Wetland Plant Evolutionary History Influences Soil and Endophyte Microbial Community Composition

SWS Research Grant – Final Report

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BACKGROUND

Gaseous methane absorbs approximately twenty-seven times more heat than carbon-dioxide over hundred-year intervals (Boucher et al. 2009; Christensen et al. 2003), making it a potent and important greenhouse gas which contributes to climate change (Cao et al. 1998). Wetland ecosystems are responsible for 20-39% of global methane emissions (IPCC 2007). Methane emission rates differ among wetlands (Barlett et al. 1989), and this variability can be partially explained by differences in plant composition (Grünfeld and Brix 1999) and activity (Wang and Han 2005). While plants influence methane emissions, they do not directly produce methane in wetlands. Microbes known as methanogens, which grow in highly reduced, submerged soils, primarily produce methane (Grünfeld and Brix 1999; Laanbroek 2010). Methanogens only persist in anoxic conditions where organic matter is available for decomposition (Zeikus 1977). By reducing labile carbon compounds, chiefly acetate, methanogens can produce energy in environments where other microbes cannot survive (Jetten et al. 1992; Karakashev et al. 2006).

Plants contribute to methane production and emissions (Grunfeld and Brix 1999, Chanton et al. 1989) by providing substrates for microbial decomposition and an accelerated medium for gas diffusion between the atmosphere and soils (Denier van der Gon and Neue 1995; Kludze et al. 1993). Senesced shoot tissues differ chemically and structurally between plant species (Davis and van der Valk 1977), which likely influence microbial communities, including methane-utilizing microbes. Root exudates additionally differ between species (Bais et al. 2010) and influence soil microbial communities (Bridgham et al. 2013; Gagnon et al. 2007). These provide additional substrates which wetland microbes can metabolically utilize, and the products from microbial activity become increasingly reduced as oxygen availability decreases (Laanbroek 2010). Organic matter and low molecular-weight carbon compounds in the most reduced soil layers are metabolized to methane, which largely accumulates in flooded, stagnant soils (Tokida et al. 2005).

Flooded wetland soils which accumulate methane are typically anoxic and restrict gas flow (Cao et al. 1996; Laanbroek 2010), so ebullition in unvegetated soils presents the most effective means of gas diffusion from wetland soils (Fechner-Levy and Hemond 1996). In vegetated soils however, specialized porous plant tissues known as aerenchyma provide an alternative medium which allows for faster diffusion of gases such as methane and oxygen (Brix et al. 1992). The diffusion of oxygen into rhizosphere soils immediately adjacent to plant roots alters the soil's redox state (Colmer 2003) and allows different microbes to compete in otherwise anoxic conditions (Laanbroek 2010). By providing oxygen to adjacent soils, wetland plants can support microbes, such as nitrifying and methane-consuming, or methanotrophic, bacteria (Bodelier and Frenzel 1999; Ibekwe et al. 2003), in soils where they typically could not survive. Plant species differ in gas flow rates through aerenchyma (Brix et al. 1992, Konnerup et al. 2010); higher oxygen flow and subsequent oxygen availability will likely alter plant-associated wetland microbial communities since they are partitioned according to redox status.

The combination of substrate provision and soil oxidation influences wetland microbial communities and subsequently methane emissions (Bridgham et al. 2013; Laanbroek 2010). Plants in upland systems contain different microbes in their tissues and adjacent soil (Winston et al. 2014; Zarraonaindia et al. 2015), and different plants associate with different microbial communities (Kourtev et al. 2002; Westover et al. 1997). Closely related plant species may have more similar biochemistry (Giannasi 1978) and gas flow rates through aerenchyma in certain instances (Brix et al. 1992), so more closely related plant species may have more similar microbial communities and potentially methane emissions.

Do more closely related plants share more similar microbial communities overall and, more specifically, quantities of methanogens and methanotrophs? I anticipated that, owing to probable similarities in biochemistry and gas flow rates between related plants, microbial communities will differ according to plant species, with more similar microbial communities associating together with more closely related plants. I further hypothesized that both methanogen and methanotroph population sizes of each plant species will be more similar according to the relatedness of plant species.

