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Changes in rhizosphere bacterial gene expression following glyphosate treatment

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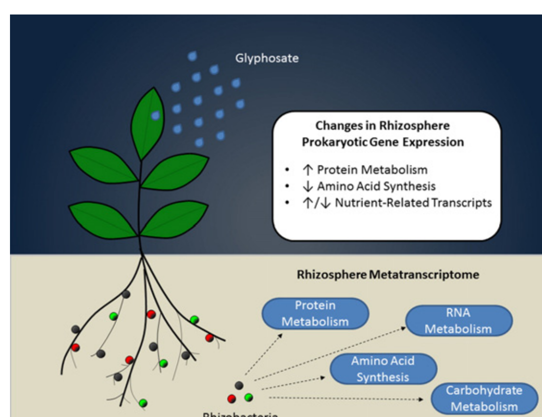
HIGHLIGHTS

- Response of rhizosphere prokaryotic metatranscriptome to glyphosate is examined.
- The metatranscriptome was dominated by RNA and carbohydrate metabolism transcripts.
- Glyphosate increased protein metabolism and decreased amino acid synthesis.
- Central carbon metabolism by bacteria was downregulated under glyphosate exposure.
- Glyphosate affects rhizosphere microbial community composition and activities.

GRAPHICAL ABSTRACT



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ABSTRACT

In commercial agriculture, populations and interactions of rhizosphere microflora are potentially affected by the use of specific agrichemicals, possibly by affecting gene expression in these organisms. To investigate this, we examined changes in bacterial gene expression within the rhizosphere of glyphosate-tolerant corn (*Zea mays*) and soybean (*Glycine max*) in response to long-term glyphosate (PowerMAX™, Monsanto Company, MO, USA) treatment. A long-term glyphosate application study was carried out using rhizoboxes under greenhouse conditions with soil previously having no history of glyphosate exposure. Rhizosphere soil was collected from the rhizoboxes after four growing periods. Soil microbial community composition was analyzed using microbial phospholipid fatty acid (PLFA) analysis. Total RNA was extracted from rhizosphere soil, and samples were analyzed using RNA-Seq analysis. A total of 20–28 million bacterial sequences were obtained for each sample. Transcript abundance was compared between control and glyphosate-treated samples using edgeR. Overall rhizosphere bacterial metatranscriptomes were dominated by transcripts related to RNA and carbohydrate metabolism. We identified 67 differentially expressed bacterial transcripts from the rhizosphere. Transcripts downregulated following glyphosate treatment involved carbohydrate and amino acid metabolism, and upregulated

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transcripts involved protein metabolism and respiration. Additionally, bacterial transcripts involving nutrients, including iron, nitrogen, phosphorus, and potassium, were also affected by long-term glyphosate application. Overall, most bacterial and all fungal PLFA biomarkers decreased after glyphosate treatment compared to the control. These results demonstrate that long-term glyphosate use can affect rhizosphere bacterial activities and potentially shift bacterial community composition favoring more glyphosate-tolerant bacteria.

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1. Introduction

Crop protection chemicals, such as pesticides, are used worldwide to protect agricultural crops from suffering dramatic losses due to pests and therefore increase crop yield and quality and optimize economic returns (Imfeld and Vuilleumier, 2012). Recent forecasts predict the global market volume for pesticides will increase to 3.2 million tons by 2019, with a market value of $75.9 billion (Mordor Intelligence, May, 2014). Herbicides constitute an average of 49% of the total amount of pesticides applied globally (Grube et al., 2011).

Glyphosate is the most widely used herbicide worldwide and is projected to reach 1.35 million metric tons used by 2017 (Global Industry Analysts, 10 October, 2011). Glyphosate is a broad-spectrum, foliar-applied herbicide that has been widely used for decades in agriculture to control weeds pre-planting and post-emergence in tolerant crops (Araújo et al., 2003; Haney et al., 2002). Its mode of action is to inhibit the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) involved in the shikimate pathway (Duke and Powles, 2008). The inhibition of EPSPS leads to a buildup of shikimate and reduced synthesis of aromatic amino acids which are necessary for plant survival (Ahrens, 1994). Although, based on its physicochemical properties, glyphosate is not expected to have a drastic effect on soil microbial community function, results of studies investigating glyphosate impacts on soil microbial communities have been conflicting. Additionally, many of these studies are relatively short term and do not assess potential effects of long-term glyphosate application. Given these incongruous results, the unprecedented and widespread use of glyphosate over the past 40 years, and its predicted increase in use globally (Benbrook, 2016), studies are needed that examine the potential ecological effects of long-term glyphosate use on the soil microbial community.

