Title: Direct and indirect effects of herbicide application on the soil microbiome

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**Abstract**

Chemical herbicides, like glyphosate and others, are commonly used to control unwanted vegetation in agroecosystems and boost yield. While their effects on aboveground weedy vegetation are well documented, by comparison, the influence of herbicide on below-ground microbial communities is poorly understood. Given the importance of the soil microbiome for providing ecosystem services, understanding microbial responses to herbicide treatment is of pressing interest. In this study, we examine the non-target effects of herbicide application by comparing three commonly used chemical herbicides: glyphosate, dicamba, and a tank mix of atrazine and mesotrione- to handweeded and non-treated controls. To do so, we assessed changes in soil microbial function, nutrient pools, and microbiome composition in *Zea mays* fields over 20 days post-treatment. The examined herbicides were shown to have limited and transient effects on soil edaphic properties and microbial function. Changes in enzymatic activities, nutrient concentrations, and fungal community structure were predicted by weedy vegetation cover, but not weed removal treatment, suggesting that these responses are indirectly mediated by herbicide application. Conversely, we show that weed removal treatment was a significant predictor of bacterial community composition and that chemical herbicide application resulted in homogenization of the bacterial community, a trend not observed in the non-treated and handweeded controls. Together, our results suggest that chemical herbicide application has a limited effect on microbial diversity and function in agroecosystems. However, the trend of biotic homogenization in agroecosystems warrants further examination.

**Introduction**

Genetically modified herbicide-resistant crops were introduced to the United States in 1996, prompting a shift in herbicide use patterns. Driven primarily by the application of glyphosate (N-(phosphonomythel)glycine), herbicide usage in the US increased by nearly 240 million kg from 1996 to 2011 (1), making these chemical compounds a staple in conventional agriculture. In this way, producers remove unwanted vegetation to reduce crop-weed competition and optimize their systems for maximum annual yield. However, the birth of the soil health movement has encouraged a new way of thinking about agriculture with a greater appreciation for long-term agroecosystem productivity. With this new school of thought, researchers and producers alike are realizing the importance of considering the non-target effects that herbicide and other agricultural amendments might have on soil and the ecosystem services that it provides (2, 3).

While commonly applied to control unwanted weedy plant populations, herbicides have the potential to affect the structure and function of the soil microbiome (2, 4–7). The microbial components of agroecosystems provide countless benefits including plant growth promotion (8), pathogen suppression (9), enhanced nutrient cycling (10–13), as well as aggregate formation and maintenance of soil structure, thus contributing an estimated $330 to $500 per ha/yr in ecosystem services (14). Given their critical importance in agroecosystems, understanding how microbial communities respond to herbicide application is necessary to optimize agricultural systems for yield and sustainability.

Reviews aimed at understanding the effects of herbicide application on the soil microbiome reveal a lack of generalizable trends (5, 15–18) and instead show that the consequences are dependent upon the characteristics of the soil, plant, application rate, and the herbicide’s mode of action (2, 5, 19). Further complicating efforts to accurately model the influence of chemical intervention is the fact that herbicides indirectly affect the soil microbiome by changing vegetation cover, thus altering patterns of root exudation (20). To better understand the short-term direct and indirect effects of chemical herbicide application on the soil microbiome, we compared three herbicides, each with a unique mode of action (Table 1), along with handweeded and non-treated controls. We hypothesized that each treatment would produce a unique response in microbiome structure and function due to differences in mode of action (2). Specifically, previous research has shown that the application of a triazine class of herbicides (simazine) disrupts nitrogen cycling in agricultural soils by affecting ammonia oxidizers (21). With this, we hypothesized that the application of atrazine-mesotrione (a triazine herbicide) would affect nitrogen (N) cycling microbes (21), resulting in significant shifts in N-pools and N-acquiring enzyme activities. Additionally, we hypothesized that the glyphosate and dicamba herbicide treatments would affect the soil microbiome by disrupting amino acid synthesis and signaling pathways present in both microbes and plants.

**Results**

*Vegetation cover*

Herbicide treatment reduced weedy vegetation cover at 10- and 20-days post-treatment (p < 0.01, F4,15 > 8.75). Specifically, chemical herbicide application reduced lamb’s quarter (*Chenopodium album*) and redroot pigweed (*Amaranthus retroflexus*) cover at 10- and 20-days post-application when compared to the nontreated plots (p < 0.05). Handweeded plots experienced an initial decrease in weed cover relative to the non-treated plots (p < 0.05). However, at 20 days post-treatment, there was no difference between the two control plots.

*Physicochemical responses*

Before treatment, no differences in edaphic conditions were detected across our plots (Supplementary Table A1). Weed removal treatment (i.e., chemical herbicide or handweeded) was a significant predictor of nitrate and total inorganic N content at both post-treatment sampling points (p < 0.05), with total weedy vegetation significant only at the 20-day sampling time (Supplementary Table A2). Glyphosate-treated plots were found to have higher levels of nitrate and total inorganic N than the handweeded plots at both post-treatment sampling points (p < 0.05) (Supplementary Table A3).

