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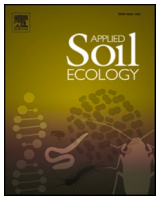

Adverse impacts of Roundup on soil bacteria, soil chemistry and mycorrhizal fungi during restoration of a Colorado grassland

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ABSTRACT

Glyphosate is a widely used herbicide in agricultural, domestic, and restoration settings to manage weeds and invasive plants and is the active ingredient in the herbicide formulation Roundup. Concurrently with its drastic increase in usage, concern over indirect ecosystem effects and effects on non-target species has grown. In restoration, glyphosate is often used to remove invasive plants so native plants may be re-introduced. However, successful reintroductions require soils and microbial communities that support native plant growth, and it is critical that glyphosate applications do not harm soil microbes such as mycorrhizal fungi. Despite previous studies investigating the effects of glyphosate on soils and microbial communities, comprehensive field experiments combining soil chemistry and next generation sequencing technologies to describe both bacterial and eukaryotic responses to glyphosate are limited, especially in the contexts of ecosystem restoration and soil health. We studied the effects of the glyphosate-based herbicide Roundup Promax at frequencies of 0, 2, 4, and 5 applications over the course of 12 months on soil biotic and abiotic soil health indicators in a Colorado prairie dominated by the invasive cool-season grass *Bromus inermis*. Here we report cascading effects on soil chemistry, with increases in nitrate and acidity and consequent decreases in calcium content and cation exchange capacity. Bacterial and archaeal communities were more affected by Roundup Promax than eukaryotic communities, with decreases in phylogenetic diversity and changes in community structure following Roundup Promax applications, particularly after five applications. More critically, the colonization of plant roots by arbuscular mycorrhizal fungi decreased significantly in plots receiving even just two applications of Roundup Promax, and dark septate endophytes decreased after four applications. Our work shows that Roundup Promax had multiple negative effects on soil biota in this field study due to either direct effects or indirect effects mediated through plant removal. Our results suggest that repeated herbicide applications are especially damaging to soil health and microbe-plant associations. These effects in turn could severely hamper the ability of native plants to establish during ecosystem restoration projects.

1. Introduction

Glyphosate (*N*-(phosphonomethyl) glycine) is the most abundant herbicide used worldwide (Benbrook, 2016; Woodburn, 2000), killing plants by inhibiting 5-enolpyruvoylshikimate-3-phosphate synthase (EPSPS) in the shikimate pathway in the chloroplast, which is essential for aromatic amino acid and secondary metabolite production (Steinrücken and Amrhein, 1980). First implemented in the Monsanto product Roundup in the 1970s, in the last several decades glyphosate has proven to be an extremely effective herbicide in agricultural, domestic, and restoration settings, in terms of the breadth of weed and invasive plant control, supposedly low toxicity (but see Myers et al., 2016), and lack of

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evolved resistance (Baylis, 2000; Sammons and Gaines, 2014). Its use has also skyrocketed in recent years with the development of glyphosate-resistant crops (Dill, 2005). As a consequence of its increased use, the number of studies examining the ecological impacts of glyphosate has steadily increased over the last 40 years (Zyoud et al., 2017). An important theme in this body of work is the effects of glyphosate on soil microbial communities, which are important for nutrient cycling, plant health, and overall ecosystem functioning (Berendsen et al., 2012; Graham et al., 2016; Schlesinger and Bernhardt, 2013; van der Heijden et al., 2008; Zak et al., 2003). While the EPSPS pathway is absent in mammals, fish, birds, and insects, it is present in many microorganisms, leading to concern about glyphosate side effects on the archaea, bacteria, fungi, algae, and protists that make up the soil microbiome (Kepler et al., 2019). However, information for land managers interested in preserving soil microbial health while implementing glyphosate-based herbicides is relatively scarce.

In practice, glyphosate is almost always applied as a mixture of glyphosate and adjuvants designed to increase the effectiveness of the herbicide. A vast diversity of glyphosate-containing products exist, each with adjuvants that alter the physical qualities of the spray in a different way. For example, some adjuvants are designed to decrease aerial drift, others increase the surface connection of the spray with the plant, and several improve waterproofing to prevent the herbicide from being washed off the plant during rain events. Several studies have shown that ecological impacts of glyphosate-based formulations are different than glyphosate alone with several demonstrating substantial increases in toxicity with the addition of adjuvants (Coalova et al., 2014; Gill et al., 2018; Mesnage et al., 2014; Mesnage and Antoniou, 2018).

The literature to date has shown mixed effects of glyphosate and glyphosate-based products on soil microbial biomass, activity, richness, and community structure. Certain concentrations of glyphosate have been shown to increase the abundance of some bacterial (e.g., Proteobacteria, *Bulkholderia*) and fungal taxa and decrease others (e.g., Acidobacteria) (Araújo et al., 2003; Imparato et al., 2016; Lancaster et al., 2010; Newman et al., 2016; Wardle and Parkinson, 1990). Glyphosate products have either had no effect on or decreased microbial biomass, across a wide range of concentrations (Busse et al., 2001; Haney et al., 2000; Lane et al., 2012; Tejada, 2009). Glyphosate products have increased microbial activity, suggesting that some microbes can degrade glyphosate and use it as a carbon source for metabolism (Busse et al., 2001; Haney et al., 2000; Sprankle et al., 1975; Weaver et al., 2007) or as a source of phosphorus and nitrogen (Moore et al., 1983; Pipke and Amrhein, 1988). Field concentrations of glyphosate (0.5–7.02 kg ha^{-1}) have been shown to have no effect on microbial community structure (Barriuso et al., 2011; Hart et al., 2009; Kepler et al., 2019; Weaver et al., 2007; Zabaloy et al., 2012), but high concentrations simulating a spill (e.g., 100 times field rate) can stimulate some bacteria (Ratcliff et al., 2006; Weaver et al., 2007). Some of the detrimental effects of glyphosate reported on soil microbes include a decrease in arbuscular mycorrhizae spore viability and root colonization (Druille et al., 2013a, 2013b, 2016), a decrease in dark septate endophyte colonization and growth (Druille et al., 2016; Spagnoletti and Chiocchio, 2019), a decrease in nitrogen fixers and growth-promoting taxa (Druille et al., 2016; Zobiole et al., 2010, 2011), and an increase in plant pathogens such as *Fusarium* (Johal and Huber, 2009; Zobiole et al., 2011). These previous studies are limited by the broad taxonomic descriptions (e.g., bacteria, fungi, actinomycetes), focus on one taxonomic group, or focus on agricultural settings, while glyphosate-based herbicides are also commonly used in native plant community restoration projects to control non-native and invasive plant species (Bell, 1997; Bohn et al., 2011; Irvine et al., 2013; Kulmatiski and Beard, 2006; Leahy et al., 2018; Mozdzer et al., 2008; Robertson et al., 2013; Simmons et al., 2007; Stover et al., 2017).

