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Science of the Total Environment 443 (2013) 725–732

Contents lists available at SciVerse ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Characterization of soil bacterial community structure and physicochemical properties in created and natural wetlands

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HIGHLIGHTS

- Soil properties showed differences by site without a specific age-trajectory.
- Soil bacterial communities were distinguished by wetland sites.
- *Proteobacteria* contributed to the majority of the community composition of all soils.
- The relative abundance of different phylogenic groups differed by wetland site.
- Bacterial community structures were significantly associated with C:N ratio and pH.

ARTICLE INFO

Article history:
Received 15 August 2012
Received in revised form 7 November 2012
Accepted 8 November 2012
Available online xxxx

Keywords:
Wetland soil
Soil bacterial community
Multi-tag pyrosequencing
Soil physicochemistry
Created mitigation wetlands

ABSTRACT

We used multi-tag pyrosequencing of 16S ribosomal DNA to characterize bacterial communities of wetland soils collected from created and natural wetlands located in the Virginia piedmont. Soils were also evaluated for their physicochemical properties [i.e., percent moisture, pH, soil organic matter (SOM), total organic carbon (TOC), total nitrogen (TN), and C:N ratio]. Soil moisture varied from 15% up to 55% among the wetlands. Soil pH ranged between 4.2 and 5.8, showing the typical characteristic of acidic soils in the Piedmont region. Soil organic matter contents ranged from 3% up to 6%. Soil bacterial community structures and their differences between the wetlands were distinguished by pyrosequencing. Soil bacterial communities in the created wetlands were less dissimilar to each other than to those of either natural wetland, with little difference in diversity (Shannon's H') between created and natural wetlands, except one natural wetland consistently showing a lower H'. The greatest difference of bacterial community structure was observed between the two natural wetlands ($R = 0.937$, $p < 0.05$), suggesting these two natural wetlands were actually quite different reflecting differences in their soil physicochemistry. The major phylogenic groups of all soils included *Acidobacteria*, *Actinobacteria*, *Bacteroidetes*, *Chloroflexi*, *Firmicutes*, *Gemmatinomadetes*, *Nitrospira*, and *Proteobacteria* with *Proteobacteria* being the majority of the community composition. *Acidobacteria* group was more abundant in natural wetlands than in created wetlands. We found a significant association between bacterial community structures and physicochemical properties of soils such as C:N ratio ($\rho = 0.43$, $p < 0.01$) and pH ($\rho = 0.39$, $p < 0.01$). The outcomes of the study show that the development of ecological functions, mostly mediated by microbial communities, is connected with the development of soil properties in created wetlands. Soil properties should be carefully monitored to examine the progress of functional wetland mitigation.

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). Even after more than two decades of wetland mitigation practices, the developmental trajectory of mitigation wetlands is highly variable (Zedler and Callaway, 1999; Morgan and Short, 2002; Matthews et al., 2009) and some wetlands never develop the structural and/or functional attributes of their natural counterparts (Erwin, 1991; Kentula et al., 1992; Hoeltje and Cole, 2007). Failures of created/restored wetlands are often attributable to a lack of soil development (Bishel-Machung et al., 1996; Stolt et al., 2000; Cole et al., 2001; Campbell et al., 2002).

Soil development is closely related to vegetation development that is often the single attribute used to evaluate the success of wetland mitigation (Spieles, 2005). Soil physicochemical attributes, including bulk density, moisture, soil organic matter (SOM), total organic carbon (TOC), and texture are all inevitably linked to the development of plant communities in wetlands (Ballantine and Schneider, 2009; Ehrenfeld et al., 2005) and often heavily impacted by construction processes used in wetland construction (Whittecar and Daniels, 1999; Stolt et al., 2000;

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http://dx.doi.org/10.1016/j.scitotenv.2012.11.052

Moser et al., 2007). Created wetlands are thus often limited in soil nutrients and organic matter that are important to the development of plant and microbial community (Craft et al., 2002; Anderson et al., 2005; Bruland et al., 2009; Moser et al., 2009). Proper development of the soil physicochemical properties is critical for the development of more complex functional attributes of wetlands (Hossler and Bouchard, 2010; Wolf et al., 2011).

Soil microbial communities are involved in biogeochemical processes and their activities are crucial to the functions of wetland systems (Balser et al., 2002; Boon, 2006; Reddy and DeLaune, 2008). Recent use of molecular tools (Mills et al., 2003; Roesch et al., 2007; Lauber et al., 2009; Chariton et al., 2010) has provided increasing evidence that the structures of bacterial communities are related to soil processes (Tam et al., 2001; Fierer et al., 2003; D'Angelo et al., 2005; Edwards et al., 2006; Mentzer et al., 2006; Peralta et al., 2010). However, little is known about bacterial communities in created wetlands and if they are related to soil physicochemical development.

We analyzed bacterial community structure and physicochemical properties of soils in created and natural wetlands in the Piedmont physiographic region of Virginia. We used multi-tag pyrosequencing (MTPS) to investigate soil bacterial communities, the most recent sequencing technology that allows high-throughput sequencing. The main objective of the study was to characterize bacterial community structure and physicochemistry of soils from both created and natural wetlands. We hypothesized that bacterial community structures would be associated with physicochemical properties in the wetland soils.

2. Materials and methods

2.1. Site description

Four non-tidal freshwater wetlands located in the Piedmont physiographic region of northern Virginia were used in this study (mean annual precipitation 109 cm, mean temperature min 7 °C/max 18 °C; Fig. 1). Two of the wetlands were created mitigation wetlands and the other two were natural wetlands. Loudoun County Mitigation Bank (LC) is a 12.9 ha wetland and upland buffer complex, constructed by Wetland Studies and Solutions, Inc. (WSSI) in the summer of 2006 (3 years old at the time of the study) in Loudoun County, Virginia (39°1′ N, 77°36′ W). LC receives surface water runoff from an upland housing development and forested buffer, as well as minor groundwater inputs from toe-slope intercept seepage. LC consists of three wetland basins (LCs 1, 2, and 3). LCs 1 and 2 are two contiguous sites separated by a berm and connected by a drainage channel with LC1 approximately 0.4 m higher in elevation than LC2. This design causes LC1 to drain more quickly leaving it inundated for shorter periods after precipitation than LC2, while LC2 can remain under standing water (e.g., < ~12 cm) for longer periods. LC3 is another basin completely separated from LCs 1 and 2, but built within the LC complex on the same floodplain as LC 1 and 2. The part of LC3 included in this study receives groundwater input from toe-slope intercept seepage. Bull Run Mitigation Bank (BR) is a 20.2 ha wetland and upland buffer complex, constructed by WSSI in 2002 (7 years old at the time of the study) in Prince William County, Virginia (38°51′ N, 77°32′ W). The site may receive water from Bull Run seasonally (e.g., minimum once a year) from a culvert 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 wetlands and negligible groundwater. Both wetlands designed by WSSI contain at least a 0.3 m low permeability subsoil layer covered with the original topsoil from the site that was supplemented with commercially available topsoil to a depth of 0.2 m. This design creates a perched, precipitation-driven water table close to the soil surface and limits groundwater exchange in the wetland. Vegetation in the created wetlands is mostly herbaceous, interspersed with young tree saplings and shrubs in projected forested areas.

