BACTERIAL COMMUNITY COMPOSITION AND PHYSICOCHEMICAL PROPERTIES OF SOILS IN CREATED AND NATURAL WETLANDS IN VIRGINIA

by

Rita M. Peralta

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Committee:

_____	Dr. Changwoo Ahn, Thesis Director
_____	Dr. Patrick Gillevet, Committee Member
_____	Dr. Robert Jonas, Committee Member
_____	Dr. Robert Jonas, Department Chairperson
_____	Dr. Richard Diecchio, Associate Dean for Academic and Student Affairs, College of Science
_____	Dr. Vikas Chandhoke, Dean, College of Science

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By

Rita M. Peralta
Bachelor of Arts
George Mason University, 2002

Director: Changwoo Ahn, Professor
Environmental Science and Policy

Spring Semester 2011
George Mason University
Fairfax, VA

DEDICATION

This is dedicated to mom and dad. Your love and hard work is my daily inspiration.

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TABLE OF CONTENTS

Page

ABSTRACT vii

1. INTRODUCTION 1

2. SITE DESCRIPTIONS 5

2.1 General setting 5

2.2 Created wetlands 5

2.3 Natural wetlands 7

3. METHODS 8

3.1 Soil sampling design 8

3.2 Soil physicochemical analysis 8

3.3 Soil bacterial community analysis 9
3.3.1 DNA extraction 9
3.3.2 LH-PCR 9
3.3.3Multitag pyrosequencing (MTPS) 11

3.4 Hydrologic connectivity groupings 12

3.5 Data analysis 13

4. RESULTS 16

4.1 Soil physicochemistry 16
4.1.1 Soil physicochemical attributes by season 16
4.1.2 Soil physicochemical attributes by wetland site 16

4.2 Soil bacterial community composition and diversity 17
4.2.1 Bacterial community composition by LH-PCR and by MTPS 17
4.2.2 Soil bacterial community diversity 20

4.3 Association between bacterial community measures and physicochemical attributes of wetland soils 20

4.4 Characterization of HC groups and bacterial community and physicochemisry of soils by HC groups 21

5. DISCUSSION 23

5.1 Soil properties in created vs. natural wetlands 23

5.2 Bacterial composition and diversity of created and natural wetlands 24

5.3 Association between physicochemical attributes and bacterial community measures in wetland soils 25

5.4 Potential role of site hydrologic connectivity on biogeochemical patterns of wetland soils 27

5.5 Suitability of molecular methodology 28

5.5 Implications of the outcome of the study 29

LITERATURE CITED 32

Table 1. Soil physicochemistry in created and natural wetlandsduring growing and non-growing season, 2008-2009 38

Table 2. Multivariate statistical pairwise comparisons of bacterial community Bray Curtis dissimilarities between wetland sites by each type of molecular analysis used in the study. 39

Table 3. Richness, evenness, and diversity measures of bacterial communities estimated by both LH-PCR and MTPS 40

Table 4. Soil physicochemical and bacterial community diversity for hydrologic connectivity groups 41

Figure 1. Map of the study sites located in the Piedmont region of Virginia.. 42

Figure 2. Principal component analysis (PCA) of physicochemical variables of soils 42

Figure 3. Relative abundances and distribution of major bacterial phyla in each wetland site during the non-growing (October 2008) and growing (June 2009) seasons. 42

Figure 4. Relationships (Spearman rank correlations) between major bacterial phyla revealed by MTPS in the communities of all wetland soils.....42

CURRICULUM VITAE.....47

ABSTRACT

BACTERIAL COMMUNITY COMPOSITION AND PHYSICOCHEMICAL PROPERTIES OF SOILS IN CREATED AND NATURAL WETLANDS IN VIRGINIA

Rita M. Peralta, MS

George Mason University, 2011

Thesis Director: Changwoo Ahn

Soil properties are often studied along with vegetation to examine the status of ecosystem development in created wetlands. Soil bacterial communities are essential to the biogeochemical processes in wetlands, yet are rarely examined when assessing ecosystem development in created wetlands. We used two molecular methods, amplicon length heterogeneity polymerase chain reaction (LH-PCR) and multi-tag pyrosequencing (MTPS), of 16S ribosomal DNA to characterize the bacterial communities of soils collected from two created (i.e., LC and BR) and two natural wetlands (i.e., BN and BP) during the growing and non-growing season in the Piedmont physiographic province of Virginia, USA. Soil physicochemical attributes [i.e., percent moisture, pH, soil organic matter (SOM) (%), total organic carbon (TOC) (%), total nitrogen (TN) (%), and C:N ratio were also investigated. The measures of both bacterial communities and physicochemistry of soils were tested for any association or relationship, and examined within and between these wetlands, in terms of age and hydrologic connectivity to a

surrounding fluvial system. Soil moisture was significantly higher during the growing season compared to non-growing season in the wetlands, primarily due to the natural wetlands (i.e., BN and BP), and an older and hydrologically better connected created wetland (i.e., BR) holding consistently higher soil moisture over two seasons. Soil pH of the wetlands ranged between 4.2 and 5.8, typical of the acidic soils of the Piedmont. Soil organic matter content were approximately 3 to 6 % with one of the LC wetland sites (LC1) and BN being consistently higher than in the other wetland sites. TOC and TN content followed the same pattern as SOM with no major seasonal differences. Soil bacterial community patterns were more clearly distinguished with MTPS (Ribosomal Database Project level 6: genus) method compared to LH-PCR, showing the merits of the new sequencing method. Based on the MTPS results there were many significant differences found in bacterial community patterns between the wetland sites (ANOSIM $R > 0.5$, $p < 0.05$), but with no differences between created and natural wetlands. However, one natural wetland (i.e., BP) with a lower soil pH seemed to be associated with least diverse bacterial communities. Bray Curtis dissimilarities of bacterial communities from each wetland site were correlated with C:N ratio ($\rho = 0.43$, $p < 0.01$) during the non-growing season, and with pH and SOM ($\rho = 0.40$, $p < 0.01$) during the growing season. In addition, relative abundance and distribution of major bacterial taxa varied both between the wetlands and between the seasons with certain taxa more sensitively responding than the others. The results also show phyla level correlations between certain major bacterial groups with *Acidobacteria* negatively correlated with *Bacteroides* ($r = -0.70$, *Bootstrap adjusted* $p < 0.05$), *Firmicutes* ($r = -0.78$, *Bootstrap adjusted* $p <$

0.05) and *Actinobacteria* (*r = -0.48, Bootstrap adjusted p<0.05*). When all plots of the wetlands studied were classified into three hydrologic connectivity (HC) settings the group with the higher HC (i.e., BR3) seemed to support higher bacterial community diversity. However, BN, the natural wetland with low HC, showed as high a bacterial community diversity measure as that of BR3. This might be attributed to the comparable soil moisture content in BR3 and BN plots, which were higher than the rest of the wetland sites/plots. Further investigation is needed on the relationship between bacterial community measures and physicochemical attributes in wetland soils, which may help us develop an useful microbial community indicator that can be used to track the functional progress of wetland soils in created wetlands.

1. INTRODUCTION

Wetlands are often created and/or restored as a result of Section 404 'no net loss' of the Clean Water Act, which mandates the mitigation of inevitable losses of natural wetlands (National Research Council, 2001). After more than two decades of wetland mitigation, the inherent ecological question of whether wetlands can be functionally replaced still remains (Zedler, 1996; Hoeltje and Cole, 2007; Ballantine and Schneider, 2009; Matthews et al., 2009). Many created or restored mitigation wetlands fail to meet regulatory standards for replacement of the natural wetlands, (Zedler, 1996; Spieles, 2005) often due to poor soil development (Bishel-Machung et al. 1996; Stolt et al., 2000; Cole et al., 2001; Campbell et al., 2002). Created and restored wetlands tend to show lower levels of organic C and N, higher bulk densities and lower productivity than their natural counterparts (Giese et al., 1999; Craft et al., 2002; Anderson et al., 2005; Bruland and Richardson, 2006; Fajardo, 2006; Fennessy et al., 2008). Still, in most cases of wetland mitigation, vegetation has been used as the sole measure of mitigation success (Breaux and Serefiddin, 1999; Spieles, 2005). Relying on vegetation alone leaves out the role of soil physicochemical (e.g. soil moisture, pH, C content) and biological (e.g. bacterial communities) attributes in the functional development of wetlands.

Soils host diverse bacterial communities that mediate key biogeochemical processes including nutrient cycling and energy transfers (Batzer and Sharitz, 2007;

Madigan et al., 2008). Use of molecular tools has provided increasing evidence that the patterns of bacterial communities are related to soil processes (Bossio and Scow, 1995; Fierer et al., 2003; Zak et al., 2003; Merkley et al., 2004; Edwards et al., 2006; Gutknecht et al., 2006; Hunter et al., 2006; Mentzer et al., 2006; Zul et al., 2007; Strickland et al., 2009). Many of these processes occur in coupled biogeochemical reactions that are carried out by different members of the bacterial community (Batzer and Sharitz, 2007; Madigan et al., 2008). For example, the biological removal of N through denitrification is carried out by a series of bacteria (and fungi) that metabolize and reduce nitrate to gaseous N under anaerobic conditions. Identifying process-level controls thus needs an understanding of the dynamics of bacterial community structures.

