BIOTIC AND ABIOTIC FACTORS AFFECTING THE SURVIVAL OF *LISTERIA MONOCYTOGENES* IN PRAIRIE POTHOLE SOILS AND SEDIMENTS

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Title

Biotic and abiotic factors affecting the survival of Listeria monocytogenes in prairie pothole soils and sediments

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The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

MASTER OF SCIENCE

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ABSTRACT

The diversity-invasion relationship states that more diverse communities are more resistant to invasion. *Listeria monocytogenes* – a gram-positive facultative anaerobe, soil saprotroph, and opportunistic human pathogen – is capable of surviving in a diverse range of habitats, including soil, and several recent studies have shown that the prevalence of *L. monocytogenes* in soil increases with proximity to surface water. In addition, *L. monocytogenes* resides frequently in the guts of ruminants and poultry, creating many opportunities for deposition in soil. However, little work has been done to examine the effects of native soil microbiota on the survival of the pathogen. This thesis builds on previous work by examining microbial community diversity in the prairie pothole ecosystem and how it impacts the survival of *L. monocytogenes*. Results indicate that survival of *L. monocytogenes* does not seem to differ greatly as an effect of community diversity.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1. LITERATURE REVIEW

The diversity-invasion relationship

The diversity-invasion relationship, first described by Charles Elton over 50 years ago, was the name given to the observation that more diverse communities tend to be better at resisting colonization by invasive species (Elton, 1958). The leading explanation for this phenomenon is that more diverse communities are more efficient at exploiting resources, leaving less niche space for invaders to colonize (Falcao Salles et al., 2015; Tilman, 2004). A study by van Elsas and colleagues demonstrated a diversity-invasion relationship between *Escherichia coli* and soil microbial communities of varying diversity (van Elsas et al., 2012); community diversity was manipulated by serially diluting native soils in autoclaved soils, resulting in successively diminished native microbiota. *E. coli* inoculated into less diluted (more diverse) soils died faster than in less diverse treatments.

Another study conducted by Salles and colleagues looked specifically at the effect of resource pulses on the ability of communities to resist invasion (Falcao Salles et al., 2015). If efficiency of resource exploitation is the key driver of the diversity-invasion relationship, then resource pulses, even in very diverse communities, should free up extra niche space for invaders. In their study, Salles et al. found that *E. coli* survival was negatively correlated with community species richness. But upon the introduction of a resource pulse (D-Galactose), even in the most diverse treatment where *E. coli* had declined in density to nearly undetectable levels, the increased resources strongly favored the proliferation of the pathogen. In the 5-, 15-, and 30-species communities where D-Galactose was added, *E. coli* abundance rebounded to 10^4-10^5 CFU/mL. The same uptick was not observed in the control communities. This result provides

support for the hypothesis that resource availability is what drives the diversity-invasion relationship.

From the two studies mentioned above, it would appear that *E. coli*, when treated as an invasive species in soil, exhibits a diversity-invasion relationship. However, community diversity is only one possible factor among several thought to affect invasion success. As with many different areas of ecology, more work has been done in studying the invasion ecology of animals and plants than has been done studying microbes. But in regards to invasive plant species, key factors influencing invasion success include wide dispersal over short and long distances, frequency and density of invasion events (propagule pressure), a generalist physiology that allows for adaptation to many different environments, and the ability to grow quickly and outcompete native flora (Dukes & Theoharides, 2007). Native community diversity, then, may not be the most important factor, or even a significant factor, in every invader-ecosystem relationship.

A 2015 study by Hambright and colleagues focused on the organism *Prymnesium parvum*, a marine alga that has demonstrated the ability to invade freshwater lakes and cause harmful algal blooms (Hambright et al., 2015; Hambright 2012). Researchers in this study started with natural freshwater microbial communities and altered the amounts of resources that each microcosm received. In addition to monitoring community diversity and resource availability, Hambright and colleagues also looked at the effects of propagule pressure on the success of invasion. In contrast to the two *E. coli* studies mentioned previously, community diversity and resource availability were not found to have impacted *P. parvum* invasion success. Rather, only propagule pressure influenced whether or not the invasion succeeded, with higher and more frequent propagules establishing the organism more successfully.

Disagreements among the current body of research in invasion ecology suggest several possibilities with regards to the diversity-invasion relationship. Perhaps the simplest explanation for these contradictions is that microcosm studies, although informative, do not closely enough resemble the natural communities and invasion events they are being used to model. This is especially true of microbial assemblages – microcosms created by inoculating sterile soil with a mixture of several pure cultures – where microbial community members are limited only to those which can be cultured in the laboratory. But even in microcosms created from soil samples that have not been manipulated, it may not be feasible to simulate the myriad environmental conditions that occur in natural ecosystems, such as precipitation events, temperature fluctuations (both within and between days and seasons), and different chemical resources that could be entering the system.

Another possible explanation could be that the organisms being used as model invaders are not invasive species with respect to the ecosystem under study. For example, pathogenic strains of *E. coli* may prefer the mammalian gut as a native habitat, and therefore could be considered an invasive species if deposited in extrahost environments such as soil, but there are several groups of environmental *E. coli* that may do quite well in soil. Some have even claimed that certain *E. coli* phylotypes can persist in soil long-term, possibly with growth (Brennan et al., 2016). A third, and more complex explanation for the disagreement between such studies is that some invasive species are not sensitive to community diversity while others are, the implication being that the diversity-invasion relationship is not a universal phenomenon, but instead depends on the specific invader-community combination (D'Antonio & Levine, 1999). This may be especially true if resource exploitation is not the dominant factor governing invasion success. For

example, resource availability may not matter much if the invader can free up resources by selectively killing other closely-related organisms that compete for the same type of resources.

It seems then that some evidence has mounted in support of the diversity-invasion relationship, but it may not be generalizable to all invaders or native communities. If this is the case, then it would be necessary to examine each invasive organism's particular sensitivity to native community diversity in order to determine how best to prevent invasion.

Listeria monocytogenes

Listeria monocytogenes is a gram-positive facultative anaerobe, an opportunistic human and animal pathogen, and the causative agent of Listeriosis (Cossart, 2011). *L. monocytogenes* rarely causes illness in healthy individuals, but is particularly dangerous for immunocompromised populations and pregnant women, with a mortality rate between 20 and 30% (Cossart, 2011). The main route of transmission of *L. monocytogenes* in humans is through the food supply due to the ability of the pathogen to survive on food processing equipment (Cerf & Carpentier, 2011), in food (especially poultry and livestock, Wiedmann et al., 2004; Caugant et al., 2003), and in soils (Piveteau et al, 2013; Wiedmann et al, 2006). The success of *L. monocytogenes* in food processing environments has been linked to its ability to survive (and even grow) at low temperatures and form biofilms that are difficult to remove (Ahmed et al, 2015; Kathariou et al, 2006; Banks et al, 1990). However, the factors affecting *L. monocytogenes* survival in soil are not as clearly understood, due in part to the complexity and heterogeneity of soil environments.

Many studies have been conducted to assess the prevalence of *L. monocytogenes* and its ability to survive in soil. One of the earliest of these studies was conducted by Welshimer in 1960, where *L. monocytogenes* was inoculated into microcosms containing two different types of

autoclaved soil – "fertile" and "clay" – and cell density was monitored by plate counts at regular intervals (Welshimer, 1960). Provided with adequate moisture, *L. monocytogenes* was still detectable at 295 days in both soil types, indicating that soil can act as a suitable habitat for the organism. A more recent study by Hartmann and colleagues on a sample set of 100 French soils looked at a wider range of soil physical, chemical, and biotic factors in affecting the survival of *L. monocytogenes* (Hartmann et al., 2013). They found soil chemistry – specifically the saturation rates of various cations – to have the largest impact on *L. monocytogenes* survival, and that in autoclaved soils, *L. monocytogenes* was detectable through the end of the 84-day study. In a subset of 9 of the soils, they examined the effect of the soil microbiota on the survival of *L. monocytogenes* by inoculating an autoclaved and non-autoclaved microcosm for each soil. Interestingly, survival was reduced by the native microbiota, but only in those soils with a pH above 7. Below a pH of 7, no difference in survival was observed between autoclaved and non-autoclaved soils. However, *L. monocytogenes* survival, on the whole, was worse below pH 7 than it was in either treatment above pH 7. This suggests that *L. monocytogenes* survival may be governed by complex interactions between biotic and abiotic factors.

Microcosm studies have been valuable in teasing apart factors that influence *L. monocytogenes* survival when inoculated into soil, but they cannot provide the entire picture. For one, inoculum levels for these experiments are often 10^6 to 10^8 cells – likely a much higher cell density than would occur naturally through dispersal and deposition by animals. Therefore, some researchers have chosen to model *L. monocytogenes* survival by examining its prevalence at the landscape scale. One such study was conducted by Bergholz and colleagues where surface water, soil, and soil surface drag swab samples were collected on and near fresh produce farms in New York State and were tested for the presence of several different pathogens – including *L.*

monocytogenes – through enrichment-based isolation techniques (Bergholz et al., 2013). The GPS location of each sample was recorded and prevalence data was mapped spatially along with soil and water chemical data and weather data. These data were then used to create a classification tree model identifying the factors with the greatest impact in predicting the prevalence of *L. monocytogenes.* Results indicated a base prevalence of 15% for *L. monocytogenes* among all samples, and the terminal rules in the classification tree indicated local prevalence of up to 45-50%. In this case, available water storage and proximity to both surface water and grazing pastures were the most informative factors in predicting the likelihood of *L. monocytogenes* detection. A follow-up study (Strawn et al., 2016) validated the findings through repeated drag swab field sampling and found that the proximity-based rules (proximity to surface water and grazing pastures) were indeed able to predict the occurrence of *L. monocytogenes* with reasonable accuracy.

Many more studies on the survival and prevalence of *L. monocytogenes* have been conducted than can be discussed here, but the common theme is that factors relating to the availability of moisture – whether it be proximity to water sources or soil physical characteristics that affect the storage of moisture – have the greatest impact on *L. monocytogenes* survival. Although some brief examinations on the impact of the native microbiota on *L. monocytogenes* survival have revealed a potential role for the soil microbiome (Hartmann et al., 2013, Vivant et al., 2013), more detailed approaches are needed to characterize the interactions between *L. monocytogenes* and the soil microbiome with a focus on the diversity-invasion relationship. Furthermore, although *L. monocytogenes* prevalence increases with proximity to surface water, little has been done to investigate the plausibility of different freshwater sources as potential reservoirs for *L. monocytogenes* persistence and growth. Several studies have shown that *L.*

monocytogenes can be isolated from surface water and sediments in small creeks and lakes (Topp et al., 2007; Bergholz et al., 2013) and one study goes so far as to suggest that various *Listeria* species – including *L. monocytogenes* – are capable of permanently colonizing freshwater sediments (Scherer et al., 2016). Therefore, more work needs to be done to 1) determine the role of the microbiome in the ability of *L. monocytogenes* to survive in soils once deposited and 2) elucidate the role that surface water plays in providing a suitable niche for *L. monocytogenes*, both in freshwater sediments and surface water proximal zones.

The Prairie Pothole Region of North America

The Prairie Pothole Region of North America covers approximately 900,000 km^2 across Saskatchewan, Alberta, and Manitoba in Canada and North Dakota, South Dakota, Minnesota, and Iowa in the United States (Harju, et al., 2005). The region is so named for the thousands of microlakes – prairie potholes – left by glacial depressions about 12,000 years ago, and serves as the breeding grounds for a majority of the continent's waterfowl, earning it the nickname "America's Duck Factory" (Gleason et al., 2013). Because of their near ubiquity in the aforementioned regions of Canada and the United States, prairie potholes can be found embedded in a variety of different ecosystems, including forests, grasslands, grazing pastures, and agricultural fields. Furthermore, due to their small size and isolation from other bodies of water, they are sources of surface water which may act as reservoirs for foodborne pathogens.

As was mentioned in the previous section, the prevalence of *L. monocytogenes* has been shown to increase with proximity to both surface water and grazing pastures (Bergholz et al., 2013; Strawn et al., 2016). This is especially important for areas that irrigate crops using ground or surface water that could potentially be harboring foodborne pathogens. One such area is the Salinas river valley in California, a major production region for leafy greens. In a 2-year study by

Cooley and colleagues looking at lakes and rivers near farm fields in the Salinas River Valley, the average prevalence across all samples collected was 65% for *Salmonella* and 43% for *Listeria* (Cooley et al., 2014). A similar potential may exist for prairie potholes to harbor *L. monocytogenes* and other foodborne pathogens. But the question of whether or not *L. monocytogenes* can persist in and even permanently colonize wetland sediments remains to be seen, and may depend on a mosaic of biotic and abiotic factors and their combined effects. Since the prairie pothole region serves as the breeding ground for migratory waterfowl, evidence showing the ability of foodborne pathogens such as *L. monocytogenes* to survive in wetland sediments could have important implications for controlling the spread of these pathogens in produce fields.