Invasive species are not only agricultural pests, but are also one of the main drivers of global biodiversity loss (Sala et al., 2000), and represent a significant barrier to native species re-establishment (Prior et al., 2018). Herbicide treatment and removal of invasive species is one of the most frequently applied restoration management strategies worldwide (D'Antonio et al., 2016; Weidlich et al., 2020) and glyphosate is one of the main active ingredients applied (Kettenring and Adams, 2011). The concentration of glyphosate in solutions applied in restoration settings ranges from 1 to 6 % (0.5 to 7.02 kg ha^{-1} active ingredient) (Bell, 1997; Bohn et al., 2011; Kulmatiski and Beard, 2006; Leahy et al., 2018; Robertson et al., 2013; Simmons et al., 2007; Stover et al., 2017) and are comparable to those used in agricultural settings (e.g., 0.36 to 4.48 kg ha^{-1} active ingredient) (Barriuso et al., 2011; Busse et al., 2001; Cherni et al., 2015; Druille et al., 2016; Hart et al., 2009; Kepler et al., 2019; Kremer and Means, 2009; Schlatter et al., 2017, 2018; Sprankle et al., 1975; Weaver et al., 2007; Zobiole et al., 2010, 2011). Herbicides are often applied repeatedly to manage some of the new weeds that colonize after the first spray and leave space for desirable species to colonize after seeding, yet the side effects of repeated applications on ecosystems and other biota such as soil microorganisms, especially over the course of a single growing season, remain understudied. For example, most studies on this topic have either examined one single application event (Bell, 1997; Bohn et al., 2011; Cherni et al., 2015; Hart et al., 2009; Kremer and Means, 2009; Kulmatiski and Beard, 2006; Leahy et al., 2018), or one application a year for multiple years (Barriuso et al., 2011; Busse et al., 2001; Druille et al., 2016; Kepler et al., 2019; Stover et al., 2017), while only a few have studied the effects of multiple sprays in one growing season in a greenhouse (Schlatter et al., 2018; Weaver et al., 2007; Zobiole et al., 2011) or in the field (Robertson et al., 2013; Simmons et al., 2007).

Restoration projects typically do not address the direct chemical effects or indirect effects (via plant removal, lower biomass) of glyphosate on soils and microorganisms (but see Irvine et al., 2013, Duell et al., 2022), despite evidence that microbial communities, and particularly arbuscular mycorrhizal fungi, are important for successful native plant community restoration (Harris, 2009; Requena et al., 1997; Richter and Stutz, 2002; Rowe et al., 2009; Zubek et al., 2009). For example, Irvine et al. (2013) found that 0.7 mL of a 4 % glyphosate solution sprayed on 2 g of soil caused a decrease in pink-pigmented facultative methylotrophic bacteria (PPFM) and that adding methanol or PPFMs can help in native plant restoration. Duell et al. (2022) found decreases in AMF biomass following 8 years of glyphosate spraying to control the invasive grass *Bothriochloa bladhii* (Retz.) (Caucasian bluestem), and that native soil inoculations can be used to increase native plant survival and diversity.

Here we build on previous studies by sampling soils over a twelve-month period in a field experiment in which the effects of the application frequency of the glyphosate-based herbicide Roundup Promax were studied. Because glyphosate is almost always applied as a formulation, we chose to test the effects of the formulation, Roundup Promax, rather than glyphosate alone, to provide the most applicable information to land managers. We used next-generation high-throughput 16S and 18S rRNA gene sequencing to characterize alpha- and beta-diversity responses of bacterial and eukaryotic communities, combined with root fungal colonization data and a suite of soil chemistry measurements. We hypothesized that there would be (1) an increase in soil nitrogen content due to the nitrogen content of glyphosate, and an associated decrease in pH and base cations, (2) a decrease in alpha-diversity in both bacteria and eukaryotes due to direct toxic effects of glyphosate or adjuvants and competitive exclusion by species that are stimulated by glyphosate or adjuvants, (3) a significant change in beta-diversity as taxa change in abundance in response to Roundup Promax additions, and (4) a significant decrease in root colonization by arbuscular mycorrhizal fungi, fine root endophytes and dark septate endophytes, based on previous studies. In all cases, we predicted the magnitude of change to mirror the frequency of Roundup Promax application (i.e., greater effect size with more frequent Roundup Promax additions).

2. Materials and methods

2.1. Field site and experimental design

Roundup Promax additions and soil and plant sampling occurred at Denver Botanic Gardens Chatfield Farms (39.54°N, 105.10°W, 1680 m elevation), a botanic garden and working farm owned by the United States Army Corps of Engineers and managed by Denver Botanic Gardens. The property was an active farm and rangeland in the early 20th century but transitioned to management by the Gardens in the 1960s, where small-scale agriculture still occurs. This site has ongoing research on the effects of herbicides, soil treatments, and native plant seeding on the restoration of native plants and ecosystem functioning, so our experiment did not involve any additional use of herbicides. At the time of our sampling, there had been very little germination and growth of the seeded native plants, so this did not impact our study.

The Natural Resources Conservation Service has classified soils at this site as "Denver clay loam, 2 to 5 percent slopes", which are part of the "Denver" soil taxonomic series and the "fine, smectitic, mesic Torrertic Argiustolls" soil taxonomic class. Average percent content of sand, silt, and clay is 43.5 %, 26.5 %, and 30 %, respectively, average organic matter content is 4.9 %, and average pH is 6.9. The dominant plant species is the cool-season invasive perennial grass *Bromus inermis* Leyss. (smooth brome), which has invaded the native mixed-grass prairie in this area and much of the Great Plains region (DeKeyser et al., 2013). Other abundant plant species include the exotic forbs *Thlaspi arvense* L., *Convolvulus arvensis* L., and *Melilotus officinalis* (L.) Lam., and the native forbs *Ellisia nyctelea* (L.) L. and *Helianthus annuus* L.