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MATERIALS AND METHODS

Five large, rhizomatous, monoculture-forming wetland plants in the order Poales were selected for study. Two hydrologically connected wetland sites housed these five species. Two grasses, (*Phalaris arundinacea* and *Phragmites australis*), one sedge, (*Bolboschoenus fluviatilis*), one cattail (*Typha x glauca*), and one bur-reed (*Sparganium eurycarpym*) were selected: the grasses belong to the family Poaceae and are related to *Bolboschoenus* in the sedge family Cyperaceae while the cattail and bur-reed reside within a more distantly related family, Typhaceae. *Bolboschoenus, Phragmites,* and *Typha* dominated various regions of wetland one (Figure 1); *Phalaris* and *Sparganium* occupied wetland two (Figure 2).

Unvegetated soils were selected from each wetland site, over one meter away from any discernable plant shoots. Three soil samples between within fifty centimeters from a plant shoot were taken for each plant species, and three rhizosphere soil samples were gathered by placing soil attached directly to roots in polypropylene tubes. Three root and leaf tissues per plant species were thoroughly rinsed under tap water to remove soil before chopping into small pieces and inserting in tubes for surface sterilization. A series of three solutions (70% ethanol, 10% bleach, and 90% ethanol) were used to surface sterilize tissues so that only endophyte DNA remained.

The soils and surface sterilized tissue samples were extracted using a Qiagen PowerSoil DNA Kit according to the protocol included. Polymerase chain reactions (PCR) of the prokaryotic ribosomal 16S region using the 515F/806R primers and PNA clamps, to avoid amplification of eukaryotic mitochondria and plastid DNA in root and leaf samples, produced viable amplified DNA for sequencing (Caporaso et al. 2011; Caporaso et al. 2012; Lundberg et al. 2013). Sequencing was performed on the Illumina Mi-Seq platform.

To quantify the abundance of methanotrophs and methanogens in these samples, we ran qPCR on triplicate subsamples for their respective genes: *pmoA* (Bourne and McDonald 2001) and *mcrA* (Steinberg and Regen 2009). Averages and standard deviations amongst sample types and plant species were calculated, and statistical tests evaluated the data for significance.

Differences in microbial community composition were evaluated by Anosim and permanova analyses and visualized using non-metric multidimensional scaling (NMDS) plots using the vegan package in R (Team 2013). A maximum parsimony phylogeny of the host plants was developed using internal transcribed spacer sequences from GenBank, and branch lengths between each taxon were recorded. Mantel tests were used to compare differences in microbial community composition with plant phylogenetic distance.

FIGURE 1. Wetland One in late July, after samples were collected. *Bolboschoenus fluviatilis* in the foreground, *Phragmites australis* further back, and *Typha x glauca* in the distance. *Lemna* were present at this site, which appears to have once contained trees which rotted and died many years before. A prairie of *Agrimonia, Asclepias, Euthamia* (*E. graminifolia, E. gymnospermoides*), and *Solidago* grew to the northeast while *Phalaris arundinacea* occupied the southwestern and drier border.

FIGURE 2. Dr. Pamela Weisenhorn (advisor) measuring water pH, voltage, and temperature in a *Sparganium eurycarpum* stand at Wetland Two.

FIGURE 3. NMDS ordination plots of all microbial samples. All samples colored to denote sample type and plant species respectively. Adonis tests indicated that type accounted for 52% of the variation, species for 18%, and site for 7% (all significant, p=0.001).