Soil physicochemistry is often studied to track the progress of created wetlands (CWs) since it is closely related to vegetation development over time that is regularly monitored to examine the success of wetland mitigation. Soil organic matter (SOM) and carbon in particular are considered the main drivers of biogeochemical processes in wetlands (Reddy and DeLaune, 2008; Messina and Conner, 1998). Nutrient availability affects bacterial growth rates and the C:N ratio has been identified as a limiting factor in some wetland environments (Reddy and DeLaune, 2008). Yet, created wetlands have been found to lack in SOM accumulation (Craft et al., 2002; Anderson et al., 2005; Bruland and Richardson, 2006) which could directly impact the development of ecological functions (Reddy and DeLaune, 2008). The ability of wetlands to support diverse metabolic and catabolic processes depends on the ability to support anaerobic and

aerobic environments (Ogram et al., 2006; Reddy and DeLaune, 2008), which are directly affected by SOM and the resulting water holding capacity (D'Angelo et al, 2005).

Hydrology is still the most important driving force for the ecosystem development in wetlands (Mitsch and Gosslink, 2000). Hydrologic connectivity (HC) can be ecologically defined as the water-mediated transfer of matter, energy and/or organisms within or between elements of the hydrologic cycle (Pringle, 2003). Hydrologic inputs (e.g., precipitation, surface runoff, groundwater, and overbank flow) and outputs (e.g. surface runoff, groundwater recharge and evaporation) help determine the water depth, hydroperiods and other flow patterns that influence soil biochemistry and bacterial community dynamics (Batzer and Sharitz, 2007). During flooding periods water limits the amount of oxygen that can infiltrate the soil matrix and given a long enough flooding period soil microbes consume the residual oxygen until anoxia. In the absence of oxygen, metabolically diverse bacterial communities can consume other chemical electron acceptors such as nitrate, iron, magnesium, and organic substances in a series of oxidation-reduction reactions (Batzer and Sharitz, 2007), altering the morphological patterns (redoximorphic features) of the inundated soils. Redoximorphic features persist after soils become aerated and are used as hydric soil indicators, often examined for wetland development (Vepraskas et al., 2006). However, the lack of design criteria in the creation of mitigation wetlands has left many created wetlands with little to no connection to other water bodies (e.g., perched design). Wetlands with better HC to surrounding fluvial systems may better restore natural dynamics necessary for wetland soil development. A recent study (Wolf et al., 2011) found that HC changed soil

physicochemical patterns and enhanced nitrogen cycling in both created and natural wetlands, but little is known about the effects of HC on soil microbial communities.

We analyzed bacterial community composition and physicochemical parameters of soils in created and natural wetlands (NWs) in the Piedmont physiographic region of Virginia. Specifically we use LH-PCR fingerprinting and multi-tag pyrosequencing (MTPS) to build community fingerprints, define the bacterial composition and diversity, and examine their association with soil physicochemical attributes studied. Three main objectives of the study were 1) to compare bacterial community patterns and physicochemistry of soils between created wetlands of different ages and between created and natural wetlands, 2) to investigate if any relations or association between bacterial community composition and diversity, and the developments in soil attributes, and 3) to examine if a design element of created wetland (i.e., hydrologic connectivity) has any effect on bacterial communities and physicochemistry of wetland soils.

2. SITE DESCRIPTIONS

2.1 General setting

Four non-tidal freshwater wetlands located in the Piedmont physiographic region of northern Virginia were chosen for this study (mean annual precipitation 109 cm, mean temperature min 7 ºC/ max 18ºC; Figure 1). The Piedmont is characterized by rolling terrain underlain by igneous and metamorphic rock (Hook et al., 1994; Moser et al., 2009). Two of the wetlands are mitigation wetlands created on old farmland to mitigate the loss of a mixture of bottomland forested floodplain, shrub/scrub and emergent wetlands. At the time of sampling the created wetlands (CWs) had a predominantly herbaceous cover, with some open water aquatic areas, shrub-scrub areas, and/or young stands of trees. The NWs are part of formerly farmed parkland and include bottomland riparian forested wetlands and open herbaceous wetlands. Natural sites were chosen for proximity and shared tributary access to each respective created wetland as references.

2.2 Created wetlands

Loudoun County Mitigation Wetland (LC) (39°02.05' N, 77°36.5' W) is a 32-acre wetland and upland buffer complex constructed in 2006. Although LC is a large created wetlands complex there were three distinct sections that were quite different from one another, so we decided to categorize the LC into three sites. Site 1 (LC1) and site 2 (LC2)

are located adjacent to each other and separated by a berm. The third site (LC3) is separated from LC1 and LC2 by an access road. All lie within the 100 year flood plain of Big Branch Creek and Goose Creek. LC2 receives flow from an unnamed tributary of Goose Creek through a head race attached to a cross vane structure with flow impacts regulated by an Agridrain structure and gate valve. The entire wetland complex receives surface water runoff from an upland housing development and forested buffer. A small part of LC3 also receives groundwater input from toe-slope intercept seepage. Vegetation is currently dominated by herbaceous plants with small, container-grown, planted woody vegetation interspersed throughout.

Bull Run Mitigation Wetland (BR) is a 50-acre wetland and upland buffer complex, constructed in 2002 in Prince William County, Virginia (Latitude 38º51′12.74″ N Longitude 77º32′58.52″ W). The site receives water from Bull Run from an AgriDrain structure that routes water via a central ditch through the wetland, as well as overbank flow from Bull Run, which sharply bends around the corner of the site. The wetland receives limited surface water runoff from uplands and negligible groundwater. Vegetation is predominantly herbaceous, with small, planted woody vegetation throughout.

The wetland design for both sites contains at least 0.3 m low permeability subsoil layer covered with 0.2 m of commercially available topsoil. This design creates a perched, surface water driven water table close to soil surface and limits groundwater exchange in the wetland. The sites were hydro-seeded with commercially available

wetland plant seed mixes appropriate for the region and the intended hydrology (e.g., wetland meadow as opposed to obligate wetland).

2.3 Natural wetlands

Manassas National Battlefield Park (BP), established in 1940, is a 5,000 acre site with areas of natural wetland coverage located in Prince William County, Virginia (38°48.46 ″ N, 77°31.18 ″ W). An area of herbaceous wetland within a matrix of forested floodplain was selected for study and comparison to the CWs. The site is connected to Bull Run by a culvert on its eastern end and also receives groundwater and upland surface water runoff. Vegetation is mostly herbaceous with a few mature trees interspersed throughout.

Banshee Reeks Nature Preserve (BN), established 1999, is a 725-acre site with areas of natural seep and riparian wetlands located in Loudoun County, Virginia (39º1′16.44″ N, 77º35′49.10″ W). The studied floodplain riparian wetlands receive water from groundwater springs, surface water runoff, and occasional overbank flooding from Goose Greek.

3. METHODS

3.1 Soil sampling design

Study plots at the created and natural wetlands were selected so that typical hydrology and vegetation of the wetland site was represented. There were a total of 12 plots in two created wetlands (i.e., four created wetland sites) and 5 plots in two natural wetlands. Soil sampling in the wetlands occurred over a two-day period in a non-growing season (October 2008) and a growing season (June 2009) in a year period. Plots were divided into four 5 x 5 m quadrants. Within each quadrant, three soil samples were taken at the depth of 5-10 cm from the top by use of an auger (1 1/4" diameter) at random and combined in a polyethylene bag. All samples were kept in a cooler with ice packs to slow bacterial activity until further processing in the laboratory. At the laboratory, each bag was homogenized manually to mix all three samples for each quadrant. Any visible root or plant material was manually removed prior to homogenization. Once mixed, a subsample was taken from each bag and transferred to a 2 ml tube for bacterial community analysis. Tubes were then frozen and transported to a -80C refrigerator for storage until microbial analysis could be conducted.

3.2 Soil physicochemical analysis

Sub-samples taken for SOM, TOC, total nitrogen (TN) and pH were air dried for approximately 48 hours, with the exception of the wettest samples that were allowed to dry for a longer period. Once air dried, soils were macerated using a mortar and pestle and any large constituents (e.g. rocks and large organic debris) were removed. SOM was calculated by the difference between oven dried weight and LOI weight (Nelson and Sommers, 1996). A Perkin-Elmer 2400 Series II CHNS/O Analyzer (Perkin-Elmer Corporation, Norwalk, CT, USA) was used to analyze percent TOC (~TC) and percent TN. Sub-samples (2-3 grams of air dried soil) were separated for SOM, loss on ignition (LOI) method, and oven dried at 105 °C for 24 hours, weighed and placed in 405 °C for 16 hours (Blake and Hartge, 1986). Soil C:N ratio was calculated based on the above measurements of carbon and nitrogen.

