ANTHROPOGENIC STRESSORS ON FRESHWATER WETLANDS: A MICROBIAL PERSPECTIVE

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DOCTOR OF PHILOSOPHY

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ABSTRACT

Benthic microbial communities play fundamental roles in wetland ecosystems including nutrient and energy cycling, and the degradation and assimilation of pollutants. Because of these crucial roles, along with their short-life cycles and high diversity, microorganisms can also play an important role as indicators of environmental change, which is particularly relevant in the current climate of increasing anthropogenic stressors, including factors such as emerging pollutants and climate change. Consequently, understanding the responses of microbes to environmental change is critical. To assess the effects of anthropogenic stressors on microbial communities in wetland ecosystems, I examined the response of sediment microorganisms from North Dakota wetlands in both microcosm and field-scale studies. First, I used 16S rRNA gene sequencing to analyze wetland microbial community responses to glyphosate treatments using an experimental microcosm approach. I found no significant differences in microbial communities among concentrations or treatments compared to controls, suggesting microorganisms in this region may have evolved glyphosate tolerance. Second, also taking an experimental approach, I measured methane, carbon dioxide, and nitrous oxide flux and porewater concentrations in microcosms to analyze net microbial production and consumption of greenhouse gases following glyphosate and/or 2,4-D treatment. I found high glyphosate concentrations significantly increased carbon dioxide emissions potentially due to increased microbial activity from the use of glyphosate as a substrate, or due to increased respiration as a stress response. Lastly, I used 16S rRNA gene sequencing to compare microbial communities in natural and restored wetlands across the North Dakota Prairie Pothole Region to assess the effects of a physical stressor, hydrologic restoration. I found no significant differences in microbial communities across wetlands, which may be due to the lack of direct sediment disturbance from restoration, or due to

the ability of microorganisms to rapidly recover, thus showing no assemblage differences 25 years after restoration. Overall, I demonstrated that integrating microbial ecology with ecotoxicology and restoration ecology can be a beneficial and applicable research approach to understanding the impact of anthropogenic-induced environmental change on wetlands and show that the use of microbial metrics and mechanisms can provide valuable insight on pertinent issues of global concern.

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DEDICATION

This dissertation is first dedicated to my husband, Logan Cornish, who has whole-heartedly and without hesitation supported me throughout this chaotic journey. I cannot begin to express my gratitude having you as my partner, best friend, and biggest supporter. I will forever cherish our time in Fargo trying new foods, weathering many blizzards, and always finishing the day at our favorite local breweries. Cheers to our next adventure!

I would also like to dedicate this dissertation to Briana Anderson. You have believed in me since the day we met and embraced every part of me. You challenge me to be a better person, teacher, scientist, and adventurer. I could not have accomplished this milestone without you and your support. Our dark, witchy souls will forever be in sync longing to lose ourselves in the forests as Mother Earth calls to us. Thank you for navigating this journey with me from afar. To Salem, we fly!!

Rest in Peace to my little nugget, Gidget.

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LIST OF ABBREVIATIONS

ANOVA .....analysis of variance

cm.....centimeter

g.....gram

L .....liter

µg g^{-1} .....microgram per gram

µg kg^{-1} .....microgram per kilogram

µg L^{-1}.....microgram per liter

µL.....microliter

µmol L^{-1}.....micromole per liter

µS cm^{-1} .....microSiemen per centimeter

mg kg $soil^{-1}$ hr^{-1}.....milligram per kilogram of soil per hour

mg L^{-1}.....milligram per liter

mL .....milliliter

mm .....millimeter

ng.....nanogram

NMDS.....non-metric multidimensional scaling

ppm .....parts per million

yrs.....years

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INTRODUCTION

Wetlands are critical ecosystems providing many important functions such as flood protection, habitat, pollutant assimilation and transformation, and biogeochemical cycling (Euliss et al. 2006; Gleason et al. 2011). However, they are also some of the most threatened habitats due to fragmentation, urbanization, pollution, and agriculture (Doherty et al. 2018). Agrochemical contamination of wetlands is an ongoing concern as pesticide use continues to increase with population growth and climate change stress (Koli et al. 2019). Herbicides are agrochemicals that target various physiological mechanisms in noxious weeds, but approximately only 5% of herbicides applied through aerial or ground spray reach target weeds (USDA, 2024). In addition, herbicide use has substantially increased since the 1990's with the introduction of herbicide-resistant crops (Pollegioni et al. 2011; Duke 2021). Specifically, glyphosate [*N*-(phsophonomethyl)glycine] is the most widely used herbicide worldwide, where glyphosate-resistant crops account for greater than 80% of crops, and glyphosate use has more than doubled as a consequence (Benbrook 2016; Duke 2021). Rapid and widespread utilization of glyphosate-resistant crops in agriculture has substantially diminished glyphosate's efficiency as over 350 weeds around the world have developed tolerance (Green and Siehl 2021). This phenomenon has also been observed with other commonly used active ingredients, such as 2,4-D, atrazine, dicamba, and others (Heap 2024), and subsequently frequent herbicide application and the use of pre-mixed herbicides (i.e., multiple active ingredients) is common practice to combat weed tolerance (Islam et al. 2018). As a result, non-point source herbicide contamination of shallow, depressional wetlands is prevalent.

Benthic sediments often serve as sinks for herbicides either rendering them biologically unavailable from adsorption (Warren et al. 2003; McMurry et al. 2016), or facilitating

biodegradation, however residues may also remobilize into the water column (Maqueda et al. 2017). Biodegradation is the primary breakdown process of herbicides in wetlands resulting in various metabolites, where both the parent compound and metabolites could be toxic, or on the contrary could be metabolized by microorganisms (Kools et al. 2005; Borggaard and Gimsing 2008; Shushkova et al. 2009). While herbicides target higher plants, bacteria and archaea can be inadvertently affected because they contain many of the same mechanisms targeted in plants (Cox 1995; Herrmann and Weaver 1999). Glyphosate, for example, targets the shikimate pathway by inhibiting the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) (Cobb and Reade 2010), which is present in approximately 50% of bacteria and 80% of archaea making them susceptible to glyphosate (Leino et al. 2021). Whereas, other microorganisms contain a naturally glyphosate-resistant EPSPS, or have evolved tolerance, enabling these species to potentially metabolize and utilize glyphosate as a nutritive source (Powell et al. 1991; Shushkova et al. 2009). Partly due to its prevalence, in addition to its potential impact, there has been extensive research on glyphosate use and its fate in wetland ecosystems (Duke and Powles 2008; Battaglin et al. 2014; Annett et al. 2014; Bai and Ogbourne 2016; Benbrook 2016), however with high variability in microbial community composition and structure (Louca et al. 2016) and the wide range of possible effects of glyphosate, there are many conflicting studies on its effects on microorganisms.

Dissertation Outline

Wetlands are often the final destination of herbicides, where benthic sediments can act as sinks exposing microbial communities to potentially impactful residues. Glyphosate and other herbicides can both directly alter microbial communities via toxicity or nutrient enrichment, or subsequently having indirect ecosystem-level effects, such as altering wetland function. To

investigate the direct and indirect effects of commonly used herbicides on freshwater wetlands, I used a multidisciplinary approach, using multiple spatial scales, focusing on the response of benthic sediment microbial communities.

Chapter 1: How benthic sediment microbial communities respond to glyphosate and its metabolite: A microcosm experiment

Glyphosate in wetlands is primarily degraded through microbial metabolic processes resulting in its main metabolite, aminomethylphosphonic acid (AMPA). Both glyphosate and AMPA can impact microorganisms through several mechanisms including toxicity and nutrient enrichment, subsequently altering microbial community composition. Benthic microbial communities play an essential role in wetland function, thus understanding the impacts of glyphosate-based herbicides on wetland microbial communities is vital. I conducted a microcosm experiment to examine the effects of glyphosate and AMPA on benthic sediment microbial communities collected from the North Dakota Prairie Pothole Region. Treatments included a control, low, medium, or high concentration of analytical-grade glyphosate or AMPA, or a commercially available glyphosate-based formula. By comparing analytical-grade and commercial formula treatments, it provides insight into formula effects (i.e., inert ingredients). I used 16S rRNA gene sequencing to analyze sediment microbial communities pre-treatment, 2-hours post-treatment, and 2-weeks post-treatment. This experiment was used to isolate the direct effects of glyphosate-based treatments on benthic sediment microorganisms to help determine how communities with no previous agricultural disturbance respond to agrochemical stress. The findings from this dissertation chapter have been published in the journal *Microbial Ecology* (Cornish et al. 2023).

Chapter 2: A perspective on how glyphosate and 2, 4-D in wetlands may impact climate change

One of the consequences of herbicide contamination of wetlands and their potential impacts on microorganisms could be alterations in biogeochemical functioning resulting in shifts in greenhouse gas cycling. This has been observed with 2,4-D, as it is a known methane oxidation inhibitor, however less is known about glyphosate, and even less is known about the combined effects of glyphosate and 2,4-D. As wetlands are important sources and sinks of methane, a potent greenhouse gas, it is crucial to better understand how herbicides could disrupt methane production and consumption. In this chapter, I summarized the literature and provided a perspective on how glyphosate and 2,4-D contamination of wetlands may contribute to increased greenhouse gases from freshwater wetlands. This chapter has been published in *Frontiers in Environmental Science* (Cornish and Sweetman 2023).

Chapter 3: Common use herbicides increase wetland greenhouse gas emissions

Wetlands are natural sinks and sources of greenhouse gases, but are increasingly vulnerable to anthropogenic-induced environmental disturbances, such as climate change and agrochemical use. Glyphosate and 2,4-D are extensively used herbicides that have substantially increased in use since the commercialization of herbicide-resistant crops, which has resulted in herbicide tolerance of many noxious weeds. Subsequently, the combined use of glyphosate and 2,4-D to combat tolerant weeds has increased. In wetlands, glyphosate can act as a nutrient source for microorganisms and 2,4-D can inhibit oxidation processes, such as methane oxidation. Therefore, when used in combination they may synergistically impact biogeochemical cycling resulting in increased greenhouse gas emissions from freshwater wetlands. To investigate this, I conducted a microcosm experiment measuring the individual and combined effects of glyphosate

and 2,4-D on greenhouse gas emissions. I measured methane, carbon dioxide, and nitrous oxide flux and porewater concentrations in non-vegetated microcosms to isolate herbicidal effects on microbial production and consumption of these greenhouse gases. This experiment was used to provide insight into how common use herbicides could impact wetland biogeochemical cycling and have global consequences for climate change. This chapter has been submitted for peer-reviewed publication, and is currently in review at *Science of the Total Environment*. A preprint is available (Cornish et al. 2024, http://dx.doi.org/10.2139/ssrn.4745228).

Chapter 4: Do sediment microbial communities recover after wetland restoration?

Across the Prairie Pothole Region many wetlands have been lost or altered due to agricultural development and other human activities. However, for several decades wetland restoration efforts have occurred to restore and conserve the various ecosystem services they provide. Specifically, benthic sediment microorganisms contribute to overall wetland health through critical biogeochemical processes where restoration may inadvertently cause disturbance to microbial communities. Extensive research has been conducted on the impacts of restoration to abiotic properties, in addition to macrophyte and waterfowl communities. However, there is a gap in the research looking at the recovery of microorganisms after restoration. I conducted a field survey of 17 wetlands to determine how wetland restoration impacted benthic sediment microbial communities. Five wetlands were natural and twelve were restored, along a gradient of time since restoration, where early restorations occurred 32 – 33 years, mid 30 years, and late 26 – 28 years prior to sampling. I collected benthic sediments and sequenced the 16S rRNA gene to look at microbial richness, diversity (alpha and beta), and functions among natural and restored wetlands. This project was used to aid wetland management practices by providing information on microbial recovery, thus giving a more comprehensive perspective on ecosystem recovery.

This project was in collaboration with a larger EPA-funded project, *"Ecosystem Services of Restored PPR Wetlands as a Function of Restoration Age"* with Dr. Marinus Otte and Dr. Jon Sweetman, which also examined changes in other aspects of wetland ecosystems, including water chemistry, vegetation and macroinvertebrate communities within the same set of natural and restored wetlands to better understand wetland recovery following restoration.

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CHAPTER 1. HOW BENTHIC SEDIMENT MICROBIAL COMMUNITIES RESPOND TO GLYPHOSATE AND ITS METABOLITE: A MICROCOSM EXPERIMENT[1]

Abstract

Glyphosate is the most commonly used agricultural herbicide in the world. In aquatic ecosystems, glyphosate often adsorbs to benthic substrates or is metabolized and degraded by microorganisms. The effects of glyphosate on microbial communities varies widely as microorganisms respond differently to exposure. To help understand the impacts of glyphosate on the sediment microbiome we conducted a microcosm experiment examining the responses of benthic sediment microbial communities to herbicide treatments. Sediments from a prairie pothole wetland were collected and 16S rRNA gene sequencing was used to analyze community composition 2-hours and 14-days after a single treatment of low (0.07 ppm), medium (0.7 ppm), or high (7 ppm) glyphosate, aminomethylphosphonic acid (glyphosate metabolite), or a glyphosate-based commercial formula. We found no significant differences in microbial community composition across treatments, concentration levels, or day of sampling. These findings suggest that microbial species in the Prairie Pothole Region of North Dakota may be tolerant to glyphosate exposure.

Introduction

Agrochemical contamination of aquatic ecosystems is an ongoing concern due to the direct and indirect risks to environmental health. Glyphosate (i.e., Roundup®) is a non-selective,

[1]The material in this chapter was co-authored by Christine Cornish, Peter Bergholz, Kaycie Schmidt, and Jon Sweetman. Christine Cornish had primary responsibility for material preparation, data analyses, and drafting the manuscript. Peter Bergholz and Kaycie Schmidt assisted with data collection. Peter Bergholz and Jon Sweetman served as proofreaders and provided minor text additions and data analysis suggestions.

systemic herbicide that has become the most commonly used herbicide in the world since the 1990s (Benbrook 2016). The substantial use of glyphosate has resulted in its widespread and frequent detection in surface waters and groundwater (Coupe et al. 2012; Belden et al. 2012), where benthic sediments often become sinks (Warren et al. 2003). Microbial metabolism is the primary degradation mechanism of glyphosate (Battaglin et al. 2014) resulting in its metabolites, aminomethylphosphonic acid (AMPA) and glyoxylate, or phosphate and sarcosine (Zhan et al. 2018).

Glyphosate targets higher plants through the inhibition of the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) enzyme in the shikimate pathway (Cobb and Reade 2010), but this pathway is also present in some microorganisms (Herrmann and Weaver 1999). Approximately 80% of archaea and 50% of bacteria contain the class I glyphosate-sensitive EPSPS protein, whereas the class II - IV glyphosate-resistant EPSPS protein is less common (Leino et al. 2021). Therefore, it was initially presumed that glyphosate may have inadvertent effects on microbial communities. Studies have reported that glyphosate can have a wide range of negative, positive, or neutral impacts on microbial community composition and function in terrestrial and aquatic ecosystems. Aquatic microbial communities have been shown to utilize glyphosate as a nutrient source resulting in increased activity, whereas in others, microbes are inhibited by toxicological effects. Pérez et al. (2007) found 6 mg L^{-1} and 12 mg L^{-1} Roundup® caused a significant decrease in average microphytoplankton and nanophytoplankton abundance. However, Lu et al. (2020) found no community structure shifts from 2.5 mg L^{-1} glyphosate, and found significantly enhanced gene expression in mechanisms potentially related to glyphosate tolerance. Several microbial species are known to exhibit glyphosate tolerance (Zhan et al. 2018), in addition to species capable of using glyphosate directly as a nutrient source (Forlani et al. 2008; Shushkova

et al. 2009). These examples demonstrate the variety of impacts glyphosate can have on aquatic microbial communities.

In addition to the impacts of glyphosate, AMPA, glyphosate's main metabolite, can also have direct and indirect effects on aquatic ecosystems (Grandcoin et al. 2017). Similar to glyphosate, AMPA can be degraded by microorganisms, but it is more mobile and persistent (Kjaer et al. 2005; Degenhardt et al. 2012; Blake and Pallett 2018). It is also a weak phytotoxin (Reddy et al. 2008) with additional concerns regarding its potential to bioaccumulate (Bai and Ogbourne 2016). AMPA is highly dependent on the presence and concentration of glyphosate (Beecraft and Rooney 2021), where both compounds frequently co-occur in areas of high agricultural intensity (Battaglin et al. 2014; Malaj et al. 2020). Glyphosate and AMPA are most often detected in surface soil (Okada et al. 2016), and are frequently transported to surface waters, including wetlands (Coupe et al. 2012; Battaglin et al. 2014) where they could impact benthic microorganisms.

The Prairie Pothole Region (PPR) is a large complex landscape covered with shallow wetlands and prairies (Dahl 2014). Glyphosate is extensively used on corn and soybean, which are the predominant crops surrounding this region (Coupe et al. 2012; Benbrook 2016). Consequently, wetlands in the PPR are subject to prolonged glyphosate contamination, where glyphosate has been reported as the most frequently detected and the highest detected herbicide in this region (McMurry et al. 2016). These wetlands are key ecosystems providing many economic and ecological services such as, waterfowl production, flood protection, and nutrient cycling. For example, biogeochemical processes including C turnover and sequestration, N and P capture, and remediation of agrochemicals are essential ecosystem functions (Holloway et al.

2011; Dalcin Martins et al. 2017), where benthic sediment microbial communities play a fundamental role in these ecosystem processes.

Understanding the impacts of agrochemical contamination on microbial community structure is vital because shifts in microbial communities impact the whole ecosystem. This is especially important in a region with high agricultural intensity, like the PPR, because microorganisms are chronically exposed to chemical stressors like glyphosate. The objective of our research was to assess the responses of benthic sediment microbial communities from the PPR to glyphosate-based herbicide treatments. We hypothesized that 1) richness (i.e., observed Operational Taxonomic Units [OTUs]) and diversity would decrease at high herbicide concentration due to toxicological effects, and 2) microbial community composition would shift at low herbicide concentration in favor of species capable of metabolizing glyphosate.

Materials and Methods

Microcosm preparation

Surface sediment (to a depth of ~ 10 cm) was collected in July 2019 and stored in a sterilized (90% ethanol) cooler from a wetland (47.0984758 °N, -99.1018688 °W) within the Cottonwood Lake Study Area (U.S. Fish and Wildlife service managed Waterfowl Production Area) of the PPR located in Stutsman County, North Dakota. This wetland is composed of glacial till with approximately 26% organic matter content (Hu et al., unpublished). The area has minimal agricultural influence, where native prairie grasslands and wetlands cover over 80% (Winter 2003). After collection, sediment was stored refrigerated (~ 4 °C) until initiation of the experiment. Thirty-two 5.7 L microcosms were prepared with the following contents: 1 cm layer of homogenized sediment, 2.5 L of dechlorinated tap water, and covered with “no-see-um” mesh (Duluth Sport Nets, Duluth, MN). Microcosms were then stored in incubators at 20 °C with a

16:8 (light:dark) hour photoperiod and left to acclimate for one month prior to treatment. Over the entirety of the 6-week experiment, microcosms were monitored for water evaporation and were filled back to the 2.5 L volume with dechlorinated tap water when necessary.

Herbicide treatment and sediment sampling

We conducted a factorial experiment with 3 herbicide treatments x 3 concentration levels x 3 replicates and 5 controls (n = 32). Analysis was conducted at three timepoints (total n = 32 x 3 = 96). Herbicide treatments consisted of analytical grade glyphosate (*N*-(Phosphonomethyl)glycine; 98.1% purity) or AMPA (99% purity) purchased from Sigma Aldrich (Saint Louis, Missouri), or 41% glyphosate concentrate (commercial formula), which contains glyphosate isopropylamine salt. All herbicide solutions were made using serial dilutions in HPLC water to reach a final glyphosate or AMPA concentration of 0.07 parts per million (ppm), 0.7 ppm, or 7 ppm. Our concentrations were chosen based on the U.S. maximum contaminant level (MCL) of glyphosate in drinking water (U.S. EPA 2015), which is equivalent to our medium concentration of 0.7 ppm (or 7 mg L^{-1}). Treatments were added to the water, lightly stirred to evenly distribute, and then allowed to settle for two hours. A 50 mL sterile polypropylene corer (needle-less syringe) was used to collect approximately 20 g (wet weight) of sediment from each microcosm pre-treatment, two hours post-treatment, and two weeks post-treatment (total n = 96). All samples were stored in sterile 50 mL polypropylene tubes at -80 °C immediately after sampling until analyses were performed.

Analyses: Herbicide residues and microbial 16S sequencing

Samples were thawed to obtain subsamples for herbicide residue and microbial analyses, and immediately shipped or returned to -80 °C for their respective analyses. Sediment subsamples (~ 10 g wet weight) were shipped frozen on dry ice to the Agriculture and Food

Laboratory at the University of Guelph (accredited lab in Ontario, Canada) for glyphosate and AMPA residue analysis. Samples were homogenized to analyze (wet weight) a representative amount of each sediment sample using liquid chromatography/electrospray ionization-tandem mass spectrometry (LC/ESI-MS/MS) to quantify glyphosate and AMPA residues. Samples were extracted using a quick, easy, cheap, effective, rugged, and safe (QuEChERS) method, which included acidified aqueous extraction (acetic acid in acetonitrile in the presence of anhydrous sodium acetate and magnesium sulfate) and solid phase extraction. Instrumentation analysis of sample extracts were conducted using SCIEX 5550 ESI-MS/MS with Agilent 1260 HPLC in positive mode with a cation guard column for chromatographic separation, and 0.1% formic acid in water and acetonitrile as mobile phase A and B, respectively. The instrument limit of detection was 0.005 ppm and the limit of quantification was 0.02 ppm for glyphosate and AMPA. Deuterium labelled internal standards, matrix blanks, spikes, and calibration standards were analyzed for quality control (QC), and identification and quantification of both compounds.

Sediment microbial communities were analyzed after extraction of environmental DNA with the Qiagen DNeasy® PowerSoil® kit. Briefly, 0.25 g sediment (wet weight) was lysed using PowerBead Tubes in a bead-beater (Biospec Mini-beadbeater-24). Kit solutions (C1 – C6) were added stepwise to purify, wash, and elute DNA into 100 µL final volume. Microbial 16S rRNA gene was PCR amplified using universal primers, 27F and U1492R, for QC to verify suitability for sequencing. Sequencing library preparation and sequencing were performed according to Oxford Nanopore Technologies 16S Barcoding Kit (SQK-RAB204) protocol and reagents. Briefly, amplicons were cleaned using AMPure XP bead cleanup, quantified via PicoGreen™ analysis (Quant-iT PicoGreen™ Kit, modified from Invitrogen's Quant-iT PicoGreen™ dsDNA Reagent and Kits protocol), and combined into a pooled sample to obtain a final DNA

concentration of 50 – 100 ng. Pooled samples contained up to twelve uniquely barcoded samples for sequencing using an Oxford Nanopore minION™. Each library was run for approximately four hours. Raw fast5 reads were basecalled and demultiplexed using Guppy v3.4. EPI2ME 16S workflow (https: //epi2me.nanoporetech.com, rev 2.1.0) was used for QC and initial characterization. For QIIME2 analysis, sequences were pre-processed using MetONTIIME (Maestri et al. 2019) and QIIME2 was used to filter and analyze the resulting OTU table abundances. The SILVA v138 database was downloaded and utilized as a reference database for taxonomic identification (Pruesse et al. 2007; Quast et al. 2012). Taxonomy barplots, describing the composition of each sample at the desired taxonomic level were visualized at QIIME2 view (https://view.qiime2.org/). Sequence tables were filtered to remove taxonomically unassigned sequences. A table of absolute OTU abundances was exported in BIOM format for further analysis in R.

Statistical analysis

A feature-table containing absolute abundances of Family level OTUs from QIIME2 was imported into R (version 4.0.2) using the R/biomformat package (1.24.0) (McMurdie and Paulson 2015). Unassigned taxa and taxa that were present in ≤ 10% of samples were removed to minimize the risk of sequencing error carryover and due to low statistical power to analyze underrepresented OTUs, respectively. All subsequent analyses were conducted using the R/stats (3.6.2) and R/vegan (2.6-2) packages (Oksanen et al. 2017; R Core Team 2022). Shannon's H. diversity index was calculated using the "diversity" function and observed OTUs were calculated using the "specnumber" function. Differences in diversity and observed OTUs between herbicide treatments, concentration levels, and day of sampling were analyzed with a Kruskal-Wallis Rank Sum test using the "kruskal.test" function. Lastly, Bray-Curtis dissimilarity distance measures

were calculated and square root transformed for non-metric multidimensional scaling (NMDS) using the "metaMDS" function to display community structure.

Results

After unassigned and rare taxa were removed, a total of 430 OTUs were detected across sediment samples taken at three timepoints from 32 microcosms (n = 96). Relative abundances across herbicide treatments were similar (Figure 1). The three most abundant families in all treatments were Hydrogenophilaceae in the Pseudomonadota (Gammaproteobacteria) phylum, and Sulfurovaceae and Desulfobacteraceae, which are assigned to the Proteobacteria (Deltaproteobacteria) phylum. Prolixibacteraceae, a family within the Bacteroidota phylum thought to be associated with sediment, was only detected in the control and glyphosate treatments.

