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Effects of wetland design and invasive species removal on carbon and microbial communities in restored wetlands

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Effects of wetland design and invasive species removal on carbon and microbial communities in restored wetlands

By:

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Abstract

Multiple wetland ecosystem services such as carbon sequestration and nutrient removal are influenced by microbial communities and dissolved organic matter (DOM). I examined DOM composition, carbon metabolism, and microbial communities in three created wetlands, which had distinct hydrology, vegetation and antecedent land-use. To study differences between wetlands, porewater and soil were collected from each wetland in spring, summer, and fall of 2018. DOM was analyzed using NMR spectroscopy and fluorescence spectrometry, soil microbial community composition was examined using 16S ribosomal sequencing, and CO_2 and CH_4 production rates were measured in anaerobic soil incubations. Structural DOM composition varied significantly between the three wetlands but did not vary seasonally. There were distinct differences in the microbial community composition of each wetland, which correlated with soil chemistry factors but did not correlate with CH_4 or CO_2 production rates. Average CH_4/CO_2 production ratios were approximately 1:1 for all sites in the spring and summer and approached 3:1 in the fall, with no differences between sites. This suggests that while DOM characteristics and microbial communities in created wetlands are impacted by site characteristics, these differences have less effect on carbon metabolism. I also examined the effects of invasive species removal via herbicide application on microbial communities in one of the wetlands. Soil was collected in the spring and summer before and after the application of glyphosate herbicide and microbial communities were examined using 16S ribosomal sequencing. There were significant and persistent changes in the microbial community following invasive species removal through application of herbicide. Further study is needed to determine direct causal relationships between invasive species control measures and microbial community changes, to determine whether shifts in microbial communities persist past a single growing season and to identify impacts of invasive removal and herbicide application on key wetland functions.

Introduction

Wetlands are considered to be one of the most socially valuable ecosystems in the world in part due to their immense ability to provide ecosystem services such as carbon cycling and carbon sequestration (Costanza et al., 2014). Despite their value, as much as 87% of natural wetlands have been lost globally since 1700 (Davidson et al., 2014). The continental United States has had a net gain of 3521 km^2 of herbaceous wetland land cover from 2001 to 2011 (Homer et al., 2015). Given the wetland creation and replacement laws in the United States (*Federal Water Pollution Control Act*, 2002) it is reasonable to assume that a significant portion of this increase consists of man-made created wetlands. The increasing prevalence of these ecosystems underscores the importance of understanding their unique biogeochemistry and how wetland construction decisions impact these processes.

Inland water systems such as wetlands have been recognized for their role in carbon (C) cycling and important contributions to the global carbon budget (Battin et al., 2009). Wetlands contain a significant amount of dissolved organic matter (DOM) and an estimated 220 Pg of carbon is stored in North American wetland soils (Bridgham et al., 2006). However, C cycling in created wetlands often differs from natural wetlands, with lower rates of biomass production and decomposition than their natural counterparts (Fennessy et al., 2008). While biomass production removes carbon from the atmosphere, decomposition of organic matter by microbial communities generates greenhouse gasses such as CO_2 and CH_4 (Battin et al., 2009; Bridgham et al., 2006). Wetlands are generally carbon sinks but created wetlands that are designed or managed poorly can become sources of CO_2 and CH_4, and created wetlands tend to have higher net fluxes of greenhouse gases than natural wetlands (Kayranli, et al., 2010). It is therefore important that carbon storage and greenhouse gas production are considered during wetland

construction and management. However, there is a wide variation in the design of created wetlands and the effects of design decisions on microbial communities, C cycling and greenhouse gas production are not well known.

Wetland ecosystems encompass a wide range of vegetation communities and hydrologic regimes, resulting in considerable variation in soil and water chemistry and microbial community structure, which can impact the rate of organic matter decomposition, nutrient removal and greenhouse gas production. Many of these key wetland factors are partially or wholly determined during the wetland design and construction process. However, the interactions between these factors in determining key functions such as C storage, nutrient cycling and greenhouse gas production are not well understood, making it difficult to identify optimal wetland design and management. Furthermore, studies have also shown that microbial community structure and composition are significantly different between natural and created wetlands (Ansola et al., 2014; Arroyo et al., 2015; Cao et al., 2017) suggesting the need for studies that focus specifically on created wetland systems.

Taken alone, the effect of individual wetland characteristics on microbial community structure and greenhouse gas production are better understood. The design of hydrologic regimes in created wetlands can have profound effects on biogeochemical processes in wetlands. Permanently flooded conditions limit oxygen diffusion and develop anaerobic sediments that promote anaerobic processes such as methanogenesis and denitrification. Conversely, in sites that are seasonally flooded, oxygen penetration is more variable and aerobic processes can occur during periods of low water table (Baldwin & Mitchell, 2000). Changes in hydrological regime have also been shown to alter the structure of soil microbial communities in aquatic ecosystems, likely due to the changes in oxygen availability (Ahn & Peralta, 2009; Foulquier et al., 2013;

Moche, Gutknecht, Schulz, Langer, & Rinklebe, 2015). Hydrologic regimes also have a determinative impact on the plant communities that develop in created wetlands (Ahn & Dee, 2011). Plant communities in turn can have downstream impacts on DOM (Barber et al., 2001), microbial community structure (Kourtev et al., 2003), and greenhouse gas production (Inglett et al., 2012). In many ecosystems there is also evidence that there are predictable relationships between plant and microbial community structure (Angeloni, et al., 2006; Arroyo et al., 2015; Kourtev, et al., 2003).

Soil chemistry in created wetlands is also heavily impacted by construction decisions, such as the land-use history of the parcel of land used. Soil nutrients and organic matter composition is also known to be an important driver of microbial community composition (Ahn & Peralta, 2009). For example, the prevalence of methanogenic organisms, a key microbial functional group, is negatively correlated with the concentration of alternative electron acceptors (S. He et al., 2015). However, many of these dynamics may have overlapping effects on microbial community structure and their interactions require more study (Lee et al., 2019).

Understanding how differences in wetland environmental factors and management strategies influence microbial community structure and function is important for developing better wetland design and management practices. In pursuit of that goal this study had four objectives. First, to evaluate if there are differences in microbial community structure between created wetlands with different land use histories and hydrologic design. I hypothesized that each created wetland would have a unique microbial community and that those differences would correlate with soil chemistry and hydrology. Second, to evaluate if created wetlands with different land use histories and hydrology have differences in the structure of their DOM. I hypothesized that given the differences in plant community composition between wetlands there

would be differences in the composition and seasonality of DOM. Third, to evaluate if differences in microbial communities and DOM resulted in differences in carbon metabolism between the wetlands. I hypothesized that each wetland would have differences in rates of carbon metabolism and these differences would be correlated with differences in microbial community structure. Fourth, to evaluate whether invasive species removal through the use of herbicide application would alter microbial community composition. I hypothesized that invasive species control measures would have no long-term effect on microbial communities.

Methods

Study sites

This study took place in 2017 and 2018 at the High Acres Nature Area (HANA), owned and operated by Waste Management of New York LLC, in Perinton, NY, USA (43° 5' N, 77° 23' W). Three created wetlands were chosen within HANA for this study, Area One North (A1N), Area Two South (A2S), and Area Three C (A3C, Figure 1).

A1N is a shallow emergent marsh environment covering approximately 1.87 ha. A1N was constructed on the site of a former gravel mine repository, the site was abandoned and left fallow in the 1960s before being converted to a wetland in 2009 (Stantec Consulting, 2009). Water levels are controlled by a culvert located in the southern end of the wetland and consistently has standing water. The plant community in A1N is dominated predominantly by narrow and broadleaf-arrowhead (*Saggittaria*, *spp.*), pickerelweed (*Pontederia cordata*), and white pond lily (*Nymphaea odorata*). A2S is a seasonally flooded wetland covering approximately 0.37 ha. A2S has seasonal standing water that recedes in the summer in periods of low precipitation but generally retains soil moisture. A2S was excavated and constructed on the

site of a former agricultural field in 2009 (Stantec Consulting, 2009). At the start of the study, spring 2017, A2S was dominated by a near monoculture of reed canary grass *(Phalaris arundinacea)*. Rodeo (glyphosate, $C_3H_8NO_5P$) herbicide was applied in A2S on September 21st and 22nd of 2017 as an invasive species control measure at an application rate of 1.8 pints/acre by method of foliar spraying, killing nearly all of the vegetation. A2S remained relatively bare for the duration of the 2018 growing season. A3C is a seasonally flooded wetland of approximately 0.61 ha, that was constructed on a former cow pasture in 2012. A3C is seasonally flooded and generally has standing water in the spring and fall but is dry in the summer. Water plantain *(Alisma subcordatum)*, rice cut grass (*Leersia oryzoides)*, and narrow and broadleaf cattail *(Typha spp)* are the dominant plant species.