Aside from their potential role as reservoirs for foodborne pathogens, prairie pothole wetlands are also believed to play a critical role in the global carbon cycle, including production and/or sequestration of the greenhouse gases carbon dioxide, methane, and nitrous oxide (Gleason et al., 2013; Gleason et al., 2015). However, the conditions under which these wetlands may act as either carbon sources or carbon sinks remain poorly understood. Studies in similar ecosystems have revealed that small lakes and estuaries have a high potential for methane and nitrous oxide emission, and that the rate of production of these gases is driven by a combination of factors, including temperature, vegetation, and allocthonous inputs from agricultural and grazing activities (Gleason et al., 2015; Jeppesen et al., 2015; Mitsch and Bernal, 2013). Jeppesen and colleagues, in a long-term freshwater mesocosm study, found that temperature alone did not account for significant differences in greenhouse gas fluxes (Jeppesen et al., 2015). They found instead that the amount of available nutrients had a much more significant impact, with annual CO_2 and CH_4 fluxes increasing in the low nutrient treatments and N_2O fluxes

displaying the opposite trend. They attributed this effect to differences in the abundances of macrophytes and phytoplankton in each system.

Another study by Gleason and colleagues examined the effect of wetland drainage and restoration on CH_4 and N_2O fluxes (Gleason et al., 2015). Their results indicated 1) that the wetlands themselves act as sources of CH_4 whereas the associated upland soils have the potential to act as CH_4 sinks; and 2) that draining wetlands – a common practice in cropped fields – reduces carbon sequestration potential, and that this reduction in sequestration potential may persist even long after the wetlands have been restored.

These and other studies to date have placed emphasis on characterizing the water and sediment chemistry across different land classifications, but little work has been done to link gas flux measurements to the microbes driving the corresponding biogeochemical processes. By studying greenhouse gas fluxes in association with microbial community structure, new insights might emerge as to how changes in microbial community structure affect flux rates. It is likely that changes in land use, nutrient input, temperature, and the like, alter the microbial communities in these sediments, which in turn alter the production potential of the ecosystem. Therefore, the microbes driving biogeochemical processes in prairie pothole sediments could be a missing link in the relationship between spatio-chemical variation and greenhouse gas flux.

CHAPTER 2. FACTORS IMPACTING SURVIVAL OF *LISTERIA MONOCYTOGENES* IN PRAIRIE POTHOLE SOILS AND SEDIMENTS

Introduction

The diversity-invasion relationship hypothesis attempts to explain the tendency of more diverse communities to be better resisters of colonization by invasive species (Elton, 1958). Some have suggested that this phenomenon is driven by resource partitioning effects in that more diverse communities partition resources more effectively, leaving little left over for invaders to exploit (Falcao Salles et al., 2015; Tilman, 2004). Soil microcosm studies with *E. coli* have succeeded in reproducing this effect in the laboratory (van Elsas et al., 2012; Falcao Salles et al., 2015), but the diversity-invasion relationship is not necessarily a universal phenomenon among microorganisms. In a study of *Prymnesium parvum* (Hambright et al., 2015) – a marine alga known to cause harmful algal blooms (Hambright 2012) – propagule pressure was the key determinant in the establishment of a successful invasion. The diversity of the invaded community did not play a role. Conflicting findings such as these raise questions as to whether the diversity-invasion relationship is a universal phenomenon, or dependent on the particular combination of invader and community. At present, more data is needed from a wider range of organisms to determine whether or not the diversity-invasion relationship is a useful framework in the study of microbial invasion ecology.

Listeria monocytogenes is a gram-positive facultative anaerobe, a food-borne pathogen, and the causative agent of Listeriosis in humans and livestock (Cossart, 2011). The success of *L. monocytogenes* as a food-borne pathogen is owed in part to its ability to form biofilms (Ahmed et al, 2015; Kathariou et al, 2006; Banks et al, 1990), increasing the organism's survival on food processing equipment (Cerf & Carpentier, 2011). However, the pathogen also has many

opportunities to enter the soil and potentially become established there. The pathogen frequently resides in the guts of various ruminants as well as poultry, and can be shed into the environment (Piveteau et al., 2013; Wiedmann et al., 2004). Consequently, the use of manure from animals harboring and shedding *L. monocytogenes* could result in direct introduction into farm fields. In addition to residing in the guts of different species of livestock, *L. monocytogenes* can also act as a soil saprophyte, thriving in environments with dead and decaying vegetation (Freitag et al., 2009). Therefore, *L. monocytogenes* can be readily deposited into the environment and is capable of – to some extent – surviving in soil (Vivant et al, 2013; Wiedmann et al, 2006), allowing for a route of transmission to humans through fresh produce. Understanding the dynamic between *L. monocytogenes* and the native microbiota in various soils – specifically, whether the diversity-invasion relationship is an important factor in the survival and establishment of *L. monocytogenes* in soil – may aid in identifying areas that are potential hotspots of pathogen abundance.

One such area of importance to agricultural land management is the Prairie Pothole Region of North America. Consisting of almost one million square kilometers of ponds and microlakes spread across Southern Canada and the Midwestern United States (Harju, et al., 2005), the Prairie Pothole Region provides breeding grounds for most of the continent's waterfowl (Gleason et al., 2013) and are thought to play a critical role in the cycling of greenhouse gases. But determining their contribution to global greenhouse gas fluxes is not clear cut; they have been shown to be sequesters of atmospheric carbon in some cases and emitters of atmospheric carbon in others (Gleason et al., 2013; Gleason et al., 2015), and although sequestration of atmospheric carbon reduces global CO_2, it can come at the expense of increased production of other greenhouse gases such as N_2O and CH_4 (Gleason et al., 2009). Moreover,

disturbances to prairie potholes, especially eutrophication, may increase the flux of one or more of these greenhouse gases (Jeppesen et al., 2015; Mitsch & Bernal, 2013; Gleason et al., 2015). Given the size of the prairie pothole region and the number of wetlands contained within it, prairie potholes have the potential to be a major factor in the global greenhouse gas budget. However, studies to date have focused mostly on variation in vegetation, temperature, and chemical inputs to the wetlands. Comparatively little work has been done to study the microbial communities in these ecosystems.

In addition to their role as contributors to the global greenhouse gas cycle, prairie pothole may be important components in the transmission cycle of *L. monocytogenes* and other foodborne pathogens due to their abundance in and around farm fields and their role as breeding grounds for migratory waterfowl. Being that *L. monocytogenes* is commonly found in poultry, a further look into the suitability of migratory waterfowl as a dispersal vector for the pathogen may be warranted. Furthermore, several studies have shown that *L. monocytogenes* prevalence is higher near streams and other sources of surface water, and that agricultural areas near these water sources may be impacted (Bergholz et al., 2013; Strawn et al., 2016; Topp et al., 2007). However, prairie potholes, as a result of their hydrographic isolation, vary dramatically with respect to water chemistry, especially salinity (Sloan, 1972). As a result, it is unclear whether the success of *L. monocytogenes* in riverine and lacustrine environments can be extended to prairie potholes. More research is needed to determine under what conditions prairie potholes may act as reservoirs for foodborne pathogens in general and what impact (if any) the native microbial communities have on their survival.

In order to examine the role that microbial community diversity plays in affecting the survival of *L. monocytogenes* as well as characterize the microbial communities in the prairie

pothole region and their impacts on greenhouse gas fluxes, two experiments were conducted in parallel. The first was a microcosm experiment in which *L. monocytogenes* was inoculated into prairie pothole soils and sediments from different land use types (agricultural, grazing, and natural) and the death rates for each sample calculated. The second experiment was a microbiome study examining the community structure and diversity in each of the different sample locations. These data were then combined in order to determine if microbial community diversity was a key driver of *L. monocytogenes* death rate. In addition, Canonical Correspondence Analysis was applied to the soil chemical and microbiome data to identify microbial families associated with different land types and environmental parameters.

Materials and Methods

Preparation of glucose defined minimal media for growth of *Listeria monocytogenes*

L. monocytogenes for inoculation into soil microcosms was grown in a glucose defined minimal media (Egli & Schneebeli, 2013) with several modifications. The minimal media was mixed fresh on the day of inoculation from several concentrated stock solutions and individual components. Table 1 below list the volumes of components required to make 50 mL of media. Tables 2-4 describe the makeup of the trace elements, vitamins, and amino acids solutions respectively.

Table 1. Stock components in glucose-defined minimal media (for 50 mL)

Component	Volume (mL)
1.28M Na_2HPO_4 (50X)	1.000
0.482M KH_2PO_4 (100 X)	0.500
2.67M $(NH_4)_2SO_4$ (50X)	1.000
0.629M $MgSO_4$ (100X)	0.500
0.5M EDTA (100X)	0.500
1M MOPS buffer, pH 7 (10X)	5.000
Trace Elements (200X)	0.250
Vitamins (1000X)	0.100
82.5mM Cysteine (100X)	0.500
205mM Glutamine (50X)	1.000
67mM Methionine (100X)	0.500
Amino acids (100X)	0.500
100 g/L Glucose (67X)	0.750
Sterile dH_2O	37.9

Components were combined in the order listed above. The glucose, amino acid, and vitamin solutions were kept at 4°C for stability. Individual amino acids (Glutamine, Methionine, and Cysteine) were stored dry and dissolved in water at the time of media preparation.

Table 2. Preparation of 100X Trace Elements solution

Component	Quantity
dH_2O	200 mL
32% HCl	10 mL
$CaCO_3$	4.00 g
$FeCl_3$-$6H_2O$	3.87 g
$MnCl_2$-$4H_2O$	0.57 g
$CuSO_4$-$5H_2O$	0.074 g
$CoCl_2$-$6H_2O$	0.065 g
ZnO	0.20 g
Boric Acid	0.062 g
dH_2O	QS* to 400 mL
EDTA tetrasodium dihydrate	39.1 g
$MgCl_2$-$6H_2O$	6.7 g
Na_2MoO_4-$2H_2O$	0.52 g

Components were combined in the order listed above and the solution was autoclaved to sterilize. *QS: "Quantity Sufficient" to yield a final volume of 400 mL.

Table 3. Preparation of 1000X Vitamins solution

Component	Quantity
dH_2O	100 mL
Biotin	2.0 mg
Folic acid	2.0 mg
Pyridoxine	10.0 mg
Thiamine HCl	5.6 mg
Riboflavin	5.0 mg
Niacin	5.0 mg
Cobalamin	5.0 mg
Pantothenic acid D-Calcium	10.9 mg
4-aminobenzoic acid	5.0 mg
Lipoic acid	5.0 mg
Nicotinamide	5.0 mg

Components were combined in the order listed above, stirred at ~600 rpm with a magnetic stir bar until dissolved, and sterile-filtered with a 0.22 µM filter.

Table 4. Preparation of 100X Amino Acids solution

Component	Volume (mL)
dH_2O	100 mL
Histidine	1.35 g
Tryptophan	1.00 g
Leucine	1.00 g
Isoleucine	1.00 g
Valine	1.00 g
Arginine	1.21 g

Components were combined in the order listed above, stirred at ~600 rpm with a magnetic stir bar until dissolved, and sterile-filtered with a 0.22 µM filter.

Listeria monocytogenes strain used for soil microcosm study

The strain of *L. monocytogenes* used for inoculation into the soil microcosms was strain J0161 transformed with the pNF8 plasmid (provided by Mike Doyle and Cathy Webb at UGA), engineered with an erythromycin resistance gene for selection and constitutively-expressed Green Fluorescent Protein (GFP) for screening (Fortineau et al., 2000; Ma et al., 2011). J0161 is an isolate of lineage II, a group more frequently found in non-clinical samples. But isolates from lineage II have also been the causes of several outbreaks. Strain J0161 was found to be the cause of a multi-state disease outbreak in the year 2000, originating from poultry deli meat (Olsen et al., 2005; Ma et al., 2011). As such, it is a relevant strain for this study because 1) it has been shown to cause disease in humans and 2) the 2000 outbreak was traced back to poultry, indicating that it could be a likely candidate for dispersal via migratory waterfowl residing in prairie potholes.