When examining how our edaphic variables changed over time within a single weed removal treatment, multiple differences were observed, including nitrate, nitrite, ammonium, total inorganic N, phosphate, calcium, magnesium, pH, EC, and gravimetric moisture content (p < 0.05) (Supplementary Table B1). Pairwise comparisons showed the pre-treatment sampling point to have the highest levels of nitrate, total inorganic N, calcium, and magnesium, though only nitrate and inorganic N were statistically significant (p < 0.05) (Supplementary Tables B2 and B3). In all plots, gravimetric moisture content increased over the 20-day sampling period (p<0.05). Across all treatment and sampling times, the concentration of nitrate was found to be negatively correlated with total vegetation cover, redroot pigweed cover, and lamb’s quarter cover (p < 0.05, r < -0.14). Ammonium, calcium, and magnesium showed the opposite trend and were positively correlated with total vegetation and redroot pigweed cover (p < 0.01, r > 0.19). Only redroot pigweed produced a significant correlation between vegetation cover and phosphate concentration (p < 0.001, r = -0.25).

*Extracellular enzyme responses*

We report no significant differences in total enzymatic profiles among treatments at any of the sampling times (p > 0.25, F4,14 < 1.261, R2 < 0.195). However, total weedy vegetation cover was a significant predictor of differences in enzymatic profiles at the 10- and 20-day post-treatment sample times (p < 0.05, F1,14 > 4.411, R2 > 0.195). For individual enzymes, only alkaline phosphatase (PHOS), ß-glucosidase (BG), and the ratio of N:P cycling enzymes showed significant differences among herbicide treatments (p < 0.05) (Supplementary Table C1). Significant pairwise comparisons at the pre-treatment sampling were limited to PHOS and the ratio of N:P cycling enzymes. Here, the nontreated plots showed higher initial PHOS activity as compared to the handweeded plots, and a lower N:P ratio as compared to the handweeded and atrazine-mesotrione treated plots (Supplementary Tables C2 and C3). While not statistically significant, this trend flipped at sampling time two and three with handweeded plots exhibiting the highest mean PHOS activity across all treatment types. Likewise, the activity of BG spiked at time two in the handweeded plots compared to the other treatments, with a similar, though non-statistically significant, trend observed for the glyphosate-treated plots (Figure 1). Total weedy vegetation was a significant predictor for BX, AG, and PHOS at time one, and for BG, LAP, and the ratio of C:P cycling enzymes at sampling time two. In all cases, β- coefficients of the regressions were positive, indicating that total weedy vegetation had a positive effect on enzyme activity.

When examining the effect of time within a single weed removal treatment, only the handweeded plots showed a significant difference in total enzymatic profile over the 20-day sampling period (p < 0.01, F2,11 = 6.88, R2 = 0.607). The atrazine-mesotrione, dicamba, and nontreated plots showed no differences in individual enzyme activities or overall enzymatic profiles throughout the sampling period (p > 0.05). Phosphatase activity was the most variable of the measured enzymes over the 20 days, showing a statistically significant increase in activity at 10-days after treatment followed by a reduction 20-days after treatment in both the handweeded and glyphosate-treated plots (p < 0.05) (Figure 1, Supplementary Table D1). No correlation was found between phosphate ion concentration and PHOS activity when considering all plots (p > 0.9, r = 0.011). Additionally, both the handweeded and glyphosate-treated plots displayed similar trends in the activity of BG, AG, BX, and the ratio of C:N cycling enzymes, with the lowest activity pre-treatment and the highest at time two (10 days) followed by a return to pretreatment levels at sampling time three (20 days). However, this was only statistically significant for the handweeded plots (p < 0.05) (Supplementary Tables D2 and D3). Finally, mantel testing of edaphic conditions and enzymatic activities showed that distance matrices of nutrient availability and total enzymatic profile were not correlated (p > 0.75, r = -0.037).

Linear mixed effects modeling revealed enzyme-specific responses to weed removal treatment, initial enzyme activity, total weedy vegetation, and the interaction of sampling time and weed removal treatment. BG, LAP, and the ratio of C:P cycling enzymes were predicted by total weedy vegetation (p < 0.05). Positive β-coefficients for weedy vegetation cover indicate that higher weed cover led to increased enzyme activity. Both BG (p = 0.055) and LAP (p < 0.05) were predicted by the interaction of treatment and time, though this was only marginally significant for BG. The level of PHOS activity before treatment was deemed a significant predictor of PHOS activity at times two and three (p < 0.05). Both herbicide treatment (p = 0.057) and the treatment by time interaction (p = 0.052) were marginally significant for predicting PHOS activity as well. All other mixed models for enzyme activity produced no significant predictors (Supplementary Table E1).