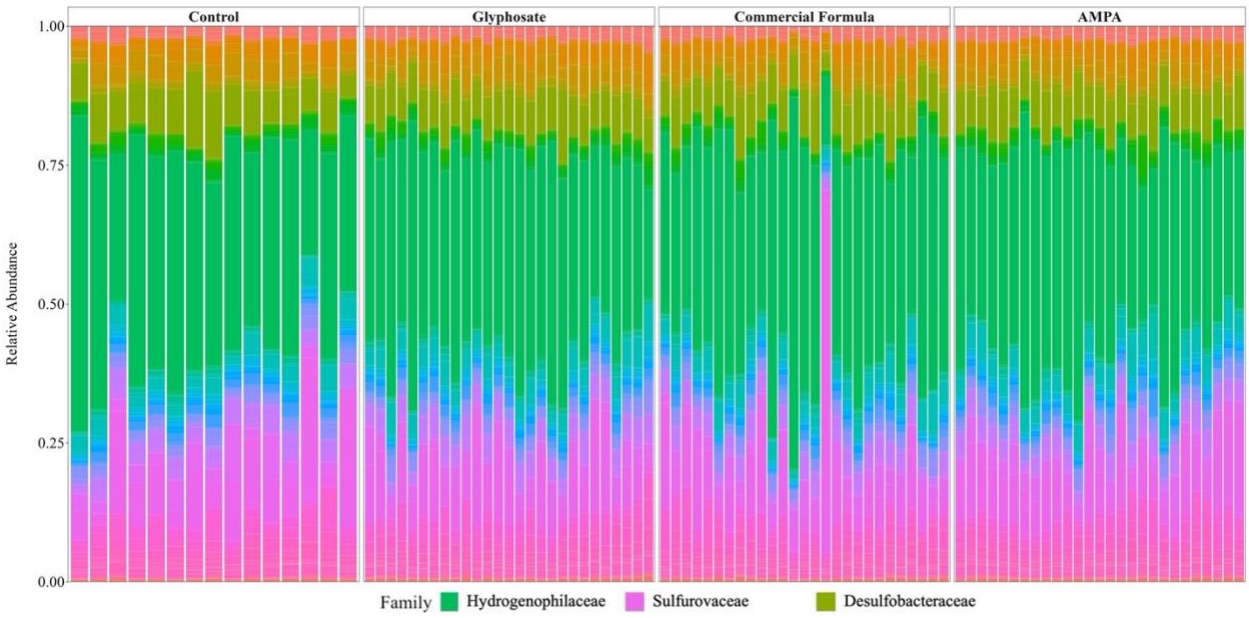

Figure 1 Relative abundance of microbial families within microcosm sediments of each herbicide treatment at all sampling timepoints (n = 96); legend shows the top three most abundant families across all herbicide treatments (full legend can be found in Figure A1)

Shannon H index and observed OTUs were similar among all treatments and controls over the entirety of the experiment (Figure 2 and 3, Table 1). Kruskal-Wallis tests showed no significant differences in alpha diversity metrics across herbicide treatments, concentration levels, or day of sampling (Table 2). A two-dimensional ordination solution was reached (stress = 0.1793708) using NMDS. Microbial communities were similar in multivariate space, which showed there were no compositional differences across treatments (Figure 4).

Table 1 Average ± standard deviation richness and diversity detected in microcosm sediments of each herbicide treatment over the entirety of the experiment

Treatment	**Shannon Diversity**	**Observed OTUs**
Control	2.7 ± 0.24	266 ± 58
Glyphosate (purity = 98.1%)	2.9 ± 0.26	267 ± 50
Commercial Formula	2.7 ± 0.41	264 ± 45
AMPA (purity = 99%)	2.9 ± 0.29	276 ± 47

Table 2 Summary of the Kruskal-Wallis rank sum test on the efects of treatments on Shannon's diversity and observed OTU. X^2, chi-square value; *df*, degrees of freedom; *P*, the *p*-value where alpha = 0.05

Species Metric	**Treatment**	χ^2	**df**	***P***
Shannon Diversity	Day of Sampling	1.81	2	0.40
	Herbicide Treatment	4.67	3	0.20
	Concentration	3.48	3	0.32
Observed OTUs	Day of Sampling	2.25	3	0.52
	Herbicide Treatment	0.22	2	0.90
	Concentration	2.45	3	0.48

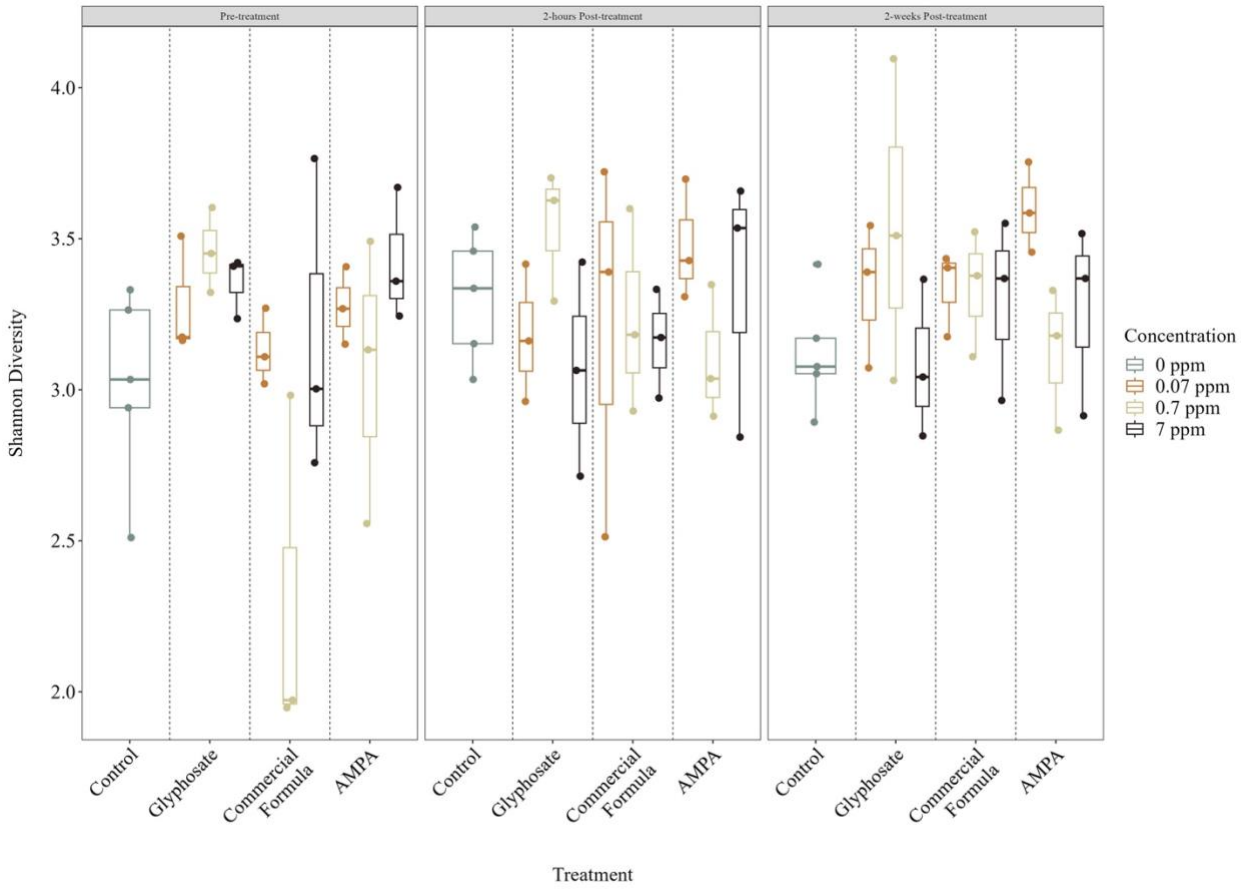

Figure 2 Shannon's H diversity quantified from microcosm sediments by herbicide treatments and concentration and paneled by day of sampling (control treatment, n = 5; herbicide treatments, n = 3). Boxplots represent: 25th quartile = bottom of the box; 75th quartile = top of the box; median = line across each box; minimum and maximum = whiskers; and data points = individual data points

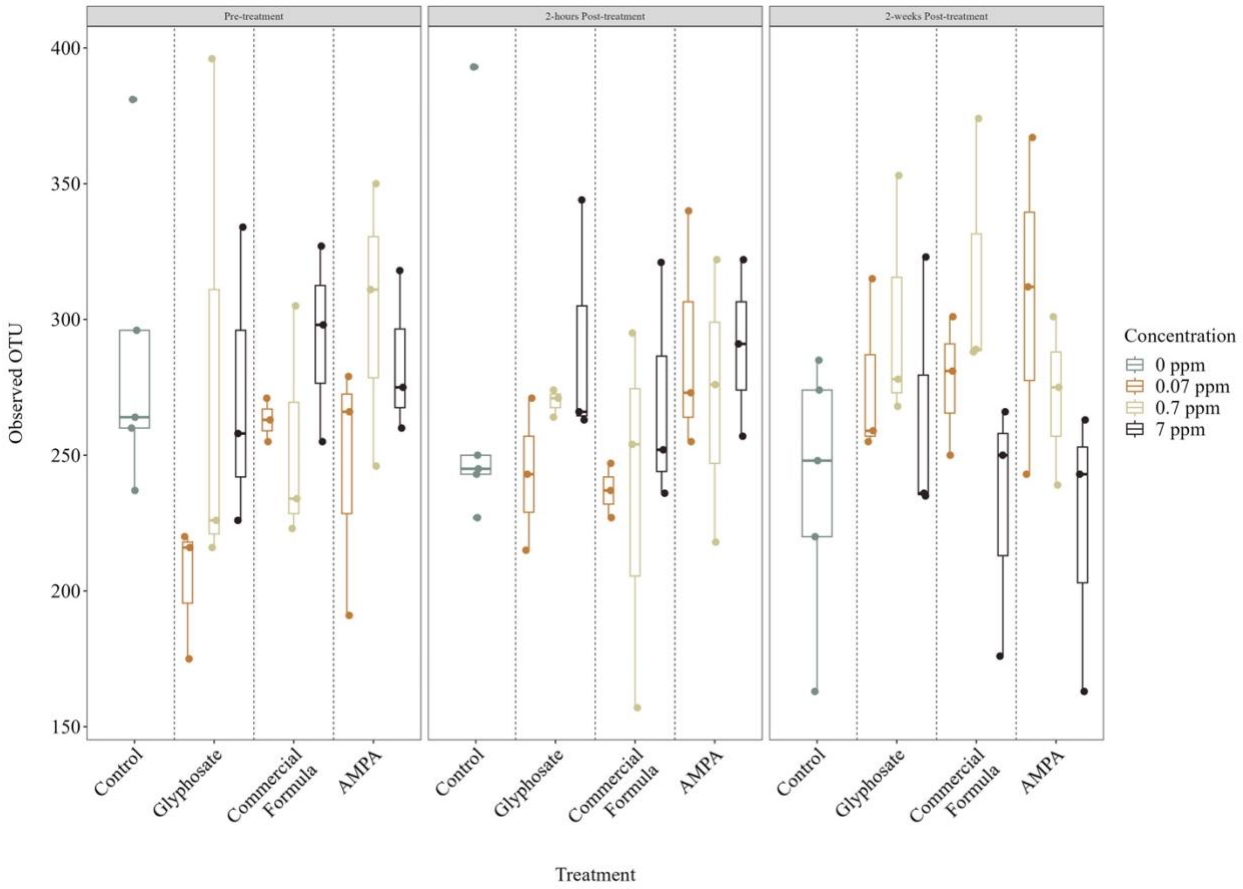

Figure 3 Observed OTUs quantified from microcosm sediments by herbicide treatments and concentration and paneled by day of sampling (control treatment, *n* = 5; herbicide treatments, *n* = 3). Boxplots represent: 25th quartile = bottom of the box; 75th quartile = top of the box; median = line across each box; minimum and maximum = whiskers; and data points = individual data points

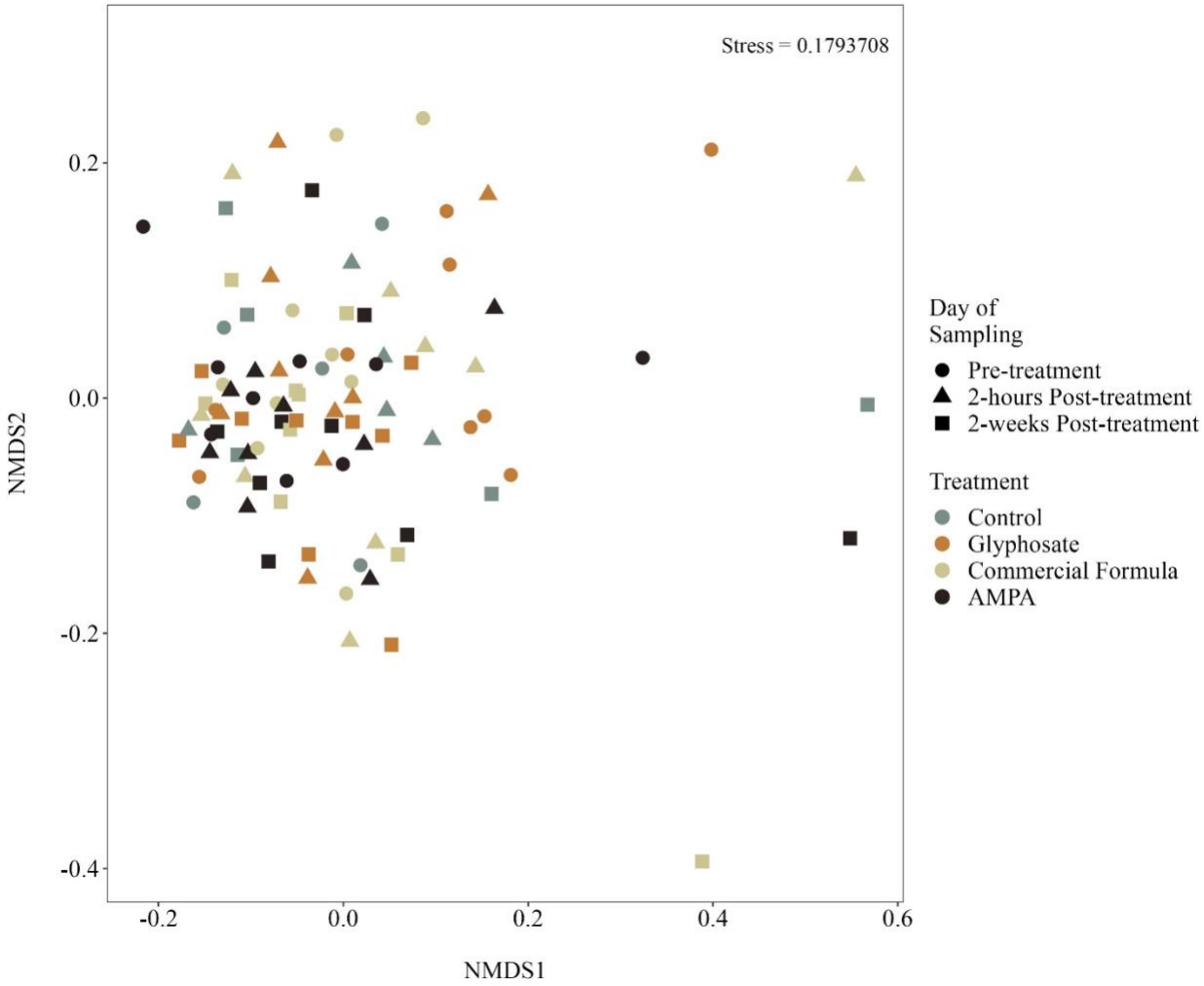

Figure 4 NMDS ordination of sediment microbial community structure across all samples (*n* = 96) where shapes represent the day of sampling and colors represent the herbicide treatment

Both glyphosate and AMPA concentrations in sediments increased over time within each herbicide treatment, but not in control treatments (Tables 3 and 4). This demonstrated residues were dissipating out of the water column shortly after treatment and settling into the sediments. Additionally, AMPA was detected in microcosms where glyphosate or commercial formula only was added, confirming that glyphosate degradation did occur (Table 4).

Table 3 Average ± standard deviation glyphosate concentrations (ppm) detected in microcosm sediments of each herbicide treatment and concentration at each sampling timepoint. Concentrations: L = 0.07 ppm, M = 0.7 ppm, and H = 7 ppm; ND = not detected

		Pre-treatment	**2-hours Post-treatment**	**2-weeks Post-treatment**
Treatment	**Concentration**	**Residues detected (ppm)[a]**		
Control		< LOQ	ND	ND
Glyphosate (purity = 98.1%)	L	0.03 ± 0.04	ND	0.05 ± 0.08
	M	ND	0.03 ± 0.04	0.28 ± 0.1
	H	ND	0.67 ± 0.35	4.37 ± 0.35
Commercial Formula	L	ND	ND	ND
	M	ND	ND	0.31 ± 0.36
	H	ND	0.41 ± 0.46	2.77 ± 1.30
AMPA (purity = 99%)	L	ND	< LOQ	ND
	M	ND	ND	ND
	H	< LOQ	ND	ND

[a]LOD = 0.005 ppm; LOQ = 0.02 ppm

Table 4 Average ± standard deviation AMPA concentrations (ppm) detected in microcosm sediments of each herbicide treatment and concentration at each sampling timepoint. Concentrations: L = 0.07 ppm, M = 0.7 ppm, and H = 7 ppm; ND = not detected

		Pre-treatment	**2-hours Post-treatment**	**2-weeks Post-treatment**
Treatment	**Concentration**	**Residues detected (ppm)[a]**		
Control		ND	ND	ND
Glyphosate (purity = 98.1%)	L	ND	ND	ND
	M	< LOQ	< LOQ	0.04 ± 0.02
	H	ND	0.05 ± 0.05	0.35 ± 0.15
Commercial Formula	L	< LOQ	ND	< LOQ
	M	ND	ND	< LOQ
	H	ND	0.03 ± 0.02	0.19 ± 0.03
AMPA (purity = 99%)	L	< LOQ	< LOQ	0.04 ± 0.03
	M	ND	0.11 ± 0.04	0.48 ± 0.18
	H	ND	1.08 ± 0.57	6.07 ± 7.90

[a]LOD = 0.005 ppm; LOQ = 0.02 ppm

Discussion

The current study used microcosms to examine the impact of glyphosate-based herbicide treatments on benthic sediment microbial communities from a prairie pothole wetland. Both glyphosate and AMPA were detected in microcosm sediments shortly following treatment indicating that residues rapidly dissipate out of the water column (Degenhardt et al. 2012; Battaglin et al. 2014). AMPA was also detected in sediments of microcosms that only received glyphosate (i.e., glyphosate or commercial formula treatment), but no direct AMPA treatment, indicating that glyphosate degradation occurred (Gill et al. 2017). In our experimental microcosms, we found that microbial community diversity and composition were not significantly affected by glyphosate, AMPA, or commercial formula at any concentration, which was contrary to our original hypotheses. Our highest treatment level (7 ppm) was a magnitude higher than the U.S. EPA's MCL for drinking water, and approximately 41 times higher than the

average concentration that have been reported in wetland sediments in the PPR (McMurry et al. 2016). Some research suggests that microbial responses are more dependent on previous glyphosate exposure history and application rates (Bai and Ogbourne 2016; Tang et al. 2019). The present experiment reflects conditions of acute glyphosate and AMPA exposure, rather than long-term exposure on the sediment community. Sediments used in our study were collected from a wetland in North Dakota with no routine pesticide usage in the immediate catchment. Thus, agricultural inputs within this area are minimal to none compared to other areas of the PPR. However, we found no differences between treatments and controls suggesting that glyphosate-based herbicides may not have adverse effects on sediment microbial communities from wetlands in this area of the PPR. This observed lacked effect may be the result of glyphosate- and AMPA-based selection pressure.

A lack of a response from microbial communities following glyphosate exposure has also been observed previously in the literature. For example, Pesce et al. (2009) exposed natural spring- and summer-collected riverine microbial communities to 10 μg L^{-1} glyphosate and found no effect on bacterial activity or diversity. Lane et al. (2012) conducted a six-month soil incubation experiment with monthly glyphosate treatments at 59 μg g^{-1} and 118 μg g^{-1}, and found no significant glyphosate effect on community structure represented by the relative abundances of functional microbial groups. Muturi et al. (2017) used 20 mg L^{-1} glyphosate treatment in microcosms and found no differences in water microbial diversity or richness after 3- and 7-days. Dennis et al. (2018) found no significant effects of a single glyphosate treatment at the recommended field application rate (33.03 mg L^{-1}) on bacterial or archaeal evenness, richness, and composition after 60-days incubation. These studies in addition to the current study represented a wide range of glyphosate concentrations, which all showed that glyphosate does

not always have direct toxic effects on microbial communities. Presumably, this may be due to the presence of glyphosate-tolerant microbes.

Several studies have, however, shown shifts in microbial communities after either short- or long-term glyphosate exposure. Lu et al. (2020), for example, found increases in Shannon and Simpson diversity of lacustrine microbial communities, in addition to differences in community structure 10- and 15-days post-treatment of 2.5 mg L^{-1} glyphosate. Widenfalk et al. (2008) also found that an environmentally relevant glyphosate concentration (150 μg kg^{-1} dry weight) caused significant shifts in lake sediment bacterial community composition in treated microcosms relative to controls after 31-days. In mesocosms, Pérez et al. (2007) found Roundup® treatment significantly decreased phytoplankton and periphyton abundance, but increased picocyanobacterial abundance and primary production. Sura et al. (2012) found pelagic and biofilm bacterial production in outdoor mesocosms was significantly inhibited by 225 μg L^{-1} glyphosate compared to controls. Microbial communities in our study and Sura et al. (2012) were both collected from wetlands within the PPR, however our results were not consistent potentially due to differences in sediment- versus water-associated communities, land use within our collection site's watersheds, or incubation versus outdoor experimental design. These studies all showed that glyphosate can have various negative and positive effects on microbial communities, which may be partially attributed to heterotrophic versus autotrophic species; whereas our results showed glyphosate can also have no effect. This discrepancy indicates that there are many underlying complexities on the effects of glyphosate at the microbial level, which may be more related to molecular mechanisms and/or environmental variables.

Some microorganisms naturally express a glyphosate-resistant form of the EPSPS enzyme of the shikimate pathway (Leino et al. 2021), and there are many glyphosate-tolerant

microbial species listed in the literature (Priestman et al. 2005; Firdous et al. 2018). Specifically, *Agrobacterium* strain CP4 was the bacterium used for the original glyphosate-resistant EPSPS gene in glyphosate-resistant crops (Padgette et al. 1995). Whereas, other species have evolved tolerance through mutations of the EPSP synthase, or metabolic or detoxifying processes (Hertel et al. 2021), which can be a result of prolonged or repeated glyphosate exposure. For example, Tang et al. (2019) added 100 mg L^{-1} glyphosate to sediments with high, low, and no previous glyphosate exposure, and found that microbes degraded glyphosate quicker in sediments with high exposure history compared to low exposure history. Additionally, Lane et al. (2012) found significantly higher microbial respiration in soils after glyphosate treatment, specifically in soils with previous glyphosate exposure history. The sediments used in the present study were collected from an agriculturally-undisturbed wetland located in an area with no known extensive glyphosate use (David Mushet, Research Wildlife Biologist, USGS Northern Prairie Wildlife Research Center, email communication June 21, 2022), thus microbial communities should not have any prolonged exposure history. We did find multiple species across all treatments that are capable of glyphosate degradation and mineralization including Cyanobiaceae, Enterobacteriaceae, Pseudomonadaceae, and Rhizobiaceae (e.g., genus *Agrobacterium*). While our sediment collection site has no known history of glyphosate use within the immediate watershed, the majority of the PPR is in an agriculturally intensive area where glyphosate use is common. Therefore, microbial species from the regional species pool may have evolved tolerance despite our study wetland and its catchment having no existing agricultural pesticide use. On the contrary, microbial communities with and without a history of glyphosate exposure may not differ in glyphosate tolerance (Allegrini et al. 2015) potentially due to the presence of the glyphosate-resistant class II EPSPS protein naturally found in some prokaryotes (Leino et al.

2021). Therefore, our findings may be the result of selection pressure and dispersal, or biochemical makeup.

Glyphosate is highly water soluble, which also allows it to be easily transported into wetlands, where environmental variables can play a role in its bioavailability and toxicity to microorganisms. Glyphosate has an affinity for oxides, metal cations, and organic matter content resulting in its rapid adsorption to sediments (Dion et al. 2001). However, glyphosate and phosphate can compete for sediment binding sites due to their chemical structural similarities (de Jonge et al. 2001). Thus, lower phosphate can increase glyphosate's sediment adsorption capacity (Guijarro et al. 2018), subsequently decreasing its bioavailability. Temperature can also affect glyphosate's environmental persistence, where higher temperatures facilitate degradation due to increased microbial activity (Helander et al. 2012; Muskus et al. 2022). Sediments used in the present study were glacial till composed of approximately 26% organic matter content (Hu et al., unpublished). Due to glyphosate's strong adsorption to organic matter, sediment microorganisms in our study may have had limited exposure to herbicide residues, thus preventing major toxicological effects. While the present study did not measure sediment physicochemical characteristics, homogenized sediments were used for all microcosms, and temperature and light conditions were controlled to help minimize variability.

Conclusion

We evaluated the responses of benthic sediment microbial communities to a single addition of low, medium, or high glyphosate-based herbicide treatment after 14-days incubation. We expected to see toxicological-induced shifts potentially in favor of tolerant species, however we found no differences in sediment microbial communities among treatments or concentrations after 2 weeks. Our results may be explained by the lower concentration of bioavailable

glyphosate in the sediments compared to the actual concentrations added. Additionally, our results may suggest glyphosate-tolerance in the benthic sediment microbial communities, but that is inconclusive without further investigation of the presence of the glyphosate-resistant EPSP gene within the microbiome. Our research suggests that in the PPR the direct effects of glyphosate on sediment microorganisms may not be as severe as initially presumed. However, the literature continues to reveal new implications of the extensive use of glyphosate on aquatic ecosystems. Therefore, further research is still necessary to determine the full range of potential effects of glyphosate on sediment microbial communities.

