that much of this "treated" stormwater is infiltrated to underlying soils and may reach downstream waterbodies with minimal N removal, especially in areas with a shallow restrictive soil layer, or where little space is available. As denitrification is the only process to fully remove DIN from the environment, the larger denitrification removal in Wet Basin1 makes this system more advantageous for DIN removal than Dry Basin2. Although stormwater monitoring was not conducted at the other two basins, the metagenomics results suggest that these systems may follow similar trends. Thus, at the watershed scale where the ultimate fate of N is concerned, the wet basins are likely to provide greater DIN internal processing and are less reliant on hydrologic benefits to achieve DIN treatment.

Results from the paired stormwater monitoring, soil metagenomics and denitrification potential analyses revealed that slow-draining often saturated wet basins had higher normalized denitrification reads and denitrification potential, which correlated to a larger reduction in DIN through denitrification than the quick-draining dry basins. We hypothesized that basins with greater denitrification genes and activity rates would produce better stormwater N treatment. These

results support this hypothesis, and give some indication that microbial activity measurements of denitrification like those by Bettez and Groffman (2012) may prove useful in assessing overall ability to reduce N from incoming stormwater. In assessing the efficacy of GI it is therefore important to consider the ultimate fate of the target pollutant. Considering denitrification along with traditional inflow-outflow monitoring would greatly improve our understanding of how these systems impact N loading to downstream waterways. We can then work to improve N treatment within GI and mitigate water quality impairment by engineering based on actual pollutant transformation processes rather than the too common black-box approaches that are currently employed.

SUPPORTING INFORMATION

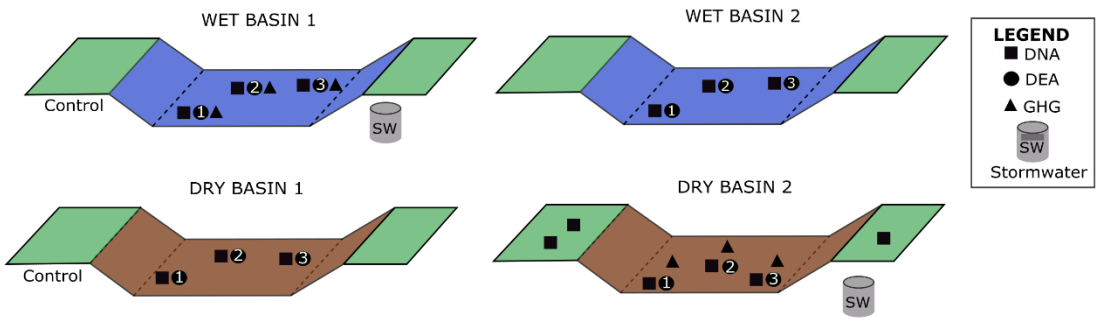

Figure S1. Stormwater basin sampling scheme. Each symbol denotes approximate location of sample collection point (not to scale). DNA = DNA metagenomics, DEA = potential denitrification enzyme assays, GHG = static chamber GHG sampling, SW = stormwater sampling at inflow and outflow of basin.

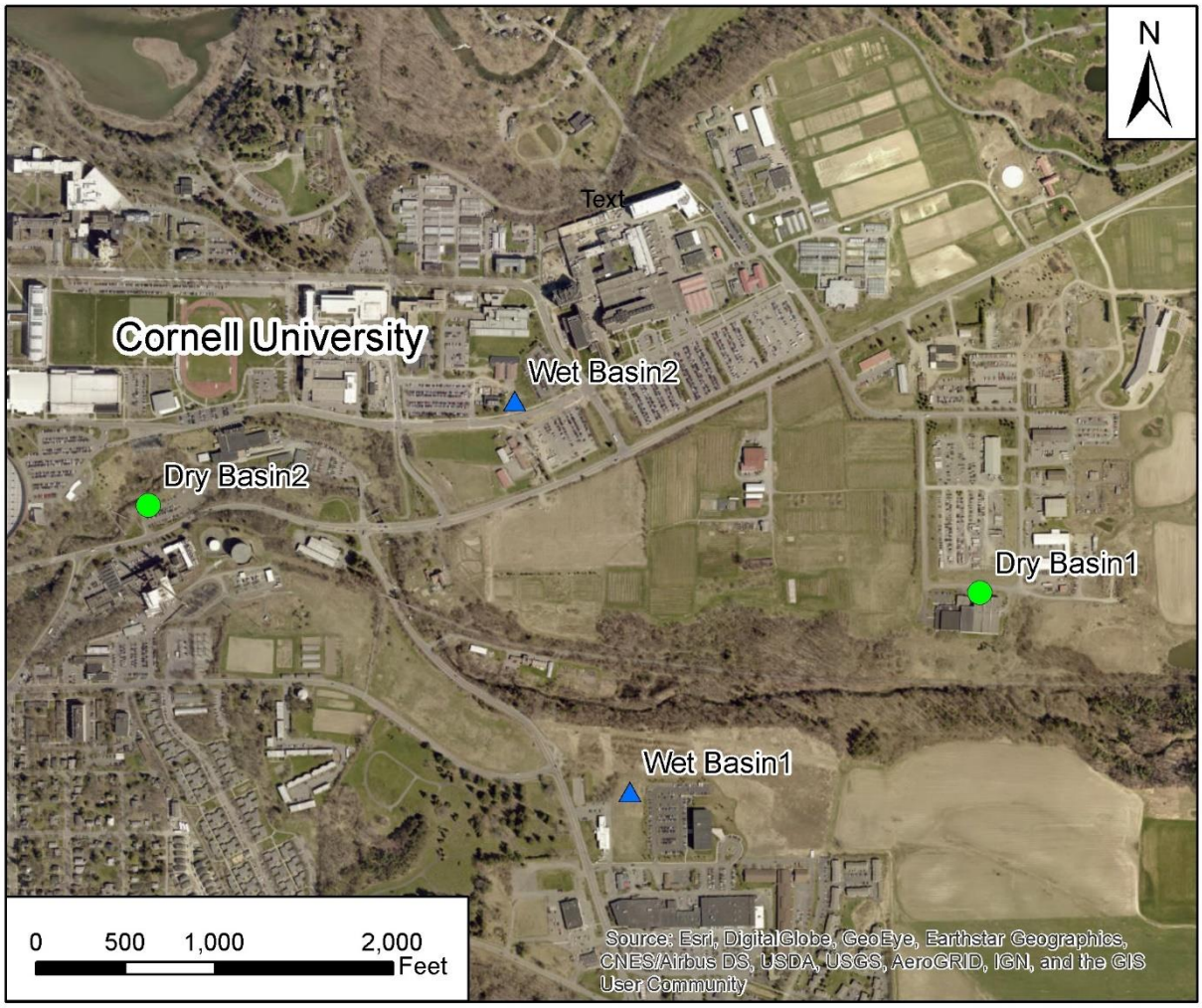

Figure S2. Site location map

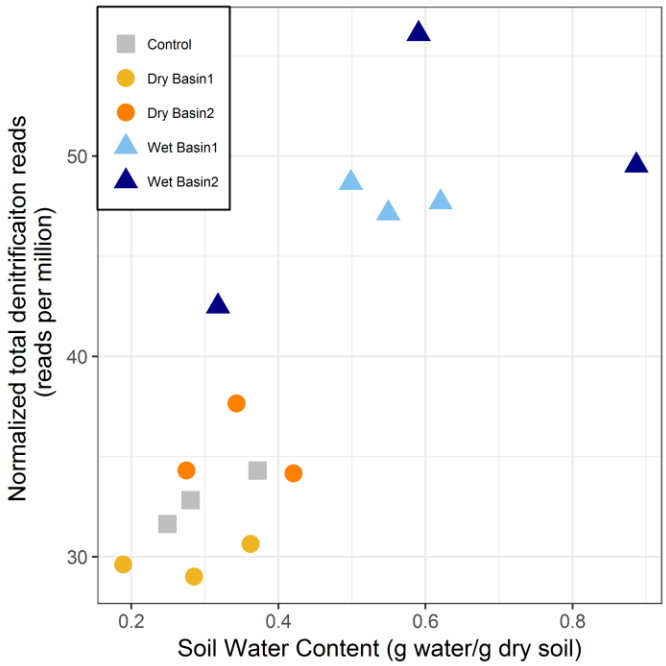

Figure S3. Normalized total denitrification reads vs soil water content at time of sample collection.

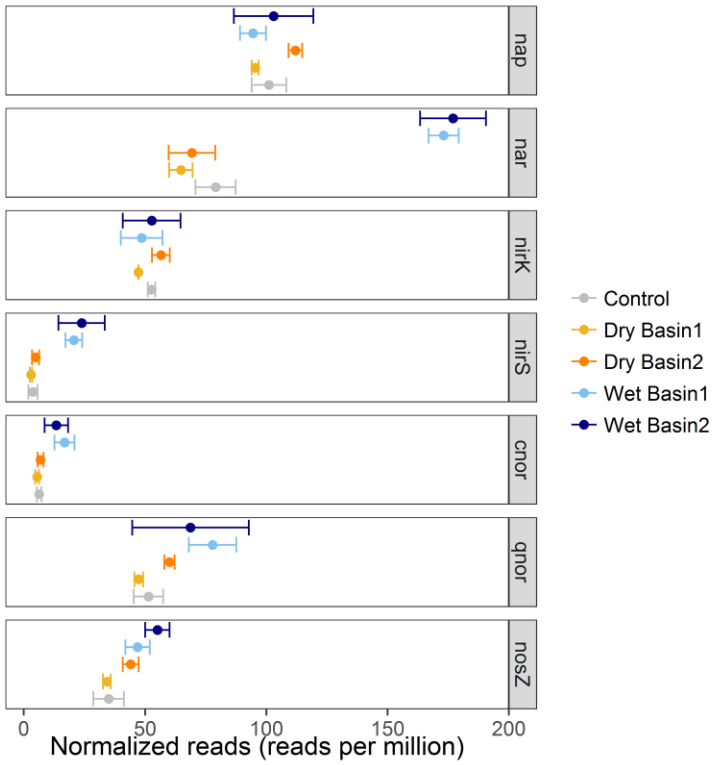

Figure S4. Individual denitrification gene reads normalized by total reads per sample. Error bars are 1 SD.

Table S1. Site average and standard error (se) of N_2O fluxes, n=3 per sample date.

Site	Date	N_2O Flux	se N_2O flux
		g N m^{-2} yr^{-1}	g N m^{-2} yr^{-1}
Dry Basin2	4/20/2016	0.145	0.277
	4/27/2016	0.413	0.068
	5/11/2016	-0.183	0.058
	6/2/2016	0.307	0.151
	6/20/2016	-0.039	0.043
	6/27/2016	0.064	0.106
	7/8/2016	0.293	0.022
	7/13/2016	0.153	0.051
	7/22/2016	-2.617	0.560
	7/25/2016	0.436	0.394
	8/2/2016	0.258	0.185
	8/10/2016	0.107	0.202
	8/23/2016	0.709	0.288
	9/14/2016	0.216	0.065
	9/19/2016	0.195	0.127
	9/21/2016	-0.162	0.201
	10/8/2016	-0.105	0.291
	Site Average	**0.011**	**0.173**
Wet Basin1	4/20/2016	0.130	0.148
	4/27/2016	-0.214	0.154
	5/11/2016	-0.295	0.268
	6/2/2016	0.126	0.116
	6/20/2016	0.305	0.434
	6/27/2016	0.407	0.325
	7/8/2016	1.856	1.770
	7/13/2016	0.897	0.348
	7/22/2016	NA	NA
	7/25/2016	1.207	0.790
	8/2/2016	-0.096	0.155
	8/10/2016	0.185	0.305
	8/23/2016	-0.048	0.091
	9/14/2016	0.180	0.130
	9/19/2016	0.063	0.085
	9/21/2016	0.074	0.132
	10/8/2016	-0.035	0.187
	Site Average	**0.296**	**0.142**

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CHAPTER 2: PLANT-MICROBE INTERACTIONS DRIVE DENITRIFICATION RATES, DISSOLVED NITROGEN REMOVAL, AND THE ABUNDANCE OF DENITRIFICATION GENES IN STORMWATER CONTROL MEASURES

ABSTRACT

The microbial community and function along with nitrogen (N) partitioning and denitrification rates were studied in soil bioretention mesocolumns (7 unique plant species). Total denitrification gene reads per million (rpm) were positively correlated with % denitrified ($r = 0.69$), but negatively correlated with total NOx removal following simulated rain events ($r = -0.79$). This is likely due to plant-microbe interactions. Plant species with greater root volume, plant and microbial assimilation %, and NOx removal %, had lower denitrification genes and rates. This implies that although microorganisms have access to N, advantageous functions, like denitrification, may not increase. At the conclusion of the 1.5 year experiment, the microbial community was strongly influenced by plant species within the Top zone dominated by plant roots, and the presence or absence of a saturated zone influenced the microbial community within the Bottom zone. *Leptospermum continentale* was an outlier from the other plants and had much lower denitrification gene rpm (average 228) compared to the other species (range: 277 to 413). The antimicrobial properties and large root volume of *Leptospermum continentale* likely caused this denitrification gene depression.

INTRODUCTION

Humans have disrupted the global nitrogen (N) cycle by applying fertilizers, burning fossil fuels, and disturbing the landscape (Schlesinger, 2009). The main environmental concerns with excess N are water quality degradation (*e.g.*, eutrophication, habitat loss, etc.) and increased

nitrous oxide (N_2O) emissions associated with global climate change (Conley et al., 2009; Firestone et al., 1980; Howarth, 1998). High N deposition from fossil fuel combustion, fertilizer applications, septic systems, and leaky sanitary sewers contribute to urban N pollution (Bettez and Groffman, 2013; Law et al., 2004; Lovett et al., 2000; Sercu et al., 2008). As urbanization is only expected to increase, there is a strong need to mitigate N pollution from urban areas (Kaushal et al., 2008; United Nations, 2015).

Bioretention, or biofilters, are popular green infrastructure (GI) practices employed throughout the world to reduce urban pollutant loads and provide hydrological benefits to downstream waterways (*e.g.*, Hatt et al., 2009; Li and Davis, 2009; Hunt et al., 2012). However, the N treatment performance within these systems is highly variable (e.g., LeFevre et al., 2015). Plant uptake and microbial denitrification are two mechanisms to internally treat N within bioretention cells.

Denitrification is a stepwise, anaerobic microbial process reducing nitrate to N_2, which is catalyzed by several enzymes: *nap*, *nar*, *nirS*, *nirK*, *nor*, and *nosZ* (Shapleigh, 2013). Denitrification rates are generally highest under anoxic conditions (Smith and Tiedje, 1979; Firestone and Davidson, 1989). Thus, bioretention cells with anaerobic saturated zones (SZ) overlaid by aerobic zones have been suggested as a strategy to reduce N water pollution (Kim et al., 2003). Lab studies reported that the inclusion of a SZ enhanced NO_3^- removal (Kim et al., 2003; Glaister et al., 2014; Payne et al., 2014c). However, to date, field studies on bioretention with SZ have shown variable results. Some studies (Dietz and Clausen, 2006; Hunt et al., 2006; Davis, 2007) have reported that a SZ did not markedly improve N treatment over conventional bioretention design, and Passeport et al. (2009) reported that two bioretention cells with SZ failed to reduce NOx loads, despite significantly reducing total outflow N loads.

In addition to SZ designs, the soil microbial community regulates denitrification and N treatment within bioretention cells. The soil microbial community diversity and composition have been shown to regulate denitrification in the environment (Cavigelli and Robertson, 2000; Rich and Myrold, 2004), but the abundance of denitrification gene and denitrification rates are not always correlated, possibly due to the facultative nature of denitrifiers (Graf et al., 2016; Hallin et al., 2009). Plant-microbe interactions also alter the microbial community and subsequent functions (Hartmann et al., 2009). However, less research has examined the role of plants on denitrification genes, especially in engineered environments like bioretention cells. Patra et al. (2006) noted that nitrate reducer community diversity (via denaturing gradient gel electrophoresis [DGGE]) were affected by plant species within grassland soils. Ruiz-Rueda et al. (2009) reported that plant species affected *nosZ* diversity in constructed wetlands. Bremer et al., (2007) noted that *nirK* diversity was affected by plant species and environmental conditions within grasslands. Graf et al. (2016) noted that soil type was more influential than plant type on the potential denitrification rates and denitrification genes (*nirS*, *nirK* and *nosZ* via PCR) within an agricultural study. Chen et al. (2013) reported that the abundance of denitrification genes (qPCR) in the soils was correlated with inundation time in a bioretention cell, but they did not examine design features or the plant-microbe interactions. Morse et al. (2017) reported that denitrification genes (metagenomics) were greater within often saturated stormwater basins than dry basins. These studies used PCR based techniques (except Morse et al., 2017), which could overlook some of the diverse denitrification genes, and largely neglected plant-microbe interactions or design considerations in stormwater systems. Work is needed to determine how our designs and plant selections influence the microbial community and particularly denitrification, especially in engineered systems designed to remove N.

The objectives of this work were to determine whether: (1) plant species altered the microbial community and the denitrification process, (2) more abundant denitrification genes translated to better N removal from simulated rain events, and (3) a SZ enhanced the relative abundance of functional genes responsible for denitrification, or altered the microbial community at large within bioretention. By using ^{15}N tracers, DNA metagenomics, and 16S profiling, we quantified the microbial community structure, abundance of denitrification genes, and N uptake pathways and overall N removal rates. A better understanding of the interplay between the various physical and biological factors should allow us to optimize bioretention system design to favor the conditions suitable for denitrifier proliferation and, hence, long-term, permanent N removal.

METHODS

This research builds on an extensive mesocolumn study conducted over a 1.5-year period in 2014 (Payne et al., 2014c, 2014b). The water quality monitoring and N portioning, via isotope tracers was completed in 2014. Additional analysis was performed on these systems in 2016 to examine the microbial population within the columns. Soil samples collected in 2014 from the columns were frozen at -80ºC until DNA extraction in 2016; samples were likely unaffected by storage conditions (Lauber et al., 2010). By pairing previously completed monitoring results with the microbial analysis herein, we aimed to provide a comprehensive view of how these systems process N.

Experimental Mesocolumns

This study examines the effects of seven representative plant species and one soil-only control on microbial communities and denitrification within mesocolumns. The plant species studied in these experiments were: Buffalo (BUF), *Carex appressa* (CAR), *Dianella tasmanica*

(DIA), *Allocasurina littoralis* (ALL), *Leptospermum continentale* (LEP), *Juncus krassii* (JUN), and *Gahnia siberiana* (GAH).

The mesocolumn construction and experimental methods are comprehensively described in (Payne et al., 2014c). Single-plant bioretention columns were grown in a greenhouse for a 6-month establishment period and a 1-year monitoring period. Columns were 600 mm high, with a "Top" 300-mm zone consisting of loamy sand and a "Bottom" 300 mm zone consisting of sand and a gravel drainage area. Columns with a saturated zone (SZ) kept the lower 300 mm saturated with an elevated outlet and "Non-saturated zone" (NS) columns allowed free drainage at the bottom of the columns (Figure S5). Table 6 shows the column design layout with the plant species and one soil-only control; there were 27 SZ and 27 NS columns.