The rhizosphere is the narrow zone of soil surrounding plant roots that is specifically influenced by plant root activities and/or is in association with root hairs and plant-produced materials (Walker et al., 2003). Rhizosphere soil contains a diverse microbial community, with bacteria being the most dominant members (Saharan and Nehra, 2011). Phospholipid fatty acid (PLFA) biomarkers have been used to assess the effects of pesticides on microbial community composition (Nye et al., 2014; Rosenbaum et al., 2014; Widenfalk et al., 2008). Although these studies provide insight into the effects of glyphosate on microbial community composition, they provide no information on its effects on microbial activity. In order to examine the effects of pesticides, such as glyphosate, on microbial activities both the structural and functional responses of the microbial community need to be assessed (Widenfalk et al., 2008; Zabaloy et al., 2008).

Functionally, bacteria within the rhizosphere can influence plant health in many ways. Many soilborne, bacterial plant pathogens can reside within the rhizosphere and have deleterious effects on plant growth and health. These effects are often a result of the production of metabolites or by nutrient competition and inhibition of beneficial rhizobacteria (Sturz and Christie, 2003). Conversely, rhizobacteria, such as plant growth-promoting rhizobacteria (PGPR), have been shown to exert a variety of beneficial effects on plants, including improved seed germination, seedling vigor, plant growth and development, biocontrol, and productivity (Mendes et al., 2013). Rhizobacteria are also involved in biogeochemical processes within the soil such as carbon, nitrogen, and phosphorus cycling. Given the impact of these microbial functions on overall plant health and subsequent crop yield, it is important to determine whether or not these functions are impaired as a result of the addition of pesticides such as glyphosate.

Several studies have noted variable effects when reviewing glyphosate impacts on soil microbial community function (Bünemann et al. (2006) and Duke et al. (2012)). Araújo et al. (2003) collected soil of two different types with and without a prior history of glyphosate exposure, applied 2.16 mg kg^{-1} of technical glyphosate, and measured microbial activity via respiration and FDA hydrolysis at 2, 4, 8, 16, 24, and 32 days after treatment. The soils with a history of glyphosate exposure (6 and 11 years) had a 10–15% higher increase in respiration when treated with glyphosate than soils with no glyphosate history. Microbial activity measured by FDA hydrolysis increased significantly (9–19%) with time and glyphosate addition. Zabaloy et al. (2008) applied glyphosate at a rate of 150 mg kg^{-1} to agricultural soil with a history of glyphosate exposure. Soil microcosms were sampled 3, 7, 14, and 21 days after treatment to measure respiration, FDA hydrolysis, and dehydrogenase activity. Although the response varied by soil type, a 42% and 28% increase in respiration was observed initially for the glyphosate-treated soils but then dissipated over time. Glyphosate had no discernable effect on FDA hydrolysis in one soil, but caused reduced FDA hydrolysis within the first two weeks after treatment in the other soil. And, dehydrogenase activity showed no consistent changes following glyphosate treatment.

Haney et al. (2000) found C and N mineralization increased in agricultural soils with increasing glyphosate application rates of 47, 94, 140, and 234 μg g^{-1} (Haney et al., 2000). Zobiole et al. (2011) observed only transient effects of glyphosate on dehydrogenase, β-glucosaminidase, β-glucosidase, and respiration following a single application of glyphosate at the rates of 800, 1200, and 2400 g a.e. ha^{-1}. Sannino and Gianfreda (2001) studied the effects of multiple pesticides at a mean application dose ranging from 40 to 200 mg kg^{-1} on invertase, urease, and phosphatase activity using several different soil types. In this study, glyphosate stimulated invertase and urease activity but showed up to a 98% reduction in phosphatase activity. These results indicate a variable response to glyphosate by the microbial community and highlight the impact of soil type, time, glyphosate history, and dosage rate on the microbial community's response to glyphosate.

All of the functions mentioned above are conventional, broad-scale measures of microbial activity. Although these measures provide useful information about glyphosate's effects on major indicators of microbial activity (e.g. respiration, nutrient cycling), these measures result in limited knowledge of bacterial functions possibly affected by glyphosate. Hence, there is a wide range of possible functional responses among rhizobacteria to glyphosate treatment that may be missed using these conventional methods (Imfeld and Vuilleumier, 2012; Jacobsen and Hjelmsø, 2014; Zabaloy et al., 2012).