The specific herbicide formulation tested, the location of treatment plots, the frequency of herbicide applications, and application type were predetermined by concurrent research at the site. Roundup Promax (48.7 % glyphosate) was applied as a 5 % solution in water to 3 m × 30.5 m plots for a final glyphosate concentration of 2.4 % (33 g L^{-1}) either by backpack or truck. We applied herbicide either 0 (control) 2, 4, or 5 times per year. Each frequency treatment was replicated 14 times each across the landscape as part of larger ongoing projects at the field site (Fig. 1). The final concentration of truck-based spraying (1st and 5th spray applications) was 173 L water + glyphosate solution ha^{-1}, and 22 L water + glyphosate solution ha^{-1} for backpack-based spraying (2nd through 4th spray applications). This is equivalent to 5.7 kg ha^{-1} and 0.726 kg ha^{-1} of active ingredient, which are near the upper and lower bounds for use in restoration (Kulmatiski and Beard, 2006; Leahy et al., 2018). Previous studies on *B. inermis* control in particular have used 1.12 kg ha^{-1} (Waller and Schmidt, 1983) and 1.84 kg ha^{-1} (Bahm et al., 2011) of glyphosate. The spraying dates were: 1st spray – May 23, 2018; 2nd spray - Jul 31 - Aug 1, 2018; 3rd spray - Sep 30 - Oct 1, 2018, 4th spray - Feb 28 - Mar 1, 2019, and 5th spray - April 4, 2019. Seeding with mixes of native prairie species was conducted in May 2019 after all Roundup Promax applications. Roundup Promax is a mixture of glyphosate (48.7 %) as potassium salt ($C_3H_7KNO_5P$) and a proprietary blend of surfactants (51.3 %).

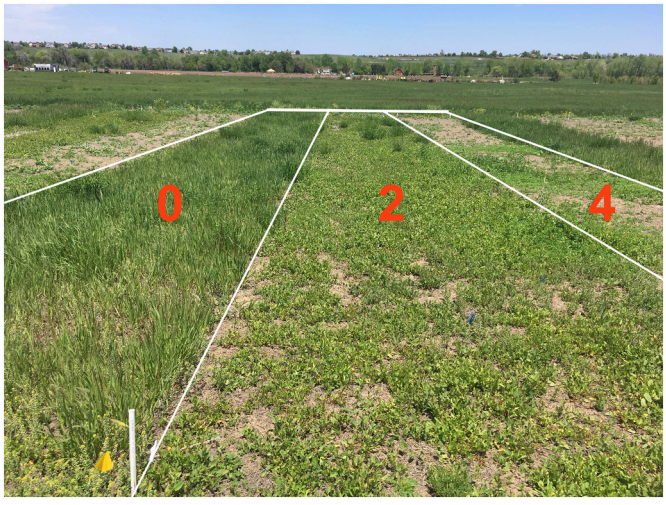

Fig. 1. Photo of the study site and the large experimental plots receiving 0 (control), 2, 4 or 5 (not shown) applications of glyphosate (Roundup Promax) per year. *Bromus inermis* dominates the control plots and declines with Roundup Promax application such that forbs dominate the 2 application plots. The 4 and 5 application plots are characterized by a higher proportion of bare ground. Photo by C. Bueno de Mesquita.

We sampled soils (randomly selected 4 of the 14 replicates each from the 0, 2, and 5 frequency treatments) for biogeochemical and microbial analyses on June 27, 2018, August 28, 2018, April 2, 2019, and June 22, 2019. This corresponds to 35, 27, 32, and 79 days after an herbicide spray. In each of the four replicate treatment plots, soil was collected from three locations that were chosen by randomly selecting distances between 2 and 10, 11–20, and 21–30 m from the north side of the plot. The same distances were used at each sampling time point. A sterile trowel was used to collect approximately 200–250 g of the top 4 cm of soil into sterile Whirl-Pak bags (Nasco Sampling, Madison, WI, USA), which were stored in a cooler on ice for transport to the lab. An aliquot of each of the three individual soil samples per plot was immediately taken for DNA extraction and stored at −20 °C. A second aliquot of the three soil samples was taken and combined and homogenized into 1 composite sample per plot and sent to Midwest Laboratories (Omaha, NE) for organic matter, total nitrogen, nitrate, ammonium, total phosphorus, available phosphorus, pH, potassium, magnesium, calcium, and cation exchange capacity (CEC) analyses. We also sampled plants for root staining and microscopy on June 27, 2019, destructively harvesting one individual of *B. inermis* and *E. nyctelea* from each of six plots receiving the 0, 2, and 4 frequency of Roundup Promax addition. The plants were selected to represent a grass and a forb, and a non-native and native plant. Roots were cleaned and stored in formaldehyde-acetic acid-alcohol (FAA) at 4 °C until staining.

2.2. Staining and microscopy

Staining and microscopy were performed following established protocols (Koske and Gemma, 1989; McGonigle et al., 1990; Schmidt et al., 2008). Roots were rinsed 3 times with deionized water to remove FAA, cleared with 10 % KOH for 1 h in a 90 °C water bath, rinsed with water to remove KOH, soaked in 1 % HCl at room temperature for 20 min, and then soaked overnight in acidic glycerol with 0.05 % trypan blue. In the morning, roots were destained with acidic glycerol and stored in acidic glycerol at 4 °C until microscopy. Several fine root segments and their branches, totaling 10–20 cm of root, were placed horizontally across slides, covered with a cover slip, and viewed at 160-200× magnification under a microscope with a crosshair on the ocular. Passes were made up and down the slide at random intervals and the presence of arbuscular mycorrhizal fungi (AMF), dark septate endophyte (DSE), or fine root endophyte (FRE) structures at each of 100 intersections with the crosshair was recorded (Bueno de Mesquita et al., 2018). Percent colonization for each fungal group is the number of times it was present at each of the 100 intersections.

2.3. DNA extraction and processing

DNA was extracted from 0.3 g soil using the Qiagen DNeasy soil extraction kit (Hilden, Germany). Each extraction was amplified in triplicates using the universal bacterial/archaeal primer set 515F/806R and the eukaryotic primer set 1391f/EukBr as done by the Earth Microbiome Project (Thompson et al., 2017). Although the 515F/806R primer pair is commonly used for bacterial/archaeal surveys and has been updated to enhance archaeal read abundances, these primers are still biased against archaea (Bahram et al., 2019). However, because the

Euryarchaeota and Thaumarchaeota phyla are still captured, we still present results of archaeal taxa from these groups. Amplicons were diluted to equimolar concentration, pooled, and sequenced on the Illumina MiSeq platform (Illumina, Inc., San Diego, CA) at the University of Colorado BioFrontiers Institute (Boulder, CO) using paired-end 2 × 150 base pair chemistry.