3.3 Soil bacterial community analysis

3.3.1 DNA extraction

Total bacterial community DNA was isolated from approximately 0.5-1 g of soil per sample using the Bio 101 FastDNA ® SPIN Kit for soil (MP Biomedicals, Inc., Carlsbad, CA). Extracted DNA and ten-fold dilutions (5 µL DNA in 45 µL buffer) were stored at -20C.

3.3.2 LH-PCR

LH-PCR bacterial community fingerprints were created by amplifying two variable regions of the 16S rRNA gene using 27F (FAM labeled) and 355R universal bacterial primers in 20 µL Polymerase Chain Reactions (PCR) as described by Ahn and

Peralta (2009). Briefly, the reactions were performed using 20µL mixtures containing a final concentration of: 1× PCR-GOLD buffer, 25mM MgCl2, deoxynucleoside triphosphates at a concentration of 200µM each, primers at the concentration of 0.5µM and 0.5U (5 units/µL) of AmpliTaqTM-GOLD DNA polymerase (Applied Biosystems, Foster City, California). GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA) was programmed for initial denaturation and polymerase activation at 95 ◦C for 11 min. It was followed by 35 cycles of denaturation at 95 ◦C for 30 s, annealing at 48 ◦C for 30 s, and extension at 72 ◦C for 2 min (+ 5 s/cycle), with a final extension step at 72 ◦C for 30 min to ensure the complete extension of all the fragments. PCR products were checked for sufficient amplification using a 1% agarose gel with ethidium bromide. They were stored at 4 ◦C until used in fingerprinting. A 1:10 dilution of the PCR products was made and mixed with 1:20 ratio of internal size standard (ILS 600, Promega) and HiDi Formamaide (Applied Biosystems, Foster City, CA). The mixtures, containing amplicons of variable lengths, were then separated and visualized using capillary electrophoresis on an SCE 9610 capillary (Spectrumedix LLC, State College, PA). Analysis of the community fingerprints was done using the GenoSpectrum (Spectrumedix LLC, State College, PA) software package (version 2.08) to convert fluorescence data into electropherograms. The resulting LH-PCR fingerprints are composed of peaks representing operational taxonomic units (OTUs) where the peak area is proportional to the abundance of that amplicon. The identified OTUs may represent various bacterial taxa and cannot be interpreted as distinct species (Acinas et al., 2004). The LH-PCR

fingerprint data was analyzed using a custom PERL script to normalize the data and calculate the relative abundance of each amplicon size for each sample.

3.3.3Multitag pyrosequencing (MTPS)

MTPS is a high throughput sequencing by synthesis technique that expands on the 454 pyrosequensing protocol (Gillevet et al., 2010). The MTPS technique uses specifically designed emulsion PCR fusion primers that contain the 454 emulsion PCR adapter, joined to a 7 base tag along with the appropriate target primers (Gillevet et al., 2010). The addition of the tagged primers allows for pooling of several samples in each pyrosequencing run. The data then is deconvoluted by sorting the sequences into bins based on the barcodes using custom PERL scripts (Gillevet et. al., 2010).

While MTPS analysis allows for lower cost per sample than regular pyrosequencing, price continues to be a limiting factor. Therefore, we performed MTPS analysis using two samples per plot chosen at random using a randomization generator rather than the four used for LH-PCR. The same PCR conditions used to amplify the bacterial community for LH-PCR were used, with the exception that tagged fusion primers (containing linkers and tags) were used for the MTPS based analysis of the V2 and 3 of the 4 16S rRNA regions (Gillevet et al, 2010). The PCR products were then purified using Ampure magnetic beads (Agencourt Bioscience Corp.) and combined for pyrosequencing on a Roche GS-FLX instrument.

The 454 pyrosequencing protocol uses oil emulsion PCR to amplify the DNA strands and immobilize all amplicons on each bead. After sufficient amplification, the oil

emulsion is broken and the beads released and placed on a picotiter plate. Picotiter plates are etched with wells that allow for only one bead per well, packed with the enzymes to synthesize the DNA and allow for other crucial enzymatic reactions. This set up allows for all reactions in the plate to occur in parallel but isolated from one another. The pyrosequencing reaction is achieved by laminar flow of one of the four deoxynucleotide triphosphates over the slide at a time. As each nucleotide is incorporated into the synthesis of the new strand, pyrophosphate is released in proportion to the number of nucleotides added. The pyrophosphate released is then converted to ATP in the presence of adenosine 5´ phosphosulfate. The final reaction uses ATP to fuel the conversion of luciferin to oxyluciferin by the firefly enzyme luciferase and subsequently releases light in proportion to the number of bases added. The light is detected by a camera in the Roche GS-FLX instrument to produce peaks in a pyrogram. The sequence data were compared to sequences in the Ribosomal Database Project release 10 (RDP 10) using a custom PERL script. MTPS consisted of RDP matched sequences and was hierarchically classified from phyla (RDP level 1) to the genus level (RDP level 5).

3.4 Hydrologic connectivity groupings

Hydrologic connectivity is most often studied in terms of categorical groupings of open or closed systems to the surrounding hydroscape (Hopkinson, 1992; Craft and Casey, 2000). Wolf (2010) derives HC as a PCA component of measured hydrologic variables. For the purpose of this study the PCA component was limited to

sedimentation, standing water and redox measures that account for surface and sub-surface hydrology and the variability within those measurements.

3.5 Data analysis

Data were analyzed separately for each seasonal grouping (e.g. non-growing and growing) to analyze wetland and site specific data differences. Normality was checked by generating box plots, probability plots and quantile-quantile plots. If the normality of a data set was deemed severely non-normal it was transformed by applying a log transformation. Soil attributes, including SOM, TOC, TN, percent moisture and pH were tested for differences among wetlands, sites and plots with the one-way 'analysis of variance' (ANOVA). Pairwise comparisons were made with Dunnett's T3 post-hoc test which does not require the assumption of homogeneity of variance and is better suited to handle unbalanced designs (e.g. different number of samples per group). Scatterplots were used to check for relationships between variables and linear regressions were conducted on any set demonstrating strong relationships. TOC and TN were found to be very strong covariates (adjusted $R^2 = 0.9$) and since TOC is a component of SOM, it was omitted from further statistical analysis. Statistical analyses were conducted using SYSTAT V.12 software (Systat, 2007).

Principal component analysis (PCA) of the soil physicochemical variables was conducted to identify groups of variables contributing most to variance between samples. The PCs were then used to visualize the data on a two dimensional plane. Site differences in bacterial community fingerprints (OTUs) and MTPS (taxa) determined

compositions were tested using the rank similarity matrices and the 'analysis of similarities' (ANOSIM) test which is a non-parametric analogue of the one-way ANOVA (Clarke and Gorley, 2001). Both p-values and an R statistic value are generated for each pairwise test. The R values, which are an absolute measure of differences between the groups, are interpreted in the results (Clarke and Gorley, 2001). The relationship between the SBCs and the respective physicochemical attributes was probed using RELATE, non-parametric comparative (Mantel-type) tests, on matched similarity matrices to calculate the Spearman rank correlation (ρ). Statistical analyses were conducted using Primer V6 software (Clarke and Gorley, 2001).

Additional analysis of the relationships between community patterns and soil physicochemistry was conducted using scatterplots to plot diversity indices against physicochemical PCs. Plots that showed possible correlations were tested using non-parametric Spearman rank correlation test (Zuur et al., 2007). HC groups were identified using HC variables studied by Wolf (2010) that included all the wetland sites investigated in this study. The hydrologic variables defined to be representative of HC status (Wolf, 2010); including the coefficient of variance (CV) for redox potential, CV of standing water, average standing water, average sediment mass accumulation and CV of sediment mass accumulation for, were analyzed by a cluster analysis . Study plots were separated at 40% resemblance by HC, using PRIMER software (Clarke and Gorley, 2001).

Three bacterial community diversity indices were calculated using both fingerprint (OTUs) and pyrosequencing (taxa) data. Richness (S) is equal to the number of OTUs or taxa in each sample. The Shannon Diversity Index (H') is equal to $\sum$(pi(ln

pi)), where pi is the peak area (i.e., a relative abundance of amplicon size) in the ith species. Evenness (*E*) is equal to *H'*/ln S. Site averages of the three indices were compared using one-way ANOVA using SYSTAT V.12 software (Systat, 2007).

4. RESULTS

4.1 Soil physicochemistry

4.1.1 Soil physicochemical attributes by season

The average moisture (%) of soils in the four wetlands was significantly greater during the growing season (40.2% ± 1.8) than the non-growing season (29.2% ± 2.2) ($p < 0.001$). The soil pH levels were marginally lower during the non-growing season (4.9 ± 0.1) than the growing season (5.1 ± 0.1) (*Bonferroni adjusted* $p = 0.09$). Soils did not demonstrate significant seasonal differences in SOM ($p = 0.86$), TOC ($p = .63$), TN ($p = 0.89$) or C:N ($p = 0.70$).