Growth of *Listeria monocytogenes* for inoculating soil microcosms

To prepare inoculum for the soil microcosm study, a frozen stock of J0161 was removed from -80°C, streaked for isolation onto Brain Heart Infusion (BHI) agar containing 40 µg/mL Erythromycin and grown at 37°C for 48 hours. This strain had been previously transformed with a plasmid bearing genes for erythromycin resistance and Green Fluorescent Protein (GFP). Isolated colonies were screened for fluorescence using a blue LED transilluminator. Positive colonies were used to inoculate 10 mL each of BHI broth containing 40 µg/mL erythromycin for plasmid maintenance. Tubes were shaken at 215 rpm for 48 hours at 37°C. Following growth on BHI, 100 µL of culture was transferred to 10 mL of glucose-defined minimal media + erythromycin and grown on a shaker as before. From this growth, a final passage was performed by taking 100 µL of culture and placing it into 10 mL fresh glucose-defined minimal media +

erythromycin. After 48 hours of growth on the shaker, the culture (now in a tertiary passage) was considered ready for inoculation into soil microcosms.

Soil sample collection

Prairie pothole soils and sediments were collected from three different areas near Jamestown, ND, representing natural, agriculturally impacted, and grazing impacted prairie pothole wetlands. Potholes classified as natural (or protected) were located in the Cottonwood Lake Study Area (CLSA), a Long Term Research area maintained by the United States Geological Survey (USGS, 2012) focused on studying the prairie pothole ecosystem. Potholes classified as agriculturally impacted and grazing impacted were located on private land, and permission for sample collection was obtained in cooperation with the USGS. At each pothole, eight gas flux monitoring stations are installed, five within the wetland and three at increasing distances away from the wetland (Gleason et al., 2013).

Soil and sediment samples were collected adjacent to four different gas flux stations at each of 12 different wetlands on two different dates. Table 5 shows the samples collected on each of the two sampling dates, along with wetland designation, wetland ID, and gas flux station where the sample was collected. Figure 1 shows the relative locations of the different stations within a wetland that samples were collected at. For each sample number in Table 5, the letter and first number in the sample ID designates the wetland ID and the last number designates the gas flux station. Station 1 was in the submerged wetland zone, station 2 at the wetland margin, station 3 at the toe slope of the basin, and station 4 at the shoulder slope. The toe and shoulder slopes were 2-5m and 70-100m from the wetland margin, respectively (linear distance, not elevation). All wetlands starting with "P" were natural/protected. Wetlands R2, R4, and R5 were grazing-impacted while R8, B5, and B6 were agriculturally-impacted. For example, sample

P3003 was taken at protected wetland 3 at the wetland basin's toe slope whereas sample R2001 was from submerged sediment in a grazing-impacted wetland. The below set of samples was collected twice – once on May 16, 2016 and again on July 26, 2016.

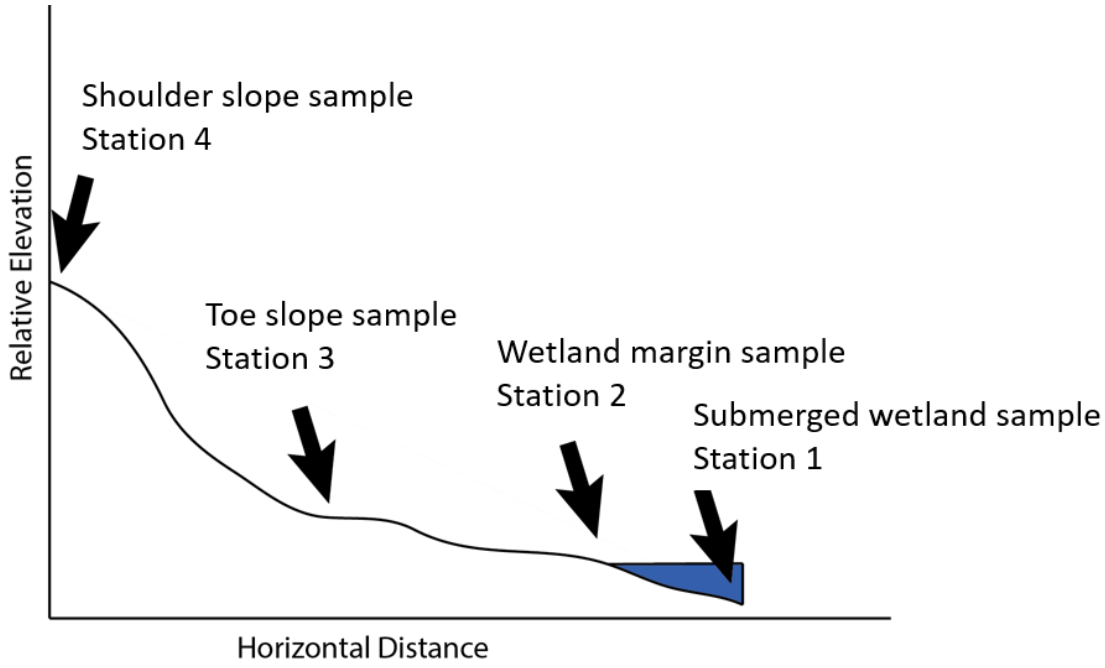

Figure 1. Wetland sampling scheme.

Table 5. Listing of samples taken at each wetland.

Samples	Wetland Designation
P1001-1004	Natural/Protected
P3001-3004	
P4001-4004	
P6001-6004	
P7001-7004	
P8001-8004	
R2001-2004	Grazing impacted
R4001-4004	
R5001-5004	
R8001-8004	Agriculturally impacted
B5001-5004	
B6001-6004	

Samples were collected with sterile plastic scoops and placed in 8 oz. Whirl-Pak® bags. Submerged sediments were first unearthed with a shovel and the sample was then taken from the removed sediment. All samples were kept in a cooler to protect from sunlight until returned to the laboratory for processing. Upon returning to the laboratory, the cooler was kept at room temperature overnight until samples could be processed.

Sample processing

The morning following sampling (beginning less than 24 hours after the first sample was taken), soils and sediments were each separated into three fractions. The first fraction consisted of approximately 1.5 mL in each of two 2-mL cryovials which were labeled with the sample number and immediately frozen at -80^{o}C for DNA extraction at a later date. The second fraction consisted of 40 mL of either soil or sediment placed in a 160-mL sterile container. These containers served as microcosms for analysis of *L. monocytogenes* survival, and were moved to a 20^{o}C incubator to equilibrate to temperature prior to inoculation the following day. The

remaining material for each sample was moved to brown paper bags, weighed to get a starting mass, and moved to 55^{o}C for drying. Drying served the dual purpose of obtaining a dry weight for soil moisture calculations as well as preparing the soils for chemical analysis.

Inoculation of soil microcosms with *Listeria monocytogenes*

L. monocytogenes broth cultures (prepared as described previously in "Growth of *Listeria monocytogenes* for inoculating soil microcosms") were removed from the shaker at the 48-hour mark and were transferred to 15-mL conical centrifuge tubes. Cultures were pelleted by centrifugation at 4,500 rpm, 4^{o}C for 8 minutes and then resuspended in 5 mL 1X PBS to wash cells. The centrifugation was repeated and the pellet was once again suspended in 1X PBS, this time 10 mL. Cells in PBS were transferred to a sterile 500 mL flask for dilution to the target cell density.

From previous test growths, it was known that *L. monocytogenes* in this media and growth conditions reached a density of 1.2 x 10^{8} CFU/mL after 48 hours in the final passage. This cell density was used to calculate the volume of diluent needed to reach the target cell density of 1.0 x 10^{7} CFU/mL. The two tubes of cells in 1X PBS (total 20 mL at a theoretical cell density of 1.2 x 10^{8} CFU/mL) were added to the 500 mL flask and diluted to a total volume of 240 mL 1X PBS, yielding a theoretical concentration of 1.0 x 10^{7} CFU/mL.

The diluted cell suspension was used to inoculate the soil microcosms at a volume of 4 mL per 40-mL microcosm, yielding an expected microcosm cell density of 1.0 x 10^{6} cells per mL of microcosm. The inoculum was carefully mixed into the soil for each microcosm and the microcosms were weighed. This was done to determine what the density of each microcosm was, since the soil texture and moisture content varied widely between samples. After this, microcosms were incubated at 20^{o}C.

Measurement of *Listeria monocytogenes* death rate by plate count

To enumerate surviving *L. monocytogenes*, microcosms were plated 5 times in the first 10 days post-inoculation and once every 5-7 days thereafter until *L. monocytogenes* counts declined below the limit of detection of 1 colony per plate (< 200 CFU/mL soil). To plate out microcosms at each time point, a 0.5 mL aliquot of the soil from each microcosm was transferred to a 50-mL graduated centrifuge tube. The amount of microcosm weighed to obtain 0.5 mL was determined by dividing the total mass of the microcosm (representing 40 mL of microcosm) by 80. Then, working in a Biological Safety Cabinet (BSC), 10 mL of 1X PBS (e.g. a 1:20 dilution) was added to each tube and tubes were mixed by vortex for 20-30 seconds, then allowed to settle for at least 10 minutes.

For each sample, 1 mL of supernatant was taken by pipette without disturbing the settled soil and was moved to a fresh 1.5-mL microfuge tube. This suspension was then serially diluted twice 1:10 in 1X PBS to create the dilution series. Each dilution was plated in triplicate on CHROMagar® (CHROMagar Media – Paris, FR) at a volume of 100 µL per plate using a sterile hockey-stick spreader to evenly distribute the inoculum across the surface of the agar. Agar was prepared according to package instructions and supplemented with a final concentration of 40 µg/mL of Erythromycin. Plates were allowed to dry open in the hood and were then covered. Plates were placed at 37°C and allowed to incubate for 5-7 days. At the end of the incubation, colonies were counted using a blue LED transilluminator.

Death curve analysis of *Listeria monocytogenes* count data

Replicate plate counts of survivors were averaged, and Log10 CFU/mL was estimated. When the time series study was complete, log-transformed plate count data was exported as a comma separated file and imported in *R, v3.3.0* (R Core Team, 2016). For each microcosm, the

log CFU/mL values were plotted as a function of time (days post-inoculation) and were fit to a Geeraerd model using the R package *nlsMicrobio* (Delignette-Muller & Baty, 2013). The full Geeraerd model is a negative logistic function with three phases: 1) a flat lag phase where cells are dying at very low rates (the shoulder); 2) a region of increasing and then decreasing rates of cell death, with the inflection point being equal to the maximum death rate (*kmax*); and 3) a tail where the death rate levels off, indicating either that all cells have died or that they have reached a stable state supportable by their environment. Three different variations of the Geeraerd model were available: 1) a model containing all three parameters, 2) a model containing only the shoulder and *kmax* parameters, and 3) a model containing only the tail and *kmax* parameters. All three models were applied to each curve, but in most cases, only one of the models converged to a solution. In cases where more than one model fit the data, the model with the lowest sum of squared errors was chosen. The key piece of information derived from the Geeraerd model fitting was the model *kmax,* or rate parameter, which is the maximum death rate.

Extraction of DNA from soils for microbiome sequencing

DNA was extracted from the soil and sediment aliquots that had been stored at -80ºC. Extractions were performed using a two stage purification consisting of the MoBio PowerSoil® kit followed by the MoBio PowerClean® kit, both according to the kit instructions. Half of the samples were purified from the May sampling time point and half from the July time point. Samples for extraction from the first time point were chosen in a pseudorandom manner such that an equal number of samples would be sequenced from each sample site and from each hydrographic zone within sample sites. At the July time point, samples were chosen that were not sequenced the first time to complete the set of one microbiome per sample. Table 6 shows

which samples were sequenced from the May time point and which samples were sequenced from the July time point.

Table 6. Extraction time points of each sample

Sample	Time point	Sample	Time point	Sample	Time point
P1001	May	P7001	July	R5001	July
P1002	July	P7002	May	R5002	May
P1003	July	P7003	July	R5003	July
P1004	May	P7004	May	R5004	May
P3001	July	P8001	May	R8001	May
P3002	May	P8002	July	R8002	July
P3003	July	P8003	July	R8003	July
P3004	May	P8004	May	R8004	May
P4001	May	R2001	May	B5001	May
P4002	July	R2002	July	B5002	July
P4003	May	R2003	May	B5003	May
P4004	July	R2004	July	B5004	July
P6001	May	R4001	July	B6001	July
P6002	July	R4002	May	B6002	May
P6003	May	R4003	May	B6003	July
P6004	July	R4004	July	B6004	May

DNA was extracted for half of the soil/sediment samples at the May time point and half at the July time point.