Raw sequencing reads were de-multiplexed using the open source "idemp" tool (https://github.com/yhwu/idemp) and adapters were cut from the sequences using the open source "cutadapt" (Martin, 2011) tool (https://cutadapt.readthedocs.io/en/stable/). Sequences were then analyzed with the DADA2 pipeline (Callahan et al., 2016) to quality filter reads, merge forward and reverse reads, assign amplicon sequence variants (ASVs, Callahan et al., 2017), remove chimeras, and assign taxonomy using the SILVA version 132 databases for 16S and 18S (Quast et al., 2013). Sequences were aligned with the MUSCLE (Edgar, 2004) and phylogenetic trees constructed using the FastTree algorithm in the QIIME program (Caporaso et al., 2010). Chloroplast and mitochondria were removed from the 16S dataset while plants, bacteria, and archaea were removed from the 18S dataset; then data were rarefied to the lowest number of sequences per sample (8889 for 16S, 5226 for 18S) with the *mctoolsr* R package (Leff, 2017) before downstream analyses. ASV sequences are available on NCBI under the project accession number PRJNA699325.

2.4. Statistical analyses

Differences in microbial community composition were tested with a repeated measures permutational multivariate analysis of variance (PERMANOVA, Anderson, 2001) implemented with the 'adonis2' function in the *vegan* R package (Oksanen et al., 2019) on a weighted UniFrac distance matrix (Lozupone et al., 2007), calculated with the *phyloseq* R package (McMurdie and Holmes, 2013). Sampling location nested in plot was defined with the 'setBlocks' function in the *permute* R package (Simpson, 2019) to restrict permutations to take into account repeated measures. Furthermore, we used the argument by = "margin" to calculate the marginal effects of the variables time and Roundup Promax application frequency, and their interaction. Pairwise PERMANOVAs were calculated with the *RVAideMemoire* R package (Hervé, 2019). Faith's phylogenetic diversity (Faith, 1992) was calculated with the *picante* R package (Kembel et al., 2010). SIMPER analysis in the *vegan* package was used to determine the top taxa contributing to community dissimilarities. To further explore taxonomic responses, we made heatmaps and performed Kruskal-Wallis tests with Bonferroni *p*-value correction on the abundances of major bacterial families (> 1 % relative abundance) and eukaryotic taxa (SILVA level 5) using *mctoolsr*. Soil chemistry data and microbial alpha-diversity metrics were analyzed with repeated measures ANOVA models with fixed effects of time (four sampling time points), Roundup Promax application frequency and their interaction, and either plot (for soil chemistry) or sampling location nested in plot (for alpha-diversity) as random factors. These models were implemented with the 'lmer' function in the *lme4* R package (Bates et al., 2015) using Helmert contrasts and tested for significance using the 'Anova' function in the *car* R package (Fox and Weisberg, 2011), with Type III sum of squares. Significant models were followed by Tukey posthoc tests implemented with the *multcomp* R package (Hothorn et al., 2008). Fungal colonization data were analyzed with generalized linear models with a binomial distribution (number of fungal structures hit out of 100 trials), and Tukey posthoc tests implemented with *multcomp*. All analyses were performed in the software R (version 3.5.3, R Core Team, 2019).

3. Results

3.1. Soil chemistry

Roundup Promax additions resulted in several changes to soil chemical properties, including increases in nitrate and decreases in pH, calcium, and cation exchange capacity (Table 1, Fig. 2). In each case the magnitude of the effect increased with increasing Roundup Promax application frequency (Fig. 2). On the other hand, organic matter, available phosphorus, total phosphorus, potassium, magnesium, ammonium, and total nitrogen were not significantly affected by Roundup (repeated measures ANOVA, $p > 0.05$).

3.2. Soil bacterial and archaeal communities

Combined bacterial and archaeal ASV richness was unaffected by Roundup Promax application frequency but changed significantly over time (Table 1, Fig. 3). Bacterial and archaeal phylogenetic diversity declined significantly with increasing Roundup Promax application frequency and changed significantly over time (Table 1, Fig. 3). The significant effect of Roundup Promax additions was driven by differences between controls and plots receiving Roundup Promax five times (Tukey HSD, $p < 0.05$).

Bacterial and archaeal community structure changed significantly both over time and with Roundup Promax application frequency (Table 1, Fig. 4). Within sample type community variation was homogenous for Roundup Promax treatments ($F = 0.71$, $p = 0.5$) but significantly different for sampling timepoint ($F = 6.7$, $p < 0.001$). Pairwise PERMANOVA showed that effects of Roundup Promax were driven by a significant difference between control plots and plots receiving five Roundup Promax applications per year ($p = 0.02$). According to SIMPER analyses, the top five ASVs contributing to this difference belonged to the Pyrinomonadaceae, *Tychonema*, *Udeaeobacter*, Nitrososphaeraceae, and *Nitrososphaera* groups (Table 2). Other major

Table 1
Statistical results showing the degrees of freedom (DF; numerator, denominator), Chi-square (χ2) (from ANOVA) or pseudo-F (from PERMANOVA) test statistics, P values, and significance levels (. = marginal $p < 0.10$, * = $p < 0.05$, ** = $p < 0.01$, *** = $p \leq 0.001$) for the two variables and their interaction. PD = Faith's phylogenetic diversity; Prok. = Prokaryotes, Euk. = Eukaryotes; Comp. = composition (Bray-Curtis dissimilarity).

Dep. Variable	Ind. Variable	DF	χ2	P	Sig.
Prok. Richness	Roundup Promax	2, 138	4.43	0.109	
	Sample Date	3, 137	9.07	0.028	*
	Interaction	6, 134	5.14	0.526	
Prok. PD	Roundup Promax	2, 138	7.48	0.024	*
	Sample Date	3, 137	12.42	0.006	**
	Interaction	6, 134	6.46	0.373	
Euk. Richness	Roundup Promax	2, 139	0.81	0.667	
	Sample Date	3, 138	236.20	< 0.001	***
	Interaction	6, 135	5.77	0.449	
Euk. PD	Roundup Promax	2, 139	1.45	0.486	
	Sample Date	3, 138	182.33	< 0.001	***
	Interaction	6, 135	3.14	0.791	
Calcium	Roundup Promax	2, 45	8.41	0.015	*
	Sample Date	3, 44	45.48	< 0.001	***
	Interaction	6, 41	4.78	0.572	
pH	Roundup Promax	2, 45	10.65	0.005	**
	Sample Date	3, 44	17.44	< 0.001	***
	Interaction	6, 41	8.80	0.185	
CEC	Roundup Promax	2, 45	2.37	0.306	
	Sample Date	3, 44	62.05	< 0.001	***
	Interaction	6, 41	12.48	0.052	.
Nitrate	Roundup Promax	2, 45	51.41	< 0.001	***
	Sample Date	3, 44	59.11	< 0.001	***
	Interaction	6, 41	19.93	0.003	**
			Pseudo-F	P	
Prok. Comp.	Roundup Promax	2, 138	2.29	0.001	***
	Sample Date	3, 137	17.91	0.001	***
	Interaction	6, 134	0.860	0.624	
Euk. Comp.	Roundup Promax	2, 139	1.28	0.001	***
	Sample Date	3, 138	7.33	0.001	***
	Interaction	6, 135	0.77	0.896	