Columns were dosed with semi-synthetic stormwater (0.99 mg L^{-1} (NO_3/NO_2)-N, 0.41 mg L^{-1} NH_4-N, 0.38 mg L^{-1} particulate organic N, 0.44 mg L^{-1} dissolved organic N, 0.36 mg L^{-1} total phosphorus (TP), and 150 mg L^{-1} total suspended solids). A dosing volume (3.7 L) was set to reflect a bioretention cell sized to 2.5% of the drainage area, with average precipitation observed for Melbourne (Australia) across a twice-weekly frequency. Outflow concentrations of TN, TP, total dissolved phosphorus and NO_3/NO_2 were measured monthly at the National Association of Testing Authorities, Australia (NATA) lab (Water Studies Centre, Monash University, Australia). At the conclusion of the experiment, root characteristics were destructively sampled. Total root length (cm), volume (cm^3), mass (g), and surface area (cm^2) were determined for each column by scanning 3 subsamples (EPSON Flatbed Scanner EPSON Expression 10000XL 1.8 V3.49) and then analyzing them with WinRHIZO software (v. 2009c, Regent Instruments Canada Inc.). The ratio of the sub-sample dry mass to total dry mass was used to calculate total root length, volume, and surface area for the entire root system.

Table 6. Column design and soil DNA sampling layout. Configuration indicates whether the column had a non-saturated (NS) bottom zone or a saturated (SZ) bottom zone. Soil samples collected for DNA extraction were taken from the bottom (BOT) or top (TOP) zones.

Plant Species	Abbreviation	Configuration	Column Replicates	Sampled for Metagenomics	Sampled for 16S
Allocasurina littoralis	ALL	NS	3	0	0
		SZ	3	0	3 BOT; 3 TOP
Buffalo	BUF	NS	3	1 BOT; 2 TOP	3 BOT; 3 TOP
		SZ	3	2 BOT; 3 TOP	2 BOT; 3 TOP
Carex appressa	CAR	NS	3	3 TOP	3 BOT; 3 TOP
		SZ	3	2 BOT; 3 TOP	2 BOT; 2 TOP
Dianella tasmanica	DIA	NS	3	0	3 BOT; 3 TOP
		SZ	3	3 BOT; 2 TOP	2 BOT; 3TOP
Gahnia siberiana	GAH	NS	3	0	0
		SZ	3	0	3 BOT; 3 TOP
Hypocalmma angustifolium	HYP	NS	3	0	0
		SZ	3	0	3 BOT; 3 TOP
Juncus krassii	JUN	NS	3	0	0
		SZ	3	3 BOT; 3 TOP	3 BOT; 3 TOP
Leptospermum contintale	LEP	NS	3	2 TOP	3 BOT; 3 TOP
		SZ	3	3 BOT; 3 TOP	3 BOT; 3 TOP
Soil Only Control	Soil	NS	3	3 TOP	3 BOT; 3 TOP
		SZ	3	2 BOT; 3 TOP	3 BOT; 3 TOP

Isotope Tracers

^{15}N isotope tracers were used to partition incoming N among plant and microbial assimilation ("assimilation"), microbial denitrification, and soil porewater. Full details are described in (Payne et al., 2014b). Briefly, a 1:1 $Na^{14}NO_3$:$Na^{15}NO_3$ nutrient solution was applied to the columns during two trials. Prior to tracer addition and thereafter every 6 hours, samples were collected from inflow solution and porewater and analyzed for NH_4^+ and NO_3^-. Dissolved N_2O, $^{28}N_2$, $^{29}N_2$, and $^{30}N_2$ were also analyzed from the samples to determine denitrification rates. N_2O and N_2 concentrations were analyzed by gas chromatography using a NCA GSL2 elemental analyzer coupled to a Hydra 20-22 isotope ratio mass-spectrometer (IRMS; Sercon Ltd., UK).

Linear regression of $^{29}N_2$ and $^{30}N_2$ production calculated ^{14}N denitrification and ^{15}N denitrification rates, respectively. The total amount of ^{15}N denitrified was calculated by integrating the rate of denitrification over the 12-hour monitoring period. The amount of $^{15}NO_3^-$ remaining in the pore water was calculated based on the final $^{15}NO_3^-$ concentration at 12 hours. The proportion of $^{15}NO_3$ assimilated was calculated as the difference between ^{15}N denitrified and ^{15}N remaining in the pore water.

Soil Metagenomics and 16S

We collected soil samples from the middle of the Top (TOP) and Bottom (BOT) zones of the columns at the end of the 1.5-year experiment. Soils were frozen at -20ºC prior to DNA extraction. Total DNA was extracted from each sample using the Mo-Bio PowerSoil® DNA Isolation Kit as per manufacturer's instructions. The effluent was frozen at -80ºC until sequencing. Prior to sequencing, DNA extract concentrations were quantified using a Quant-It hsDNA assay kit (ThermoFisher).

Metagenomic and 16S rRNA sequencing was conducted at Micromon (Monash University, Australia). Metagenomic sequencing libraries were prepared using the Nextera XT library preparation kit (Illumina) according to the manufacturer's instruction. A total of 1ng of DNA was used as input, and the libraries were purified with 0.6V of AxyPrep Mag PCR Cleanup beads (Axygen). All library types were quantitated using a Qubit fluorimeter and Quant-IT DNA HS kit (Invitrogen) as per the manufacturer's instructions, and sized using an Bioanalyzer 2100 and DNA HS kits (Agilent) according to the manufacturer's instructions. The libraries were denatured and diluted for sequencing using a NextSeq 500 (metagenomic libraries) and NextSeq 500 Mid-Output V2 (150cycles) kit or a MiSeq and MiSeq V2 (600 cycle) Reagent Kit (16S amplicon libraries) according the manufacturer's instructions.

Samples were selected to cover a range of plant species types, and also to coincide with the isotope tracer study previously conducted (Payne et al., 2014b). Forty-nine samples were sequenced for DNA metagenomics based on quantification results (Table 6; each sample was from an individual replicate column), thus some treatments did not have the full n=3 as DNA was not successfully extracted from each column. Approximately 10M 150 base pair (bp) single-strand reads were sequenced for each sample (49 samples x 10M reads = 490M reads total). Raw data can be accessed via the European Sequencing Archive (Study: PRJEB24343). Next, DIAMOND (double index alignment of next generation sequencing data), a high throughput alignment program comparing DNA sequence reads to reference sequences, was performed with the sample DNA sequences against a custom, manually curated database of reference proteins critical for N cycling. This is analogous, but faster, than BLASTx (Buchfink et al., 2015). DIAMOND returned a matrix of matches for each sequence, within each sample; results were filtered where percent sequence identity (pid) >50, and assigned read length >25 amino acids. Once functional gene count reads were obtained, they were normalized by total reads per sample and multiplied by one million to obtain reads per million reads (rpm), which is a relative abundance term.

Denitrification was the process of interest for complete N removal herein. Therefore, analyses focused on functional genes associated with denitrification: nitrate reductase (*nap* and *nar*), nitrite reductase (*nirK* and *nirS*), nitric oxide reductase (*cNor* and *qNor*), and nitrous oxide reductase (*nosZ*). The sum of all these genes is herein referred to as 'total denitrification genes', which are expressed as rpm. While all these genes are indeed part of the denitrification pathway, *nap* and *nar* are also used in other dissimilatory nitrate reduction processes.

Taxonomic assignment with the metagenomics sequences was conducted via kmer mapping and matched with known genes from the JGI/IMG database; additional details are given

in the Supplementary Materials. The percentage of reads that could be assigned to a gene varied, but ranged from 50-70%. The name of the source of the gene sequences were extracted and then used to query NCBI to receive complete taxonomic description of the source.

16S rRNA amplicon sequencing was conducted within all plant treatments and the soil-only control. Samples were selected to coincide with the DNA metagenomics samples, and also include additional plant treatments to increase statistical power among treatments. Eighty-four samples were sent for 16S sequencing, although 3 samples failed to amplify, therefore a total of 81 samples are included in this analysis. 16S rRNA amplicon libraries were prepared by amplifying the V3+4 region in 50µL reactions: 1µM forward (TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG) and reverse (GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC) primers, 5uL of genomic DNA, 2x HiFi HotStart ReadyMix (KAPA) made up to volume with UltraPure water (Invitrogen). The PCR program considerations are given in Supplementary Materials. Approximately 40,000 16S sequences were completed per sample. Quality filtering and OTU picking procedures have been described elsewhere (Henry, 2016; Schang et al. 2016). The assembled reads were analyzed using the QIIME 1.8.0 closed-reference OTU picking workflow with UCLUST for de novo OTU picking and the GreenGenes 13_8 release (Caporaso et al., 2010). Data for 16S samples are available on the Short Read Archive, project reference PRJNA309092 (ncbi.nlm.nih.gov/bioproject/PRJNA309092). Following removal of singletons (18% of OTUs) and doubletons (9% of OTUs), and rarefraction without replacement to 30,000 reads per sample, 8,122 OTUs remained in the 81 samples. Summarization of relevant OTUs included only those with a relative abundance ≥0.2%. All other analyses used the entire rarefied dataset. Alpha and

Beta diversity assessments were done with rarefied data including singletons and doubletons. The phyloseq package in R was used for 16S analysis.

Statistical Analyses

We used R software (version 3.1.1; R Development Core Team, 2014), and a criteria of 95% confidence (a=0.05) was applied for all statistical analyses. Nitrogen treatment and isotope tracer results through the columns approximately 3 months prior to soil DNA collection were used to compare against DNA data.

For metagenomics results, we used analysis of variance (ANOVA) to test if plant species significantly affected denitrification gene rpm between the plant treatments. Tukey honest significant differences (HSD) post-hoc tests determined what plant species, if any, differed significantly from each other. The influence of a SZ on total denitrification genes rpm was assessed with a mixed effects model. Total denitrification gene rpm was the dependent variable, sample location (Top vs Bottom zone) was the independent fixed effect, and plant species was the random effect variable. This controlled for random variation from species to species, while allowing an overall effect of SZ to be determined. Spearman correlations were calculated for total denitrification gene rpm, % denitrified, % assimilated, and % NOx removal to determine relationships among these variables.

ANOVA was used to compare observed and Chao1 α-diversity differences between groups. Principal coordinate analysis (PCoA) from pairwise Unifrac and weighted Unifrac distances for the 16S data were created to visualize differences between groups, and analysis of similarity (ANOISM) tested differences between groups.

RESULTS AND DISCUSSION

Soil Metagenomic Denitrification Genes

Total denitrification genes rpm within the SZ columns were significantly and positively correlated with % denitrified N (r = 0.69) (Table 7). Table 7 illustrates the correlations between the columns; all values are the measured value for the entire column (no TOP or BOT distinction), except for the total denitrification reads, which are the average for that column. This indicated that the denitrification genes rpm corresponded to increased denitrification function, thus supporting the use of total denitrification genes rpm as a biological marker for actual denitrification. The % denitrified in NS columns could not be determined, as the tracer method required sampling from porewater, which was unavailable in the NS columns.

Table 7. Spearman correlation values for total denitrification gene rpm, % NOx reductions, and partitioning of N as % denitrified and % assimilated determined via ^{15}N tracer. Correlations with total denitrification genes were calculated using DNA results from the sum of both Top + Bottom zones of SZ columns (*n*=17). Root length, surface area, volume and mass are all total values from the column. Values in **bold** are significant ($p<0.05$).

	NOx removal	**Denitrified**	**Assimilated**	**Total Denitrification genes**	**Root Length**	**Root Surface Area**	**Root Volume**	**Root Mass**
	%	%	%	rpm	cm	cm^2	cm^3	g
NOx Removal	1	**-0.94**	**0.90**	**-0.79**	0.50	**0.64**	**0.80**	**0.61**
Denitrified		1	**-0.91**	**0.69**	**-0.58**	**-0.66**	**-0.73**	**-0.69**
Assimilated			1	**-0.77**	**0.66**	**0.79**	**0.84**	**0.73**
Total Denitrification Genes				1	-0.20	-0.33	**-0.55**	-0.32
Root Length					1	**0.92**	**0.78**	**0.77**
Root Surface Area						1	**0.93**	**0.86**
Root Volume							1	**0.79**

Plant effects on denitrification genes and microbial community

We examined the effect of plant species on total denitrification genes rpm in SZ columns (NS had too few samples for analyses). Plant species effects were significant only within the Top zones ($p<0.001$), and not within the Bottom zones ($p=0.17$) (Figure 4). The Top zone was influenced by plant roots, which were largely absent from the Bottom zones (Payne et al., 2014c). Thus, the plant effects were relegated to the upper 30 cm of the soil column (Top Zone). These metagenomic results align with other research where plant roots and their exudates are likely key drivers in soil microbial communities (Chandrasena et al., 2017; Grayston et al., 1998; Haichar et al., 2008).

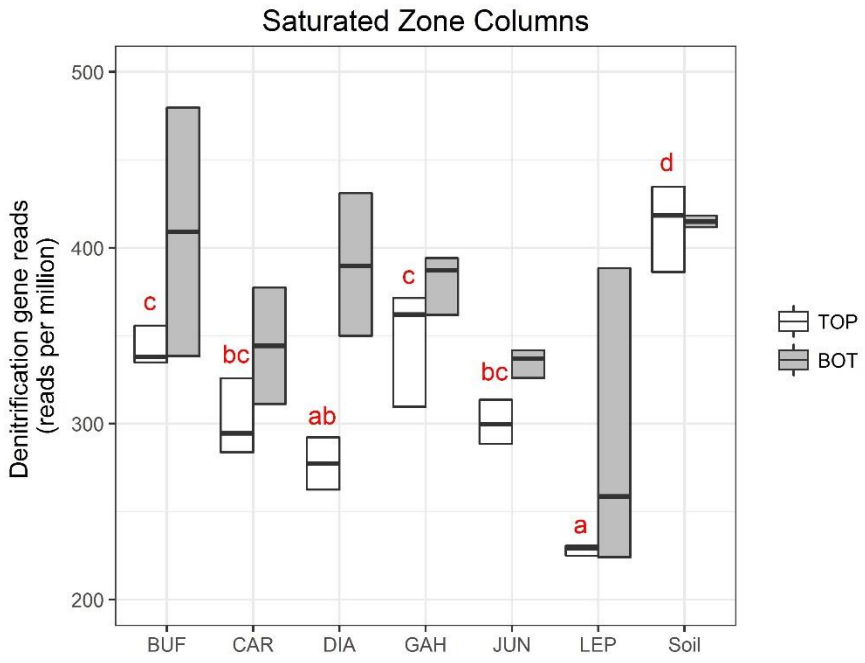

Figure 4. Total denitrification gene reads for SZ columns. TOP = Top zone, BOT = Bottom zone. Differences between plant species within Top zone treatments are shown by differing letters as calculated by Tukey HSD ($p<0.05$). No statistical differences between Bottom zone treatments were observed.

Plant species effects on individual denitrification genes were also explored to determine if specific genes within the pathway were driving observed differences (Figure S6). Analysis of variance was conducted with the seven individual denitrification genes rpm on the Top zone of SZ columns (Table 8). *Nar*, *nirK*, *qNor*, and *nosZ* rpm were all significantly affected by plant species type (p-values <0.05). Generally, these genes and the total denitrification genes rpm followed the same trend where LEP had the lowest (224-230 rpm), DIA, CAR, and JUN were mid-level (262-325 rpm), and GAH, BUF and Soil had the highest (309-434 rpm). Because the entire pathway was significantly affected by plant species type, it is unlikely that individual genes were selected preferentially by any plant species. Rather, it is plausible that certain plants fostered an environment agreeable to denitrifiers in general. These results align with Rich et al. (2003) and Bremer et al. (2007), who used qPCR to show that *nirK* and *nosZ* gene abundance were influenced by specific plant species.

Table 8. Analysis of variance (ANOVA) results for individual and total denitrification genes tested against plant species. Tukey HSD differences (p<0.05) classified which plant species were significantly different from each other; a=lowest relative abundance of gene, and d=highest relative abundance. All samples were from the Top zone of SZ columns.

	ANOVA	Tukey HSD plant differences					
	p-value	*a*	*ab*	*b*	*bc*	*c*	*d*
Nap	0.2	NA	NA	NA	NA	NA	NA
Nar	**<0.001**	LEP	DIA, CAR, JUN	GAH, BUF	NA	Soil	NA
NirK	**<0.001**	LEP	DIA, CAR, JUN	GAH, BUF, Soil	NA	NA	NA
NirS	0.43	NA	NA	NA	NA	NA	NA
cNor	0.15	NA	NA	NA	NA	NA	NA
qNor	**0.02**	LEP	DIA, CAR, JUN	GAH, BUF, Soil	NA	NA	NA
NosZ	**<0.001**	LEP	DIA	JUN	CAR	GAH, BUF	Soil
Total Denit	**<0.001**	LEP	DIA	NA	CAR, JUN	GAH, BUF	Soil

The soil-only control had the highest total denitrification gene rpm (mean of 413 rpm; Figure 4), which could be due to lack of plant competition for available N. Without plants using N, a soil microbial community may adapt to encourage N utilization (as is the case with denitrification). Indeed, the total root length, surface area, volume and mass were all negatively correlated with % denitrified and positively correlated with % assimilated (Table 7). Total denitrification genes were also negatively correlated with total root volume (Table 7, r = -0.55), which could indicate that plants with greater root volume outcompeted microbes for N and led to a decrease in denitrification genes. LEP had the lowest total denitrification genes rpm (mean 228 rpm), and the greatest root volume (mean 164.16 cm3). The greater root volume in LEP could explain the depressed denitrification reads, although the antimicrobial properties of LEP (Demuner et al., 2011; Prosser et al., 2014) might also be depressing denitrifiers, or giving advantages to more competitive microorganisms. Chandrasena et al. (2014) and Li et al., (2016) both found that LEP significantly enhanced *E. coli* die-off relative to other plant species. It is unclear if denitrifiers are as susceptible to antimicrobials from LEP, but it could explain the lower rpm we observed in this treatment.

Within the 16S data, the overall abundance of unique Operational Taxonomic Units (OTUs) via observed and Chao1 α-diversity (Table 9) were not significantly different for plant species in the Top zone (ANOVA p=0.119). This could indicate the presence or absence of OTUs (α-diversity) is less affected by plants than the proportion of specific groups, such as denitrifiers.

Genus level differences within the most abundant OTUs above 0.2% relative abundance were examined between the plant species (Figures 5A and 5B). Interestingly, *Nitrospira* relative abundances in the Top zone of both NS and SZ LEP treatments (0.007% and 0.005%, respectively) were an order of magnitude lower than all other plant species (ranged from 0.07% to 2.6%; Figure

5A). *Nitrospira* was significantly positively correlated with total denitrification genes rpm in Top zone samples ($r = 0.77$, $p < 0.001$). Thus, we suspect the depression of this taxa by LEP could depress denitrification within this plant treatment. *Nitrospira* are nitrite-oxidizing bacteria that convert nitrite (NO_2^-) to NO_3^- during nitrification (Altmann et al., 2003). Siripong and Rittmann (2007) noted that *Nitrospira* were the dominant NO_2^- oxidizing bacteria in activated sludge systems. It is possible reductions in this upstream nitrification process via reduction of the *Nitrospirae* phylum and *Nitrospira* genus is limiting available NO_3^- used in denitrification, and depressing denitrification genes and rates in LEP. Denitrifiers are phylogenetically diverse, occurring in a wide array of phyla (Chèneby et al., 2000), so we could not do a similar analysis with a specific "denitrification" genus.

