Title:

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Keywords:

Target: Scientific Reports or Applied and Environmental Microbiology

**Abstract – 200-word guideline**

Patterns of pesticide use have changed dramatically over the past 25 years, specifically since the adoption of glyphosate resistant crops. While consideration is often given to the effects of herbicide on aboveground diversity, our understanding of its influence belowground lags. 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. Previous research has revealed mixed responses in microbial diversity and function following herbicide application, spurring calls for further research to clarify direct and indirect effects. In this study, we examine 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 nontreated controls. We examine shifts in soil microbial function, soil nutrient pools, and microbial community composition in *Zea mays* fields over twenty-days post-treatment. We show the examined herbicides to have limited and short-lasting effects on soil edaphic properties and microbial function. Shifts in enzymatic activities, nutrient concentrations, and fungal community structure were predicted by weedy vegetation cover, but not weed removal treatment, suggesting that these responses may instead be indirectly mediated by herbicide application. However, we show that weed removal treatment was as significant predictor of bacterial community composition. Additionally, we report homogenization of bacterial community composition following chemical herbicide application, a trend not observed in the non-treated and handweeded controls. Our results suggest that chemical herbicide application may have limited effects on microbial diversity and ecosystem functions.

**Main Text – 4500-word guideline not including methods**

**Introduction**

Genetically modified herbicide-resistant crops were introduced to the United States in 1996, prompting a shift in herbicide use patterns. Primarily driven by the application of glyphosate (N-(phosphonomythel)glycine), herbicide application in the US increased by nearly 240 million kg from 1996 to 2011 (1). Classically, producers were concerned 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 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 may also affect the structure and function of the soil microbiome (2, 4–7). This unseen component of agroecosystems provides 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 maximum yield and sustainability.

Reviews and syntheses of the effects that herbicide application has microbiome structure and function reveal a lack of generalizable trends (5, 15–18) and instead show that the effects are dependent upon the characteristics of the soil, plant, application rate, and mode of action (2, 5, 19). Further complicating efforts to accurately model the influence of chemical intervention is the fact that herbicides have the potential to indirectly affect the soil microbiome through changes to vegetation and subsequent shifts in the patterns of root exudation (20). To better understand the short-term effects chemical herbicide application has on the soil microbiome, we compared three herbicides, with unique modes of action, alongside hand weeded and non-treated control plots, allowing for the direct and indirect effects of herbicide application to be assessed. With each chemical herbicide acting on a unique mode of action (Table 1), we hypothesized that each would elicit a unique response in microbial function and microbiome structure (2). Specifically, we hypothesized that the atrazine-mesotrione treated plots would affect nitrogen (N) cycling microbes (21) and result in significant shifts in N-pools and N-associated enzymes. Additionally, we expected glyphosate and dicamba containing herbicides to affect the soil microbiome through disruption of amino acid synthesis pathways present in both microbes and plants.

**Results (with subheadings)**

**Discussion (without subheadings)**

**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 prior to corn planting in accordance with 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 – 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) (22) 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 hand weeded control (herein referred to as hand weeded), 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 hand weeded 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 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 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 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 (23). 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 (24–28). 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) (29). 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 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 (27) 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.

*Microbiome library preparation*

Subsamples of the same soils used for edaphic measurements were extracted using PowerSoil extraction kits (MO BIO, Carlsbad, CA) according to 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. 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 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” (39). For all statistical comparisons, we report statistical significance at α = 0.05 and marginal significance at α = 0.1.

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**Acknowledgements** (optional)

**Author contributions** (names must be given as initials)

**Data availability statement** (mandatory)

**Additional Information** (including a Competing Interests Statement)

**Figure legends** (these are limited to 350 words per figure)

**Tables**

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

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| --- | --- | --- | --- |
| **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 |