Abstract

Patterns of herbicide use have changed dramatically over the past 25 years. While great consideration is often given to shifts in aboveground diversity, the belowground component of ecosystems has been largely neglected. Previous research has revealed a mixed response of edaphic parameters, microbial diversity and function to herbicide application and has spurred calls for further research as well as disentanglement of the direct and indirect effects of herbicide application. With microbes being largely responsible for nutrient cycling and other pivotal ecosystem processes, understanding the direction and magnitude of their responses to vegetation removal in agro-ecosystems is of the utmost concern. In this study, we compare three chemical herbicides: Roundup (glyphosate), Clarity (dicamba), and a tank mix of Aatrex and Calisto (atrazine and mesotrione), to handweeded and nontreated control plots to understand how herbicide application may affect soil diversity and function over twenty days post-treatment in *Zea mays* fields. Our results show that the examined herbicides have limited and short-lasting effects on soil edaphic properties and microbial function measured by extracellular enzyme analysis. Furthermore, weedy vegetation was shown to be a significant predictor of enzymatic activities, nutrient concentrations, and fungal community structure, suggesting that the effects of herbicide application may instead be mediated via indirect pathways. Additionally, we report weed removal treatment to be significant for bacterial assemblages and show a homogenization of the bacterial compartment following chemical treatment.

Introduction

In 1996, genetically modified herbicide resistant crops were introduced to the United States, prompting a shift in herbicide use patterns. Driven mainly by the use of glyphosate (commonly known as Roundup; ﻿N-(phosphonomethyl)glycine), herbicide application increased by 239 million kilograms from 1996 to 2011 in the United States alone (1). In the past, growers were concerned, and rightfully so, with a single parameter: crop yield. However, the birth of the soil health movement has prompted a new way of thinking about production agriculture with a newfound focus on long-term yield and a holo-ecosystem approach to farming. With this new school of thought, researchers and producers alike are realizing the importance of understanding the 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 may also affect microbial community structure and function (2, 4–7). Microbes provide countless benefits in agricultural systems, including: plant growth promotion (8), pathogen suppression (9), nutrient cycling (10–13), as well as aggregate formation and maintenance of soil structure, contributing an estimated $330 to $500 per ha/yr in ecosystem services (14). Due to the fact that microbes are such important members of agroecosystems, understanding the multifaceted microbial response to herbicide application is necessary in order to optimize agricultural systems for yield and sustainability. Difficulties in elucidating the effects of herbicide application on microbial populations have been demonstrated in reviews of the topic (5, 15–18). This is underscored by the fact that herbicides are a very broad group of chemicals, and their effects on microbes may differ depending on soil (5) and plant characteristics (19) as well as rate of application (5) and mode of action (2). Furthermore, herbicides have the potential to indirectly affect the soil microbiome through changes to vegetation and subsequent root exudation (20), making it even more difficult to understand how microbial systems might respond.

Studying the effects of herbicide on soil properties and processes is not a new endeavor, with research dating as far back as the 1960’s (4). Until recently, the methods used to examine the effects of herbicide on soil microbes, like denaturing gradient gel electrophoresis (DGGE) or soil respiration studies, have limited the ability of researchers to uncover fine scale changes in microbiome structure or function following application, and this resulted in the general consensus that herbicides have limited to no effect on soil microbial assemblages (16). However, over the past ten years, the use of modern molecular tools including high throughput sequencing (HTS), metagenomics and even enzyme assays have afforded researchers a new level of detail when studying the response of soil communities to agricultural amendments. Despite the new level of detail, results from studies examining the effects of herbicide on the soil microbiome remain mixed and only highlight the need for further research (16, 21–24) .