4.1.2 Soil physicochemical attributes by wetland site

The NWs (i.e., BN and BP) significantly differed from each other in all soil physicochemical variables except pH ($p=0.12$) and C:N ratio ($p = 0.88$) (Table 1). The natural forested wetland soils (e.g. BN) retained the highest percent moisture levels ranging between 38.2% in the non-growing season to 53.9% in the growing season, of all wetlands studied (Table1). The 6 year old created wetland (e.g. BR) and the natural herbaceous wetland (e.g. BP) had similar soil moisture (%) levels during both seasons. The lowest soil moisture values were in LC (2 year old created wetland) where LC2 and LC3 averaged 15.7 and 16.7%, respectively during the non-growing season. However,

LC1 moisture levels more similarly resembled the natural sites, ranging between 27.7 % and 55.2 %. The SOM content of the CW soils ranged between 3.3 to 6.2 % (Table 1). Soil organic matter ranged between 4.9 to 6.1% for the forested wetland (i.e., BN) and 2.9 to 3.6 % for the herbaceous wetland (i.e., BP). Pairwise comparisons revealed that soils collected in BN and LC1 have similar SOM, TOC and TN content in both seasons (Table 1).

The PCA results for the non-growing season showed that 68.5 % of the variance was explained with two components (Figure 2a). Component 1 (PC1) accounted for 46.8 % and component 2 (PC2) for 21.7 % of the variance of the samples. PC1 is negatively related to all variables except C:N and strongly correlated with N and SOM. PC2 is strongly negatively related to the soil pH and C:N and slightly negatively related to SOM. The PCA for the soil physicochemical variables during the growing season showed a cumulative percent of 67.5 % explained with two components (Figure 2b). PC1 is 40.8 % and PC2 is 26.7 %. The former is positively related to all variables but highly correlated to moisture, TN and SOM. PC2 is highly positively related to pH and to a lesser extent SOM. It is negatively related to C:N as well as moisture and N.

4.2 Soil bacterial community composition and diversity

4.2.1 Bacterial community composition by LH-PCR and by MTPS

Bacterial communities scoped by MTPS led to higher number of differences than by LH-PCR between wetland sites (Table 2). During the growing season there were 7

strongly dissimilar ($R > 0.5$, $p < 0.05$) pairwise comparisons of Bray Curtis dissimilarities of MTPS derived taxa compared to 9 during the non-growing season. ANOSIM results showed strong Bray Curtis dissimilarity of bacterial composition (genus level by MTPS) between sites during the non-growing season (*Global* $R = 0.649$, $p < 0.05$) and weaker, but sure dissimilarity during the growing season as well (*Global* $R = 0.26$, $p < 0.05$). LH-PCR derived Bray Curtis dissimilarities generated ANOSIM results that showed a marginal dissimilarity between wetlands during the non-growing season (*Global* $R = 0.137$, $p < 0.05$), but a slightly higher dissimilarity during the growing season (*Global* $R = 0.238$, $p < 0.05$).

Generally, the SBCs in the CWs were more similar to each other than to the SBCs of either NWs, especially during the growing season (Table 2). The non-growing season ANOSIM pair-wise comparisons of the sites showed the biggest difference between the two natural wetlands, BP and BN ($R = 0.937$, $p < 0.05$). The measures of SBCs in BN consistently showed significant differences from those in the other wetland sites, except BR during the growing season (Table 2). BP was different from any created site in SBCs. Among the created wetland sites (i.e., LCs and BR) LC3 was consistently different from both LC 1 and LC2 (Table 2). The SBC measures in BR were different from all LC sites only during the non-growing season (Table 2).

Figure 3 shows overall community patterns between the two seasons with the relative abundances of bacterial phyla plotted using a stacked bar chart of the percentage that each phylum contributes to each site. Generally the *Proteobacteria* contributed to the majority of the bacterial composition of all soils during both seasons (Figure 3).

However, seasonal variability of the *α-Proteobacteria* sub-phylum was pronounced, ranging between 18 to 34% in the non-growing season and 32 to 52 % in the growing season (Figure 3). While *δ-Proteobacteria* and *γ-Proteobacteria* contributed to a higher percentage of the bacterial population during the non-growing season (7 to 13 % and 3 to 5 % respectively). Seasonal patterns were also noted in increased percent composition of *Bacteroidetes, Bacteria incertae sedis,*and *Nitrospira* in the growing season. On the other hand, *Chloroflexi* and *Actinobacteria* percent composition was lower during the growing season.

Site specific changes were also observed in the relative abundances of some of the major bacterial phyla. While *Acidobacteria* percent composition remained constant for most sites during both seasons, it increased in LC1 and BP during the growing season (Figure 3). LC1 and BP also experienced a decrease in percent composition of *Actinobacteria* during the same time. *Bacteria incertae sedis* populations appeared in LC1 and BN and disappeared in LC2 during the growing season. LC1 and BP also saw the appearance of *Nitrospira* while the population decreased in BN during the growing season.

In addition, some relationships were clearly found between the dominant phyla (Figure 4). The relative abundance of *Acidobacteria* are negatively correlated with *Bacteroides* ($r = -0.70$, *Bootstrap adjusted* $p < 0.05$), *Firmicutes* ($r = -0.78$,*Bootstrap adjusted* $p < 0.05$) and *Actinobacteria* ($r = -0.48$, *Bootstrap adjusted* $p < 0.05$). While *Firmicutes* and *Bacteroides* were positively correlated with each other ($r = 0.88$, *Bootstrap adjusted* $p < 0.05$) (Figure 4).

4.2.2 Soil bacterial community diversity

The species richness values ranged from 16 to 20 OTUs for LH-PCR and 10 to 21 taxa (genus level) based on the MTPS data (Table 3). The latter contained a total of 110 different taxa and the former 57 OTUs. No differences in the community diversity were found between created and natural sites, but there were significant differences in all diversity measures between the two seasons (*Bonferroni adjusted* $p < 0.001$) (Table 3). All diversity indices were higher in the non-growing season consistently in the measures by both LH-PCR and MTPS (Table 3). LH-PCR based diversity measures showed significant differences among sites ($p < 0.05$) in both seasons, with the exception of evenness during the growing season ($p = 0.25$) (Table 3). For MTPS based diversity measures, J was different between sites during the non-growing season and S and H´ were different with BP being consistently less diverse ($p < 0.05$) (Table 3).

4.3 Association between bacterial community measures and physicochemical attributes of wetland soils

Multivariate statistical analyses revealed some significant association between SBC measures and the patterns of soil physicochemical attributes in the wetlands. Association between SBC fingerprints and physicochemistry were not significant ($\rho = 0.02$ with C:N, $p = 0.9$ during the non-growing season ; $\rho = 0.21$ with pH, $p = 0.1$ during the growing season). MTPS derived communities did relate to physicochemical attributes. During the non-growing season C:N was correlated with the Bray Curtis dissimilarities of bacterial taxa composition ($\rho = 0.43$, $p < 0.01$) while during the

growing season pH ($\rho = 0.39$, $p < 0.01$) were the highest correlated variables to the SBC. To further probe the relationship between community structure and physicochemical variables, the physicochemical PC1 and PC2 scores (Figure 2) were tested for correlations with the bacterial community diversity measures (Table 3). During the non-growing season PC1 (most variance explained by SOM and TN) was slightly correlated with the J of bacterial communities ($\rho = 0.31$, $p < 0.05$) while PC2 (variance explained by pH) was fairly correlated ($\rho > 0.40$, $p < 0.05$) with all three community diversity measures (Table 3) during the growing season.

4.4 Characterization of HC groups and bacterial community and physicochemisry of soils by HC groups

The cluster analysis (grouped at 40 % resemblance) of the wetland sites/plots in this study by the hydrologic variables representative of HC (see Wolf, 2010) led to three HC groups, with BR3 being of high HC (HCG1), BN plots 4 and 5 being of low HC (HCG3), and the rest of the included wetland plots being of medium HC (HCG2). Specifically, HCG1 had high average sediment accumulation; HCG2 had higher CV redox potential and average standing water; HCG3 had the lowest average standing water and sediment accumulation.

Gravimetric soil moisture was highest in the high sedimentation HCG1 and the forested wetland plots that made up HCG3 ($p < 0.05$; Table 4). There were no differences in soil TOC, TN and SOM between any groups. Bacterial community

diversity (*H'*) using MTPS data was higher in HCG1 and HCG3 than HCG2 ($p < 0.05$; Table 4).

5. DISCUSSION

Ahn and Peralta (2009) previously found that TOC and TN tend to increase with age and the C:N (which was negatively related to moisture content) has a significant association with SBC structures in three CWs in the Piedmont physiographic region of Virginia. The present study expands on those results by incorporating MTPS analysis to explore the bacterial composition in two created mitigation and two natural non-tidal freshwater wetlands.