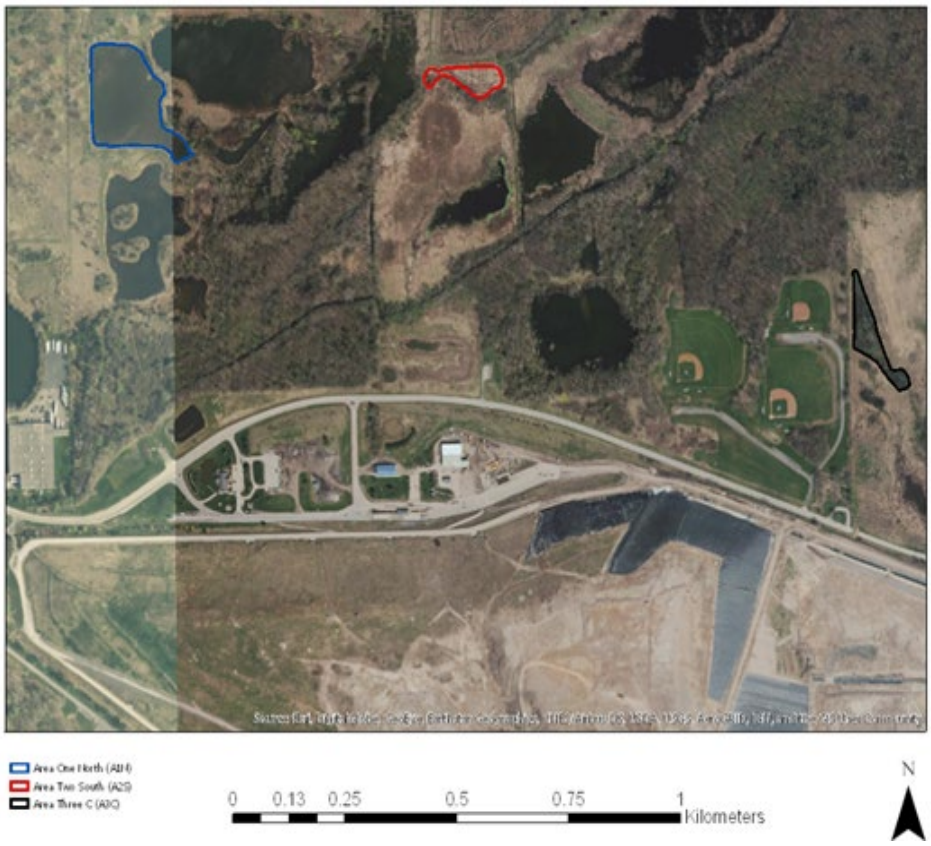

Figure 1. Map of the three created wetlands used in this study. Area One North (A1N) is outlined in Blue, Area Two South (A2S) is outlined in red and Area Three C (A3C) is outlined in black.

Soil Collection and Porewater Collection

Eight 1x1m plots were established in each of the wetlands studied. Soil cores were taken to a depth of ten centimeters in each of the plots in A2S in the spring, and summer of 2017, and in each plot in all of the three wetlands in the spring, summer, and fall of 2018. Each soil core was homogenized in the field and stored on ice for transport. A subset of each core was frozen for nutrient and microbial analysis on the day of collection. In 2018 a subset of each core was sieved and used for anaerobic soil incubations. Porewater was collected using lysimeters at a depth of 10cm in each wetland in the spring, summer, and fall of 2018. Porewater was stored on ice for transport, filtered within 12 hours and immediately frozen for later analysis on the day of collection. Porewater was collected based on groundwater availability and therefore not collected in every plot in every season.

DOM analysis

The fluorescent portion of the porewater DOM was analyzed using a Carey Eclipse Fluorescence Spectrometer. Excitation of each sample was performed every five nm from 295 to 600 nm and emission was measured every five nm from 295-600 nm. The freshness index of each sample was calculated as the emission intensity at 380 nm divided by the maximum intensity of emission between 420 and 435 nm at the excitation of 310 nm (Parlanti, Wörz, Geoffroy, & Lamotte, 2000; Wilson & Xenopoulos, 2009). This index provides information about relative freshness of the organic matter, with larger values indicative of more recently derived DOM. The Fluorescence index of each sample was calculated as the emission intensity of wavelengths 470nm/520nm at excitation of 370 nm. This index provides information about the source of the DOM, from terrestrially derived sources (plant and soil organic matter) having

a value of ~1.2 and microbially derived (bacteria and algal by-products) having a value of ~1.8 (Cory & McKnight, 2005).

A portion of each porewater sample was freeze-dried to isolate the DOM. Freeze dried samples were analyzed using 500Mhz NMR. Four structural groups were identified in the DOM, Aromatic, Carbohydrate, CRAM (Carboxylic rich alicyclic molecules), and Alykl groups (Figure 2). The relative proportion of each structural group, measured against a Sodium trimethylsilylpropanesulfonate (DSS) standard, was quantified for each sample.

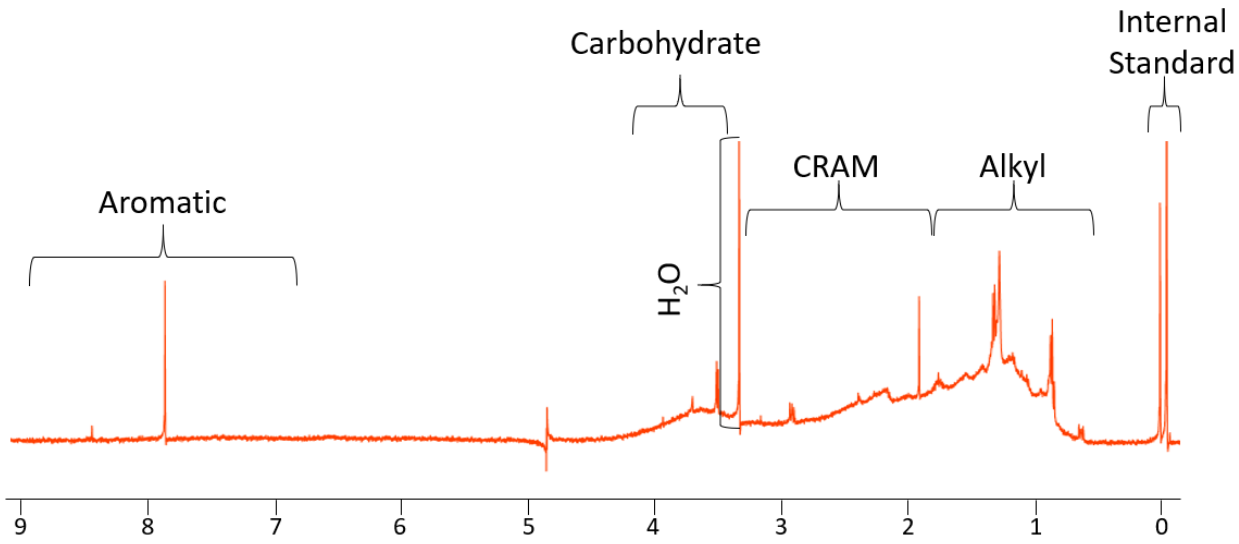

Figure 2. The regions of integration for each of the structural components that were examined using NMR spectroscopy, overlaid on a sample spectrum.

Soil Chemistry Analysis

Soil percent moisture was calculated by immediately drying soil from the field at 60ºC and recording the weight lost. Soil organic matter content (%OM) was calculated by the loss on

ignition method (Heiri et al., 2001). Inorganic phosphorus was extracted from dried soil through dissolution in 1M HCl, total phosphorus was extracted from dried soil by adding 0.5 ml $MgNO_{2(3)}$ to ~0.1g of dry soil and then ashing the soil at 550ºC for 1.5 hours followed by dissolution in 1M HCl. The dissolved phosphate levels were then measured using the ammonium molybdate method (Murphy & Riley, 1962). Soil extractable nitrate+nitrite and ammonium were extracted by shaking soil in 2M KCl and filtering the supernatant through a 45 um filter. Nitrate+nitrite concentrations were analyzed using the method described by Doane and Horwath (2003). Soil ammonium concentrations were measured using the Phenol-hypochlorite method (Solórzano, 1969). Extractable ammonium and nitrate+nitrite were combined to obtain total extractable N. Soil percent carbon (%C), percent nitrogen (%N) and C:N was measured using a Perkin Elmer CNHS/O 8000 analyzer.