To better understand how herbicide application influences microbial function and diversity in Wyoming, we compared the effects of three different herbicides along with handweeded and nontreated controls on the structure and function of the soil microbiome in *Zea mays* over twenty days post-application. Each of the three examined herbicides act on a different mode of action (Table 1), and as such, we hypothesized that the effects of each herbicide on microbial diversity and function would differ (2). Specifically, we hypothesized the Aatrex and Calisto tank mix would affect nitrogen cycling microbes, as other members of this family of herbicides have been demonstrated to do so (25). As such, we hypothesized that this herbicide combination may affect enzymatic activates as it contains organic N in the chemical structure of the herbicide. Roundup Powermax and Clarity are also likely to affect the structure and function of the soil microbial community as they inhibit amino acid synthesis and signaling pathways that are present in both plants and microbes. Furthermore, Roundup and other glyphosate-based herbicides contain organic phosphorus and may affect phosphorus mineralization rates following application. Inclusion of handweeded checks allowed for comparison of the effects of herbicide application to mechanical weed removal. With this experimental design we are able to examine whether shifts in microbial function and diversity are the direct result of herbicide application or whether changes are indirectly mediated through vegetation removal (26).

Methods

*Site description and experimental design*

In the summer of 2018, 20 experimental plots at the Sustainable Agriculture Research and Extension Center (SAREC) in Lingle, WY (MAT 8.4° C) were planted with *Zea mays* variety ‘P9188AMX’ at 34,000 seeds per acre. Plots were fertilized using a dry fertilizer prior to corn planting according to best practices (40 rock fertilizer- 125Nitrogen-35Phosphorus-0Potassium-35Elemental Sulfur-13Sulfate-2Zinc blend -J.R. Simplot Company, Boise, ID). Plots were irrigated every several days using lateral pivot irrigation according to best practices. 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) (27) 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 control (nontreated), handweeded plots, and three different chemical herbicide applications. The three chemical herbicides used in the study were Roundup PowerMax (glyphosate, group 9), Aatrex and Callisto (atrazine and mesotrione tank mix, group 5 and 27 respectively, herein referred to as Aatrex), and Clarity (dicamba, group 4). Herbicides were applied at the following recommended field rates: Roundup PowerMax 2338.5 mL/Ha, Aatrex 1169.25 mL/Ha, Callisto 219.23 mL/Ha, and Clarity 584.62 mL/Ha (Table 1). In the handweeded plots, above ground vegetation was cut using a hoe and left in the plot on the soil surface. Belowground biomass was not disturbed and remained in the soil. A pre-treatment sampling was conducted in early June, on the morning of herbicide application and hand weeding. Following herbicide application and hand weeding, two post-treatment samplings were conducted. The first was 10-days post-treatment and the second at 20-days post-treatment.

*Sampling and analysis*

To facilitate sampling, a grid was laid out over each plot and coordinates of sample location were determined using a random number generator. The corn stalk closest to the coordinate was selected, and a 0.25 m2 Daubenmire plot was placed so that stalk was in the center. Cover classes of the main weeds including, redroot pigweed (*Amaranthus retroflexus*), Nightshade (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.

Per treatment plot, 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 processed within 24 hours. Soil samples were first thoroughly mixed in the Whirl-Pak bag used for collection and then sieved though 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) prior to analysis and filtered through a 0.45 μm hydrophilic filter as per (28). Anions were ran 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 ran 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 (29–33). 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) (34). 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). In order to minimize difference between buffer and soil pH, we used a buffer pH 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 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-amido-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 (32) 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 in a similar manner as 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 sample at each time point and 60 samples across the three time points.

*Soil microbiome analysis*

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

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. In order to amplify the V4 region of the bacterial 16S rRNA gene the modified 515F (5’-GTGYCAGCMGCCGC GGTAA-3’) (35) and 806R (5’-GGACTACNVGGGTWTCTAAT-3’) (36) were used. For the ITS1 region of the fungal genome, the primers ITS1F (5'-CTTGGTCATTTAGAGGAAGTAA-3') (37) and ITS2 (3'-CGTAGCTACTTCTTGCGTCG-5') (38) were used. Environmental DNA (eDNA) was normalized to 10 ng/μL prior to 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 Illumnia 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 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 consist of 10 μL of cleaned PCR product from the first step and 5 μL of FlowCell mastermix 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 the Axygen’s AxyPrep Mag PCR Clean-up Kit. Following cleaning, sample concentration was checked using a Synergy HTX Take 3 trio plate reader. Samples 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*

*Statistical analysis*

While soil samples collected from within the same treatment plot could be considered pseudoreplication and would 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 that were collected from the same treatment plot. With aboveground vegetation being 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” (39). For all statistical comparisons, we report statistical significance at α=0.05 and marginal significance at α=0.01.