Table 9. 16S observed and Chao1 (mean ±se) alpha diversity for the Top zones of SZ columns

	Observed		**Chao1**	
Plant Species	**mean**	**SE**	**mean**	**SE**
ALL	2,451	62.4	3,304	123.3
BUF	2,393	114.1	3,195	181.3
CAR	2,304	88.5	3,032	96.6
DIA	2,240	112.4	2,997	203.2
GAH	2,358	42.0	3,173	75.2
HYP	2,456	12.6	3,327	52.0
JUN	2,220	78.7	2,867	159.0
LEP	2,169	23.4	2,805	34.8
Soil-Only	2,342	63.8	3,237	65.3

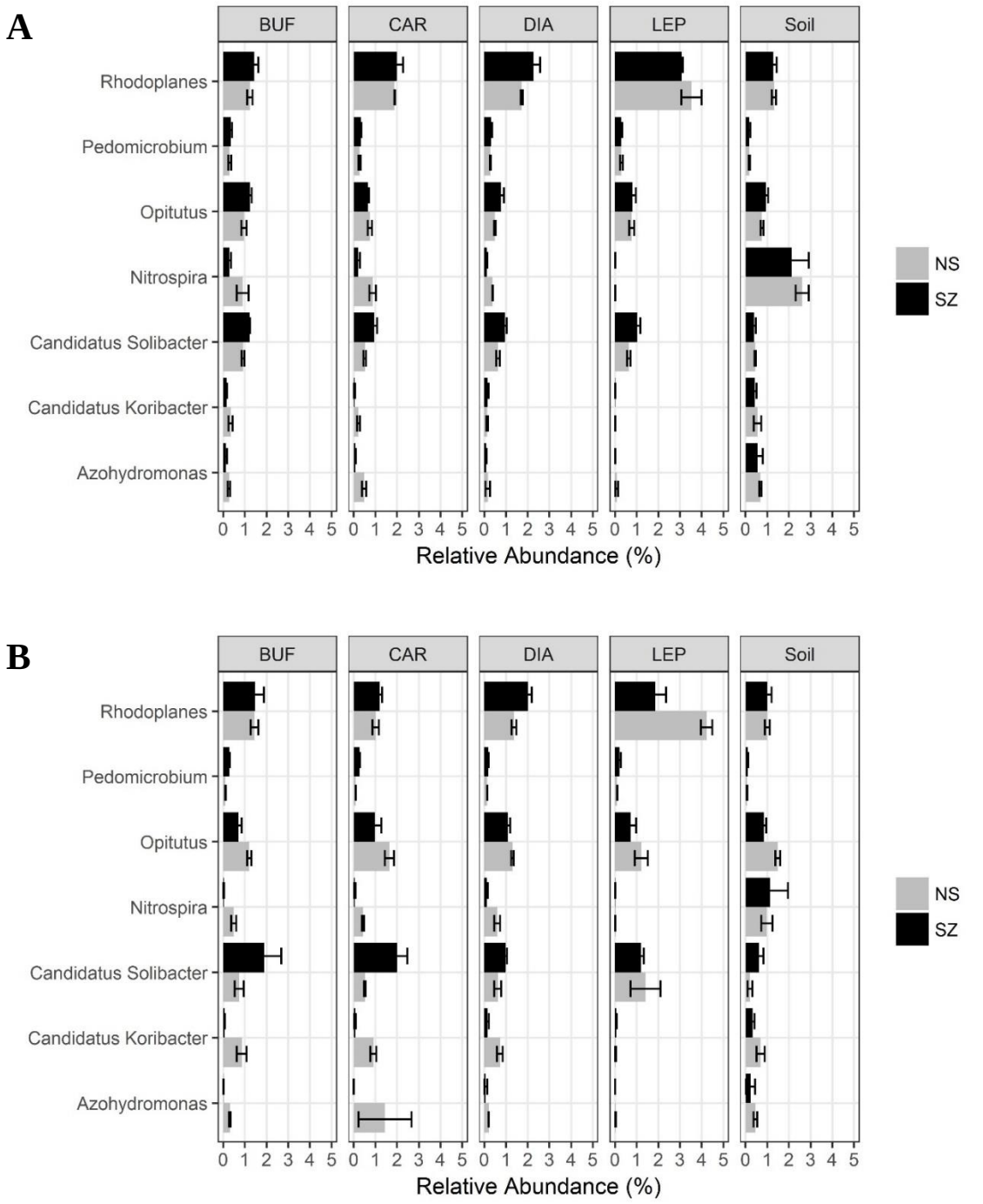

Figure 5. Mean relative abundance of genus across plant species with a saturated zone (SZ) or without (NS) in the (**A**) Top zone or (**B**) Bottom zone. Genus shown are those with relative abundances >0.2%. Only plant species with both NS and SZ samples are shown.

We investigated the taxonomic distribution of *nirK* and *nosZ* to determine if plant species affected the members of the community responsible for these processes. Among the assigned reads, the *Proteobacteria* phylum remained dominant across all plant species accounting for ~70% of the *nirK* reads (Figure S7A). For *nirK*, the *Nitrospirae* phylum relative abundance (% of reads within that sample belonging to that phylum) was depressed within the LEP treatment while those assigned to the *Actinobacteria* phylum increased. It is unclear why *Nitrospirae* organisms have *nirK* genes, since these organisms are thought to be NO_2 oxidizers, not reducers. The *Actinobacteria* phylum is a large and diverse group of bacteria, which is capable of antibiotic production in soils (Barka et al., 2016). Perhaps because of their ability to produce antibiotics, these organisms were unaffected by the antimicrobials produced by LEP. Of the assigned *nosZ* reads, the *Bacteroidetes* and *Proteobacteria* phyla were dominant across plant species, accounting for ~40% and 30% of the total assigned reads, respectively (Figure S7B). The LEP treatment had the lowest proportion of reads assigned to *Acidobacteria.* It seems likely these bacteria were impacted by plant produced antimicrobials. However, the environmental controls on this diverse phylum are poorly understood (Kielak et al., 2016).

Microbial effects on NOx treatment

NOx reductions were greater in vegetated SZ columns (89 % average) than NS columns (72 % average) (Figure S8) (Payne et al., 2014c). It is worth noting these high removal rates may be greater than we would expect in field trials because the columns were dosed with relatively small water volumes, which is not likely to flush N out of the systems, unlike real-world storms. The N isotope tracer results within the SZ columns revealed that assimilation (plant and microbial) was the predominant fate of incoming N to these columns (77-98%) (Payne et al., 2014c). The soil-only control had approximately 38% assimilation, presumably all due to microbial uptake

(Payne et al., 2014b). Therefore, plant assimilation rates were likely around 39-60%, and were the largest driver of N removal within vegetated columns. In contrast, the proportion denitrified was significantly less (1-7%) (Payne et al., 2014b). According to ANOVA results, *Juncus krassii* (JUN), *Buffalo* (BUF), *Carex appressa* (CAR), *Allocasurina littoralis* (ALL), and *Leptospermum continentale* (LEP), (average 1-3%) had significantly less denitrification than *Dianella tasmanica* (DIA) (average 7%), and the soil-only control had the highest relative denitrification rates (average 15%).

Overall, plant species with high assimilation, NOx removal, and large root volume were correlated with lower denitrification gene rpm, and denitrification % (Table 7). This contradicts our hypothesis that greater NOx removal would correlate with higher denitrification genes rpm. Plants with higher root volume likely outcompeted microbes for N, and/or altered the microbial community to reduce denitrification genes. The mechanisms and effects of plant and microbial competition of available N is an active area of research. It is widely accepted that microbes are key regulators of N cycling, but plants influence the microbial communities and their subsequent abilities to cycle N (Knops et al., 2002; Van Der Heijden et al., 2008). In this study, the observation that plants with large root volume had high NOx removal rates, high plant and microbial assimilation % and lower denitrification % (and total denitrification genes rpm) leads us to speculate that highly effective plant species out-compete denitrifiers for available N, and depress denitrification genes. This is aligned with the results from (Moreau et al., 2015), where plants with greater N uptake decreased the abundance of nitrate reducing bacteria observed via qPCR. While we did not divide assimilation into plant or microbial mechanisms, it appears that plants fostering an environment where either (or both) plant or microbial assimilation flourish also diminish denitrification.

Studies suggest that microbes are better short-term competitors for N, but that plants may be superior in the long term (Jackson et al., 1989; Hodge et al., 2000). These were mature columns (~1.5 years after establishment), and it is likely the partitioning of incoming N may vary during periods of plant establishment, or with higher N loading. Consequently, a larger denitrifier community may indeed facilitate better N treatment during alternative conditions not tested in this experiment. Further research in this area could elucidate how microbial N cycling affects water quality over time.

Even though assimilation was the largest driver of NOx removal, it is important to remember microbial and plant assimilation is not a permanent removal strategy unless plants are harvested. Consequently, we should look to promote the complete removal of NOx through denitrification by avoiding highly competitive plants that decreased denitrification genes rpm, like *Leptospermum continentale.*

Comparison of saturated and non-saturated zones

Figures 6A and 6B display the PCoA plots for the Top and Bottom 16S zone samples, respectively; note post-hoc tests between the 10 groups were not possible due to small sample size. In the Bottom zones, the NS and SZ groups were significantly different for both Unifrac and weighted Unifrac distances (ANOSIM: $p<0.001$, $R=0.58$ and 0.48, respectively). This relatively high R-value indicates a robust difference between the NS and SZ groups. *Candidatus Solibacter* relative abundance was significantly greater (t-test $p<0.001$) within SZ columns (4.3%) than NS columns (2.1%) (Figure 5B). This genus is associated with carbon metabolism and NO_3^- and NO_2^- reduction, although denitrification capabilities were not found (Pearce et al., 2012). Both processes are pertinent for denitrification and, perhaps, the SZ is fostering an environment conducive for denitrification by reducing NO_3^- and making it available to denitrifiers.

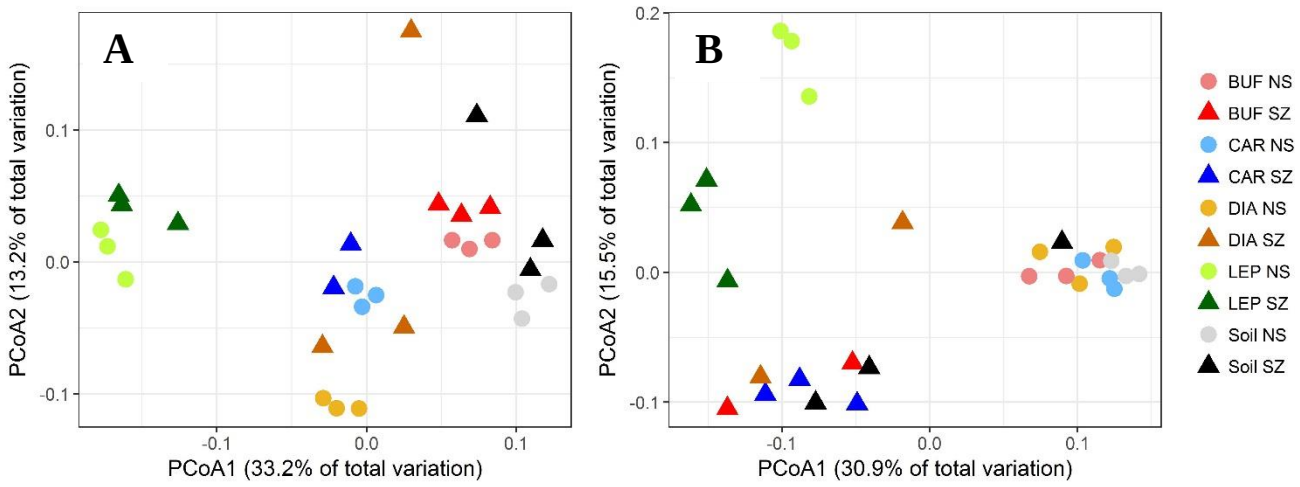

Figure 6. PCoA of weighted Unifrac distances from the 16S results within (**A**) Top zone samples and (**B**) Bottom zone samples. NS = non saturated zone and SZ = saturated zone. Significant differences between NS and SZ groups were shown with ANOISM for Top zone samples but not the Bottom zone; post-hoc tests between groups were not possible due to small sample size.

Conversely, within Top zones neither the unweighted or weighted Unifrac distances were significantly different between NS and SZ groups (p=0.06, p=0.09, respectively), and the R-values (0.08 and 0.07, respectively) were considerably lower than observed for the Bottom zones. This indicates the saturated Bottom zone is having little effect on the overlying aerobic Top zone. Previously discussed results of significant plant species differences and the clustering of samples by plant type indicate plant effects on the Top zone samples are more pronounced than the Bottom zone.

Ideally, comparisons of denitrification genes rpm within the Bottom zones of columns of SZ and NS would highlight the effect of this design alteration. However, we were unable to extract adequate DNA from NS Bottom zones for metagenomics sequencing. The greater abundance of DNA suggests SZ increased the entire microbial community and could explain the observed higher NOx removal rates (Payne et al., 2014c). Total denitrification genes rpm were similar across the

Top zones of NS and SZ columns (Figure S9); only LEP had significantly less in SZ columns (t-test $p=0.01$). This suggests that the underlying SZ is not influencing the denitrification community within the rhizosphere of the roots within the top zones.

In general, Bottom zones within SZ columns had higher total denitrification genes rpm than Top zones (Figure 4). Sample location (Top vs. Bottom zone) was a significant predictor of total denitrification genes ($p<0.001$) within a mixed effects model for SZ columns. On average, total denitrification genes rpm increased from 318 to 367 rpm when going from the Top to Bottom zone. This supports our hypothesis that a SZ enhances denitrification, otherwise the relative abundances would have been approximately equally distributed throughout the column, which was not the case. This is expected because the Bottom SZ may encourage the anaerobic process of denitrification, while the aerobic Top zone may not. SZ columns also had better overall NOx treatment than their NS counterparts. Direct comparisons of % denitrified from SZ and NS columns was not possible because the isotope tracer could not be used on the NS columns. However, because % denitrified and total denitrification genes rpm were correlated, it is likely the SZ columns had better NOx treatment due to increased denitrification. The results reported here suggest a SZ is enhancing permanent removal of N through denitrification within these systems. Consequently, SZ design modifications in areas targeting N removal should be promoted.

ACKNOWLEDGEMENTS

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SUPPORTING INFORMATION:

Metagenomic Kmer matching methods

DNA sequences for a particular gene of interest were obtained from sequenced genomes at JGI/IMG. Kmers were extracted from each sequence using kcompress using default settings (BBMap - Bushnell B. - sourceforge.net/projects/bbmap/). Bbduk was then used to match kmers from reads to the kmer data set produced from kcompress using a kmer length of 21. The reads extracted from this step were clustered using cluster_fast program of vsearch with an identity of 0.97 (Rognes et al., 2016). This set of reads was then matched to sequences in the DNA data set using usearch_global in vsearch.

16S rRNA PCR considerations

The reactions were subjected to a PCR program consisting of an initial denaturation for 3 minutes at 95ºC, followed by 25 cycles of denaturation at 95ºC for 30s, annealing at 55ºC for 30 seconds and extension at 72ºC for 30 seconds. A final extension was carried out at 72ºC for 5 minutes. The reactions were purified using 0.6 volumes of AxyPrep Mag PCR Cleanup beads (Axygen), washed twice with 200µL 96% ethanol and resuspended in 25µL of UltraPure water (Invitrogen). The purified amplicon were subjected to a limited-cycle secondary PCR amplification to add Illumina-compatible adapters and per-sample indexes. Reactions were assembled to contain 25µL KAPA HiFi HotStart ReadyMix (KAPA), 5µL each of the Nextera XT Index 1 and Index 2 primer and from the Nextera XT Index Kit (Illumina), 5µL of purified V3+4 amplicon, then made up to 50µL with UltraPure Water (Invitrogen). The reactions were subjected to a PCR program consisting of an initial denaturation for 3 minutes at 95ºC, followed by 8 cycles of denaturation at 95ºC for 30s,

annealing at 55ºC for 30 seconds and extension at 72ºC for 30 seconds. PCR reactions were purified as indicated above. The purified amplicons constituted the final sequencing library for each sample.

Sequencing data were demultiplexed using MiSeq Reporter V2.4.60 and quality trimmed and adapter filtered using Trimmomatic (Bolger et al. 2014). Reads were filtered and trimmed to remove adapters and any terminal stretches of bases at or below Q30 (Bolger et al. 2014). Reads shorter than 180 nt were discarded. Pre-cluster read pairs were trimmed and filtered to produce single reads using PEAR (Zhang et al. 2014).

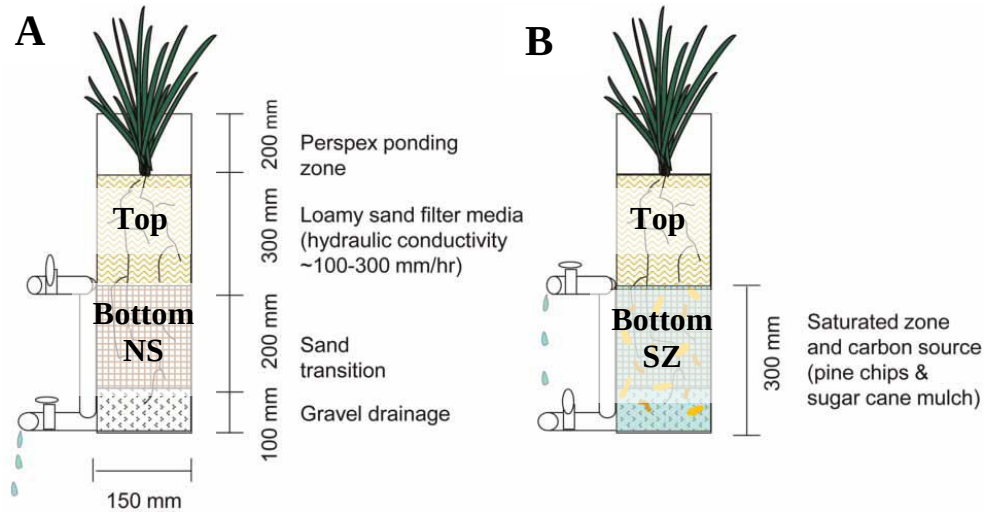

Figure S5. Mesocolumn set-up: (A) non-saturated (NS) and (B) saturated zone (SZ)

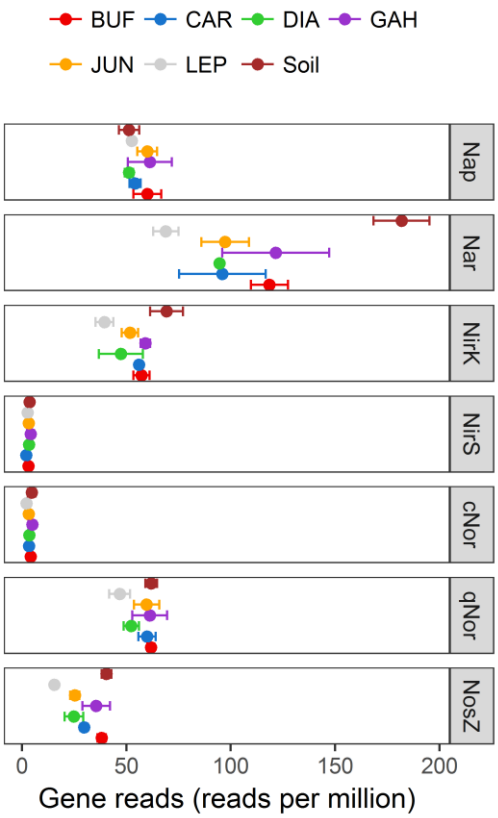

Figure S6. Gene reads of individual denitrification genes (rpm) for the Top zone of SZ columns.