Manassas National Battlefield Park (BP), established in 1940, is a 2000 ha site with areas of natural wetland coverage located in Prince William County, Virginia (38°49′ N, 77°30′ W) right next to BR. An area of herbaceous wetland within a matrix of forested floodplain was selected for study and comparison to the created wetlands. 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) is a 290 ha site with areas of seep and riparian wetlands located in Loudoun County, Virginia (39°1′ N, 77°35′ W), right next to LC complex. These floodplain riparian wetlands receive water from groundwater springs, surface water runoff, and occasional overbank flooding from Goose Creek. Vegetation is a mixture of herbaceous plants with some mature bottomland forest surrounding our study areas.

2.2. Soil sampling

There were a total of 12 plots (10 m × 10 m each) established in four created wetland sites (i.e., LCs 1, 2, 3, and BR) and 5 plots in two natural wetlands (i.e., BP and BN). Soil sampling was conducted twice at each site, once in October 2008 and the second time in June 2009. Plots were divided into four 5 × 5 m quadrants. Within each quadrant, three soil samples were taken at the depth of 5–10 cm from the surface by use of an auger (3.2 cm diameter) at random spots and combined in a polyethylene bag. Soils were mostly saturated with little standing water (<1–2 cm). 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 stored at a freezer (at −80 °C) until microbial molecular analysis was conducted.

2.3. Soil physicochemical analyses

To determine SOM, TOC, TN and pH, soils were air dried. Once air dried, soils were macerated using a mortar and pestle and large constituents (e.g. rocks and large organic debris) were removed. A Perkin–Elmer 2400 Series II CHNS/O Analyzer (Perkin–Elmer Corporation, Norwalk, CT, USA) was used to analyze TOC (~TC) and percent TN. Sub-samples (2–3 g of air dried soil) were separated for SOM and oven dried at 105 °C for 24 h, weighed and placed at 405 °C for 16 h. SOM (%) was measured using weight loss on ignition method (Wilson and Sanders, 1996). For gravimetric soil moisture, field-wet mass was measured and samples dried at 105 °C for 48 h. Percent soil moisture was calculated by the difference between field moist mass and oven dried mass [(wet mass − dry mass)/(dry mass) × 100] (Gardner, 1986). For pH determination, 10 g air dried soil samples were combined with 10 mL of deionized water, swirled and left to stabilize for 10 min prior to measurement (Thomas, 1986). Soil texture analysis was conducted using a LISST-100X laser particle size analyzer (Sequoia Scientific, Inc., Bellevue, WA, USA) (Wolf et al., 2011).

2.4. Soil bacterial community analysis

2.4.1. 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). Products were verified by gel electrophoresis and diluted in 1:10 dilutions (5 μL DNA in 45 μL buffer). Extracted DNA and ten-fold dilutions were stored at −20 °C.

2.4.2. PCR amplification of 16S ribosomal DNA

Bacterial 16S ribosomal DNA fragments were amplified by PCR using the universal bacterial primer set of forward primer 27F