Metatranscriptomic analyses, such as RNA-Seq, measure the expression of many genes and functions at once by detecting mRNA transcripts rather than focusing on a single functional response to a given treatment. Metatranscriptomics has been used in many instances to investigate microbial community functional diversity in various habitats, as well as to examine the microbial community response to a given environmental alteration (Damon et al., 2012; Goffredi et al., 2015; Mason et al., 2012; Qi et al., 2011; Shrestha et al., 2009; Urich et al., 2008; Zakrzewski et al., 2012). For instance, Urich et al. (2008) used a metatranscriptomic approach to simultaneously study the structural and functional diversity of a soil microbial community, and Shrestha

et al. (2009) applied an RNA-Seq approach to compare transcriptional activities between oxic and anoxic paddy soils. These studies demonstrate the feasibility and usefulness of the metatranscriptomic approach in studying soil microbial communities. In addition, Mason et al. (2012) examined the prokaryotic metatranscriptome from seawater to reveal the microbial functional response to the Deepwater Horizon oil spill, indicating that metatranscriptomes provide a sensitive measure of the microbial community response to a given treatment.

The objective of the present study was to investigate the long-term effects of glyphosate treatment on rhizosphere microbial community composition and bacterial gene expression. Specifically, PLFA analysis was used to detect broad-scale changes in rhizosphere microbial community composition following glyphosate exposure, and RNA-Seq was used to examine changes in bacterial gene expression in the rhizosphere of corn and soybean in response to repeated glyphosate application. Our overall hypothesis was that microbial community composition and bacterial gene expression in the rhizosphere of crops treated long-term with glyphosate would differ from untreated crops, specifically with respect to functions related to glyphosate utilization and resistance.

2. Methods

2.1. Greenhouse study

A detailed description of the rhizobox greenhouse study and sampling in this experiment can be found in Newman et al. (2016). A Blount silt loam (fine, illitic mesic Aeric Epiaqualf) soil was collected in 2-cm increments from soil pits at a farm undergoing organic management in Delaware County, OH to a depth of 39 cm (37 cm from the A horizon and the remaining 2 cm from the O horizon). This field site was previously under an alfalfa-orchard grass-corn, oats-alfalfa-orchard grass, spelt-timothy-clover, and timothy-clover rotation and had never been exposed to glyphosate. The soil was stored in sealed plastic bags, returned to the lab on ice, and placed in rhizoboxes, constructed as described by Bott et al. (2008), starting with the 38–39-cm increment, using ~62 g of soil per cm fill height. The soil was evenly distributed in each rhizobox and compacted to a bulk density of 1.3 g cm^{-3} with a total fresh soil weight of 2500 g per rhizobox. A total of eight rhizoboxes were planted with four rhizoboxes each of two crops, Roundup Ready corn (*Zea mays*; DeKalb hybrid seed brand DKC62-54 (VT3)) and soybean (*Glycine max*; OX 20-8 RR). Two rhizoboxes per crop were treated with glyphosate (PowerMAX™ Glyphosate, Monsanto Company, MO, USA; Active ingredient: Glyphosate, N-(phosphonomethyl) glycine, in the form of its potassium salt), and two rhizoboxes served as untreated plant controls. These eight rhizoboxes were part of a larger ongoing research project that utilized all available rhizoboxes, leading to two rhizoboxes per treatment combination in this study.

Plants were grown in eight growth periods over three years, with each growth period lasting 58 days. Fertilization was conducted twice per growth period by applying 25 mL of fertilizer solution per rhizobox. Fertilizer solution was prepared by dissolving 3.745 g of Peters® 20/20/20 Professional fertilizer per liter, which equaled 0.749 mg N, 0.749 mg P, and 0.749 mg K mL^{-1}. Trace element concentrations were magnesium (0.019 mg mL^{-1}), boron (0.749 μg mL^{-1}), copper (0.002 mg mL^{-1}), iron (0.004 mg mL^{-1}), manganese (0.002 mg mL^{-1}), molybdenum (0.019 μg mL^{-1}), and zinc (0.002 mg mL^{-1}). Fertilizer was applied on days 30 and 50.

Roundup PowerMAX was applied to all rhizoboxes, except for the controls, in the form of its potassium salt on day 1, before planting, at the recommended field rate of 300.79 mL ha^{-1} (equivalent to 163 g of glyphosate [Glyphosate, N-(phosphonomethyl) glycine] ha^{-1}). Corn and soybean seedlings were germinated on cotton tissue and transplanted into the rhizoboxes (2 plants/box) on day 10. The Ontario Agronomy Guide (Baute et al., 2002) was used to estimate growth stage for corn and soybean. When plants reached the V3-5 and V6-7 growth stages (days 30 and 51, respectively), a cell spreader was used to apply glyphosate to plant leaves, and all aboveground plant material was harvested on day 58. This schedule was then repeated for eight growth periods, and the rhizosphere soil samples used in this study were collected on day 58 of the fourth growth period.