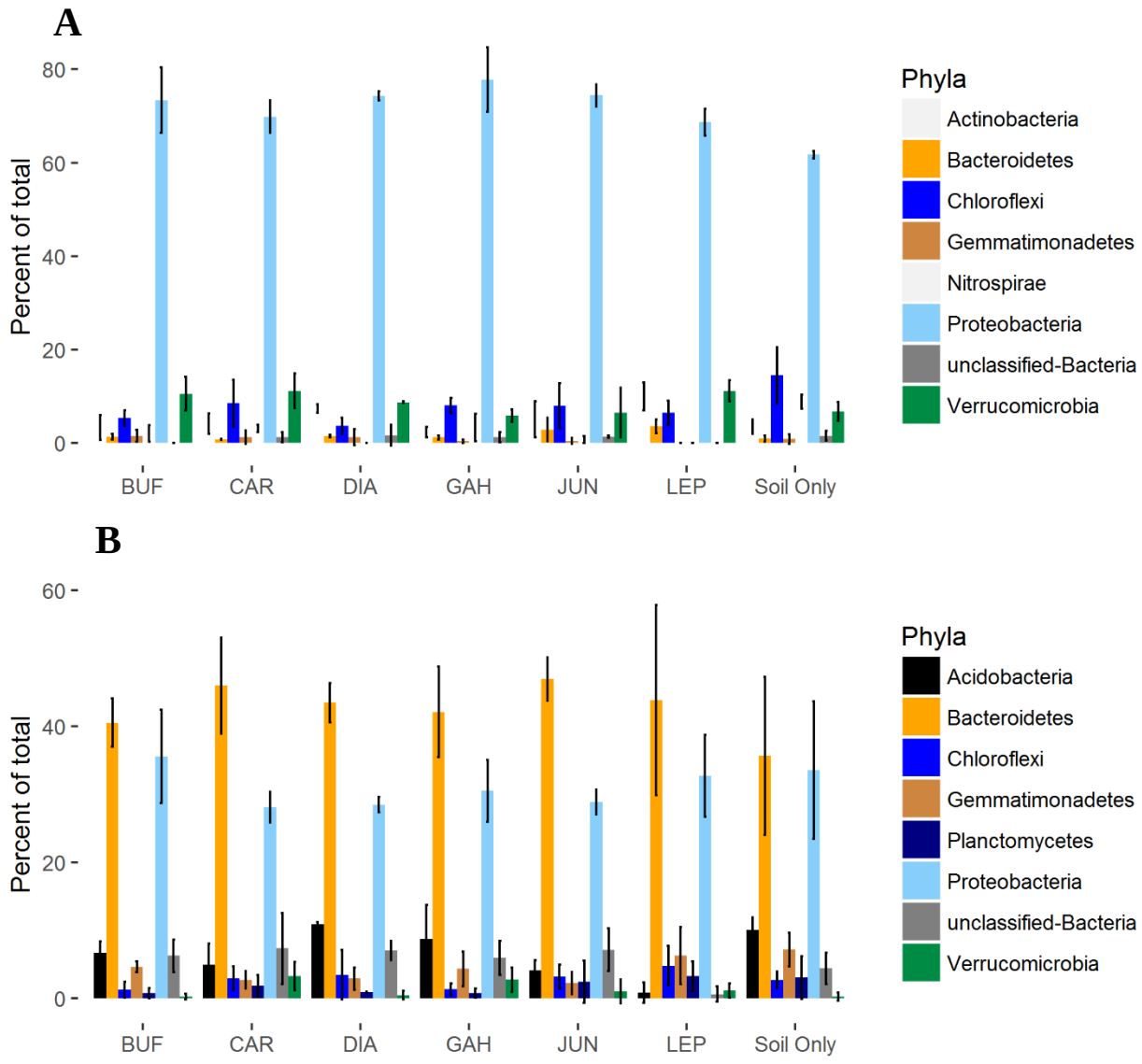

Figure S7. Relative abundance of (A) *NirK* gene reads (% of gene reads within that sample belonging to that phylum), and (B) *NosZ* gene reads within the Top zone of SZ columns for each plant species. Error bars are SD.

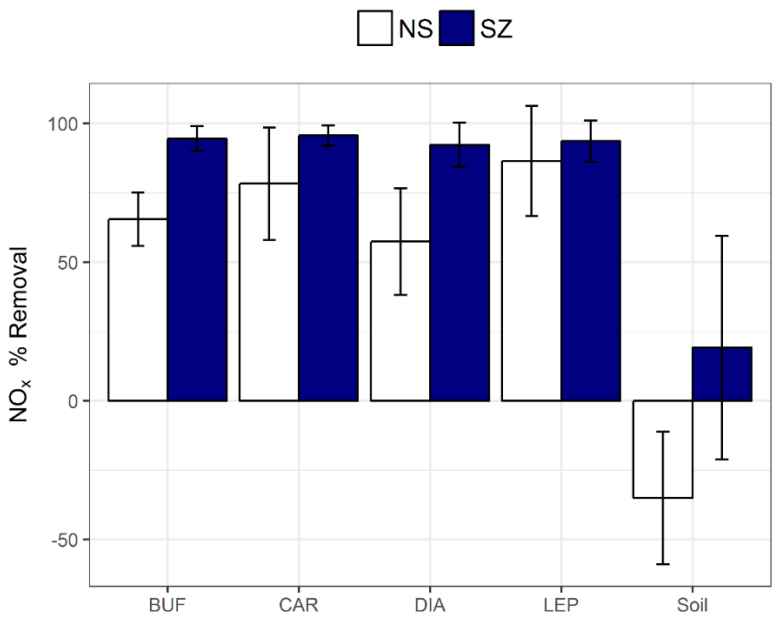

Figure S8. Average NOx removal rates from columns with a SZ and without NS. Error bars are standard deviations (n=9).

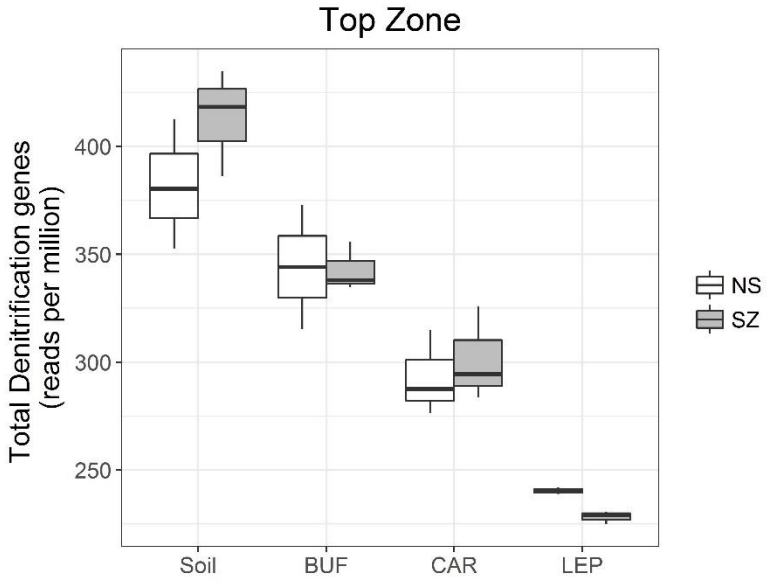

Figure S9. Total denitrification genes (rpm) within the Top zones of columns with a SZ and without NS. NS=no saturated zone, SZ = saturated zone.

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CHAPTER 3: COMPARING GREENHOUSE GAS FLUXES FROM PASSIVE URBAN STORMWATER MANAGEMENT TO CONVENTIONAL WASTEWATER TREATMENT

ABSTRACT

Green infrastructure (GI) is a popular tool to improve city resilience to climate change and improve water quality. However, because these systems receive pulses of nutrient rich stormwater, they may be contributing excess greenhouse gas (GHG) emissions. This study quantifies the amount of $CO_{2\text{-eq}}$ emitted per L of stormwater treated for both GI basins and conventional wastewater treatment plants (WWTP). GHG and stormwater monitoring were conducted at two stormwater basins in Ithaca, NY: one site was often saturated (Anaerobic Basin), and the other was quick draining (Aerobic Basin). In opposition to our hypothesis, rain events did not produce larger CH_4 or N_2O fluxes at the basins. Compared to literature values of field-scale monitoring at WWTPs (220.3 mg $CO_{2\text{-eq}}$ L^{-1}), the Anaerobic Basin produced similar $CO_{2\text{-eq}}$ (230.0 mg $CO_{2\text{-eq}}$ L^{-1}), but the rates were 55X lower in the Aerobic Basin (4.0 mg $CO_{2\text{-eq}}$ L^{-1}). Using EPA calculations, SW sent to a WWTP would produce 7X more CO_2 than if it were sent to a Dry Basin. Conversely, SW sent to a Wet Basin instead of the WWTP would lead to an approximately 8X increase in CO_2. This shows that some GI (aerobic treatments) may be a benefit to reduce GHG emissions instead of the alternative WWTP. However, anaerobic GI (Wet Basin) my actually increase GHG emissions over the traditional WWTP. These results are especially relevant to combined sewer systems, where decisions must be made to upgrade decaying infrastructure and/or invest in GI. From a GHG perspective, installing aerobic GI practices would likely reduce the GHG emissions from cities.

INTRODUCTION

Global climate change is expected to radically alter the global environment by increasing temperatures, augmenting rainfall patterns, increasing drought and flood conditions in varying locations, disrupting habitats and decreasing biodiversity. More than half the global population lives in urban areas (United Nations, 2015), and urban spaces have a significant carbon footprint (Dodman, 2009). Many cities have recently acknowledged their large greenhouse gas (GHG) emissions and are working to curtail emissions. For example, the Carbon Neutral Cities Alliance (CNCA) represents 20 international cities who propose an 80% GHG emission reduction by 2050 (Urban Sustainability Directors Network, 2018). In a similar vein, cities are working to combat frequent and large flooding events associated with climate change (Rosenzweig et al., 2010). Thus, they are installing Green infrastructure (GI) to create resilient infrastructure and manage flooding in urban areas (Ahern, 2011; Foster et al., 2011).

GI has been shown to reduce nuisance flooding, and improve water quality (Wilson et al., 2014; Loperfido et al., 2014; LeFevre et al., 2015), but the GHG fluxes from these systems are less understood. Because urban GI are designed and manipulated to store and treat excess stormwater runoff, GI systems may experience prolonged wetting and drying regimes. This altered hydrology may make them hot-spots (McClain et al., 2003) for biogeochemical processes such as methanogenesis and denitrification. The high rates of biogechemical processes may release harmful GHGs like nitrous oxide (N_2O) or methane (CH_4). Consequently, cities interested in lowering their carbon footprint while also combatting flooding from climate change may be at odds with determining which stormwater infrastructure to install.

A very popular GI practice, stormwater basins (or bioretention), are engineered depressions in the landscape with specific media and plants designed to detain and treat stormwater

runoff. Because of the basin-like design, and their ability to retain high soil moisture, these systems may be inclined to facilitate biogeochemical processes that release excess GHGs. (Grover et al., 2013) showed occasionally high N_2O and CH_4 fluxes from stormwater basins following storm events. (McPhillips and Walter, 2015) also showed that basins with consistently high soil water content emitted higher CH_4 (2,755 ug CH_4-C m^{-2} hr^{-1}) than drier basins (-11 ug CH_4-C m^{-2} hr^{-1}). These results indicate that stormwater basins may be sources of GHG fluxes. However, an annual budget or comparisons to alternative treatment methods is lacking. In combined sewer systems (CSS) stormwater is sent to wastewater treatment plants (WWTP) for treatment. In the USA, CSS serve an estimated 40 million people in 772 cities (U.S. EPA, 2004). Consequently, the CSS has a large footprint and potential to alter water quality and GHG emissions from major cities.

The stormwater has to go somewhere (or flooding occurs), and thus planners must choose between sending it to conventional WWTP or GI. Cities and decision makers need to better understand how GHG emissions are altered with their choice in stormwater management. This research asked the question, if stormwater usually sent to WWTPs was sent to GI, would GHG fluxes be decreased? This study aimed to compare GHG fluxes from stormwater basins to conventional WWTPs) which are the alternative treatment technology for stormwater runoff.

METHODS

Study Sites

This study utilized two stormwater basins (one slow draining 'Anaerobic Basin' and one fast draining 'Aerobic Basin') on Cornell's campus in Ithaca, New York. At seven occasions in 2013, these sites were monitored for N_2O and CH_4 fluxes (McPhillips and Walter, 2015), and for soil metagenomics and nitrogen cycling processes in 2016 (Morse et al., 2017b). This study builds off these earlier works, with a deeper focus on the GHG budget. While both basins were designed

following New York Department of Environment and Natural Resources (NYDENR) guidelines, either due to construction error or ecosystem succession, the Anaerobic Basin developed into a wetland like basin, while the Aerobic Basin remained dry. The basins were all originally planted with turfgrass (primarily perennial ryegrass- *Lolium perenne*) and have 10-15 cm topsoil underlain by native silt loam and a bottom layer of sand. Below the sand is an underdrain (perforated pipe) that helps drain the system. Consequently, this study exploited these differing wetness regimes to infer how alternative designs may influence stormwater treatment performance and GHG fluxes. Table 10 depicts the site characteristics.

Table 10. Study site characteristics.

		Anaerobic Basin	Aerobic Basin
Drainage Area	m^2	11,049	4,249
Basin Area	m^2	1,500	480
Drainage Area: Basin Area	ratio	7.37	8.85
Year Constructed	Year	2004	2006
Impervious Drainage Area	%	95	100
Contributing Drainage Area		Parking Lot	Parking Lot

Soils were previously characterized for texture, bulk density, total carbon (C), metals, pH, nitrate, and salinity (Morse et al., 2017b). The soil characteristics are shown in Supplementary Information Table S2.

Greenhouse Gas Monitoring

Greenhouse gas monitoring was conducted at three locations within the basin. *In-situ* static chambers monitored GHG fluxes, CH_4, N_2O and CO_2, at each basin (Hutchinson and Mosier, 1981). Monitoring was conducted approximately weekly from April 2016 to October 2016, and May 2017 to October 2017. Rainfall events could influence GHG fluxes in basins because the

influx of water and nutrients may stimulate biogeochemical processes releasing GHGs (Xu et al., 2004; Molodovskaya et al., 2012). Therefore, this study specifically monitored GHG fluxes from ambient dry weather conditions and immediately after storm events (within 24-hours). Storms ranged in size and duration, but storms had to be at least 2 mm in depth and have at least one dry day between storm events to be categorized as "storms".

A single gas flux (g m^{-2} min^{-1}) measurement consisted of four gas samples collected at 0, 10, 20 and 30 minutes after the chamber was closed. These samples were injected into pre-evacuated 10-ml glass serum vials. Gas samples were analyzed via gas chromatography (GC; Agilent 6890N). Flux was calculated as the rate of change of concentration over the chamber deployment time. Fluxes were converted from volumetric to mass-based units (μg gas m^{-2} hr^{-1}) using the ideal gas law.

During GHG monitoring soil volumetric water content (VWC), soil temperature, and electrical conductivity were monitored at each of the 3 sample locations at each site. A FieldScout TDR 100 Soil Moisture Meter monitored soil VWC, and a FieldScout Direct Soil EC Meter Testr11+ monitored soil temperature and electrical conductivity.

Outliers

Outliers were defined as observations beyond four standard deviations away from the mean flux value. Data points that were identified as outliers were removed for further calculations. Less than 3% of fluxes were identified as outliers. This was done so that excessive outliers would not bias the mean and standard deviation.

Calculation of GHG mass per volume of stormwater

Because GHG monitoring was not continuous, we summed the GHGs emitted over the monitoring period and divided this mass by the total stormwater runoff for the same period to calculate the CO_2 fluxes per L of water treated ($CO_{2\text{-}eq,L}$; mg $CO_{2\text{-}eq}$ L^{-1}).

The total $CO_{2\text{-}eq}$ fluxes over the monitoring period were calculated by multiplying the average flux rate at each timepoint by the duration between samples at each sample location (Equation 1).

$$CO_{2-eq,S*} = \sum_{t=i}^{t=n} \frac{CH_{4,ti+1}+CH_{4,ti}}{2} \times (t_{i+1} - t_i) \times 25 + \frac{N_2O_{ti+1}+N_2O_{ti}}{2} \times 298 \quad [1]$$

Where $CO_{2\text{-}eq,S*}$ = total $CO_{2\text{-}eq}$ fluxes over the monitoring period at each sample location (mg $CO_{2\text{-}eq}$ m^{-2} monitoring $period^{-1}$), *S** indicates distinct sample locations (* =1, 2, or 3 within each basin), *t* = timepoint of sample collection (day) from initial (*i*) to final sample date (*n*), N_2O = nitrous oxide flux (mg N_2O-N m^{-2} day^{-1}), CH_4 = methane flux (mg CH_4-C m^{-2} day^{-1}). CH_4 and N_2O fluxes were converted to $CO_{2\text{-}eq}$ using EPA recommended values of 25 and 298, respectively (U.S. Environmental Protection Agency, 2015). We then averaged the three $CO_{2\text{-}eq,S*}$ values, and multiplied it by the stormwater basin area (m^2), for a total mass of $CO_{2\text{-}eq}$ emitted. Then, this value was divided by the total stormwater runoff volume over the monitoring period (Equation 2) to calculate the $CO_{2\text{-}eq}$ per L of water treated ($CO_{2\text{-}eq,L}$; mg $CO_{2\text{-}eq}$ L^{-1}).

$$CO_{2-eq,L} = \left[CO_{2-eq,S1} + CO_{2-eq,S2} + CO_{2-eq,S3}\right] \times \frac{1}{3} \times BA \div SWV \quad [2]$$

Where *BA* = basin area (m^2), and *SWV* = stormwater volume runoff (L).