*Edaphic, enzymatic and vegetation statistical analyses*

Edaphic parameters were examined using multiple model structures. To accomplish this, the entire 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, ANCOVA was used for our statistical analyses. As weedy vegetation is a driver of belowground exudation and resource competition, it is likely to contribute to the concentration of nutrients and enzyme activities. As such, it was collected as a covariate and included in our models. 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 order to examine changes in extracellular enzyme activities, the same subsets and model structures were used as were implemented with the edaphic parameters. 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).

For multivariate responses to weed removal treatment or time since treatment, PerMANOVA testing (Adonis) was utilized to determine statistical differences among total enzymatic profiles (40, 41), using Bray-Curtis dissimilarity. Non-metric multidimensional scaling was utilized to visualize differences in total enzymatic profiles. 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 with similar statistical models as were used for comparisons of enzymatic and edaphic properties. As these measurements were collected as cover classes and converted to a continuous covariate, we do not report percent change or means following treatment, only directionality of change.

*Microbiome statistical analyses*

Differences in microbial alpha-diversity (α) between herbicide types were assessed using ANVOA and rarefied taxon counts 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 modeled point estimates generated by the CNVRG package. Significant effects of herbicide treatment were determined by PERMANOVA testing using beta\_dispr() and adonis() functions in the vegan package (40). Again, total weedy vegetation was included in these models (Y ~ Herbicide \* Time + total weedy vegetation, permutations = 1,000). Heterogeneity in microbiome composition was assessed using Bray-Curtis dissimilarities and visualized with boxplots of pairwise dissimilarities to all other members of that treatment group at that time point. All chemical treatments were analyzed separately and then again when combined as single treatment group (e.g., chemical herbicide). Shifts in community composition were visualized using NMDS, implemented in the vegan and ggordiplots packages. Shifts in the abundance of dominant taxa within a treatment across the three sampling points, determined by total abundance, were assessed using the rarefied taxon count tables.

Results

*Vegetation responses*

As was expected, herbicide application controlled total weedy vegetation at 10-days (time two) and 20-days (time three) post-treatment (p < 0.05). When the cover of weedy species was examined individually, only lamb’s quarter and pigweed had sufficient cover to estimate differences among time points. Like total weedy cover, herbicide application reduced lamb’s quarter and redroot pigweed cover at 10- and 20-days post-application when compared to the nontreated plots. At 10-days post-treatment, the handweeded controls also showed a significant drop in total weedy vegetation, lamb’s quarter, and redroot pigweed cover as compared to the nontreated plots (p < 0.05). However, the observed decreases in cover disappeared at 20-days post-treatment, and weedy vegetation levels were no longer different from the nontreated plots as compared to the nontreated plots.

*Edaphic properties*

Examination of edaphic parameters within in a single time point revealed no significant differences pre-treatment (time one) among the weed removal treatments when the effect of total weedy vegetation was accounted for (Table 2 and Supplementary Tables 1a and 1b). At 10-days (time two) and 20-days (time three) post-treatment, nitrate and total inorganic nitrogen (N) concentration were significantly different among treatment types (p < 0.05) (Table 2), with weed removal treatment being a significant predictor at both time points but total weedy vegetation being significant only for sampling time two. Plots treated with RoundUp Powermax had higher levels of nitrate and total inorganic N than the handweeded plots at time point two and three (p < 0.05). At time three, Roundup treated plots also had significantly higher nitrate concentrations as compared to the nontreated and Aatrex treated plots (p < 0.05). We observed the same trend at sampling time two, but the differences were only marginally significant (p < 0.09).