2.2. Rhizosphere soil sample collection

Rhizosphere soil samples for PLFA and RNA extraction were collected in the fourth growth period (on day 58) of corn and soybean. For the collection of rhizosphere soil samples for PLFA and RNA extractions, rhizoboxes were placed horizontally on the lab bench, screws, clamps and the top acrylic plate were removed, and three 5 g subsamples of soil were randomly collected from throughout the rhizosphere and stored at −80 °C until further processing.

2.3. Phospholipid fatty acid (PLFA) analysis

Phospholipid fatty acids were extracted from 2 g of rhizosphere soil with a single-phase mixture of chloroform, methanol and aqueous citrate buffer (Bligh and Dyer reagent). The organic, lipid-containing, phase was collected and the lipids were separated into neutral, glyco- and phospholipid fractions using silicic acid columns. The phospholipids were then converted to their methyl-esters by alkaline methanolysis (Bardgett et al., 1996; Frostegård et al., 1993). Methyl nonadecanoate served as an internal standard, which allowed calculation of FAME concentrations (Zelles, 1996). For FAME detection and quantification, a Hewlett-Packard 5890 Series II gas chromatograph (GC) equipped with a HP Ultra 2 capillary column and a flame ionization detector were used. PLFA peak detection was performed with the MISystem, Version 4.5 (MIDI Inc., Newark, DE), using the Eukary method. The GC temperature program ramped from 170 to 300 °C at 5 °C min^{-1}. The reports generated by the MISystem software provided peak areas (response) and peak names (according to the peak match with the Eukary method library). Standard nomenclature for the FAMEs includes the number of C atoms counted from the omega (Ω) end (i.e., opposite the carboxyl end), followed by the number of double bonds after the colon; *cis* conformations are designated with the suffix c, and the prefixes i and a are given for iso- and anteiso-branched FAMEs, respectively. The suffix 10ME indicates a methyl group at the 10th C atom and cy indicates cyclopropane groups. Altogether, 14 PLFAs were detected in every sample and used for further analysis. PLFA biomarkers for Actinobacteria were 16:0 10ME and 18:0 10ME. Markers for Gram-positive bacteria were a15:0, i15:0, i16:0, a17:0 and i17:0. Selected monounsaturated and cyclopropane FAMEs such as 16:1Ω7c, 18:1Ω7c, cy17:0 and cy19:0 served as biomarkers for Gram-negative bacteria (Zelles, 1999). The PLFAs 18:2 Ω6,9c and 18:1Ω9c served as fungal biomarkers (Zelles, 1999), and 16:1Ω5c served as a biomarker for arbuscular mycorrhiza fungi (Olsson et al., 1995).

2.4. RNA extraction

Total RNA was extracted from 1 g of rhizosphere soil using the RNA PowerSoil Total RNA Isolation™ kit (MO BIO Laboratories, Inc., CA, USA) resulting in 100 μl of total RNA extract. A portion of this total RNA extract was then DNase treated using the RTS DNase™ kit (MO BIO Laboratories, Inc., CA, USA) with a final reaction volume of 50 μl. DNase-treated RNA extracts were visualized on 1% agarose gels using GelRed™ (Biotium, Inc., CA, USA) and quantified using a Qubit® fluorometer and the RNA HS Assay™ kit (Life Technologies, NY, USA). All subsamples were extracted separately using the methods above, and then combined prior to the RNA-Seq analysis below to account for variability in bacterial community composition within the rhizosphere of a given replicate rhizobox.