*Bacterial diversity*

Bacterial count tables were rarefied to 3,000 reads per sample. After outlier removal, 164 independent samples were included in the downstream analysis. Rarefied results are reported unless otherwise noted. No differences in bacterial α-diversity (Shannon, richness, or Chao1) were found between the treatment types at any of the sampling times (p > 0.26). Adonis testing of the rarefied and Dirichlet Multinomial (DMN) transformed taxon tables showed a significant, but minor effect of weed removal treatment (p < 0.05, F4,147 = 1.101, R2 = 0.0268), and an effect of the interaction of weed removal and treatment\*time (p < 0.001, F8,147 = 1.189, R2 = 0. 0579) (Figure 2, column 1). The effect of sampling time on community dissimilarity was not significant for the rarefied taxon count tables (p = 0.11, F2,147 = 1.094, R2 = 0.0133) but was significant, though minor, for the DMN table (p < 0.01, F2,152 = 2.1183, R2 = 0.02247). Total weedy vegetation was not a significant predictor of bacterial dissimilarity for either of the taxon tables.

We then assessed the difference in multivariate dispersion to understand how herbicide application may affect biotic homogenization in the soil microbiome. Glyphosate and atrazine-mesotrione treated plots showed homogenization in their bacterial community over the 20-day sampling period (p < 0.057) (Figure 2, column 2). The handweeded and nontreated plots showed a trend of increased heterogeneity, though this was not statistically significant. When all chemical treatments were combined into a single chemically treated herbicide group, we found higher levels of multivariate spread as compared to the non-treated and handweeded plots at sampling time one (p < 0.01). However, the dispersion of the chemically treated group decreased over the next 20 days, and at the final sampling, all groups showed similar levels of multivariate spread (Figure 2, column 3).

Changes in the abundance of dominant families over time were detected in all treatment groups. Of particular interest, the family Sphingomonadaceae showed a statistically significant increase in abundance in all chemical herbicide-treated plots (p < 0.001, F2,30 = 9.469). No other consistent taxon enrichments or depletions were detected across herbicide or treatment types. Linear modeling of bacterial taxa identified by simper and the Boruta machine learning classification algorithm showed that weed removal treatment, sampling time, and total weedy vegetation explained at most a total of 15% of the variation in taxon abundance (Supplementary Table F1).

*Fungal Diversity*

Fungal count tables were rarefied to 1,000 reads per sample and resulted in 157 independent samples being included in downstream analysis, after outlier removal. Again, rarefied results are reported unless otherwise noted. No differences in α-diversity (Shannon, richness, or Chao1) were found between the treatment types at either of the first two sampling times (p > 0.3). At 20 days post-application, Shannon diversity (H`) was marginally significant (p = 0.051, F4,48 = 2.551), with no significant pairwise differences between treatments. PERMANOVA testing of the rarefied and DMN taxon tables revealed significant effects of sampling time (p < 0.05, F2,140 < 1.521, R2 = 0.0139), total weedy vegetation (p < 0.01, F1,140 = 1.845, R2 = 0.0116), and the interaction of sampling time\*weed removal treatment (p < 0.05, F8,140 = 1.185, R2 = 0.0259) (Figure 3, column 1). No clear trends in dispersion within treatments through time were found for the fungal community (Figure 3 – columns 2 & 3).

Shifts in dominant fungal families revealed increased abundance of Ceratobasidiaceae in the glyphosate-treated plots over the three sampling times (p < 0.001, F2,30 = 9.469). Additionally, the family Spizellomycetaceae showed an increase in abundance in the two controls as well as the glyphosate-treated plots over the sampling period (p < 0.05, F2,28  > 3.351). Linear modeling of fungal taxa identified by simper and the Boruta classification algorithm showed that weed removal treatment, sampling time, and total weedy vegetation explained -at most- a total of 33% of the variation in taxon abundances (Supplementary Table G1). However, these taxa were rare and often not detected.

**Discussion**

The application of chemical herbicides in conventionally managed agricultural systems is widespread. However, a comprehensive understanding of how these agrochemicals affect below-ground microbial processes remains lacking. Our experimental results aid in this appreciation and suggest that soil microbial function in conventionally managed agroecosystems is largely resistant and resilient to herbicide application over the 20 days post-treatment. Furthermore, we show that the factors affecting bacterial and fungal diversity differed, with bacteria showing a significant effect of weed removal treatment and fungi responding to changes in total weedy vegetation. Additionally, the convergence of bacterial communities following chemical herbicide applications suggests that, regardless of the herbicide type, chemical intervention homogenizes the bacterial compartment in soil and leads to a “chemically disturbed” microbiome, contrary to our initial hypothesis. Finally, and perhaps most importantly, experimental designs like ours and others (22) are extremely valuable as they allow for direct and indirect effects of herbicide application to be teased apart. This improved understanding offers valuable insight into the dynamics of the soil microbiome following vegetation removal and permits an accurate assessment of the effects of chemical herbicide application.