When examining how our soil variables changed over time within a single weed removal treatment, multiple differences were observed. Significant models include nitrate, nitrite, ammonium, total inorganic N, phosphate, calcium, magnesium, pH, EC, and gravimetric moisture content (p < 0.05) (Table 3 and Supplementary Tables 2a and 2b). Pairwise contrasts showed time one to have the highest levels of nitrate and total inorganic N across all treatments (p < 0.05). This was also true for the concentration of magnesium and calcium, with a trend of decreasing concentrations over the 20-day sampling period. Total weedy vegetation was only a significant predictor of nitrate concentration in the handweeded plots (p < 0.05). Moisture content was also significantly different across time in all treatment types including controls (p < 0.05) and showed the opposite trend of many nutrients, with the later sampling times having higher moisture content in all cases.

Across all treatment types 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 % cover and phosphate concentration (p < 0.001, r = -0.25).

*Extracellular enzymes*

We report the response of extracellular enzyme activity to weed removal treatment to be limited as well as enzyme and sampling time specific. There were 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-days post-treatment sample times (p < 0.05, F1,14 > 4.411, R2 > 0.195). With respect to individual enzymes, only alkaline phosphatase (PHOS), ß-glucosidase (BG), and the ratio of N:P cycling enzymes produced significant differences among herbicide treatments (p < 0.05) (Table 4, and Supplementary Tables 3a and 3b). Significant pairwise comparisons at the pre-treatment sampling were limited to PHOS activity and the ratio of N:P cycling enzymes, with the nontreated plots having higher initial PHOS activity as compared to the handweeded plots, and lower N:P ratio as compared to the handweeded and Aatrex treated plots. 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-significant trend being observed for the Roundup treated plots (Figure 1). While herbicide treatment was not a significant predictor of activity for many enzymes, 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 the examining the effect of time within in a single weed removal treatment, significant differences in enzyme activities over the 20-day sampling period were limited to the handweeded and RoundUp treated plots. The Aatrex, Clarity, and nontreated plots showed no significant differences in individual enzyme activities or overall enzymatic profiles over time (p > 0.05). 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). Pairwise comparisons revealed time one to be significantly different from time two (p < 0.05) and marginally significant from time three (p = 0.055). Phosphatase activity was the most variable of the measured enzyme activities over time, showing a statistically significant increase in activity at 10-days after treatment followed by a reduction 20-days after treatment for both the handweeded and Roundup Powermax plots (p < 0.05). No correlation was found between phosphate ion concentration and PHOS activity when considering all plots (p > 0.9, r = 0.011). In addition to PHOS activity, both the handweeded and Roundup treated plots had similar trends over time for BG, AG, BX, and the ratio of C:N cycling enzymes, with the lowest activity at time one, the highest at time two and a return to pretreatment levels at sampling time three (Figure 1). However, this was only statistically significant for the handweeded plots (p < 0.05) (Table 5 and Supplementary Tables 4a and 4b).

Our conservative linear mixed models of the entire dataset revealed enzyme specific results with respect to the significance of weed removal treatment, initial enzyme activity, total weedy vegetation, and the interaction of 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 higher enzyme activity. Both BG and LAP were also predicted by the interaction of treatment and time (p < 0.05), though this was only marginally significant for BG (p = 0.055). PHOS activity at time one was deemed a significant predictor of PHOS activity at time 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 5).

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).