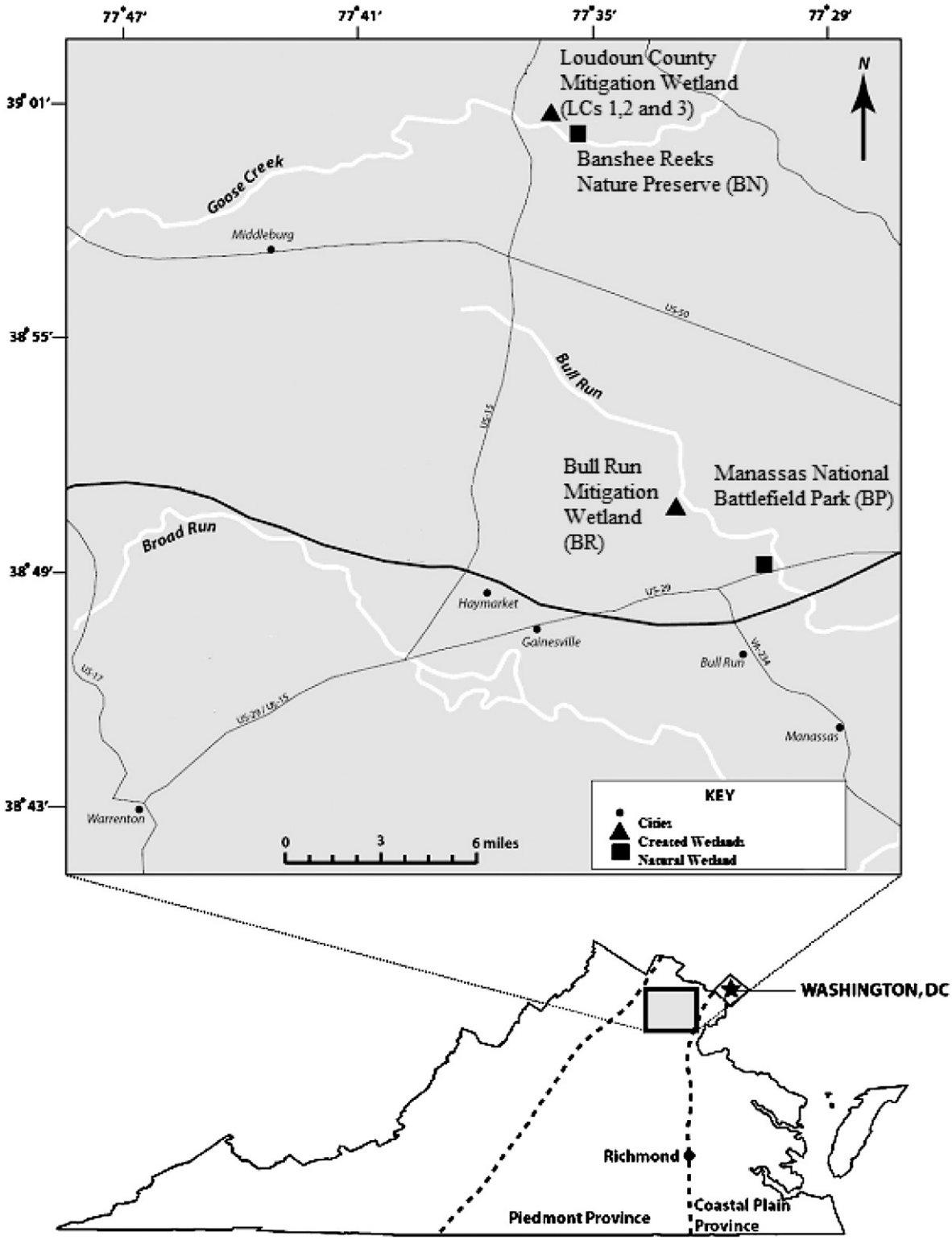

Fig. 1. Map of the study sites located in the Piedmont region of Virginia. There were two natural (BN and BP) and two created mitigation wetlands (BR and LC) included in the study. LC has three sites (LCs 1, 2 and 3).
Modified from Wolf et al. (2011).

(5′-AGAGTTTGATCCTGGCTCAG-3′) and reverse primer 355R (5′-GCT GCCTCCCGTAGGAGT-3′). Details of 20 μL PCR reaction parameters are described by Ahn and Peralta (2009). Products were confirmed by electrophoreses of 5 μL of each reaction on 1% agarose gel. Negative controls were included in each experimental run.

2.4.3. Multi-tag pyrosequencing

Multi-tag pyroquencing (MTPS) is high throughput sequencing by synthesis technique that expands on the 454 pyrosequencing protocol (Acosta-Martinez et al., 2008; Lauber et al., 2009; Gillevet et al., 2010). The 454 pyrosequencing platforms provide about 400 bp reads, which

allows a reliable annotation of the DNA fragments (Liu et al., 2012). MTPS 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. 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, the cost for MTPS is still fairly expensive (Margulies et al., 2005; Lauber et al., 2009), thus we ran MTPS analysis on two out of four samples used for soil properties analysis per plot, picked using a random number generator. A secondary PCR was performed using tagged fusion primers (containing linkers and tags). Specifically, we amplified the V2 and V3 regions of 16S rRNA gene. The PCR products were then denatured to produce single-stranded DNA templates, and purified using Ampure magnetic beads (Agencourt Bioscience Corp.) and pyrosequenced on a *Roche GS-FLX* instrument (Nutley, New Jersey) (see Gillevet et al., 2010). Taxonomic identity of pyrosequencing data were determined with the Ribosomal Database Project (RDP)10 Bayesian classifier (Wang et al., 2007) using a custom PERL script, down to RDP level 6 (i.e., genus) (Gillevet et al., 2010).

2.5. Data analysis

Prior to statistical analysis, the cumulative data sets were each tested for normality and the data were log-transformed whenever normality was not met. 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 part of SOM, it was omitted from further statistical analysis.

Three bacterial community diversity indices were calculated using pyrosequencing data (i.e., taxa). Richness (S) is equal to the number of taxa defined at RDP level 6 (i.e., genus) for MTPS results in each sample. The Shannon–Weiner Diversity Index (H') is equal to $\sum(\text{pi}(\ln \text{pi}))$, where pi is the relative abundance of taxa for MTPS. Evenness (J) is equal to $H'/\ln S$. Site averages of the three indices were compared using one-way ANOVA. The averages were then further compared by using a modification of Bonferroni 95% confidence intervals. All statistical analyses were conducted using SYSTAT V.12 software, and tests were considered significant at $\alpha = 0.05$, unless otherwise noted. In addition, we plotted the relative abundance of the twelve most abundant bacterial phylogenic groups (i.e., defined at RDP level 1 for all groups, and further defined at RDP level 2 for *Proteobacteria* groups) to compare the composition of the communities between the wetland sites.

Principal component analysis (PCA) was conducted for soil physicochemical variables to identify groups of variables contributing most to variance between sites. Analysis of Similarities (ANOSIM) (Clarke and Warwick, 2001), a non-parametric analog of the one-way ANOVA, was used to compare sample assemblages of bacterial community data between sites. The association between bacterial community structures and physicochemical attributes of wetland soils was evaluated using RELATE, a non-parametric comparative (i.e., Mantel-type) test (Clarke and Warwick, 2001; Zuur et al., 2007), on matched similarity matrices to calculate the Spearman rank correlation (ρ). All multivariate statistical analyses were conducted using PRIMER-E V6 software (Clarke and Gorley, 2001).

3. Results and discussion

3.1. Soil physicochemistry

Little difference was found between the wetland sites in soil physicochemical properties measured (Table 1). No significant differences in soil attributes such as SOM, TOC, and percent soil moisture were found among created wetlands (i.e., LC site and BR) (Table 1). It seemed there was higher spatial heterogeneity in the soil properties measured. Spatial variability of soil properties in both created and natural wetlands were noted and discussed in previous studies (Bruland and Richardson, 2005; Dee and Ahn, 2012), and the findings of this study were comparable with those reported. Soil texture classification for all soils investigated was determined as 'silt loam' with no difference between the sites.