Stormwater Monitoring

Stormwater quantity was completed at each site from May 2016 to November 2016, and from May 2017 to October 2017. The stormwater monitoring is discussed in (Morse et al., 2017b).

Briefly, inflow volume was determined based on the U.S. Soil Conservation Service (SCS) Curve Number method and precipitation measured on site via rain gauges, and outflow volume was determined via a v-notch weir-box fitted with HOBO pressure-transducer water depth loggers.

Calculated and literature values for WWTP fluxes

EPA and IPCC guidelines were used to calculate the CH_4 and N_2O fluxes per volume of water treated from WWTPs (EPA, 2015; IPCC, 2006). The equations used to calculate the CH_4 and N_2O fluxes for different WWTP systems are given in the Supplementary Materials. Then, depending on the system (aerobic, anaerobic, and with or without denitrification) the CH_4 and N_2O fluxes were converted to $CO_{2\text{-eq}}$ and summed for a total $CO_{2\text{-eq,L}}$ estimate per volume of water treated.

Because CH_4 fluxes are driven by BOD load within the EPA guidelines, we investigated the possible consequences of a combined wastewater and stormwater load. (Wang et al., 2013) noted the variability in wastewater and stormwater compositions, and thus used the same pollutant concentrations for wastewater and CSO events associated with mixed stormwater and wastewater wet weather flows. (Gasperi et al., 2008) reported that dilution by runoff in a CSS reduced the median BOD concentration from 140 mg L^{-1} to ~70 mg L^{-1}. Thus, we compared GHG fluxes from full strength wastewater, a mix of wastewater and stormwater (WW+SW) where BOD concentration was set to half that of wastewater, and a 100% stormwater scenario where BOD concentration was set to the national stormwater runoff average (20.3 mg L^{-1}) reported in the NURP study (Makepeace et al., 1995).

Because WWTPs vary in size and design, an effort was made to quantify the general range of GHG fluxes from a wide variety of WWTPs. Descriptions of the field-scale monitoring studies used herein are presented in Table 11.

Table 11. Literature used to quantify WWTP GHG emissions. All studies were full-scale field monitoring studies, except Monteith et al. 2005 which did full-scale monitoring but used these values to model results of generic WWTP properties as presented in this table.

Author	Date	WWTP Description	Monitored GHGs	Location
Monteith et al.	2005	Primary treatment and anaerobic digestion Activated sludge + anaerobic digestion	CH_4, CO_2	Canada
Ahn et al.	2010	separate-stage biological nitrogen removal plug-flow	N_2O	USA
Wang et al.	2011	step-feed non-biological nitrogen removal anaerobic/anoxic/oxic	CH_4	China
Daelman et al.	2013	activated sludge, anoxic, aerobic	CH_4, N_2O	Netherlands
Yan et al.	2014	Orbital oxidation ditch anoxic/anaerobic/aerobic anaerobic/anoxic/aerobic	CH_4, N_2O, CO_2	China

Statistical Analyses

The statistical analysis was conducted using R software (version 3.1.1; R Development Core Team, 2014). A criteria of 95% confidence (α=0.05) was selected for all analyses herein. Paired t-tests were used to compare differences in N_2O or CH_4 fluxes between the basins; site average fluxes were used for each sample date. The fluxes themselves were non-normal, but the differences between the fluxes (as is tested in a paired t-test) were normal, and thus a paired t-test instead of nonparametric tests was used for this comparison. Wilcoxon rank sum tests were used to test whether GHGs during ambient or rain conditions were significantly different at each site. Mixed-effects models were used to determine which factors were significant predictors of N_2O or CH_4 fluxes. Because fluxes were non-normal ranked values of fluxes were used as the dependent variable within these models. Model AIC scores were compared to select the best model and

determine which factors (Site, precipitation, soil temperature, and soil water content) were significant.

RESULTS

Soil Characteristics

Average soil characteristics are presented in Table S2. Both Anaerobic and Aerobic sites had near-neutral pH, 7.3 and 7.5, respectively. The Anaerobic Basin had slightly higher total C (10.7 %) than the Aerobic Basin (5.3%). All sites were classified as sandy loam based on the U.S. Department of Agriculture (USDA) soil classification.

Climatic Conditions

Over the monitoring period, a total of 18 ambient and 15 rain or storm GHG sampling events were collected. The average rain event prior to GHG sampling was 10.44 mm (7.15 sd). The average air temperature during ambient and rain events was fairly similar at 18.05ºC (5.72) and 18.49 ºC (5.38), respectively. During the 2016 and 2017 monitoring periods, rainfall patterns remained fairly consistent (Figure 7).

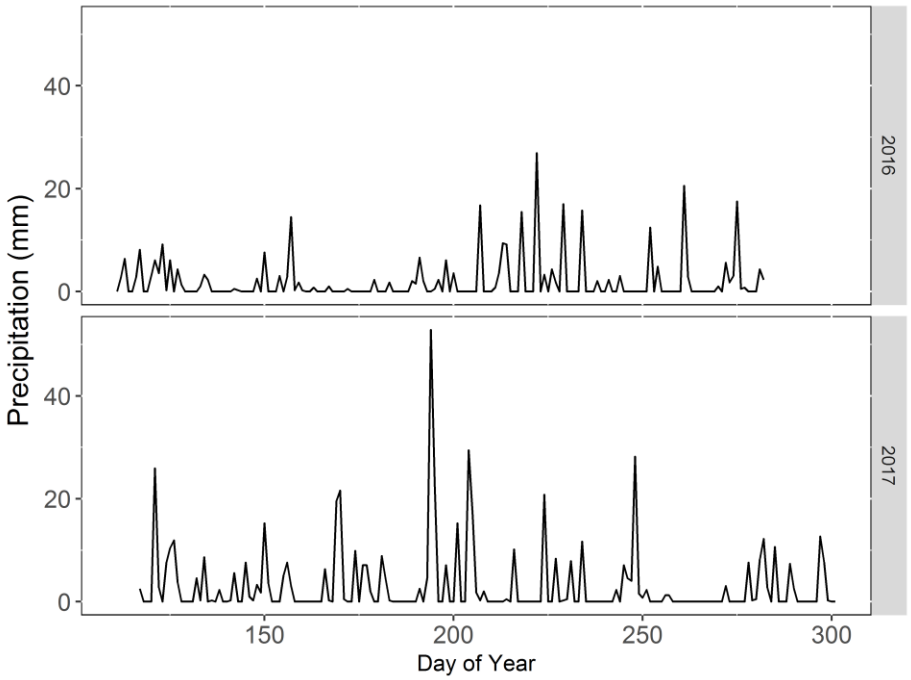

Figure 7. Rainfall patterns during the 2016 and 2017 monitoring periods.

Greenhouse Gas Monitoring

Approximately 1.6% and 0.6% of CH_4 and N_2O fluxes respectively, were removed as outliers since their absolute values were greater than 4sd from the mean (Table S3). Figure 8 displays the N_2O $CO_{2\text{-eq}}$ and CH_4 $CO_{2\text{-eq}}$ fluxes over the monitoring period. The Anaerobic Basin had significantly greater CH_4 fluxes than the Aerobic Basin (paired t-test $p<0.001$). The average CH_4 $CO_{2\text{-eq}}$ fluxes at the Anaerobic and Aerobic Basins were 106.3 (±95.1 sd), and -1.36 (±5.6) mg m^{-2} hr^{-1}, respectively. N_2O fluxes from the Anaerobic Basin and Aerobic Basin were not significantly different (paired t-test $p= 0.65$). However, the average N_2O $CO_{2\text{-eq}}$ flux at the Anaerobic Basin (8.21 mg m^{-2} hr^{-1} (±15.0) was approximately twice that of the Aerobic Basins (4.0 mg m^{-2} hr^{-1} (±18.34). Combining the CH_4 and N_2O fluxes on each sampling date, the average $CO_{2\text{-eq}}$ fluxes were 116.0 (±95.0) mg m^{-2} hr^{-1} and 2.64 (19.1sd) mg m^{-2} hr^{-1}, for the Anaerobic and Aerobic Basins, respectively. Overall, the Anaerobic Basin had greater $CO_{2\text{-eq}}$ fluxes than the

Aerobic Basin ($p<0.001$). This higher $CO_{2\text{-eq}}$ flux rate within the Aerobic Basin was largely driven by CH_4 fluxes (Figure 8).

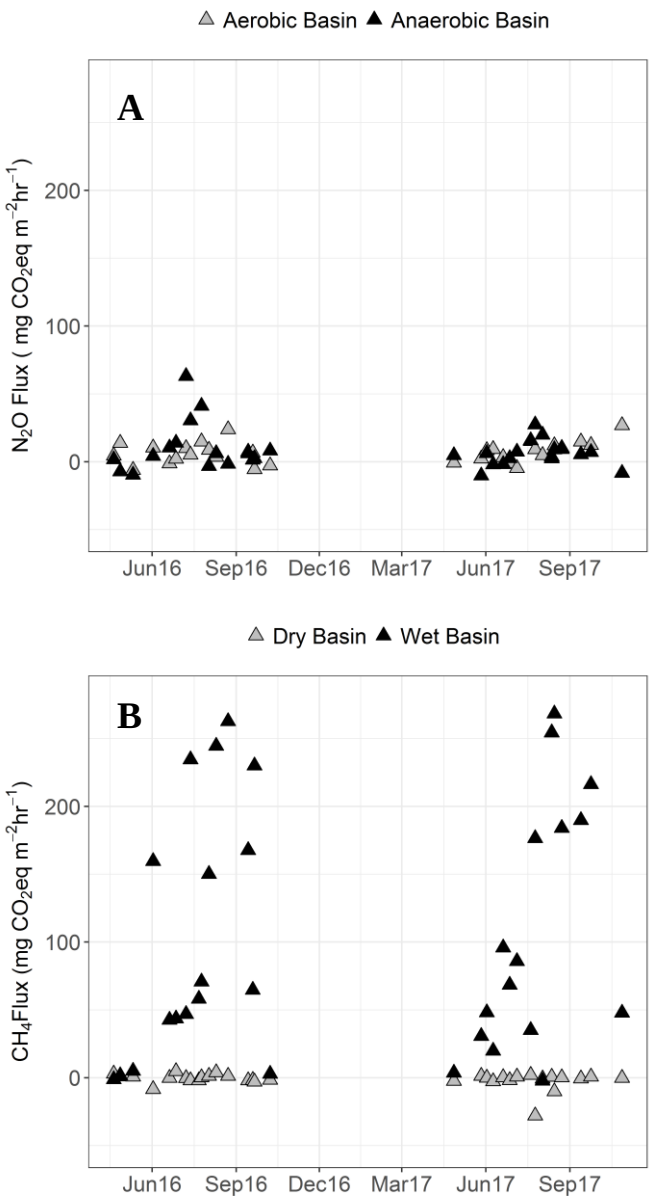

Figure 8. (A) N_2O $CO_{2\text{-eq}}$ m^{-2} hr^{-1} and (B) Site average CH_4 $CO_{2\text{-eq}}$ m^{-2} hr^{-1} over the monitoring period at each site. Each point is the site average at that sample date (n=3). Dates are given in month year format (Jun16 = June 2016).

Drivers of GHG Fluxes

Neither CH_4 nor N_2O fluxes were statistically different during ambient or rain conditions (Wilcoxon rank sum tests p-values > 0.1). This was counter to our hypothesis that GHG fluxes

would be higher immediately following a rain event. Figure 9 illustrates the range of CH_4 and N_2O fluxes (in $CO_{2\text{-eq}}$) for both basins during ambient or rain conditions.

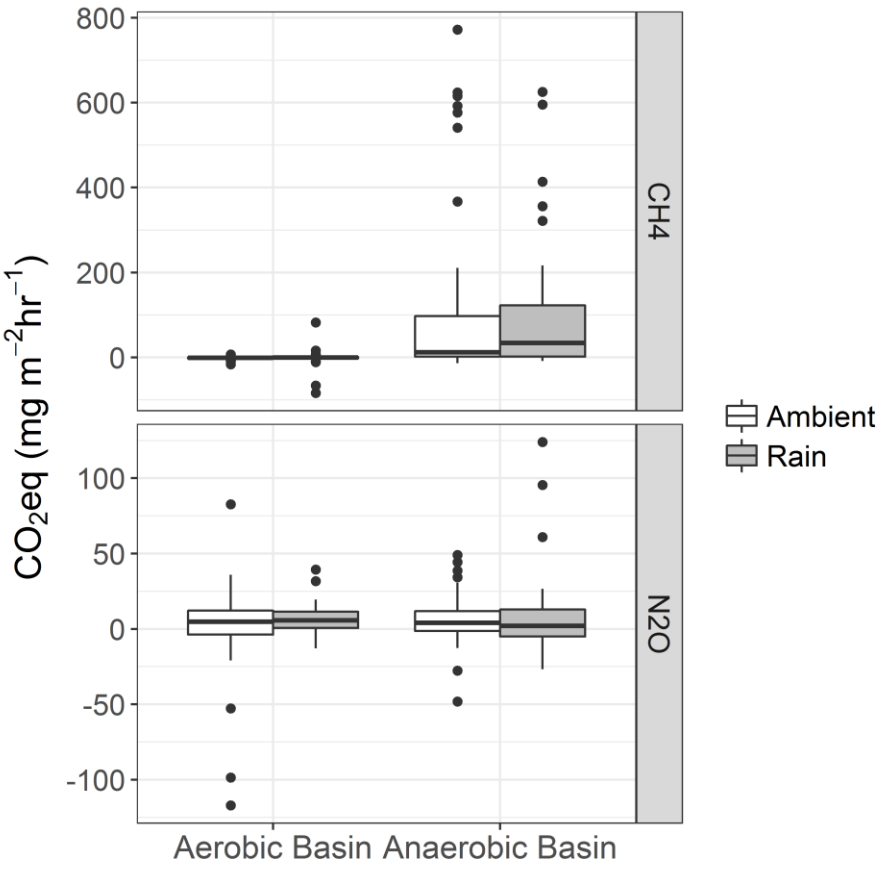

Figure 9. Boxplot of individual flux rates measured ambient conditions and immediately following a rain/storm event. Fluxes for CH_4 and N_2O are given in $CO_{2\text{-eq}}$. No differences were noted between the Ambient and Rain groups at either site.

GHG fluxes were likely unaffected by rain events because the soils at the time of sampling were not wetter than during ambient conditions. Comparisons of soil wetness on individual sample points during ambient or rain conditions were all insignificant (t-tests, $p>0.05$). This is likely due to the soil characteristics at each site. The soils at the Aerobic Basin stayed fairly dry due to their quick draining nature, conversely at the Anaerobic Basin, soils were usually very wet regardless of rainfall (Figure 10).

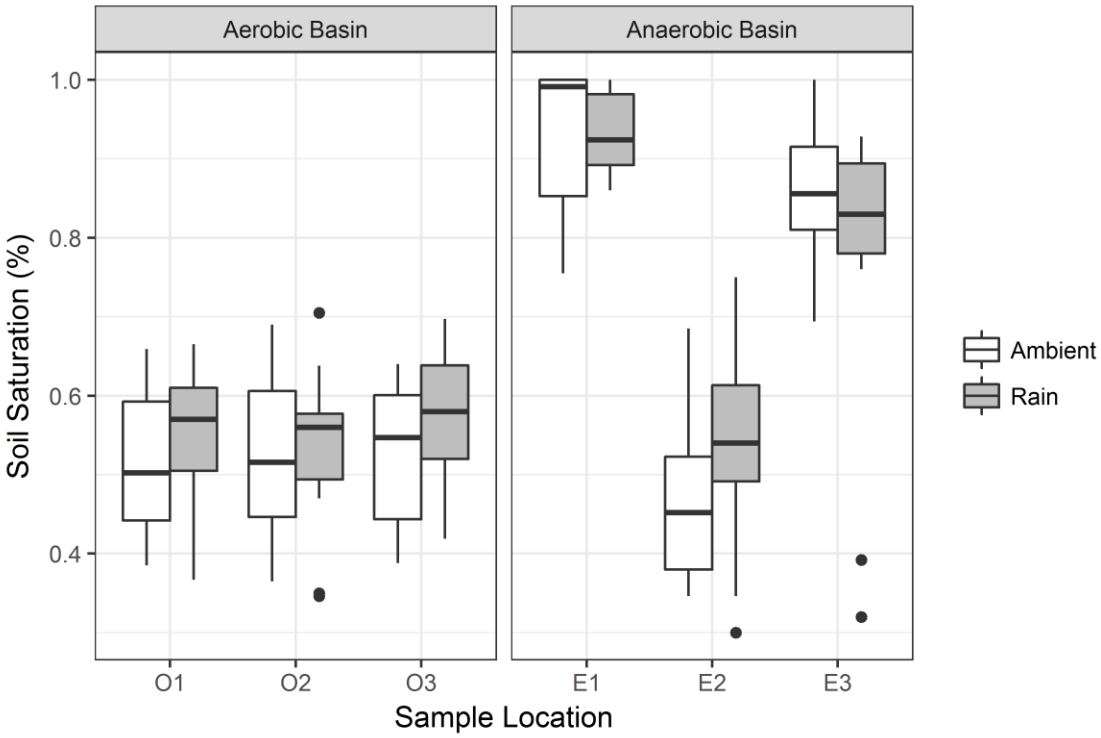

Figure 10. Soil saturation (%) measurements at each sample location within the Aerobic and Anaerobic Basin during Ambient or Rain conditions.

Mixed-effects models were used to determine if site (Anaerobic vs Aerobic), precipitation depth (mm), soil wetness VWC (%), soil electrical conductivity (mS/m) or soil temperature (ºC) were significant predictors of CH_4 or N_2O fluxes. Because flux values were non-normal, and transformations failed to produce normal data, rank values of the fluxes were used as the dependent variable within these models. Table 12 shows the model output results. For CH_4, site ($p=0.032$),

soil temperature ($p<0.001$), and soil VWC ($p<0.001$) were significant predictors. The best performing model (that with the lowest AIC score) removed precipitation as an exploratory factor (Table 12). For N_2O, only soil temperature was a significant predictor ($p=0.032$), although site, precipitation and electrical conductivity remained in the model they were not significant predictors. This supports our earlier finding that rain events did not produce higher flux rates for N_2O or CH_4. CH_4 fluxes were also higher in the Anaerobic Basin (positive value for Site parameter, Table 3), which supports our earlier finding that the Anaerobic Basin had significantly greater CH_4 fluxes. Notably, soil temperature was a significant predictor of both N_2O and CH_4. This is supported by previous research where increased temperatures were associated with increased N_2O and CH_4 emissions (Segers, 1998; Smith et al., 1998).

Table 12. Mixed-effects model parameters and p-values. Note, due to non-normal data the dependent variables of interest were rank transformed. Site = Anaerobic or Aerobic, Precip = precipitation depth (mm) immediately prior to sampling, Temp = soil temperature (ºC), EC= soil electrical conductivity VWC = soil volumetric water content (%). Values with "NA" indicate that these parameters were excluded from the model based on model AIC score.