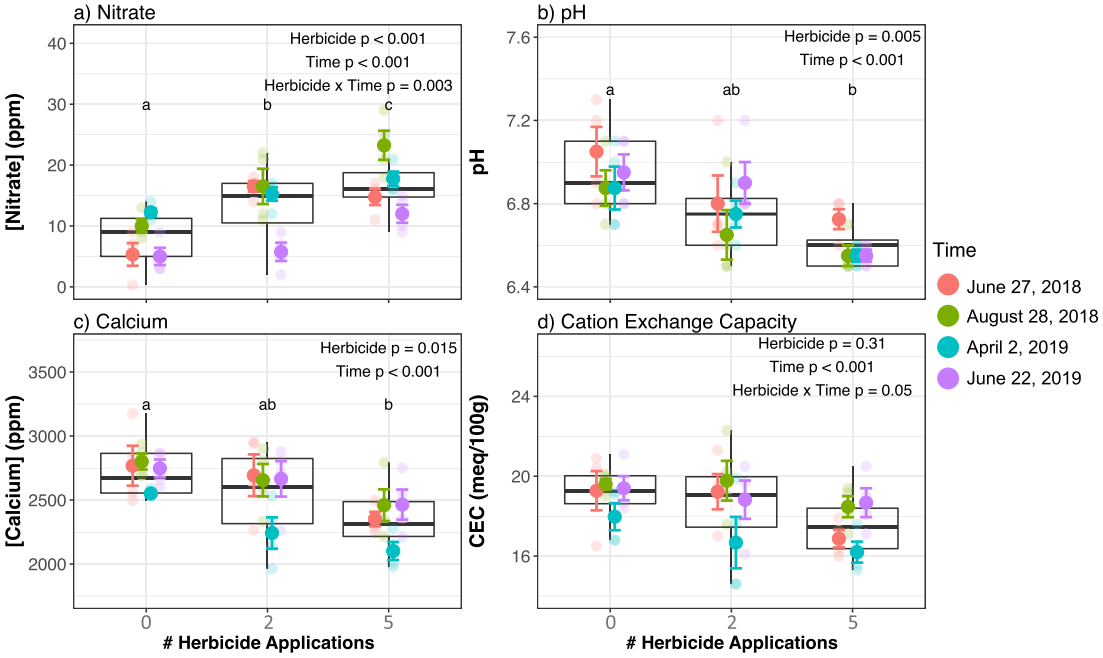

Fig. 2. Effects of time and Round Promax herbicide application frequency on soil a) nitrate, b) pH, c) calcium, and d) cation exchange capacity. *P* values are from repeated-measures ANOVA and different letters signify significant pairwise differences among the herbicide treatments (Tukey posthoc, $p < 0.05$). Boxplots summarize all of the data within each herbicide frequency. Faded points show raw data within each time point, with solid points showing means with standard error bars within each time point.

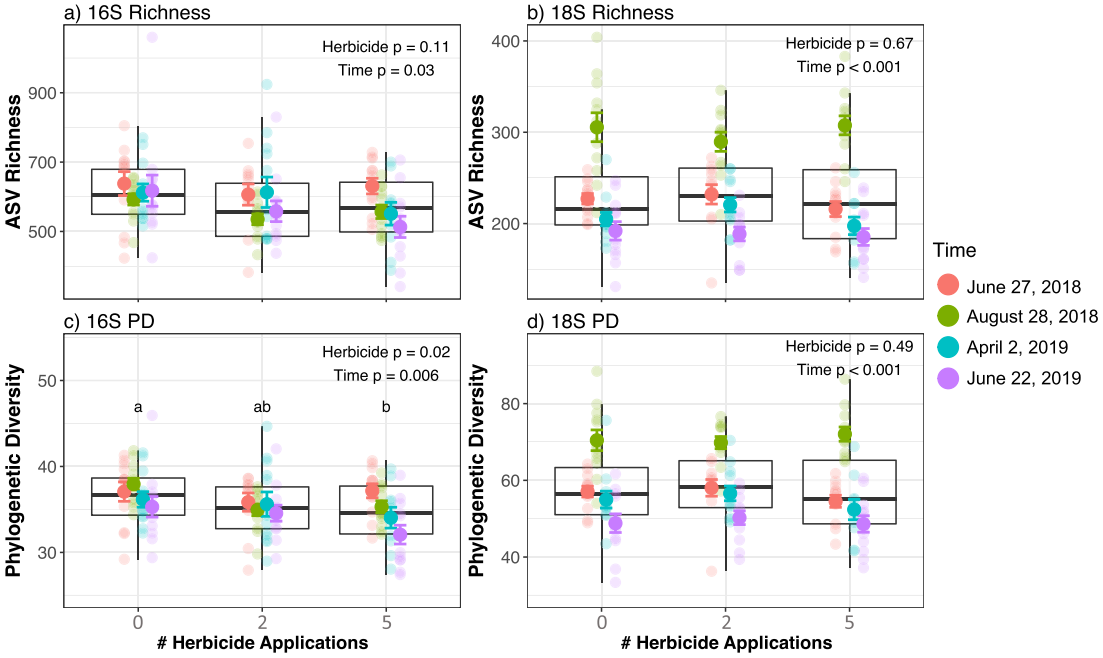

Fig. 3. Effects of time and Roundup Promax herbicide application frequency on a) bacterial and archaeal (16S) ASV richness, b) eukaryotic (18S) ASV richness, c) bacterial and archaeal (16S) phylogenetic diversity, and d) eukaryotic (18S) phylogenetic diversity. P values are from repeated-measures ANOVA and different letters signify significant pairwise differences among the herbicide treatments (Tukey posthoc, $p < 0.05$). Boxplots summarize all of the data within each herbicide frequency. Faded points show raw data within each time point, with solid points showing means with standard error bars within each time point.

taxa responding to Roundup Promax additions in the final sampling period included Azospirillaceae, Chthoniobacteraceae, and Micromonosporaceae (Kruskal-Wallis $p < 0.05$, Fig. 5).