16S microbiome sequencing using the V3-V4 Dual Indexing (DI) approach

At each of the two timepoints, DNA samples (24 per time point) were sent to the University of Minnesota Genomics Center (UMGC) for 16S microbiome sequencing. The UMGC uses a dual indexing approach (Beckman et al., 2016) based on the Earth Microbiome Project (EMP) primer set (Gilbert et al., 2014) which targets variable regions 3 and 4 (V3-V4) of the bacterial 16S rRNA gene, frequently used in microbial taxonomic studies such as this. For each sequencing run, all 24 samples were multiplexed on a single Illumina MiSeq® lane using 300 bp paired-end sequencing.

Microbiome sequence analysis using QIIME

Sequence data was received from the UMGC already demultiplexed, with forward and reverse reads contained in separate FASTQ files for each sample. Reads were first quality trimmed using *trimmomatic* in paired-end mode (Usadel et al., 2014). Trimmed reads for which both read pairs survived were used as input for *QIIME v1.9* (Quantitative Insights Into Microbial Ecology; Knight et al., 2010). Reads were first joined using the *join_paired_ends.py* script with a maximum percent mismatch threshold of 10%. Joined reads were then assigned sample names according to a mapping file using the script *multiple_split_libaries_fastq.py* which performs more quality trimming and places all sequences (with sample identifiers) into a single FASTA sequence file, *seqs.fna*.

Using the *seqs.fna* file as input, operational taxonomic units (OTUs) were assigned using the script *pick_open_reference_otus.py*. As the name suggests, the OTU picking strategy used by this script is an open reference strategy. In open reference OTU picking, sequences are first matched against a database of identified sequences – in this case, the *Greengenes* database, *v13.8* (Andersen et al., 2006) with a percent OTU identity cutoff of 97%. Sequences that do not match

anything in the database are then clustered *de novo* and these new sequences are matched again to the database. In most instances, the taxonomy assigned to these second round OTUs is not as specific as that assigned to closed reference database hits. The output resulting from this operation is an OTU table containing OTU IDs and assigned taxonomy.

The final step in a basic *QIIME* analysis is the alpha- and beta-diversity analyses which are accomplished with the *core_diversity_analyses.py* script using the OTU table from the previous step as input. By default, *QIIME* calculates three different measures of alpha-diversity: observed OTUs, *Chao1* richness (Chao, 1984), and Faith's Phylogenetic Diversity (PD, Faith, 1992). The three metrics measure diversity in very different ways. Faith's PD is the most informative of the three because it is calculated based on the sum of branch lengths in the community-wide phylogenetic tree. The tree is generated by *QIIME* based on the OTU tables using the program *FastTree* (Price, Dehal, & Arkin, 2009). In this way, it measures not just the number of different species (the case with observed OTUs), but it measures the degree of relatedness between the members present. Additionally, the phylogenetic tree from which Faith's PD is calculated is the basis of *UniFrac* distance, the community dissimilarity metric preferred by the developers of *QIIME*. *UniFrac* represents the "Unique Fraction" of phylogenetic tree branch lengths between two samples (Knight et al., 2009) and is used as the default measure of distance for β-diversity in *QIIME*.

Faith's PD stands in contrast to *Chao1* richness, which is a calculation based on the ratio of singleton to duplicate OTUs. As such, *Chao1* richness performs better as a metric for completeness of sequence coverage rather than it does as a metric for community diversity. Taken together, the measures help to provide a complete picture of diversity encompassing effectiveness of sequence coverage, total number of different members, and the relatedness of

the different members of the community. However, to avoid redundancy among predictor variables used in modeling *L. monocytogenes* death rate, only PD was included in the model selection to the exclusion of *Chao1* and observed number of OTUs.

Chemical analysis of dried soils

Dried soils (see "Sample processing") were weighed to get a dry mass and then were sent to the North Dakota State University soil testing laboratory for chemical testing. The set of characteristics tested for included pH, electrical conductivity, percent organic matter, nitrate, phosphorus, and iron. Moisture content measurement was performed in our laboratory and was calculated by taking (1 – (dry weight / original weight)) * 100.

Analysis of *L. monocytogenes* death curves using backward model selection

In order to determine whether microbial community diversity was a key determinant of *L. monocytogenes* death rate, a linear model was generated using the *lm* function in the *R* stats package (R Core Team, 2016) with *kmax* (death rate) as the response variable and numerous predictor variables, including soil chemical, moisture, and community diversity (PD). Although there was chemical and soil moisture data available for 96 samples (48 at each of 2 time points), only the soil data corresponding to the 48 microbiome samples was used. The linear model was then used as input for a backwards model selection using the *MASS* package (Ripley & Venables, 2003) in *R* to identify the factor or set of factors that had the greatest influence on *L. monocytogenes* death rate in soil. In total, 4 linear models and subsequent model selections were performed. The first model contained all predictors. The second model was identical to the first, but with *PD_whole_tree* (phylogenetic diversity, PD) and hydrographic zone included as a combined effect. The third and fourth models built on the second, but added moisture + zone and electrical conductivity + zone, successively. This was done to account for the observation that

the wetland and upland zones represent two distinct ecosystems, leading to a great deal of covariation among the predictors.

Canonical Correspondence Analysis of Bacterial and Archaeal Families

Canonical correspondence analysis (CCA) was used to identify trends in bacterial and archaeal family abundance driven by site and environment. Briefly, CCA is an ordination technique that aims to identify the environmental optima for each taxon in multivariate datasets (ter Braak, 1986; Verdonschot & ter Braak, 1995). The required data inputs for this method are tables containing environmental data by sampling site and taxonomic counts by sampling site, and the method assumes that the data are normally distributed. As a result, prior to CCA, the data must be transformed and standardized such that they conform to the assumption of normality, which can be accomplished using a standardization function.

To prepare the inputs for CCA, the OTU table from the QIIME analysis mentioned above (*out_table_mc2_w_tax_no_pynast_failures.biom*) was converted to tab delimited format using the *biom* command line utility (Caporaso et al, 2012) and was loaded into *R*. Taxonomic assignment data was parsed into separate columns yielding a single data frame with each taxonomic level represented as a unique column. All fields for which taxonomy could not be assigned were standardized as "Unassigned" for consistency and to allow for querying of important summary statistics, such as percentage unassigned OTUs at each taxonomic level.

Environmental data were assembled next, which consisted of the results of the soil chemical testing added to gas flux data for CO_2, CH_4, and N_2O. Gas flux measurements were collected by the USGS at all the same sample sites where soils were collected. The dates of measurement varied, with some occurring on the same day as soil sampling and some occurring several days before or after. The difference between the dates of soil sampling and gas flux

measurement never exceeded one week. For both CO_2 and CH_4, a single outlier was present requiring removal of the associated sample. This brought the total number of observations to 46. For the N_2O data, two outliers were present, which if removed, would have decreased the total number of observations to 44. Furthermore, these two observations were from the same land cover type (Agricultural), so the N_2O data was excluded for the CCA in order to minimize loss of samples.

Soil and gas flux data (minus N_2O) were merged into a single table and transformed for analysis. Proportional data (percentages of organic matter and moisture) were square-root *arcsin* transformed first, and then all data were transformed using the standardizing function as implemented in the *R* package *vegan* (Dixon, 2003). The OTU data were aggregated by taxonomic Family and transformed from absolute to relative by dividing the count for each OTU by the total number of OTUs in the respective sample. Relative abundance data were Hellinger (square root) transformed, again using the implementation in *vegan*, and all OTUs with a maximum relative abundance less than 0.0025 post-transformation were removed. The two data tables (environmental and taxonomy data) were used as the inputs for CCA using the *cca* method in *vegan*. In order to reduce complexity of the resulting ordination plot, specific taxa were highlighted corresponding to their reported ecological role (e.g. methanogens, sulfur reducers, etc.) and sites were color coded by the two different available metrics: hydrographic zone and land use type. Environmental variables were represented as vectors with direction and magnitude corresponding to the taxa and sample sites that they most characterized.

Results

Soil and sediment chemical attributes

Kruskal-Wallis (Kruskal & Wallis, 1952) and pairwise Wilcoxon rank-sum tests (Wilcoxon, 1945) revealed several significant differences between soil and sediment chemical attributes, both across different hydrographic zones and across different land use classes (Tables 7 and 8, respectively). With respect to hydrography, NO_3 was significantly different between zones 2 and 4, and Fe, electrical conductivity, and moisture were all significantly higher in zones 1 and 2 compared with upland soils. Focusing on the different land use classes, the impact of land management practices in agricultural areas becomes clear: NO_3 was significantly higher and organic matter significantly lower in agricultural soils and sediments as compared to natural wetlands. Furthermore, P and Fe were higher in agricultural wetlands than in both grazed and natural wetlands while pH was significantly lower.

Table 7. Differences in prairie pothole chemistry by hydrography

	Hydrographic zone – mean (s.d.)				
Chemical property	1	2	3	4	Kruskal-Wallis *p-value*
NO_3 (lbs/acre)	10.86 ^{AB} (10.51)	6.83 ^{A} (8.81)	12.83 ^{AB} (9.47)	26.88 ^{B} (32.57)	0.00040
P (ppm)	22.52 ^{A} (17.96)	14.04 ^{A} (10.83)	17.25 ^{A} (9.57)	14.13 ^{A} (10.49)	0.078
pH	7.47 ^{A} (0.68)	7.37 ^{A} (0.65)	7.55 ^{A} (0.60)	7.33 ^{A} (0.49)	0.37
Fe (ppm)	139.04 ^{A} (76.71)	137.98 ^{A} (86.08)	56.25 ^{B} (33.04)	27.46 ^{C} (18.07)	1.92e-11
Organic matter (%)	12.08 ^{A} (8.76)	10.01 ^{A} (10.06)	12.05 ^{A} (5.23)	9.31 ^{A} (2.33)	0.12
Electrical conductivity (mmhos/cm)	1.25 ^{A} (0.59)	1.10 ^{A} (0.49)	0.87 ^{B} (1.00)	0.42 ^{C} (0.12)	4.44e-10
Moisture (%)	51.53 ^{A} (14.77)	41.87 ^{AB} (15.03)	29.88 ^{B} (8.20)	18.18 ^{C} (4.44)	2.19e-12

For each chemical property, pairs of values without at least one letter in common (i.e. A, B, C) were statistically different from each other by Wilcoxon rank-sum test (Bonferroni-corrected *p-value* less than 0.05).

Table 8. Differences in prairie pothole chemistry by land use

	Land use – mean (s.d.)			
Chemical property	protected	agricultural	grazing	Kruskal-Wallis *p-value*
NO_3 (lbs/acre)	9.35 ^{A} (9.16)	26.83 ^{B} (33.22)	11.92 ^{AB} (8.85)	0.011
P (ppm)	11.98 ^{A} (6.45)	30.96 ^{B} (16.70)	12.5 ^{A} (5.94)	1.23e-7
pH	7.73 ^{A} (0.42)	6.82 ^{B} (0.55)	7.48 ^{A} (0.50)	9.75e-8
Fe (ppm)	61.55 ^{A} (45.47)	155 ^{B} (105.62)	76.21 ^{A} (51.80)	0.0019
Organic matter (%)	8.74 ^{B} (6.53)	15.20 ^{A} (8.74)	10.56 ^{AB} (4.55)	0.0022
Electrical conductivity (mmhos/cm)	0.79 ^{A} (0.56)	0.91 ^{A} (0.54)	1.13 ^{A} (1.00)	0.16
Moisture (%)	32.71 ^{A} (17.63)	39.90 ^{A} (16.93)	34.97 ^{A} (14.97)	0.18

For each chemical property, pairs of values without at least one letter in common (i.e. A, B, C) were statistically different from each other by Wilcoxon rank-sum test (Bonferroni-corrected *p-value* less than 0.05).

16S sequencing and taxonomic assignment

The number of OTUs for each sample ranged from a low of 33,904 (sample R5001) to a high of 280,328 (sample R4003). The reason for the large range of OTUs per sample was due to variation between the two sequencing runs. The sequencing run for the May timepoint was slightly over-clustered during the library preparation stage while the sequencing run for the July timepoint was under-clustered. Such a difference in numbers of OTUs between samples has been shown to be a problem when counts are rarefied prior to β-diversity analysis, but this can be overcome by using compositional analysis techniques instead of random sampling techniques like rarefaction (McMurdie & Holmes, 2014; Gloor et al., 2016). The data preparation used for the CCA mirrors that required by compositional analysis.

Sequencing the V3-V4 segment of the 16S rRNA gene, or even sequencing the entire gene, does not guarantee species- or even genus-level coverage. In fact, a vast majority of OTUs in this study were not classified down to either of these two levels. Figure 2 shows the percentage of OTUs for which positive identification could be made at each taxonomic level. After the Order level, the rate of positive taxonomic assignment drops off considerably, with slightly over 50% being assigned a Family, less than 20% assigned to a genus, and around 2% assigned to a species.