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CHAPTER 2. A PERSPECTIVE ON HOW GLYPHOSATE AND 2,4-D IN WETLANDS MAY IMPACT CLIMATE CHANGE[2]

Abstract

An increase in herbicide use is occurring due to a growing population and herbicide-resistant crops in agriculture, which has resulted in more herbicide tolerant target species. Glyphosate and 2,4-Dichlorophenoxyacetic acid (2,4-D) are two of the most commonly used herbicides worldwide and are more recently being used in combination in pre-mixed commercial formulas. Subsequently, herbicide contamination of wetlands will increase exposure of microorganisms to multiple chemical stressors. Methane is a potent greenhouse gas naturally emitted from wetlands, but herbicides may disrupt biogeochemical processes leading to an unbalanced methane cycle. We review the impacts of these herbicides on aquatic microbial communities from glyphosate-derived nutrient enrichment and 2,4-D inhibition of methane oxidation, and examine how these altered metabolic processes may lead to increased methane production in wetlands. The response of wetland ecosystems to herbicide contamination will vary across regions, in part due to the complexity of microbial communities, however, this perspective gives a glimpse into the potential global implications of continuing herbicide use on wetlands and demonstrates the importance for research on ecosystem-level co-stressors.

Introduction

Climate change is an ongoing global concern as greenhouse gas (GHG) emissions continue to increase (IPCC 2021). Methane (CH_4) is the second most abundant GHG, after

[2]The material in this chapter was co-authored by Christine Cornish and Jon Sweetman. Christine Cornish had primary responsibility for topic conception and drafting the manuscript. Jon Sweetman served as proofreader and provided text additions and suggestions.

carbon dioxide (CO_2), but is about 25 times more potent (Islam et al. 2018; EPA 2023). Wetlands are a large natural source of CH_4 as they play a significant role in carbon (C) sequestration and cycling (Andresen et al. 2017), and it has recently been suggested that agrochemicals may impact GHG emissions from freshwater ecosystems (Stehle and Schulz 2015). In particular, herbicide use has substantially increased over the past 30 years due to the introduction and rapid adoption of herbicide-resistant crops worldwide (Coupe and Capel 2016; Bai and Ogbourne 2016; Peterson et al. 2018). As a result, target plants have developed herbicide-resistance and the use of pre-mixed formulas that contain multiple active ingredients (i.e., multiple modes of action) has become more common (Freydier and Lundgren 2016; Schütte et al. 2017). Herbicide use is also projected to increase due to ongoing climatic change (Delcour et al. 2015), where higher temperatures can enhance toxicity and alter biodegradation processes (Noyes et al. 2009; Koleva and Schneider 2010; Matzrafi 2019). Subsequently, wetland biota are subjected to combinations of more severe physical and chemical stressors.

Glyphosate and 2,4-D are two of the most commonly used herbicides globally, and are also used in pre-mixed formulas such as Enlist Duo® (1:0.95 glyphosate:2,4-D) and Landmaster™ II (1:0.83 glyphosate:2,4-D) (Benbrook 2016; Zuanazzi et al. 2020; EPA 2022). Their extensive use is cause for environmental concern within aquatic ecosystems as herbicides are already substantial contributors to wetland pollution (Casado et al. 2019). Wetlands are often located in the lowest drainage points of agricultural fields, where they can serve as critical sinks for herbicides transported through spray drift, runoff, groundwater leaching, and wind and sediment erosion (Annett et al. 2014; Bento et al. 2017). However, their fate is dependent on many landscape- and ecosystem-level components such as, precipitation patterns, herbicide application dates, surrounding land use, and plant and animal composition. For example,

glyphosate and 2,4-D are highly water soluble, thus a rainfall shortly after application would rapidly transport substantial amounts of these herbicides into nearby wetlands (Bertuzzo et al. 2013). These agrochemicals often persist in sediments of temperate and northern climates (Helander et al. 2012; Mierzejewska and Urbaniak 2022), can bioaccumulate in organisms such as biofilms (Beecraft and Rooney 2021), and can be transported between habitats via emerging insects (Roodt et al. 2023). Herbicides are frequently detected within aquatic ecosystems around the world, where they have even been found in protected conservation areas (Wolfram et al. 2023).

Consequently, "pesticide cocktails" can affect microorganisms that are important contributors to wetland biogeochemical cycling and overall ecosystem function (Sun et al. 2013; Aparicio et al. 2013; Islam et al. 2018; Baker et al. 2020). Microorganisms are sensitive to disturbances (Sun et al. 2013), thus as both herbicide use and climate change intensifies it is critical to assess the potential effects of herbicides on GHG emissions. Methanogens (i.e., CH_4 producers) and methanotrophs (i.e., CH_4 consumers), in addition to plant and algal-mediated transport, play a critical role in the global CH_4 budget of wetlands, which may be impacted by glyphosate and 2,4-D. In this article we highlight previous research on glyphosate-derived nutrient enrichment and 2,4-D inhibited CH_4 oxidation to suggest that herbicides entering wetlands could alter CH_4 emissions via synergistic effects on microbial communities and consequently impact climate change.

Glyphosate

Glyphosate's impacts on wetland microorganisms are often dose- and species-dependent (Bai and Ogbourne 2016), therefore it can be detrimental or advantageous to different microbial species. While glyphosate's mode of action was developed to target the shikimate pathway in

higher plants (Hetrick and Blankinship 2015), many archaea and bacteria also utilize this pathway resulting in non-target effects (Herrmann and Weaver 1999). Despite the potential negative impacts on microorganisms, in many instances increased growth, respiration, and enhanced metabolism in wetland microbial communities have been observed as a result of glyphosate biodegradation (Vera et al. 2012; Lu et al. 2020) and linked with the use of glyphosate as a nutritive source (Saxton et al. 2011; Wang et al. 2016). Due to glyphosate's chemical structure, its degradation often contributes substantial amounts of phosphorus (P), which has been found to be favored and more rapidly utilized by microorganisms compared to other sources of soil P (Hébert et al. 2019; Sun et al. 2019). Specifically, stimulated cyanobacterial growth and cyanotoxin production has been recorded from glyphosate-derived P enrichment (Vera et al. 2010; Qiu et al. 2013; Zhang et al. 2016; Hernández-García and Martínez-Jerónimo 2020; Wang et al. 2021; Lin et al. 2023). Glyphosate degradation was found to be positively correlated with total P concentrations in surface waters (Carles et al. 2019). Glyphosate additions to aquatic ecosystems can contribute to water quality issues, such as eutrophication, which has been demonstrated to be an important driver of CH_4 emissions (Sepulveda-Jauregui et al. 2018; Beaulieu et al. 2019; Yang et al. 2019; Bertolet et al. 2020). Ultimately, increased glyphosate use could shift microbial community dynamics towards copiotrophs and algae, altering important C biogeochemical processes and resulting in an indirect increase in CH_4 production in wetlands (Figures 5A, B).

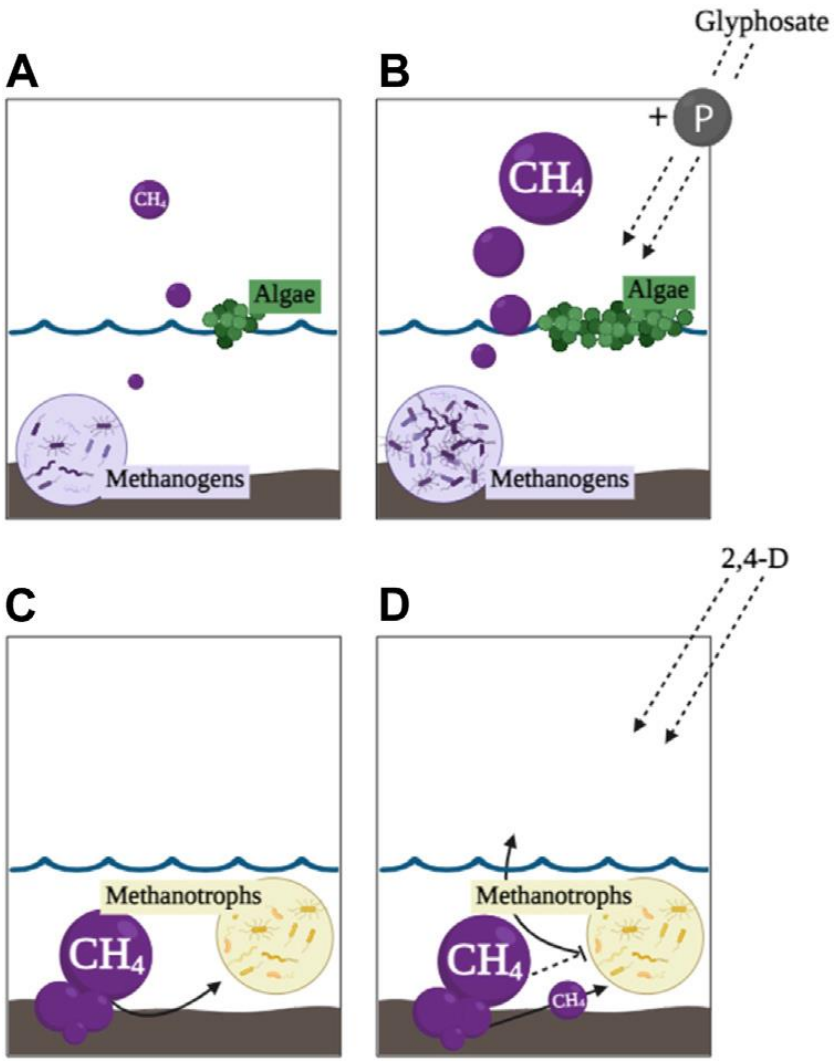

Figure 5 Conceptual diagrams of a wetland with methanogens, methanotrophs, and algae. **A** represents balanced CH_4 production; **B** represents glyphosate contamination stimulating methanogens and algae causing higher CH_4 emissions; **C** represents balanced CH_4 oxidation; **D** represents 2,4-D contamination inhibiting oxidation (i.e., removal) of CH_4 by methanotrophs causing higher CH_4 emissions (created with BioRender.com)

2,4-D

Despite 2,4-D being the first synthetic herbicide, compared to glyphosate, relatively little research has been conducted on its effects on aquatic microorganisms (Donald et al. 2018; Malaj et al. 2020). However, similar to glyphosate, 2,4-D can have a variety of impacts on wetland microbial communities. It targets broadleaf plants through mimicking the plant growth hormone, indol-3-yl-acetic acid (IAA or auxin), resulting in plant overgrowth (Cobb and Reade 2010), but

auxin synthesis and usage in microorganisms is also well known making them vulnerable non-target organisms (Spaepen and Vanderleyden 2011). In addition, 2,4-D can be applied as an aquatic herbicide resulting in species being exposed to higher concentrations compared to terrestrial transport (Mierzejewska and Urbaniak 2022). While 2,4-D is already a widespread environmental contaminant frequently detected in aquatic ecosystems (Malaj et al. 2020) and its use has also increased in recent decades with the development of herbicide-resistant crops, its use will likely continue to increase in the future (Freydier and Lundgren 2016). Consequently, wetland microorganisms could be highly susceptible to its toxic effects with limited capacity to degrade it. Previous research has found some species use 2,4-D as a C source, whereas other species are toxicologically inhibited (Benndorf et al. 2004; Zabaloy et al. 2008; Sachu et al. 2022). Research in microcosms has also found that increased 2,4-D concentrations resulted in inhibition of CH_4 oxidation, decreases in CH_4 removal time, and increased CH_4 emissions (Syamsul Arif et al. 1996; Kumaraswamy et al. 1997; Top et al. 1999). Where studies from Top et al. (1999) and Seghers et al. (2003) suggested decreases in CH_4 removal could be due to 2,4-D inhibition of methanotroph-mediated oxidative metabolism. Research on the effects of 2,4-D on CH_4 oxidation is extremely limited, however these studies do indicate that 2,4-D loading into wetlands could potentially alter the CH_4 cycle by suppressing the removal of CH_4 via the food web, resulting in greater concentrations within the water column and higher emissions (Figure 5C, D).

Pesticide cocktails: Glyphosate plus 2,4-D

The increased use of pre-mixed glyphosate and 2,4-D herbicides further exposes wetland microorganisms to combinations of chemical stressors, which could lead to unforeseen long-term effects. Research on the combined effects of pesticides has been conducted since the 1970's, but

the majority of the focus has been on the direct toxicological impacts to aquatic flora and fauna (Lichtenstein et al. 1973; Faust et al. 1994; Gardner and Grue 1996; Hayes et al. 2006; Relyea 2009; Moreira et al. 2020). These studies included compounds such as atrazine, chlorpyrifos, fipronil, etc., whereas research on the combined effects of glyphosate and 2,4-D is limited, especially at the aquatic microbial level. Additive and/or synergistic effects of glyphosate and 2,4-D have been found on fish and amphibian growth, fertilization, survival, and behavior (Carvalho et al. 2020; Pavan et al. 2021; Bernardi et al. 2022; Peluso et al. 2022), and zooplankton emergence (Portinho et al. 2018). Lozano et al. (2018) found additive impacts of glyphosate and 2,4-D on phytoplankton composition, abundance, and chlorophyll *a* after 7 days in microcosms, but also found an antagonist effect on total and live abundance of *Staurastrum* spp. In outdoor mesocosms, Lozano et al. (2018) found a decrease in phytoplankton respiration and gross primary production from a high glyphosate (applied as Roundup Max®), low 2,4-D (applied as AsiMax 50®) treatment after 4 hours. Additionally, after 7 days in mesocosms with high glyphosate, an increase in primary production, chlorophyll *a*, and micro- and nanophytoplankton was observed (Lozano et al. 2020). Sura et al. (2015) researched the effects of a herbicide mixture including glyphosate, 2,4-D, MCPA, clopyralid, dicamba, dichlorprop, mecoprop, and bromoxynil on pelagic and benthic communities in nutrient-sufficient and nutrient-deficient wetlands. They found pelagic bacterial productivity significantly increased after treatment in the nutrient-sufficient wetland, but benthic bacterial productivity did not change, which suggests the stimulatory effect of these herbicides may be related to nutrient bioavailability. These results demonstrate the complexity of the direct effects of herbicide mixtures on aquatic microorganisms, but the potential indirect effects are still poorly understood. As pre-mixed glyphosate and 2,4-D herbicides become more common it is important to consider

the extent of their effects on aquatic ecosystems. Glyphosate can easily be used as a nutrient source stimulating microbial activity, specifically algal communities, whereas 2,4-D may inhibit methanotrophic communities from oxidizing CH_4. As these compounds enter aquatic ecosystems their impacts on microorganisms may become synergistic and/or additive resulting in eutrophication and inhibition of methanotrophs from glyphosate and 2,4-D, respectively. Subsequently, eutrophic conditions and decreased CH_4 removal could cause increased CH_4 production via an unbalanced CH_4 cycle (Figure 6).

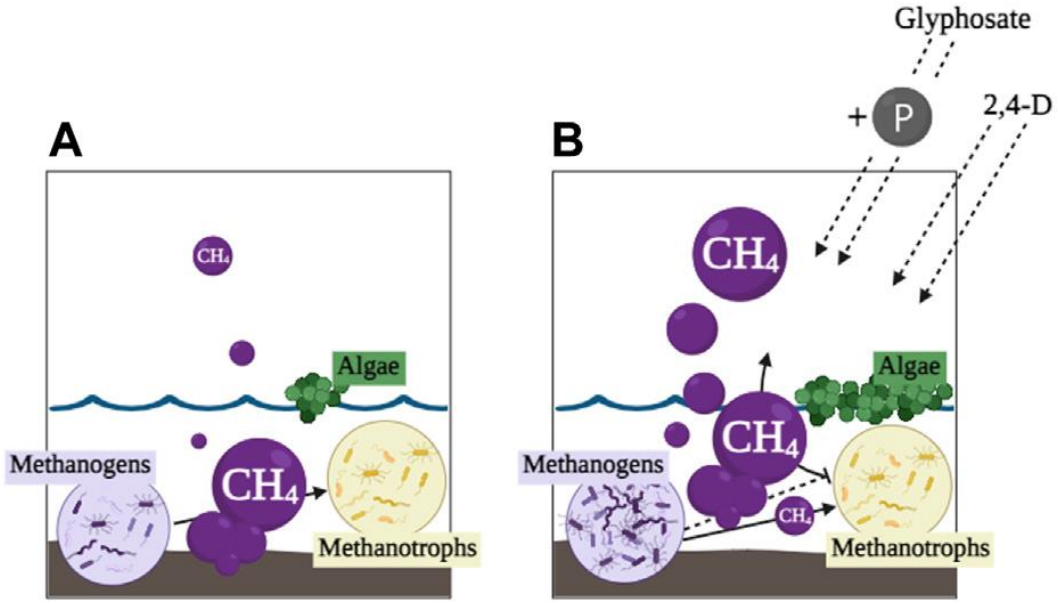

Figure 6 Conceptual diagrams of a wetland with methanogens, methanotrophs, and algae. **A** represents a balanced CH_4 cycle, where algae and methanogens produce CH_4, methanotrophs oxidize CH_4; **B** represents an unbalanced CH_4 cycle where glyphosate and 2,4-D contamination stimulate methanogens and algae and inhibit CH_4 oxidation, respectively, and thus result in higher CH_4 emissions (created with BioRender.com)

Pollution-Induced Community Tolerance (PICT)

Aquatic ecosystems are subjected to year-round herbicide contamination, where herbicide use differs across crop, season, habitat, and region. Microorganism structure and function can be impacted by herbicides, but toxicity is often dependent on the mode of action, concentration, and duration of exposure, as well as microbial species and environmental factors (DeLorenzo et al.

2001). For example, glyphosate stimulated *Chlorella vulgaris* growth 24-hours after exposure, but then inhibited growth after 48-hours at the same concentrations (i.e., hormesis) (Reno et al. 2014). In addition to the duration of exposure, the exposure to a different mode of action could also impact microorganisms by causing a community shift often appearing as changes in gene expression or diversity (Feld et al. 2015). Pollution-Induced Community Tolerance (PICT) refers to the response of a community to a pollutant, which results in an increased tolerance to that pollutant (Blanck 2002). The use of PICT analysis is extensive in the toxicology literature, especially on phototrophic microorganisms, which are often more susceptible to herbicidal effects due to their similarities with target species (DeLorenzo et al. 2001; Larras et al. 2016). Bérard and Benninghoff (2001) found phytoplankton were significantly more sensitive to atrazine after one day, but then significantly less sensitive after at least 11 days. Phototrophic biofilms were found to be increasingly more sensitive to diuron as contamination levels decreased 1-3 years after its ban in the European Union (Pesce et al. 2016). It has also been shown that selection pressure from multiple stressors can lead to more opportunistic species and higher tolerances (Rotter et al. 2013). Ultimately, PICT results suggest that more sensitive species are being replaced by less sensitive species creating a more tolerant community (Blanck 2002). This has also been seen with both glyphosate and 2,4-D. Microbial communities from sediments with high glyphosate exposure were able to degrade glyphosate faster and had higher diversity compared to sediments with low to no previous exposure (Tang et al. 2019). Zabaloy et al. (2008) saw an increase in a 2,4-D degrading population in soils for approximately one month after treatment and found that agricultural soils had higher 2,4-D tolerance compared to reference soils via PICT analysis. In a study by de Lipthay et al. (2002) 2,4-D treatment induced transcription of the gene responsible for 2,4-D degradation (*tfdA*) which demonstrates a survival

response from the microbial community. These studies demonstrate PICT can occur when communities are exposed to a herbicide, therefore contamination by an additional herbicide could further alter communities that have not been exposed before. It could be presumed that wetland microbial communities within a glyphosate-dominant region may substantially change when 2,4-D is introduced in combination with glyphosate and species are replaced. This potential shift would impact the biogeochemical functions of the community, subsequently altering herbicide degradation or metabolism.

Conclusion

Glyphosate and 2,4-D are frequently cited as having minimal to no environmental impacts (Peterson et al. 2016; Duke 2020; Singh et al. 2020), however there is increasing evidence that their indirect effects may be of more substantial global concern. Wetlands naturally emit CH_4 via diffusion, ebullition (i.e., bubbles), and plant-mediated transport, and are the highest natural sources of CH_4 in the environment (Andresen et al. 2017; Aben et al. 2017), but emissions may be increasing due to agrochemical use adversely impacting CH_4 sink potential (Seghers et al. 2005). Glyphosate could stimulate microbial processes resulting in increased CH_4 production, in addition to 2,4-D inhibiting CH_4 oxidation further resulting in increased CH_4 production. Ultimately, this would lead to higher CH_4 production versus removal from freshwater creating elevated CH_4 in the atmosphere. Due to the widespread and extensive use of glyphosate and 2,4-D, these herbicides are frequently found in wetlands (Islam et al. 2018; Malaj et al. 2020). To our knowledge there has been no research investigating the combined impacts of glyphosate and 2,4-D on wetland microbial communities. The potential bottom-up effects of glyphosate and 2,4-D could be detrimental to a changing climate, thus improving our understanding of how these herbicides can impact GHG emissions is crucial.

Future research

To investigate the effects of glyphosate and 2,4-D on CH_4 emissions from freshwater ecosystems, micro- or mesocosm experiments could be conducted. Experiments under controlled conditions could help determine how wetland microbial communities are affected by glyphosate and 2,4-D. Specifically, this research would give insight into the CH_4-related mechanisms that may be enhanced or disrupted in microorganisms. In addition to incubation experiments, pesticide loading data could be incorporated into GHG models. These data are currently not included in estimations of CH_4 emissions from wetlands, but could be an important source of variation, and could be useful for future climate modeling. These potential impacts are crucial to research as herbicide use is only expected to increase over time, where chemical selection pressure on microbial communities could contribute to climate change.

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CHAPTER 3. COMMON USE HERBICIDES INCREASE WETLAND GREENHOUSE GAS EMISSIONS[3]

Abstract

Wetlands play a disproportionate role in global climate as major sources and sinks of greenhouse gases. Herbicides are the most heavily used agrochemicals and are frequently detected in aquatic ecosystems, with glyphosate and 2,4-Dichlorophenoxyacetic acid (2,4-D), representing the two most commonly used worldwide. In recent years, these herbicides are being used in mixtures to combat herbicide-tolerant noxious weeds. While it is well documented that herbicide use for agriculture is expected to increase, their indirect impacts on downstream wetland greenhouse gas dynamics are virtually unknown. To fill this knowledge gap, we conducted a factorial microcosm experiment using low, medium, and high concentrations of glyphosate or 2,4-D, individually and in combination to investigate their effects on wetland methane, carbon dioxide, and nitrous oxide fluxes. We predicted that mixed herbicide treatments would have a synergistic impact on greenhouse gases compared to individual herbicides. Our results showed that carbon dioxide flux rates and cumulative emissions significantly increased from both individual and mixed herbicide treatments, whereas methane and nitrous oxide dynamics were less affected. This study suggests that extensive use of glyphosate and 2,4-D may increase carbon dioxide emissions from wetlands, which could have implications for climate change.

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[3]The material in this chapter was co-authored by Christine Cornish, Olivia Johnson, Sheel Bansal, Jake Meier, Ted Harris, and Jon Sweetman. Christine Cornish had primary responsibility for topic conception, data analyses, and drafting the manuscript. Text additions were provided by Olivia Johnson, Sheel Bansal, and Jake Meier, and proofreading with minor text additions and suggestions were provided by Sheel Bansal, Ted Harris, and Jon Sweetman.

Introduction

Wetlands are crucial GHG sinks, but are also sources for methane (CH_4), carbon dioxide (CO_2), and nitrous oxide (N_2O) via diffusive, ebullitive, and plant-mediated gas fluxes (Andresen et al. 2017; Aben et al. 2017; Tangen and Bansal 2022). Anthropogenic activities, such as agrochemical use may alter GHG emissions from wetland ecosystems via impacts to biogeochemical cycling (Stehle and Schulz 2015; Rosentreter et al. 2021). Herbicides, insecticides, and fungicides have been found to effect GHG emissions from aquatic systems, attributed to changes in vegetation or microbial metabolic processes (Kumaraswamy et al. 1998; Kinney et al. 2004; Seghers et al. 2005; Kyaw and Toyota 2007; Das et al. 2011; Badiou et al. 2019). Tridemorph (fungicide) has been found to increase CH_4 production in flooded tropical rice soils at lower concentrations (5-20 $\mu g\ g^{-1}$) by increasing substrate and methanogen abundance, and decrease oxidation at higher concentrations (50-100 $\mu g\ g^{-1}$) by inhibiting methanotrophs (Bharati et al. 1999). Whereas, the herbicide butachlor inhibited CH_4 production by 15% at 5 $\mu g\ g^{-1}$ to 98% at 100 $\mu g\ g^{-1}$ compared to controls due to low methanogen abundance, especially at higher concentrations (Mohanty et al. 2004). Das et al. (2011) compared the individual and mixed effects of herbicides bensulfuron methyl and pretilachlor on GHGs and found significant decreases of CH_4 and N_2O emissions when applied separately, but significantly increased emissions when combined. Herbicide use in agriculture is only projected to increase due to growing population demands (Tilman et al. 2011) and climate change (Koli et al. 2019).