*Bacterial diversity*

Bacterial count tables were rarefied to 3,000 reads per sample and resulted in 164 independent samples being included in downstream analysis, after outlier removal; rarefied results 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). PERMANOVA testing of the rarefied and DMN taxon tables revealed a significant effect of weed removal treatment (p < 0.05, F4,147 = 1.101, R2 = 0.0268), and a significant effect of the interaction of weed removal 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), and significant 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. Pairwise PERMANOVA testing showed the Clarity and handweeded plots to be significantly different at all three time points (p < 0.05). Additionally, the handweeded and Aatrex treated plots showed different community structure at time points one and three (p < 0.05). Comparison of pairwise Bray-Curtis dissimilarity among treatment replicates revealed different trends among treatment types. Pre-treatment, the handweeded plots displayed a more homogenous bacterial assemblage as compared to the plots that would receive chemical herbicide treatment (p < 0.01). However, at 20-days post application, Roundup and Aatrex treated plots showed a homogenization of their bacterial community, while the handweeded and nontreated plots showed an increase in heterogeneity (Figure 2 – column 2). Additionally, when all chemical herbicide treatments were grouped together and pairwise dissimilarities recalculated, chemical herbicide treated plots, despite a high level of initial dissimilarity, converged and displayed lower heterogeneity as compared to either of the control treatments (Figure 2 – column 3). Changes in the abundance of dominant taxa over time were detected in all treatment types, with Shpingomonadacae showing a statistically significant increase in abundance in all chemical herbicide treated plots (p < 0.001, F2,30 = 9.469) (Supplementary figure XXX).

*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; rarefied results 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, the global model showed Shannon diversity (H`) to be 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 a 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). Additionally, no clear trends in dissimilarity 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 Roundup Powermax treated plots over the three sampling times (p < 0.001, F2,30 = 9.469) (Supplementary figure X).

Discussion

Our examination of soil properties and microbial function following weed removal treatment revealed few statistically significant differences over 20-days post-treatment. Our results suggest soil function in traditionally managed agroecosystems is largely resistant and resilient to herbicide application. Factor affecting bacterial and fungal diversity differed, with bacteria showing a significant effect of weed removal treatment and fungi showing a response to total weedy vegetation as well as a temporal effect. We show that the examined edaphic parameters and most enzyme activities, other than PHOS, were not different among treatments at sampling time one (pre-treatment), suggesting that our initial conditions were similar across plots and that any observed differences at sampling times two and three are a result of herbicide application and/or changes in weedy vegetation, with the latter finding more support. With this, it appears that the act of killing vegetation, instead of the direct effects of herbicide (19), may be the primary driver of microbiome shifts in agroecosystems. Additionally, convergence of dissimilarity in bacterial assemblages following chemical herbicide applications suggests that, regardless of herbicide type, chemical intervention homogenizes the bacterial compartment in soil and results in a single “chemically disturbed” assemblage type, contrary to our initial hypothesis.

By splitting our enzymatic dataset by weed removal treatment, we examined the effect of time since treatment on edaphic parameters and found changes in the concentrations of nutrients like ammonium, phosphate, nitrate, magnesium and calcium. However, these shifts were correlated with total weedy vegetation cover. We hypothesize that competition for nutrients, among plants and microbes, is driving the observed decreases as opposed to a direct effect of herbicide. As the trend of increased extractable phosphate was observed in all treated plots (i.e., nontreated controls excluded), we suggest that the effects of herbicide treatment are indirectly mediated through increased litter inputs, instead of a sign of organophosphate degradation, as would be the case in the Roundup Powermax treated plots (42). For example, during initial stages of decomposition, soluble organic carbon can be carried by moisture pulses from irrigation into the soil profile and would feed microbial activity, potentially explaining the increase in activity we observed at sampling time two (43).

Previous work has examined the effects of glyphosate on soil N and showed no difference in inorganic N between vegetation removal plots (e.g., handweeded and Roundup) over twenty weeks (26). The authors did however report significant differences between the handweeded plots and the 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. Conversely, our results showed nitrate levels to be lowest in our handweeded and nontreated plots, with concentrations being higher in our Roundup plots. While our results differ from Hagner et al. (26), we show that nitrate concentrations were significantly correlated with vegetation cover, and at 20-days post-treatment, the handweeded plots experienced an increase in weedy vegetation cover and *Zea mays*. Given the lack of difference between their handweeded and Roundup treated plots and the observed correlations between nitrate concentration and vegetation cover in our experiment, we again suggest that differences in inorganic nitrogen between glyphosate, handweeded, and nontreated plots are indirectly mediated through changes in vegetation instead of a direct effect of herbicide application (19, 20, 44). Experimental designs like ours and the one implemented by Hagner et al. (26) allow for direct and indirect effects of herbicide application and vegetation removal to be accounted for when considering the implications of chemical herbicide application.