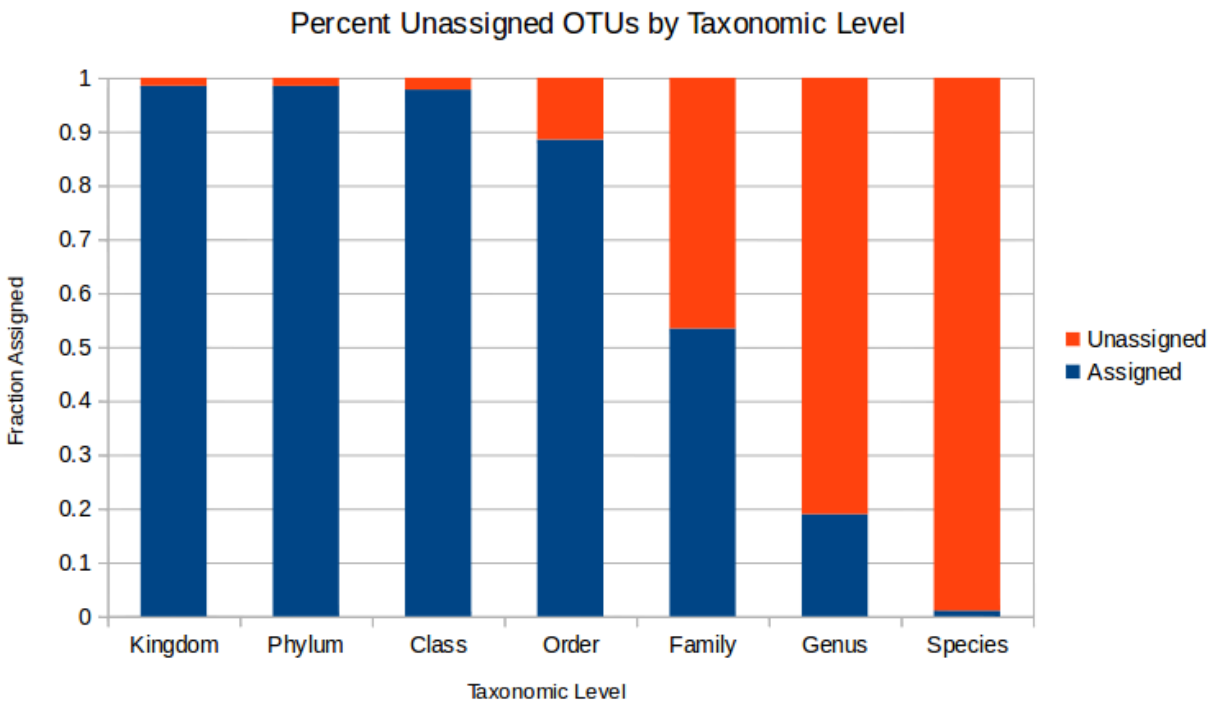

Figure 2. Percentage unassigned OTUs by taxonomic level. Less than 20% of OTUs could be assigned a genus and only 2% were assigned a species.

Microbial community alpha diversity

As mentioned previously, *QIIME* calculates three different measures of alpha diversity by default: number of observed OTUs, *Chao1* richness (Chao, 1984), and Faith's Phylogenetic

Diversity (PD, Faith, 1992). The different measures displayed some disagreement with each other, particularly with respect to changes in hydrography. Tables 9 and 10 show how alpha-diversity differs by hydrographic zone and land use class, respectively. Figures 3 and 4 show the alpha rarefaction plots for Faith's PD (designated *PD_whole_tree*). Faith's PD displays a strong negative trend with increasing distance away from the wetland center while there is no significant difference when it comes to land use. The reverse is true of *Chao1* richness and observed OTUs, where there is not much difference between hydrographic zones, but there are significant differences between land use classes. However, as discussed in the methods section, Faith's PD stands out from the other two measures because it is based on the diversity of the communities' phylogenetic trees, whereas *Chao1* and observed OTUs are count-based.

Table 9. Differences in alpha-diversity by hydrography

	Hydrographic zone – mean (s.d.)				
Alpha-diversity measure	1	2	3	4	Kruskal-Wallis *p-value*
Observed OTUs	4975 ^{AB} (979)	5074 ^{A} (780)	4408 ^{AB} (570)	4383 ^{B} (315)	0.018
Chao1 richness	7648 ^{A} (1945)	7858 ^{A} (1681)	6757 ^{A} (1146)	7153 ^{A} (638)	0.19
Phylogenetic diversity	323 ^{A} (60)	320 ^{A} (41)	244 ^{B} (43)	225 ^{B} (11)	1.95e-5

For each measure of alpha-diversity, pairs of values without at least one letter in common (i.e. A, B, C) are statistically different from each other by Wilcoxon rank-sum test (Bonferroni-corrected *p-value* less than 0.05).

Table 10. Differences in alpha-diversity by land use

	Land use – mean (s.d.)			
Alpha-diversity measure	protected	agricultural	grazing	Kruskal-Wallis *p-value*
Observed OTUs	4430 [A] (761)	5076 [B] (701)	4904 [AB] (608)	0.026
Chao1 richness	6612 [A] (1181)	8198 [B] (1358)	7995 [B] (1396)	0.0018
Phylogenetic diversity	271 [A] (65)	284 [A] (51)	287 [A] (63)	0.479

For each measure of alpha-diversity, pairs of values without at least one letter in common (i.e. A, B, C) are statistically different from each other by Wilcoxon rank-sum test (Bonferroni-corrected *p-value* less than 0.05).

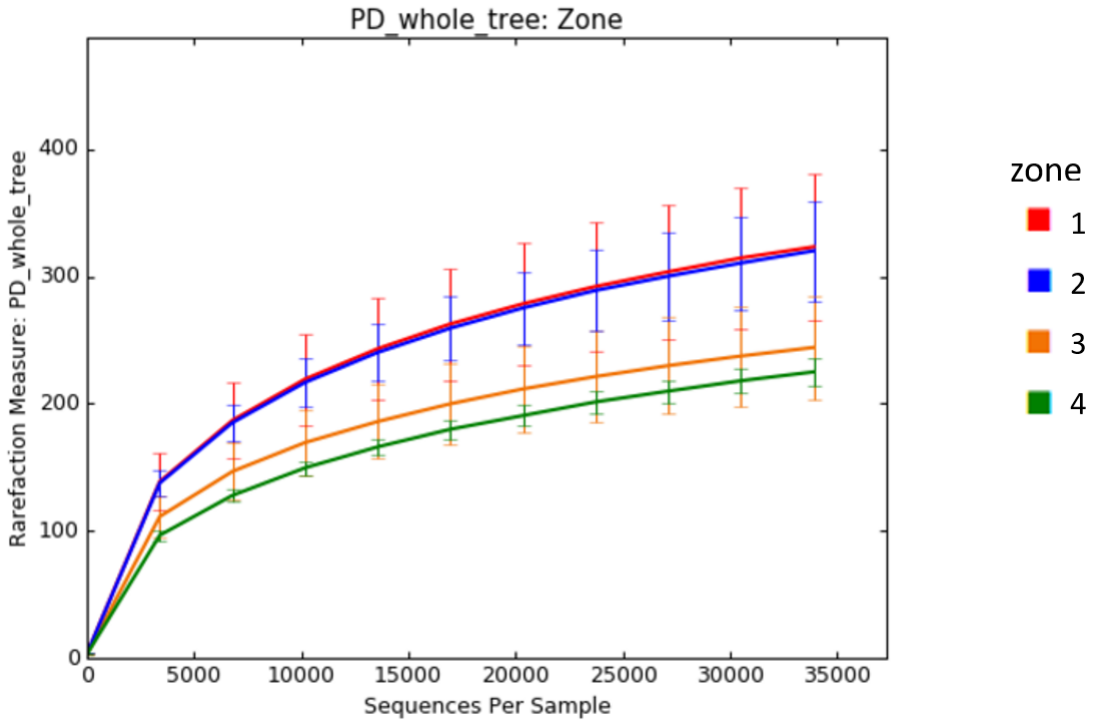

Figure 3. Difference in Faith's Phylogenetic Diversity between hydrographic zones. The submerged sediments (zones 1 and 2) are more diverse than their associated upland soils.

Figure 4. Difference in Faith's Phylogenetic Diversity between land use classes. There is little difference in phylogenetic diversity between the different land use types.

Factors with the greatest impact on *L. monocytogenes* death rate

Death rates of *L. monocytogenes* displayed little variation between either hydrography or land use types (Figures 5 and 6, respectively). Death rate by hydrographic zone did show a slight negative trend, matching that seen in Faith's PD. But among all predictor variables in the four different *L. monocytogenes* death rate models, phosphorus, electrical conductivity, and moisture were consistently deemed to be the most important (Table 11). Models 1 and 2 contained only those three variables and displayed better (i.e. lower – in this case, more negative) Akaike Information Criterion scores (AIC – a measure of model fit; Akaike, 1973) than models 3 and 4, which were more complex and did not fit the data as well. In fact, community diversity (PD) was not included in any of the 4 models. Therefore, it appears as if community diversity does not have an impact on *L. monocytogenes* death rate. However, it may also be that a diversity effect

was not observed because PD is too closely intertwined with other environmental variables to be selected as a key predictor.

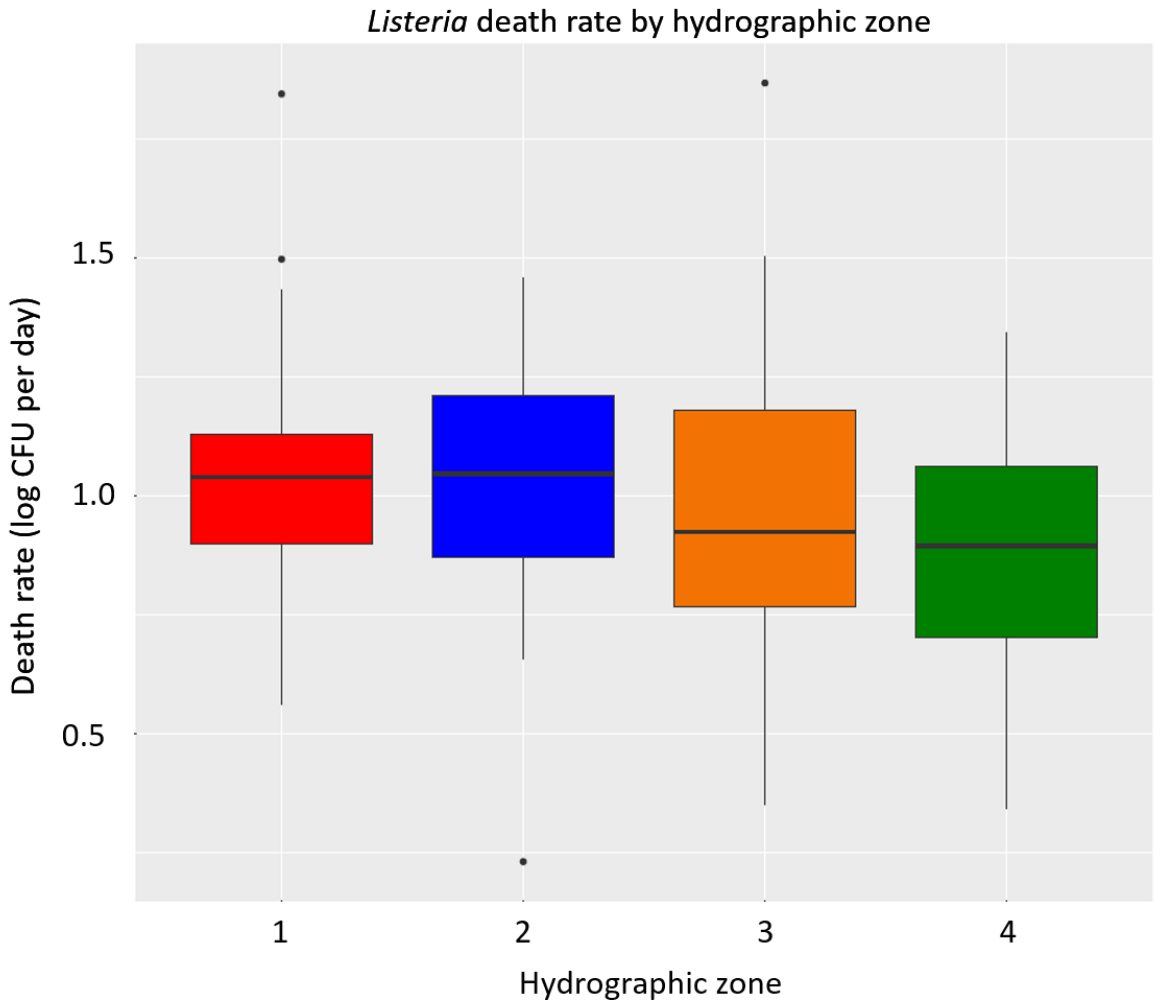

Figure 5. Death rate of *L. monocytogenes* by hydrography.