Two of the most commonly used herbicides worldwide are glyphosate and 2,4-Dichlorophenoxyacetic acid (2,4-D), and as a result of herbicide tolerance in target weed species, these herbicides are often combined in "pesticide cocktails", such as Enlist Duo® (1:0.95 glyphosate:2,4-D) and Landmaster™ II (1:0.83 glyphosate:2,4-D) (Benbrook 2016; Schütte et al.

2017; Heap and Duke 2018; Zuanazzi et al. 2020). Due to the increased use of mixed herbicides along with global intensification of agriculture, depressional wetlands, particularly in agriculturally-dominated landscapes, are receiving increasing amounts of glyphosate and 2,4-D loading (Xu et al. 2009; Messing et al. 2011; Battaglin et al. 2014; McMurry et al. 2016). Glyphosate and 2,4-D are primarily degraded by microorganisms, where select species can use them for nutrients (Benndorf et al. 2004; Wang et al. 2016b), and other species are inhibited by their toxicity (Tsui and Chu 2004). Even minor herbicide-induced shifts in wetland microbial communities can potentially alter ecosystem function and GHG emissions.

The paucity of research on the effects of glyphosate and 2,4-D on GHGs demonstrates the lack of understanding on the potential global implications of these common use herbicides. Previous research conducted in aquatic and terrestrial systems have found glyphosate and 2,4-D can disrupt biogeochemical cycling. For example, in agricultural soils, 12.7 $\mu g\ g^{-1}$ analytical grade glyphosate and Roundup® decreased N_2O reduction (i.e., consumption), and 635 $\mu g\ g^{-1}$ stimulated N_2O production; overall, Roundup® was found to have greater effects than glyphosate indicating potential inert ingredient effects (Carlisle and Trevors 1986). Whereas, in flooded tropical rice soil 2,4-D stimulated methanotrophs and CH_4 oxidation at 25 $\mu g\ g^{-1}$, but significantly decreased methanotrophic activity at concentrations 50 $\mu g\ g^{-1}$ and greater (Kumaraswamy et al. 1997). Inhibition of CH_4 oxidation and decreased oxidation rate from 2,4-D has also been observed in microcosms. Syamsul Arif et al. (1996) found a significant decrease in CH_4 oxidation rate at 5 $\mu g\ L^{-1}$ and complete suppression of oxidation at 25 $\mu g\ L^{-1}$. Additionally, Top et al. (1999) found 0.5 $\mu g\ L^{-1}$ 2,4-D inhibited CH_4 oxidation. While the isolated effects of glyphosate and 2,4-D on wetlands and their biota have been extensively

researched, their combined effects on ecosystem function and GHG emissions are understudied (Zabaloy et al. 2012; Drzyzga and Lipok 2018; Sun et al. 2019; De Oliveira et al. 2021).

In this study, we investigated the independent and synergistic effects of glyphosate and 2,4-D across a range of concentrations on GHG fluxes in freshwater microcosms by measuring flux and porewater concentrations of CH_4, CO_2, and N_2O. We calculated cumulative emissions to gain insight into the broader effect of these herbicides over a longer time period. We focused on non-vegetated wetland sediments under constant water depth to exclude the effects of plants on the production and consumption of GHGs (Whalen 2005). Our predictions were that mixed herbicide treatments would have a greater effect on GHGs, and specifically that CH_4 would be the most affected due to 2,4-D inhibition of CH_4 oxidation (Top et al. 1999; Seghers et al. 2003).

Materials and Methods

Pilot studies

Prior to the current study, microcosm pilot experiments were conducted to determine which herbicide concentrations to use in the experiment. Glyphosate (pilot #1) was tested at 0, 0.3, 50, 1000, 4000, and 8000 mg L^{-1}, and 2,4-D (pilot #2) was tested at 0, 0.1, and 1.35 mg L^{-1}.

Microcosm preparation and treatment

We conducted a randomized factorial 21-day microcosm experiment to assess the effects of glyphosate and 2,4-D on GHG fluxes from prairie wetland sediments. Microcosms were polypropylene containers (25 height × 12 diameter cm) with 4 cm of standing water (deionized water) and 10 cm of sediment collected from an experimental wetland at the Northern Prairie Wildlife Research Center (NPWRC, Jamestown, North Dakota, 46.88 °N, -98.64 °W). Water level was maintained at 4 cm above sediment surface with deionized water. In October 2021, sediment from the top 20 cm was collected from the shallow region of the experimental wetlands

(30 cm water depth, wetlands dominated by hybrid cattail (*Typha* × *glauca*) and reed canary grass (*Phalaris arundinacea*)). Sediment was stored outside in sealed containers and allowed to freeze until January 2022, then was thawed and sieved (6 mm) for homogenization prior to microcosm preparation (see Bansal et al. [2020] for additional details on sediment processing). In the center of each microcosm, a 2.5 cm diameter PVC pipe with slits in the bottom 4 cm was installed to collect porewater (i.e., a mini-piezometer, Geoprobe, Salinas, KS, USA). Mini-piezometers were capped with Parafilm® to prevent gas exchange and equilibration with ambient air. Throughout the experiment, microcosms were kept in a room temperature grow room at NPWRC under controlled environmental conditions (~ 23 °C, light only when sampling).

The randomized factorial design consisted of 11 herbicide treatments and a no herbicide, deionized water control (n = 4). Microcosms acclimated for three days prior to treatment addition. Both glyphosate and 2,4-D were applied at low (L), medium (M), and high (H) concentrations separately and in combination to observe individual and combined effects. Herbicide treatments consisted of: glyphosate-isopropylamine salt (Roundup®) at 0.3 mg glyphosate L^{-1} (L; maximum concentration detected in ponds or wetlands, Battaglin et al. 2014), 10 mg L^{-1} (M; approximate acute toxicity to nonvascular and vascular plants (Environmental Protection Agency 2023)), and 1000 mg L^{-1} (H; pilot study concentration found to have an immediate effect for all GHGs); 2,4-D-dimethylamine salt (Weedar® 64) at 0.04 mg L^{-1} (L; similar concentration to maximum contaminant level in drinking water Gervais et al. 2008), 4 mg L^{-1} (M; 2-times concentrations in Lozano et al. 2018), and 400 mg L^{-1} (H; high concentration chosen as exponential increase from L and M). Mixtures of glyphosate and 2,4-D included various combinations of L, M, and H concentrations (Table 5). Control microcosms only received deionized water. Roundup® and Weedar® 64 were diluted with deionized water to

obtain target concentrations, and all treatments were added to microcosm surface water, gently stirred to evenly distribute, and then allowed to settle for three days before the first sampling. Microcosms were left open (no lid), except during gas flux measurements.

Table 5 Factorial experimental design of treatment concentrations and combinations, where each treatment consisted of four replicates. Gray boxes indicate the mixture combinations that were not included in this experiment[a]. Treatment concentrations are represented as C = control, L = low, M = medium, and H = high

		Glyphosate (Roundup®)			
		C = 0 mg L^{-1}	L = 0.3 mg L^{-1}	M = 10 mg L^{-1}	H = 1000 mg L^{-1}
2,4-D (Weedar® 64)	C = 0 mg L^{-1}				
	L = 0.04 mg L^{-1}				
	M = 4 mg L^{-1}				
	H = 400 mg L^{-1}				

[a]All possible mixture combinations were not tested due to logistical constraints (i.e., limited grow room space and time to measure all replicates)

Gas measurements

All gas measurements described below were collected on Days 0, 3, 7, 14, and 21, where Day 0 represents pre-treatment sampling, and all other time points represent days since treatment application. Instantaneous CH_4, CO_2, and N_2O flux was measured using a high-frequency GHG analyzer with Fourier Transform Infrared spectroscopy technology (Gasmet DX4015 Analyzer, Gasmet Technologies Oy, Finland). For each flux measurement, a closed system was created using a modified lid with inlet and outlet ports connected to the GHG analyzer. Gas concentrations were continuously measured over a 15-minute interval to calculate gas flux rate. Flux rates were calculated based on microcosm headspace volume and surface area, barometric pressure, and air temperature using the ideal gas law using the *HMR* package in R 3.5.1

(Pedersen et al. 2010; 2022). In some cases, a sudden increase in gas concentration was observed immediately after placing the lid on the microcosm, indicating artificially induced ebullition. If artificial ebullition occurred, the microcosm was allowed to vent with the lid off for a minimum of 30 minutes, and then the flux measurement was repeated. Flux rate data were standardized by microcosm soil mass by dividing flux rate by final dry soil weight. Dry soil weight was calculated by allowing microcosms to dry out at the end of the experiment until a consistent weight was reached, weighing the empty microcosms, and subtracting the empty microcosm weight from the dried microcosm weight. Lastly, the difference in standardized flux rates from the start to the end of the experiment was calculated at microcosm-level by subtracting Day 0 values from Day 21 values, where these data were used for all downstream statistical analyses.

Following flux measurement, porewater was collected to determine dissolved gas concentrations using the headspace equilibration method (Bansal et al. 2023). Approximately 25 mL of porewater was collected from the mini-piezometers using clean 50 mL polypropylene syringes with attached Teflon tubing and three-way valves. 35 mL of nitrogen gas was added to each syringe and shaken for at least three minutes to equilibrate. A gas chromatograph (SRI Instruments, CA, USA), equipped with a flame ionization detector and electron capture detector was used to determine headspace gas concentrations, and dissolved gasses were calculated using Henry's constant. Differences in Day 0 and Day 21 were also calculated for porewater concentrations to be used for all statistical analyses.

Cumulative emissions, the total amount of gas emitted over the entirety of the experiment, were calculated by averaging flux rates between consecutive time points (Days 0, 3, 7, 14, and 21), multiplying by time (in hours) between those time points (e.g., Day 0 to Day 3 =

72 hours), and then summing over all intervals. Cumulative emissions after 21 days were used for statistical analysis as those emissions incorporate fluxes over the entirety of the experiment.

Statistical analysis

The primary goals of these analyses were to determine if herbicide treatments affected, 1) GHG flux rates and porewater concentrations after 21 days, and 2) cumulative GHG emissions. We also investigated if, 1) flux rates and porewater concentrations were correlated by treatment, 2) mixed herbicide treatments had a combined effect (synergistic, antagonistic, or additive) on GHGs compared to individual herbicide treatments, and 3) treatment and time affected GHG flux rates, porewater concentrations, and cumulative emissions (Appendix B). All data analyses were conducted in RStudio version 4.3.0 with R version 4.2.0 (R Core Team, 2022). To determine whether parametric or non-parametric analyses were appropriate, difference in flux rates, difference in porewater concentrations, and Day 21 cumulative emissions were checked for normality using the *shapiro.test* function from the "stats" package (version 4.2.0, R Core Team 2022) and homoscedasticity using the *leveneTest* function from the "car" package (version 3.1.1, Fox and Weisberg 2019).

Difference (i.e., Day 21 – Day 0) in flux rates and porewater concentrations

Prior to analysis, repeated flux rate measurements where artificially induced ebullition events occurred were assessed per GHG, and outliers were removed. When an outlier was removed for one GHG, but not others, or if the repeated measurements did not contain an outlier, measurements were averaged to obtain one flux value per microcosm and sampling time point. CH_4 were not normally distributed, thus a non-parametric Kruskal-Wallis test was performed using the *kruskal.test* function from the "stats" package (R Core Team 2022). CO_2 and N_2O were normally distributed and homoscedastic, therefore one-way analysis of variances (ANOVA)

were performed using the *aov* function in the "stats" package (R Core Team 2022). When $p < 0.05$, Least Significant Difference test was performed to compare treatment means using the *LSD.test* function from the "agricolae" package (version 1.3.6, de Mendiburu 2023). For difference in porewater concentrations, a one-way ANOVA was used on CH_4, and Kruskal-Wallis tests were conducted on CO_2 and N_2O, as described above. Gas measurement data collected on Days 3, 7, and 14, were excluded from the main manuscript as individual time points did not change the overall conclusions for flux rates or porewater concentrations (Appendix B).

Cumulative emissions

To test for the effect of herbicide treatment on cumulative emissions after 21 days: a one-way ANOVA was performed on CH_4 and a Kruskal-Wallis was performed on CO_2. N_2O data were not normally distributed and were heteroscedastic, thus Welch's ANOVA was performed using the *oneway.test* function with *var.equal=FALSE* from the "stats" package (R Core Team 2022). When $p < 0.05$, a Dunn's post-hoc test was performed using the *dunnTest* function from the "FSA" package (version 0.9.5, Ogle et al. 2023).

Discussion and Results

In this study, we used microcosms to test the effects of glyphosate- and 2,4-D-based herbicides on freshwater GHG flux. We found that both individual (i.e., glyphosate or 2,4-D) and mixed (i.e., glyphosate plus 2,4-D) herbicide treatments significantly increased CO_2 flux, with high glyphosate being the apparent driving factor affecting cumulative emissions. However, there was no distinct treatment effect on porewater concentrations and thus, no differences in sediment GHG production.

To our knowledge this is the first study to investigate the combined effects of glyphosate and 2,4-D on non-vegetated freshwater GHG fluxes as previous research has often used different treatment types, combinations, and environmental parameters (Kinney et al. 2004; Seghers et al. 2005; Kyaw and Toyota 2007; Das et al. 2011; Badiou et al. 2019). Similar to previous research, however, we suggest our findings are likely due to substrate and metabolic changes of the microbial community as our sediments were not sterilized and our response variables were isolated from other flux factors such as changing hydrologic regimes, temperature, and vegetation (Bansal et al. 2016; Knox et al. 2021).

CO_2

Carbon dioxide flux rates significantly increased from Day 0 to Day 21 in all treatments containing high herbicide concentrations, where the H-Gly + H-24D treatment had the overall highest mean difference (mean ± standard deviation = 0.51 ± 0.12 mg kg $soil^{-1}$ hr^{-1}) (Figure 7). All treatments that contained high glyphosate had significantly higher CO_2 emissions after 21 days compared to the control and many other herbicide treatments (Figure 8). Specifically, all treatments containing high glyphosate were net sources of CO_2, whereas all other treatments were net sinks of CO_2. Microorganisms readily degrade glyphosate and 2,4-D using it as a nutritive source, where glyphosate has shown to increase microbial growth and respiration, and enhance metabolic processes (Saxton et al. 2011; Vera et al. 2012; Wang et al. 2016b; Lu et al. 2020). Specifically, glyphosate degradation results in bioavailable phosphorus, which can be rapidly utilized resulting in stimulated microbes producing more CO_2 via respiration (Sun et al. 2019). Higher CO_2 flux and emissions after 21 days in microcosms receiving 1000 mg L^{-1} of glyphosate suggest the potential use of glyphosate-derived substrate or a stress response. This is consistent with other studies that found significant increases in aquatic microbial community

respiration from glyphosate exposure (Vera et al. 2012; Lu et al. 2020), where utilization of glyphosate as a phosphorus, carbon, and nitrogen source has been found (Saxton et al. 2011; Wang et al. 2016a). Lane et al. (2012) found that significant increases in respiration paralleled increases in glyphosate concentrations in microcosms, particularly in sediments with a history of glyphosate exposure. While sediments used in our study did not have a history of glyphosate exposure, we still observed significant effects on CO_2 fluxes, which suggests glyphosate and 2,4-D may elicit a stress response in microorganisms (Xiao et al. 2023), thus increasing respiration.

The difference between Day 0 and Day 21 porewater concentrations were similar among treatments for all GHGs, though it is notable that the mixed herbicide treatments all generally resulted in decreased porewater CO_2 (Figure 9). We found the highest difference in porewater concentrations in individual treatments H-Gly (mean ± standard deviation: 205 ± 248 μmol L^{-1}) and L-24D (130 ± 410 μmol L^{-1}), in addition to the combination of those concentrations (H-Gly + L-24D = 119 ± 196 μmol L^{-1}) were the only treatments greater than 0. While CO_2 in porewater was not significantly different among treatments, our flux rate and cumulative emission results still demonstrate that glyphosate in wetlands, individually or in combination with 2,4-D may contribute to atmospheric CO_2.

CH_4

Differences in CH_4 flux rates and porewater concentrations did not significantly differ among treatments (Figures 7 and 9). The highest flux rate means were L-24D (0.012 ± 0.011 mg kg $soil^{-1}$ hr^{-1}) and H-Gly + L-24D treatments (0.012 ± 0.012 mg kg $soil^{-1}$ hr^{-1}), where H-Gly + L-24D was also the only treatment with mean porewater concentrations greater than the control (H-Gly + L-24D = 45 ± 56 μmol L^{-1}, Control = 9 ± 155 μmol L^{-1}). It is notable, however, that all the mixed herbicide treatments generally resulted in decreased porewater CH_4 (Figure 9). CH_4 flux

rates suggest a potential antagonistic relationship between glyphosate and 2,4-D, particularly in mixed treatments containing L-Gly (Figure B5). Antagonistic effects of glyphosate and 2,4-D have been observed on different organisms including phytoplankton abundance and beaked toad mortality (Lozano et al. 2018; Peluso et al. 2022). However, antagonistic effects found by Lozano et al. (2018) on phytoplankton abundance was from high glyphosate plus low 2,4-D (6 mg L^{-1} + 0.135 mg L^{-1}), which is opposite of our results, where antagonistic effects looked more apparent with low glyphosate. The antagonistic effect on the lethal concentration to 50% (LC50) of beaked toads was lower in mixed glyphosate and 2,4-D treatments compared to the respective individual treatment, where Peluso et al. (2022) used a wide range of concentrations and combinations including glyphosate at 9.6 – 96 mg L^{-1} and 2,4-D at 84 – 840 mg L^{-1}. Additive effects of glyphosate and 2,4-D have also been observed on phytoplankton abundance and chlorophyll *a* after 7 days in microcosms, and on gross and net primary production after 23 days in outdoor mesocosms (Lozano et al. 2018, 2020). We noted phytoplankton in some of our microcosms, however, anecdotally, there was no excessive phytoplankton growth in one treatment or treatment type, indicating this would have minimal to no effect on our CH_4 results. Even though there were no significant treatment effects, our study may still suggest potential antagonistic and additive effects of glyphosate and 2,4-D on CH_4-mediated processes. It is also still notable that all treatments were sources of CH_4 over the experiment, and that the average cumulative emissions for mixed treatments were approximately twice as high as the control (Figure 8). Some literature has shown that 2,4-D can inhibit CH_4 oxidation, thus increasing CH_4 emissions (Top et al. 1999; Seghers et al. 2003). Thus, we predicted mixed herbicide treatments would significantly increase CH_4 in our microcosms, however we did not see differences in flux or porewater (production). This suggests that CH_4 oxidation or methanotrophs may have not

been substantially affected (Seghers et al. 2005), although slightly higher cumulative emissions from mixed treatments may indicate rapid transport of CH_4 to the atmosphere after production in sediments.

N_2O

Lastly, N_2O flux rates, porewater concentrations, and cumulative emissions did not significantly differ among treatments, and high variability was observed (Figure 7 – 9). Previous research has found that glyphosate and 2,4-D can impact nitrification processes. For example, in soils amended with organic matter content 2 L glyphosate hectare^{-1} significantly decreased N_2O production by 20% and 49% in chitin amendments, and 50% and 92% in rice straw amendments after 6-weeks (Kyaw and Toyota 2007); whereas in soybean microcosms 250 mg L^{-1} 2,4-D significantly increased N_2O flux (Lifeng et al. 2000). These results and our findings contrast potentially due to differences in organic matter content (total mean ± standard deviation, 5.7% ± 0.17, Table B1), plant presence and water content, and subsequent oxygen levels. Additionally, increased CH_4 oxidation has been found to be coupled with increased N_2O emissions (Kinney et al. 2004; Raghoebarsing et al. 2006), which could also help explain the lack of N_2O differences in our study as we did not see decreases in CH_4. We did observe N_2O uptake in most herbicide treatments, and in controls (Figure 8), demonstrating that wetlands can be sinks of N_2O (Majumdar, 2013). M-Gly, L-Gly + H-24D, and M-Gly + M-24D were the only treatments found to be sources of N_2O, not only was variation high, but also emissions were very low and would be negligible on a global scale. Some literature demonstrates wetlands are sources of N_2O and that herbicides can impact nitrogen cycling and N_2O emissions (Kinney et al. 2004; Galloway et al. 2004; Frankenberg et al. 2005; Das et al. 2011; Jiang et al. 2015). However, our results

suggest that freshwater wetlands would minimally contribute to atmospheric N_2O, even under changing herbicides and concentrations.

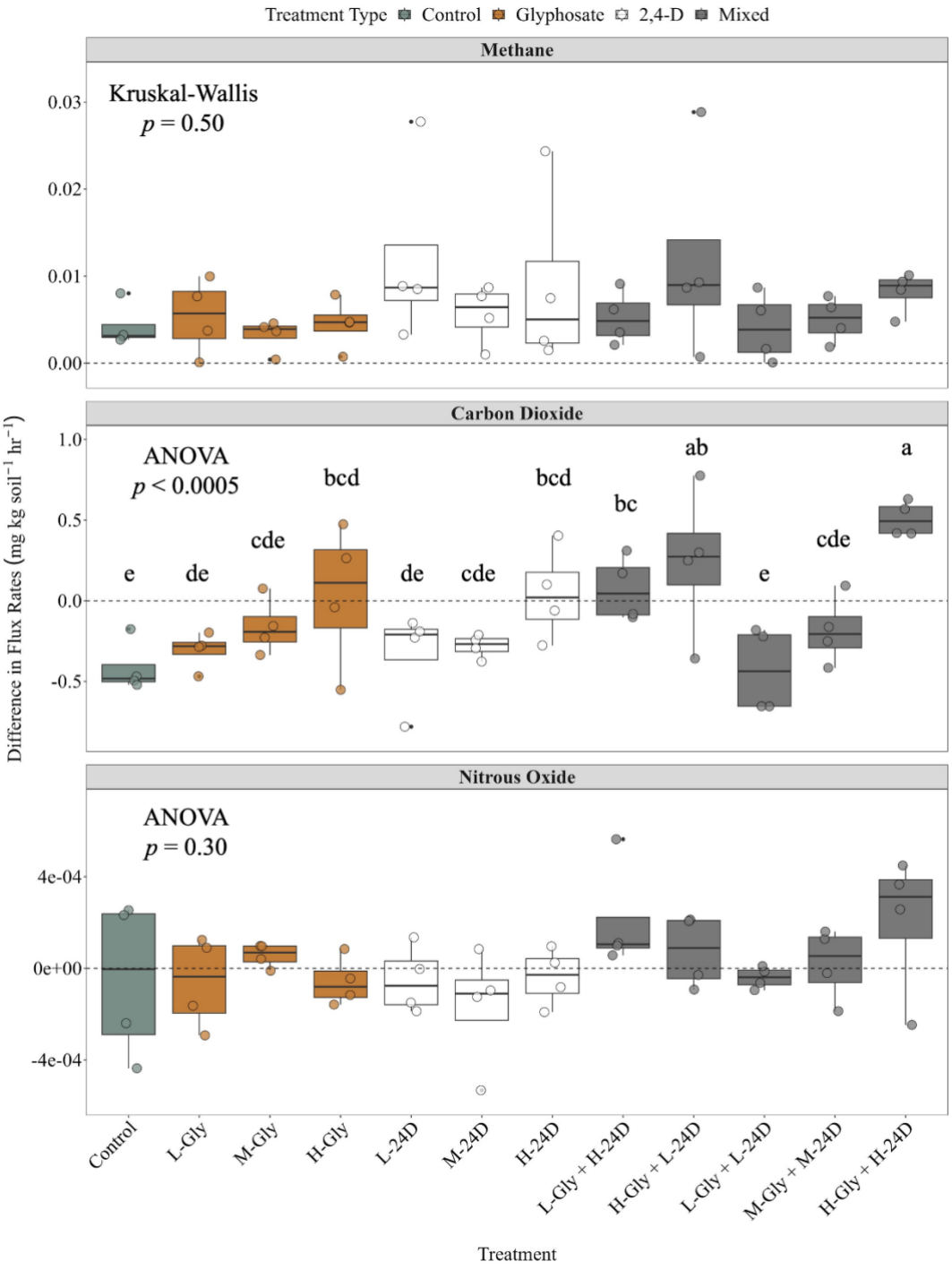

Figure 7 Difference (i.e., Day 21 - Day 0) in CH_4, CO_2, and N_2O flux rates by treatment, where boxes are colored by treatment type (see Figure B7 for time series data). Boxplots show data first and third quartile, median, minimum, and maximum, and individual data points. The x-axis is coded as follows: Herbicide concentrations, L = low, M = medium, H = high; Herbicide treatment, Gly = glyphosate and 24D = 2,4-D. Different letters indicate statistically significant differences between herbicide treatment means based on alpha = 0.05 using Least Significant Differences Post-Hoc test