Soil incubations

Soil greenhouse gas production potential was evaluated by measuring the production of CO_2, and CH_4 from the soil samples collected in each wetland in the spring, summer and fall of 2018. The soil from each plot was homogenized, sieved at 2.38 mm to remove rocks and roots, placed in airtight jars with equal weight nanopure water, and flushed with N_2 to establish anaerobic conditions. An equal amount of soil from each plot was dried at 60ºC to find the dry weight of the soil added. Each jar was incubated at 22ºC for an anaerobic period of 14 days to allow the microbial community to acclimate and was then flushed with N_2 to re-establish a CO_2 and CH_4 free headspace. Jars were then incubated at 22ºC and gas samples were collected 6-8 times over the next 12-14 days. The concentration of CO_2 and CH_4 in gas samples was measured using a Shimadzu-2014 gas chromatograph with a methanizer and FID detector and the change in concentration over time per gram of dry soil was used to determine potential production rates.

Soil DNA Extraction and analysis

Soil microbial DNA was extracted from ~0.5g of soil from each plot sampled using MoBio Powersoil® DNA extraction kits. DNA quality was confirmed using a nanodrop spectrometer and frozen at -20ºC until analysis. The variable V3-V4 region of the 16S bacterial gene was amplified using the primer pair MiSeq341F (5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3') and MiSeq805R (5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3'). Sequencing was performed on a Illumina MiSeq System, samples were loaded at a concentration of 8pM and sequenced using the MiSeq V3 600 cycle kit for 2 x 250 bp cycles. Microbial DNA was processed using Qiime 2 software (Bolyen et al., 2019). Microbial Operational Taxonomic Units (OTUs) were defined for analysis as having 99% sequence similarity. An even sampling depth of 9137 OTUs was selected to account for differences in read numbers between extractions and exclude as few samples as possible. Taxonomy of each sample was assigned using a naive Bayes classifier, trained with the Greengenes reference database at 99% sequence similarity.

Statistical analysis

Statistical tests were performed using R-statistical software (R Core Team, 2017). Normality of soil greenhouse gas production potential, DOM fluorescence indices as well as each of the soil chemistry characteristics measured was assessed using shapiro-wilks test, homogeneity of variance was assessed using levene's test. Data that was non normally distributed was log transformed. Two-way ANOVA was used to analyze the effects of wetland site, season,

invasive species removal via herbicide application, and their interactions on the data that was normally distributed and met the assumptions for homogeneity of variance. Tukey HSD post hoc tests were performed where significant effects were observed using the "agricolae" package (Mendiburo, 2019). For data that was non normally distributed but had homogeneity of variance, Kruskal-Wallis tests were used with Dunn post hoc test and Benjamini-Hochberg P-value adjustment.

DOM structural characteristics were split into four structural chemical groups, "Aromatic," "Carbohydrates," "Carboxylic Rich Alicyclic Materials" (CRAM), and "alkyl," groups. These groups were then bound for each sample and differences were tested between site and season with a two-way MANOVA test.

OTU diversity for each site and season was measured using Shannon's diversity index and significant differences between sites and seasons as well as treatment and season were measured using Kruskal-Wallis test with Dunn post hoc test and Benjamini-Hochberg p-value adjustment. Differences in microbial community structure were evaluated using the PermANOVA test found in the adonis function of the "vegan" package in R statistical software (Oksanen, 2010). The envfit function of the "vegan" package was used to fit soil chemistry and CO_2 and CH_4 production vectors to a nonmetric multidimensional scaling ordination plot (NMDS plot) constructed from Bray-Curtis dissimilarity distances. The significance of each factor was evaluated using a goodness of fit statistic tested against 999 permutations of the environmental variables (Oksanen, 2010).

Results

Wetland Type

Soil chemistry

Soil %OM, total phosphorus, soil moisture, soil %C, soil %N, and soil C:N differed significantly between each site (Table 1). Soil %OM was highest in A2S and lowest in A1N. Soil total phosphorus was similar in A2S and A3C ($p=0.3$), but significantly lower in A1N than A2S ($p=0.04$) or A3C ($p<0.001$), with a significant seasonal interaction (Table 1 and 2). Soil moisture content was higher in Spring and Fall than Summer ($p=0.002$, $p=0.008$), soil moisture in A1N was significantly higher than A3C ($p=0.013$). Soil %C ($p=0.001$) and %N ($p<0.001$) differed between wetlands, for %N, A2S=A3C>A1N, while for %C, A2S>A3C=A1N, resulting in soil C:N ratio differences between wetlands, with A1N>A2S>A3C (Table 1 and 2). The soil extractable N in each wetland was similar, but varied by season ($p=0.041$, Table 1 and 2).

Table 1. Soil physical chemistry factors in each wetland in each season expressed as means ± standard error.

Soil Characteristics	A1N			A2S			A3C		
	Spring	Summer	Fall	Spring	Summer	Fall	Spring	Summer	Fall
Organic matter (%)	10.20 ± 0.81	9.79 ± 0.53	10.42 ± 0.78	15.66 ± 0.68	17.93 ± 1.08	16.86 ± 1.58	13.64 ± 1.60	13.30 ± 1.57	12.84 ± 1.58
Total Phosphorous (mg/kg)	874.9 ± 45.1	808.8 ± 35.3	854.3 ± 22.1	871.6 ± 37.7	1033.4 ± 34.9	870.9 ± 40.4	886.3 ± 55.3	1103.2 ± 43.1	921.2 ± 31.5
Soil extractable N (mg/kg)	40.19 ± 3.85	11.69 ± 6.21	25.43 ± 3.35	31.90 ± 4.67	33.86 ± 5.64	42.56 ± 3.34	37.88 ± 8.42	32.31 ± 3.31	24.10 ± 4.26
Soil Moisture (%)	50.40 ± 2.15	50.14 ± 2.08	50.56 ± 3.36	52.46 ± 1.81	44.71 ± 1.90	47.92 ± 1.61	45.50 ± 3.18	32.20 ± 2.70	50.15 ± 0.16
Carbon (%)	4.56 ± 0.23	5.15 ± 0.39	5.26 ± 0.43	5.70 ± 0.46	6.15 ± 0.33	8.17 ± 0.69	4.96 ± 0.46	4.47 ± 0.34	6.07 ± 0.65
Nitrogen (%)	0.28 ± 0.02	0.30 ± 0.03	0.31 ± 0.03	0.40 ± 0.03	0.40 ± 0.02	0.55 ± 0.06	0.38 ± 0.02	0.33 ± 0.02	0.46 ± 0.04
C:N Ratio	18.98 ± 0.29	20.63 ± 2.12	20.42 ± 1.19	17.59 ± 0.19	17.88 ± 0.20	18.04 ± 0.81	15.96 ± 0.93	15.68 ± 0.84	15.25 ± 0.69

Table 2. Results of two-way ANOVA and Kruskal-Wallis tests comparing the effects of each area and season on soil chemistry characteristics. Significant p-values (<0.05) are bolded, p<0.001 are starred.