The lack of differences in total enzymatic profiles between treatments at sampling time one indicates that our plots were functionally homogeneous before treatment, and as such, any differences at the subsequent time points can be attributed to our weed-removal treatments. At ten days post-treatment, weed removal did not affect enzymatic profiles, but total weedy vegetation did. This provides additional support to the idea that the effects of herbicide application on soil function are indirectly mediated through changes in vegetation (19, 20). This effect was also observed for the fungal community, with total weedy vegetation significantly affecting the composition of the fungal microbiome. As plant diversity is known to influence fungal diversity (23) and fungi are thought to be largely responsible for the degradation of plant litter (24), it is not surprising that total weedy vegetation cover affects microbial enzymatic profile (25) though its influence on soil fungi.

Furthermore, although we report transient increases in the enzymatic activities in glyphosate-treated plots, we also observed this trend in our handweeded controls, suggesting that an increase in available above- and below-ground plant detritus may be responsible, as opposed to a direct effect of glyphosate application. With the observed spike in enzyme activities and a return to pre-treatment levels, we believe that labile litter inputs would stimulate decomposition. However, once the detritus became more recalcitrant or its C:N ratio increased, it may no longer be favorable for decomposition, resulting in the observed decrease in extracellular enzyme activity from 10 to 20-days post-application, with others observing similar trends (26).

The response of microbial enzymatic activity to glyphosate-based herbicides in clay-loam soils has been shown to display temporal variability, with alkaline phosphatase being more sensitive (27) than other members of the phosphatase enzyme family. Using a controlled mesocosm experiment to examine the effect of glyphosate on phosphatase activity, Cherni et al. (28) showed no differences between the recommended field rate group (1 L/HA) and untreated controls and only a mild increase in activity at 10X field application rates (10 L/HA). As our application rate was only slightly above the 1X and well below the 10X rates used by Cherni et al. (28), it is not surprising that shifts in PHOS activity were minimal and only short-lived. To this end, others have also shown a high glyphosate dose requirement to observe significant effects on microbial activity (29–31) and hypothesize that at these high rates, increased microbial activity may be a result of detrimental effects on lithotrophic microbes (26). If true, this would shift the competitive balance among soil microbes, favoring heterotrophs. The observed increase in the abundance of Shpingomondacae provides support for this hypothesis and suggests a competitive advantage for these putative herbicide degraders following chemical herbicide application (32, 33). ~~It is worth noting, that even though we observed differences in PHOS activity in our experiment, our mixed modeling found the activity of PHOS at time one to be a significant predictor of activities at time two and three, suggesting the existence of a legacy effect in phosphatase activity. Thus, our ability to say whether PHOS activity was affected by weed removal treatment is hindered by pretreatment differences.~~

Given the importance of nitrogen availability for agricultural productivity (34), understanding the influence of chemical herbicides on nitrogen-cycling microbes should remain a primary concern. Although our sequencing approach did not specifically target these microbes, measures of inorganic nitrogen availability and nitrogen cycling enzymes provide clues to their response. Previous work examining the effects of glyphosate on soil N showed no differences in inorganic N pools between vegetation removal plots over twenty weeks (e.g., handweeded and glyphosate) (22). Here, the authors did however report significant differences between their handweeded and nontreated plots, showing nitrate levels, as measured by resin ion exchange strips, to be higher in their handweeded plots as compared to their nontreated controls. Their result indicates that the removal of vegetation may lead to increased soil nitrate levels. We report nitrate levels to be lowest in our handweeded and nontreated plots, with concentrations being highest in our glyphosate plots. While our results may appear superficially different from Hagner et al. (22), we show that nitrate concentrations were significantly correlated with vegetation cover, and at 20 days post-treatment, the handweeded plots experienced increased weedy vegetation and crop cover, potentially contributing to decreased inorganic N availability. Given the lack of difference between their handweeded and glyphosate-treated plots and the observed correlations between nitrate concentration and vegetation cover in our experiment, we suggest that differences in inorganic nitrogen between glyphosate, handweeded, and nontreated plots are mediated through changes in vegetation instead of a direct effect of herbicide application (19, 20, 26).

Beyond glyphosate, differences in N-cycling have also been reported following triazine application, with a general trend of short-term reduction in the rate of N mineralization following application (21, 35). Accordingly, we show a decrease in the inorganic N pool in the atrazine-treated plots over the 20-day post-application period, providing support for our hypothesis and further evidence that this herbicide affects nitrogen-cycling microbes. Importantly, however, the trend of decreased inorganic N was observed in other treatments as well. Together with the observed correlations with total weedy vegetation, we are unable to determine if this effect is direct or indirect as the crop, *Zea mays*, and other weedy vegetation may be partially responsible for a reduction in inorganic N through uptake and immobilization in plant biomass.

Although the effects of chemical herbicides on soil microbial function and physicochemical parameters appear to be limited in scope, the observed homogenization of the bacterial community following chemical treatment is potentially cause for concern. Biotic homogenization may severely limit the ability of ecosystems to respond to global change scenarios, thus negatively affecting long-term stability (36). In particular, in conventional agricultural systems, there is evidence that the intensive use of agrochemicals and disturbance (e.g., tillage) to boost productivity may result in a reduction in β-diversity as compared to organic practices (37). As we face increasingly common climatic extremes (38), maintaining the resiliency of agroecosystems is imperative for the continued provisioning of a stable global food supply (39).