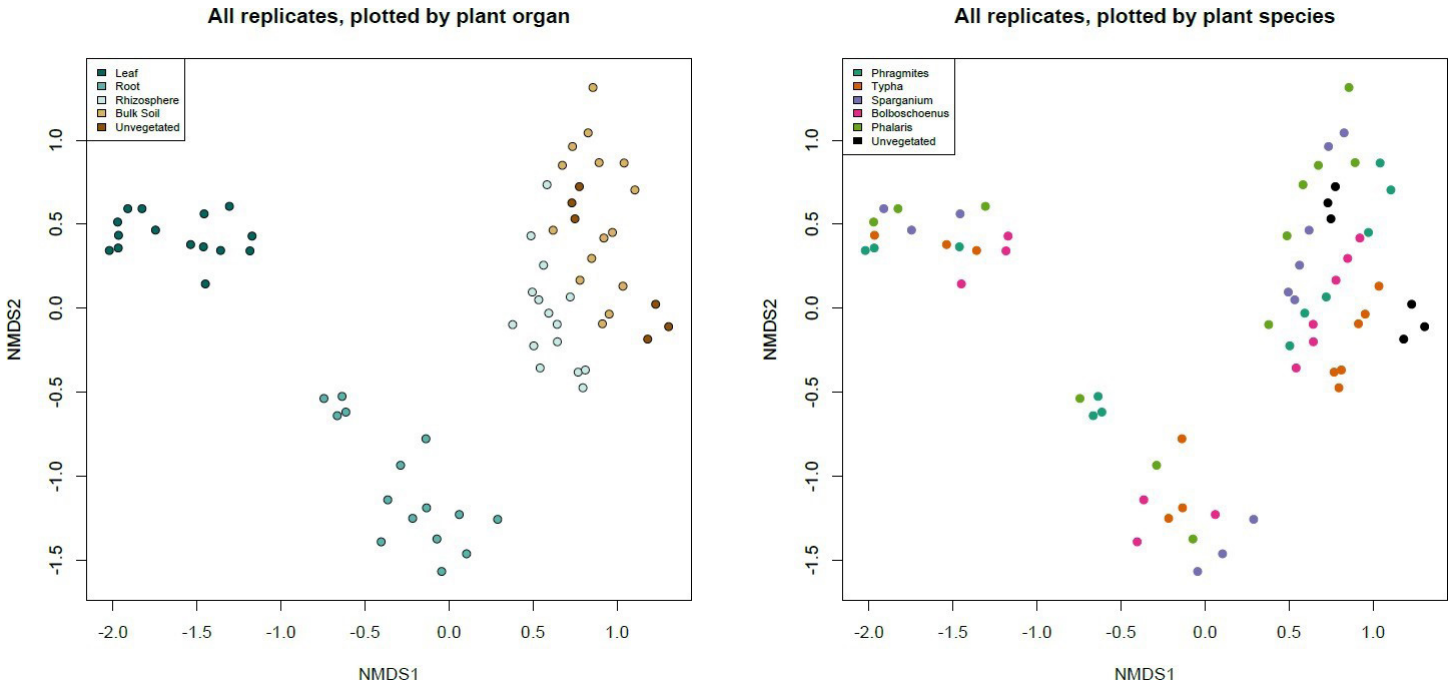


FIGURE 4. NMDS ordination plots of bulk soil samples, colored to denote wetland site and plant species respectively. Site and species accounted for 37% and 76% of the variation observed, respectively (both significant, p=0.001)