Dependent Variable = rank(CH_4)			
	Value	df	p-value
Site	43.54	4	**0.032**
Precip	NA	NA	NA
Temp	2.51	182	**<0.001**
EC	-4.38	182	0.54
VWC	1.12	182	**<0.002**
Dependent Variable = rank(N_2O)			
	Value	df	p-value
Site	5.40	4	0.615
Precip	-0.75	183	0.206
Temp	2.26	183	**0.032**
EC	-1.22	183	0.841
VWC	NA	NA	NA

Greenhouse Gas Fluxes per Volume of Water Treated

Because GHG fluxes were not driven by rain events, we used all the GHG fluxes over the study period regardless of when they were collected. Using Equations 1 and 2, the total $CO_{2\text{-eq}}$ per L of stormwater treated were calculated for the Anaerobic and Aerobic Basins (Table 13). The Anaerobic Basin had a much larger flux rate of 230.0 mg $CO_{2\text{-eq}}$ L^{-1}, than the Aerobic Basin, 4.0 mg $CO_{2\text{-eq}}$ L^{-1}. This is in-line with our previous findings that larger CH_4 fluxes, and overall $CO_{2\text{-eq}}$ L^{-1} occurred in the Anaerobic Basin. Comparisons to actual field monitoring studies reveal the average literature rate of 220.3 mg $CO_{2\text{-eq}}$ L^{-1} to be very similar to the Anaerobic Basin rate from this study. The Aerobic Basin is emitting approximately 55X lower $CO_{2\text{-eq}}$ than the literature WWTPs. Using the EPA guidelines, a CO_2-eq L^{-1} rate was calculated for full strength wastewater, a 50/50 mix of wastewater and stormwater, and 100% stormwater in a WWTP (Table 13). A mock scenario where 50L of wastewater and 50L of stormwater must be treated is also shown in Table 13. With this scenario, the total emissions are dominated by the wastewater (54.9 $gCO_{2\text{-eq}}$), which we assumed to always be treated in a WWTP (*i.e.*, no wastewater is sent to a stormwater basin). The estimated emissions for treating the 50L of stormwater in a WWTP, the Wet Basin, or the Dry Basin are 1.5, 11.5, and 0.2 g $CO_{2\text{-eq}}$, respectively. This indicates that shunting stormwater to a Wet Basin could lead to a nearly 8X increase in $CO_{2\text{-eq}}$ emissions instead of a WWTP. Conversely, sending the stormwater to the Dry Basin would reduce $CO_{2\text{-eq}}$ emissions by a factor of 7.

Table 13. Average and standard deviation (sd) values of $CO_{2\text{-eq}}$ per L of water treated. The average CO_2-eq emissions for an idealized scenario of treating 50L of water is also presented. Literature values are from sources in Table 11. "50/50 Wastewater and Stormwater" indicates that the WWTP has an equal proportion of wastewater and stormwater. The source water for the literature values is assumed to be 100% wastewater, although some mixing with stormwater is possible.

Source Water	Treatment Location	Calculation	Volume L treated	Mean Rate mg CO_2 L^{-1}	SD Rate mg CO_2 L^{-2}	Mass g CO_2
Wastewater	WWTP	EPA Calculation	50	1097.1	973.0	54.9
Stormwater	WWTP	EPA Calculation	50	30.0	5.6	1.5
Stormwater	Wet Basin	Measured in this study	50	230.0	264.8	11.5
Stormwater	Dry Basin	Measured in this study	50	4.0	7.8	0.2
Wastewater	WWTP	Monitored in the Literature	50	220.3	250.5	11.0
50/50 Wastewater and Stormwater	WWTP	EPA Calculation	50	555.5	486.5	27.8

CONCLUSIONS

The objective of this study was simple: to compare $CO_{2\text{-eq}}$ fluxes per volume of water treated in stormwater basins and WWTPs. This research highlights the $CO_{2\text{-eq}}$ flux variability, both within WWTPs and stormwater basins. The Anaerobic Basin clearly emits less $CO_{2\text{-eq}}$ per L than the Aerobic Basin. Consequently, stormwater management system designs to improve water quality and curtail GHG fluxes may consider strategies to minimize saturated conditions.

In the absence of monitoring data, municipalities and cities may use the US EPA and/or IPCC guidelines (EPA, 2015; Metz et al., 2007) to estimate WWTP $CO_{2\text{-eq}}$ fluxes. In this study, if

1 L of stormwater went to an Aerobic Basin instead of a conventional WWTP, it would reduce $CO_{2\text{-eq}}$ fluxes by approximately 7X. Unfortunately, this same level of CO_2 reduction was not observed for the Anaerobic Basin, which produced approximately 8X the $CO_{2\text{-eq}}$ as the WWTPs. In this scenario, where stormwater within the WWTP could dilute the BOD concentration by half, the wastewater and stormwater mix (WW+SW) would produce approximately 140X and 2X more $CO_{2\text{-eq}}$ than the Aerobic Basin or Anaerobic Basin, respectively. These results are especially relevant to CSS, where decisions must be made to upgrade decaying infrastructure and invest in new technology like GI.

For example, Wang et al. (2013) conducted a life cycle assessment on scenarios where stormwater was managed with a CSS (base case) or by diverting the stormwater to newly constructed GI. In their analysis the effect of constructing GI resulted in excess GHG emissions over the base case where the CSS treated both wastewater and stormwater. However, their analysis did not consider on-site GHG emissions from either WWTPs or GI. Consequently, if these emissions were factored in, the small increase of CO_2 from installing GI could possibly be negated. For instance, the yearly $CO_{2\text{-eq}}$ emissions associated with installing an aerobic stormwater basin, 70 kg $CO_{2\text{-eq}}$ $year^{-1}$, would be added to the total $CO_{2\text{-eq}}$ emitted treating the yearly runoff (1,012,000L $year^{-1}$ x 4.0 mg $CO_{2\text{-eq}}$ L^{-1} = 4.0 kg $CO_{2\text{-eq}}$ $year^{-1}$) for a total of 74.0 kg $CO_{2\text{-eq}}$ $year^{-1}$. Similarly, the 30 kg $CO_{2\text{-eq}}$ $year^{-1}$ associated with maintaining a WWTP would be added to the total $CO_{2\text{-eq}}$ emitted from treating the runoff in the WWTP over that year (1,012,000L $year^{-1}$ x 1,097.1 mg $CO_{2\text{-eq}}$ L^{-1} = 1,110.3 kg $CO_{2\text{-eq}}$ $year^{-1}$) for a total of 1,140.3 kg $CO_{2\text{-eq}}$ $year^{-1}$.

Cities have recently become aware of their impact on the global carbon budget, and are working to reduce emissions. For example, the Carbon Neutral Cities Alliance (CNCA) represents 20 international cities who aim to reduce GHG emissions by 80% by 2050 (Urban Sustainability

Directors Network, 2018). Adopting GI technology could be one potential way to reduce GHG emissions within these cities. As waste and wastewater constitute ~3% of anthropogenic GHG emissions (Metz et al., 2007), any improvements to this sector could greatly reduce the GHG footprint of cities.

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SUPPLEMENTARY INFORMATION

Table S2. Average basin soil characteristics.

		Anaerobic Basin	Aerobic Basin
pH		7.3	7.5
Carbon	%	10.7	5.3
Cu	mg kg^{-1}	12.8	11.9
Pb	mg kg^{-1}	8.4	23.3
Zn	mg kg^{-1}	76.0	87.7
sand	%	83.7	76.4
silt	%	3.0	6.4
clay	%	13.3	17.2
soil moisture	g water g dry $soil^{-1}$	0.56	0.35
soil NO_3	mg kg^{-1}	1.01	3.82

Table S3. Outlier fluxes (greater than 4sd) removed prior to analyses in this work.

Sample ID	Date	Site	GHG Type	Flux rate (mg $CO_{2\text{-}eq}/m^2/hr$)
E3	6/2/2016	Anaerobic Basin	CH_4	1063.06
E1	7/20/2017	Anaerobic Basin	CH_4	1519.75
E1	7/25/2017	Anaerobic Basin	CH_4	874.25
E1	8/2/2017	Anaerobic Basin	CH_4	1297.89
E1	8/23/2017	Anaerobic Basin	CH_4	1306.25
E3	7/8/2016	Anaerobic Basin	N_2O	1539.94
E1	7/22/2016	Anaerobic Basin	N_2O	-983.58
E2	7/22/2016	Anaerobic Basin	N_2O	-251.94
E3	7/22/2016	Anaerobic Basin	N_2O	-270.32

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CHAPTER 4: URBAN STORMWATER DOES NOT DRASTICALLY ALTER THE SOIL MICROBIOME, ALTHOUGH METHANOGENESIS AND V-ATPASE ARE ENHANCED WITHIN A WET STORMWATER BASIN

ABSTRACT

Understanding how urbanization and human alteration of the environment will affect the soil microbial population is an important task as the global urban footprint expands. We investigated the effects of urban stormwater on the soil microbial community to assess how urbanization might alter the microbial community and key biogeochemical processes. Soils were placed within three study sties, two treatment sites receiving stormwater (Wet Basin and Dry Basin), and one reference field site (Reference) and monitored over a 2-year period. Stormwater runoff was relatively unpolluted, Nitrate, SRP, Cu, Pb, and Zn all <0.5 mg L^{-1}, although Na levels were higher (115 mg L^{-1} in Wet Basin and 20 mg L^{-1} in the Dry Basin). Soil samples used for DNA metagenomics and soil health monitoring were collected after 2-months and 2-years exposure to these treatments. Overall, the microbial communities did not shift dramatically within either treatment. Microbial sub-pathways of methanogenesis and V-ATPase were increased within the Wet Basin treatment, likely due to excess Na and soil moisture. While denitrification is also an anaerobic process like methanogenesis, the denitrification genes did not increase within the often saturated Wet Basin treatment. The soil type within each treatment influenced the abundance of denitrification genes, even after 2-years. This indicates that denitrification may take longer to adjust to new environments, and practices like stormwater basins that rely on denitrification to remove nitrogen and improve water quality may be initially limited. Genes associated with metal stress and regulation were not increased in either stormwater treatment relative to the Reference.

This indicates stormwater exposure was likely not detrimental to the microbial community, and that urbanization (in this relatively unpolluted environment) will not significantly alter the soil microbial community, within the initial 2-years.

INTRODUCTION

How microbial populations respond to environmental change and pollution is an important component of ecosystem response and resiliency. The urban landscape is increasing throughout the globe (Lambin and Meyfroidt, 2011), but the impact of this landuse change on the in-situ microbial community and subsequent biogeochemical cycling is rarely studied. Studies on the urban soil microbiome have largely focused on pathogens and waste treatment (Dobrowsky et al., 2014; Werner et al., 2011) degradation of valuable building surfaces (e.g., (Papida et al., 2000), and recently a few on biogeochemical processes in urban settings (Bettez and Groffman, 2012; Scharko et al., 2015; Wang et al., 2017). However, we know very little about the urban soil microbiome overall, and even less about the role human's play on this microbial community (Pointing et al., 2016). As the majority of humans live and work in urban areas (United Nations, 2015) we must work to understand how we impact the soil microorganisms which regulate biogeochemical processing, plant growth, soil health and ultimately human health (e.g., Barnard et al., 2005; Turnbaugh et al., 2007; Zhou et al., 2012).

Of particular concern in the urban environment is water management. Wastewater treatment is the most notable process where human health is concerned. Elevated levels of organic matter, nutrients (nitrogen and phosphorus), metals, salinity, antibiotics, and endocrine-disrupting products, are found within wastewater (Carey and Migliaccio, 2009; Gasperi et al., 2008). In addition to wastewater, managing urban stormwater runoff is a critical aspect of city planning. Increased runoff from impervious surfaces leads to receiving waterbody degradation (*e.g.*,, 'urban stream syndrome'), property damage associated with flooding, and harmful algae blooms (Carpenter et al., 1998; Mitchell et al., 2003; Rabalais et al., 2002; Walsh et al., 2005). Green infrastructure (GI) distributes small management practices throughout the urban watershed to detain and treat

stormwater runoff. GI practices, like stormwater bioretention, greenroofs, constructed wetlands, permeable pavements, etc., became increasingly popular in the 1990's and are now the de-facto stormwater management tools used in developed countries (U.S. Environmental Protection Agency (EPA), 2014).

While stormwater is less contaminated than treated wastewater, it may still contain elevated nutrients, metals, salinity and oil compounds (LeFevre et al., 2015). Directly shunting stormwater to GI practices may alter the soil microbial community tasked with treating this runoff. As GI continues to expand in urban areas, it may have a considerable impact on the biogeochemical processes in the urban environment. Some research has examined the greenhouse gas (GHG) emissions from urban soils, and a few even examined emissions from the stormwater environment (Grover et al., 2013; McPhillips and Walter, 2015; Ström et al., 2007). However, little research has examined the urban soil microbiome within the stormwater environment. Chen et al. (2013) noted that the abundance of denitrification genes (qPCR) positively correlated with inundation in a bioretention cell. Morse et al. (2017) reported that denitrification genes (metagenomics) were greater within often saturated stormwater basins than dry basins. Bai et al. (2014) found that the microbial community within rhizopsheric soil of a constructed wetland was more diverse than influent water, and suggests the constructed wetland is supporting microbial growth. Saxton et al. (2016) used 16S rRNA to show that the microbial community in stormwater basins developed as a function of the environment and specific basin characteristics. Badin et al. (2012) reported that the bacterial community diversity within a stormwater basin differed among the macropores and solid particles. These studies conducted surveys of microbial populations within existing GI practices. However, field studies on the impacts of stormwater runoff to the soil microbial community are lacking.

As the GI footprint is expected to increase in the future, and this environment is explicitly designed to treat urban runoff we must better understand how the stormwater it seeks to treat impacts the soil microbiome. Consequently, this study examined how the stormwater environment in GI influenced the urban soil microbiome over a 2-year period.

METHODS

Treatment Sites

Exposure to stormwater and the bioretention environment was accomplished by locating sites within actual functioning stormwater basins. As stormwater design and function vary, two stormwater treatment sites were used: a "Wet Basin", which was slow draining, and a "Dry Basin, which was rarely saturated. These sites, located on Cornell University campus in Ithaca, New York, were previously monitored for N_2O and CH_4 fluxes (McPhillips and Walter, 2015; Morse et al., 2017). Additionally, a "Reference" treatment site not receiving stormwater was included to serve as a control site where the influence of stormwater inundation was not present. This reference site consisted of a field adjacent to residential homes. A description of site characteristics is given in Table 14.

Table 14. Study site characteristics.

		Wet Basin	Dry Basin	Reference
Drainage Area	m^2	11,049	4,249	NA
Basin Area	m^2	1,500	480	NA
Drainage Area: Basin Area	ratio	7.37	8.85	NA
Year Constructed	Year	2004	2006	NA
Impervious Drainage Area	%	95	100	0
Contributing Drainage Area		Parking Lot	Parking Lot	Field

At each treatment site, three plots were established using the soil and vegetation from the three treatment sites, i.e., each treatment site had the same 3 soils placed within it. Therefore, any specific soil variations that could alter the treatment effects were controlled for by using these 3 different soils. The soils were essentially swapped from one site to the others. Figure 11 displays the general site layout. These plots were exposed to treatment conditions for the 2-year experiment. Surface vegetation and soil (2 feet [ft] x 1 ft x 0.5 ft) were excavated and placed within the treatment sites. Soils were then left exposed to the treatment conditions (Wet Basin, Dry Basin, or Reference) over the 2-year experiment. Thus, possible influences to the microbial community are the treatment and soil type.

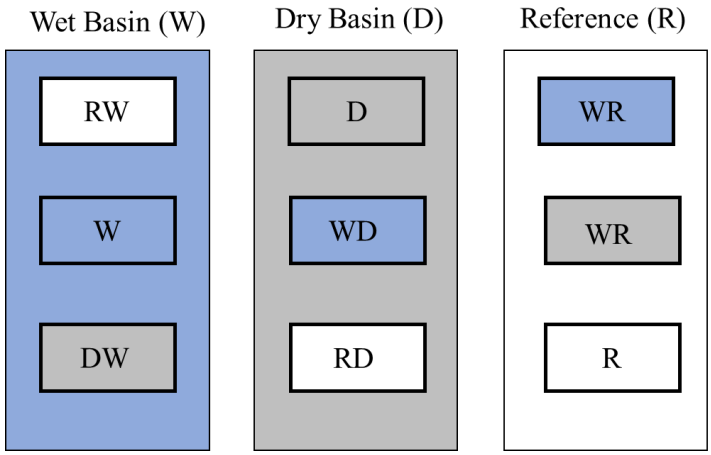

Figure 11. Treatment site layout. The Treatment site is the large rectangle, and the small individual rectangles indicate the soil type placed within that treatment site. "D" = Dry Basin, "W" = Wet Basin, "R" = Reference. Site names reflect where the soil originated with the first letter, and the treatment site location with the second letter, e.g., "WD" indicates Wet Basin soil within the Dry Basin treatment site.

Plots were monitored periodically (~monthly) for temperature, conductivity, and volumetric water content (VWC). A FieldScout TDR 100 Soil Moisture Meter monitored soil VWC, and a FieldScout Direct Soil EC Meter Testr11+ monitored soil temperature and electrical conductivity.

Stormwater Monitoring

Stormwater quantity and quality monitoring was completed at each site from July – November 2015. A total of seven storms at each site were monitored. Inflow volume was determined based on the U.S. Soil Conservation Service (SCS) Curve Number method and precipitation measured on site via rain gauges. A v-notch weir-box was fitted with HOBO pressure-transducer water depth loggers at 1-minute intervals to quantify outflow volume. ISCO automated samplers (ISCO 6712) collected stormwater samples over the storm duration. To capture the general inflow and outflow water quality, the individual samples were flow-weight composited for an event mean concentration, prior to analysis. Consequently, one inflow and one outflow sample characterized each storm at each site.

Water quality analyses included total suspended solids, nitrite-nitrate, ammonium, orthophosphate, and total metals following general water quality guidance (American Public Health Association, American Water Works Association, and Water Environment Federation (APHA, AWWA, and WEF), 1998; USEPA, 2007). A Latchet Quickchem measured N species, the molybdenum blue method (USEPA 1978) with an auto-sampler (O.I. Analytical FS3000) was used to measure phosphate (PO_4^{-3}), and total metals were extracted with nitric acid and measured via inductively coupled plasma (ICP) spectrometry.

Soil Metagenomics

High-throughput next-generation sequencing was used to analyze the soil DNA genome (metagenomics) at each stormwater basin and the reference site. Soils were collected on September 28, 2015, and July 10, 2017, approximately 2-months and 2-years after treatment exposure (referred herein as timepoints 2-months and 2-years). At each site two soil cores were collected and pooled for DNA extraction and soil characterization (9 sites x 2 sample periods = 18 total

samples). Soils were collected with a steel push probe (2.5 cm diameter) from the top 5 cm of soil. Soils were kept on ice during sampling, and stored at -20ºC until DNA extraction. Soil DNA was extracted with Mo-Bio PowerSoil® DNA Isolation Kit. For each of the 18 samples, DNA was extracted in triplicate and then pooled to reduce variability within the soil sample and provide ample DNA for sequencing. Concentration of extracted DNA was assessed using a Qubit fluorometer with dsDNA BR and HS assay kits. Extractant was frozen at -20ºC until sequencing.