2.5. RNA preparation and sequencing

DNase-treated total RNA extracts were submitted to Otogenetics Corporation (Norcross, GA, USA) for RNA-Seq assays. Briefly, the integrity and purity of total RNA were assessed using an Agilent Bioanalyzer 2100 and OD260/280. Total RNA extracts were subjected to rRNA depletion using the RiboZero Human/Mouse/Rat kit (Epicentre Biotechnologies, Madison, WI USA) and the RiboZero Meta-Bacteria kit (Epicentre Biotechnologies, Madison, WI USA) to enrich for prokaryotic mRNA. OligodT-primers were used to deplete eukaryotic mRNA in order to enrich for prokaryotic, non-polyA mRNA present in the total RNA extracts. The remaining mixture containing prokaryotic mRNA and eukaryotic and prokaryotic rRNA cDNA was generated from the depleted RNA using the NEBNext mRNA Sample Prep kit (New England Biolabs, Ipswich, MA USA) with ultra-low protocols. cDNA was profiled using an Agilent Bioanalyzer 2100 and subjected to Illumina library preparation using NEBNext reagents (New England Biolabs, Ipswich, MA USA). The quality, quantity, and size distribution of the Illumina libraries were determined using an Agilent Bioanalyzer 2100. The libraries were then submitted for Illumina HiSeq2000 sequencing according to the manufacturer's recommended protocol. Paired-end 100 nucleotide reads were generated and checked for data quality using FASTQC (Babraham Institute, Cambridge, UK).

2.6. Data analysis

All PLFA data were expressed on the basis of soil dry weight and were tested for normal distribution (Kolmogorov–Smirnov test) before statistical analysis. Significant differences between glyphosate and non-glyphosate treatments within each crop were tested by an Analysis of Variance (ANOVA) using the Scheffé test (SAS Vers. 9.3). Principal component analysis (PCA) of bacterial PLFA biomarkers was performed using PC-ORD for Windows (Version 6.0, MjM Software, Gleneden Beach, OR).

For the RNA-Seq data, paired-end reads from each cDNA library were uploaded to the MG-RAST pipeline (Meyer et al., 2008) and joined using FastqJoin (Aronesty, 2011). The joined reads were then further processed using the MG-RAST pipeline, including trimming using a modified DynamicTrim algorithm (Cox et al., 2010) to remove low quality sequences.

Reads were functionally annotated by comparing them to the M5NR database using a maximum e-value of $1e-5$, a minimum identity of 60%, and a minimum alignment length cutoff of 15. The functionally annotated reads obtained from MG-RAST were classified according to the SEED subsystems functional hierarchy, and the number of hits for each annotation were compared using edgeR v.2.0.5 (Robinson et al., 2010) in R v.2.12.0 (R Core Team, 2008) to evaluate differential gene expression between treatments for each crop using an alpha of 0.05 to determine significance. EdgeR is a Bioconductor software package that uses an overdispersed Poisson model to account for biological and technical variability and empirical Bayes methods to moderate the degree of overdispersion across transcripts, making it a useful tool for comparing transcript counts in RNA-Seq studies where replication is often minimal (Robinson et al., 2010). The transcript reads were deposited in the NCBI Sequence Read Archive (SRA) under the accession number SRP060211.

3. Results

Table 1 shows the concentration (nmol g^{-1}) of PLFA biomarkers in the rhizosphere of plants exposed to glyphosate and control plants with no exposure to glyphosate. PLFA biomarkers indicated that Gram-positive bacteria, Actinobacteria, mycorrhiza, and fungi were significantly decreased ($p < 0.05$) after plant treatment with glyphosate. The PCA plot given for fungal and bacterial PLFA biomarkers (Fig. 1) shows that the rhizosphere soil samples of glyphosate-treated plants are mostly separated from non-treated control plant rhizosphere soils along axis 1, which explained 70.5% of the variance of the dataset.

Table 2 contains a summary of the sequencing statistics for the cDNA libraries generated from both control and glyphosate-treated samples. Sequencing yielded an average of 24,912,442 ≥ 100-base long reads per sample for a total of 199,299,540 reads (16.7 Gbp), and an average quality (Phred score) per read of 37, where a Phred score of 30 corresponds to a probability of error of 10^{-3} (Ewing and Green, 1998). Following joining of paired-end reads and trimming, samples contained an average of 17,014,959 reads (2.1 Gbp average). An average of 6.7 million predicted coding regions were identified per sample, with an average of 1.2 million (16.5%) that could be annotated using the M5NR database. On average, approximately 329,225 of these predicted coding regions (52.7%) were then matched to proteins in the SEED subsystems database.

Sequences linked to RNA metabolism (36–48%), carbohydrate metabolism (15–37%), and phages, prophages, and transposable elements (11–17%) had the highest relative abundance among the Level 1 functional categories in the control and glyphosate-treated rhizospheres of corn and soybean (Fig. 2). Sequences within the carbohydrate and RNA metabolism categories were dominated by those involved in sugar utilization and encoding Group II intron-associated genes. The carbohydrate metabolism category also contained many sequences involved in monosaccharide metabolism. Within the phages, prophages, and transposable elements categories, the majority of sequences (approx. 99%) were associated with phages, specifically r1t-like streptococcal phages and phage capsid proteins. Transcripts involved in glyphosate degradation pathways were also identified. Specifically, transcripts of genes within the *phn* operon encoding for the carbon-phosphorus lyase pathway were present, including those for proteins PhnA, PhnB, PhnE, PhnJ, PhnK, and PhnP. Additionally, transcripts for the gene *thiO* encoding the glycine oxidase involved in oxidation of glyphosate to form aminomethylphosphonic acid (AMPA) and glyoxylate were observed. However, the abundance of these transcripts involved in glyphosate degradation was not significantly different between control and glyphosate-treated samples.