3.3. Soil eukaryotic communities

Eukaryotic ASV richness and phylogenetic diversity changed significantly over time but were unaffected by the frequency of Roundup Promax applications (Table 1, Fig. 3). Eukaryotic community structure changed significantly both over time and with Roundup Promax application frequency ($F = 1.28$, $p = 0.13$, Fig. 4). However, pairwise PERMANOVAs among the application frequencies were not significant ($p > 0.05$). Within sample type variation was homogenous among Roundup Promax treatments ($F = 0.73$, $p = 0.48$), but significantly different

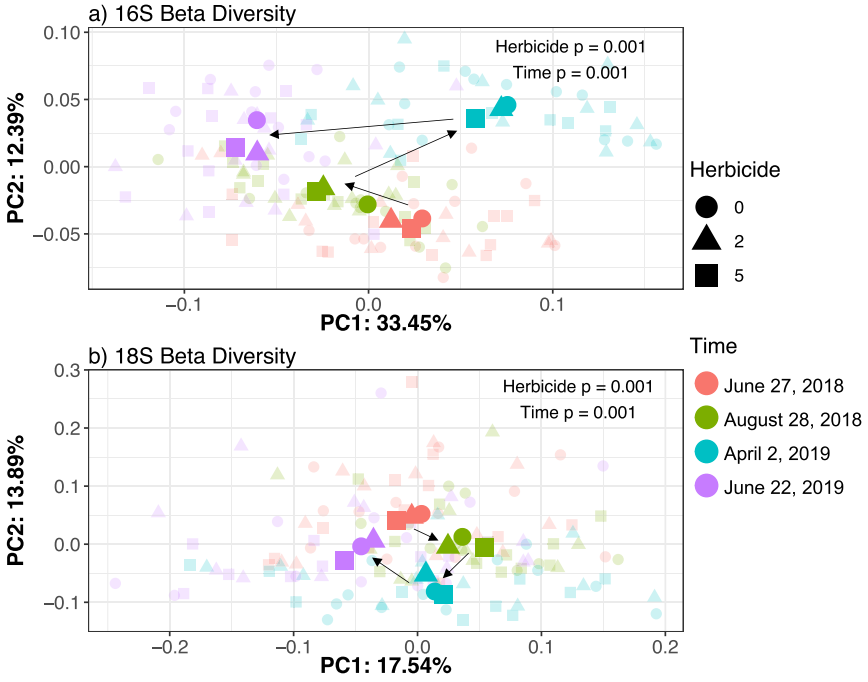

Fig. 4. Principle coordinates analysis of a) bacterial and archaeal (16S) beta diversity and b) eukaryotic (18S) beta diversity. Beta diversity was calculated as weighted UniFrac distances. Faded datapoints represent each individual sample. Large bold datapoints are the centroids of each Roundup Promax herbicide application frequency at each sample time. Arrows follow the changes over time. P values are from PERMANOVA.

Table 2
Top five amplicon sequence variants contributing to community composition differences between the control and 5× Roundup Promax treatments across all time points. Shown are the direction of the effect of Roundup Promax, the taxonomy of the amplicon sequence variant, the percent contribution of that taxa to dissimilarity, and the cumulative percent contribution.

Effect	ESV ID	Kingdom	Phylum	Class	Order	Family	Genus	% Cont.	Cum. %
Positive	ASV_1	Bacteria	Acidobacteria	Blastocatellia	Pyrinomonadales	Pyrinomonadaceae	*RB41*	1.03	1.03
Positive	ASV_8	Bacteria	Cyanobacteria	Oxyphotobacteria	Nostocales	Phormidiaceae	*Tychonema*	0.97	2.00
Positive	ASV_2	Bacteria	Verrucomicrobia	Verrucomicrobiae	Chthoniobacterales	Chthoniobacteraceae	*Udaeobacter*	0.88	2.88
Positive	ASV_3	Archaea	Thaumarchaeota	Nitrososphaeria	Nitrososphaerales	Nitrososphaeraceae	*Nitrososphaera*	0.48	3.36
Negative	ASV_4	Archaea	Thaumarchaeota	Nitrososphaeria	Nitrososphaerales	Nitrososphaeraceae		0.43	3.79

among time points ($F = 4.1$, $p = 0.008$). In the last sampling point, the one major taxon that responded to Roundup Promax additions was Mortierellaceae, which increased with increasing Roundup Promax application frequency (Kruskal-Wallis $p < 0.05$, Fig. 5).

3.4. Root endophytes

Bromus inermis roots were colonized by arbuscular mycorrhizal fungi (AMF), dark septate endophytes (DSE), and to a lesser extent, fine root endophytes (FRE) with a mean total fungal colonization across all plots of 16 % ± 2.75 SE (Fig. 6). Percent colonization by AMF declined significantly with two Roundup Promax additions per year and even more with four Roundup Promax additions per year (Tukey posthoc, $p < 0.05$, Fig. 6a). Percent colonization by DSE was significantly lowered only in the plots receiving four Roundup Promax applications per year (Tukey posthoc, $p < 0.05$, Fig. 6b). There were no differences in FRE colonization (Logistic regression, $p > 0.05$, Fig. 6c). *Ellisia nyctelea* was also colonized but to a much lesser extent (mean total colonization across all plots = 4 % ± 1.75) and there were no significant effects of Roundup Promax on any of the three fungal groups in *E. nyctelea* (Logistic regression $p > 0.05$).

4. Discussion

Taken together, our results show some important detrimental effects of Roundup Promax additions at application rates normally used in restoration projects on abiotic and biotic soil characteristics. The most drastic effects were on soil chemistry, including increases in nitrate and decreases in pH, calcium, and cation exchange capacity (Fig. 2), on bacterial communities, which declined in phylogenetic diversity and shifted community structure with increasing Roundup Promax application frequency (Figs. 3a, c, 4a), and on fungal endophyte communities (Fig. 6), which declined in abundance in plant roots. In most cases, plots receiving two applications of Roundup Promax per year, a more typical frequency of application for restoration projects, were not significantly different than controls, while plots receiving four or five applications of Roundup Promax per year showed significant changes. In contrast, eukaryotic soil microbial communities appeared more resistant to the effects of Roundup Promax, as they did not show significant declines in diversity and experienced more minor shifts in community structure.