Figure 6. Death rate of *L. monocytogenes* by land use.

Table 11. Predictor variables chosen by backwards model selection.

Model #	Final model	Model AIC value
1	*kmax* ~ P + EC + moisture	-122.20
2	*kmax* ~ P + EC + moisture	-122.20
4	*kmax* ~ P + pH + Fe + EC:location + location:moisture	-121.53
3	*kmax* ~ P + EC + Fe	-119.31

EC: electrical conductivity; PD_whole_tree: phylogenetic diversity, OM: organic matter; P: phosphorus. Models are arranged by AIC value, from lowest (best fit) to highest (worst fit). P, EC, and moisture were consistently among the most important variables. Community diversity (as measured by phylogenetic diversity) was not included as an explanatory variable in any of the models.

Patterns of microbial diversity in prairie wetlands with hydrography and land use

Canonical correspondence analysis based on the family-level taxonomic assignments revealed which microbial communities were most closely related to each other (Beta diversity) as well as where different functional microbial groups were likely to be found in relation to environmental characteristics. In figures 7 and 8, the same plot is represented with two different color coding schemes – one with communities coded by hydrography and the other coded by land use. In the CCA plots, four functional groups are highlighted: methanogens, methanotrophs, anaerobic methane oxidizers (ANME-2D), and sulfate reducers.

In the first plot, several patterns emerge with respect to hydrography, the most prominent being the spreading pattern of the communities along the two axes as they come closer to the wetland center. The upland-most soils (zone 4) are the most tightly clustered, followed by the toe-slope soils (zone 3). This indicates that these communities are more closely-related to each other than they are to the sediment communities, even across the three different land use types. Furthermore, the samples from the wetland centers and margins (zones 1 and 2) are, in some cases, more separated from each other than from the upland soils. In other words, prairie pothole sediment communities are much more diverse than their associated upland soils. This is corroborated in the calculations of Faith's PD previously. Looking at the chemical attributes loaded as vectors onto the ordination, increased soil NO_3 and CO_2 flux are associated with the upland zones (3 and 4) while CH_4 flux and soil Fe and electrical conductivity are associated with sediments. Again, this is in agreement with statistical tests performed with the chemical data.

In the second plot, colored by land use class, additional insights are drawn out of the data. Among agricultural samples, sediment communities cluster separately from the other sediments at the bottom of the plot, and are characterized by increased Fe and P concentrations and lower

pH, as is evident by the pH vector aiming in the opposite direction of the plot. Likewise, grazing-impacted sediments cluster together along the vectors for CH_4 flux and electrical conductivity, and natural sediments tend towards higher pH.

The final piece of data in these ordinations is the location of several specific groups of microbial families. Sulfate reducers (triangles) are loaded along the vectors for CH_4 flux and electrical conductivity. Most methanogens and the one group of methanotrophs are oriented in the direction of increased Fe concentration and moisture. Unique among these is a family of Anaerobic Methane Oxidizers (ANME-2D), commonly found in marine environments (Haroon et al., 2013).

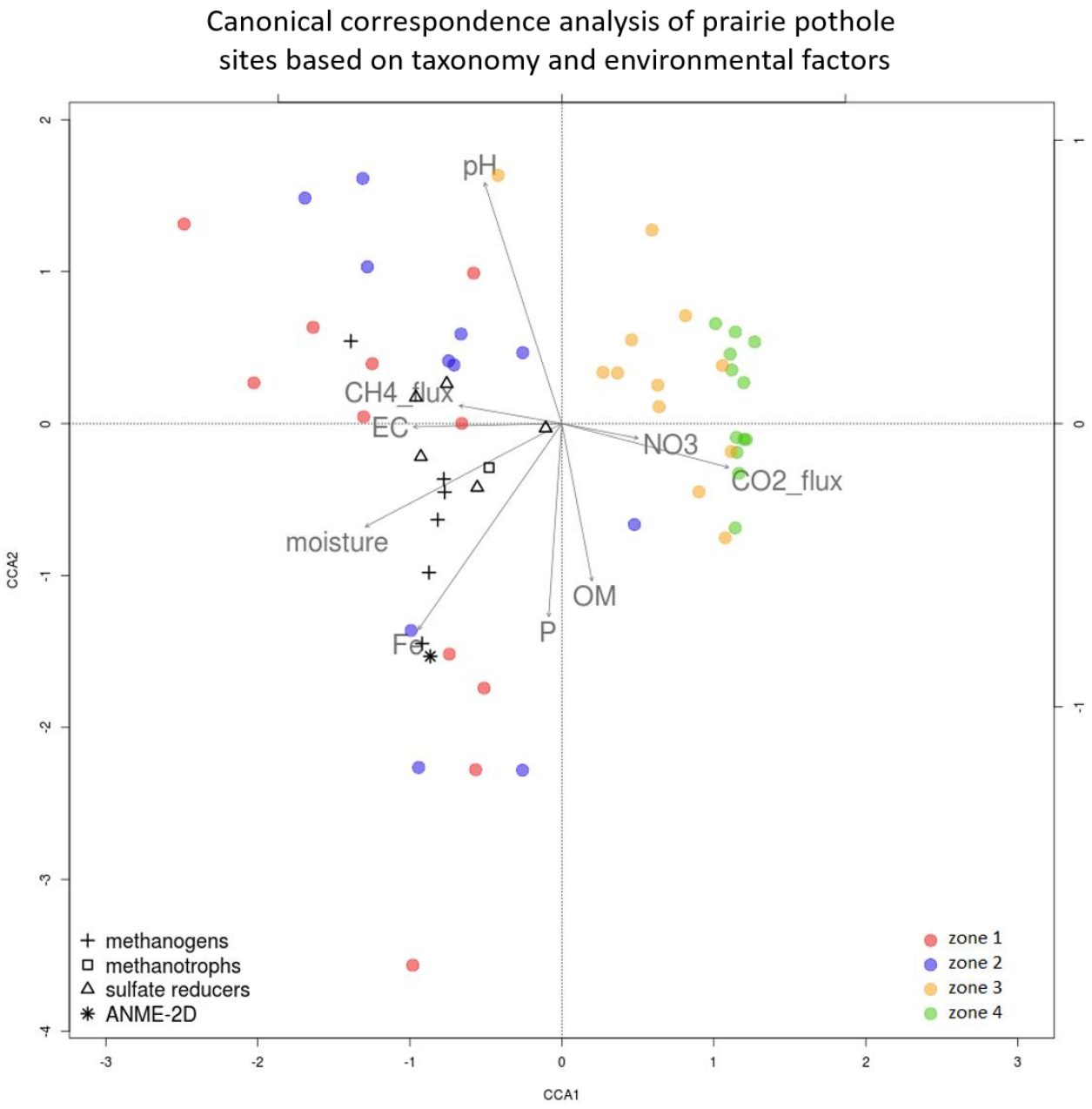

Figure 7. CCA triplot with samples coded by hydrography. Upland soils are more closely related to each other and are much less variable compared with submerged sediments, consistent with Faith's PD measurements. Upland soils are also characterized by increased NO3 content and CO2 flux.

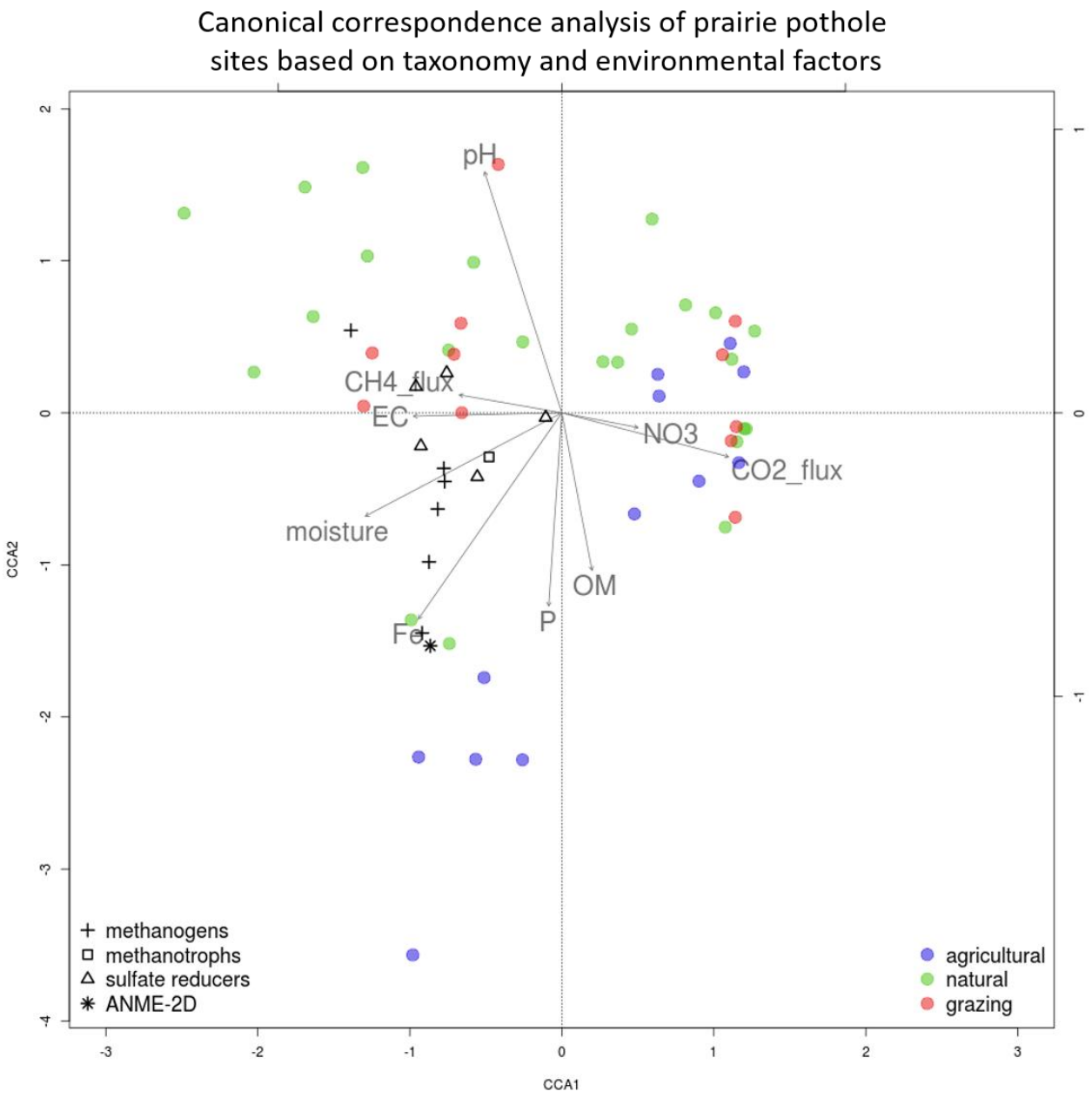

Figure 8. CCA triplot with samples coded by land use. A subset of agricultural sediments cluster near the bottom, and are associated with higher phosphorus content, consistent with soil chemical data. Grazed sediments are characterized by increased electrical conductivity, methane flux, and abundance of sulfate reducers.

When zooming in on specific groups of microbes, more phyla displayed variable abundance across different hydrographic zones than across different land use types. Some of the phyla that changed most dramatically with hydrography were *Acidobacteria*, *Actinobacteria*, *Chloroflexi*, *Euryarchaeota*, and *Proteobacteria* (Figures 9-13, respectively). Most interesting among these are the *Euryarchaeota* which contain all known families of methanogens. From the plot of *Euryarchaeota* by hydrographic zone, a dramatic shift is evident, with the abundance of methanogens being highest (and most variable) in the submerged sediments and decreasing to near undetectable levels in the upland-most soils. This observation is consistent with the two CCA triplots.