SOM and TN were significantly higher in the forested natural wetland (i.e., BN) and one of the youngest created wetland (i.e., LC1) with SOM content of up to 6.2% and TN of up to 0.24% (Table 1). Compared to the other wetlands, TOC content was highest in LC1 and BN with values averaging 2.1 and 2.4%, respectively. LC1 showed a significantly higher TOC content than the same-aged LCs 2 and 3, showing that soil properties varied both within and among sites (Table 1). Soil pH ranged from 4.2 to 5.8 (Table 1), showing a typical, acidic characteristic of the soils of Virginia Piedmont (Farrel and Ware, 1991). There was no distinctive difference in soil pH between the sites (*Bonferroni adjusted* $p = 0.09$), except that one of the natural wetlands, BP, showed a consistently lower pH than the other sites (Table 1). There was no difference by sampling periods for soil attributes measured [i.e., pH ($p = 0.42$) SOM ($p = 0.86$), TOC ($p = 0.63$), TN ($p = 0.89$) or C:N ($p = 0.70$)]. The

Table 1
Soil physicochemistry (Mean ± SE)* in created (i.e., LCs and BR) and natural wetlands (i.e., BN and BP) by two sampling periods.

Wetland site	Moisture (%)	pH	SOM (%)	TOC (%)	TN (%)	C:N
October 2008						
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
June 2009						
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 ± 0.001c	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 ± 0.001c	12.8 ± 1.73 a

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

two natural wetlands (i.e., BN vs. 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 6 year old created wetland (i.e., BR) and a natural herbaceous wetland (i.e., BP) displayed similar soil physicochemical characteristics overall (Table 1). Soil moisture content was higher (40.2% ± 1.8) in soils collected in June compared to soils collected in October (29.2% ± 2.2) ($p < 0.001$), especially for created wetlands (i.e., LCs 1, 2, and 3). Soil moisture (%) was higher in BN soils consistently during the two sampling periods where the average was 46%, (Table 1). Percent soil moisture was significantly higher in LC1 compared to the same-aged sites, LCs 2 and 3, being comparable to that of natural wetlands, BP and BN (Table 1).

The PCA of soil physicochemical variables showed that on average 68% of the data variability was explained with two principal components (i.e., PC1 and PC2), consistently for both sampling periods (Fig. 2a, b). Overall, PC1 accounted from 41 to 47% and PC2 from 22 to 27% of the variability (Fig. 2a, b). The PCA pattern was fairly consistent for soils collected in both sampling periods, showing SOM, TN, and percent soil moisture relatively grouped together with the same directionality (Fig. 2a, b). The variability of SOM, TN, and percent moisture of soils were mostly explained by PC1 whereas the variability of C:N ratio and pH of soils were better explained by PC2 (Fig. 2). In June samples (Fig. 2b), SOM, TN, and percent soil moisture were all positively related to PC1 with correlation coefficients of 0.55, 0.63, and 0.54, respectively. Overall, LC1 and BN showed the characteristics of more mature wetland soil development as measured by relatively higher SOM, TN, and percent soil moisture than those in the other wetlands. C:N was negatively related to PC2 (−0.64) whereas soil pH was positively correlated to PC2 (0.69) in June samples (Fig. 2b).

Soil properties develop through the accumulation of SOM which is usually closely associated with seasonal plant senescence over the years (Wolf et al., 2011). However, soil development trajectories have been found to be highly variable (Wolf et al., 2011; Dee and Ahn, 2012). SOM (and TOC) accumulation can vary due to variables that may facilitate or impede autochthonous (e.g. seasonal plant senescence) and allochthonous or allogenic (e.g. sediment brought by flooding or runoff) sources of organic matter. The construction process itself can compact soils, increasing bulk density and decreasing microtopography, and leading to a loss in water holding capacity and of SOM (Moser et al., 2009). Moreover, created mitigation wetlands are usually constructed by removing the top layer of soil and creating a 'bath tub' like structure that is engineered to maintain the desired hydrology. Because the construction process usually removes top soil without replacement, created wetlands often tend to demonstrate low levels of organic matter (Confer and Niering, 1992; Bishel-Machung et al., 1996; Campbell et al., 2002; Bruland and Richardson, 2006). Therefore comparison of soil development within and between wetland sites may be better achieved by identifying a set of soil attributes that contribute to soil development. Accumulation of SOM, TOC, and TN along with increased soil moisture content have been identified as structural attributes of soil development (Craft et al., 2002; Hossler and Bouchard, 2010; Wolf et al., 2011) and showed previously significant correlation with plant community establishment (Dee and Ahn, 2012) and nitrogen cycling (Wolf et al., 2011).

As a young created wetland, LC1 showed relatively higher SOM and TOC levels which was comparable to those of a natural wetland (i.e., BN) (Table 1). A significant level of spatial heterogeneity was found in all soil attributes within each wetland site as reported in Dee and Ahn (2012). It might also have been due to the drift of some of the organic amendments that had been applied to the wetland as a whole excluding our study plots since our plots were right next to the areas of the wetland amended (personal communication with WSSI). All the other created wetland sites demonstrate TOC and SOM levels more comparable to BP (Table 1). As shown in the PCA results (Fig. 2) LC1 and BN consistently cluster together, indicating that SOM (and TOC implicitly) and TN are reliable attributes for soil maturation since they consistently showed higher levels in the natural forested wetland (i.e., BN) and LC1 influenced by organic amendments.

3.2. Bacterial community structure

All soils were dominated by five major bacterial phylogenic groups (*Acidobacteria*, *Actinobacteria*, *Bacteroidetes*, *Firmicutes* and *Proteobacteria*). These groups made up more than 80% of the sequences for every sample. The relative abundances of these groups in the soils corresponded roughly to the dominant groups found in wetland soils with lower pH in North Carolina (see Hartman et al., 2008). Fig. 3 shows the relative abundances of bacterial phylogenic groups per wetland site. Generally, the *Proteobacteria* contributed to the majority of the community composition of all soils, indicating that sequences affiliated with the *Proteobacteria* contributed to a higher percentage (~up to 50%) of the community DNA (Fig. 3). The relative abundance of α-*Proteobacteria* sub-group varied between the sites, ranging between 18 and 52% (Fig. 3). The other *Proteobacteria* sub-groups contributed to about the half of the entire *Proteobacteria* groups (Fig. 3). Other phylogenic groups