Soil Characteristic	Season		Site		Season x Site	
	F, χ^2	p	F, χ^2	p	F	p
Organic matter (%)	$\chi^2_{2,71}$=0.14	0.9	$\chi^2_{2,71}$=23.2	**<0.001***	-	-
Total Phosphorous (mg/kg)	$F_{2,71}$=6.8	**0.002**	$F_{2,71}$=7.8	**<0.001***	$F_{4,71}$=4.3	**0.004**
Soil Extractable N (mg/kg)	$F_{2,71}$=3.4	**0.04**	$F_{2,71}$=3.1	0.05	$F_{4,71}$=3.8	**0.007**
Soil Moisture (%)	$\chi^2_{2,71}$=13.1	**0.001**	$\chi^2_{2,71}$=8.3	**0.02**	-	-
Carbon (%)	$\chi^2_{2,71}$=6.5	**0.04**	$\chi^2_{2,71}$=13.7	**0.001**	-	-
Nitrogen (%)	$\chi^2_{2,71}$=5.8	0.05	$\chi^2_{2,71}$=20.2	**<0.001***	-	-
C:N Ratio	$\chi^2_{2,71}$=1.2	0.5	$\chi^2_{2,71}$=39.8	**<0.001***	-	-

Microbial distribution

The microbial communities clustered distinctly within each wetland and were significantly different from each other (PERMANOVA, p = 0.001). Although less clearly clustered, season also had a significant effect on microbial community structure across all wetlands (PERMANOVA, p = 0.023). Soil %OM, soil moisture, soil %C, soil %N, soil C:N and total soil phosphorus were all significantly correlated with the ordination of the microbial communities (Figure 2, Table 5). There was no significant correlation of greenhouse gas production with microbial community ordination. The Shannon diversity index of microbial diversity in each wetland was significantly higher in the spring (A1N: 10.05 ± 0.25, A2S: 10.52 ± 0.1, A3C: 9.14 ± 0.06, mean ± se), than the summer (A1N: 9.09 ±0.08, A2S: 8.95 ± 0.09, A3C: 8.93 ± 0.05) or the fall (A1N: 9.03 ± 0.09, A2S: 9.23 ± 0.23, A3C: 9.05 ± 0.25), there were no significant differences in the Shannon diversity index between sites..

The microbial communities in all wetlands were dominated predominantly by the phyla Proteobacteria, Bacteroidetes, Verrucomicrobia, and Chlorobi (Figure 4). A1N had a lower portion of Proteobacteria, Planctomycetes, and Nitrospirae than A2S or A3C, and had a higher abundance of Bacteroidetes, Firmicutes, Gemmatimonadetes, and Euryarchaeota. A2S had a

lower abundance of Verrucomicrobia and a higher abundance of Chlorobi than A1N or A3C.

A3C had a much lower proportion of rare phyla than A1N or A2S.

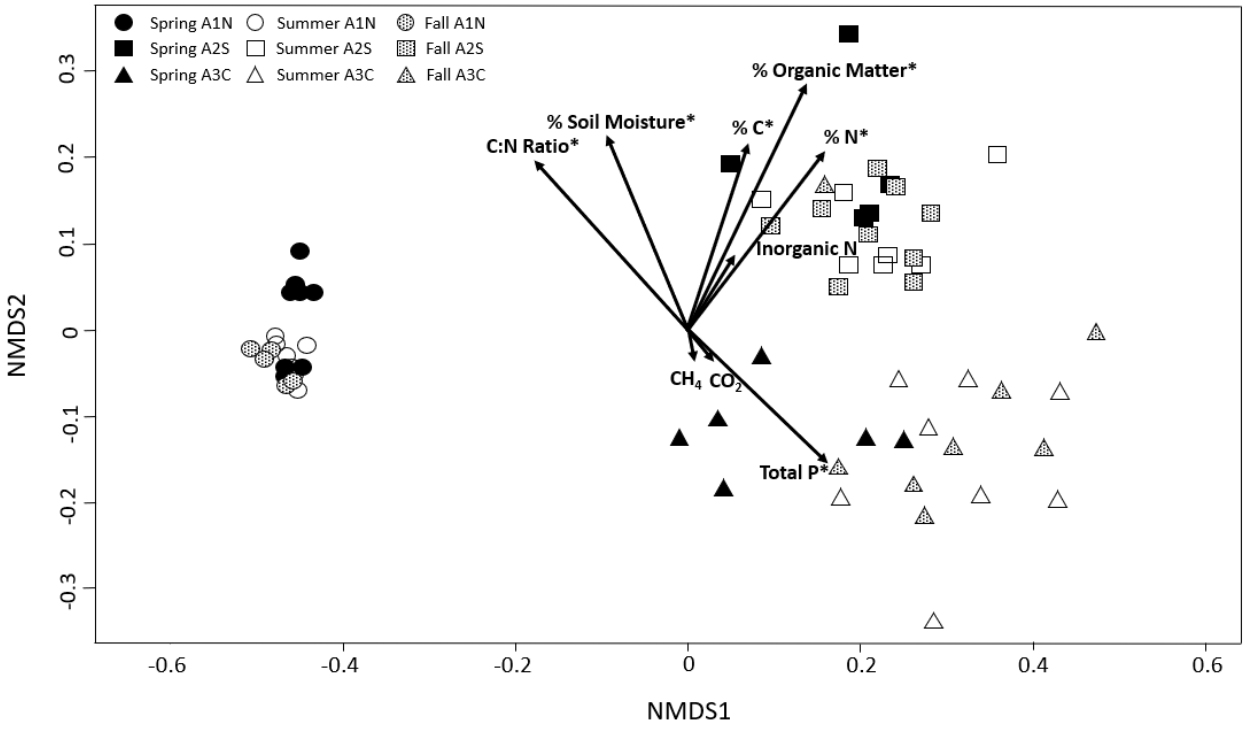

Figure 3. The NMDS ordination comparing the bacterial community structure between each of the wetlands in 2018. Stress values of the NMDS ordination are below 0.2 indicating an acceptable fit. The length of each vector on the graph is proportional to the strength of the correlation between each of the soil chemistry factors and the microbial community structure. Significant correlations ($p<0.05$) are starred.

Table 3. Results of ENVFIT permutation test comparing soil characteristics and greenhouse gas production potential with microbial community ordination.

Soil Characteristic	R^2	p
Organic matter (%)	0.46	**<0.001***
Total Phosphorus (mg/kg)	0.26	**<0.001***
Soil Extractable N (mg/kg)	0.05	0.193
Soil Moisture (%)	0.46	**<0.001***
Carbon (%)	0.22	**0.002***
Nitrogen (%)	0.30	**<0.001***
C:N Ratio	0.32	**<0.001***
CH_4 Production	<0.01	0.995
CO_2 Production	<0.01	0.896

Table 4. Results of PERMANOVA and Kruskal-Wallis comparing the effects of area and season on microbial distribution and microbial diversity. Significant p-values are bolded, p<0.001 are starred.

Characteristic	Season		Site		Season x Site	
	F	p	F	p	F	p
Community structure	$F_{2,61}$=0.13	**0.023**	$F_{2,61}$=6.8	**<0.001***	$F_{4,61}$=3.4	0.071
Shannon diversity	$\chi^2_{2,61}$=20.8	**<0.001***	$\chi^2_{2,61}$=5.6	0.058	-	-

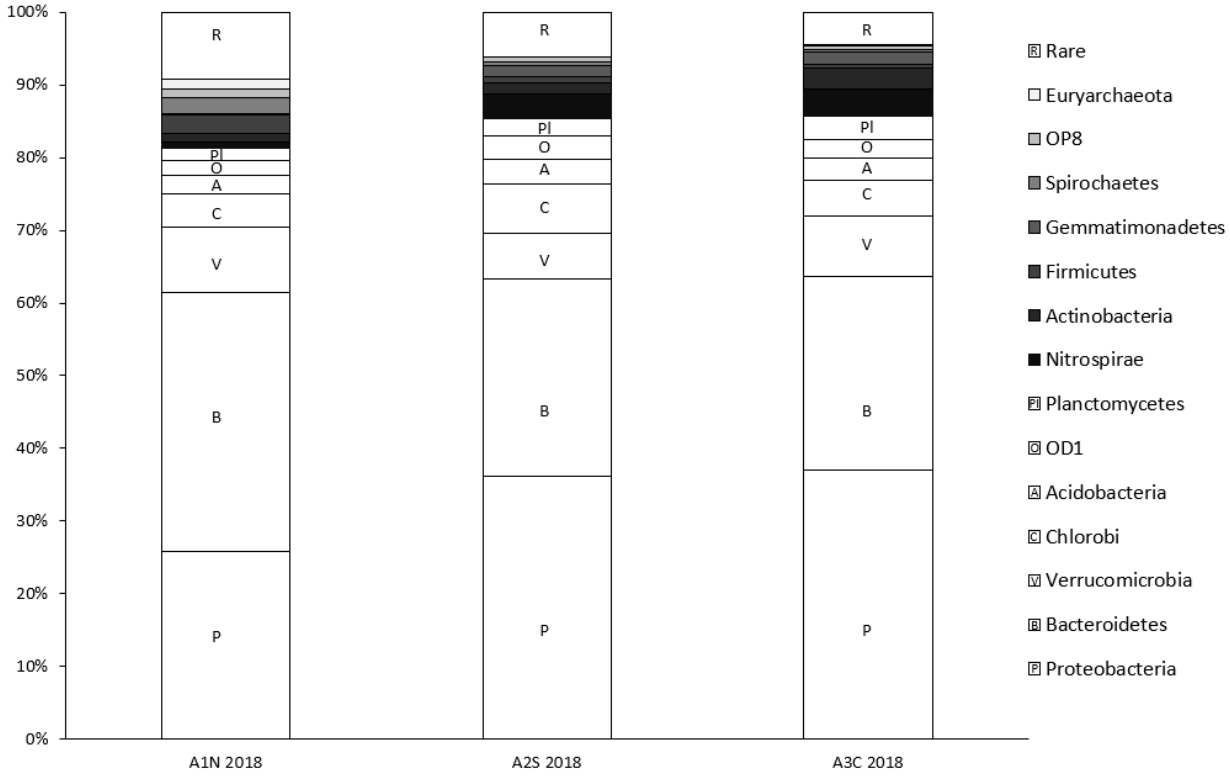

Figure 4. The relative abundance of each phylum taken from each wetland. All phylum that did not make up more than one percent of the abundance in any season were combined into a category denoted as rare.