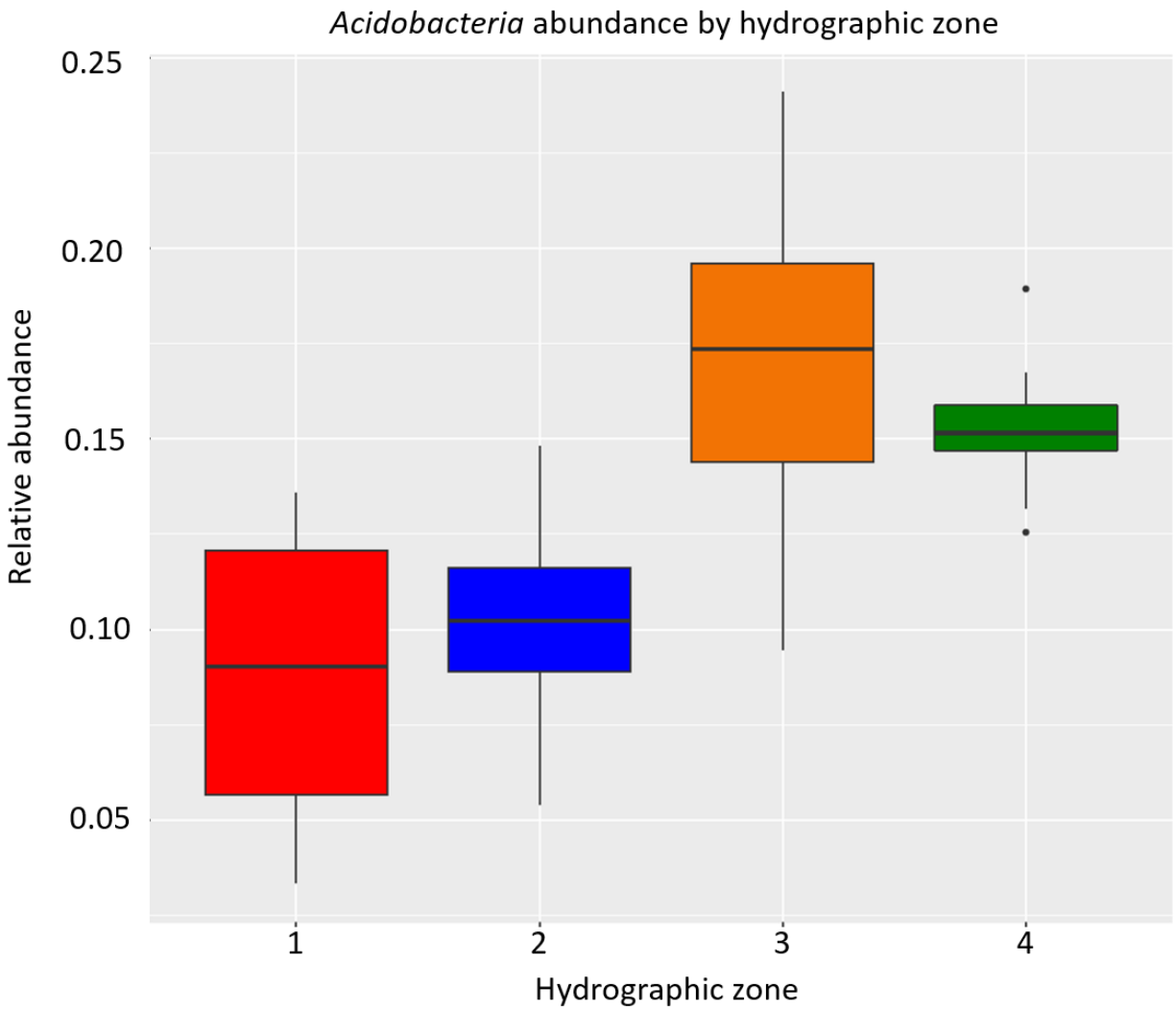

Figure 9. Change in abundance of *Acidobacteria* with hydrography.

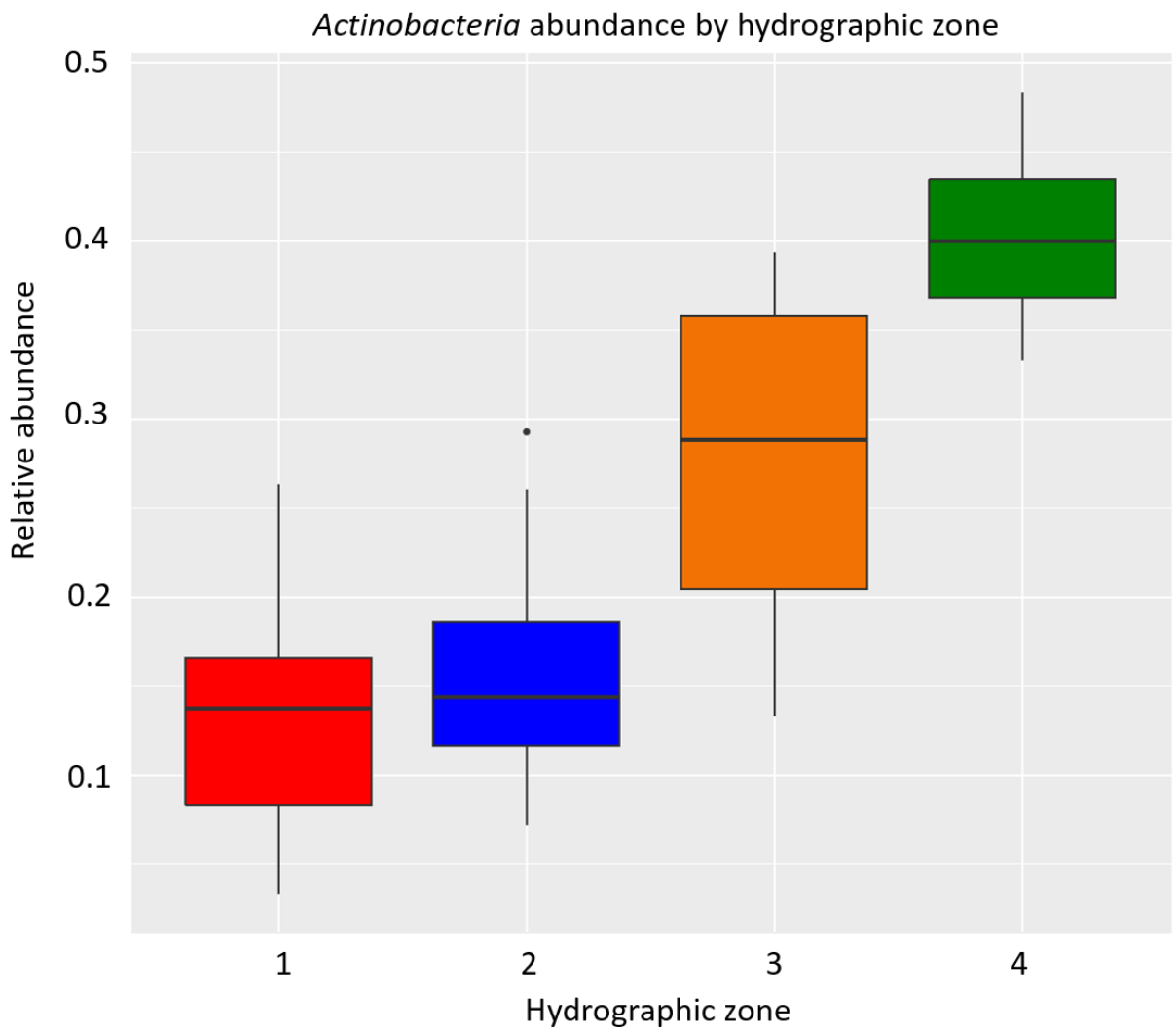

Figure 10. Change in abundance of *Actinobacteria* with hydrography.

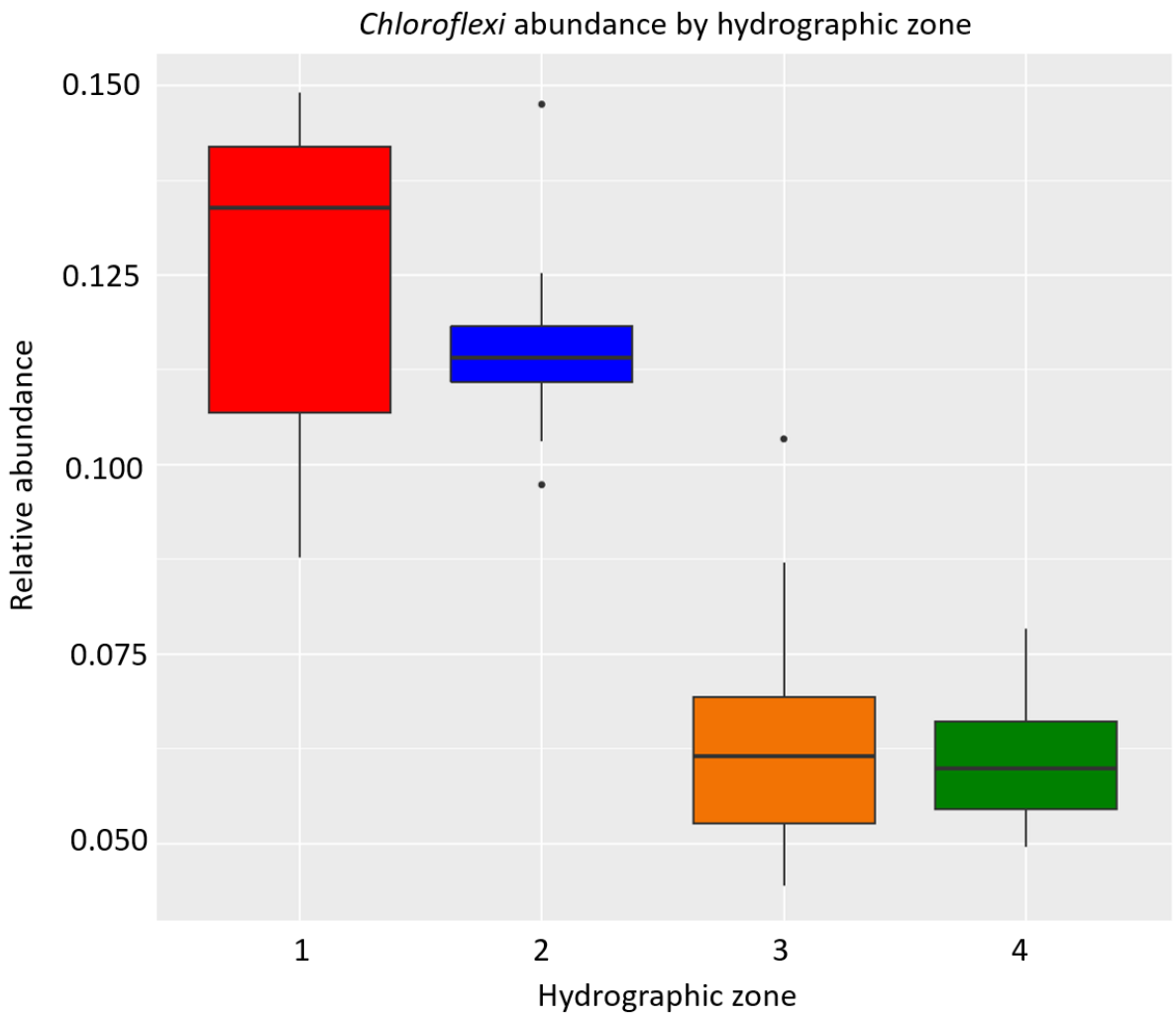

Figure 11. Change in abundance of *Chloroflexi* with hydrography.