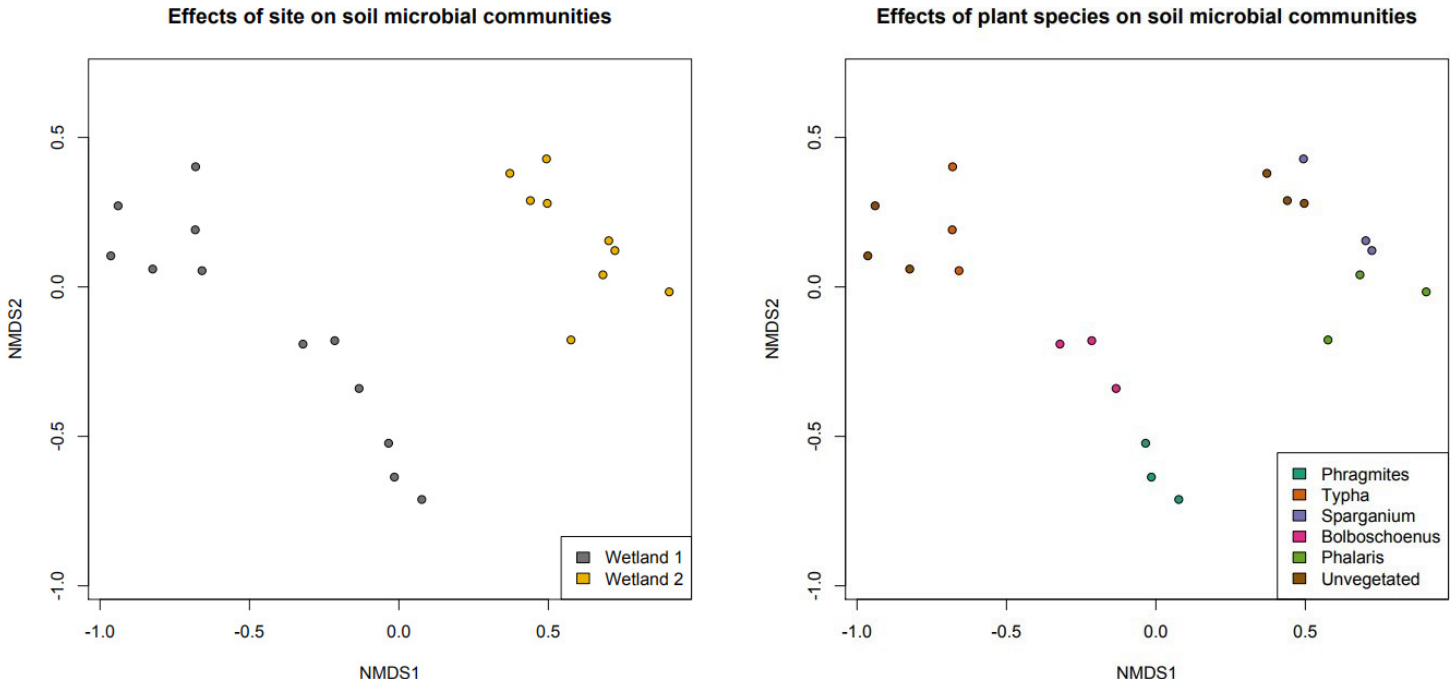


TABLE 1. Anosim and permanova tests evaluated if significant differences between sample types existed. Mantel tests take evolutionary distance between plant hosts (Figure 5) into account.

	Anosim p	Anosim R	Permanova p	Permanova r^2	Mantel p	Mantel r^2
Bulk	0.001	0.8919	0.001	0.722	0.001	0.4034
Rhizosphere	0.001	0.8222	0.001	0.624	0.002	0.4034
Root	0.002	0.6459	0.001	0.542	0.001	0.5345
Leaf	0.001	0.4296	0.001	0.518	0.007	0.2022

RESULTS

Taxa were assigned based on similarity to sequences provided in the microbial database

GreenGenes. Entire communities from each sample were compared to evaluate wholistic differences and determine which factors primarily influenced community compositions. Sample type (soils or plant tissues) influenced community differences more than plant species or wetland site (Figure 3); plant species explained most of the variation observed within each sample type, particularly soils (Figure 4). Each sample type differed significantly from one another according to Anosim and permanova tests, and phylogenetic distance of plant hosts correlated with phylogenetic distance of the plant hosts according to Mantel tests (Table 1; Figure 5). Rhizosphere samples were the most diverse, followed by bulk soil samples; endophytic communities were less diverse, with leaves displaying the lowest diversity of any sample type. The ten most abundant microbial orders are displayed in Figure 6 to help visualize across sample differences.

Amplification of the *pmoA* gene via qPCR and subsequent statistical analyses calculated that methanotroph population sizes did not differ substantially between plant species when considering all sample types together. Plant species and sample type had an interactive effect ($p=0.0016$, $F=4.3$) on population sizes, estimated by the *pmoA* gene. Methanotrophs were the most abundant in root samples and least abundant in leaf tissues across each plant species tested (Figure 7). *Typha* and *Sparganium* respectively contained fifteen and nine times more methanotrophs in their roots than unvegetated soils while *Bolboschoenus, Phalaris,* and *Phragmites* contained three times more than unvegetated soils.