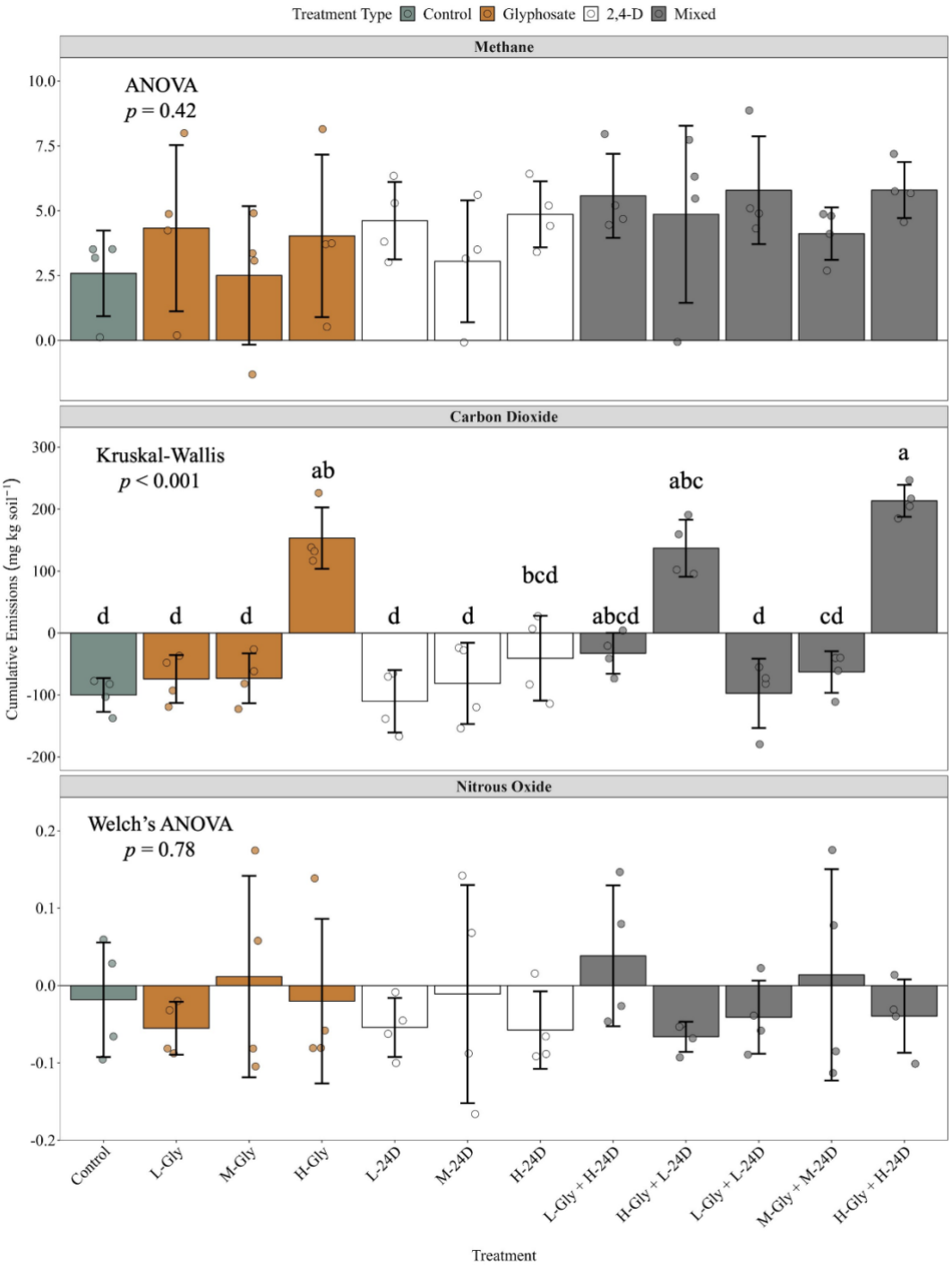

Figure 8 Average ± standard deviation cumulative CH_4, CO_2, and N_2O emissions after 21 days by treatment (see Figure B8 for time series data). Columns are colored by treatment type. Columns greater than zero (i.e., above the line) represent GHG sources to the atmosphere, whereas columns less than zero (i.e., below the line) represent GHG sinks. The x-axis is coded as follows: Herbicide concentrations, L = low, M = medium, H = high; Herbicide treatment, Gly = glyphosate and 24D = 2,4-D. Different letters indicate statistically significant differences between herbicide treatment means based on alpha = 0.05 using Dunn's Post-Hoc test

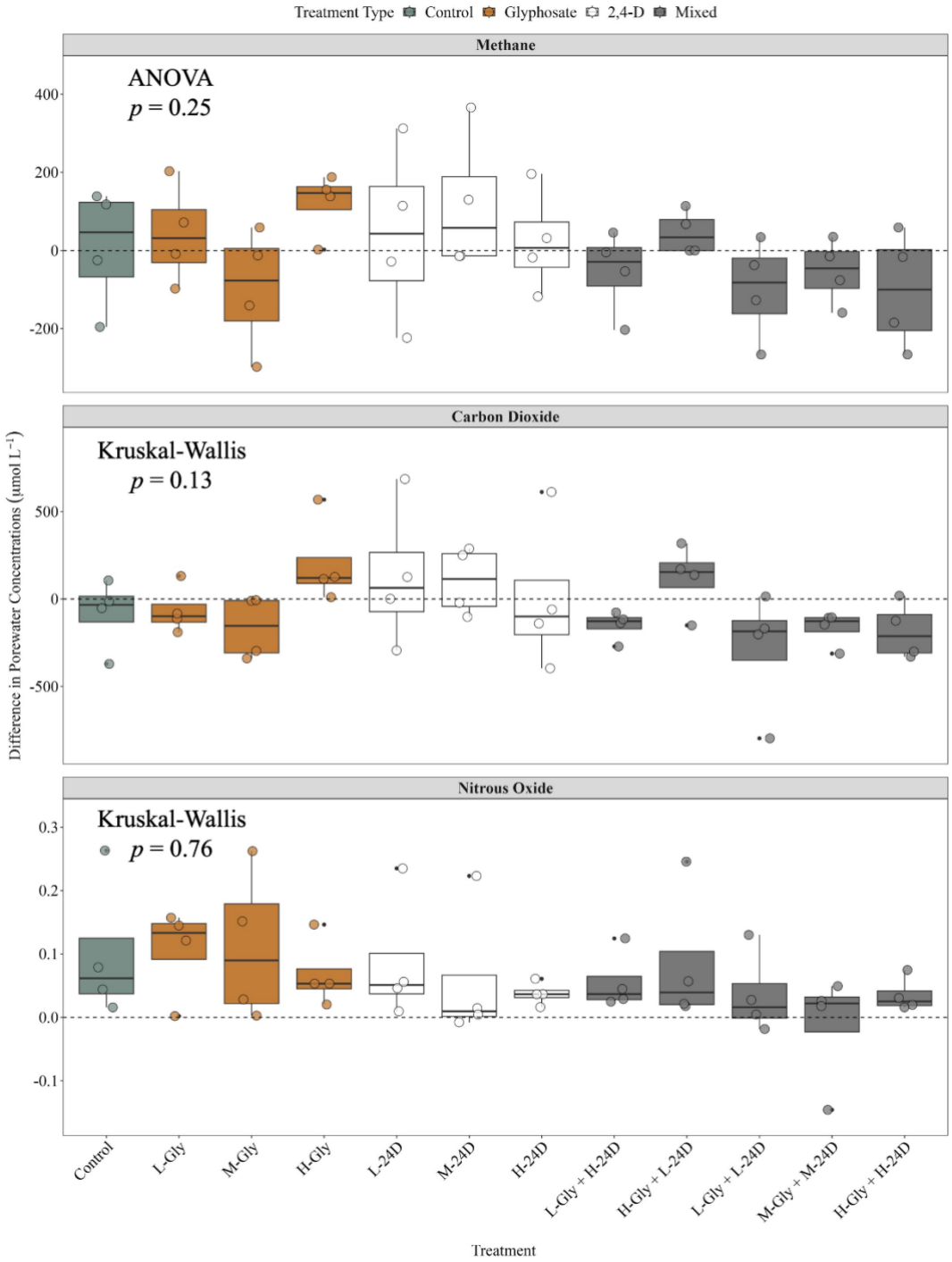

Figure 9 Difference (i.e., Day 21 - Day 0) in porewater CH_4, CO_2, and N_2O concentrations by treatment, where boxes are colored by treatment type (see Figure B9 for time series data). Boxplots show data first and third quartile, median, minimum, and maximum, and individual data points. The x-axis is coded as follows: Herbicide concentrations, L = low, M = medium, H = high; Herbicide treatment, Gly = glyphosate and 24D = 2,4-D

Conclusions

Freshwater wetlands are substantial contributors to global GHG budgets, therefore understanding the potential role herbicides play in affecting GHGs is essential for national GHG budgets and developing management strategies to mitigate climate change. Our results demonstrate that aerobic microorganisms producing CO_2 may be most impacted by glyphosate and 2,4-D. Thus, herbicide loading into wetlands could impact C biogeochemical cycling, subsequently contributing to atmospheric CO_2, whereas impacts to CH_4 and N_2O could be minimal when considering only sediment diffusive flux under consistent water level conditions. Wetland sediments are substantial C sinks, yet there are still uncertainties regarding wetland GHG budgets, especially as climate change alters environmental conditions. The research on how agrochemical use may impact climate change is still extremely limited. Additional research investigating the effects of herbicides on GHG coupled with biogeochemical functional genes would provide insight into the microbial mechanisms potential being affected. In addition, future pond-scale experiments with treatments at recommended field application rates will better represent wetlands within agricultural catchments, as well as ecosystem interactions with vegetation and hydrology. Spatial and temporal herbicide use estimates could also be factored into modeling wetland GHG budgets to help control for non-point source contamination. Overall, due to increases in herbicide use and changes in the climate, understanding chemical stress on wetlands is critical to determining potential global consequences.

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CHAPTER 4. DO SEDIMENT MICROBIAL COMMUNITIES RECOVER AFTER WETLAND RESTORATION?[4]

Abstract

Wetland restoration in the Prairie Pothole Region is a common management practice to facilitate habitat and ecosystem recovery after degradation or drainage. While recovery of vegetation, fish, and macroinvertebrates are often studied, very little is known about the recovery of sediment microorganisms, which are critical for overall ecosystem health. We collected benthic sediments from natural and restored wetlands, where restorations had occurred 26 – 33 years ago, to analyze microbial communities. We used 16S rRNA gene sequencing to investigate differences in microbial structure and function among natural and restored wetland groups. Water pH, conductivity, temperature, and major ions were also measured to investigate differences among wetland groups, as well as to evaluate their potential influences on microbial community composition. We found no significant differences in any microbial metric or environmental variable among wetland groups. There were also no significant environmental influences on community composition. Overall, our results suggest that 26 years allows ample time for microorganisms to recover and resemble natural wetland communities. These findings could indicate that subsequent ecosystem functions may also recover within this time frame.

Introduction

The Prairie Pothole Region (PPR) is an extensive prairie-wetland complex of approximately 777,000 km^2 within five U.S. states and three Canadian provinces (Dahl 2014)

[4]The material in this chapter was co-authored by Christine Cornish, Marinus Otte, and Jon Sweetman. Christine Cornish had primary responsibility for field work, data collection and analyses, and drafting the manuscript. Marinus Otte and Jon Sweetman served as proofreaders and provided minor text additions and suggestions.

(Figure 10). This region serves as critical habitat for biodiversity, breeding and migratory waterfowl, flood protection, nutrient control, and carbon storage (Gleason et al. 2011; Euliss et al. 2006). The PPR, however, is considered the most critically threatened habitat in North America (Doherty et al. 2018) as over 50% of its wetlands have been lost to agricultural conversion (Euliss et al. 2006; Ross & McKenna 2023), which is a substantial contributor to loss of biodiversity and ecosystem functions (Zedler & Kercher 2005; Barral et al. 2015). As a result, rehabilitative efforts such as wetland restoration have been implemented to conserve habitat and ecosystem function (Lu et al. 2021). Many PPR wetlands have been hydrologically restored via plugging drainage ditches, and have subsequently been monitored for biological, chemical, and physical recovery (Card & Quideau 2010). Several approaches have been used to measure and monitor wetland restoration success, such as sediment and water quality, vegetation, fish, macroinvertebrates, and diatoms (Swartz et al. 2019; Bruland et al. 2003). Oftentimes macroinvertebrates are used as indicators of restoration success because of their abundance, rapid response to disturbance, somewhat sedentary life, and critical role in wetland ecosystems (Sartori et al. 2014; Yang et al. 2017; Lu et al. 2021). However, microorganisms fit this niche too, but are notoriously overlooked in the literature as useful proxies of wetland restoration success (Sims et al. 2013; Pesce et al. 2017).

Microorganisms are a foundational component of wetland health and function as they can influence many biological, chemical, and physical properties (Sims et al. 2013; Urakawa & Bernhard 2017). Microorganisms are extremely prevalent and widespread, have high biomass, can undergo rapid selection pressure, and are highly sensitive to disturbance making them potentially useful indicators of ecosystem changes (Zhu et al. 2019; Harris 2003; Paerl et al. 2003). Previous studies that investigated microbial structure and function in wetlands have found

restoration can decrease abundance and diversity, and alter composition (Schimel et al. 2007; Hartman et al. 2008; Card & Quideau 2010), but microorganisms may be able to recover and resemble natural conditions rapidly (Xu et al. 2017). For example, Zhu et al. (2019) used a microbial community-based index of biotic integrity to evaluate wetland health and found that restored wetlands were generally considered healthy, however they used microorganisms in water, which can be transient. Sediment microbial recovery after restoration, in addition to length of recovery period, is an understudied area in wetland management relative to other biological communities (Hartman et al. 2008; Sims et al. 2013). As microorganisms play many critical roles that can impact overall wetland health, such as nutrient cycling, decomposition, and pollutant degradation (Portier & Palmer 1989; Mellado & Vera 2021), integrating microorganisms into evaluation of recovery may give a more comprehensive assessment of restoration success.

Assessment of wetland recovery after restoration is a common management practice, however most research evaluates macro-communities. As restoration practices continue on PPR wetlands to combat anthropogenic-induced changes, including agricultural conversion and climate change, it is important to better understand sediment microbial recovery, and subsequently ecosystem recovery, after wetland restoration. To provide insight on this we investigated the structure and function of benthic sediment microorganisms in natural and restored wetlands within the North Dakota PPR, where we focused on restorations 26 – 33 years old. The objective of our study was to investigate if benthic sediment microbial richness, diversity (alpha and beta), and composition differed among natural (no restoration), and restored wetlands.

Materials and Methods

Study sites and sampling

Eighteen wetlands located in the Prairie Pothole Region of North Dakota, United States (Figure 10), were sampled for benthic sediment microbial communities and environmental variables in July 2020. Five of the wetlands were natural to be used as reference sites and thirteen were restored wetlands where restorations were conducted between 1987 – 2016. All of our sites were located in protected areas, such as Wildlife Management Areas, Wetland Management Districts, and Waterfowl Production Areas. To allow for comparison among restorations, only hydrologic restorations that were completed via filling drainage ditches were included, and more recent restorations where sediment excavation occurred were excluded (Larson et al. 2020). One restoration site was removed from the dataset (Beu1, 47.4319222 °N, -101.9569294 °W) because it was dry at the time of sampling, resulting in five natural and twelve restored sites (n = 17) with restorations occurring between 1987 – 1993 (Table 6).

Sediments were collected using a Universal sediment corer (Aquatic Research Instruments, Idaho, USA) deployed at five random locations within each wetland. Sediments were extruded on-site where the upper 5 cm of sediment was collected in Whirl-Pak® bags, and samples were transported on ice to the lab. A composite sample was made from sediment samples from each wetland to obtain one representative sediment sample, which was stored at -80 °C until subsequent microbial analysis.

At each wetland, water quality data were also collected. At three locations within each wetland a YSI multiparameter probe (YSI Inc, Ohio, USA) was used for pH, conductivity, and temperature, and ~1 L of water was collected (~5 – 10 cm below the surface) for analysis of major ions. Water samples were transported on ice to the lab and stored at 4 °C for no longer

than 48 hours before processing. Samples were filtered through 0.45 µm and submitted to the North Dakota State University Soil Laboratory (Fargo, North Dakota) for analyses of (Calcium [Ca^{2+}], Chloride [Cl^{-}], Potassium [K^{+}], Magnesium [Mg^{2+}], Sodium [Na^{+}], and Sulfate [SO^{2-}_{4}]).

Microbial analysis

Environmental DNA was extracted from sediment samples to analyze for microbial community metrics. The Qiagen DNeasy® PowerSoil® Kit (Qiagen, Germantown, MD, USA) was used according to protocol for extraction of microbial genomic DNA. A 0.25 g (wet weight) sample was lysed using PowerBead Tubes in a bead-beater (Biospec Mini-beadbeater-24). Kit solutions were added to samples, vortexed, centrifuged, and decanted stepwise to purify, wash, and elute DNA into 100 µL final volume. All final DNA extracts were stored in -80 °C until sequencing. DNA extracts were shipped frozen on dry ice to Novogene Corporation Inc. (Beijing, China) for high-throughput/next-generation sequencing which included, amplicon library preparation, DNA purification, NovaSeq PE250, PCR amplification, and bioinformatics via QIIME2. All downstream analyses were conducted in RStudio (version 4.3.1, R Core Team 2022) on Observational Taxonomic Units (OTU) and species-level taxonomic units received from Novogene.

Data analysis

For analyses, natural and restored wetlands were categorized into four restoration categories using the time since restoration relative to the sampling year (i.e., 2020 − 1987 = 33 years [yrs]). Restoration categories consisted of natural = 0 yrs (n = 5), early = 32-33 yrs (n = 5), mid = 30 yrs (n = 4), and late = 26-28 yrs (n = 3). Prior to statistical analyses, response variables were checked for normality and equal variances using the *shapiro.test* function (“stats” package,

version 4.3.1, R Core Team 2022) and *leveneTest* function ("car" package, version 3.1.2, Fox & Weisberg 2019), respectively.

Richness and alpha diversity

Richness and alpha diversity were calculated on species taxonomic units with the "vegan" package (version 2.6-4, Oksanen et al. 2017). The *specnumber* function was used to compute richness, and Shannon diversity was computed using the *diversity* function. To test for differences in these metrics between restoration categories, Analysis of Variances (ANOVA) were performed using the *aov* function ("stats" package, version 4.3.1, R Core Team 2022). Additionally, Pearson correlations were performed to test for relationships in richness and Shannon diversity with time since restoration using *cor.test* ("stats" package, version 4.3.1, R Core Team 2022).

Beta diversity

Total beta diversity and contribution of richness difference and species replacement were also calculated on species taxonomic units by restoration category using *beta.div.comp* with the quantitative coefficient of Sörenson-based indices (Legendre 2014). Richness difference can be explained as one restoration category containing more species than other categories, versus replacement, which is defined as species turnover (Legendre 2014). Local Contribution to Beta Diversity (LCBD) was calculated for each site and tested for significance using 999 permutations within the *beta.div* function. The "adespatial" package was used for all beta diversity calculations (version 0.3-23, Dray et al. 2023). LCBD was tested for differences between restoration categories using ANOVA, and for a relationship with time since restoration using Pearson correlation.

Bray-Curtis distances were calculated on raw OTUs to compare microbial communities across restoration categories using an Analysis of Similarities (*anosim* function). In addition, non-metric multidimensional scaling (NMDS) was conducted using *metaMDS*, to display composition differences within multivariate space. The *envfit* function was used to conduct multiple regressions of environmental variables with ordination axes and permutation tests to investigate significant explanatory variables of microbial composition. All community composition analyses were conducted using the "vegan" package (version 2.6-4, Oksanen et al. 2017).

Functional traits

The Functional Annotation of Prokaryotic Taxa (FAPROTAX) database was used to predict microbial functional profiles from OTUs (Louca et al. 2016). The FAPROTAX database uses prokaryotic clades obtained from the literature to assign OTUs a function based on identified taxa. The "microeco" package (version 1.3.0, Chi et al. 2021) was used to remove taxa assigned to "mitochondria" and "chloroplast" (*microtable$filter_pollution*), map and assign OTUs to functional profiles (*trans_func$cal_spe_func*), and then calculate species percentages based on functional traits (*trans_func$cal_spe_func_perc*). Functional profiles assigned to "Other" were removed, and only carbon-, nitrogen-, and sulfur-cycling, and energy source profiles were used for statistical analysis (32 functional traits). Functional trait percentages within the profiles were then grouped by wetland site and functional traits, and then summed. To test for differences in functional traits across restoration categories, a Kruskal-Wallis test was performed. Additionally, non-parametric multiple linear regression was conducted to assess relationships between functional traits and water chemistry variables using the *gsm* function within the "npreg" package (version 1.0-9, Helwig 2022).

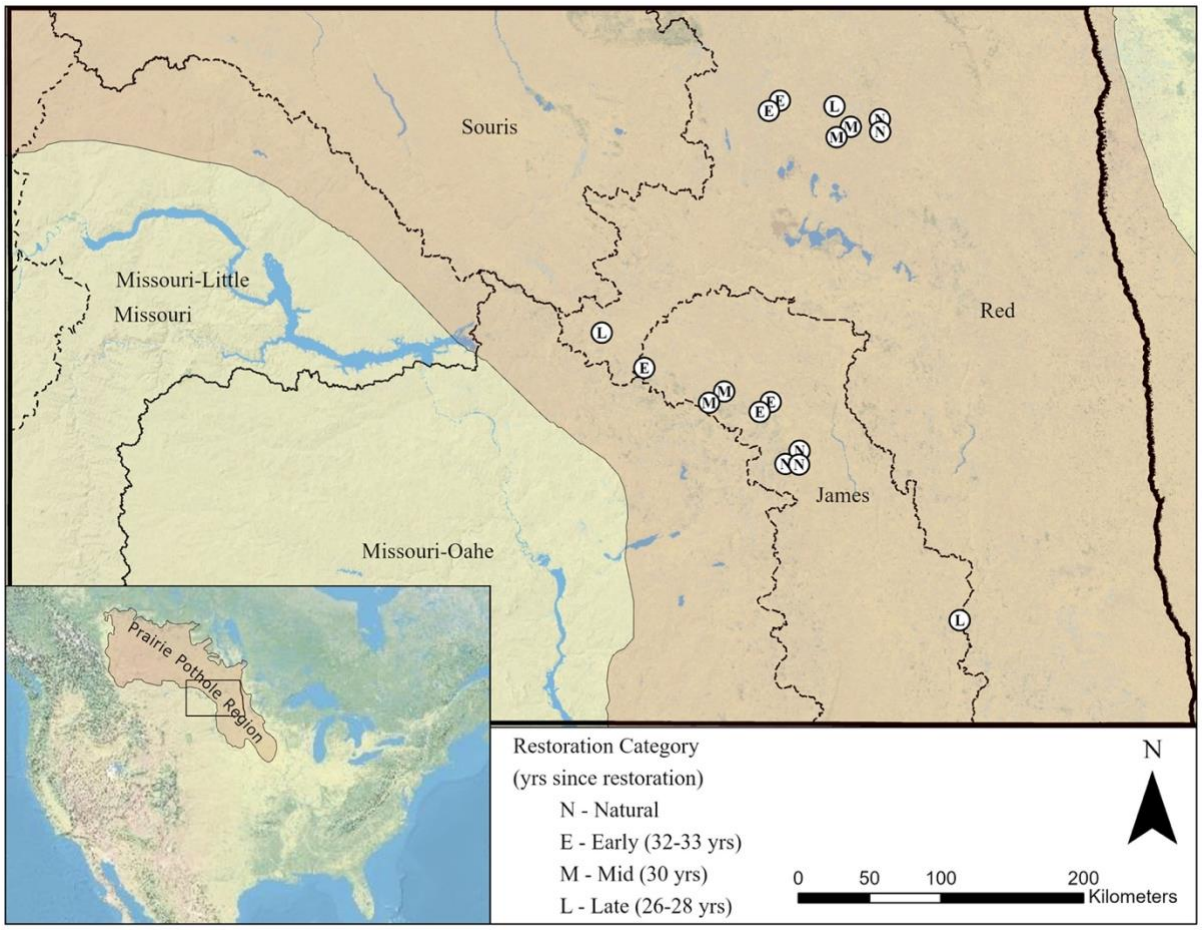

Figure 10 Map of North Dakota showing sampling sites as wetland restoration categories where, N = natural (*n* = 5) , E = early (*n* = 5), M = mid (*n* = 4), and L = late (*n* = 3) with watershed boundaries as dotted lines (National Map Hydrography Dataset) (https://www.usgs.gov/national-hydrography/national-hydrography-dataset) and a tan shaded boundary of the Prairie Pothole Region (Mann 1974)

Table 6 Site specific information including restoration category based on TSR = time since restoration, site name, water quality (pH, conductivity, and temperature), and location. Water quality variables show averages ± standard deviations (n = 3), where NAs are present if only one measurement was taken. Average rows show the averages of each variable per restoration category, where **bold** values represent the highest average among categories

Restoration Category	Site Name	TSR (yrs)	pH	Conductivity (µS cm^{-1})	Temperature (°C)	Latitude (°N)	Longitude (°W)
None (natural)	CWP3	–	6.3 ± 0.01	6191 ± 26	20.4 ± 0.17	47.1018007	-99.1015394
	CWP8	–	6.7 ± 0.02	2196 ± 32	23.6 ± NA	47.0990382	-99.1033780
	CWT1	–	6.8 ± 0.01	5971 ± 23	25.3 ± 0.25	47.0987805	-99.1025329
	Aus1	–	7.3 ± 0.01	2510 ± 83	20.7 ± 0.12	48.5008986	-98.5961917
	Aus2	–	7.1 ± 0.02	2173 ± 24	19.7 ± 0.12	48.5024675	-98.5938663
	Average	--	6.8 ± 0.38	3808 ± 1927	21.7 ± 2.27	--	--
Early	Hwk1	33	6.5 ± 0.02	1870 ± 19	20.2 ± 0.00	47.3154517	-99.2737209
	Hwk2	33	6.6 ± 0.05	2028 ± 9	21.3 ± 0.30	47.3177726	-99.2759772
	DNC1	32	6.9 ± 0.03	6216 ± 28	22.0 ± 0.10	47.4608059	-100.0687251
	Niko1	32	7.5 ± 0.03	5968 ± 102	21.1 ± 0.87	48.5840644	-99.2157319
	Niko5	32	7.6 ± 0.06	8988 ± 775	21.0 ± 0.25	48.5849891	-99.2185846
	Average	--	7.0 ± 0.48	**5014 ± 2829**	21.1 ± 0.70	--	--

Table 6 (continued) Site specific information including restoration category based on TSR = time since restoration, site name, water quality (pH, conductivity, and temperature), and location. Water quality variables show averages ± standard deviations (n = 3), where NAs are present if only one measurement was taken. Average rows show the averages of each variable per restoration category, where **bold** values represent the highest average among categories

Restoration Category	Site Name	TSR (yrs)	pH	Conductivity (µS cm^{-1})	Temperature (°C)	Latitude (°N)	Longitude (°W)
Mid	Swt1	30	6.6 ± 0.06	4723 ± 18	23.4 ± 0.21	47.3604385	-99.5798609
	Swt2	30	6.7 ± 0.01	3629 ± 26	23.1 ± 0.15	47.3570435	-99.5816070
	Moo1	30	7.0 ± 0.03	3605 ± 245	18.5 ± 0.15	48.4752144	-98.7830953
	Moo2	30	7.0 ± 0.02	2881 ± 10	21.2 ± 0.17	48.4754465	-98.7785717
	Average	--	6.8 ± 0.19	3710 ± 695	21.6 ± 2.07	--	--
Late	Kin1	28	9.4 ± NA	6619 ± NA	25.8 ± NA	47.6101331	-100.3367995
	Pil1	27	6.9 ± 0.05	3539 ± 3	25.0 ± 0.20	46.3773601	-98.0835900
	Ban1	26	7.2 ± 0.02	2785 ± 16	20.9 ± 0.15	48.5606421	-98.8057538
	Average	--	**7.4 ± 0.90**	3656 ± 1360	**23.3 ± 2.34**	--	--

Results

Richness and alpha diversity

Species richness and Shannon diversity both showed no significant differences among restoration categories (richness: F = 0.26, p = 0.85; diversity: F = 0.45, p = 0.72; Figure 11). The highest average richness was in late restored wetlands (mean ± standard deviation = 244 ± 12), whereas the highest average Shannon diversity was in natural wetlands (0.71 ± 0.17). We observed much higher variability in early restorations compared to late. For richness, the standard deviation was 13.3% of the mean for early restorations, and 4.9% of the mean for late restorations; and for Shannon diversity, the standard deviation was 33.5% of the mean for early restorations, and only 1.5% of the mean for late restorations (Figure 11). There was also no significant relationship between richness or Shannon diversity and time since restoration, but they both displayed a slight decrease with increasing years since restoration (richness: R = -0.15, p = 0.64*;* diversity: R = -0.21, p = 0.52; Figure 12A and B).