Differences in N-cycling under Aatrex and other atrazine based herbicides treatment over time have also been observed in previous work, with a general trend of short term reduction in the rate of N mineralization following application (45). Accordingly, we report a decrease in the inorganic N pool over the twenty days post-application in the Aatrex treated plots, providing support for our hypothesis and further evidence that this herbicide has short term effects on N-cycling. However, the trend of decreased inorganic N was observed in other treatments as well, and together with the observed correlations with total weedy vegetation, it is difficult to tell whether this effect is direct or indirect as the crop, Zea mays, and other weedy vegetation may also be partially responsible for a reduction in inorganic N via uptake and immobilization in plant biomass. Other similar herbicides, e.g., members of the ﻿triazine family, have also been shown to inhibit nitrification and decrease the abundance of specific ammonia oxidizers, leading to short term alterations to N-cycling (25).

As for differences in soil function based on extracellular enzymatic activities, we showed that there was no difference in total enzymatic profile between treatments at sampling time one, indicating that our plots were relatively homogeneous prior to treatment. At ten days post-treatment, weed removal treatment did not affect enzymatic profiles, but total weedy vegetation did, again providing support to the idea that the effects of herbicide application on soil function may be indirectly mediated through changes in vegetation (19, 20). This was further supported by the significance of total weedy vegetation as a predictor of fungal community composition and lack of significance for weed removal treatment. As plant diversity has been shown to influence microbial diversity, albeit the effect on bacteria and fungi is not equal, with a larger effect of vegetation being reported for fungal communities (46), it is not surprising that total weedy vegetation influences microbial enzymatic profile (47). This result, 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.

When examining changes in enzyme activities over the 20-days post-treatment within a single treatment type, we found phosphatase (PHOS) activity to be significantly different within the Roundup Powermax and handweeded plots. In these treatments, PHOS activity displayed a humped pattern, with an increase in enzyme activity at day 10 and a drop at day 20. It is possible that the increase in anthropogenic soil P and chemical by-products of microbial degradation of glyphosate (48) led to the decreased activity observed in the Roundup Powermax treated plots between sampling times two and three. As extracellular enzymes are costly to produce for microbes, it could be hypothesized that the production and secretion of alkaline phosphatase might increase following the addition of an organophosphorus compound like Roundup (glyphosate) but decrease once there is sufficient P in the soil (49), as was shown with the increasing trend of soil phosphate over the 20-day period. However, given the lack of correlation between phosphate concentration and PHOS activity and observing the same patterns in the handweeded plots, we hypothesize forces besides herbicide application may be responsible (e.g., redroot pigweed cover, that was shown to be negatively correlated with phosphate concentration).

Though not following the same humped pattern that we observed, others have found the response of PHOS to glyphosate based herbicides in clay-loam soils to be related to time since application, with alkaline phosphatase being more sensitive (50) than other members of the phosphatase enzyme family. Further, Cherni et al. (51) used a controlled mesocosm experiment to examine the effect of Roundup on phosphatase activity and showed no differences between the recommended field rate group (1 L/HA) and controls and only a mild increase in activity in the 10X field rate application (10 L/HA), suggesting that the application of glyphosate based herbicide at rates of up to 10X field rate has limited effects on PHOS activity. As our application rate was only slightly above the 1X and well below the 10X rates used by Cherni et al. (51) it is not surprising that the effects of Roundup Powermax on PHOS activity were minimal and short lived. Others have also shown a high glyphosate dose requirement in order to observe significant effects on microbial activity (52–54), and hypothesize that at these high rates, increased microbial activity following herbicide application is a result of detrimental effects on lithotrophic microbes (44). If true, this could shift the competitive balance, with heterotrophic microbes being favored. Observed shifts in the abundance of Shpingomondacae in all chemical herbicide treated plots support this hypothesis and show an increase in this family of putative herbicide degrader following chemical herbicide application (55, 56). However, as we observed increased activity in several extracellular enzymes, both in the handweeded and Roundup (glyphosate) treated plots, it is possible that an increase in available above- and belowground plant detritus could explain the shifts in enzymatic activity as well. With the observed hump shape in enzyme activities and a return to pre-treatment levels, it could be hypothesized that labile litter inputs would stimulate decomposition, but 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 similar results shown by Damin and Triveli (44).