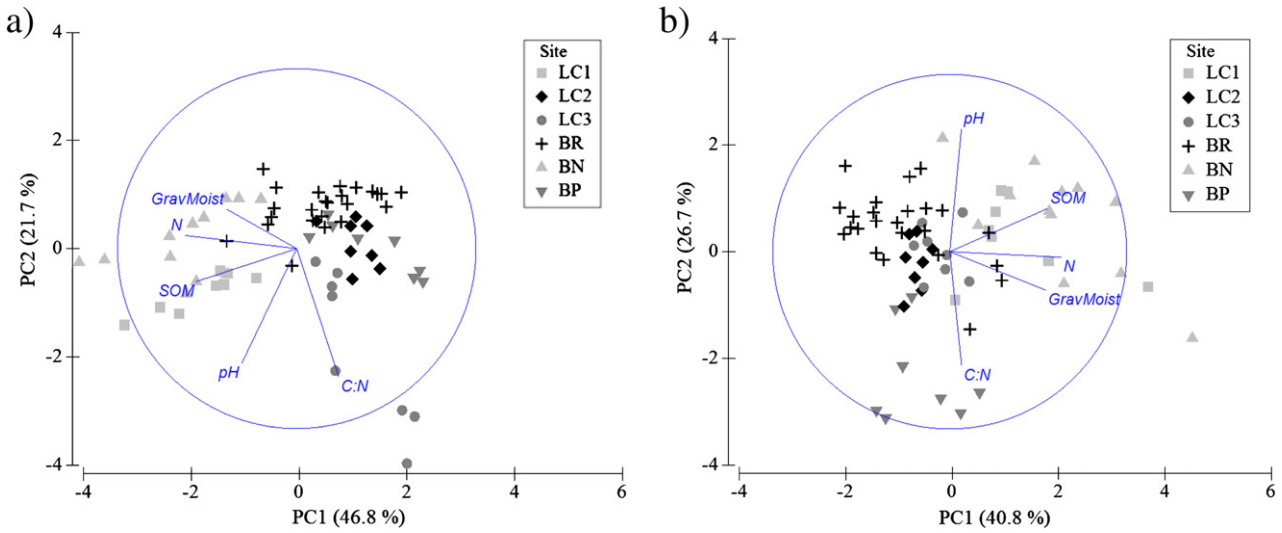

Fig. 2. Principal component analysis (PCA) of physicochemical variables of soils collected during: a) October 2008 and b) June 2009. Arrows represent the correlation between the physicochemical variables. Variables that are angled more than 90° of each other have the least correlation. Variables that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

included *Acidobacteria*, *Actinobacteria*, *Bacteroidetes*, *Chloroflexi*, *Firmicutes*, *Gemmatinomadetes*, *and Nitrospira*. Overall, *Acidobacteria* group was more abundant in natural wetlands (i.e., BP and BN) than in created wetlands (i.e., LCs 1–3 and BR). It also was slightly more abundant in the older created wetland (i.e., BR) than the younger ones (i.e., LC) (Fig. 3). The relative abundance of *Actinobacteria* was higher in the October samples in all sites than in the June ones, showing some variation of abundance of each group by the timing of sampling. *Bacteria incertae sedis* group appeared distinctively in BP while being negligible or absent in the other sites (Fig. 3). *Firmicutes* were consistently higher in abundance in LC3 compared to all the other sites in both sampling periods (Fig. 3). *Nitrospira* appeared only in the older created wetland site, BR, and the two natural wetlands with none detected for all LC sites (Fig. 3).

There were several pairwise comparisons of soil bacterial taxa that were found to be highly different ($R>0.5$, $p<0.05$) between the sites (Table 2). ANOSIM results showed strong Bray Curtis dissimilarity of soil bacterial composition between wetland sites in both the October samples (*Global* $R=0.65$, $p<0.05$) and the June samples (*Global* $R=0.26$, $p<0.05$). Generally, the soil bacterial communities in the created wetlands were less dissimilar to each other than to those of either natural wetland (especially during the June sampling) (Table 2). The greatest difference of bacterial community structure was observed between the two natural wetlands, BP and BN ($R=0.937$, $p<0.05$), suggesting these two natural wetlands were quite different reflecting differences in their soil physicochemistry. The structure (i.e., S and H′) of soil bacterial communities of BP also showed significant differences from all created wetland sites (i.e. BR and LC) (Table 3).

We obtained 228,023 tagged sequences that were over 100 bp pairs in length and had an average coverage of 3354 reads per soil sample, but we may not assume that the full extent of bacterial diversity within all soils was determined (Lemos et al., 2011). Based on our sampling efforts, we could assume that more than 110 different taxa, as identified at RDP level 6 (i.e., genus level), were found on the soils we sampled. The species richness (S) per each site ranged from 8 to 24 taxa with little difference between the sites, except BP being the lowest (Table 3). Evenness (J) was slightly different between sites in October samples, but the difference was not observed in June samples. Shannon–Weiner's diversity index (H′) ranged between 1.74 and 2.93 with no distinctive difference between the wetland sites,

Table 2
Multivariate statistical pairwise comparisons (ANOSIM[a]) of bacterial community Bray Curtis dissimilarities between wetland sites by two sampling periods. Significant difference at $\alpha=0.05$.

	October 2008		June 2009	
	R	p-value	*R*	p-value
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

[a] ANOSIM *R* statistic values closer to 1 indicate community dissimilarity among groups.

except BP being less diverse (Table 3). The levels of S and H′ found in this study were relatively lower than expected based on the studies that used a high throughput pyrosequencing (Acosta-Martinez et al., 2008; Will et al., 2010), but fall within an acceptable range of those values in acidic soils previously studied (Hartman et al., 2008; Lauber et al., 2009). We used the traditional indices (i.e., S, H′, and J) to present and compare community diversity of the wetland soils in this study, but caution needs to be taken in interpreting these indices with microbiological data. Microbial ecologists are often faced with the dilemma of deciding if these traditional indices are appropriate measures for microbial community profiles since they are designed for discrete macro-community analyses (Mills et al., 2006). The traditional ecological indices such as H′ that are based on the clear definition and ecological description of an individual species as an entity are often difficult to define in microbiology (Mills et al., 2006). These indices, though, have been used extensively to present fingerprints and pyrosequencing results in a number of studies of microbial communities