5.1 Soil properties in created vs. natural wetlands

Age related soil development through improved wetland soil quality (higher SOM, TOC, TN and moisture content) was not apparent when comparing the wetlands (Table 1). Created mitigation wetlands are usually constructed by removing the top layer of soil and leaving a 'bath tub' like structure that is engineered to maintain the desired hydrology. Because the construction process usually removes top soil without replacement, CWs tend to demonstrate lower levels of organic matter (Confer and Niering, 1992; Bishel-Machung, 1996; Campbell et al., 2002; Bruland and Richardson, 2006) and modified soil texture (Zedler 1996, Shaffer and Ernst 1999, Whittecar and Daniels 1999, Stolt et al., 2000). Texture was found to be similar among all sites by a concurrent study (Wolf, 2010). Organic matter accumulation is one defining feature of

wetlands due to high productivity and slow decomposition rates (Mitsch and Gosselink, 2000). However, the two created wetland sites observed in this study were built with replacement and addition of topsoil and induced microtopography through disking. The latter factor was found to influence above ground productivity in freshwater wetlands in Virginia (Moser et al., 2007). In the case of LC1, drift of organic amendments (applied to the wetland as a whole, but not to the study plots) may account for higher than expected TOC, TN and SOM (ranging from 1.7 – 2.3 %; 0.14 – 0.21 %; 4.9 – 6.1 % respectively) (Ahn and Peralta, 2009).

A number of studies have suggested that forested wetlands take longer (15 – 50 years) to develop than non-forested wetlands (Niswander and Mitsch, 1995; D'Angelo et. al., 2005). While one of the goals of this study is to evaluate age related patterns between created sites and between CWs and NWs, admittedly both CWs are still very young (LC and BR were 2 and 6 years old respectively during October 2008 sampling). In this regard, the created sites (with the exception of LC1) demonstrate TOC and SOM levels more comparable to BP (a mainly herbaceous site) during both seasons (Table 1). While LC1 and BN, at least when it comes to soil physicochemistry, consistently cluster together (Figure 2). The PCA results indicate that TN and SOM (TOC implicitly) are reliable maturation indices since they consistently (PC1 for both non-growing and growing seasons) (Figure 2) show the highest levels in the natural forested wetland site (and LC1).

5.2 Bacterial composition and diversity of created and natural wetlands

While differences in diversity indices were not always significant, all diversity indices were higher and more variable during the non-growing season (Table 2). Seasons differed significantly in moisture content with the growing season having a wetter status that may have decreased the heterogeneity of the soil environment (e.g., inundation reducing microtopography) thus affecting the SBCs. Bacterial communities have been shown to be sensitive to dry-wet stress (Fierer et al., 2003) phosphorus loading (Ahn et al., 2007), variability in microtopography (Ahn et al., 2008) and C:N and moisture content (Ahn and Peralta, 2009) indicating a role of seasonal hydrologic change in the SBC structures. Additionally, SBC diversity measures were correlated with SOM and TN (PC1 of physicochemical variables) during the non-growing season which has a role in nutrient availability and water holding capacity.

Table 3 shows that there were significant differences in SBC Bray Curtis dissimilarities of the two natural wetlands. BN and BP represented two very different natural wetland settings; a forested wetland and a mainly herbaceous wetland respectively. Additionally, BP is impacted by stream flooding events that are more frequent than those experienced by BN which is perched.

5.3 Association between physicochemical attributes and bacterial community measures in wetland soils

When comparing the SBCs, the greatest differences were between the two types of NW (e.g. BP- herbaceous vs. BN- forested) and between the created sites and BP regardless of the molecular method (Table 3). While communities would have been

expected to more closely resemble BP than BN, if taking soil physicochemistry results alone, it seems that the sensitivity of organisms to pH levels may have been a larger driver. Still, BIOENV results did show a moderate relationship between the MTPS resemblance matrix and C:N ($\rho = 0.43$) during the non-growing season. This relationship was identified during the previous non-growing season as well (Ahn and Peralta, 2009). During the growing season the communities were moderately related to pH and SOM ($\rho = 0.40$). These results are congruent with other published results finding pH to be the biggest indicator of bacterial community structure in a range of soils (Hartman et al., 2008, Lauber et al., 2009).

Biogeochemical wetland processes including decomposition of organic matter and nutrient cycling are mediated by microbial communities and moderated by the coupling of aerobic and anaerobic conditions (Reddy and DeLaune, 2005). Although this study does not explicitly measure function (e.g. denitrification); identification of community members along with physicochemical conditions can be used as a tool to further our understanding of these processes. Recent work using molecular amplification of functional genes has shed some light into the controls over community structure (Wallenstein et al., 2006). One of the most frequently studied processes is denitrification, where bacterial community structure has been found to be influenced by moisture, substrate availability (organic matter) and pH (Wallenstein et al., 2006; Flores-Mireles et al., 2007; Peralta et al., 2010). While denitrificatiers are a diverse community that includes bacteria from different phyla, we can look at the community dynamics in relation to the above mentioned parameters. We collected our samples during two

seasons that were very different in moisture content (non-growing season range: 15 % - 46 %; growing season range: 33 % - 54 %) and to a lesser extent pH (Table 1). The overall diversity of the growing season was lower than that of the non-growing season (Table 2). This might have occurred as a result of increased moisture and presumably increase in anoxic conditions, leading to selection of anaerobic or facultative anaerobic organisms. Specifically, during the wetter season we observed changes in percent composition of several phyla including an increase of anaerobic *Bacteroides*, and chemolitho-autotrophic nitrite-oxidizing *Nitrospira*, suggesting variable oxic/anoxic conditions. MTPS data shows that percent composition of *Actinobacteria* (important cellulose and chitin decomposers; Madigan et al., 2008), *Bacteroides* and *Firmicutes* (includes obligate anerobic *Clostridia*) decrease while *Acidobacteria* population increases (Figure 4). These relationships can notably be shown even as the correlation between physicochemistry and bacterial communities does not appear as clearly.

5.4 Potential role of site hydrologic connectivity on biogeochemical patterns of wetland soils

Soil physicochemical variations between HCGs indicate that the higher surface hydrology (HCG1) does increase sub-surface hydrology (e.g. soil moisture) as well as the accumulation of SOM relative to plots within the same site but defined in HCG2 (e.g. BR4-6; Table 4). While HCG3 was defined by low surface hydrology measures and thus lower connectivity to the adjacent stream, it also had higher soil moisture than HCG2 which had higher standing water levels. This difference may indicate the role of

accumulated SOM and (presumed lower bulk density) in the forested wetland plots (HCG3) allowing for better water holding capacity in this natural site (Noe and Hupp, 2005). The clustering of SBCs by the HCG 1 and 3 were not surprising as SBCs generally displayed closer resemblance within sites than between sites (Table 2). A concurrent study found increased ammonification rates (N-mineralization) in soils with greater HC (Wolf, 2010), suggesting a carbon limited environment (Reddy and DeLaune, 2008). Additionally, while HC did not directly enhance nitrification and denitrification rates, they were found to increase with soil moisture, which was highest in HCG1 (e.g., highly connected created plot) and HCG3 (e.g., BN natural forested wetland).

5.5 Suitability of molecular methodology

The characterization of SBCs presents a challenge and is limited by the methodology available. While in previous studies the use of LH-PCR was found to be sufficiently sensitive to characterize soil communities (Ahn et al., 2007; Ahn et al., 2008; Ahn and Peralta, 2009), this study highlights its limitations and showed higher sensitivity and effectiveness of the MTPS method in better distinguishing and identifying SBC patterns as found in Hartman et al. (2008) and in Acosta-Martinez et al. (2008). The LH-PCR fingerprints are composed of peaks representing operational taxonomic units (OTUs) where the peak area is proportional to the abundance of that amplicon. The identified OTUs may represent various bacterial taxa and therefore the LH-PCR fingerprints are better used to estimate community diversity dynamics rather than true composition. The value of fingerprinting a bacterial community is that it is a low cost

tool to evaluate and compare whole bacterial structure from various sources that has been repeatedly used for soil bacterial community analysis (Ritchie et al., 2000; Ahn et al., 2007; Ahn and Peralta, 2009).

5.5 Implications of the outcome of the study

Soil development was found to be variable in terms of soil physicochemical parameters and SBCs in created wetlands (Ahn and Peralta 2009, Wolf et al., 2011). Physicochemical properties tended to show an age related progression in development with SOM accumulation leading to higher moisture content (Campbell et al., 2002; Ballantine and Schneider, 2009). However, the evaluation of SBCs showed that their structural patterns may relate to some soil physicochemical attributes as well as community-level interactions (Allison et al., 2005; Hartman et al., 2008). SBCs were sensitive to C:N ratio during the drier non-growing season, which was also observed by our previous study (Ahn and Peralta, 2009). We were also able to discern the potential impact of HC on SBCs in this study (Table 4). The plot with the greatest level of HC (e.g., BR plot 3) had higher soil moisture and SBC diversity that were comparable to the natural forested wetland (e.g., BN).

The state of wetland creation and mitigation of the losses is based on the premise of replacement of function. If the function being lost involves biochemical reactions, then SBCs are integral in the determination of proper soil development. While these biochemical processes can be regulated by a myriad factors (e.g. oxygen content of soil, temperature, pH, nutrient influx), they are ultimately carried out by the soil microbial

communities. In the case of impacted soils, such as created wetlands, the patterns of bacterial communities may be the best indicator of this function (Allison et al., 2005; Hartman et al., 2008; Acosta-Martinez et al., 2008).