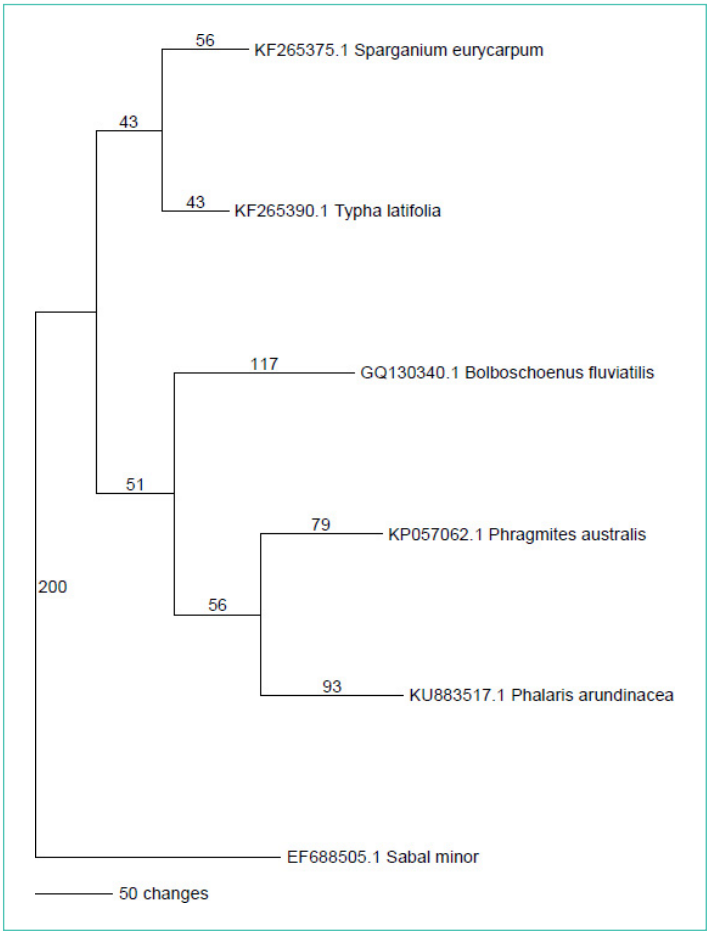
Unlike methanotroph population sizes, methanogen populations were almost always smaller than the average unvegetated soil populations from both localities based on amplification of the *mcrA*. Plant species and plant part had a significant interactive effect on methanogen population sizes ($p=0.02$, $F=2.3$). Methanogens were the most abundant in rhizosphere samples and least abundant in root and leaf tissues across each plant species tested (Figure 7). *Typha* bulk soil, *Bolboschoenus* rhizosphere soil, and *Sparganium* rhizospheres soil were the only samples to contain more methanogens than unvegetated soils.

DISCUSSION

Sample type was the single largest influence on microbial communities as noted in the NMDS ordination plots, Anosim, and permanova tests. Plants species influenced bulk soil communities, although the influence of plants upon their microbial communities at the order level overall decreased from bulk to rhizosphere to root to leaf samples. Microbial alpha diversity was higher in soils than plant tissues, with rhizosphere soils and leaf tissues representing the most and least diverse habitats respectively.

Organic matter accumulates in anoxic, wetland bulk soils and may become thermodynamically difficult to degrade (Tanner and Sukias 1995), requiring particular syntrophic microbes to decompose (McInnery et al. 2009). While specific, cooperative microbial interactions exist which extract energy from organic matter, the anoxic conditions within bulk soil limit total microbial diversity. The wide array of organic matter at various states of decomposition within bulk soils allow many anaerobic microbes to survive, but bulk soils are likely less diverse than rhizosphere soils since the latter contain greater redox heterogeneity. Microbes in rhizosphere soils are additionally exposed to root exudates rather than organic matter derived from senesced shoots in bulk soil. The presence of low molecular-weight, labile chemicals deposited to the substrate likely provide more easily metabolized com-

FIGURE 5. Evolutionary relationships between the five plants sampled, all within the order Poales with Sabal minor (Arecales) as an outgroup. Most parsimonious tree found from 10,000 trees of the internal transcribed spacer (ITS) region from GenBank.



pounds for generalist microbes to utilize, likely increasing microbial community diversity.