Beta diversity

LCBD across restoration categories were similar (F = 0.67, p = 0.59), however there were significantly unique sites (Figure 11C). Specifically, four sites were significantly unique, where the two highest contributors to beta diversity were an early restored and natural wetland (E: Niko1 = 0.075, N:CWT1 = 0.071), and a late restored and natural wetland contributed similarly to beta diversity (L:Pil1 and N:Aus1 = 0.065) (Figure 11C). LCBD was not significantly correlated with time since restoration, but contrary to richness and Shannon diversity, LCBD slightly increased with years since restoration (R = 0.25, p = 0.43; Figure 12C).

There were no significant differences in community composition among restoration categories as confirmed by Analysis of Similarities (R = 0.08, p = 0.21), where two-dimensional

NMDS also demonstrated species composition was similar across sites in multivariate space (stress = 0.17) (Figure 13). While stress within two-dimensional ordination space was high, a stressplot indicated a good fit of the non-metric model ($R^2 = 0.97$) and three-dimensions did not provide new or contradictory conclusions to our findings. Permutation tests resulted in no significant environmental drivers of microbial community composition (Table 7, Figure 13).

Functional traits

Microbial functional traits did not significantly differ across restoration categories ($X^2 = 0.5$, $p = 0.92$, and there was relatively high variability (Figure 14). However, some trends were observed at site-level. The abundance of denitrification traits were low across most sites except Aus1 where they were higher than the mean, and Swt1, Niko1, CWT1, and Moo1 where they were lower than the mean. Aus1, Aus2, Swt1, and Pil1 had higher abundance in all functional traits, whereas Niko1, CWP8, and CWT1 were lower, especially for traits associated with nitrogen cycling (Figure 14). There were also no significant relationships between functional traits and surface water ions (Table C2).

Table 7 Multiple regressions and permutation tests (permutations = 999) of environmental variables with ordination axes, where r^2 = strength of relationship to the first two ordination axes and *p*-value with significance based on alpha = 0.05

Variable	**r^2**	***p*-value**
pH	0.10	0.49
Conductivity	0.17	0.25
Temperature	0.08	0.55
Ca^{2+}	0.16	0.30
Cl^{-}	0.06	0.68
K^{+}	0.26	0.14
Mg^{2+}	0.10	0.51
Na^{+}	0.02	0.84
SO^{2-}_{4}	0.01	0.94

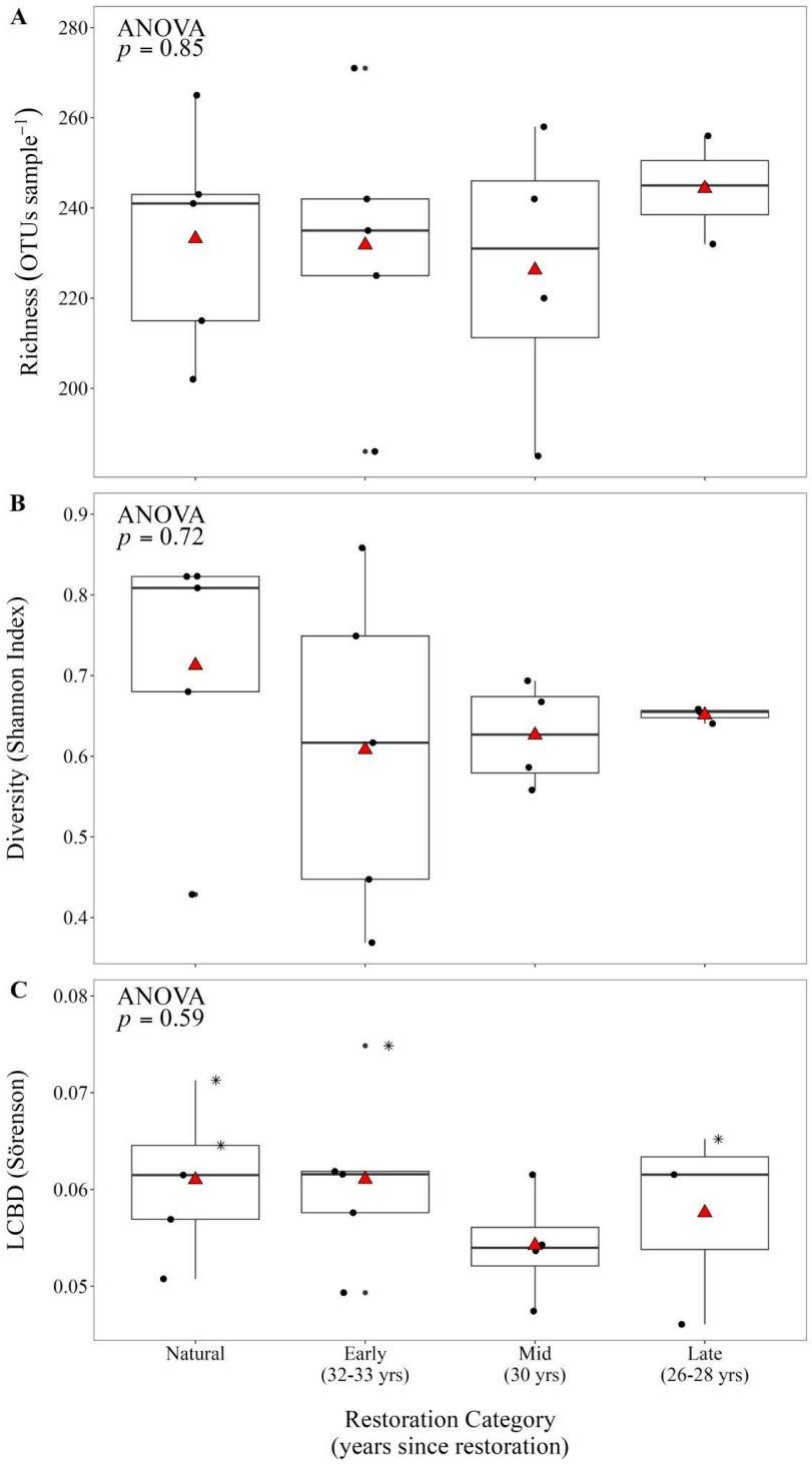

Figure 11 Species taxonomic unit **A** richness, **B** alpha diversity, and **C** LCBD among natural and restored wetlands, where restoration categories represent years since restoration. Boxplots show data first and third quartile, median, minimum, and maximum, and individual data points, and red triangles represent the mean. Test of significance performed on LCBD indices with 999 permutation, where asterisks indicate a significant ($p < 0.05$) LCBD

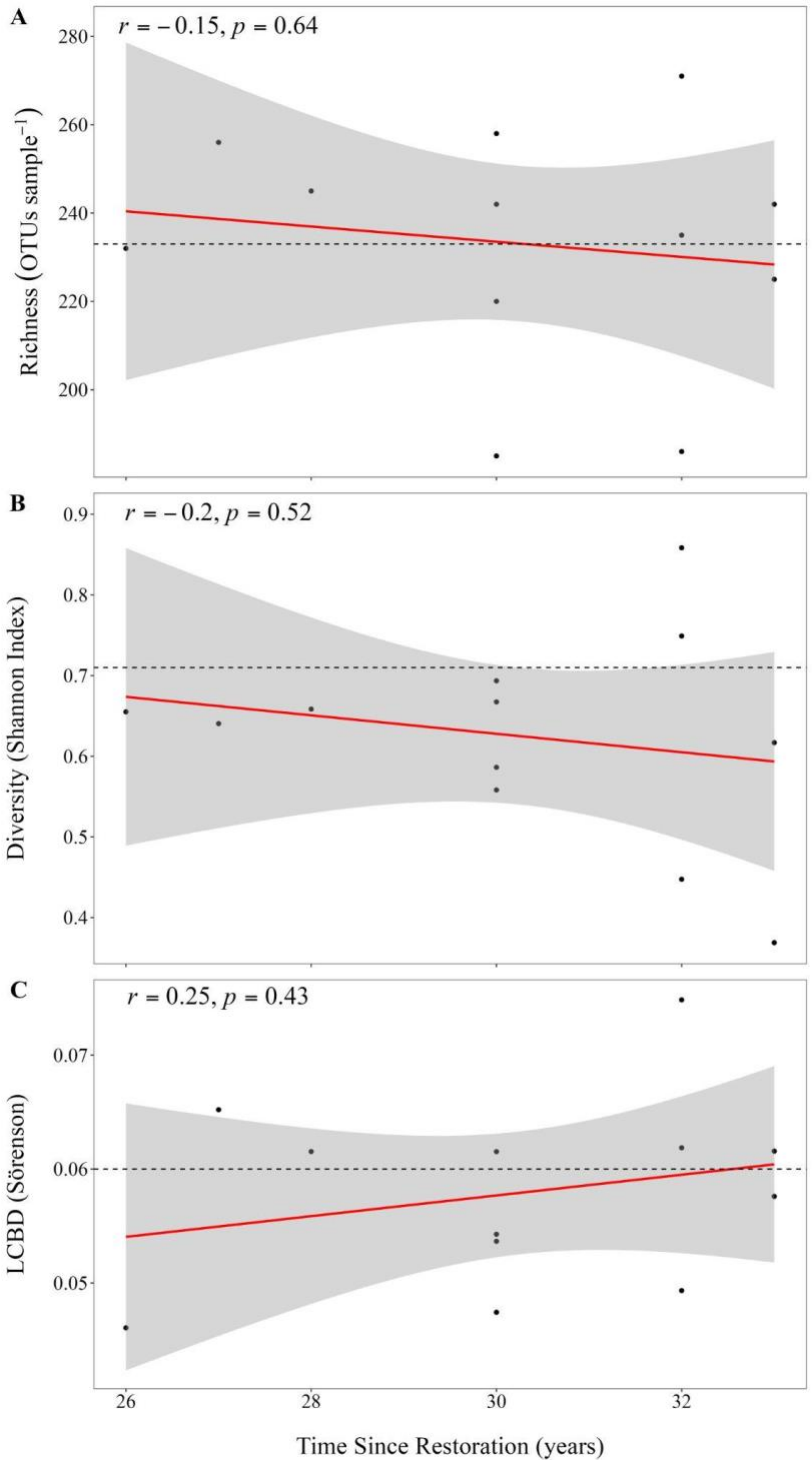

Figure 12 The linear relationship between species taxonomic unit **A** richness, **B** alpha diversity, and **C** LCBD and time since restoration in years. Pearson correlations were performed to obtain the strength and significance of the relationship between restored wetlands and species metrics, where r = correlation coefficient and p = p-value with significance based on alpha = 0.05. The dashed line represents the average of natural wetlands (n = 5) for each respective metric. Gray zone around the red trend line represents the 95% confidence interval

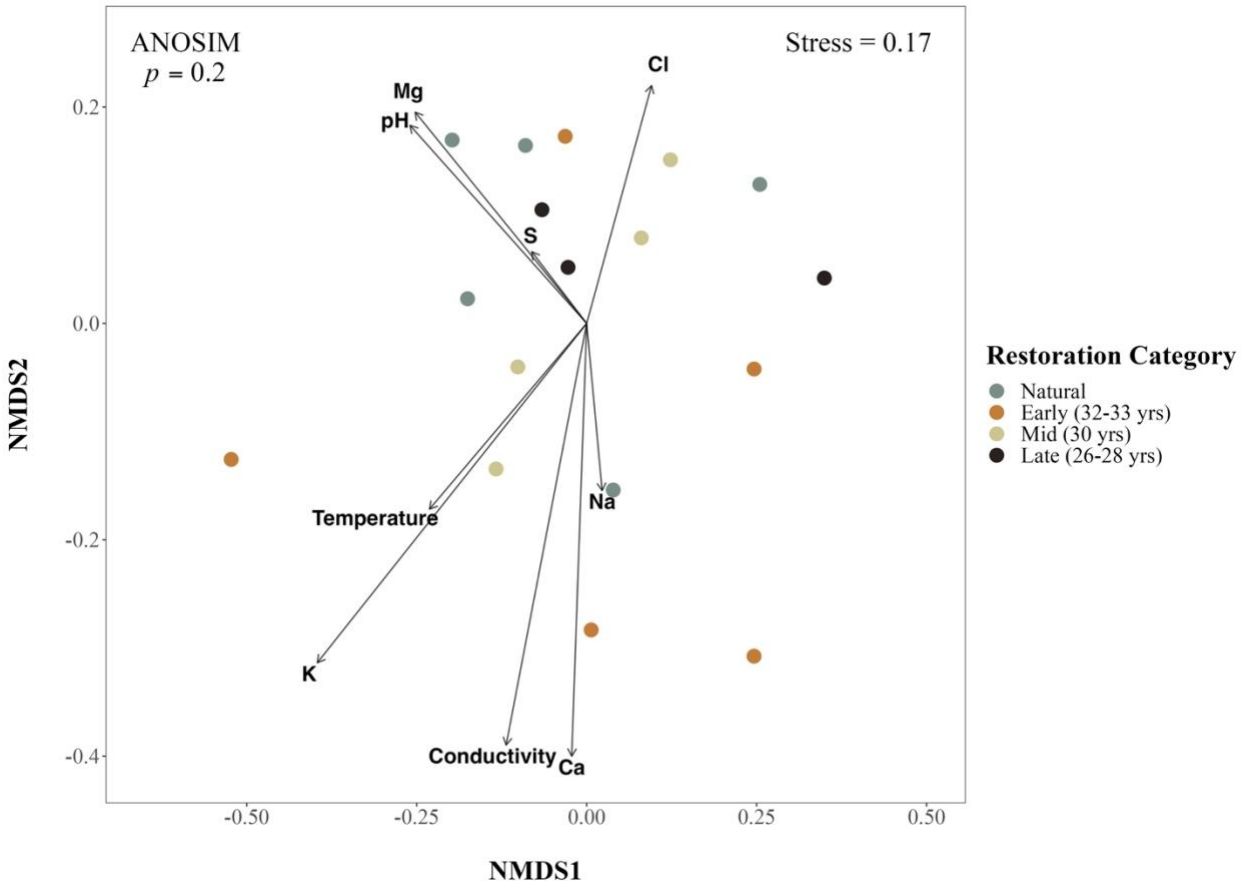

Figure 13 NMDS ordination of OTUs colored by restoration category with environmental variables as vectors

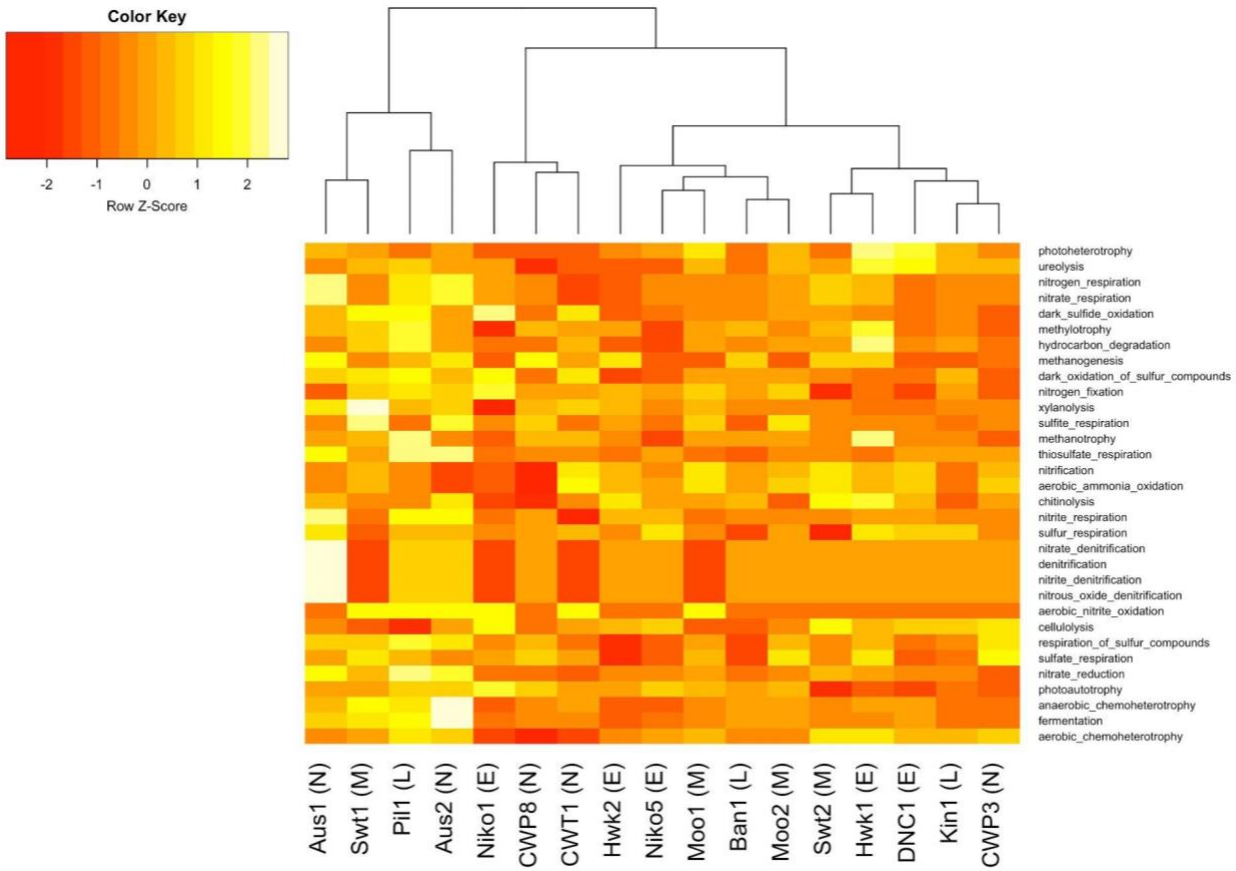

Figure 14 Heatmap of standardized microbial functional profiles across individual sites (restoration categories: N = natural, E = early, M = mid, and L = late). Functional groups represented are the carbon-, nitrogen-, and sulfur-cycles, and main energy source. Dendrogram shows functional profile relatedness among sites

Discussion

Our study used benthic sediment microbial communities collected from five natural and twelve restored wetlands in the North Dakota PPR to investigate microbial recovery along a gradient of time since restoration. We found that microbial richness, diversity, composition, and function, as well as environmental variables, of all restored wetlands closely resembled natural wetlands, and there were no apparent differences with respect to time since restoration. Our results indicate that microbial communities in restored wetlands can resemble natural wetland communities after at least 26 years post-restoration. The lack of differences we found in

microbial communities among our restoration categories demonstrates a positive outcome for ecosystem health following hydrologic restoration of PPR wetlands.

Richness and diversity

Previous research comparing microbial communities in natural and restored wetlands have often found distinct community differences. For example, in bottomland forested wetlands in Kentucky, U.S., D'Angelo et al. (2005) found significantly higher biomass of anaerobic bacteria in wetland restorations greater than 10 years old versus younger restorations. Hartman et al. (2008) found in North Carolina, U.S. coastal plains that restoration significantly decreased bacterial diversity, and that natural wetlands had lower diversity than agricultural wetlands. Card & Quideau (2010) compared younger (1 – 6 yrs) and older (7 – 11 yrs) wetland restorations to reference sites in the Canadian PPR and found significantly lower microbial biomass, evenness, and diversity in younger restorations compared to reference sites. The restored wetlands we examined in our study were all greater than 25 years old at the time of sampling compared to less than 15 years old in the aforementioned studies. This considerable temporal gap may explain the lack of significant differences we found among our restored and natural wetlands, as microorganisms had a longer recovery period (Moreno-Mateos et al. 2012). It has been suggested that ecosystems may take anywhere between 20 years to centuries to fully recover after restoration (Jones & Schmitz 2009; Moreno-Mateos et al. 2012), where Xu et al. (2017) indicated that microbial community structure in soybean cultivated marshes in China needed 12 years or more to mimic natural conditions. We found similar microbial richness, alpha and beta diversity, and composition patterns among all categories of restored wetlands and reference wetlands, where restorations occurred 26 to 33 years prior.

Our results do align with some studies, including comparisons between younger restored wetlands and natural wetlands. Despite Xu et al. (2017) suggesting a 12-year recovery period is needed for microorganisms, they still found similar community composition in restored marshes 6 years post-restoration and natural marshes. In 7-11 year old restorations, Card & Quideau (2010) found microbial community composition was similar to reference wetlands, but contrary to our results, richness increased with increasing time since restoration. Contrarily, age of restoration was not found to significantly influence microbial communities in coastal marshes, where microbial biomass was similar between created and natural marshes within 8 years post-construction (Abbott et al. 2022). This is inconsistent with terrestrial, prairie ecosystems in Illinois, U.S. that showed prairie age had the greatest influence on microbial community composition in soils (McKinley et al. 2005). While our sites consisted of shallow, temperate freshwater wetlands that were impacted by hydrological restoration, our results still resemble findings collected in various systems indicating that microbial community recovery may not be as influenced by restoration technique, climate, or biome, but may be more affected by time since restoration. Recovery is often organism-dependent (Meli et al. 2014), thus microorganisms should recover within a shorter period of time compared to other macrofauna due to differences in reproduction, life cycles, and habitat. Our restored wetlands were relatively old, between 26 – 33 years post restoration, providing a long period of time for microbial communities to recover. The homogeneity of microorganisms across our sites suggests that ecological restoration of semi-permanent and permanent wetlands in the PPR may not impact microbial communities long term.

Functional traits

Taxonomic composition of microorganisms can be highly variable through space and time, while functional community composition of sites can be similar (Louca et al. 2016). Functional diversity patterns are major contributors to wetland ecosystems via biogeochemical cycling (Escalas et al. 2019). The major biogeochemical cycling groups, carbon (C), nitrogen (N), and sulfur (S), were similar across 13 of our sites spanning all restoration categories, however two natural wetlands (Aus1 and Aus2), one mid restoration (Swt1), and one late restoration (Pil1) had notably higher abundance in most functional traits. In two meta-analyses (Moreno-Mateos et al. 2012; 2015), C storage in soils was analyzed as a proxy for biogeochemical function, and they found that it remained significantly lower in restored wetlands than reference wetlands up to 30 years post-restoration and was still approximately 25% lower after 100 years. Swt1 and Pil1 were 30- and 27-years post-restoration, respectively, and both sites had higher functional trait abundance, whereas this pattern was not seen in any of our early restorations (i.e., 32-33 years since restoration). We also observed consistently average to lower abundance of denitrification traits, including nitrate, nitrite, and nitrous oxide denitrification in most sites, which is surprising as wetlands within the PPR are frequently subjected to excess nutrient inputs from surrounding agricultural land (Martin et al. 2019). Many of our restoration sites were adjacent to cropland or grazing, whereas our natural sites were in areas with less agricultural disturbance. As restored wetlands often represent transitional systems between agricultural land and natural wetlands, they may exhibit biological, chemical, or physical characteristics of both (Li et al. 2022), especially if restorations did not consist of sediment excavation, which may explain the high variability in functional traits across sites. Variance in functional community composition can be partially driven by environmental filtering

and biotic interactions (Louca et al. 2016). We found similar water chemistry, microbial assemblage, and macroinvertebrate communities (Sauskojus et al. *unpublished*), which could also explain similarities in functional profiles across sites. Additionally, our sites were all located within two major watershed basins, James River and Red River Valley, which could facilitate long distance microbial dispersal via water or other organisms, therefore homogenizing the regional species pool and facilitating recovery (Philippot et al. 2021).