DOM Results

There were no significant differences in the fluorescence index between sites or seasons (Table 5 and 6). The freshness index was significantly higher in A1N than A2S (Table 5 and 6). NMR analysis revealed differences in DOM structure by site (MANOVA, p=0.01), but no significant differences in DOM structure by season (MANOVA, p=0.3, Figure 5).

Table 5. Results of Kruskal-Wallis comparing the effects of season and wetland on DOM fluorescence indices. Significant p-values <0.05 are bolded, p<0.001 are starred.

Soil Characteristic	Season		Site	
	F	p	F	p
Fluorescence index	χ^2_2=1.71	0.425	χ^2_2=2.21	0.329
Freshness index	χ^2_2=2.64	0.267	χ^2_2=11.26	**0.004**

Table 6. Fluorescence index and freshness index values expressed as averages ± standard error

	Fluorescence index	Freshness index
A1N		
Spring	1.49 ± 0.06	0.61 ± 0.02
Summer	1.63 ± 0.03	0.6 ± 0.01
Fall	1.54 ± 0.09	0.71 ± 0.04
A2S		
Spring	1.63 ± 0.06	0.57 ± 0.01
Summer	1.57 ± 0.03	0.59 ± 0.01
Fall	1.51 ± 0.03	0.57 ± 0.01
A3C		
Spring	1.55 ± 0.07	0.60 ± 0.03
Summer	-	-
Fall	1.56 ± 0.06	0.62 ± 0.03

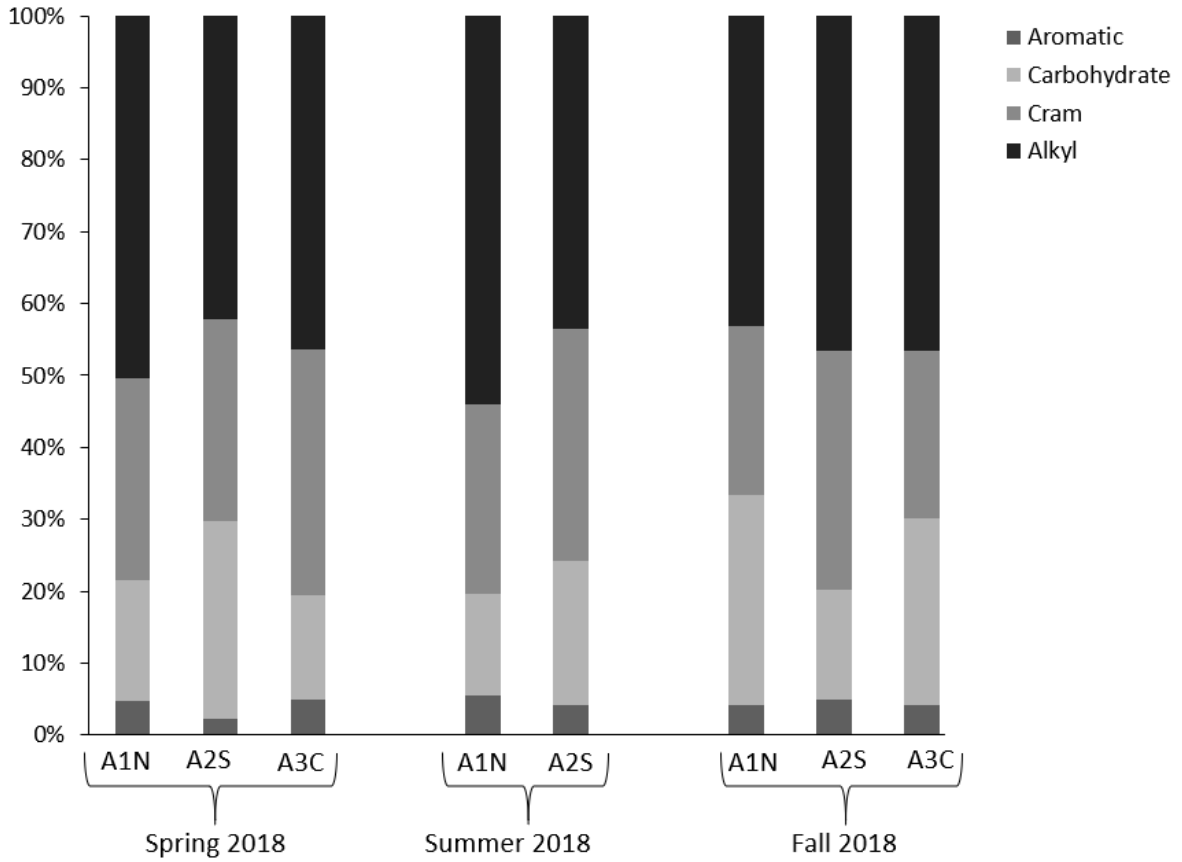

Figure 5. The relative proportions of structural components of DOM taken from A1N and A2S in fall of 2017, and DOM taken from each of the three created wetlands in spring of 2018, summer of 2018 and fall of 2018.

Potential Carbon Metabolism Rates

There were no significant differences in soil CO_2 or CH_4 production potential between the wetlands (Figure 6). However, CO_2 production potential was significantly higher in the summer than the spring ($p=0.007$) and the fall ($p=0.004$) and CH_4 production rates were significantly higher in the fall than the spring ($p<0.001$ Figure 6). The CH_4:CO_2 production rate ratio ranged from 0.76 mg C g soil^{-1} d^{-1} in A3C in the summer to 3.31 mg C g soil^{-1} d^{-1} in A1N in the fall and was significantly higher in fall than the spring ($p<0.001$) or summer ($p<0.001$).

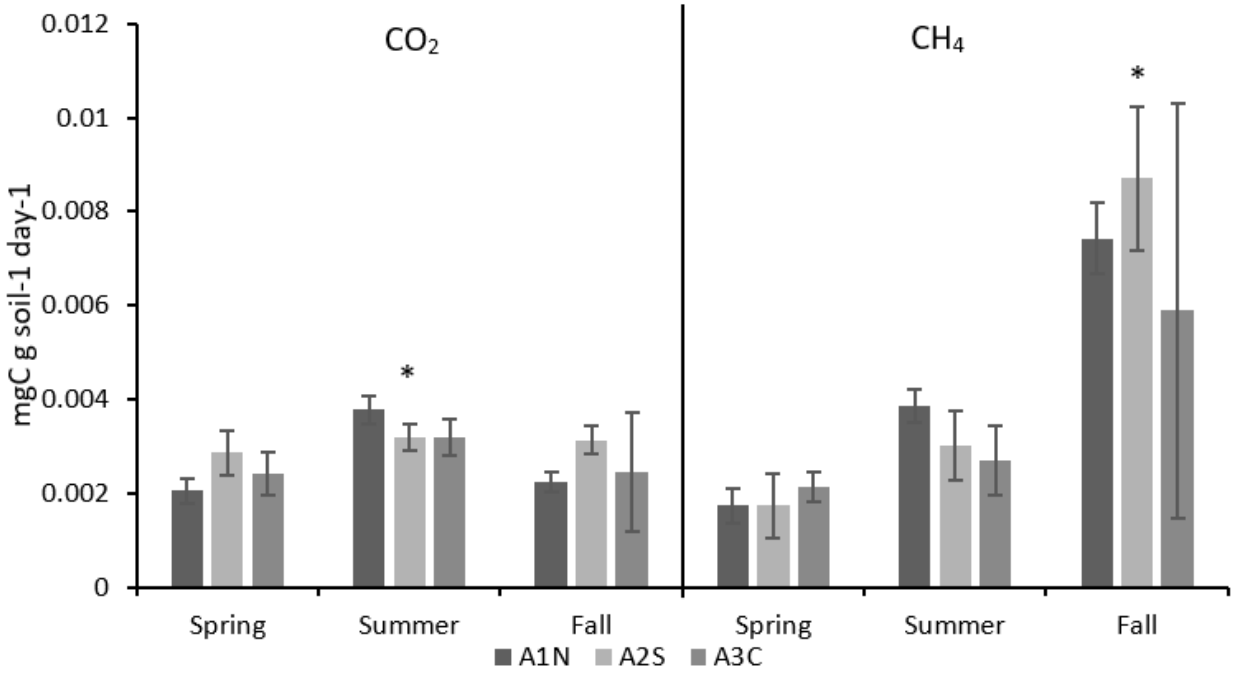

Figure 6. Soil CH_4 and CO_2 production potential values in the spring, summer, and fall. One star indicates a significant difference in production potential between seasons.