Based on our conservative mixed models, four enzymes or enzyme ratios produced significant models, however, each model had different significant predictors, suggesting that soil enzyme activity may respond to herbicide application and environmental ques in an enzyme specific fashion. In particular, the activity of BG, LAP and the ratio of C:P cycling enzymes were shown to be predicted by total weedy vegetation. As both BG and LAP are produced by both plants as well as microbes, it is not surprising that their activities are predicted by total weedy vegetation cover (57–59). It is also possible that the mechanical disruption of soil during the enzyme assay prep could have also led to intracellular enzymes, contained in plant material, to be released into the soil slurry (60). 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 PHOS activity. Thus, our ability to say whether PHOS activity was affected by weed removal treatment is limited.

Unintended effects of herbicide application, whether that be non-target effects on local plant communities or shifts in pathogen abundance are a cause for concern and underscore the need for a systems-based approach to production agriculture. While our results suggest most effects from herbicide application to be indirectly mediated and relatively short lived, we also report increased abundance of the fungal family Ceratobasidiaceae under specific herbicide treatments. Members of Ceratobasidiaceae, like *Rhizoctoina,* are plant pathogens responsible for costly diseases in both crops and turf grass (61). Previous research has shown application of glyphosate-based herbicides to increase disease severity of rhizoctonia on sugar beet in glasshouse settings (62), though conflicting results exist and show a cultivar specific response (63). However, given that we did not measure lesion formation, we cannot say whether the statistically significant increase in Ceratobasidiaceae following application translated to a biologically meaningful increase in disease severity.

Together, our results suggests that herbicide application has limited direct effects on the soil microbiome and edaphic conditions over 20-days post-application. We suggest that the observed differences are most likely mediated through indirect effects caused by changes in vegetation cover. Our findings are not all that surprising due to the high degree of functional redundancy in the soil microbiome (64, 65) and the fact that we used a relatively low rate of herbicide in our applications. With respect to herbicide application in agricultural systems, it appears that direct effects on microbial function are few and short lasting and that in heavily managed, traditional agricultural systems, the choice of herbicide has little direct effect on soil function and microbial diversity.

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Tables

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

|  |  |  |  |
| --- | --- | --- | --- |
| **Weed removal treatment** | **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 |
| Handweed | NA | Mechanical removal | NA |
| Nontreated | NA | NA | NA |

Figures

Figure 1. Enzyme activities of the a) handweeded and b) Roundup Powermax treatment plots over three sampling times. Enzyme activities were significant as per global models for AG, BG, BX, PHOS, and the ratio of C:N cycling enzymes for the handweeded plots. Only PHOS activity was significant in the Roundup Powermax plots, but the same trend was observed for many enzymes in both treatment types. Thick middle lines in boxes of box and whisker represent the median, with 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 lack of significant pairwise differences.

Diagram

Description automatically generated

Figure 2. 16S ordination of rarefied count tables with 95% SE ellipses of mean. Dissimilarity within treatment types show a homogenization of herbicide treated plots through time (column 2 and 3). When all chemical treatments grouped, we observe a decrease in dissimilarity not observed in the handweeded or non-treated plots. More details on box plot structure etc.