Roundup Promax additions increased soil nitrate content and decreased pH and calcium content, in partial support of our first hypothesis. On the other hand, Roundup Promax additions did not increase ammonium or aluminum content. Glyphosate is a nitrogen, phosphorus, and carbon containing compound, and laboratory incubation studies have shown that glyphosate can increase both carbon and nitrogen mineralization (Haney et al., 2002), but the present study is to our knowledge the first to show increases of soil nitrate levels under field conditions utilizing glyphosate application rates typical of restoration projects. Studies of nitrogen deposition to field soils have demonstrated

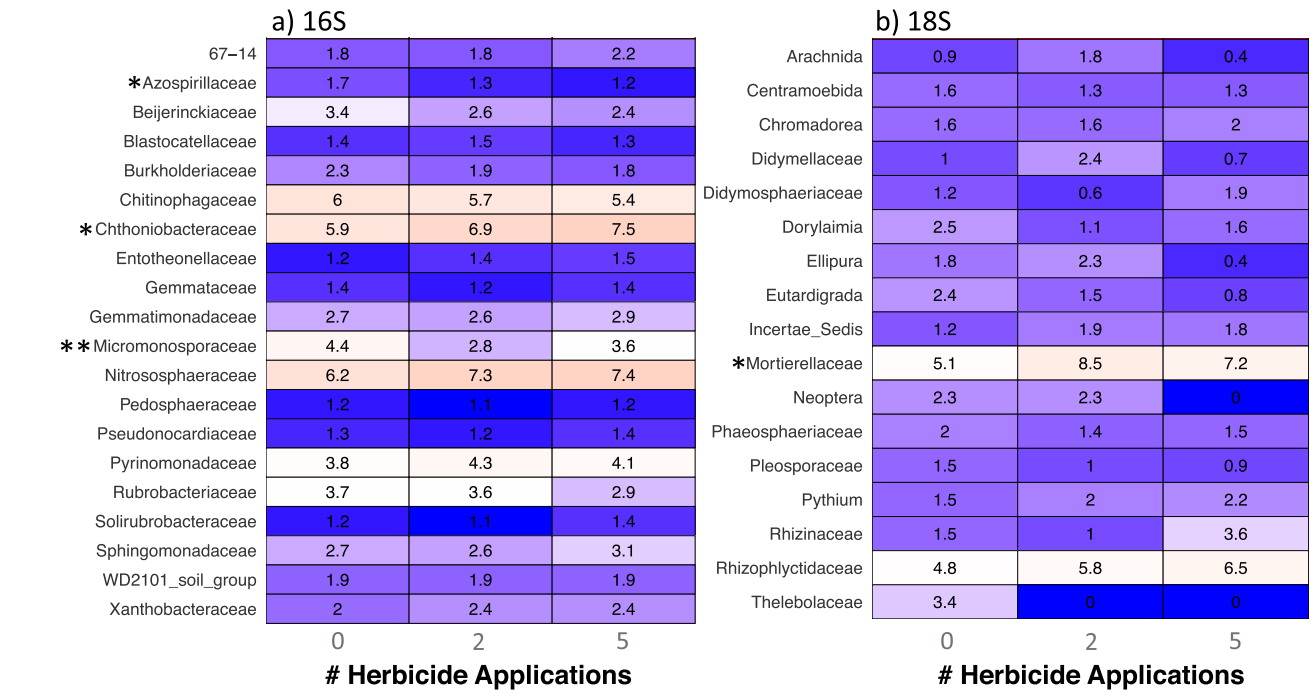

Fig. 5. Heatmap of percent relative abundances across three Roundup Promax herbicide application frequencies for the final sampling period (June 2019) for a) bacterial and archaeal (16S) families with at least 1 % relative abundance and b) eukaryotic (18S) taxa (genus to class level - SILVA database level five) with at least 1 % relative abundance. *Kruskal-Wallis $p < 0.05$; **Kruskal-Wallis $p < 0.05$ after Bonferroni correction.

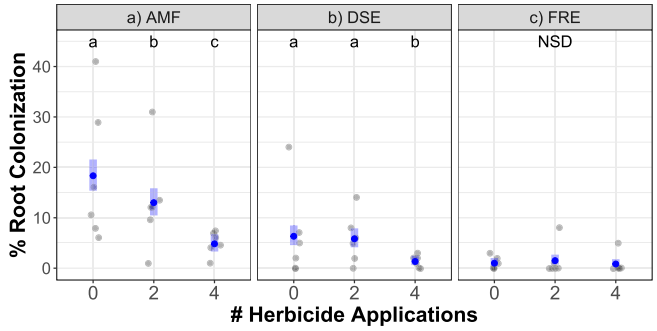

Fig. 6. Percent root colonization of *Bromus inermis* by a) arbuscular mycorrhizal fungi, b) dark septate endophytes, and c) fine root endophytes across different application frequencies of Roundup Promax herbicide. Faded black points show the percent colonization for each individual plant sample. Solid blue points are the modeled estimates for percent colonization from binomial regression models, and blue shaded segments represent the 95 % confidence intervals for the estimates. Different letters signify significant pairwise differences among the herbicide treatments (Tukey posthoc, $p < 0.05$).

that increases in nitrate lead to decreases in pH and a loss of soil base cations and cation exchange capacity (Bowman et al., 2008; Lieb et al., 2011). However, calcium is the only base cation that decreased in this study, and we found no changes in magnesium and sodium. Nonetheless, these findings are troublesome because high soil nitrate levels persisted in soils even after winter snows and spring rains likely caused leaching of some of the nitrate out of the system (Fig. 2). Ongoing work at this site will document whether soil parameters such as nitrate levels will eventually return to more normal levels, but we are not monitoring the magnitude or effects of nitrate leaching from these soils to downstream ecosystems. In addition to direct effects of the glyphosate and adjuvants in Roundup Promax, some of the observed effects on soil chemistry may also be attributed to indirect effects via changes in plant cover and composition, as the plots receiving four or five applications of Roundup Promax were characterized by a much higher proportion of bare ground (Fig. 1). For example, nitrate levels could be elevated due to a combination of an increase in nitrification and a lack of uptake by plants. Whatever the cause, nitrate is very mobile in soil solution and increased nitrate may lead to increased pollution of groundwater and downstream ecosystems (Vitousek et al., 1997).

The lack of increase in ammonium as a result of Roundup Promax application suggests that ammonium produced by mineralization of glyphosate was nitrified to nitrate. This is also consistent with the increases in the relative abundance of the members of the genus *Nitrososphaera* (ASV 3, Table 2), which are known obligate aerobes that can live chemoautotrophically by oxidizing ammonia to nitrite (Kerou and Schleper, 2016; Kozlowski et al., 2016). Soil *Nitrososphaera* have also been shown to prefer high concentrations of ammonium (Stieglmeier et al., 2014; Tourna et al., 2011) which is consistent with their being enriched for in soils receiving high concentrations of ammonium from glyphosate. The Nitrososphaeraceae as a whole slightly increased with herbicide application frequency (Fig. 5), but contained ASVs with both positive and negative responses, highlighting the existence of species-specific responses to Roundup Promax additions and/or the resulting changes in soil chemistry and plant composition within the same microbial family and functional group. Nitrososphaeraceae have also been studied in other plant restoration projects that involved weed control, and were found to increase over time throughout the course of revegetation (Yan et al., 2020).