Impacts of invasive species control

Soil chemistry

There was a significant increase in soil organic matter content, and total soil phosphorus and a decrease in soil %C in the year following invasive species control through herbicide application (Table 7 and 8). There were also significant differences in the soil moisture content of the soil before and after herbicide application (Table 7 and 8). Soil extractable N, and soil C:N ratio were unchanged by invasive species control measures (Tables 7 and 8). Each soil chemistry parameter showed significant seasonal variability except for soil C:N, soil moisture content, and soil extractable N (Table 8).

Table 7. Soil physical chemistry factors before and after herbicide application in each season expressed as means ±standard error.

Soil Characteristics	Pre-Herbicide		Post-Herbicide	
	Spring 2017	Summer 2017	Spring 2018	Summer 2018
Organic matter (%)	12.01 ± 0.92	17.22 ± 0.63	15.22 ± 0.68	17.93 ± 1.08
Total Phosphorous (mg/kg)	715.67 ± 22.47	791.38 ± 26.67	883.06 ± 37.67	1033.43 ±34.8
Soil Extractable N (mg/kg)	14.25 ± 2.26	17.42 ± 2.01	32.40 ± 4.67	33.86 ± 5.64
Soil Moisture (%)	36.65 ± 1.80	42.66 ± 2.24	52.70 ± 1.81	44.71 ± 1.90
Carbon (%)	6.24 ± 0.47	7.40 ± 0.32	5.85 ± 0.46	6.15 ± 0.33
Nitrogen (%)	0.39 ± 0.03	0.49 ± 0.02	0.38 ± 0.03	0.40 ± 0.02
C:N Ratio	18.88 ± 0.54	17.87 ± 0.19	17.74 ± 0.19	17.88 ± 0.20

Table 8. Results of two-way ANOVA and Kruskal-Wallis comparing the effects of invasive species removal via herbicide application and season on soil chemistry characteristics in Area two south. Significant p-values of <0.05 are bolded, values of p<0.001 are bolded and starred.

Soil Characteristic	Season		Treatment		Season x Treatment	
	F, χ^2	p	F, χ^2	p	F	p
Organic matter (%)	$F_{1,30}$=18.4	**<0.001**	$F_{1,30}$=6.4	**0.018**	$F_{1,30}$=3.00	0.094
Total Phosphorous (mg/kg)	$F_{1,30}$=13.3	**0.001**	$F_{1,30}$=42.4	**<0.001***	$F_{1,30}$=1.1	0.315
Soil Extractable N (mg/kg)	$F_{1,30}$=0.51	0.48	$F_{1,30}$=17.8	**<0.001***	$F_{1,30}$=0.18	0.675
Soil Moisture (%)	$\chi^2_{1,30}$=1.7	0.192	$\chi^2_{1,30}$=11	**<0.001***	-	-
Carbon (%)	$F_{1,30}$=4.3	**0.046**	$F_{1,30}$=6.6	**0.030**	$F_{1,30}$=0.79	0.3793
Nitrogen (%)	$F_{1,30}$=4.5	**0.045**	$F_{1,30}$=3.2	0.086	$F_{1,30}$=1.46	0.238
C:N Ratio	$\chi^2_{1,30}$=0.6	0.429	$\chi^2_{1,30}$=1.1	0.286	-	-

Microbial community structure

The microbial community structure as described by the BrayCurtis dissimilarity index in A2S underwent a significant shift after herbicides were applied to control invasive species. There were also significant differences in the microbial community based on season and significant interactions between season and treatment (Table 9). The structure of the microbial community in the spring after invasive species control measures were used appears to be similar to the community structure in the summer before herbicide was applied, and the community structure appears to diverge further from initial conditions in the summer after herbicide application (Figure 7). There were significant changes in the Shannon diversity score related to both season and treatment (Table 9). The Shannon diversity score was significantly higher the year before herbicide was applied to control invasive species (before application 10.5 ± 0.08, after application 9.7 ± 0.23) and significantly higher in the spring than in the summer (spring 10.5 ± 0.07, summer 9.7 ± 0.21). The microbial community was dominated predominately by the phyla Proteobacteria, Bacteroidetes, Verrucomicrobia, and Chlorobi across years and seasons.

Qualitative analysis of the phyla present in the two years showed that after herbicide was applied to manage invasive species the relative abundance of organisms in the phyla of Chlorobi and OD1 were higher and the relative abundance of organisms in the phyla of Nitrospirae, Actinobacteria, Gemmatimonadetes, Firmicutes, and Spirochetes were lower (Figure 8).