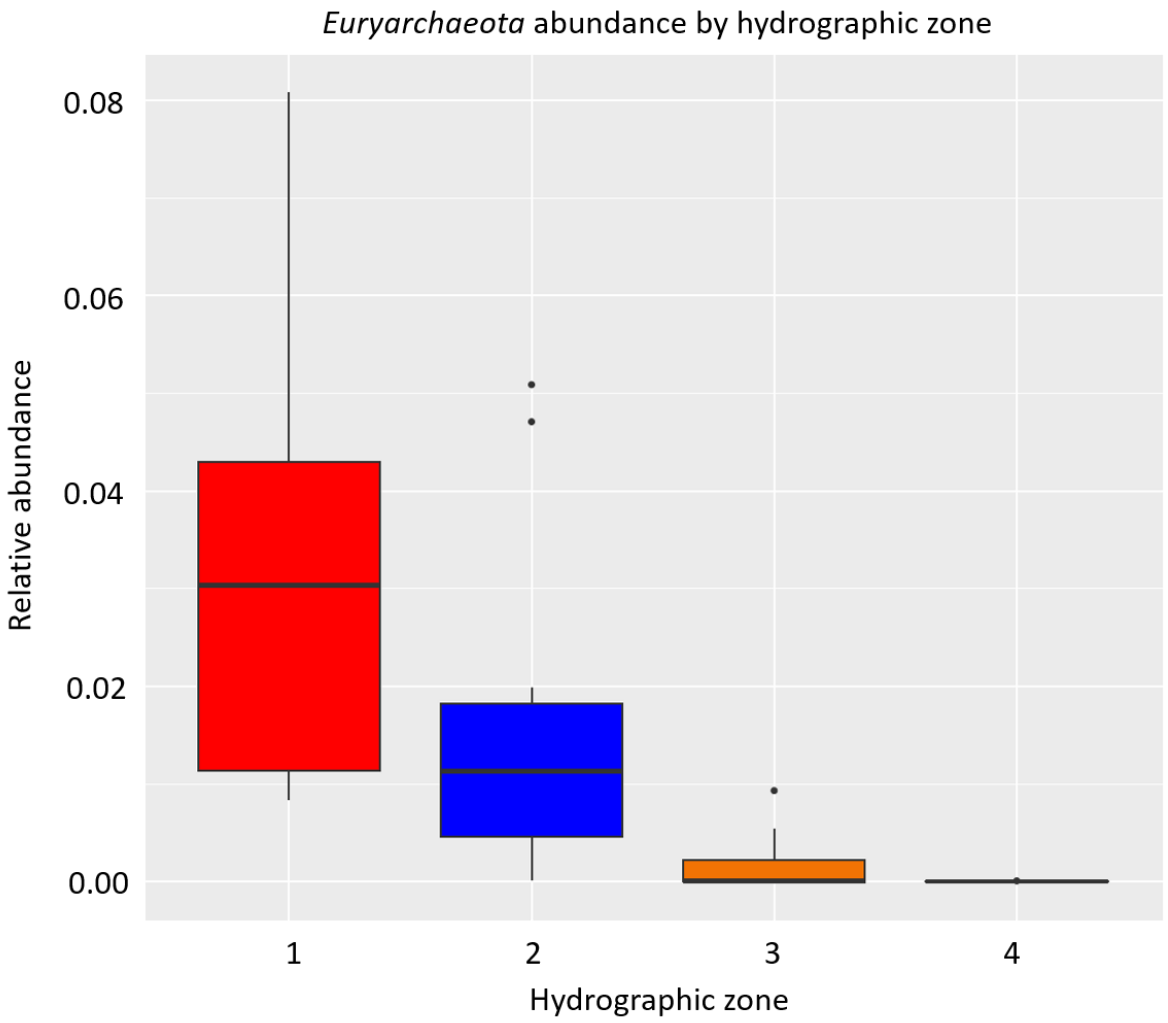

Figure 12. Change in abundance of *Euryarchaeota* with hydrography.

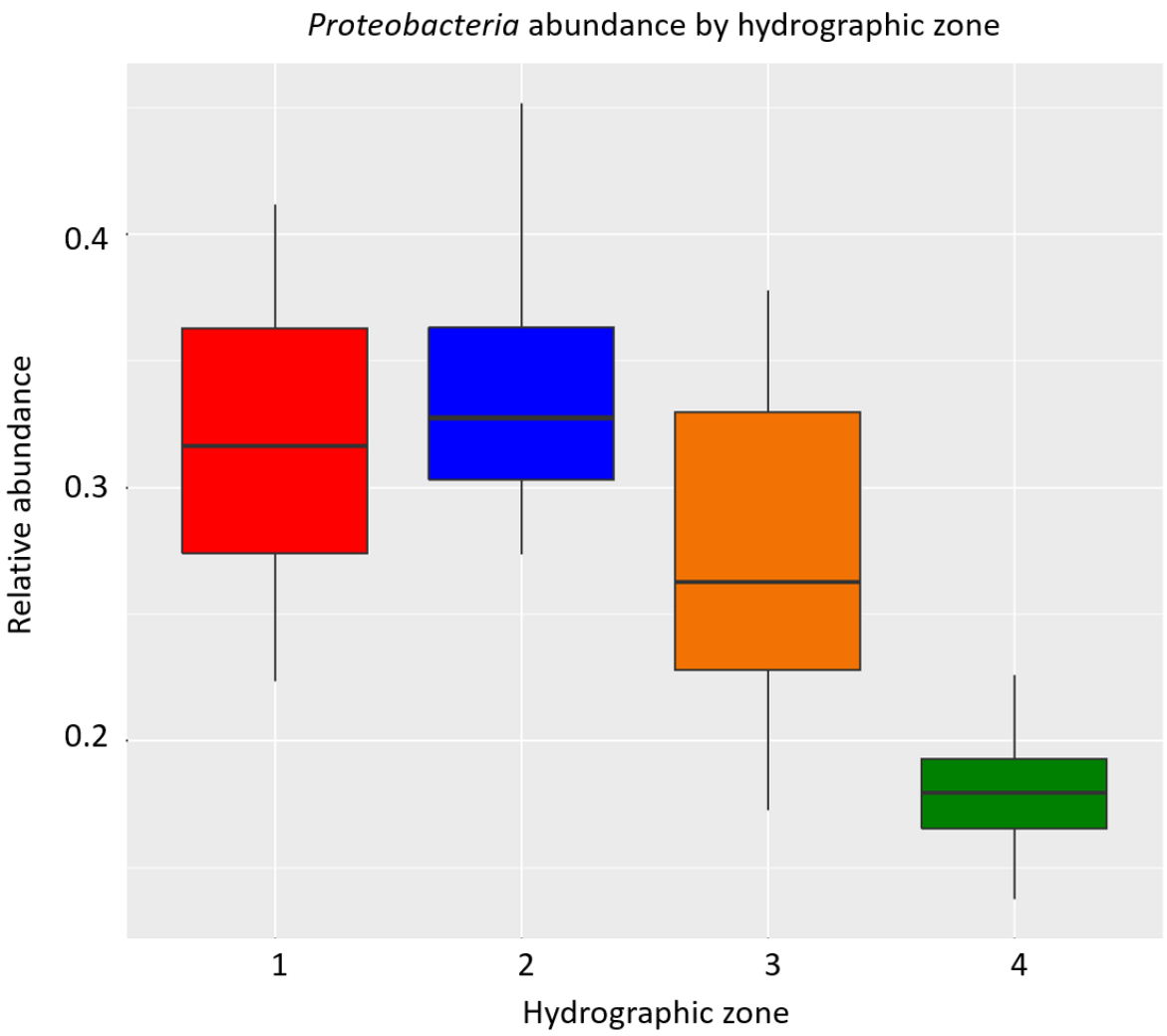

Figure 13. Change in abundance of *Proteobacteria* with hydrography.

Discussion

Microbial community diversity does not account for much variation in *L. monocytogenes* death rate

The results from the backward model selection of variables associated with *L. monocytogenes* death rate indicate that microbial community diversity does not greatly affect its ability to survive in wetland soils and sediments. The predictor variables consistently deemed to be most important in determining the death rate of *L. monocytogenes* were phosphorus, electrical

conductivity, and moisture; phylogenetic diversity (PD) was not included in any of the final models. *L. monocytogenes* did display a slight negative trend in death rate associated with hydrography similar to the trend seen in Faith's PD, but this apparent correlation could be caused by many different factors working synergistically. For instance, some properties, such as electrical conductivity and moisture, were associated both with community diversity and *L. monocytogenes* death rate. Using the data gathered in this study, it is not possible to infer which factor or set of factors drove the effects observed. In order to determine the causal relationships underlying trends in community diversity and *L. monocytogenes* death rate, a more controlled study is required.

Several studies using microcosms have indicated that the native soil microbial community does reduce the survival of *L. monocytogenes* (Hartmann et al., 2013; Vivant et al., 2013; McLaughlin et al., 2011; Welshimer, 1960), which provides support for a diversity-invasion relationship between *L. monocytogenes* and soil microbiota. However, most of these studies are performed with microcosms in which diversity is manipulated in some way in order to simulate a gradient of diversity. A popular way of doing this is the "dilution to extinction" method, wherein native soils are serially diluted with autoclaved soil, resulting in increasingly diminished microbial diversity with increasing proportions of sterile soil incorporated (van Elsas et al., 2012; Falcao Salles et al., 2015; Vivant et al., 2013). The result is a reduction not just in the abundances of certain groups that might be affected by a perturbance, but a reduction in the abundance of all species simultaneously. In this study, community diversity was not manipulated, but rather, the natural differences in diversity among the different samples were included as one variable among many. Therefore, it is understandable that the effect of microbial

community diversity may be more subtle in this type of study than in a controlled experiment in which moisture, soil chemistry, and community diversity can be manipulated in specific ways.

The four models generated using backward model selection instead indicated that phosphorus, electrical conductivity, and moisture are most important in determining the survival of *L. monocytogenes* in wetland soils and sediments. Several studies have indicated the importance of moisture to the survival of *L. monocytogenes* (Bergholz et al., 2013; Strawn et al., 2016, Welshimer 1960). But in these studies, higher moisture content and availability of surface water have led to increased survival of the pathogen whereas in this study, *L. monocytogenes* death rate was higher in the submerged sediments than it was in upland soils. This suggests, perhaps, that increased soil moisture is a benefit provided that the soils are not completely submerged. Even though *L. monocytogenes* is a facultative anaerobe, it could be that the chemistry of the anoxic wetland sediments creates an environment that is less hospitable to the organism than high moisture non-submerged soils. Electrical conductivity was increased in the sediments, and although the role of electrical conductivity in the survival of *L. monocytogenes* has not been extensively studied, one group did find decreased lag times and increased cell densities associated with low conductivity growth medium, defined as < 1.3 mS (Bhunia et al., 2006). To place this in perspective, in the prairie potholes surveyed, electrical conductivity ranged from a minimum of 0.30 mMhos/cm (equivalent to 0.3 mS) to a maximum of 4.30 mMhos/cm (4.3 mS). Therefore, it is possible that is some of the sediments, excess moisture is not itself the driver of increased *L. monocytogenes* death rate, but rather, the higher salinity.

Among the available literature – especially in microcosm studies – the factors that have the greatest impact on *L. monocytogenes* death rate are available moisture and soil texture. In this study, the two factors other than moisture that contributed most to increased death rates were

phosphorus and electrical conductivity. Further controlled microcosm experiments examining these and other factors potentially associated with different land management strategies could shed light on the dynamics of *L. monocytogenes* survival in soils of extreme salinity and chemical perturbation.

Wetlands are sources of methane while uplands are sources of carbon dioxide

Measures of Faith's PD and clustering patterns in the CCA both indicate that prairie pothole sediments are more diverse than their associated upland soils. This was surprising, since it was expected that the effects of land use on microbial community composition would be more pronounced in samples most distant from the wetland center. For example, the zone 4 sample locations in the agriculturally impacted areas were in fields tilled and planted with row crops, as compared to those in the same zone in natural areas, which were characterized by grassy groundcover and higher soil compaction. In this same zone in grazing pastures, the effects of grazing, both in terms of reduced grassy ground cover and cattle fecal deposits were apparent. And yet, the zone 4 communities are almost indistinguishable from each other in the CCA plots with regards to land use.

One explanation as to why the sediment communities were observed to be more diverse than their upland counterparts could be that prairie potholes are geographically isolated from each other, leading to a situation, where members from one sediment community have few pathways by which they can be introduced to a neighboring sediment community. Alternatively, it may be that since sediments are by their nature submerged, chemical inputs to the system accumulate in sediments whereas in upland soils, precipitation and drainage working hand in hand might serve to prevent those same inputs from accumulating – at least at the soil surface.

CCA also showed that upland soils were associated with increased CO_2 flux while the submerged sediments were associated with increased CH_4. This is consistent with other studies of these same potholes looking at the relationship between hydrography and greenhouse gas flux. A study conducted by Gleason and colleagues showed that wetlands are much greater sources of CH_4 than the nearby upland soils (Gleason et al., 2015), and that even though prairie pothole wetlands often act as sinks of atmospheric CO_2, the global warming potential of CH_4 is higher than CO_2, increasing the contribution of prairie potholes to planetary warming (Gleason et al., 2015; Jeppesen et al., 2015; Mitsch & Bernard, 2013). Another finding of this study related to CH_4 was the association in the CCA triplot between grazing-impacted sediments and increased CH_4 flux. This makes sense given research showing that cattle themselves act as sources of methane, leading to increased methane production associated with grazing lands (McGeough et al., 2014; Byers et al., 1999). However, the presence of methanogenic families of archaea arranged in the same direction as the methane flux vector suggests a potential contribution from biogeochemical sources as well.

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