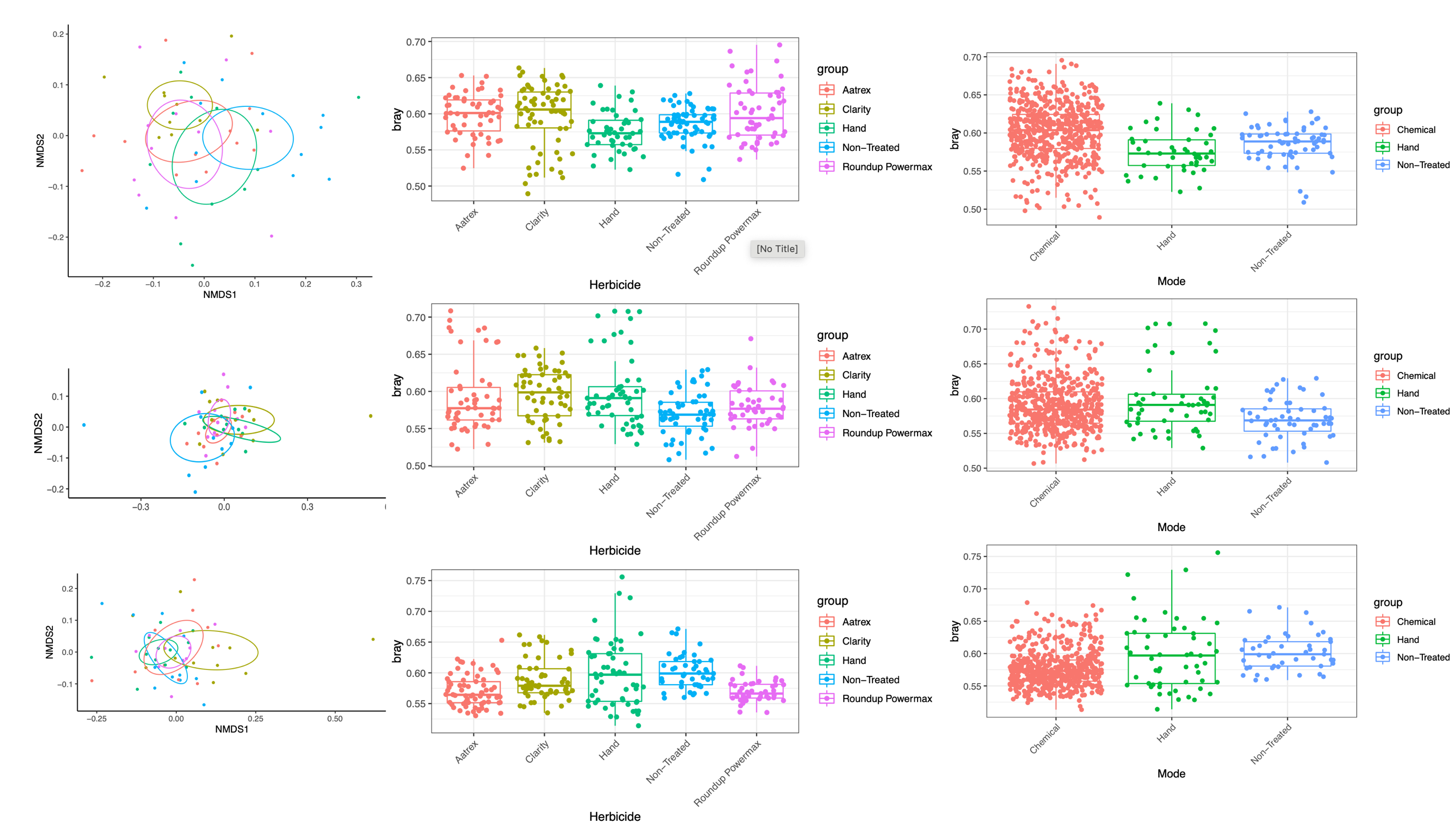


Figure 3. ITS ordination of rarefied count tables with 95% SE ellipses of mean. Dissimilarity within treatment types (column 2). When all chemical treatments grouped (column 3). More details on box plot structure etc.

Diagram

Description automatically generated