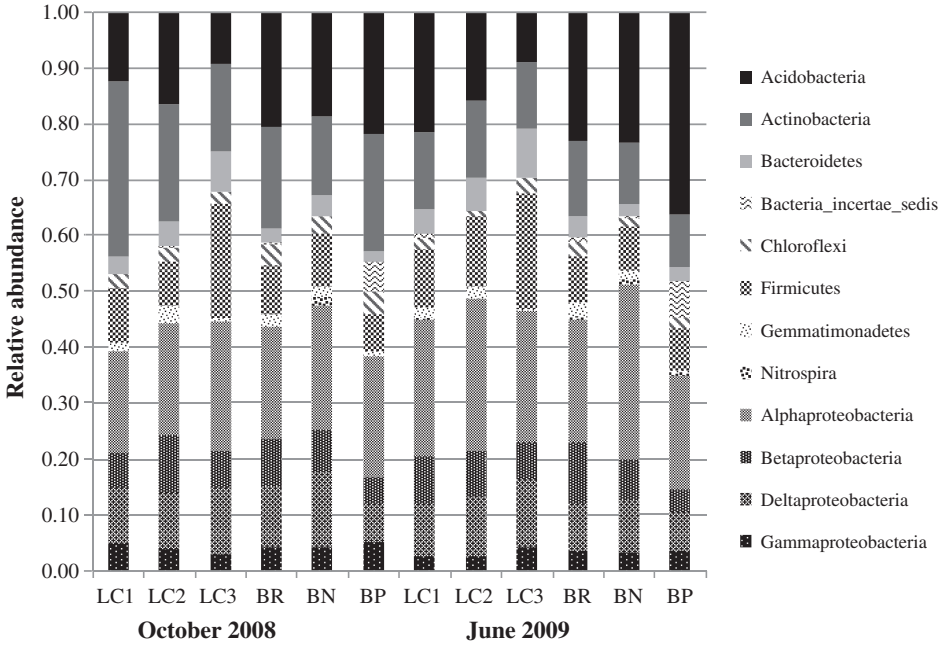

Fig. 3. Relative abundances and distribution of major bacterial phyla in wetland soils collected in two sampling periods.

Table 3
Richness, evenness, and diversity measures of bacterial communities estimated using genus level identification of multi-tag pyrosequencing fragments.

Site and Season	Richness (S)	Pielou's evenness (J)	Shannon diversity (H')
October 2008			
LC1	21 ± 2.6 a	0.95 ± 0.008 b	2.84 ± 0.09 a
LC2	16 ± 2.5 a	0.96 ± 0.006 b	2.60 ± 0.17 a
LC3	19 ± 2.4 a	0.94 ± 0.003 c	2.77 ± 0.12 a
BR	17 ± 0.8 a	0.97 ± 0.003 a	2.72 ± 0.05 a
BN	18 ± 0.8 a	0.97 ± 0.005 ab	2.80 ± 0.05 a
BP	15 ± 2.9 a	0.94 ± 0.018 c	2.52 ± 0.26 a
June 2009			
LC1	15 ± 0.8 a	0.95 ± 0.007 a	2.55 ± 0.05 a
LC2	13 ± 1.2 a	0.94 ± 0.013 a	2.36 ± 0.10 b
LC3	14 ± 0.3 a	0.91 ± 0.010 a	2.36 ± 0.01 b
BR	16 ± 0.9 a	0.94 ± 0.014 a	2.58 ± 0.06 a
BN	16 ± 1.2 a	0.94 ± 0.013 a	2.59 ± 0.11 a
BP	10 ± 2.3 b	0.89 ± 0.040 a	2.03 ± 0.29 c

Values with different letters are significantly different with Bonferroni adjusted p-values ($p<0.05$).

and useful especially to compare the communities from different environmental conditions (Ahn et al., 2009; Hartman et al., 2008; Liu et al., 2012).

It has been known that bacterial communities can be sensitive to dry–wet gradient (Fierer et al., 2003) and soil moisture content (Ahn and Peralta, 2009). Soil moisture content differed significantly in LC sites (i.e., LCs 1, 2, and 3) between the two sampling periods with October being drier and June being wetter (Table 1). The high moisture condition in June might have stressed on the extant soil bacterial communities, triggering the relatively lower diversity of the community (Table 3). Ahn and Peralta (2009) found that the dry site (i.e., LC1) had consistently higher diversity and evenness than the wet site (i.e., LC2) in a previous study. We may not be conclusive about this though because we found the opposite trend of bacterial community being more diverse in wetter areas (i.e., hollows) than drier areas (i.e., hummocks) of a natural palustrine forested wetland in another study (Ahn et al., 2009). One thing to note in this study is that soils collected during June, the wetter period, compared to those collected in October, the drier period, showed slight changes in percent composition of several phyla, including a slight increase of anaerobic *Bacteroides* and chemolitho-autotrophic nitrite-oxidizing *Nitrospira* (Fig. 3), which confirms that soil bacterial community structure may respond to a gradient of oxic/anoxic conditions of soil microsites. Further study is needed to know how soil bacterial community structure and diversity in wetlands may respond to their hydrologic regimes with quantitative monitoring of the redox potential status of soils.

3.3. Association between bacterial community structures and physicochemical properties in wetland soils

We found a significant association between soil bacterial community structures and physicochemical properties in the wetlands. Especially, C:N ($\rho = 0.43$, $p<0.01$) and pH ($\rho = 0.39$, $p<0.01$) showed the highest correlation with the Bray Curtis dissimilarities of bacterial taxa composition in each sampling period (i.e., October and June, respectively). These results are congruent with previous studies where C:N or pH was associated with changes in bacterial community compositions in a range of soils (Hartman et al., 2008; Ahn and Peralta, 2009; Lauber et al., 2009). To further probe the relationship between community structure and physicochemical attributes, the physicochemical PC1 and PC2 scores (Fig. 2) were tested for correlations with the bacterial community diversity measures (Table 3). PC1 (most variance explained by SOM and TN) was significantly correlated with the evenness (J) of bacterial communities ($\rho = 0.12$, $p<0.01$) of soils whereas PC2 (variance mostly explained by pH) was significantly correlated with all three community diversity measures (i.e., S, J, and H′) [$0.42<\rho$ *(correlation coefficient)*>0.56, $p<0.02$]. Soil pH has been found as a controller or influencing factor for soil biogeochemical processes such as denitrification (Groffman, 1994; Hunter and Faulkner, 2001), an important ecological function often expected to develop in created wetlands. Wallenstein et al. (2006) noted that soil pH is a key factor along with carbon availability and soil moisture content in determining denitrifying community structure that is responsible for denitrification function of wetlands. Further study is needed to fully examine which physicochemical variable is the most closely associated with the changes in bacterial community structures.