Environmental variables

Environmental conditions are important drivers of microbial communities, where changes in land use, water levels, nutrient availability, temperature, and other factors can significantly impact assemblage and activity (Bossio et al. 2006; Peralta et al. 2012; Ratzke et al. 2020). We measured chemical and physical characteristics of surface water in our sites, but none of our environmental variables were found to be significant drivers of microbial community structure across wetlands. In contrast, Abbott et al. (2022) found that environmental conditions were more influential than age of restoration in determining microbial community assemblages, and Moreno-Mateos et al. (2012) found that wetland size and regional climate impacted overall wetland recovery, including biota and biogeochemical function, specifically larger wetlands (> 100 hectares) and tropical climates had faster recovery times. Wetlands in our study were all relatively small (< 5 hectares) with only one site ~25 hectares (Kin1) and all sites had similar microbial communities and environmental conditions suggesting that small wetlands may also be capable of quicker ecosystem recovery. The PPR has experienced a wet period since the early 1990s (McKenna et al. 2019), which could facilitate microbial dispersal via hydrologic connectivity (Stres et al. 2010). The sediments in our sites were not directly disturbed from restoration, therefore under wetter conditions the regional sedimentary species pool may be

contributing to the homogeneity of microbial communities across our sites and allow for easier and faster recovery. Land use, specifically agriculture, can have significant effects on microbial communities and ecosystem function (Bruland et al. 2003; Bossio et al. 2006), but local environmental conditions may have a stronger influence on microbial communities (Peralta et al. 2012). Agriculture is deeply integrated within the PPR where both natural and restored sites could be impacted directly or from non-point source pollution, which may have shifted all wetlands in our study into a similar alternative state (Moreno-Mateos et al. 2012). However, similar environmental variables across our sites may better explain the lack of differences in microbial community composition.

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CONCLUSIONS

Benthic microorganisms play foundational roles in freshwater wetlands including moderating energy, nutrient cycling, regulating many biogeochemical processes, and assimilating and transforming pollutants, and they can be important sentinels of ecological disturbance (Sun et al. 2013; Wang et al. 2020; Yi et al. 2021). Despite this, very little is known about the effects of chemical and physical stressors on freshwater microbial communities relative to other ecosystems. Microbial richness, diversity, composition, and function can serve as important bioindicators of ecological change as they can be highly sensitive to disturbance (Paerl et al. 2003). For example, decreases in microbial abundance have been found to correlate with decreases in ecosystem function (Philippot et al. 2021), presumably due to their influence on water quality and biogeochemical cycling (Roulet 2000). Anthropogenic-derived stressors including herbicide contamination and climate change could directly affect microbial structure and function, and subsequently impair wetland health.

Herbicides are widespread contaminants that are frequently detected in wetlands, where they can often persist in sediments (Helander et al. 2012; Battaglin et al. 2014). Herbicides can directly affect microorganisms via toxicity, nutrient enrichment, or Pollution Induced Community Tolerance, which shifts a community from herbicide-sensitive to herbicide-tolerant (Blanck 2002; Shushkova et al. 2009; Van Bruggen et al. 2018). In addition, herbicide use may increase as a result of climate change, where higher concentrations, frequency of application, and number of active ingredients used are expected (Delcour et al. 2015; Cornish and Sweetman 2023). As wetlands are subjected to herbicide contamination and climatic changes, which may continue to intensify, it becomes particularly important to understand the potential direct and indirect effects of these stressors on benthic microorganisms.

The objective of my dissertation was to investigate the effects of common use herbicides on sediment microorganisms as a proxy for wetland health. My first chapter was an incubation experiment to isolate the direct effects of glyphosate and its main metabolite AMPA on sediment microbial communities from a PPR wetland with no known history of glyphosate use. I also used an incubation experiment for my third chapter to isolate the effects of glyphosate and 2,4-D on microbial-mediated greenhouse gas production and consumption, where chapter two outlines my speculations on those processes potentially being affected. Lastly, my fourth chapter was a field study within the PPR in North Dakota to survey herbicide concentrations and sediment microbial communities among natural and restored wetlands. While analysis of herbicides resulted in non-detectable residues for 2,4-D, acetochlor, atrazine, deethyl-atrazine (atrazine metabolite), glyphosate, AMPA (glyphosate metabolite), and metolachlor, I still analyzed microbial communities to investigate their long-term recovery after wetland restoration.

Despite their sensitivity and potential to be useful indicators of environmental change, I found microbial communities to not be significantly different in both my chapter 1 microcosm experiment, and when comparing between restored and natural, undisturbed sites in chapter 4. In the present day, the use of microorganisms as indicators of environmental disturbance may be more challenging due to ubiquitous anthropogenic-derived stressors, in addition to the array of already complex ecological interactions. Microorganisms have high taxonomic variability (Louca et al. 2016) and it has been suggested that the use of taxonomic-based metrics may be insufficient when extrapolating for management or policy (Fierer et al. 2021). However, the use of microorganisms as bioindicators for water quality, long-term pollution, and genetic resistance to stressors has proven successful in some studies (Paerl et al. 2003; Sagova-Mareckova et al. 2021). It has been argued that microbial DNA-based approaches should be utilized, but these

analyses may come with barriers such as requirement of precise analytical skills and equipment, and money. However, if a DNA-based approach is feasible, the application of microorganisms for ecotoxicological application may be more beneficial. The use of incubation experiments to investigate the isolated effects of stressors on microorganisms aids in the understanding of potential bottom-up effects.

I used a DNA-based approach to examine the effects of anthropogenic stressors on benthic sediment microorganisms in freshwater wetlands from several different spatial scales for my dissertation research. My research provides an ecotoxicological perspective into wetland microbial ecology, which incorporated global issues of rising concern. Academics, industry, agriculture, government, and the public could translate my findings into management, application, or future research. My research could have substantial contributions to better understanding wetlands in a changing world.

Future research

Microbial ecology has undergone dramatic improvements with the use of higher quality molecular methods, which can provide contextual information to help answer complex ecosystem level questions. I used metabarcoding with 16S rRNA gene sequencing in chapters 1 and 4 of my research, which provided taxa identification from environmental DNA. This gave me a broad overview of species presence and abundance, and enabled further quantification of species metrics, such as richness and diversity. However, caveats to this analysis is that it cannot differentiate rates of change in the 16S gene (Clarridge 2004), which would be useful in an evolutionary context. Due to ongoing environmental changes, in addition to the constant emergence of new pollutants, targeting genes would provide a deeper understanding of how microbial communities are being impacted by various stressors. This method could also be

coupled with paleo-ecotoxicology to reconstruct disturbance, such as chemical pollution, to investigate evolutionary responses of microbial communities over time. In particular, pesticide effects could translate as changes in gene expression, but not changes in richness or taxonomic diversity (Feld et al. 2015). Targeting microbial genes can also provide information on biogeochemical cycling through functional groups, including carbon and nitrogen cycles. In chapter 3, I used GHG flux and porewater concentrations as a proxy for carbon- and nitrogen-related cycling responses to individual and combined herbicide treatments. While these metrics are commonly used to determine the net effect of microbial production and consumption of GHGs, there is still a question on the particular mechanisms being directly affected by chemical stress. Investigating synergistic effects can be particularly challenging, especially at larger scales, however analysis of microbial communities is a great way to aid in the understanding of wetland processes.

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APPENDIX A. HOW BENTHIC SEDIMENT MICROBIAL COMMUNITIES RESPOND TO GLYPHOSATE AND ITS METABOLITE: A MICROCOSM EXPERIMENT [5]

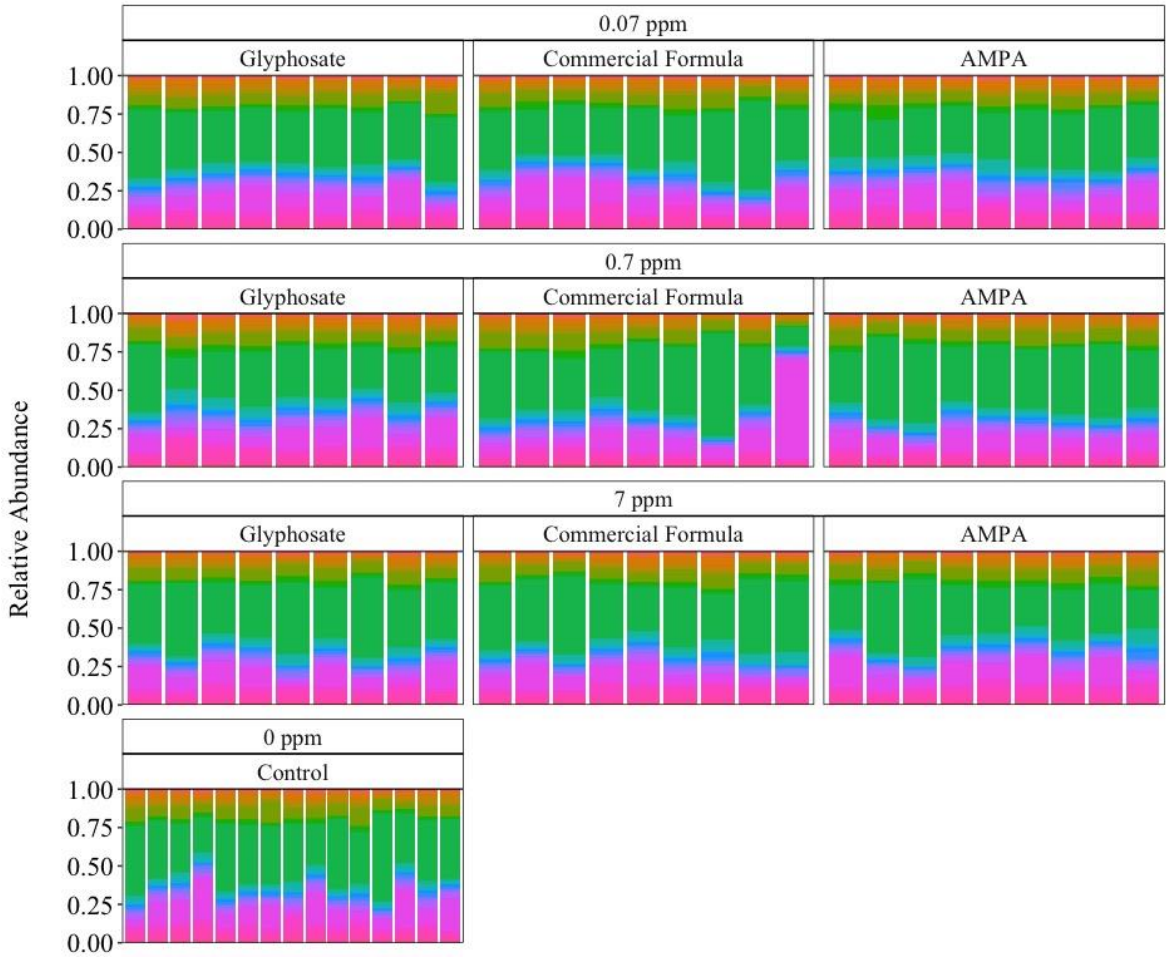

Figure A1 Relative abundance of microbial families within microcosm sediments at all sampling timepoints (n = 96)

[5]The material presented here is supplementary information for Chapter 1

Figure A1 (continued) Relative abundance of microbial families within microcosm sediments at all sampling timepoints (n = 96)

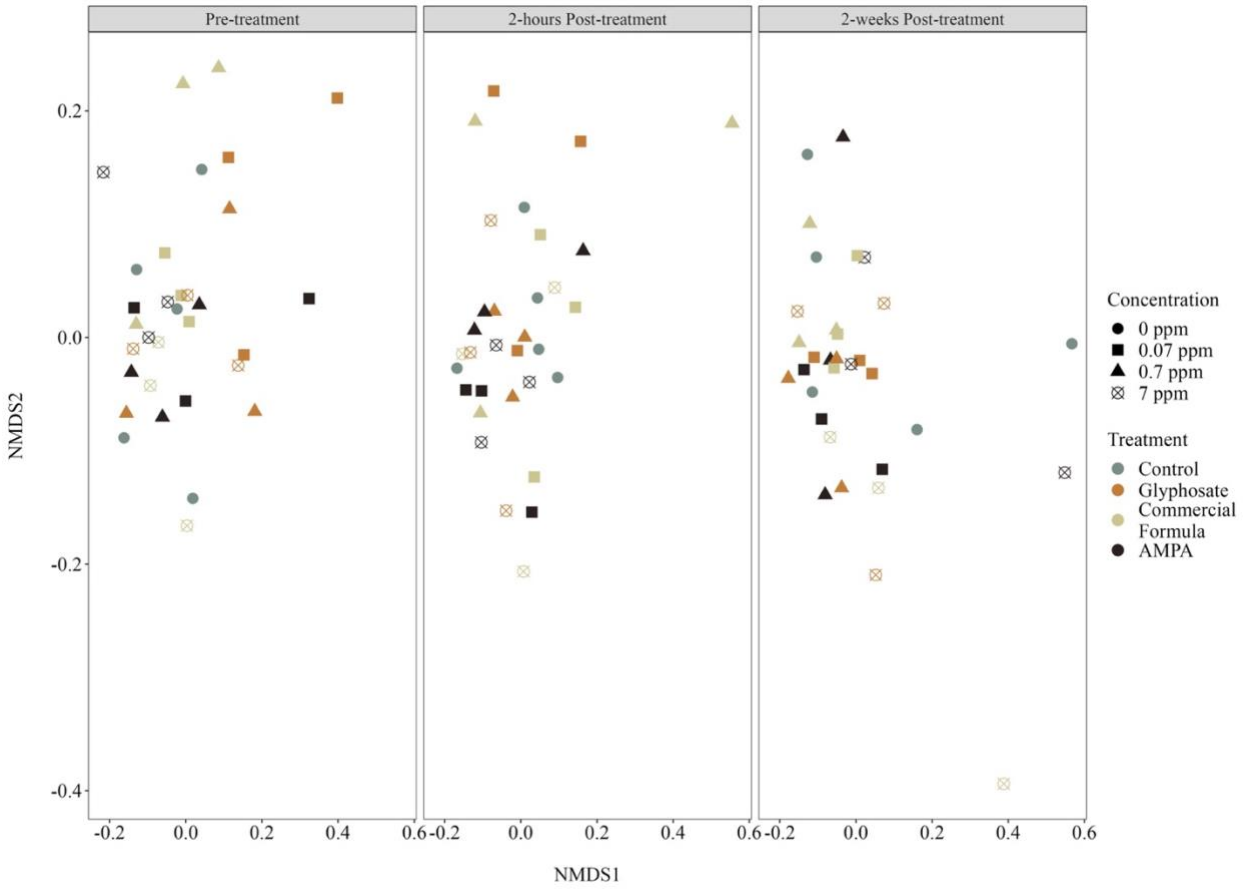

Figure A2 NMDS plot of sediment microbial community structure across all samples (n = 96) where shapes represent concentrations, colors represent herbicide treatments, and individual panels show day of sampling

APPENDIX B. COMMON USE HERBICIDES INCREASE WETLAND GREENHOUSE GAS EMISSIONS[6]

Methods and Materials

Correlation: Flux rates and porewater concentrations

To investigate the monotonic relationship between flux rates and porewater concentrations, Spearman Rank correlation analyses were performed. Flux rates and porewater concentrations from days 0, 7, 14, and 21 were used. Day 3 measurements were removed to fully align datasets by microcosm (n = 171). Data were grouped by treatment and then correlations were performed using the *cor.test* function from the "stats" package (version 4.2.0, R Core Team, 2022).

Additive, antagonistic, or synergistic effects

To investigate whether mixed herbicide treatments had an additive, antagonistic, or synergistic effect on GHG fluxes we compared expected (i.e., individual) effects with observed (i.e., mixed) effects. We used a simple additive model (Figure B1) and Bansal et al. (2013) for calculations, where expected effects were calculated in three steps. First, the average of controls (n = 4) was subtracted from the difference in flux rates for individual herbicide treatments, and then divided by the average of controls. Second, the average of each treatment was calculated (e.g., L-, M-, H-Gly). Lastly, the sum of the treatment averages were calculated to correspond with each mixed herbicide treatment.

The observed effects were calculated by first subtracting the control average from the difference in flux rates for mixed herbicide treatments, and then dividing by the control average.

[6]The material presented here is supplementary information for Chapter 3

The expected value was then subtracted, and values were averaged by treatment (e.g., L-Gly + H-24D). These calculates were also conducted on Day 21 cumulative emissions. The mixed effects were evaluated on overlap of expected and observed standard deviations, where they were considered antagonistic when the observed effect was less than the expected effect, and synergistic when greater than the expected effect.

Sediment organic matter content

After all gas measuremenst at the end of the experiment (Day 21), approximately 10 g (wet weight) of sediment was collected from microcosms using a mini-PVC sediment corer. Sediment was only collected on Day 21 because there was limited sediment available to remove without modifying microcosm characteristics and affecting gas measurements. Sediment was stored frozen (-20 ℃) until being processed for organic matter content. Frozen sediments were freeze dried (FreeZone® Catalog #: 7934021, Labconco Corporation, Kansas City, Missouri) at -70 ℃ for 48 hours to remove all water content and then homogenized using mortar and pestle. Approximately 5 g (dry weight) of sediment was weighed into a pre-weighed porcelain crucible and placed in a 1200 ℃ box furnace (Model #: BF51732PC-1, Lindberg/Blue M™, Riverside, Michigan) at 550 ℃ for 4 hours. After sediments cooled, organic matter content was calculated as Loss on Ignition (LOI), as follows.

$$\text{Organic Matter Content (\%)} = \frac{(Weight_{dry} - Weight_{550})}{Weight_{dry}} \times 100 \times 0.93754^{a}$$

Where:

$Weight_{dry}$ = homogenized dry sediment weight

$Weight_{550}$ = sediment weight after 4 hours at 550 ℃

[a]LOI correction factor from North Dakota State University soil testing lab calculated using regression analysis on soils spanning the state of North Dakota

Results

Correlation: Flux rates and porewater concentrations

CH_4 flux rates were positively correlated with porewater concentrations in M-Gly (p = 0.03, R = 0.58; Figure B2), whereas CO_2 flux rates were negatively correlated with porewater concentrations in H-Gly + H-24D (p = 0.01, R = -0.65; Figure B3). No other significant relationships were observed for CH_4, CO_2, or N_2O (Figures B2 – B4).

Additive, antagonistic, or synergistic effects

Comparisons between expected effects of individual herbicide treatments and observed effects of mixed herbicide treatments showed various antagonistic and synergistic effects. For flux rates, L-Gly + L-24D had an antagonistic effect on CH_4, and L-Gly + H-24D, M-Gly + M-24D, and H-Gly + H-24D had synergistic effects on CO_2 (Figure B3). Whereas, for cumulative emissions, H-Gly + L-24D and H-Gly + H-24D had a synergistic effect on CO_2, and L-Gly + H-24D and L-Gly + L-24D had an antagonistic effect on N_2O (Figure B4).

Sediment organic matter content

Sediment organic matter content (%) was similar across treatment types, where all averages were ~ 6% (Table B1).

Table B1 Average ± standard deviation sediment organic matter content (%) for each treatment

Treatment	Organic Matter Content (%)
Control	5.8 ± 0.57
L-GLy	5.6 ± 0.25
H-Gly	5.5 ± 0.10
L-24D	5.8 ± 0.27
H-24D	5.7 ± 0.12
L-Gly + H-24D	5.6 ± 0.53
H-Gly + L-24D	5.8 ± 0.28
L-Gly + L-24D	6.0 ± 0.62
H-Gly + H-24D	5.6 ± 0.10
Overall	**5.7 ± 0.17**

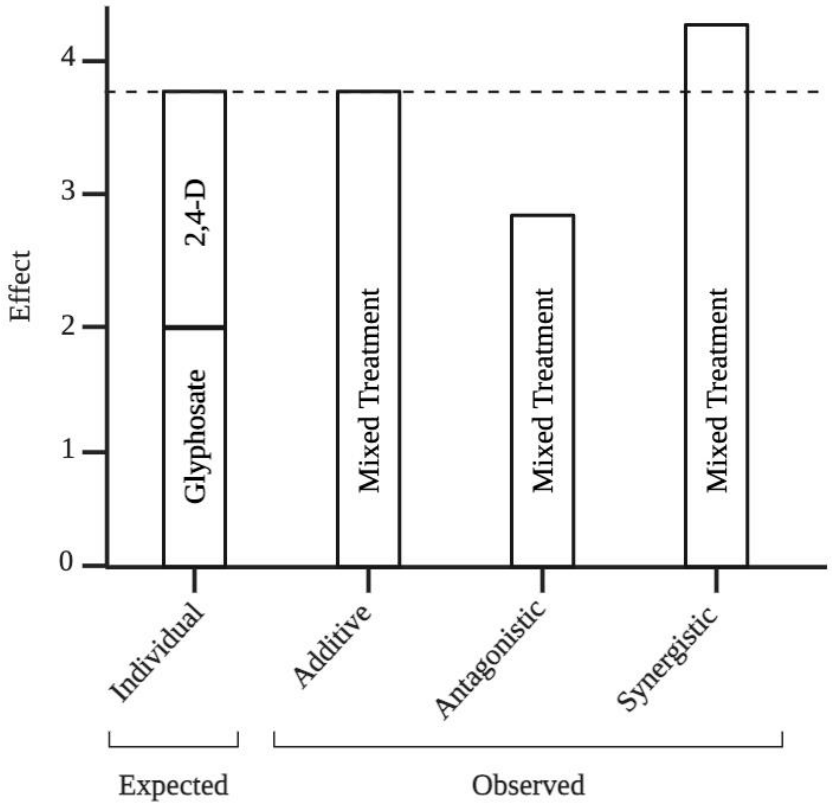

Figure B1 Conceptual diagram comparing expected additive effects and potential observed effects (Created with BioRender.com)

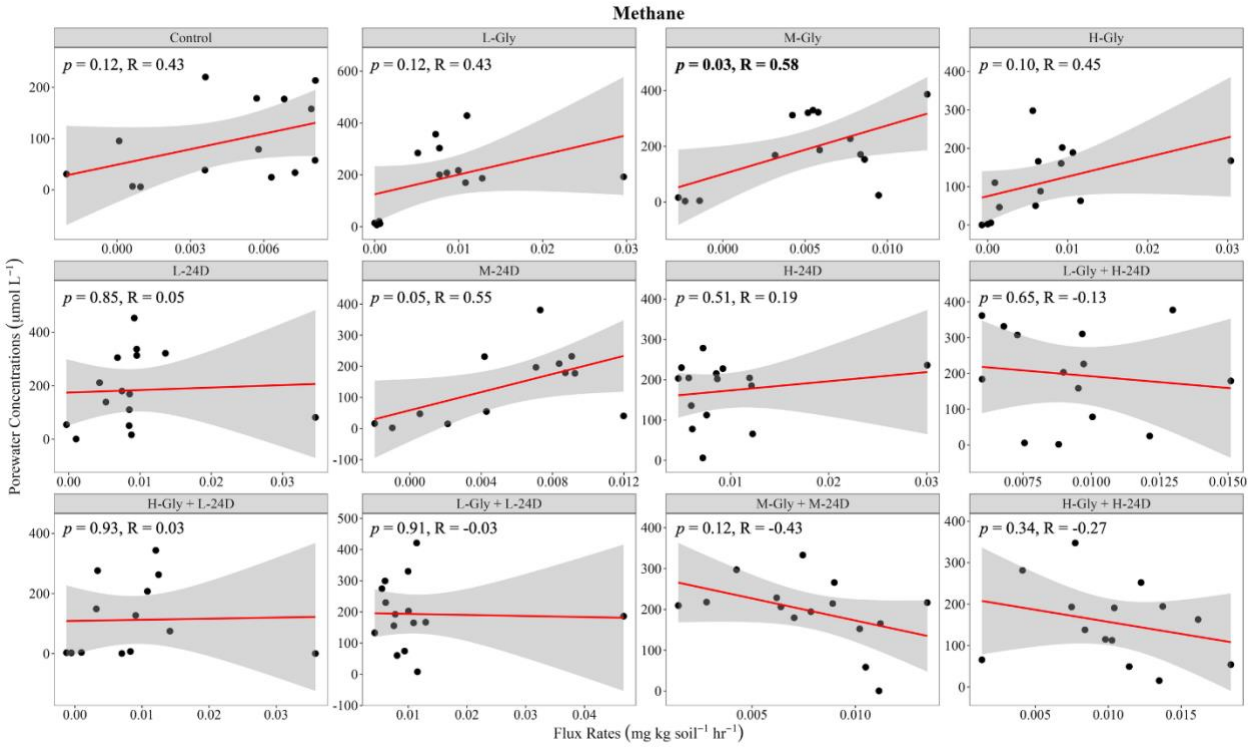

Figure B2 Scatterplots showing the linear relationships of CH_4 flux rates and porewater concentrations. Gray zones around red trend lines represent the 95% confidence interval. Spearman Rank Correlations were performed with corresponding *p*-values (*p*) and correlation coefficients (R) printed in the upper left of each panel. A **bold** *p*-value represents a significant relationship based on alpha = 0.05