Taken together, the limited functional and fungal responses, along with bacterial community homogenization following chemical treatment directly refutes our hypothesis that each mode of action would result in a unique functional and community response. Instead, we show that herbicide application resulted in either no or a homogenizing effect on soil microbial diversity. Additionally, the importance of total weedy vegetation suggests that instead of direct effects, herbicide application affects the soil microbiome via indirect mechanisms. With this, our findings are not all that surprising due to the high degree of functional redundancy in the soil microbiome (40, 41) and the fact that we used a relatively low rate of herbicide application. With respect to agricultural systems, it appears that the direct effects of chemical herbicides on microbial function are few and short-lasting. In heavily managed, conventional agricultural systems, the choice of herbicide has no lasting effect on soil function, and the observed effects are instead mediated through indirect pathways.

**Methods**

*Site description and experimental design*

In the summer of 2018, 20 experimental plots were established at the Sustainable Agriculture Research and Extension Center (SAREC) in Lingle, WY (MAT 8.4° C). Plots were fertilized using a dry fertilizer before corn planting following best farming practices (40 rock fertilizer- 125Nitrogen-35Phosphorus-0Potassium-35Elemental Sulfur-13Sulfate-2Zinc blend -J.R. Simplot Company, Boise, ID). Plots were then planted with *Zea mays* variety ‘P9188AMX’ at 34,000 seeds per acre and irrigated using a lateral pivot system every three to five days. The soil at this site has an alkaline pH (~ 8) with CaCO3 content between 1 and 3 %. The soil can be characterized as silty clay loam (27 % - 40 % clay) (42) and is mapped as a well-drained Haverson and McCook loam (i.e. HnA), 0-3% slope (Web Soil Survey, USDA-NRCS). Each plot, 3 m x 10 m, consisted of five evenly spaced rows of *Zea mays*. The experimental design included five different treatments with four replicate plots each: a non-treated control (herein referred to as non-treated), a handweeded control (herein referred to as handweeded), and three different chemical herbicide applications applied at recommended field rates: glyphosate (group 9), dicamba (group 4), and a tank mix consisting of atrazine and mesotrione (group 5 and group 27, respectively) (Table 1). In the handweeded plots, above-ground vegetation was cut using a hoe and left on the soil surface. Belowground biomass was not disturbed and remained in the soil. A pre-treatment sampling was conducted on the morning of weed removal treatment in early June. Following herbicide application and hand weeding, two post-treatment samplings were conducted: 10 days post-treatment and 20 days post-treatment.

*Sampling and analysis*

To facilitate sampling, a grid was laid out over each plot, and the coordinates of sample locations were determined using a random number generator. The corn stalk closest to the randomly generated coordinate was selected, and a 0.25 m2 Daubenmire plot was placed so that the stalk was in the center. Cover classes of the main weeds including, redroot pigweed (*Amaranthus retroflexus*), Nightshades (Solanaceae), lamb’s quarter (*Chenopodium album*), and any graminoid, were recorded for each Daubenmire plot. Total weedy vegetation cover was estimated by converting weedy cover classes to a continuous predictor and summing.

Three plot replicate soil samples were collected at each of the time points for a total of 180 samples (5 treatments x 4 replicate plots x 3 plot replicates x 3 time points). Each plot replicate soil sample was a composite of three soil cores from a single Daubenmire to ensure sufficient soil for laboratory analyses.Soil samples were collected using flame-sterilized soil corer to a depth of 5 cm and stored on ice in sterile Whirl-Pak bags. *In situ* soiltemperature was measured at a depth of 2.5 cm using a temperature probe. Soil samples were transported back to the University of Wyoming on ice for further processing.

In the lab, field-fresh soil samples were processed within 24 hours. Soil samples were first thoroughly mixed in the Whirl-Pak bag used for collection and then sieved through an ethanol-cleaned 2 mm sieve. Gravimetric moisture content was determined by weight difference of a five-gram subsample of sieved field-fresh soil after oven drying at 105° C for 48 hours. Next, ~11 g of sieved soil was weighed out for extraction in 50 mL of 0.5 M K2SO4 for assessment of extractable levels of cations (ammonium, calcium, sodium, and magnesium) and anions (nitrate, nitrite, phosphate, and chloride) on a Thermo Scientific Dionex Integrion HPIC system (Thermo Fisher Scientific, Waltham, MA). Briefly, samples were shaken in extractant for 30 minutes, stored at 4 °C overnight, and filtered through Fisherbrand Q5 filter paper the next day. Filtered extracts were diluted with nanopore water (1:10) before analysis and filtered through a 0.45 μm hydrophilic filter as per (43). Anions were run on a Dionex IonPac AS18 4 um 4x150 mm column for 20 minutes with a flow rate of 1.0 mL/min at 35 °C. Cations were run on a Dionex IonPac CS16-Fast 4um 4x150mm column for 20 minutes with a flow rate of 0.64 mL/min at 40°C. Raw values of anions and cations were reported as ppm and converted to mg/kg dry soil. The remaining soil was then split into two portions, of which one was frozen at -20 °C for microbial analysis (e.g., DNA extraction for determination of microbial community structure and extracellular enzyme analysis for microbial function), and the other portion was air-dried. Air-dried soil was used to measure pH and electrical conductivity using an Oakton PC700 benchtop meter (OAKTON instruments, Vernon Hills, IL) with a soil to DI water ratio of 1:2 (w:v).