Approximately 10M 100 base single-strand reads were sequenced for each sample (18 samples x 10M reads = 180M reads total) by the Cornell University Genome Center on an Illumina HiSeq 2500. The Functional Mapping and Analysis Pipeline (FMAP) was used to align reads to functional genes using DIAMOND against a KEGG reference cluster (Kim et al., 2016). DIAMOND (double index alignment of next generation sequencing data), is a high throughput alignment program analogous, but faster, than BLASTx (Buchfink et al., 2015). DIAMOND returned a matrix of matches for each sequence, within each sample; results were filtered where percent sequence identity (pid) >50, and assigned read length >25 base pairs.

This study focused on reads corresponding to functional genes, instead of phylogenetic classifications common with 16S rRNA amplicon sequencing. Focusing on functional genes within the soil microbial metagenomes allows us to better understand microbial nutrient cycling functions and drivers in this diverse soil ecosystem(Lombard et al., 2011). The functional gene count reads were normalized by total reads per sample to compensate for the slight variation in sample to sample reads (8.8M to 18.5M per sample), and multiplied by one million to obtain reads per million reads (rpm).

Metagenomic Pathway Analysis

Significant differences in microbial pathways were identified with FMAP (Kim et al., 2016). FMAP aligned reads against a KEGG reference cluster, and analyzed differentially abundant genes with Kurskal-Wallis rank-sum tests (Kim et al., 2016). This study focused on the effects of stormwater exposure on microbial metabolism and nutrient cycling, consequently the oxidative phosphorylation (KEGG pathway K00190), methane metabolism (K00680) and nitrogen metabolism (K00910) were investigated. The genes involved with each pathway are listed in Supplementary Material.

Once significant differences in pathways were identified in FMAP, we then drilled down within the pathway to determine what specific KEGG modules (analogous to a sub-pathway) were most affected. Investigation into sub-pathway differences centered on environmental and biological relevancy. For example, the 'methane metabolism' pathway in KEGG has 181 enzymes, but the Methanogenesis, CO_2 => methane module (M00356) has only 20. Because sub-pathways are smaller and more specified, we used them to determine how treatments affected the biology of microorganisms in a more detailed way. The FMAP analysis only supports two-group comparisons, (*i.e.,* Wet Basin treatment vs. Reference), but since we have 3 treatments we also compared overall treatment and soil type effects with two-way ANOVA. Once pathway differences were identified in FMAP, the sum of these genes within each pathway were summarized and normalized to each individual plot. Then the differences between the treatments (n=3 plots) for these pathways was assessed with two-way ANOVA.

Taxonomy

Metagenomic DNA reads (fastq files) were uploaded to the U.S. DOE Systems Biology Knowledgebase (KBase). The KBase platform allows users to annotate, assemble and model

genomes. The web-browser interface supports many analysis tools. We used the Kaiju taxonomic classifier to assign reads to taxa {Citation}. The abundance of taxa within each sample were then exported and analyzed in R.

Metal regulating genes

We focused on 6 functional genes involved in metal regulation to give a general overview of possible stormwater stress upon the microbial community (Hemme et al., 2010). The 6 genes of interest were: cueR (Cu efflux), cutF (Cu homeostasis), pcoD (Cu resistance), ycnJ (Cu uptake), mgtE (Mg transporter), and czcD (Zn/Cd/Co efflux). The number of sequences assigned to these genes via FMAP were extracted, along with the total number of sequences assigned to any gene, and used to calculate odds ratios. We compared odds ratios for Reference soils placed within the Wet Basin or Dry Basin treatment at the 2year timepoint.

Soil Characteristics

Each soil core used for DNA analysis was also analyzed for bulk density, total carbon (C), metals, pH, nitrate, and salinity. The cores were oven-dried at 105°C over a 24-hour period and weighed to determine bulk density (Blake and Hartge, 1986) for each sample. A subsample was ground to less than 250 µm and analyzed for % total C (g C g^{-1} dry soil), which was measured at the Cornell University Stable Isotope Laboratory (Ithaca, NY) through dry combustion on a Conflo III Elemental Analyzer.

Hot plate assisted acid digestion (EPA method 3051) was used to extract total metals (US EPA, 1996), which were quantified via ICP at Cornell Nutrient Analysis Laboratory. Soil pH was determined with a 1:1 soil to water ratio.

Denitrification Enzyme Assay

Denitrification potential was monitored within each plot at each timepoint via denitrification enzyme assay (DEA) (Groffman et al., 1999). This acetylene-block method is well suited to compare site-to-site differences, but does not quantify actual denitrification rates *in situ* (Attard et al., 2011; Groffman et al., 1999). Two soil cores at each plot were pooled and run in duplicate for each sample point (2 time-points x 9 sites x 2 replicates = 36 samples total).

Statistical Analyses

The statistical analysis was conducted using R software (version 3.4.0; R Development Core Team, 2017). A criteria of 95% confidence (a=0.05) was selected for all analyses herein. Due to evidence of heteroscedasticity, non-parametric tests were used for stormwater quality assessments. Comparisons between the two sites used the Mann-Whitney test, and comparisons of paired data used the Wilcoxon signed rank test. Two-way analysis of variance (ANOVA) was used to assess treatment and soil type effects. Principal coordinate analysis (PCoA) on a distance matrix from the metagenomics data was used to visualize differences between treatments. Pearson correlations were used to assess how variables related to each other. The abundance of taxa within each sample were compared between treatments via ANOVA with the Bonferroni correction.

The effect of Wet Basin or Dry Basin treatment upon the Reference soil type was assessed by comparing the odds ratio on relevant metal regulating genes for either treatment. We assessed the likelihood of observing the number of sequences via odds ratio for a particular gene within the treatment site compared to the Reference site using the same soil type. The odds ratios were calculated (*i.e.*, for cueR gene within Wet Basin treatment) as follows:

$$Odds\ Ratio = \frac{A/B}{C/D}$$

Where:

A = # sequences assigned to cueR gene within Reference soils within Wet Basin treatment

B = # sequences assigned to all genes within Reference soils within Wet Basin treatment

A = # sequences assigned to cueR gene within Reference soils within Reference treatment

B = # sequences assigned to all genes within Reference soils within Reference treatment

P-values were calculated for each odds ratio using two-tailed Fisher's Exact Test to determine significant deviations from equilibrium (odds ratio = 1). Values were plotted as ln (odds ratio) to better visualize trends within the data.

RESULTS

Stormwater Monitoring

Average stormwater runoff (or inflow to the basins) characteristics are given in Table 15. Overall, the stormwater was relatively low in nutrients and metals. The average runoff nitrate levels were 0.21 mg L^{-1} and 0.18 mg L^{-1} for the Wet Basin, and Dry Basin, respectively. Stormwater concentrations at the Wet and Dry Basin were not significantly different for nitrate (Wilcoxon p = 0.32) or soluble reactive phosphorus (SRP; p = 0.34). Metal contamination was also low, where the average Cu, Pb, and Zn concentrations were below 0.5 mg L^{-1} at both sites. The average concentrations of Cu, Pb, and Zn were also not significantly different between the sites (p = 0.19, 0.36, and 0.65, respectively). This indicates that each stormwater basin received relatively similar stormwater runoff. Sodium is the notable exception, where the Wet Basin had significantly higher inflow concentrations (115.16 mg L^{-1}), compared to the Dry Basin (20.54 mg L^{-1}); Wilcoxon p-value <0.001. This indicates that the incoming stormwater is similar at both Basins, except for

Na+. Thus, the microbial community at either Basin is exposed to similar stormwater pollutant loads, except Na+ which is much higher in the Wet Basin. The microbial community at the Reference site is exposed to rainwater only, and is not exposed to the stormwater pollution seen at the Basin sites.

Table 15. Average stormwater runoff concentrations.

		Wet Basin	Dry Basin
TSS	mg/L	13.90	13.22
Nitrate	mg/L	0.21	0.18
Ammonium	mg/L	0.20	0.17
SRP	mg/L	0.04	0.06
Cu	mg/L	0.01	0.01
Na	mg/L	115.16	20.54
Pb	mg/L	0.01	0.01
Zn	mg/L	0.23	0.21

The pollutant concentrations within stormwater runoff at these sites was slightly lower than the national averages observed by the National Urban Runoff Program (NURP) (Makepeace et al., 1995). NURP reported average nitrate concentrations ranged from 0.01-12 mg L^{-1}, and SRP levels of 0.04-3.52 mg L^{-1}. The metal concentrations observed in this study were also slightly lower than average Cu (0.01-0.15 mg L^{-1}), Pb (0.02-1.56 mg L^{-1}) and Zn concentrations (0.02-0.58 mg L^{-1}) (Makepeace et al., 1995). The Na+ concentration observed in this study are within the range of mean Na+ levels observed by the NURP of 0.18-660 mg L^{-1}. They note that Na+ is likely present in stormwater from NaCl deicing salts. As these Basin sites are located near parking lots in New York, it is likely NaCl was used as a deicing salt.

Soil Characteristics

Table 16 shows the average soil characteristics for each treatment site at the 2-month and 2-year timepoints. Overall, soils did not differ substantially between the treatment sites, except for VWC, conductivity and Na+ levels. The Wet Basin treatment site average conductivity and Na+ levels

were an order of magnitude above the Reference and Dry Basin treatments. Two-way ANOVA results with "Treatment" and "Soil type" revealed that only VWC, Na+, and conductivity had significant treatment effects at the 2-month and 2-year timepoints (Table 17). Soil metal concentrations, total C, and N were not affected by treatment at either timepoint. Only denitrification potential (DEA) had a significant treatment effect at the 2-month timepoint, although it was not present at the 2-year timepoint. This suggests that the 2-month treatment effect on DEA was either spurious or short-lived. These results suggest that the treatment effects were limited to VWC, conductivity and Na+ within the soils.

Table 16. Average soil characteristics at each treatment site (n=3).

	Treatment	Dry Basin		Reference		Wet Basin	
	units	2 months	2 years	2 months	2 years	2 months	2 years
Total C	%	5.49	4.20	4.27	3.15	4.15	3.91
Total N	%	0.32	0.30	0.29	0.26	0.24	0.32
VWC	%	28.08	23.78	30.31	25.48	71.58	65.95
DEA	mg N kg $soil^{-1}$ hr^{-1}	0.21	0.38	0.10	0.30	0.42	0.30
pH		NA	7.71	NA	7.16	NA	7.83
Salinity	uS	0.15	0.11	0.07	0.08	1.73	1.98
Na	mg/kg	420.54	135.37	524.13	348.02	1,654.33	1,787.60
Cu	mg/kg	22.56	25.97	22.49	25.74	24.11	27.47
Pb	mg/kg	25.52	23.20	24.59	25.19	23.77	23.35
Zn	mg/kg	87.22	94.93	91.33	88.26	83.45	84.50
P	mg/kg	805.69	762.14	735.93	751.35	770.45	768.48

Table 17. Two-way ANOVA results for the factors of treatment (stormwater Wet Basin, stormwater Dry Basin, or Reference), and soil type on the soil characteristics and relative gene abundances (rpm) of sub-pathways.

Variable	Units	timepoint	Treatment	Soil Type
			p-values	
DEA	mg N kg^{-1} hr^{-1}	2-months	**0.02**	0.30
DEA	mg N kg^{-1} hr^{-1}	2-years	0.88	0.87
VWC	%	2-months	**<0.001**	0.14
VWC	%	2-years	**<0.001**	0.26
Conductivity	uS	2-months	**0.01**	0.85
Conductivity	uS	2-years	**<0.001**	0.39
Total C	%	2-months	0.49	0.12
Total C	%	2-years	0.35	0.22
Total N	%	2-months	0.15	**0.02**
Total N	%	2-years	0.25	**0.01**
Cu	mg/kg	2-months	0.63	**0.01**
Cu	mg/kg	2-years	0.89	**0.06**
Zn	mg/kg	2-months	0.42	**0.05**
Zn	mg/kg	2-years	0.30	0.24
Na	mg/kg	2-months	**0.01**	**0.01**
Na	mg/kg	2-years	**0.02**	1.00
V-ATPase	rpm	2-months	0.06	**0.01**
V-ATPase	rpm	2-years	**<0.001**	0.07
denitrification	rpm	2-months	0.96	0.07
denitrification	rpm	2-years	**0.03**	**0.05**
methanogenesis	rpm	2-months	**0.05**	**0.04**
methanogenesis	rpm	2-years	**0.01**	0.14

Metagenomic Results

Overall Treatment Effects

Overall, treatments did not greatly alter the entire DNA microbiome at either the 2 month or 2 year timepoints. Principal coordinate analysis (PCoA) revealed no clustering by treatment (Figure S10). This indicates that prolonged exposure to the stormwater or reference treatments, did not alter the DNA enough to cause the microbial community to resemble each other. This lack of overall

treatment effect on the soil DNA could signify (1) DNA recalcitrance (Carini et al., 2017), where DNA persists even though it may not be active or (2) exposure to the stormwater environment was not a strong enough 'treatment' to drive microbial differentiation.

To better understand if there was a likely stormwater treatment effect (scenario 2), we examined the effect of the stormwater exposure on the soils themselves. Two-way ANOVA with soil type and treatment factors showed that treatment effects existed for soil parameters VWC, Na+, and conductivity at 2-months and 2-years (Table 17). This indicates that the stormwater treatment effects on soil moisture and salinity occurred quickly and persisted till the end of the 2-year experiment. Other soil characteristics, like total C, total N, and metal concentration were unaffected by treatments (Table 17 treatment effects $p>0.05$). Soil type effects on total N, and Cu and Zn were significant at 2 months and 2 years (Table 17 soil type effects $p<0.05$). This indicates that the soil type controlled the concentration of N and metals initially and after 2 years of exposure to these treatments. Consequently, changes in total N and metals were not governed by treatments and instead the soil type retained these levels throughout the experiment.

Thus, VWC and conductivity (and Na+) are the likely treatment effects influencing the soil microbial community. Figure 12 illustrates the overall treatment effect on VWC and conductivity over the 2-year period (n=110). Because the overall stormwater treatment effect on the soils was limited to VWC and conductivity (and Na+), we hypothesized that these were the main drivers of microbial changes. Unfortunately, VWC and conductivity (and Na+) were also highly correlated (Table 18), and determining which of these factors was most responsible for altering the microbial community was not possible.

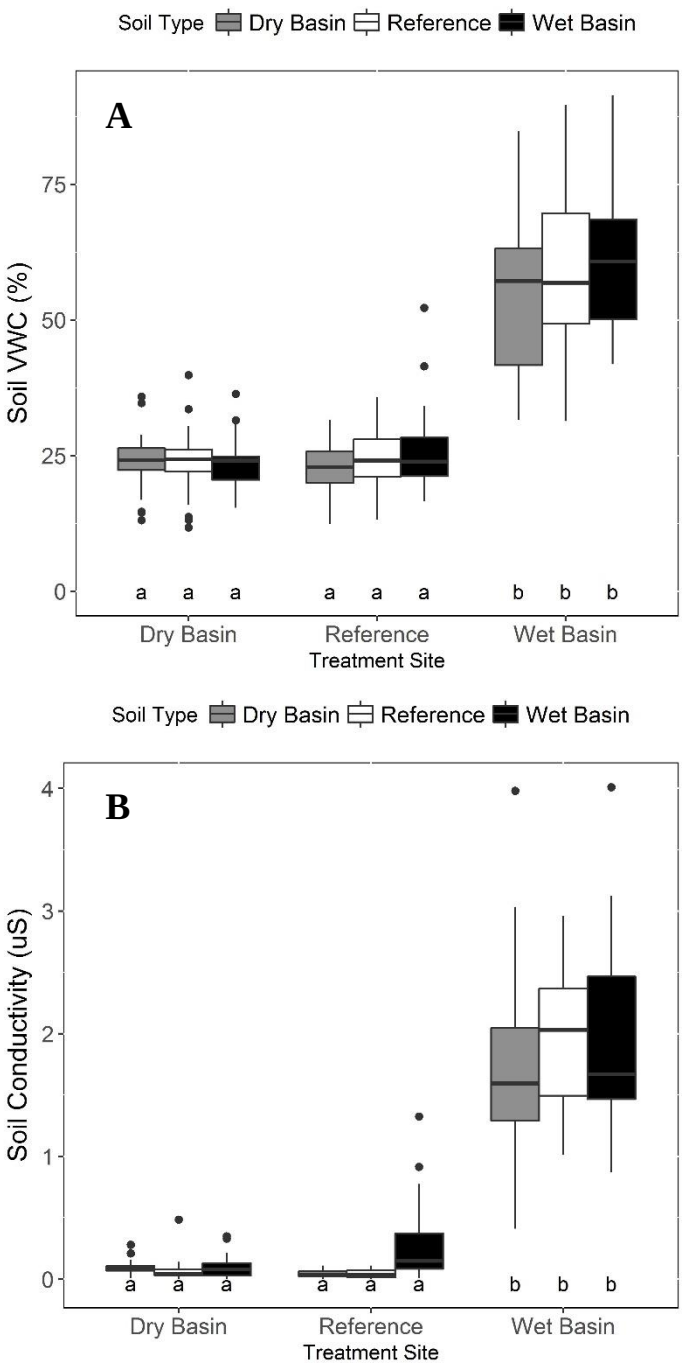

Figure 12. (A) Soil volumetric water content (VWC) and (B) Soil conductivity measured over the 2-year experiment (n=38). Letters denote significant differences between 9 plots as determined by Tukey HSD tests ($p<0.05$).

Table 18. Pearson correlations for soil characteristics and microbial DNA sub-pathways (n=18). Correlation coefficient (r) values in bold are significant ($p<0.05$).

	Total C	Total N	VWC	Conductivity	Na	Zn	Cu	Ca	DEA	V-ATPase	Methanogenesis	Denitrification
	%	*%*	*%*	*(uS)*			*mg Kg^{-1}*		*mg N kg^{-1} hr^{-1}*		*rpm*	
Total C	1	0.45	0.01	-0.12	0.23	0.25	0.25	-0.03	-0.03	0.28	0.20	0.38
Total N		1	0.00	-0.06	0.10	-0.34	-0.34	**-0.54**	-0.23	0.01	0.13	-0.16
VWC			1	**0.94**	**0.89**	-0.41	0.08	-0.26	0.28	**0.76**	**0.83**	0.27
Conductivity				1	**0.77**	-0.41	0.04	-0.19	0.22	**0.69**	**0.76**	0.25
Na					1	-0.34	0.17	-0.22	0.13	**0.82**	**0.87**	0.39
Zn						1	**0.66**	**0.62**	0.22	-0.07	-0.19	0.22
Cu							1	0.40	**0.56**	**0.47**	0.38	**0.51**
Ca								1	-0.04	-0.07	-0.19	0.15
DEA									1	0.28	0.27	0.14
V-ATPase										1	**0.97**	**0.73**
Methanogenesis											1	**0.57**
Denitrification												1

Taxonomic Differences Between Treatments

The Kaiju taxonomic classifier used in KBASE (Menzel et al., 2016) classified approximately 30% of reads to the order level. Fifty-eight unique orders were identified among the 18 samples. ANOVA, with Bonferonni correction, revealed treatment differences in 11 orders at the 2-year timepoint. At the 2-month timepoint, no significant differences between orders were observed. This indicates (1) the treatment effect was not large even at the 2-year timepoint, as only 11 of 58 orders showed changes with treatment, and (2) the treatment effect was not present at the early 2-month timepoint.