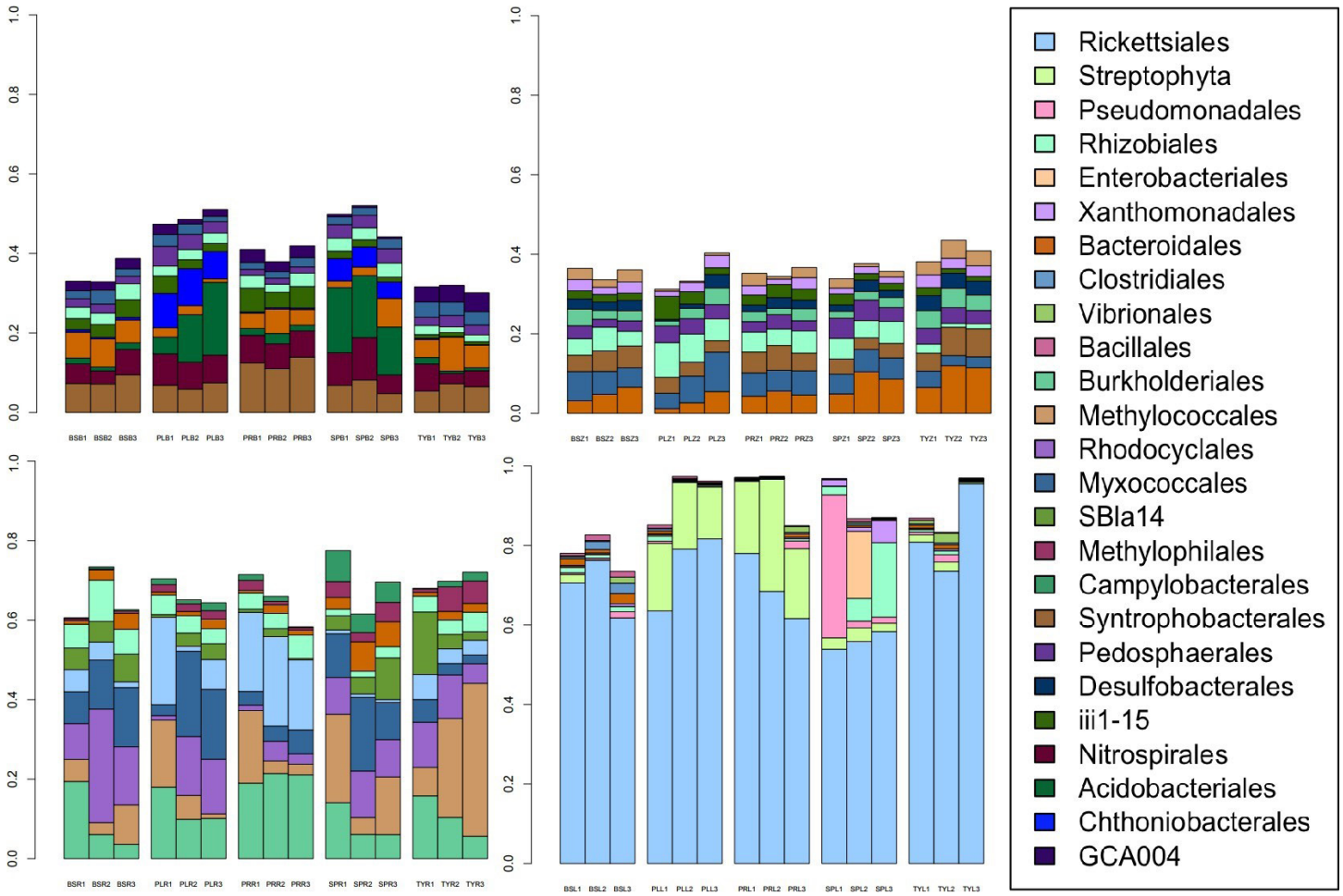
Endophytes have immediate access to additional substrates, but they must also avoid destruction by their host's immune system. Subsequently, less diversity would be expected, and were found in these data, within plant tissues. Endophytes also likely differ from soil-associated microbes since root and leaf tissues contain high oxygen concentrations even though root endophytes are surrounded by anoxic soils in wetlands. The large disparity in redox states between rhizosphere soils and root tissues indicates that the microbes may not be able to survive in both environments. Leaf tissues present additional light, heat, redox, and water stresses in addition to host defenses. Fewer microbes, including members of the orders Rickettsiales, would be able to tolerate these environments, hence showing decreased diversity but increased abundance. Furthermore, particular microbes appeared to specialize in certain plant parts and species. A cyanobacterial order was prominently found within grass (*Phalaris* and *Phragmites*) roots and particularly leaves, the latter cases being the only instances of a non-Rickettsiales order being consistently above 15% abundance in leaf tissues and may represent a shared evolutionary symbiosis within Poaceae.