*Extracellular enzyme analysis*

Extracellular enzyme activities (EEA) were measured for seven enzymes involved in the cycling of carbon (C), nitrogen (N), and phosphorus (P) as outlined in (44–48). The enzymes measured include ß-glucosidase (BG), β-xylosidase (BX), α-glucosidase (AG), cellobiohydrolase (CBH), alkaline phosphatase (PHOS), N-acetyl-ß-glucosaminidase (NAG), and leucine aminopeptidase (LAP). A preliminary assay was performed using four different substrate concentrations (ranging from 200 μM to 4000 μM) at five different measurement times (1, 2, 4, 6, 8, and 10 hours). This was done to ensure non-limiting substrate availability and maximum potential enzyme activity (Vmax) (49). Three soil samples encompassing the expected variability in enzyme activity were used for these preliminary assays. Briefly, a soil slurry was created by homogenizing 1 g of soil with 100 ml of sodium acetate buffer (50 mM, pH 7.95) for 30 seconds using a Magic Bullet blender (Homeland Housewares LLC). To minimize the difference between buffer and soil pH, we used a buffer pH of 7.95 which represents the mean pH of all soil samples within 1 standard deviation. In a 96-well microplate, 200 μL of soil slurry homogenate was combined with 50 μL substrate and incubated at 20 °C for 1-10 hours. Four technical replicates per soil sample were used to measure fluorescence after the addition of substrates. Hydrolytic enzyme activity was measured via fluorescence on a SYNERGY HTX multi-mode reader (BioTek® Instruments Inc., Winooski, VT) at an excitation wavelength of 360 nm and an emission wavelength of 450 nm. Background fluorescence was corrected using a negative control (sample homogenate with buffer), as well as a quench control (sample homogenate with standards). This was done to correct for interference of soil particles with fluorescence intensity. Fluorescence conversions were based on measurements of standards (10 μM): 7-amino-4-methylcoumarin hydrochloride (AMC) for LAP and 4-methylumbelliferone (MUB) for BG, BX, AG, CBH, NAG, and PHOS. Final enzyme activities were calculated using formulas outlined in DeForest (47) and were reported as nmol of substrate converted per hour per g soil dry mass (nmol h-1 g-1). The incubation time and substrate concentration, from the preliminary assay, producing the highest average enzyme activity were selected for each enzyme. This resulted in a single substrate concentration and incubation time to be used for each enzyme across all samples for final enzyme assays. Final enzyme assays for all samples were done similarly to the preliminary assays but using the substrate concentration and incubation time that resulted in the maximum potential enzyme activity (Vmax). One sample from each plot was used for enzyme analysis for a total of 20 samples at each time point and 60 samples across the three time points.

*Microbiome library preparation*

Subsamples of the same soils used for edaphic measurements were extracted using PowerSoil extraction kits (MO BIO, Carlsbad, CA) according to the manufacturer’s instructions. Soil DNA extracts were stored at -20 °C until PCR and library preparation.