Figure 13 illustrates the average normalized reads for the 11 orders with significant differences between treatments at the 2-year timepoint. The Wet Basin treatment had significantly higher abundances than the Dry Basin and Reference treatments (Tukey-HSD $p<0.05$) within the Anaerolineales, Bacteroidales, Caldilineales, Clostridiles, Desulfobacterales, Desulfovibrionales, Desulfuromonadales, and Methanosarcinales orders. Conversely, the Wet Basin treatment had significantly lower abundances than the Dry Basin and Reference treatments within the Frankiales, Solirubrobacterale, and Streptosporangiales orders. Interestingly, the Wet Basin is the only treatment with reads within the Anaerolineales, Bacteroidales, Caldilineales, Desulfobacterales, Desulfovibrionales, and Methanosarcinales orders.

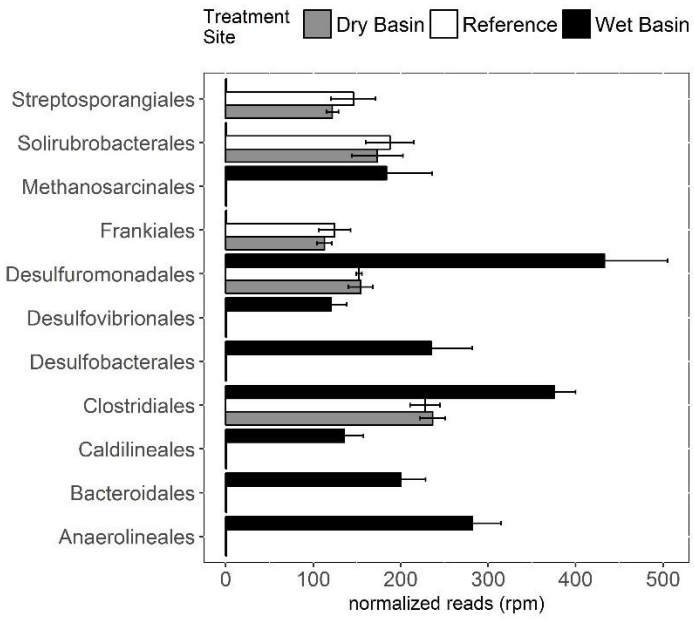

Figure 13. Taxonomic order for each treatment site at 2-year timepoint (n=3). The 2-month timepoint had no significant differences between treatments. Error-bars are standard deviations (n=3).

Sub-pathway differences

FMAP identified significant differences between treatments for genes within the methanogenesis sub-pathway, but not within the denitrification sub-pathway (KEGG module M00529). The methane metabolism pathway has 4 methanogenesis sub-pathways. We focused on one methanogenesis sub-pathway, methanogenesis methanol to methane (M00356), although the other sub-pathways followed similar trends. Additionally, significant differences within the V-ATPase, prokaryotes sub-pathway (M00159) were observed between treatments.

Figure 14 shows which treatment group had significantly different sub-pathways. Methanogenesis and V-ATPase sub-pathways were significantly greater in the Wet Basin treatment in the 2-year timepoint (Tukey-HSD $p<0.05$). At the same timepoint, the denitrification sub-pathway was not significantly greater in the Wet Basin treatment, but the average rpm (268.9) was higher than the

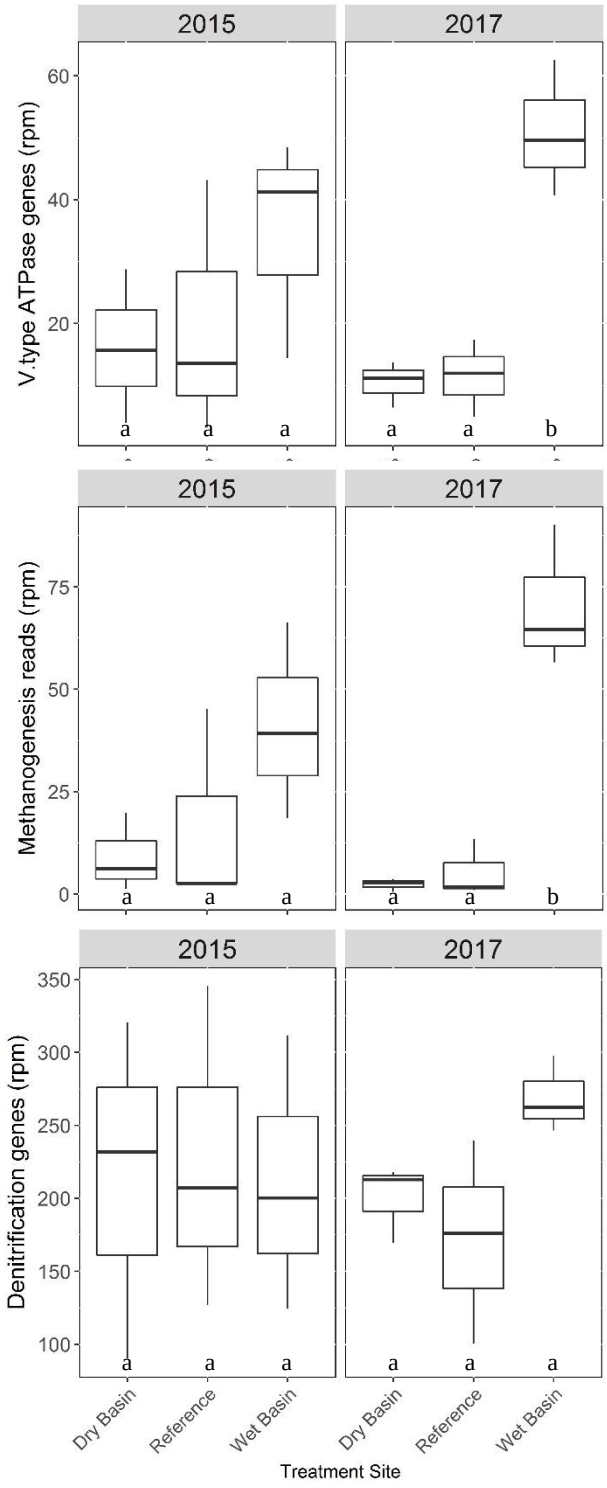

Figure 14. (A) V-ATPase sub-pathway **(B)** methanogenesis sub-pathway and **(C)** denitrification sub-pathway normalized reads (reads per million [rpm]) within the treatment site (n=3) at each timepoint. Different letters represent significant ($p<0.05$) differences between the 3 treatment sites as assessed with Tukey-HSD.

Dry Basin or Reference treatment at 200.1 and 226.5 rpm, respectively (Figure 14). This indicates that prolonged exposure to the Wet Basin treatment likely increased the abundance of methanogensis, V-ATPase, and to a lesser degree denitrification genes. Because no treatment effects were observed at the 2-month timepoint (Figure 14), this supports our earlier finding that the treatment effects on the microbial community likely required prolonged (2 years) exposure.

In an effort to determine if the soil-type affected the microbial community, we examined the effects of treatment and soil type on sub-pathways via two-way ANOVA. Treatment significantly altered V-ATPase, methanogenesis, and denitrification at the 2-year timepoint, while soil-type did not (Table 17). This indicates that at this sample point (2-years) the abundance of these sub-pathways is controlled more by the treatment (Wet Basin, Dry Basin or Reference site), than the original soil type. Therefore, the predominant treatment effects, VWC and conductivity (and Na+), are likely driving the abundance of these sub-pathways in the soil. VWC, conductivity, and Na+ were all strongly correlated with V-ATPase and methanogenesis sub-pathways (Table 18). However, the denitrification sub-pathway was not.

We hypothesize that methanogenesis was driven by greater soil moisture, as this is an anaerobic process. While conductivity is also strongly correlated with methanogenesis, VWC and conductivity covary ($r = 0.94$; Table 18), and determining which variable is more aligned with any sub-pathway is not possible. The V-ATPase sub-pathway was strongly correlated with VWC and conductivity (and Na+). We hypothesize that excess Na+ within the soil water is enhancing the abundance of V-ATPase because this enzyme can utilize Na+ to synthesize or hydrolyze ATP for energy (Mulkidjanian et al., 2007). Consequently, microorganisms adapted to use Na+ may thrive in the salty stormwater environment.

The denitrification sub-pathway is less straightforward. We hypothesized that denitrification genes and potential denitrification (DEA) would increase in the Wet Basin treatment due to the anaerobic nature of denitrification. However, at the 2-year timepoint, neither DEA or denitrification genes were significantly different than the other treatments (Figure 14 and SM2), and were not correlated with VWC (Table 18). This suggests it may take longer than 2-years of exposure to a high water content environment to induce denitrification changes. The soil type was a significant predictor of the denitrification sub-pathway at the 2-year timepoint, but not in the V-ATPase or methanogenesis sub-pathways (Table 17). The original soil type, and reservoir of DNA, was more influential on denitrification than the other sub-pathways.

The denitrification sub-pathway ($r = 0.51$) and DEA ($r = 0.56$) were positively correlated with soil Cu levels (Table 18). This could be an artifact, or because Cu is used as a cofactor in two denitrification enzymes, *nirK* and *nosZ* (Richardson et al., 2009). The Cu levels within the soils were all fairly low (16.8 to 29.2 mg kg^{-1}), near background Cu concentrations in the eastern U.S. of ~20 mg kg^{-1}, and well below EPA screening levels for plants (70 mg kg^{-1}) or invertebrates (80 mg kg^{-1}) (U.S. Environmental Protection Agency, 2007), so potential harmful effects of excess Cu are not likely. We compared the correlation of individual denitrification enzymes with soil characteristics (Table 19), and interestingly Cu was correlated with *nosZ* (and also non-Cu enzymes *nap*, *nirS* and *cnor*) but not with *nirK*. Thus, it is possible extra soil Cu is enhancing denitrification, but it is also possible these correlations are spurious. VWC was negatively correlated with *nap* ($r = -0.47$), and positively correlated with *nirS* ($r = 0.64$) (Table 19). This disparate trend could explain why VWC was not correlated with the entire denitrification sub-pathway; some genes increase with VWC (*nirS*), while others decrease (*nap*). Thus, the increased

VWC in the Wet Basin treatment may not have elevated the denitrification sub-pathway as anticipated, because individual genes were not all positively correlated with VWC.

Table 19. Pearson correlations for individual denitrification enzyme gene reads and soil characteristics (n=18). Correlation coefficient (r) values in bold are significant ($p<0.05$).

gene	Total C	Total N	VWC	Conductivity	DEA	Ca	Cu	Zn
	%	%	%	uS	mg N kg^{-1} hr^{-1}	mg kg^{-1}	mg kg^{-1}	mg kg^{-1}
nap	-0.01	-0.34	**-0.47**	-0.39	0.11	**0.68**	**0.49**	**0.61**
nar	0.42	-0.10	0.36	0.30	0.08	0.03	0.42	0.09
nirK	0.35	-0.14	-0.20	-0.14	-0.17	0.32	0.31	0.30
nirS	0.38	0.16	**0.64**	**0.57**	0.20	-0.03	**0.53**	-0.03
cnor	0.36	-0.05	0.43	0.41	0.16	0.11	**0.60**	0.17
qnor	0.29	0.11	0.24	0.29	-0.08	0.03	0.40	0.15
nosZ	0.35	-0.07	0.02	0.06	0.20	0.23	**0.66**	0.40

Effects on metal regulating genes

While overall metal concentrations did not increase substantially within the stormwater basins (Table 16), we examined the abundance of metal regulating genes to determine if these seemingly low metal concentrations were enough to stress the microbial community. We focused on the 2-year timepoint to allow treatment effects to occur.

The ln odds ratio for Reference soils within the Wet Basin or Dry Basin treatment at timepoint 2 are shown in Figure 15. Only one gene, cueR, was significantly lower in the Dry Basin site than the Reference treatment site. The ln odds ratio of -0.12 indicates that Reference soils within the Dry Basin treatment had about 11% less ($1 - e^{-0.12} = 0.11$) *cueR* than the Reference site. This gene is responsible for Cu efflux out of the cell, and it appears the necessity for Cu efflux within the Dry Basin is less than the Reference Site. At the 2-year timepoint, Cu concentrations for Reference soils within the Dry Basin and Reference treatment site were 19.3 and 19.5 mg kg^{-1}, respectively. Consequently, it is unclear why *cueR* would be slightly elevated within the Reference site. The relatively small difference in *cueR* (11%) suggests this is a modest change in gene abundances.

Otherwise, the remaining 5 genes responsible for Cu homeostasis, Cu resistance, Cu uptake, Mg transport and Zn/Cd/Co efflux were not affected by either Wet Basin or Dry Basin treatments. This suggests that metal stress from receiving stormwater is not strong enough to alter the microbial community within the Wet Basin or Dry Basin sites. This agrees with the earlier finding that the stormwater was not heavily polluted, and metal concentrations were well below allowable levels.

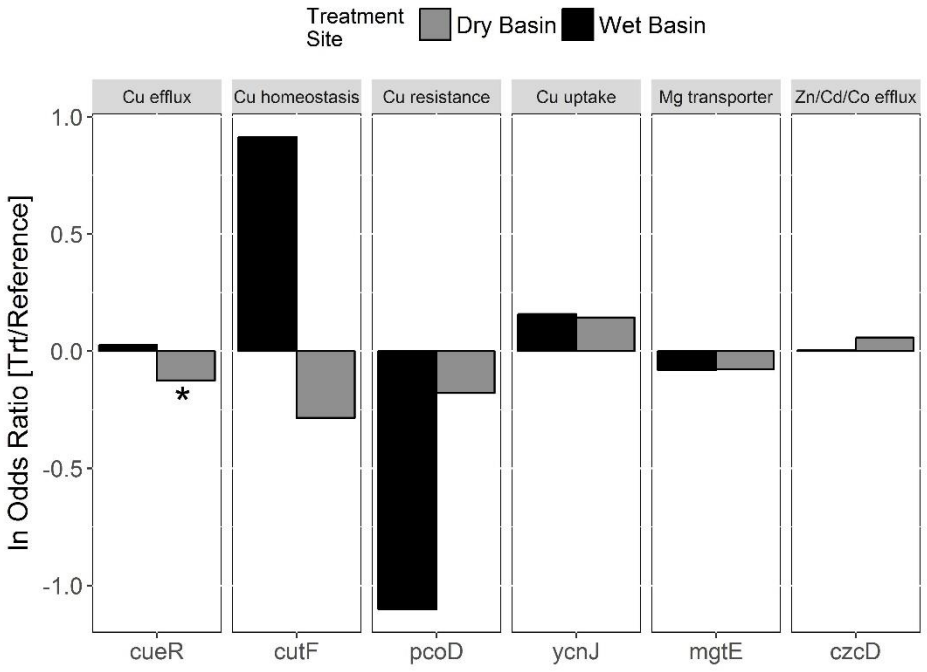

Figure 15. Ln odds ratio comparing the Reference soil type within the Dry Basin or Wet Basin treatment environment to the Reference environment. The x-axis lists representative metal regulating genes. A * indicates a significant difference between the Basin treatment and Reference treatment environment (Fisher's exact test $p<0.05$).

CONCLUSIONS

Overall, the microbial community was not drastically altered by the stormwater treatment at either the 2-month or 2-year timepoint. Soil microorganisms are not likely exposed to significant stressors (*i.e.*, metals), and thus the overall community is able to proceed with key biogeochemical processes without devoting resources to metal regulation. This implies that in this setting, parking

lot runoff in the U.S. Northeast, the stormwater was likely not polluted enough to cause a microbial shift within the 2 years of exposure. Indeed, stormwater runoff concentrations were generally low and within the national averages.

The exception for the runoff was large Na+ concentrations within the Wet Basin, and elevated Na+ and wetter soil conditions at this site lead to microbial shifts. As Na+ and soil VWC were correlated, determining which factor was more influential was not possible. The Wet Basin site had elevated V-ATPase and methanogenesis sub-pathways compared to the Dry Basin or Reference site at the 2-year timepoint (Tukey-HSD $p<0.05$). We hypothesized that denitrification, another anaerobic process, would increase within the Wet Basin treatment. However, these genes appeared more stable and less dependent on the treatment, possibly indicating that these genes are more autochthonous than the other subpathways. Prolonged exposure to wetter and/or saltier conditions likely caused the microbial community to adopt methanogenesis (an anaerobic process) and increased the use of V-ATPase, which can utilize Na+ as a protonmotive force for ATP synthesis. Thus, excess CH_4 may be emitted from soils within the stormwater environment, but N_2O may not necessarily increase. Other research within these same basins (Morse et al., 2017) noted that the Wet Basin had significantly greater denitrification reads than the Dry Basin or control after 12 years of exposure. Therefore, the long-term exposure within the Wet Basin must eventually increase denitrification reads, but the time to create this change is unknown as our experiment only recorded results for 2 years. Consequently, practices designed to improve water quality be promoting denitrification may require additional time (beyond 2-years) to enhance this process.

Appendix A includes a discussion on additional experimentation on the effects of black carbon on microbial uptake of copper and the effects on denitrification.

SUPPLEMENTARY INFORMATION

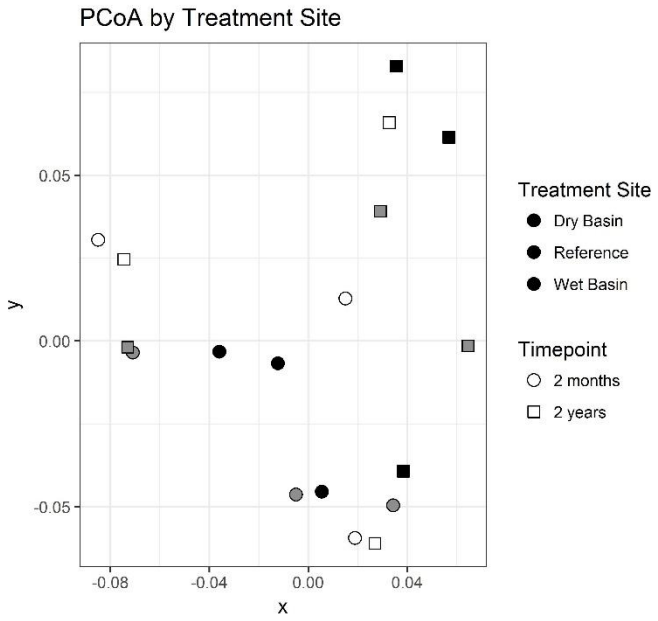

Figure S10. Principal Coordinate Analysis (PCoA) of all DNA metagenomics reads.

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