The outcome of the study shows that soil properties are linked to the structures of bacterial communities that are known to mediate a number of biogeochemical processes in wetland soils, supporting ecosystem functioning. This make it all the more important to study soil attributes in created wetlands as part of post-construction monitoring to track their functional development better to achieve not only structural, but functional success of wetland mitigation.

4. Conclusion

Bacterial communities play an important role in many biogeochemical processes that support ecological functions in created wetlands. The development of soil physicochemical properties is often used to track the progress of biogeochemical developments in created wetlands. Bacterial communities of created and natural wetlands in the Piedmont region of Virginia were investigated by high throughput pyrosequencing in this study. Soil properties such as SOM, TOC, TN, pH, and soil moisture content showed differences by site. Soil bacterial community structures and their differences between the wetlands were clearly distinguished by pyrosequencing. The outcome of the study revealed that there was a clear association between bacterial community structure and physicochemical properties of wetland soils, especially C:N ratio and pH. The development of ecological functions, mostly mediated by microbial communities, seems to depend on the development of soil properties, therefore the development of soil properties should be more carefully studied in post-construction monitoring of created wetlands.

Acknowledgments

Special thanks to Masoumeh Sikaroodi for technical assistance with molecular work. Also thanks to Wetland Studies and Solutions, Inc., for the use of their created wetlands for the study. The work was funded by the Society of Wetland Scientists South Atlantic Chapter; George Mason University Francis Heliotis Graduate Research Fund; Thomas F. and Kate Miller Jeffress Memorial Trust Fund, and USGS-NIWR Grant (G10AP00139).

References

Acosta-Martinez V, Dowd S, Sun Y, Allen V. Tag-encoded pyrosequencing analysis bacterial diversity in a single soil type as affected by management and land use. Soil Biol Biochem 2008;40:2762–70.
Ahn C, Peralta RM. Soil bacterial community structure and physicochemical properties in mitigation wetlands created in the Piedmont region of Virginia (USA). Ecol Eng 2009;35:1036–42.
Ahn C, Gillevet PM, Sikaroodi M, Wolf KL. An assessment of soil bacterial community structure and physicochemistry in two microtopographic locations of a palustrine forested wetland. Wetlands Ecol Manage 2009;17:397–407.
Anderson CJ, Mitsch WJ, Nairn RW. Temporal and spatial development of surface soil conditions in two created riverine marshes. J Environ Qual 2005;34:2072–81.
Ballantine K, Schneider R. Fifty-five years of soil development in restored freshwater depressional wetlands. Ecol Appl 2009;19:1467–80.
Balser T, Kinzig A, Firestone MK. Linking soil microbial communities and ecosystem functioning. In: Kinzig A, Pacala S, Tilman D, editors. The functional consequences of biodiversity: empirical progress and theoretical extensions. Prinction, New Jersey: Princeton University Press; 2002. p. 265–356.
Bishel-Machung L, Brook RP, Yates SS, Hoover KL. Soil properties of reference wetlands and wetland creation projects in Pennsylvania. Wetlands 1996;16:532–41.
Boon PI. Biogeochemistry and bacterial ecology of hydrologically dynamic wetlands. In: Batzer DP, Sharitz RR, editors. Ecology of freshwater and estuarine wetlands. Berkeley: University of California Press; 2006. p. 115–76.