Methanogen population sizes, as measured by the *mcrA* gene, were largest across most species in redox-heterogeneous rhizosphere soils or, in the case of *Typha,* bulk soils. Methanogen population sizes were the smallest in plant tissues. This indicates that anaerobic pockets in rhizosphere soils exist for methanogens to survive. (Hackstein 2010). Conversely, the oxidized state of plant tissues likely prevents methanogens from surviving, explaining why they were almost exclusively confined to soils.

Higher abundances in rhizospheres may indicate that methanogens grow most successfully in proximity to root exudates. Additionally, these microbes' metabolic product, methane, is quickly removed from their immediate environments, making methanogenesis more energetically favorable.

Methanotroph population sizes, as measured by the *pmoA* gene, were largest across all species in roots where oxygen is readily available and smallest in bulk soils and leaf tissues. Each plant species contained more methano-

FIGURE 6. The top ten most abundant microbial orders found amongst samples.



trophs in their roots than in any soils samples, indicating that the plants allow these microbes to grow as endophytes. The endophytes, in return, likely turn methane into carbon dioxide, which the plants can then use for photosynthesis. This would explain the exceptionally high carbon dioxide concentrations found within *Typha* leaves at night, (Constable et al. 1992) and may improve *Typha*'s photosynthetic efficiency and its subsequent ability to compete with other plants. The variation amongst methanotrophs within roots were far higher than initially expected, especially given the static nature of methanogen populations in the data presented. Methanotroph population sizes were particularly large for both members of Typhaceae (*Sparganium* and *Typha*).