While we hypothesized decreases in both bacterial/archaeal and eukaryotic alpha diversity in response to higher Roundup Promax application frequency, we only found declines in bacterial/archaeal phylogenetic diversity. Even bacterial and archaeal communities appeared to be somewhat resistant to effects of Roundup Promax, as the only significant difference was between controls and plots receiving five applications per year. Results were similar for taxonomic and phylogenetic diversity measures. Declines in phylogenetic diversity could be driven by either the direct toxic effects of glyphosate and the Promax adjuvant, indirect effects mediated through changes in soil chemistry, indirect effects mediated by changes in plant composition and cover,

and/or by indirect effects mediated by competitive exclusion by microbes that increased in abundance. Direct toxic effects of glyphosate have been demonstrated for soil bacteria grown in culture, but not in soil, perhaps because of strong adsorption of glyphosate to soil particles (Busse et al., 2000), making the indirect effects a more likely explanation. The declines in pH and calcium content could have caused a loss of certain microbes sensitive to these parameters; pH, in particular, has been shown to be a strong predictor of bacterial community composition (Lauber et al., 2009). The major decline of *B. inermis* in the plots receiving five applications per year could have had indirect effects mediated through diminished litter additions and root exudation in those plots, on which certain microbes may rely (Wardle et al., 2004). Lastly, while there were some increases in the relative abundances of a few taxa, the magnitude of these changes was not large, making it unlikely that competitive exclusion played a major role here.

With regards to beta diversity, our third hypothesis was generally supported, with significant changes in community structure for bacteria and archaea and also eukarya, though effects on the latter were more minor. One potential explanation is that bacteria and archaea are more capable of responding directly to glyphosate by breaking it down and utilizing it as a nutrient source compared to eukarya (Moore et al., 1983; Pipke and Amrhein, 1988) or by using the breakdown products (e.g. ammonium use by the Nitrososphaeraceae as described above). Another possibility is that the turnover time of the eukaryotic community in response to herbicide additions is much slower than that of the bacterial community in this system. It is also possible that more of the eukaryotic DNA is tied up in dormant cysts and spores than is the 16S community and therefore changes in the active community might not be reflected in our DNA-based approaches. These explanations are supported by our measurements of active soil communities like endophytic fungi (discussed below) which showed large significant negative responses to Roundup Promax additions, despite the 18S community data showing little change. An avenue for future research is to compare bacterial and eukaryotic responses to Roundup Promax in a microcosm setting to control for other soil and plant effects, directly test the utilization of glyphosate and adjuvants, and analyze the active microbial community with metatranscriptomics.

The decrease in both AMF and DSE root colonization in *B. inermis* is perhaps the most concerning result from this study, especially because AMF decreased after only two applications of Roundup Promax. While *B. inermis* and other invasive species and cool-season grasses may not be as dependent on AMF fungi as other plants (Pringle et al., 2009; Sherrard and Maherali, 2012; Wilson and Hartnett, 1998), AMF colonization of *B. inermis* in our study was 5–40 % in controls, and declined after repeated glyphosate additions. A growing body of work has demonstrated that arbuscular mycorrhizal fungi (AMF) are critical for successful plant restoration (Asmelash et al., 2016; Turnau and Haselwandter, 2002) and several studies have shown that inoculating soils with native AMF increases native plant diversity in grassland restoration (Koziol et al., 2018; Koziol and Bever, 2017). This may be a necessary management action if AMF levels in soil are decreased or species composition has been significantly altered. We expect that this may be the case at our field site; in the separate ongoing project on native plant restoration, all 12 of the target species that have been seeded (*Poa palustris*, *Pascopyrum smithii*, *Monarda fistulosa*, *Elymus trachycaulus*, *Coreopsis tinctoria*, *Achillea millefolium*, *Sporobolus cryptandrus*, *Solidago rigida*, *Schizachyrium scoparium*, *Penstemon secundiflorus*, *Nassella viridula*, *Erigeron speciosus*), have been reported to have associations with mycorrhizae and other endophytes and native microbial communities (Boldt-Burisch et al., 2018; Busby et al., 2011; Dhillion and Friese, 1992; Fisher and Fulé, 2004; Ioana and Roxana, 2019; Khidir et al., 2010; Maron et al., 2011; Middleton et al., 2015; Perez-Naranjo, 2009; Pérez et al., 2009; Reynolds et al., 2020; White and Cole, 1986; Wilson et al., 2016).

The mechanism by which Roundup Promax applications decreases AMF and DSE fungal colonization cannot be directly ascertained from the results of the present study. Glyphosate additions can decrease root colonization by either altering the ability of mycorrhizal associations to form (either by direct toxicity or by increasing the phosphorus nutrition of plants, which in turn lessens the need for mycorrhizal associations) or by lowering viable inocula levels. Previous work on orchid mycorrhizae demonstrated that mycorrhizal interactions can be inhibited as a result of glyphosate addition (Beyrle et al., 1995), while a field study of arbuscular mycorrhizal fungi found decreased spore viability with glyphosate additions (Druille et al., 2013b). A field study using one application of 3.5 L ha^{-1} glyphosate found no effect of glyphosate on DSE colonization in the invasive legume *Lotus tenuis* (Druille et al., 2017), while another study that applied 3 L ha^{-1} glyphosate four times found a 68 % decrease of DSE in the invasive grass *Lolium arundinaceum*. Furthermore, in the lab setting, DSE cultures isolated from wheat plants experienced diminished growth rates when grown in agar with 0.40 ppm and 2 ppm glyphosate, with a greater magnitude of decline the higher the dose of glyphosate (Spagnoletti and Chiocchio, 2019). These results are consistent with our results and highlight the importance of dose and application frequency. Obviously much more work in both microcosms and the field are needed to tease apart the direct and indirect effects of glyphosate on mycorrhizal fungi and other root endophytes and further work should also be done comparing colonization responses in invasive grasses versus native forbs.

In conclusion, we report that Roundup Promax applications can affect soil fertility, soil bacterial and archaeal communities, root endophytic fungi, and to a lesser extent, soil eukaryotic communities. Given our findings and other studies discussed above, these effects on soil microbes are likely not all due to direct toxic effects of glyphosate and adjuvants, but to indirect effects mediated by changes in plant communities and soil chemistry. These effects can also be minimized by limiting the number of application times per year (Lancaster et al., 2010), but such reductions would lower the herbicide efficacy in reducing invasive plants in plant restoration efforts. It remains to be seen how quickly microbial communities can recover to pre-herbicide states following differing application frequencies; this is an interesting avenue for future work. Furthermore, as glyphosate is almost always applied with adjuvants, future work should address the direct and indirect effects of the different adjuvants used in conjunction with glyphosate on soil properties and microbial communities, and how these changes to the soil environment affect the ability of the soil to restore native plant communities, particularly legumes and C4 grasses that depend on mycorrhizal fungi and other essential soil microbes (Wilson and Hartnett, 1998).

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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