Using edgeR, 67 sequences were identified with differential transcript abundance between the control and glyphosate-treated rhizosphere samples (Fig. 3). From these differentially expressed genes, 45 were downregulated in the glyphosate-treated rhizosphere, and 22 were upregulated (Fig. 3). The functions of the downregulated genes included carbohydrate metabolism (22%), amino acid metabolism (16%), clustering-based subsystems (11%), respiration (9%), fatty acid and lipid metabolism (7%), protein metabolism (7%), membrane transport (4%), metabolism of aromatic compounds (4%), cell division (2%), cell wall and capsule formation (2%), cofactor and vitamin metabolism (2%), iron acquisition and metabolism (2%), nitrogen metabolism (2%), phosphorus metabolism (2%), and photosynthesis (2%).

Table 1
Concentration of microbial phospholipid fatty acid (PLFA) biomarkers [nmol g^{-1}] in rhizosphere soil of glyphosate-treated and untreated plants (N = 4 per treatment).[a]

	PLFA [nmol g^{-1}]													
	Actinobacteria		Gram negative				Gram positive					Fungal		
	16:0 10ME	18:0 10ME	16:1w7c	cy17:0	cy19:0	18:1w7c	i15:0	a15:0	i16:0	i17:0	a17:0	16:1w5c	18:2w6c	18:1w9c
Control	8.500a	3.415a	6.221a	2.984a	4.419a	7.221a	8.422a	6.028a	3.614a	2.644a	2.667a	5.919a	3.851a	6.395a
Treated	7.024b	2.563b	4.810b	2.460b	3.883a	6.334a	5.684b	4.255b	2.932a	2.070b	2.033b	4.652b	2.626b	5.195b

[a] Different letters (a,b) indicate significant differences ($p < 0.05$) between glyphosate-treated and control samples.

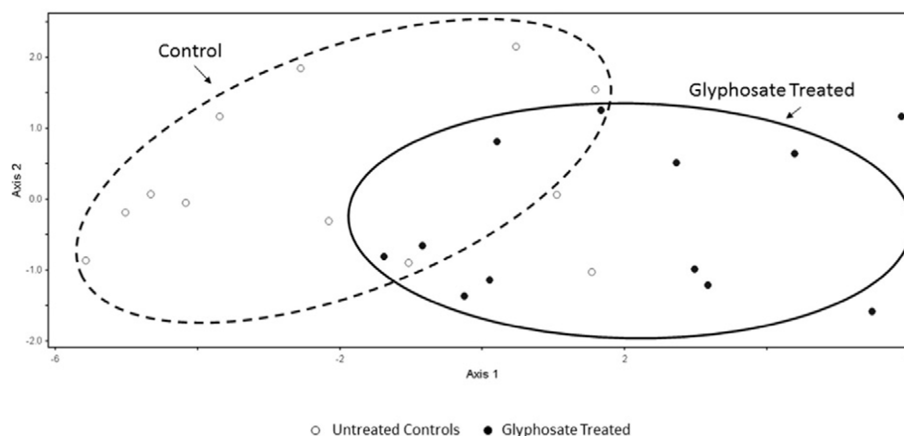

Fig. 1. Principal component analysis (PCA) plot of microbial phospholipid fatty acid (PLFA) profiles derived from rhizosphere soil of control (open symbols) and glyphosate-treated (closed symbols) plants. Variance of the data explained Axis 1: 70.5%, Axis 2: 8.1%. Circles on plot represent treatment groupings for control (dashed circle) and glyphosate-treated (solid circle) samples.

Glyphosate treatment increased expression of 22 genes in the glyphosate-treated rhizospheres (Fig. 3). The genes upregulated in the rhizosphere included protein metabolism (23%), respiration (18%), clustering-based subsystems (9%), carbohydrate metabolism (9%), amino acid metabolism (5%), membrane transport (5%), motility and chemotaxis (5%), nucleoside and nucleotide metabolism (5%), photosynthesis (5%), potassium metabolism (5%), RNA metabolism (5%), and stress response (5%). Table 3 contains a listing of all transcripts that were significantly differentially expressed in the plant rhizospheres when comparing glyphosate-treated and control samples.