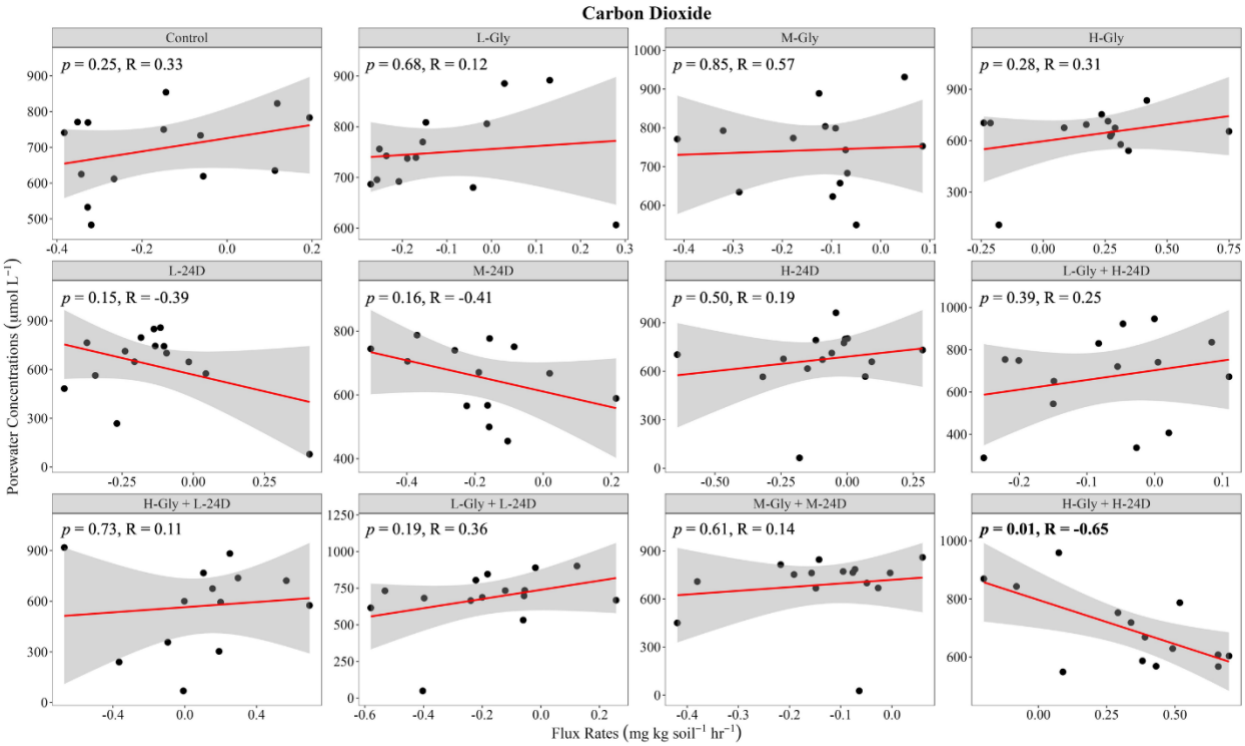

Figure B3 Scatterplots showing the linear relationships of CO_2 flux rates and porewater concentrations. Gray zones around red trend lines represent the 95% confidence interval. Spearman Rank Correlations were performed with corresponding *p*-values (*p*) and correlation coefficients (R) printed in the upper left of each panel

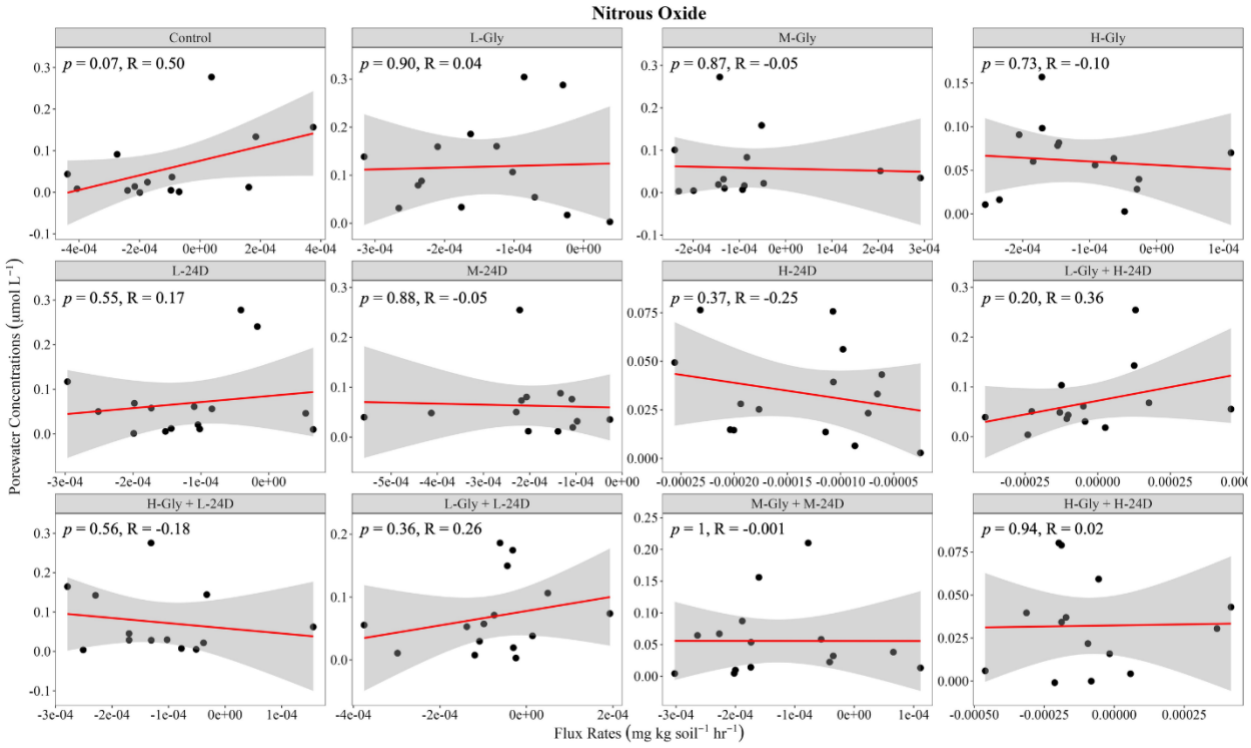

Figure B4 Scatterplots showing the linear relationships of N_2O flux rates and porewater concentrations. Gray zones around red trend lines represent the 95% confidence interval. Spearman Rank Correlations were performed with corresponding *p*-values (*p*) and correlation coefficients (R) printed in the upper left of each panel

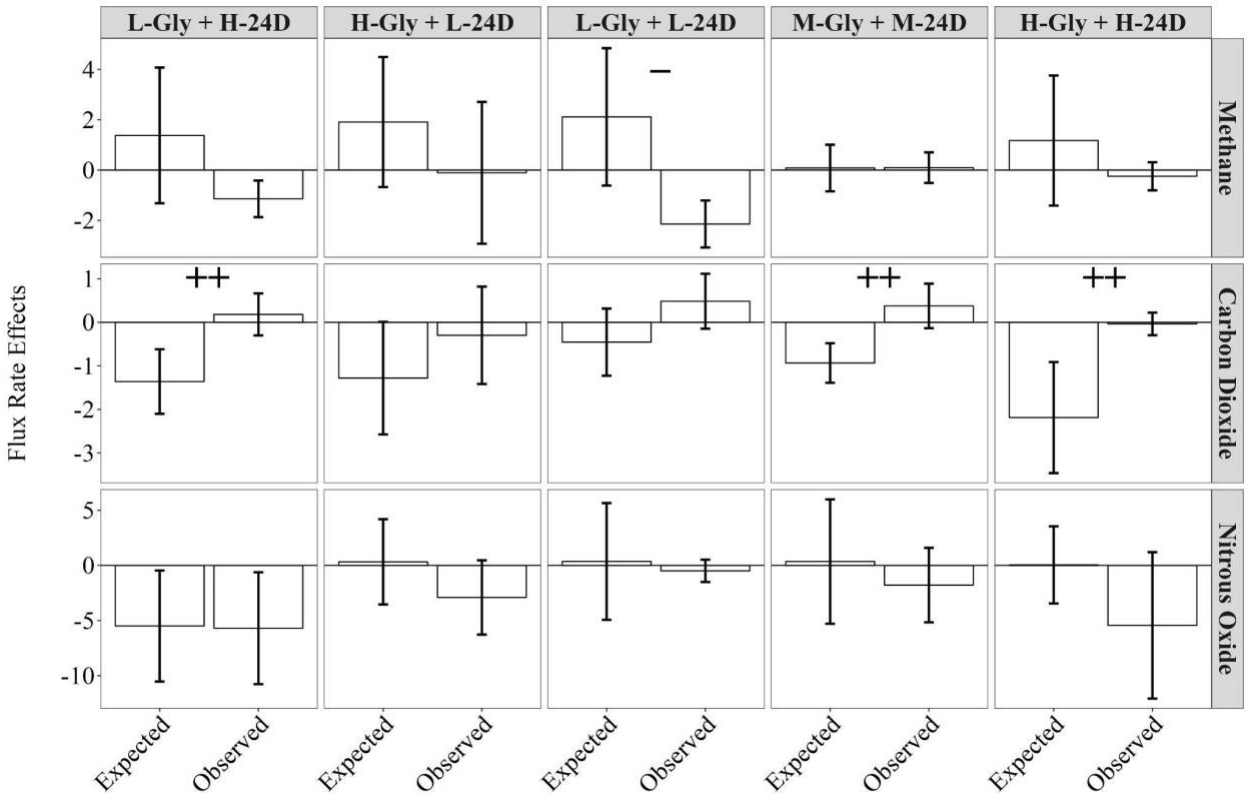

Figure B5 Expected additive and observed effects ± standard deviation flux rates for each mixed herbicide treatment (columns) and GHG (rows). Observed effects are considered antagonistic (–) when less than the expected effect, and synergistic (++) when greater than the expected effect and standard deviations do not overlap. Statistical analysis was not conducted, therefore effects were evaluated on overlap of error bars

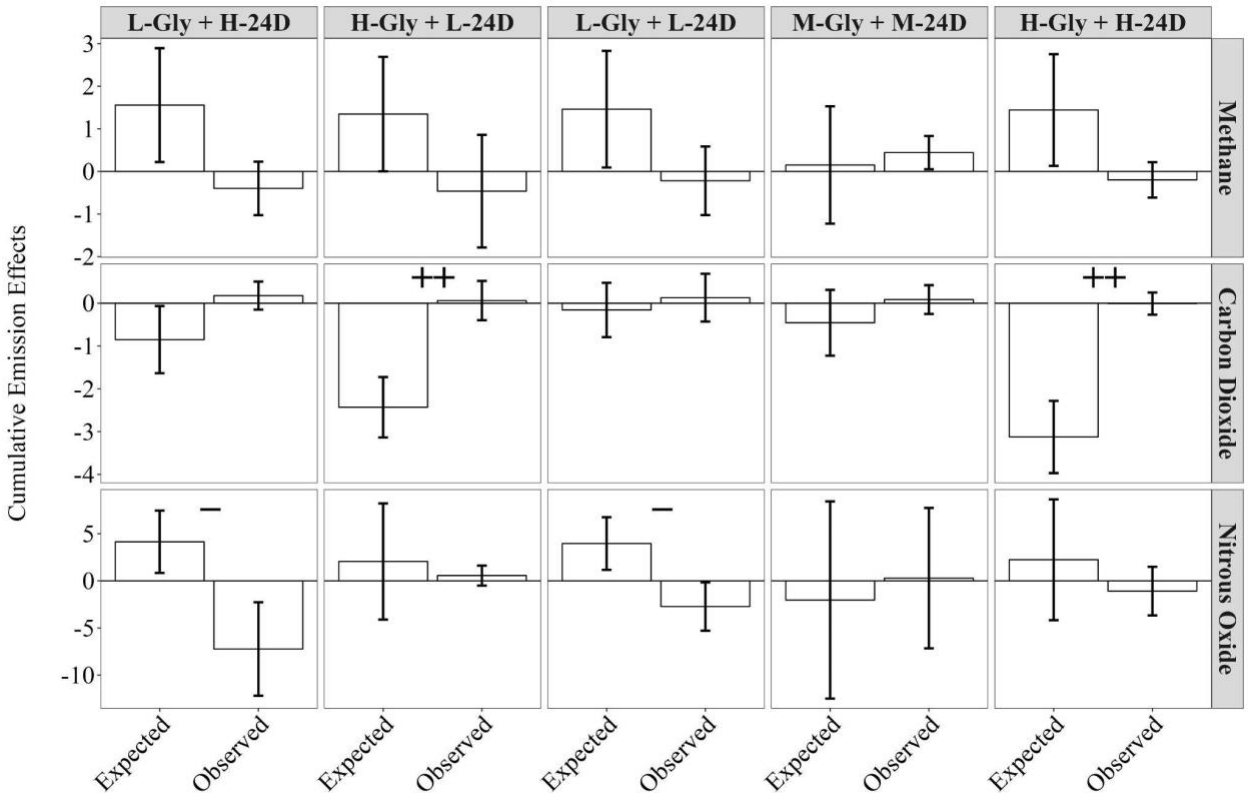

Figure B6 Expected additive and observed effects ± standard deviation cumulative emission for each mixed herbicide treatment (columns) and GHG (rows). Observed effects are considered antagonistic (**–**) when less than the expected effect, and synergistic (**++**) when greater than the expected effect and standard deviations do not overlap. Statistical analysis was not conducted, therefore effects were evaluated on overlap of error bars

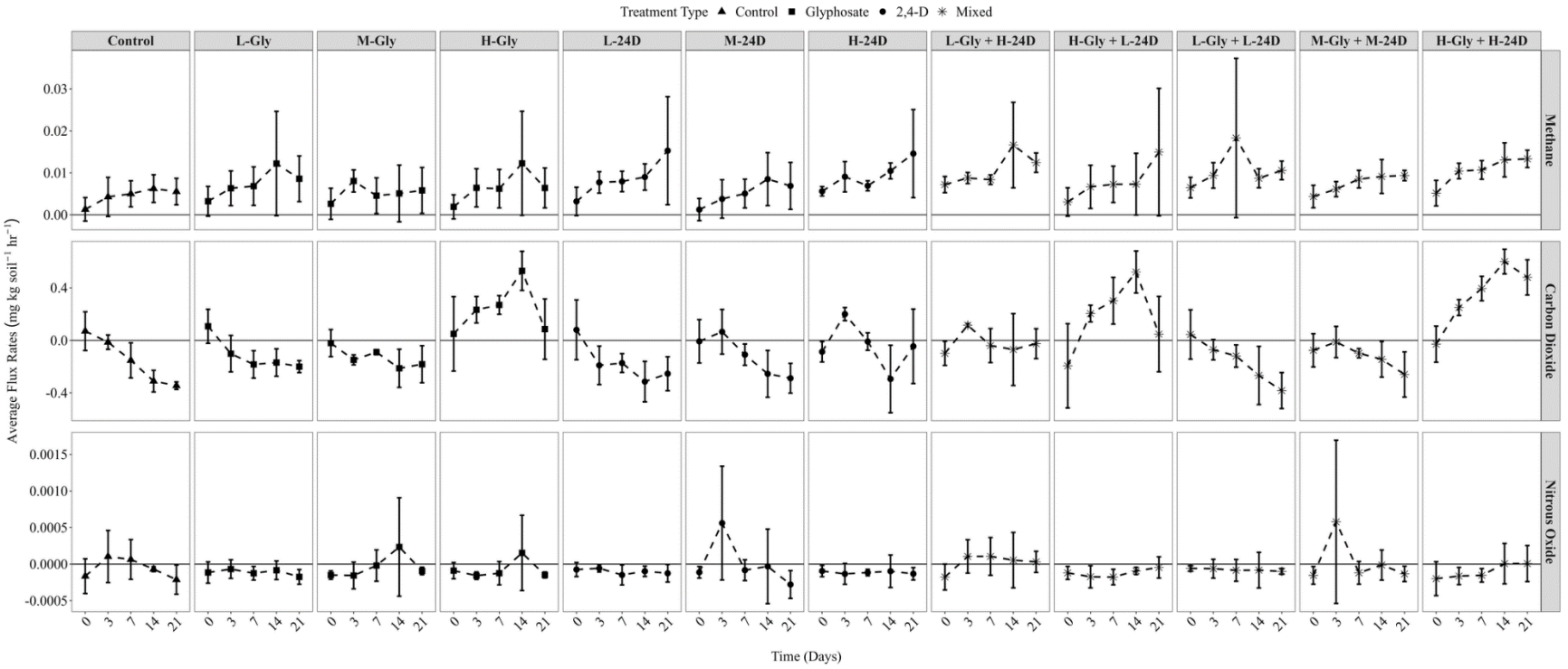

Figure B7 Time series of the average ± standard deviation flux rates for each GHG (rows) and treatment type (columns). Solid line across each panel is at y-intercept = 0

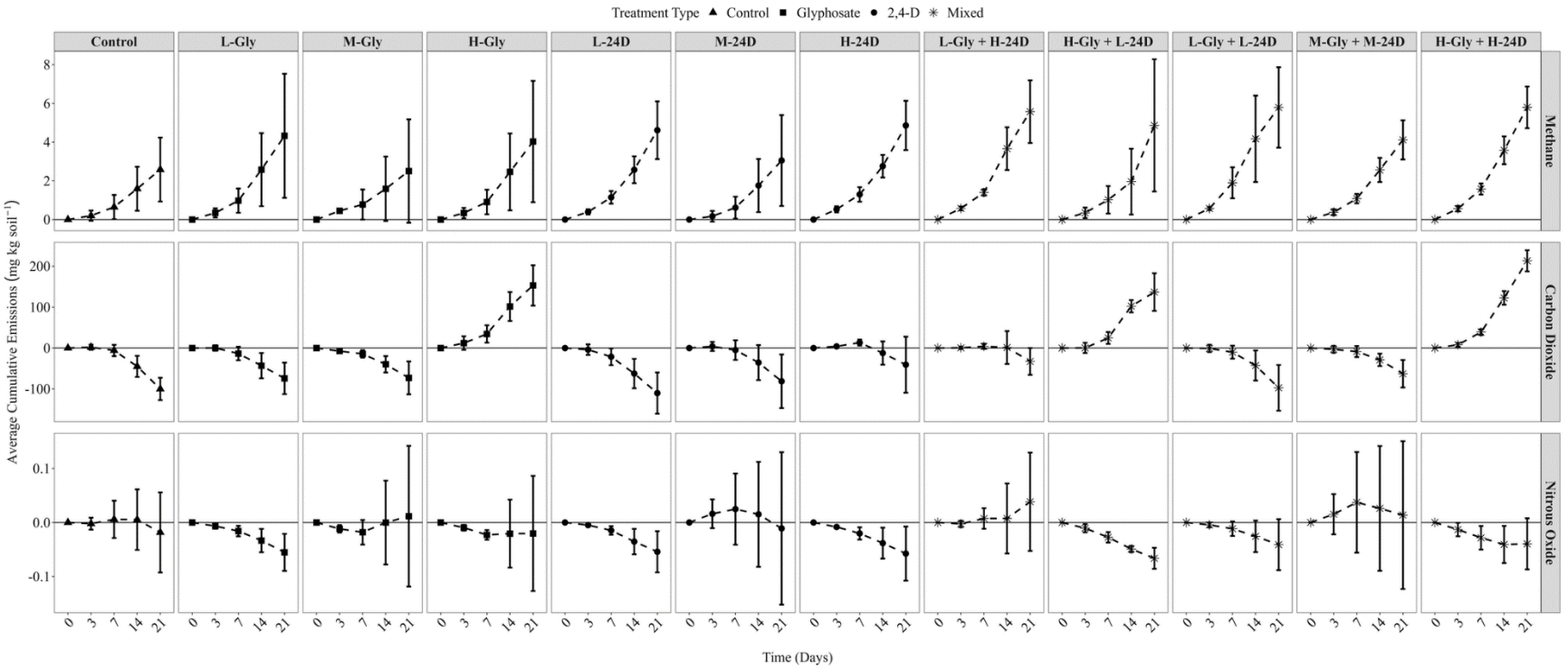

Figure B8 Time series of the average ± standard deviation cumulative emissions for each GHG (rows) and treatment type (columns). Solid line across each panel is at y-intercept = 0

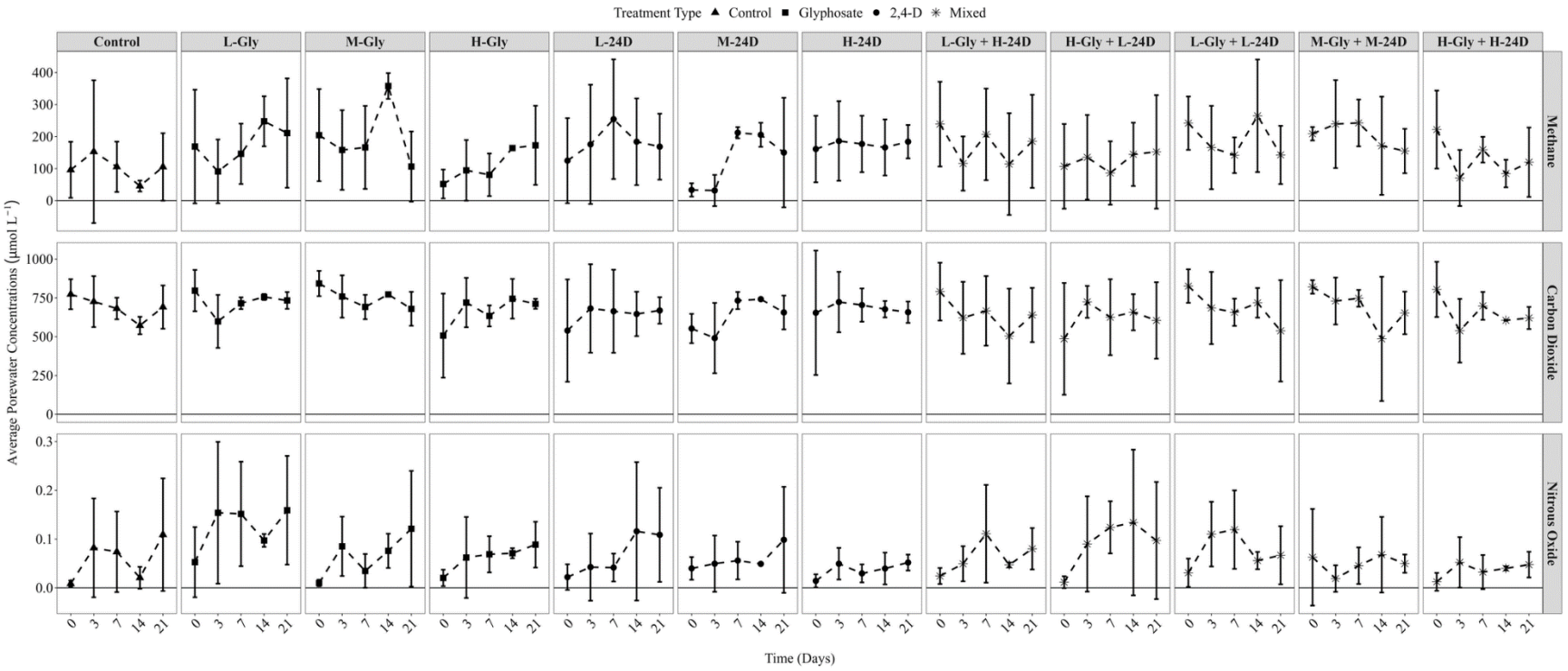

Figure B9 Time series of the average ± standard deviation porewater concentrations for each GHG (rows) and treatment type (columns). Solid line across each panel is at y-intercept = 0

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APPENDIX C. DO SEDIMENT MICROBIAL COMMUNITIES RECOVER AFTER WETLAND RESTORATION?[7]

Table C1 Ion concentrations (ppm) in surface water collected at each site

Restoration Category	Site Name	TSR (yrs)	Ca^{2+}	Cl^{-}	K^{+}	Mg^{2+}	Na^{+}	SO^{2-}_{4}
None (natural)	CWP3	–	30.5	25	18.7	35.5	314.1	598
	CWP8	–	85.8	16	13.2	8	77.6	100
	CWT1	–	15.5	26.9	45.1	35	215.6	420
	Aus1	–	66	21.2	27.5	100.1	105.6	649
	Aus2	–	52.8	31.6	26.4	83.6	55	200
Early	Hwk1	33	89.1	22.2	17.6	126.5	53.9	290
	Hwk2	33	94.6	1122.6	22	9	64.4	48
	DNC1	32	17	74.1	26.4	19.5	473	441
	Niko1	32	107.8	189.6	27.5	145.2	543.4	840
	Niko5	32	138.6	164.1	36.3	7	11	525
Mid	Swt1	30	140.8	18.4	25.3	15.5	354.8	630
	Swt2	30	148.5	32.5	23.1	14.5	169.9	250
	Moo1	30	89.1	25	16.5	107.8	259.6	100
	Moo2	30	78.1	22.2	15.4	91.3	154	944
Late	Kin1	28	14.5	49.5	52.8	19	531.8	320
	Pil1	27	124.3	27.8	15.4	12	158.4	1008
	Ban1	26	9	15.6	20.9	8.5	96.3	583

[7]The material presented here is supplementary information for Chapter 4

Table C2 Non-parametric multiple linear regressions between functional traits and surface water ions

Ion	**F statistic**	***p*-value**
Ca^{2+}	0.001	0.9812
Cl^{-}	0.005	0.9413
K^{+}	0.025	0.8748
Mg^{2+}	0.090	0.7647
Na^{+}	0.001	0.9735
SO^{2-}_{4}	0.032	0.8582