The outcome of the study also revealed the characterization of physicochemical and SBC of soils in created and natural wetlands as influenced by HC settings of wetland sites. Greater HC leads to increased water holding capacity and prolonged flooding (Wolf, 2010). These conditions increase reduced condition and allow for the biochemical reactions that facilitate the development of hydric soils for which SBCs are thus inevitably involved. The results of the study showed the effects of HC on both soil physicochemical attributes and SBC diversity. Based on the outcomes of this study future studies are necessary and should focus on evaluating changes of SBCs in both the structure and function, which may assist the development of SBC indicator to examine and track the progress of soil development in created wetlands, thus enhancing the success of wetland mitigation.

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Table 1. Soil physicochemistry (Mean ± SE)* in created (LCs and BR) and natural wetlands(BN and BP) during growing and non-growing season, 2008-2009

Wetland site	Moisture (%)	pH	SOM (%)	TOC (%)	TN (%)	C:N
Non-Growing Season						
LC 1	30.5 ± 2.80 b	5.5 ± 0.26 a	5.2 ± 0.14 a	2.1 ± 0.22 a	0.19 ± 0.02 a	11.3 ± 0.07 b
LC 2	15.7 ± 0.74 c	4.7 ± 0.14 b	3.7 ± 0.10 b	1.1 ± 0.00 b	0.11 ± 0.01 b	11.3 ± 0.79 b
LC3	16.7 ± 1.76 c	5.3 ± 0.09 a	3.9 ± 0.05 b	1.3 ± 0.02 b	0.09 ± 0.03 b	17.2 ± 6.24 a
BR	30.5 ± 1.37 b	4.7 ± 0.05 b	3.6 ± 0.29 b	1.2 ± 0.16 b	0.11 ± 0.01 b	10.8 ± 0.32 b
BN	42.1 ± 3.67 a	5.1 ± 0.35 ab	5.6 ± 0.54 a	2.2 ± 0.22 a	0.19 ± 0.01 a	11.0 ± 0.65 b
BP	30.7 ± 3.09 b	4.6 ± 0.10 b	3.2 ± 0.32 b	1.2 ± 0.19 b	0.09 ± 0.03 b	15.1 ± 2.95 a
Growing Season						
LC 1	47.6 ± 7.64 ac	5.3 ± 0.01 a	5.6 ± 0.60 a	2.0 ± 0.26 b	0.16 ± 0.02 b	12.3 ± 0.01 a
LC 2	38.0 ± 2.89 bc	5.2 ± 0.01 a	3.8 ± 0.01 b	1.1 ± 0.16 c	0.09 ± 0.01 d	12.6 ± 0.11 a
LC3	37.7 ± 2.36 bc	5.3 ± 0.02 a	3.9 ± 0.05 b	1.5 ± 0.004 bc	0.12 ± 0.001 c	12.4 ± 0.27 a
BR	35.1 ± 1.63 bc	5.3 ± 0.05 a	3.6 ± 0.28 b	1.2 ± 0.18 c	0.10 ± 0.01 cd	11.4 ± 0.37 a
BN	49.7 ± 4.19 a	5.2 ± 0.02 a	5.5 ± 0.56 a	2.5 ± 0.41 a	0.22 ± 0.02 a	11.0 ± 0.77 a
BP	38.7 ± 1.81 bc	4.2 ± 0.001 b	3.3 ± 0.25 b	1.7 ± 0.24 bc	0.13 ± 0.001 c	12.8 ± 1.73 a

*Values with different letters are significantly different with Dunnett's T3 post-hoc pairwise comparisons ($p < 0.05$).

Table 2. Multivariate statistical pairwise comparisons (ANOSIM*) of bacterial community Bray Curtis dissimilarities between wetland sites by each type of molecular analysis used in the study. Significant difference at $\alpha=0.05$

	Non-Growing Season		Growing Season	
	ANOSIM *R*	p-value**	ANOSIM *R*	p-value
LH- PCR (OTUs)				
BN, BP	0.103	0.182	**0.513**	0.001
BN, LC1	-0.028	0.567	0.085	0.099
BN, LC2	0.001	0.422	0.164	0.021
BN, LC3	-0.077	0.833	0.097	0.128
BN, BR	0.109	0.059	0.086	0.16
BP, LC1	0.051	0.271	0.23	0.006
BP, LC2	0.172	0.06	0.31	0.001
BP, LC3	0.276	0.027	0.382	0.003
BP, BR	0.302	0.012	**0.537**	0.001
LC1, LC2	0.145	0.077	-0.023	0.585
LC1, LC3	0.173	0.082	0.037	0.277
LC1, BR	0.189	0.01	0.266	0.006
LC2, LC3	0.364	0.006	0.089	0.142
LC2, BR	0.125	0.084	0.074	0.237
LC3, BR	0.156	0.04	0.174	0.036
MTPS (genus level)				
BN, BP	**0.937**	0.005	**0.837**	0.005
BN, LC1	**0.591**	0.005	0.337	0.038
BN, LC2	**0.615**	0.005	0.492	0.019
BN, LC3	0.409	0.014	**0.512**	0.010
BN, BR	**0.611**	0.001	0.201	0.077
BP, LC1	**0.698**	0.029	**0.729**	0.029
BP, LC2	**0.917**	0.029	**0.792**	0.029
BP, LC3	**0.969**	0.029	**0.927**	0.029
BP, BR	**0.981**	0.001	0.484	0.013
LC1, LC2	0.146	0.114	-0.063	0.571
LC1, LC3	0.406	0.029	**0.927**	0.029
LC1, BR	0.6	0.003	-0.159	0.758
LC2, LC3	0.427	0.029	**0.885**	0.029
LC2, BR	0.469	0.01	-0.101	0.634
LC3, BR	**0.715**	0.002	0.23	0.103

*ANOSIM *R* statistic values closer to 1 indicate community dissimilarity among groups. Values showing strong (> 0.5) significant dissimilarity of a pairwise comparison are in **bold**.

Table 3. Richness, evenness, and diversity measures of bacterial communities estimated by both LH-PCR and MTPS**.

	Fingerprinting (LH-PCR)*			Pyrosequencing (MTPS)		
Site and Season	Richness (*S*)	Pielou's evenness (*J*)	Shannon diversity (*H'*)	Richness (*S*)	Pielou's evenness (*J*)	Shannon diversity (*H'*)
Non-Growing Season						
LC1	18 ± 0.6 a	0.96 ± 0.003 a	2.78 ± 0.04 a	21 ± 2.6 a	0.95 ± 0.008 b	2.84 ± 0.09 a
LC2	19 ± 1.8 a	0.96 ± 0.012 a	2.78 ± 0.11 a	16 ± 2.5 a	0.96 ± 0.006 b	2.60 ± 0.17 a
LC3	16 ± 0.9 b	0.96 ± 0.007 a	2.74 ± 0.04 b	19 ± 2.4 a	0.94 ± 0.003 c	2.77 ± 0.12 a
BR	20 ± 0.6 a	0.96 ± 0.002 a	2.71 ± 0.03 c	17 ± 0.8 a	0.97 ± 0.003 a	2.72 ± 0.05 a
BN	17 ± 0.9 a	0.96 ± 0.003 a	2.69 ± 0.06 c	18 ± 0.8 a	0.97 ± 0.005 ab	2.80 ± 0.05 a
BP	17 ± 1.7 a	0.95 ± 0.006 b	2.69 ± 0.07 c	15 ± 2.9 a	0.94 ± 0.018 c	2.52 ± 0.26 a
Growing Season						
LC1	17 ± 1.7 b	0.95 ± 0.006 a	2.62 ± 0.11 b	15 ± 0.8 a	0.95 ± 0.007 a	2.55 ± 0.05 a
LC2	19 ± 1.8 ab	0.94 ± 0.054 a	2.61 ± 0.23 b	13 ± 1.2 a	0.94 ± 0.013 a	2.36 ± 0.10 b
LC3	19 ± 0.5 a	0.94 ± 0.004 a	2.63 ± 0.03 a	14 ± 0.3 a	0.91 ± 0.010 a	2.36 ± 0.01 b
BR	17 ± 0.4 b	0.94 ± 0.002 a	2.63 ± 0.02 a	16 ± 0.9 a	0.94 ± 0.014 a	2.58 ± 0.06 a
BN	17 ± 1.1 b	0.94 ± 0.003 a	2.63 ± 0.08 a	16 ± 1.2 a	0.94 ± 0.013 a	2.59 ± 0.11 a
BP	17 ± 1.8 b	0.93 ± 0.009 a	2.62 ± 0.16 b	10 ± 2.3 b	0.89 ± 0.040 a	2.03 ± 0.29 c

* Calculated based on the observed taxonomic units (OTUs) in the fingerprints of wetland soils (± standard error).
** Values with different letters are significantly different with Bonferroni adjusted p-values ($p < 0.05$).