The functional roles containing the most downregulated transcripts overall included amino acid and carbohydrate metabolism. The majority of amino acid-related transcripts downregulated were involved in the synthesis of amino acids, including alanine, methionine, glutamine, glutamate, aspartate, and asparagine, as well as chorismate an intermediate produced in the synthesis of aromatic amino acids. The specific transcript impacted in chorismate synthesis was 3-dehydroquinate dehydratase II (EC 4.2.1.10) which was downregulated 2.7 fold under glyphosate exposure. Transcripts involved in glycine and serine utilization and creatine and creatinine degradation were also downregulated.

Many of the carbohydrate metabolism transcripts that were downregulated are related to aspects of central carbohydrate metabolism, including the Entner-Doudoroff pathway, the pentose phosphate pathway, methylglyoxal metabolism, and anaplerotic reactions involved in pyruvate metabolism. The pentose phosphate pathway related transcripts were a ribose 5-phosphate isomerase B (EC 5.3.1.6) [12-fold decrease] and a xylulose 5-phosphate/fructose-6-phosphate phosphoketolase (EC 4.1.2.9/EC 4.1.2.22) [1.6-fold decrease]. The transcripts involved in the Entner-Doudoroff pathway that were downregulated included a PQQ-dependent glucose dehydrogenase (EC 1.1.5.2) that experienced a 6-fold decrease in expression following glyphosate treatment, and a gluconate dehydratase (EC 4.2.1.39), which had a 5.5-fold decrease in expression under glyphosate exposure.

Table 2
Metatranscriptomic sequence statistics of rhizosphere samples with and without glyphosate treatment.[a]

	Control	Glyphosate-treated
Total library size (Gbp)	2.2	2.0
Total cDNA sequences (prior to assembly)	25,388,217	24,436,668
Total cDNA sequences (post-assembly)	17,494,856	16,535,063
Total cDNA sequences (post QC)	16,384,546	15,510,898
Total rRNA sequences	5,797,802	5,783,048
% rRNA sequences	33.24	35.24
Total non-rRNA sequences	10,586,744	9,727,850
% non-rRNA sequences	60.40	58.51
Predicted protein coding regions	7,874,016	5,473,692
Protein coding regions annotated using M5NR protein database	1,481,374	988,777
% protein coding regions annotated using M5NR protein database	17.20	15.78
Total sequences matched to proteins in SEED subsystems	333,460	324,989
% annotated proteins matched to proteins in SEED subsystems	48.50	56.89

[a] Values represent means for control and glyphosate-treated samples (N = 4 each).

The majority of upregulated transcripts were involved in protein metabolism and respiration. Many of the protein metabolism transcripts that increased in expression in the glyphosate-treated samples involved ribosomal proteins involved in protein biosynthesis. For example, transcripts identified as the ribosomal proteins L16p (L10e) and Firmicutes ribosomal L7Ae family protein, involved in the synthesis of the ribosomal LSU, increased 1.4 and 2.0 fold, respectively. In addition, a transcript for the SSU ribosomal protein S11p (S14e) increased expression 1.5 fold in glyphosate-treated samples. Two transcripts involved in protein degradation were also increased in abundance in glyphosate-treated samples. These included transcripts for a proteasome beta 2 subunit (EC 3.4.25.1), which experienced a 4.3-fold increase in glyphosate-treated samples, and an aminopeptidase YpdF, which increased 3 fold in response to glyphosate exposure. Respiration-related transcripts for the gamma and delta subunits of the F_0F_1 ATP synthase both experienced a 2-fold increase in expression in the glyphosate-treated samples. In addition, expression of the putative diheme cytochrome c-553 was 1.8-fold increased, and an arsenate reductase (EC 1.20.4.1) involved in the electron accepting reactions of anaerobic respiration decreased 5.5 fold in expression.

Rhizosphere samples also showed effects of glyphosate treatment on the expression of transcripts involved in iron, nitrogen, phosphorus, and potassium metabolism. A transcript functioning as part of a ferric iron ABC transporter involved in iron acquisition decreased 5.5 fold in expression in the glyphosate-treated samples. Expression of the nitrogen regulatory protein P-II that functions in ammonia assimilation was reduced 1.7 fold under glyphosate exposure, and expression of a transcript serving as a pyrophosphate-energized proton pump (EC3.6.1.1) involved in phosphate metabolism was downregulated approximately 2 fold in the glyphosate-treated samples. Also, expression of the KefA protein composing a potassium efflux system involved in potassium homeostasis was upregulated 3.5 fold in glyphosate-treated samples.