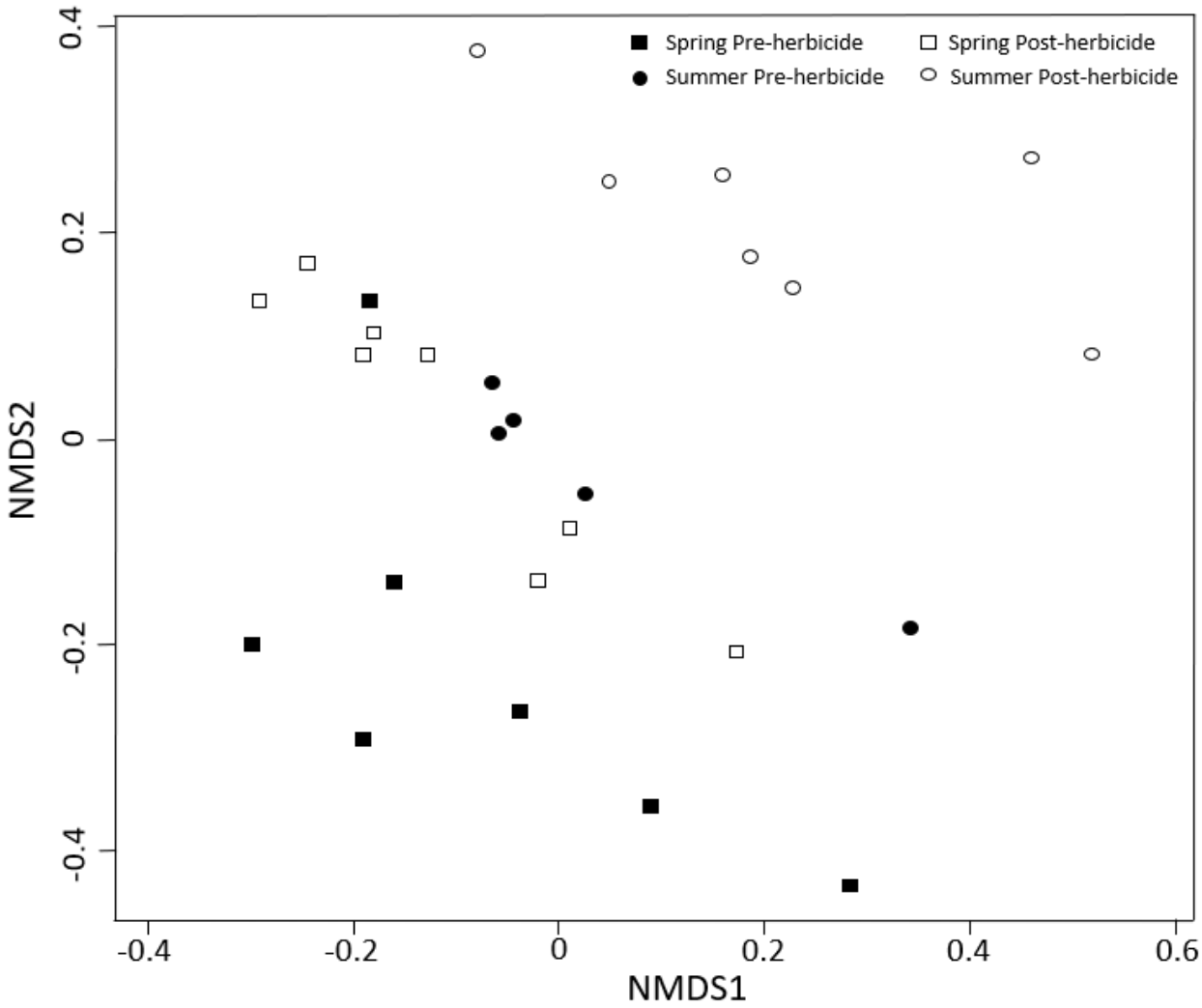

Figure 7. shows the NMDS ordination plot of microbial communities before and after herbicides were used to control invasive species cover. Stress values of less than 0.2 indicate an acceptable fit of the NMDS plot to the original Bray-Curtis values.

Table 9. Results of PERMANOVA and Kruskal-Wallis comparing the effects of treatment and season on microbial community structure and diversity. Significant p-values are bolded, p<0.001 are starred.

Characteristic	Season		Treatment		Season x Treatment	
	F, χ^2	p	F, χ^2	p	F	p
Community structure	$F_{1,27}$=1.9	**0.005**	$F_{1,27}$=2.6	**<0.001***	$F_{1,27}$=2.2	**0.001**
Shannon diversity	$\chi^2_{1,27}$=6.1	**0.01**	$\chi^2_{1,27}$=5.6	**0.02**	-	-

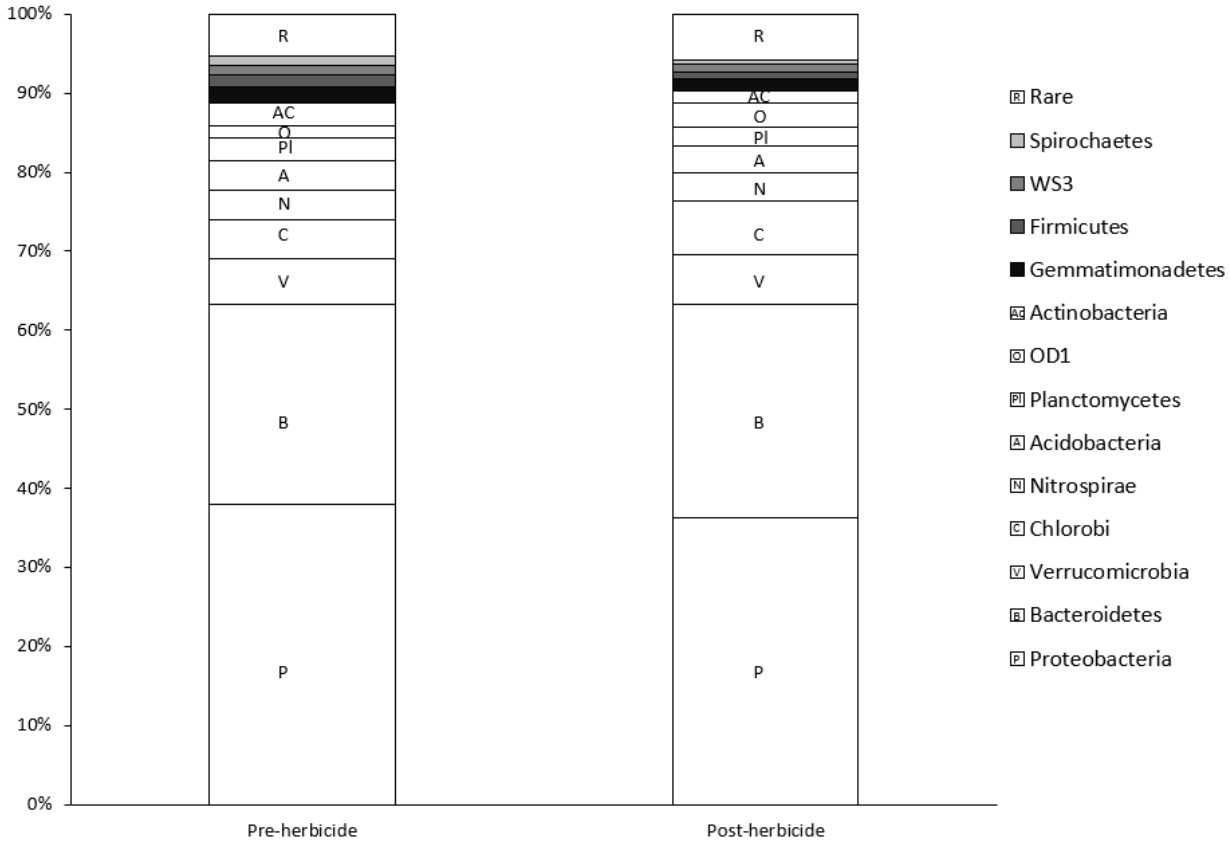

Figure 8. The relative abundance of each phylum taken from each wetland before and after herbicide was applied to control invasive species. All phylum that did not make up more than one percent of the abundance in any season were combined into a category denoted as rare.

Discussion

This study examined three created wetlands that had distinct soil chemistry, land use history, plant communities and hydrology and found that each of these wetlands had distinct microbial community composition, which correlated with soil chemistry but not carbon gas production potential. There were weaker, but significant, seasonal variations in microbial community composition in each wetland. Wetlands also had significant differences in DOM structure, which remained consistent throughout the seasons measured. The studied wetlands had similar carbon production potentials, with moderate production rates in the growing season followed by a spike in production potential in the fall. The similarity in carbon production potential between the wetlands despite differences in DOM structure suggests that the structure of DOM is not a key driver of rates of anaerobic carbon metabolism. The similarity in soil carbon production potential across sites despite the differences in microbial community structure suggest that there is a functional redundancy in the microbial communities. This study also suggests that control of invasive species using glyphosate herbicide can alter the composition of microbial communities, however, this component of the experiment was incidental and a more controlled experimental design is needed to confirm these patterns.

Wetland type

Soil chemistry

Soil chemistry regimes varied across the studied wetlands and these differences are likely related to differences in land-use history and hydrologic conditions of each wetland. The elevated organic matter and phosphorus content found in the sites with agricultural land use histories are consistent with global patterns of soil phosphorus legacies (MacDonald et al., 2012).

Agricultural enrichment of soil phosphorus tends to diminish over time (MacDonald et al., 2012), but the longevity of the elevated phosphorus levels found in this study shows that land use history can impact created wetlands for years. Past studies of these wetlands have found significant differences in soil extractable nitrogen (Lodge, 2017), however those differences were not observed in this study, suggesting that the impact of past agricultural land use on soil nitrogen levels is short lived relative to the impact on soil phosphorus levels. These results demonstrate the long term impact of land use history on soil nutrient levels in created wetlands, with agricultural land-use history resulting in higher nutrient levels. However, as time goes on these effects may become less pronounced. The effects of hydrologic conditions, particularly the ability to control water levels, permanently impacts soil moisture content.

Microbial community

Each of the studied wetlands had a distinct microbial community, however, the permanently flooded wetland (A1N) was substantially different from the two seasonally flooded wetlands (A2S and A3C). There were significant correlations between microbial community structure and soil chemistry, including moisture content, organic matter content, total phosphorus, % C, % N and C:N ratio. This reinforces the findings of other studies that showed that soil chemistry is a key driver of microbial community structure (Ahn & Peralta, 2009; Ansola et al., 2014; Arroyo et al., 2015; Foulquier et al., 2013). The divergence of A1N from the other wetlands in the study does not appear to be fully explained by the differences in soil chemistry and is likely due to it being permanently flooded whereas the other studied wetlands were only seasonally inundated. Divergence due to soil dryness would be consistent with other studies that have shown that even temporary soil dryness can lead to long term shifts in wetland

microbial communities (Fierer, et al. 2003; Foulquier et al., 2013). These shifts in the microbial community may be related to changes in the plant communities induced by the seasonal drying, or may be due to the introduction of atmospheric oxygen into the soil.