Table 4. Soil physicochemical and bacterial community diversity (mean ± standard error) for hydrologic connectivity groups (HCG), *n* = number of samples.

	HCG1* (*n* =1)	HCG2 (*n* = 8)	HCG3 (*n* = 2)
Site(Plot)	BR(3)	LC1(BB); LC2(DD,EE) BR(4,5,6); BP(1,3)	BN (4,5)
Gravimetric soil moisture (%)	38 ± 2.3 a	32 ± 8.0 b	39 ± 6.3 a
pH	4.9 ± .04 a	4.9 ± .38 a	4.7 ± .02 a
Total organic carbon (%)	1.9 ± .13 a	1.2 ± .47 a	2.2 ± .47 a
Total nitrogen (%)	0.15 ± .007 a	.12 ± .042 a	.20 ± .018 a
Soil organic matter (%)	4.7 ± 0.5 a	3.7 ± 0.9 a	6.0 ± 0.8 a
MTPS diversity (*H'*)	3.2 ± .04 a	3.0 ± .19 b	3.2 ± .02 a

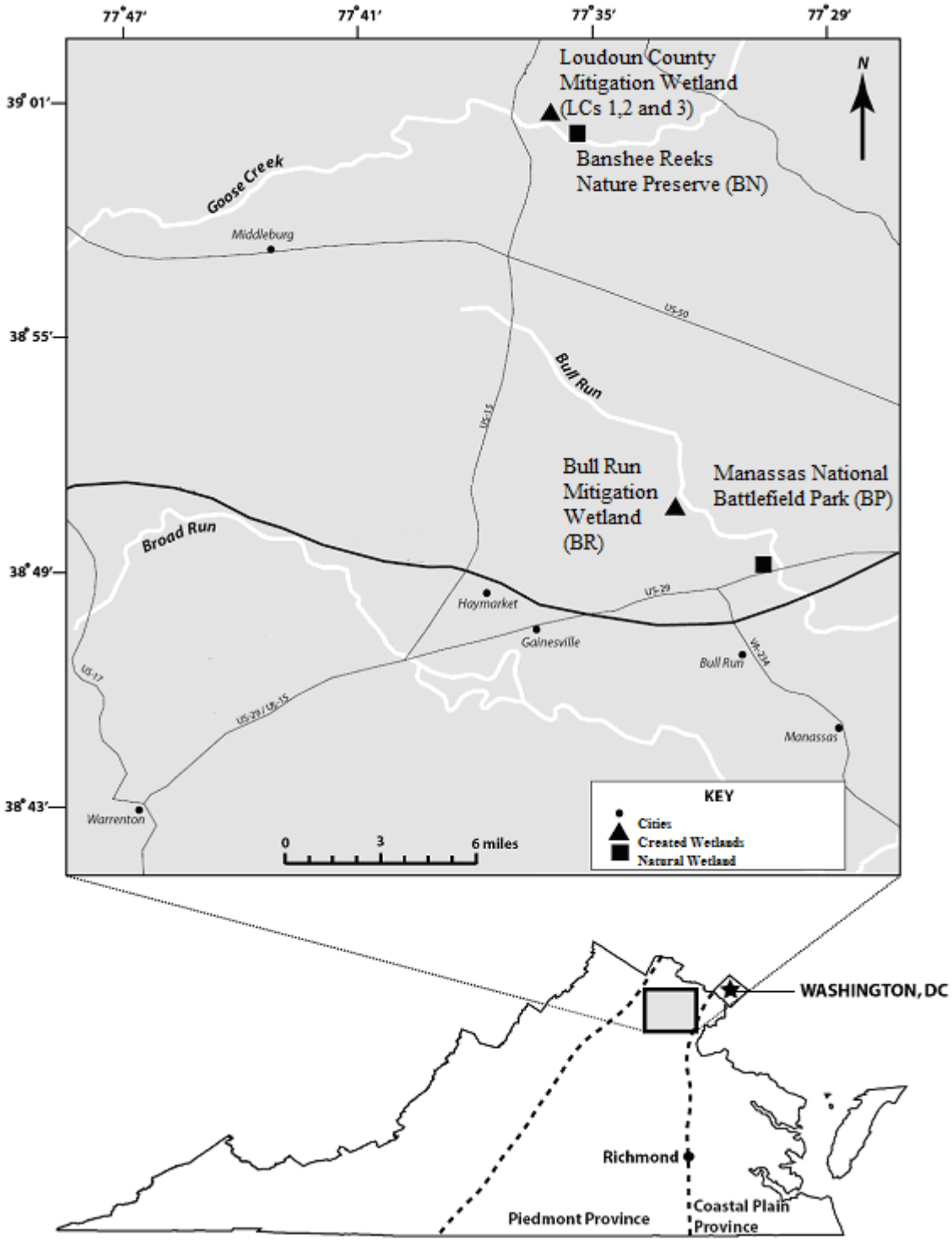
*Values with different letters are significantly different with Conover-Inman post-hoc pairwise comparisons of Kruskal-Wallis One-way Analysis of Variance ($p < 0.05$).

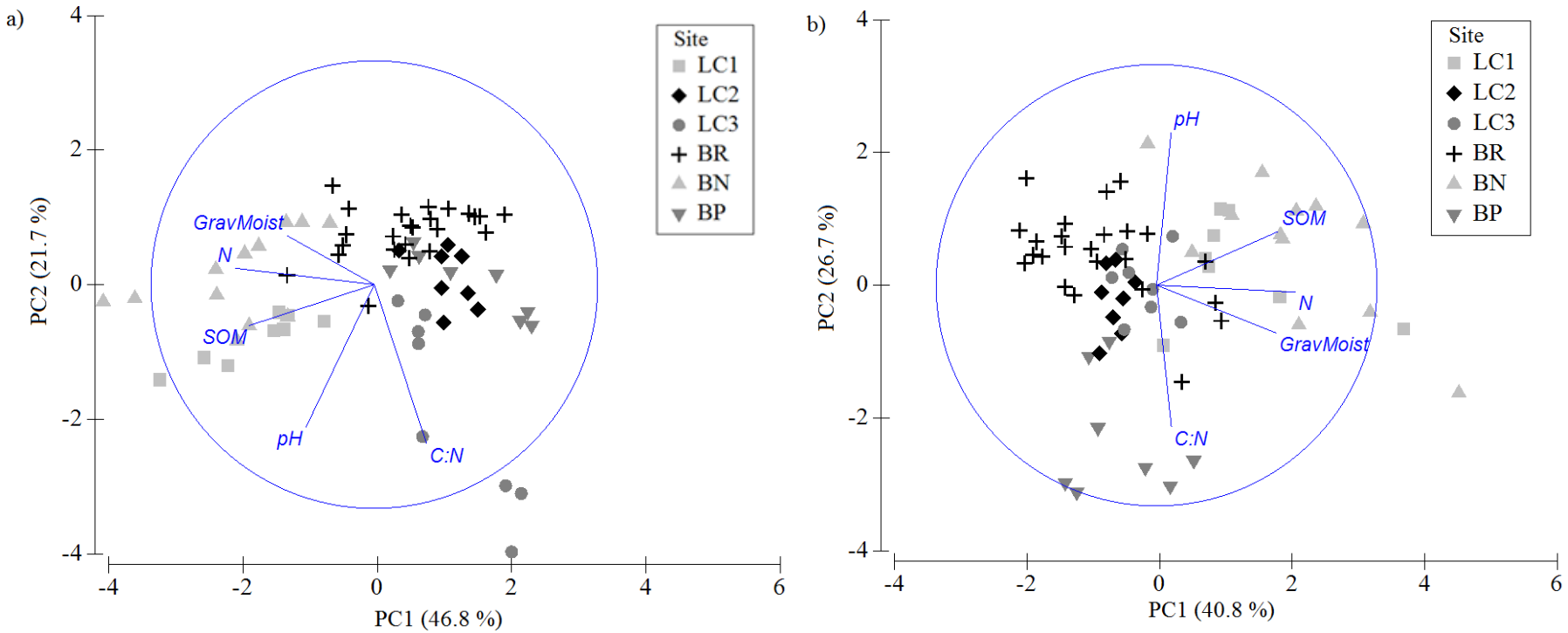
Figure 1. Map of the study sites located in the Piedmont region of Virginia. There were two natural (BN and BP) and two created mitigation wetlands (BR and LC) included in the study. LC has three sites (LC 1, 2 and 3). Modified from Wolf (2010).