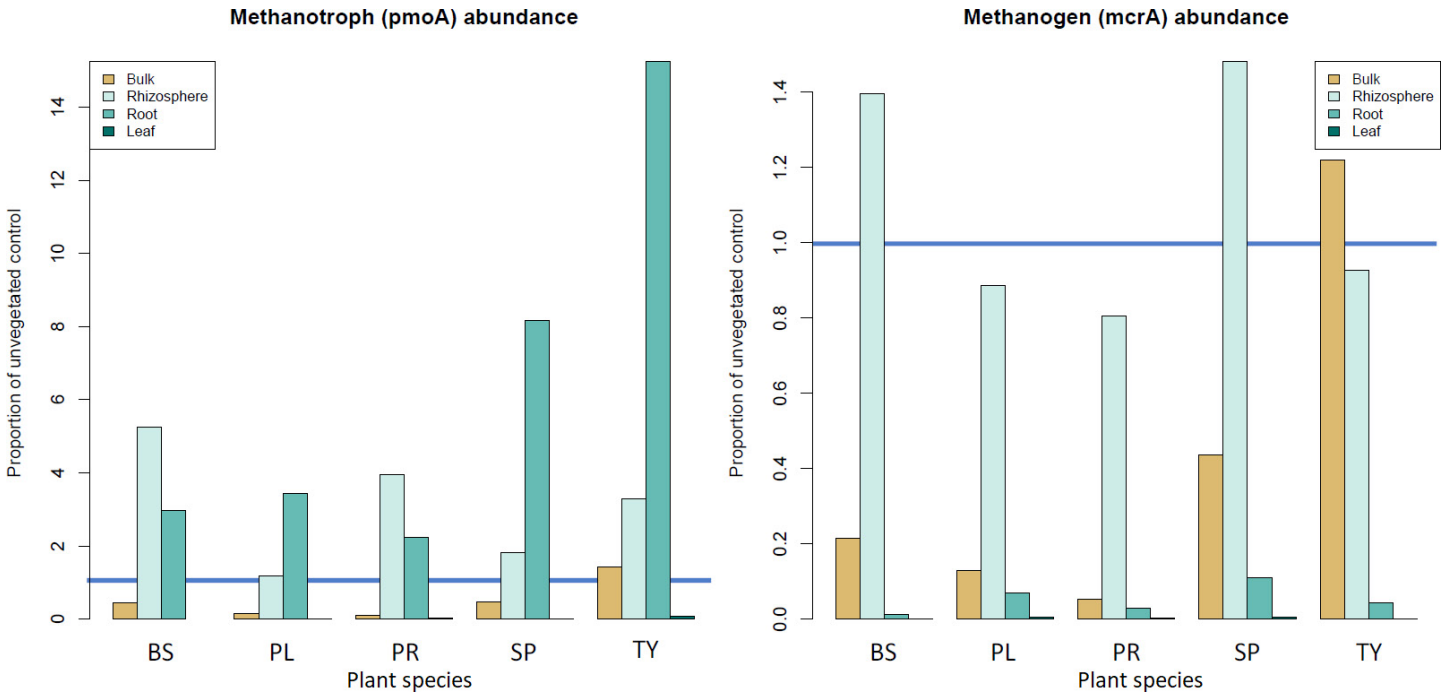
CONCLUSION

The data here corroborate past findings that sample type (leaf tissue, root tissue, rhizosphere soil, bulk soil) had the largest effect on microbial communities according to Anosim and permanova tests. Plant species had the second strongest influence on microbial communities overall. Mantel tests determined that differences between microbial communities correlated with differences in evolutionary history. Methanogen population sizes were the largest in rhizosphere samples while methanotrophs were most abundant in root samples overall. The high number of methanotrophs in Typhaceae, especially *Typha,* roots may partially explain the high carbon dioxide concentrations found in *Typha* leaves overnight from previous research (Constable et al. 1992). This would additionally indicate that *Sparganium* and *Typha* species likely emit less methane than other dominant wetland plants, such as *Bolboschoenus* or *Phragmites.*

FUTURE DIRECTIONS

The current sampling provides a limited perspective on the co-evolution between wetland plants and their microbial symbionts; an expanded sampling may help determine the influence specific soils and plant hosts have upon microbial communities. While *Typha,* and *Sparganium* to a lesser degree, in this study harbored vast numbers of methanotrophs within their root tissues, the extent of this trend in other Typhaceae species and wetland habitats remains unknown. Given the prevalent, nearly cosmopolitan nature of each species investigated, their influence upon microbial communities and subsequently global methane emissions may be substantial. *Typha* species are potentially more effective at reducing greenhouse emissions than their competitors, although additional work in additional habitats and with other species will be necessary to confirm this notion. If such is true, *Typha x glauca* and other cattail species may be a preferable wetland macrophyte rather than *Phragmites australis* or other competitors. These data are currently being synthesized into a publication for the journal *Phytobiomes*. ■

FIGURE 7. Results from qPCR of methanotroph pmoA gene (left) and methanogen mcrA gene (right). Blue bar indicates the average amount of methanotrophs or methanogens found in the unvegetated soils.



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