Bruland GL, Richardson CJ. Spatial variability of soil properties in created, restored, and paired natural wetlands. Soil Sci Soc Am J 2005;69:273–84.
Bruland GL, Richardson C. Comparison of soil organic matter in created, restored and paired natural wetlands in North Carolina. Wetlands Ecol Manage 2006;14: 245–51.
Bruland GL, Richardson CJ, Daniels WL. Microbial and geochemical responses to organic matter amendments in a created wetland. Wetlands 2009;29:1153–65.
Campbell DA, Cole CA, Brooks RC. A comparison of natural and created wetlands in Pennsylvania, USA. Wetlands Ecol Manage 2002;10:41–9.
Chariton AA, Court LN, Hartley DM, Colloff MJ, Hardy CM. Ecological assessment of estuarine sediments by pyrosequencing eukaryotic ribosomal DNA. Front Ecol Environ 2010;8:233–8.
Clarke KR, Gorley RN. PRIMER v5: User Manual/Tutorial. UK: PRIMER-E Ltd., Plymouth Marine Laboratory; 2001.
Clarke KR, Warwick RM. Change in marine communities: an approach to statistical analysis and interpretation. 2nd ed. UK: PRIMER-E Ltd., Plymouth Marine Laboratory; 2001.
Cole CA, Brooks RP, Wardrop DH. Assessing the relationship between biomass and soil organic matter in created wetlands of central Pennsylvania (USA). Ecol Eng 2001;17: 423–8.
Confer SR, Niering WA. Comparison of created and natural freshwater emergent wetland in Connecticut. Wetlands Ecol Manage 1992;2:143–56.
Craft C, Broome S, Campbell C. Fifteen years of vegetation and soil development after brackish-water marsh creation. Restor Ecol 2002;10:248–58.
D'Angelo EM, Karathanasis AD, Sparks EJ, Ritchey SA, Wehr-McChesney SA. Soil carbon and microbial communities at mitigated and late successional bottomland forest wetlands. Wetlands 2005;25:162–75.
Dee S, Ahn C. Soil physicochemical properties predict plant community development of mitigation wetlands created in the Virginia piedmont, USA. Environ Manage 2012;49: 1022–36.
Edwards KR, Cizkova H, Zemanova K, Santruckova H. Plant growth and microbial processes in a constructed wetland planted with *Phalaris arundinacea*. Ecol Eng 2006;27:153–65.
Ehrenfeld JG, Ravit B, Elgersma K. Feedback in the plant–soil system. Annu Rev Env Resour 2005;30:75-115.
Erwin KL. An evaluation of wetland mitigation in the South Florida water management district. Final Report, vol. 1. West Palm Beach (FL): South Florida Water Management District; 1991.
Farrel JD, Ware S. Edaphic factors and forest vegetation in the Piedmont of Virginia. B Torrey Bot Club 1991;118:161–9.
Fierer N, Schimel JP, Holden PA. Influence of drying–rewetting frequency on soil bacterial community structure. Microb Ecol 2003;45:63–71.
Gardner WH. Water content. In: Klute A, editor. Methods of soil analysis, part 1, physical and mineralogical methods. 2nd ed. Madison, WI: Soil Science Society of America; 1986. p. 516–7.
Gillevet P, Sikaroodi M, Keshavarzian A, Mutlu E. Quantitative assessment of the human gut microbiome using multitag pyrosequencing. Chem Biodivers 2010;7: 1065–75.
Groffman PM. Denitrification in freshwater wetlands. Curr Top Wetlands Biogeochem 1994;1:15–35.
Hartman WH, Richardson CJ, Vilgalys R, Bruland GL. Environmental and anthropogenic controls over bacterial communities in wetland soils. Proc Natl Acad Sci 2008;105: 17842–7.
Hoeltje SM, Cole CA. Losing function through wetland mitigation in central Pennsylvania, USA. Environ Manage 2007;39:385–402.
Hossler K, Bouchard V. Soil development and establishment of carbon-based properties in created freshwater marshes. Ecol Appl 2010;20:539–53.
Hunter RG, Faulkner SP. Denitrification potential in restored and natural bottomland hardwood wetlands. Soil Sci Soc Am J 2001;65:1865–72.
Kentula ME, Brooks RP, Gwin SE, Holland CC, Sherman AD, Sifneos JC. An approach to improving decision making in wetland restoration and creation. In: Hairston AJ, editor. Corvallis, OR: U.S. Environmental Protection Agency, Environmental research Laboratory; 1992.
Lauber CL, Hamady M, Knight R, Fierer N. Pyrosequencing-based assessment of soil pH as a predictor of soil bacterial community structure at the continental scale. Appl Environ Microbiol 2009;75:5111–20.
Lemos LN, Fulthorpe RR, Triplett EW, Roesch LFW. Rethinking microbial diversity analysis in the high throughput sequencing era. J Microbiol Methods 2011;86:42–51.
Liu R, Yu Z, Guo H, Liu M, Zhang H, Yang M. Pyrosequencing analysis of eukaryotic and bacterial communities in faucet biofilms. Sci Total Environ 2012;435–436:124–31.
Margulies M, Egholm M, Altman WE, Attiya S, Bader JS, Bemben LA, et al. Genome sequencing in microfabricated high-density picolitre reactors. Nature 2005;437:376–80.
Matthews JW, Spyreas G, Endress AG. Trajectories of vegetation-based indicators used to assess wetland restoration progress. Ecol Appl 2009;19:2093–107.
Mentzer JL, Goodman R, Balser TC. Microbial seasonal response to hydrologic and fertilization treatments in a simulated wet prairie. Plant Soil 2006;284:85-100.
Mills DK, Fitzgerald K, Litchfield CD, Gillevet PM. A comparison of DNA profiling techniques for monitoring nutrient impact on microbial community composition during bioremediation of petroleum-contaminated soils. J Microbiol Methods 2003;54: 54–74.
Mills DK, Entry JA, Voss JD, Gillevet PM, Mathee K. An assessment of the hypervariable domains of the 16S rRNA genes for their value in determining microbial community diversity: the paradox of traditional ecological indices. FEMS Microbiol Ecol 2006;57:496–503.
Morgan PA, Short FT. Using functional trajectories to track constructed salt marsh development in Great Bay Estuary Maine/New Hampshire, USA. Restor Ecol 2002;10: 461–73.
Moser KF, Ahn C, Noe G. Characterization of microtopography and its influence on vegetation patterns in created wetlands. Wetlands 2007;27:1081–97.
Moser KF, Ahn C, Noe G. The influence of microtopography on soil nutrients in created mitigation wetlands. Restor Ecol 2009;17:641–51.
National Research Council. Compensating for wetland losses under the Clean Water Act. Washington, DC. USA: National Academy Press; 2001.
Peralta AL, Matthews JW, Kent AD. Microbial community structure and denitrification in a wetland mitigation bank. Appl Environ Microbiol 2010;13:4207–15.
Reddy KR, DeLaune RD. Biogeochemistry of wetlands: science and applications. LLC, New York, NY: Taylor & Francis Group; 2008.
Roesch LFW, Fulthorpe RR, Riva A, Casella G, Hadwin AKM, Kent AD, et al. Pyrosequencing enumerates and contrasts soil microbial diversity. ISME J 2007;1:283–90.
Spieles DJ. Vegetation development in created, restored, and enhanced mitigation wetland banks of the United States. Wetlands 2005;25:51–63.
Stolt MH, Genthner MH, Daniels WL, Groover VA, Nagle S, Haering KC. Comparison of soil and other environmental conditions in constructed and adjacent palustrine reference wetlands. Wetlands 2000;20:671–83.
Tam L, Derry AM, Kevan PG, Trevors JT. Functional diversity and community structure of microorganisms in rhizosphere and non-rhizosphere Canadian arctic soils. Biodivers Conserv 2001;10:1933–47.
Thomas GW. Procedure for soil pH measurements: determination of pH in water. In: Bartels JM, editor. Methods of soil analysis part 3, chemical methods. Madison, WI: American Society of Agronomy, Soil Science Society of America; 1986.
Wallenstein MD, Myrold DD, Firestone M, Voytek M. Environmental controls on denitrifying communities and denitrification rates: insights from molecular methods. Ecol Appl 2006;16:2143–52.
Wang Q, Garrity GM, Tiedje JM, Cole JR. Naïve Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Appl Environ Microbiol 2007;73:5261–7.
Whittecar RG, Daniels WL. Use of hydrogeomorphic concepts to design created wetlands in southeastern Virginia. Geomorphology 1999;31:355–71.
Will C, Thurmer A, Wollherr A, Nacke H, Herold N, Schrumpf M, et al. Horizon-specific bacterial community composition of German grassland soils as revealed by pyrosequencing-based analysis of 16S rRNA genes. Appl Environ Microbiol 2010;76:6751–9.
Wilson DW, Sander LE. Total carbon, organic carbon, and organic matter. In: Sparks DL, editor. Methods of soil analysis: part 3—chemical methods. Madison, WI: SoilScience Society of America; 1996. p. 1002–5.
Wolf KL, Ahn C, Noe G. Development of soil properties and nitrogen cycling in created wetlands. Wetlands 2011;31:699–712.
Zedler JB, Callaway JC. Tracking wetland restoration: do mitigation sites follow desired trajectories? Restor Ecol 1999;7:69–73.
Zuur AF, Ieno EN, Smith GM. Analysing ecological data. New York, NY: Springer Science; 2007.