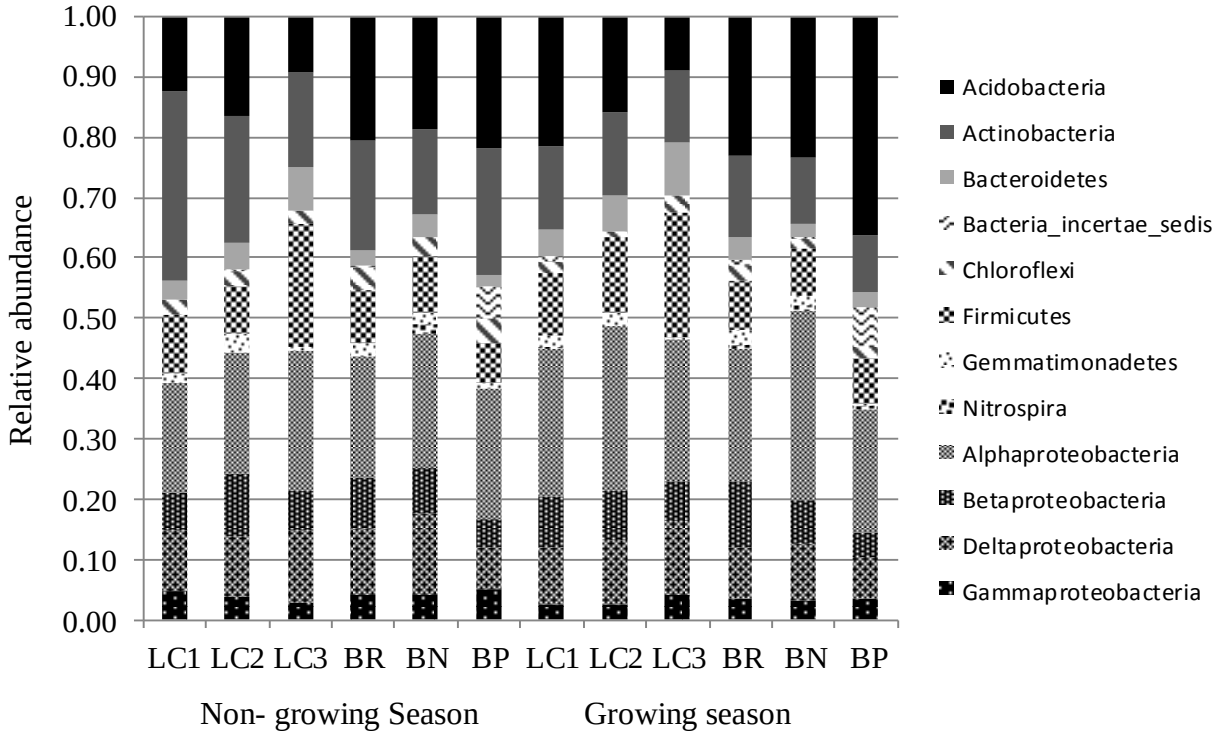
Figure 2. Principal component analysis (PCA) of physicochemical variables of soils collected during: a) non-growing season (October 2008) and b) growing season (June 2009). Arrows represent the correlation between the physicochemical variables. Variables that are angled more than 90° of each other have the least correlation. Variables that have arrows extending in opposite directions correlate negatively to each other.

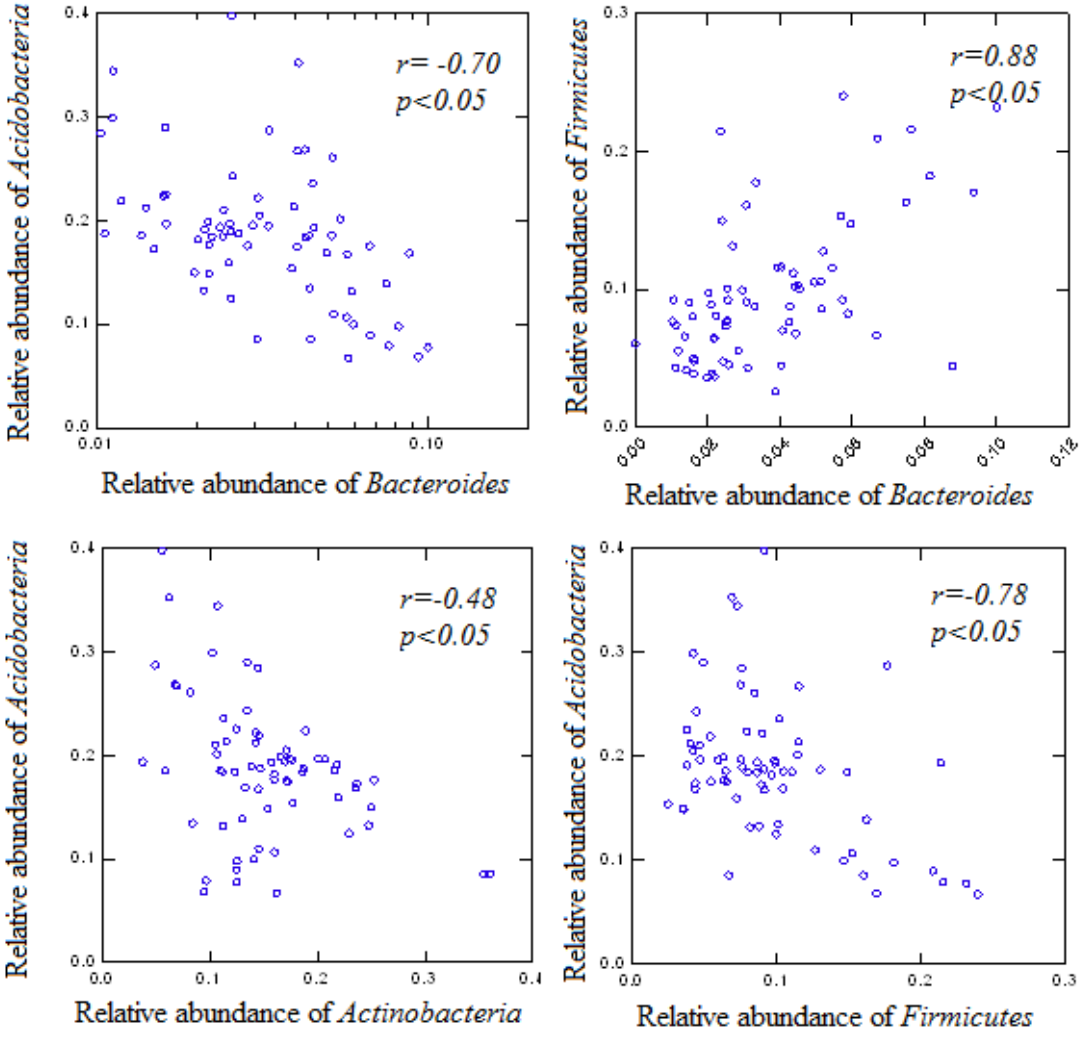
Figure 3. Relative abundances and distribution of major bacterial phyla in each wetland site during the non-growing (October 2008) and growing (June 2009) seasons.

Figure 4. Relationships (Spearman rank correlations) between major bacterial phyla revealed by MTPS in the communities of all wetland soils


77°47′
77°41′
77°35′
77°29′
39° 01′
38° 55′
38° 49′
38° 43′
Loudoun County
Mitigation Wetland
(LCs 1,2 and 3)
Banshee Reeks
Nature Preserve (BN)
N
Goose Creek
Middleburg
US-50
Bull Run
US-15
Bull Run
Mitigation
Wetland
(BR)
Manassas National
Battlefield Park (BP)
Broad Run
Haymarket
US-29
Gainesville
Bull Run
VA-234
US-17
US-29/US-15
Manassas
Warrenton
0
3
6 miles
KEY
Cities
Created Wetlands
Natural Wetland
WASHINGTON, DC
Richmond
Piedmont Province
Coastal Plain
Province


a)
b)
Site
LC1
LC2
LC3
BR
BN
BP
GravMoist
N
SOM
pH
C:N
PC1 (46.8 %)
PC2 (21.7 %)
PC1 (40.8 %)
PC2 (26.7 %)


1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
Relative abundance
LC1 LC2 LC3 BR BN BP LC1 LC2 LC3 BR BN BP
Non- growing Season
Growing season
Acidobacteria
Actinobacteria
Bacteroidetes
Bacteria_incertae_sedis
Chloroflexi
Firmicutes
Gemmatimonadetes
Nitrospira
Alphaproteobacteria
Betaproteobacteria
Deltaproteobacteria
Gammaproteobacteria


Relative abundance of *Acidobacteria*
r= -0.70
p<0.05
Relative abundance of *Bacteroides*
Relative abundance of *Firmicutes*
r=0.88
p<0.05
Relative abundance of *Bacteroides*
Relative abundance of *Acidobacteria*
r=-0.48
p<0.05
Relative abundance of *Actinobacteria*
Relative abundance of *Acidobacteria*
r=-0.78
p<0.05
Relative abundance of *Firmicutes*

CURRICULUM VITAE

Rita Peralta was born in the beautiful city of La Paz, Bolivia. Her family immigrated to the US when she was eight years old and she has lived in the Northern Virginia region ever since. She graduated from Fairfax High School in 1997 and earned her B.A. in Biology from George Mason University (GMU) in 2002. After her graduation, she worked at two biotechnology companies where she sharpened her laboratory skills and garnered her love of microbes. Feeling the need to marry her interest in microbiology and the environment, she decided to pursue a Master's degree at her Alma Mater. During her time at GMU she was a graduate teaching and research assistant.