Both bacterial (16S) and fungal (internal transcribed spacer; ITS) amplicon libraries were prepared in the same fashion, though different primers were used to amplify each region. To amplify the V4 region of the bacterial 16S rRNA gene the modified 515F (5’-GTGYCAGCMGCCGC GGTAA-3’) (50) and 806R (5’-GGACTACNVGGGTWTCTAAT-3’) (51) primers were used. For the ITS1 region of the fungal genome, the primers ITS1F (5'-CTTGGTCATTTAGAGGAAGTAA-3') (52) and ITS2 (3'-CGTAGCTACTTCTTGCGTCG-5') (53) were used. Environmental DNA (eDNA) was normalized to 10 ng/μL before amplification. The 15 μL reactions contained 3 μL 5x Kapa HiFi HotStart PCR buffer (Roche, Basel, Switzerland), 0.45 μL 10M dNTPs, 0.3 μL Kapa HiFi HotStart DNA polymerase (Roche, Basel, Switzerland), 3.25 μL HPLC H2O, 6 μL of appropriately paired barcoded primers (3 μL forward and 3 μL reverse), and 2 μL of the diluted 10 μL eDNA sample. In a two-step process, eDNA was amplified and barcoded, then Illumina adaptors were added. In the amplification and barcoding step, the following PCR conditions were used: 95 °C for 3 min (1 cycle), 15 cycles of 98 °C for 30 sec, 62 °C for 30 sec, 72 °C for 30 sec, and 72 °C for 5 min (1 cycle). PCR was performed in duplicate for each sample and combined to limit PCR biases. PCR products were cleaned using Axygen’s AxyPrep Mag PCR Clean-up Kit according to manufacturer instructions without the addition of TE buffer (Axygen Biosciences, Union City, CA). In the second step of the PCR, Illumina barcodes were added to our DNA samples. Each reaction in this step consists of 10 μL of cleaned PCR product from the first step and 5 μL of FlowCell master mix consisting of 3 μL of 5x Phusion HF buffer (Thermo Fisher Scientific, Waltham, MA, USA), 0.45 μL 10M dNTPs, 0.3 μL Kapa HiFi HotStart DNA polymerase (Roche, Basel, Switzerland), 0.5 μL 10 μM forward (AATGATACGGCGACCACCGAGATCTACACTCGTCGGCAGCGTC) and reverse (CAAGCAGAAGACGGCATACGAGATGTCTCGTGGGCTCGG) FlowCell primers, and 0.75 μL HPLC H2O. In the Illumina adaptor addition step, the following PCR conditions were used: 95 °C for 3 min (1 cycle), 19 cycles of 98 °C for 30 sec, 55 °C for 30 sec, 72 °C for 30 sec, and 72 °C for 5 min (1 cycle). PCR products from the second step were cleaned using GSAF’s modified MagBead protocol and Axygen’s AxyPrep Mag PCR Clean-up Kit. Following cleaning, sample concentration was checked using a Synergy HTX Take 3 trio plate reader. Sample DNA concentrations were then normalized and combined, and the final library concentration was checked using qPCR. The final library was sent for sequencing at Psomagen genomic sequencing and analysis facility (Rockville, MD) on their NovaSeq6000 using paired-end 2 x 250 bp chemistry with a 10% PhiX spike in. Both the fungal and bacterial libraries we sequenced together on a single lane and were separated bioinformatically.

*Bioinformatic processing of sequence data*

Sequence data were processed using vsearch 2.9.0 (54). Paired-end reads were merged and reads with more than a single estimated error were removed. Reads were clustered into operational taxonomic units using a 99% similarity threshold. Taxonomy was assigned using the sintax algorithm (55) and the Greengenes (56) database for bacteria (v13.5) and the Unite (57) database for fungi (downloaded: April 4, 2020).

The relative abundances of microbial taxa (OTUs) within a treatment group were modeled using the CNVRG algorithm (58). This model uses a Bayesian approach to estimate proportional relative abundances using the Dirichlet and multinomial distributions. Multinomial parameters are estimated for each sample and correspond to the proportional abundance of a taxon in a sample. The model is hierarchical and uses the Dirichlet distribution as a prior for this multinomial. The parameters of the Dirichlet distribution describe the proportional abundance of a taxon within a sampling group. This approach allows for a very sensitive comparison of taxon relative abundances across groups through accurate proportional abundance determination. Multinomial parameters can be used as a taxon by sample table of proportions for downstream analyses.

*Statistical analysis*

While soil samples collected from within the same treatment plot could be considered pseudoreplication and would thus violate the assumption of independent observations, we argue that pseudoreplication is scale dependent, and the scale that microbes operate on would allow us to assume independence among samples collected from the same treatment plot. With aboveground vegetation acting as a driver of belowground function and vegetation being heterogeneous meter to meter within a single treatment plot, we decided to treat each composite soil sample as an independent sample instead of averaging samples within a plot and losing the ability to assess variation within a treatment plot. All statistical analyses were carried out in R V4.1.1 - “Kick Things” (59). For all statistical comparisons, we report statistical significance at α = 0.05 and marginal significance at α = 0.1.

*Edaphic, enzymatic, and vegetation statistical analyses*

Edaphic and enzymatic measurements were analyzed using ANCOVA. Our dataset was subset into either: 1) individual time points containing all five weed removal treatment types or 2) individual weed removal treatments that contained all sampling three time points. In both cases, total weedy vegetation was included as a covariate. The first set of statistical models was used to compare the effects of weed removal treatment within a single time point (Y ~ weed removal treatment + total weedy vegetation, data = time point containing all five treatment types). The second set of models was used to examine changes over time within a single weed removal treatment (Y ~ time + total weedy vegetation, data = individual treatment type with all three time points). Pairwise differences in edaphic parameters among time points and treatment types were assessed using Tukey’s HSD. In addition, we utilized a conservative mixed modeling approach to examine the effect of time, weed removal treatment, and total weedy vegetation on enzyme activity simultaneously using linear mixed models, with fixed effects of weed removal treatment, pre-treatment enzyme activity (to account for initial differences), total weedy vegetation, and a time\*weed removal treatment interaction. The random effect in our model was limited to sampling time to account for temporal non-independence (Y ~ pre-treatment enzyme activity + treatment + time + total weedy vegetation + treatment\*time + (1|time), data = all data points from time two and three).