DOM and Carbon Metabolism

The DOM in each of the studied wetlands, as measured by the fluorescence index (Cory & McKnight, 2005), was derived approximately evenly from terrestrial and microbial sources and the source did not vary seasonally. This suggests that the differences in plant communities and potential differences in runoff patterns into each of the wetlands did not strongly influence DOM composition. These wetlands likely have similar inputs of DOM from runoff due to their geographic proximity; potential regional differences in DOM composition in runoff merits more study. There were differences in the freshness index between wetlands, indicating different degrees of degradation of the DOM (Wilson & Xenopoulos, 2009). These changes in DOM freshness did not correlate with differences in carbon production potential suggesting that the degree of degradation of DOM is not driving this portion of microbial community function in created wetlands. Differences in DOM structure between wetlands were likely related to the differences in vegetation and past land use between each of the wetlands as shown by previous studies (Barber et al., 2001; Graeber et al., 2012). However, there were no significant seasonal changes in DOM structure. This contradicts previous studies of stream systems that found changes in the chemical composition of DOM seasonally (Neff et al., 2006), but supports the finding of other studies that found that DOM structure does not shift seasonally (Graeber et al., 2012). Despite the lack of seasonal changes in DOM structure, there were seasonal changes in the microbial community structure and carbon production potential, suggesting that factors other

than DOM structure are driving community structure and carbon production in created wetlands. It is possible that non-structural components of DOM such as molecular weight are more important to microbial utilization and breakdown rates (Amon & Benner, 1996).

There were no differences in greenhouse gas production potential between wetlands, however, there was a significant increase in CO_2 production potential in the summer and CH_4 production potential in the fall. This increase contradicts the findings of previous studies in ponds that showed higher CH_4 production potential in the spring than the summer or fall (Stadmark & Leonardson, 2007). However it is consistent with field studies that show an increase in methane emissions in the fall in wetland systems (Liikanen et al., 2006; Xu et al., 2014). The increase in methane production in the fall is likely due to an influx in labile organic matter due to plant senescence. It is unclear why the CO_2 production potential was higher in the summer and this phenomenon merits more study. The similarity in greenhouse gas production potential between the wetlands was surprising and contradicts the results of other studies. Previous studies have shown significant differences in CO_2 and CH_4 production potential due to differences in plant communities (Inglett, et al., 2012), but this was not observed in the studied wetlands, despite stark differences in plant community composition. Previous studies have also found positive correlations between CO_2 production potential and soil organic matter content (Y. He et al., 2016), but the observed differences in organic content between our wetlands did not yield corresponding changes in carbon gas production rates.

Hydrology is also an important driver of greenhouse gas production in created wetlands (Mander et al., 2011), however, the incubation approach used in this study did not allow for the direct testing of the effects of water levels or soil moisture on greenhouse gas production. I was able to examine the potential of the soil microbial communities to produce greenhouse gasses

under ideal conditions. My findings that the differences in microbial community structures do not correlate with the soil greenhouse gas production potential run counter to some studies that have found a significant correlation between the structure and diversity of microbial communities with microbial activities (Foulquier et al., 2013; S. He et al., 2015; Louis et al., 2016). However the link between the structure of microbial communities and organic matter decomposition is not conclusive (Schimel & Schaeffer, 2012), and these results suggest that there is a degree of functional redundancy in the microbial community regarding carbon production in created wetlands.

Invasive species control

The application of herbicide to control invasive species in the early fall of 2017 caused a shift in the plant community from a near monoculture of *Phalaris arundinacea* (Reed Canary Grass) in the spring and summer of 2017 to a near vegetation free wetland in the spring and summer of 2018. Our examination of the microbial community in the wetland over two years showed a significant shift in the microbial community both seasonally and between years, potentially due to the effects of invasive species control measures used at the site. This supports findings in previous studies that show significant differences in wetland microbial communities in areas with invasive species (Angeloni et. al., 2006). Seasonal measurements showed that as time went on the shifts in the microbial community appeared to become more pronounced. The microbial community in the spring after the herbicide application resembled the microbial community of the summer before the herbicide application. However, in measurements of the microbial community in the summer after the herbicide application the microbial community strongly diverged from any pre-herbicide measurements. This suggests that the changes in the

microbial community in response to vegetation die-off associated with herbicide application may require time to fully develop. Alternatively, this shift could be due to other environmental changes not quantified in this study and the lack of a herbicide control, due to the fact that management decisions were outside of the control of this study, limits the ability to draw direct conclusions about the impacts of invasive species control.

Our study also found that there was a significant decrease in microbial diversity in the year following the use of herbicides to control invasive species, contrasting with studies of agricultural soils that found no significant effect of herbicide application on microbial diversity (Lupwayi et al., 2004). It is possible that the changes we observed are due to other environmental changes not quantified in this study; however, changes in plant community composition have been shown to alter microbial community structure in past studies (Angeloni et. al., 2006). Our results are similar to previous studies that found long term shifts in microbial communities on agricultural fields that have had herbicide applied over several years (Seghers et al., 2003) and also align with findings of studies that found a shift in microbial communities over short time periods of time, twenty and thirty days, due to a single herbicide application in agricultural (Sebiomo et al., 2011) and forested ecosystems (Ratcliff et al., 2006).

Alternatively, the long-term shift in soil microbial communities observed in this study may not be due to direct effects of the herbicide but to the downstream changes in the plant community and soil nutrients following invasive species eradication efforts. Vegetated and unvegetated areas of wetlands have been shown to have different microbial community compositions (Arroyo et al., 2015), suggesting that our observed shift in microbial community structure may be due in part to the removal of the plant community rather than direct effects of the glyphosate herbicide. Replacing invasive species with native species also results in changes

to the soil microbial community (Kourtev et al., 2003), suggesting that the microbial community could shift further as native plants begin to grow in the wetland. The application of herbicide was also followed by significant increases in soil organic matter and soil phosphorus, likely due to the addition of biomass to the system following plant die-off from herbicides as well as due to the herbicide itself. Numerous studies have noted correlations between soil chemistry and microbial community structure (Ahn & Peralta, 2009; Arroyo et al., 2015; Lee et al., 2019), suggesting that the changes in soil chemistry as a result of invasive species control may be in part responsible for some of the long term changes in the microbial community. The addition of fresh plant litter in particular has been shown to drive shifts in microbial community composition (Yan et al., 2018).

Herbicide is commonly used as a management tool to control invasive plant species in wetlands, but the impacts of this and other invasive species control measures on the underlying microbial communities is not well understood. The development of more advanced microbial ecology and bioinformatics tools and techniques has opened the door to further study of the impacts of herbicide on soil microbial communities (Jacobsen & Hjelmsø, 2014). Further study is merited to disentangle the direct impact of herbicides, from vegetation change and plant litter inputs associated with invasive species control, on microbial communities and ecosystem functions in created wetlands.

Broader Impacts/Conclusions

Differences in land use history, management practices, and hydrologic conditions in created wetlands can have impacts on the underlying soil chemistry and microbial communities in wetlands. Differences in land use history across the three created wetlands appeared to

contribute to distinct soil chemistry regimes. Agricultural land use history was correlated with increased soil nutrient levels in these wetlands. Elevated nutrient levels should be expected when wetlands are created on agricultural lands and are consistent with global patterns (MacDonald et al., 2012). Differences in soil nutrient levels were significantly correlated with differences in the microbial communities, however hydrology and soil water content appeared to be the strongest driver of microbial community composition. Despite the observed differences in microbial community structure there were no differences in anaerobic CO_2 or CH_4 production potential in these wetlands, indicating that there is redundancy in wetland microbial communities relating to carbon metabolism. This redundancy suggests that changes in microbial communities leading to a change in greenhouse gas (GHG) production potential may not be an important consideration in the design and management of created wetlands. There were also no apparent differences of GHG production potential in relation to differences in DOM structure between the wetlands suggesting that this should not be a significant consideration when trying to mitigate GHG emissions during wetland construction.

This study also found that invasive species control using herbicides to eradicate vegetation may lead to changes in soil chemistry and microbial community composition of a created wetland. In the year following invasive species control, there was a substantial shift in the plant community as well as an increase in soil organic matter and soil phosphorus. These changes likely contributed to the observed changes in the microbial community. These results show that the long-term impacts on microbial communities and function should be considered when making decisions about how to control invasive species in wetlands.

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