Multivariate response to weed removal treatments and time since treatment was assessed using PerMANOVA testing implemented using the Adonis function in the vegan package (60, 61). Non-metric multidimensional scaling (NMDS) was utilized to visualize differences in total enzymatic profiles (i.e., Bray-Curtis dissimilarities of all enzymes). Mantel testing was implemented to examine correlations in the distance matrices of edaphic conditions and enzymatic activities.

Differences in the cover of total weedy vegetation, lamb’s quarter, and redroot pigweed across herbicide treatments within a single time point were assessed individually using ANCOVA. These measurements were collected as cover classes and converted to a continuous covariate. Thus, we do not report percent change or means following treatment, only the directionality of change.

*Microbiome statistical analyses*

Differences in microbial alpha-diversity (α) across herbicide types were assessed using the rarefied taxon within each sampling time point. A significant effect was noted when the herbicide-treated plots diverged from either of the controls. The effect of herbicide on community dissimilarity was assessed using both rarefied taxon count tables and the Dirichlet multinomial (DMN) modeled point estimates generated by the CNVRG package (58). Significant effects of herbicide treatment were determined by PERMANOVA testing using beta\_dispr() and adonis() functions in the vegan package (60). Total weedy vegetation was included in these models (Y ~ Herbicide \* Time + total weedy vegetation, permutations = 1,000). Heterogeneity in microbiome composition at each sampling was assessed using Bray-Curtis dissimilarities and visualized with boxplots of pairwise dissimilarities to all other members of that treatment group. Significant differences in multivariate dispersion were assessed using beta\_dispr(). All chemical treatments were analyzed separately and then again when combined as a single treatment group, herein referred to as “chemical herbicide”. Shifts in community composition were visualized using NMDS implemented in the vegan and ggordiplots packages. Those taxa accounting for variation among treatments were identified via the Boruta classification algorithm (62) and simper (60). The response of these taxa was then modeled using Hellinger-transformed relative abundances and zero-inflated beta regression with time, weed removal treatment, and total weedy vegetation as fixed effects. The top 25 most abundant families were identified and shifts in their abundance over time were identified using ANOVA with sampling time as the fixed effect.

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**Tables**

Table 1. Weed removal treatment name, chemical agent, modes of action, and application rates used in experimental design.

|  |  |  |  |
| --- | --- | --- | --- |
| **Weed removal treatment – Commercial formulation** | **Chemical agent** | **Mode of action** | **Application rate** |
| Clarity ® | dicamba | Auxin mimic | 584.6 mL/Ha |
| Aatrex ® and Calisto ® | atrazine and mesotrione tank mix | Photosystem II and carotenoid pigment synthesis inhibitor | 1169.25 mL/Ha and 219.25 mL/Ha |
| RoundUp Powermax ® | glyphosate | EPSPS synthase inhibitor | 2338.49 mL/Ha |
| Handweeded | NA | Mechanical removal | NA |
| Non-treated | NA | NA | NA |

**Figures**



Figure 1. Enzyme activities of the a) handweeded and b) glyphosate treatment plots over three sampling times. Enzyme activities were significant as per global models for AG, BG, BX, the ratio of C:N cycling enzymes, and PHOS for the handweeded plots. Only PHOS activity was significant in the glyphosate plots, but the same trend was observed for many enzymes in both treatment types. Thick middle lines in boxes of box and whisker plots represent the median, with the top and bottom of each box representing the 75th and 25th quartiles, respectively. Whiskers represent 1.5 x IQR. Within each panel, letters indicate significant pairwise differences between sampling time points at α = 0.05. No letters indicate a lack of significant pairwise differences.

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Figure 2. Column 1: 16S ordination of rarefied count tables with 95% SE ellipses. Points indicate independent samples and are colored by treatment type. Column 2: Sample pairwise dissimilarity within treatment types. Letters indicate significance within a single panel (i.e., differences among treatment types). Symbols indicate differences among time points for a single treatment type. For example, in column 2, atrazine-mesotrione treated plots differed at sampling times one and three, as indicated by \* vs. +. Column 3: Sample pairwise dissimilarity when all chemical treatments were grouped as a single chemical herbicide treatment. Letters indicate significance within a single panel (i.e., differences among treatment types). Symbols indicate differences among time points for a single treatment type. For example, in column 3, the chemical-treated plots differed at sampling times one and three, as indicated by \* vs. +.



Figure 3. Column 1: ITS ordination of rarefied count tables with 95% SE ellipses. Points indicate independent samples and are colored by treatment type. Column 2: Sample pairwise dissimilarity within treatment types. No significant pairwise differences were detected across treatments within a time point or across time within in single treatment. Column 3: Sample pairwise dissimilarity when all chemical treatments were grouped as a single chemical herbicide treatment. No significant pairwise differences were detected across treatments within a time point or across time within in single treatment.