4. Discussion

Analysis of phospholipid fatty acids revealed that the concentration of several bacterial PLFA biomarkers, including those associated with Actinobacteria, Gram-negative and Gram-positive bacteria, and fungi

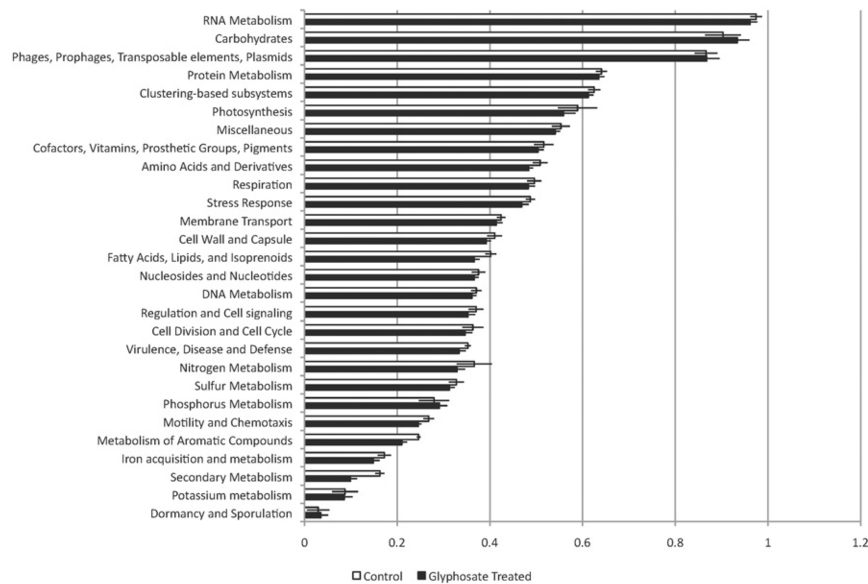

Fig. 2. Mean relative abundance (+1SE) of sequences assigned to each SEED Level 1 subsystem by MG-RAST for the control and glyphosate-treated rhizosphere metatranscriptomes.

were significantly decreased after the plant was treated with glyphosate. The decrease in Gram-positive bacteria was interesting given that Gram-positive bacteria are typically stress-tolerant, and their concentrations have been found unaltered in highly polluted soils and sediments (Pennanen et al., 1996; Rajendran et al., 1994). It should be noted that the abovementioned studies were conducted using forest soils and marine sediments.

Studies specifically examining the effects of glyphosate on agricultural soil microbial community composition have shown variable responses. Overall, phospholipid fatty acid (PLFA) biomarkers have proven to be a useful tool for assessing the effects of pesticides on microbial community composition (Nye et al., 2014; Rosenbaum et al., 2014; Widenfalk et al., 2008). It is likely that the variability of effects on PLFAs is due to differences in soil type, mineralogy, pH, organic matter and fertility, as well as variation in glyphosate formulation, application method, rate, history, fate, decomposition, and sorption of glyphosate and its metabolites (most notably aminomethylphosphonic acid (AMPA) as the major decomposition intermediate) as many of these factors have been shown to lead to variable responses in the microbial community. Thus, each soil type would have an alterable rate of decomposition and degree of sorption and toxicity of glyphosate or AMPA for susceptible populations. For example, Zhang et al. (2010) observed a differential response of this kind when using PLFA to assess the soil microbial community response to 2,4-dichlorophenoxyacetic acid butyl ester (2,4-D butyl ester) in soils with two fertility levels and using varying herbicide concentrations.

It should be also noted that the rhizosphere bacterial community is highly dynamic. For instance, a previous laboratory incubation study by Nye et al. (2014) showed transient effects of glyphosate-treated crop residue on soil Actinobacteria-associated PLFA concentrations in a soil which had never been exposed to glyphosate when compared to a control soil amended with untreated plant residues. In their study, Nye et al. (2014) observed an increase in soil Actinobacteria-associated PLFA concentrations 3 days after the start of the incubation, but this increase was not detectable after 7 and 30 days of incubation, indicating that the impacts of glyphosate on soil and rhizosphere microbial communities are temporally dynamic. In the current study, the results obtained are single-point measurements taken one week after plant exposure to glyphosate. Therefore, these PLFA results represent a snapshot of the change in bacterial community composition following glyphosate exposure.

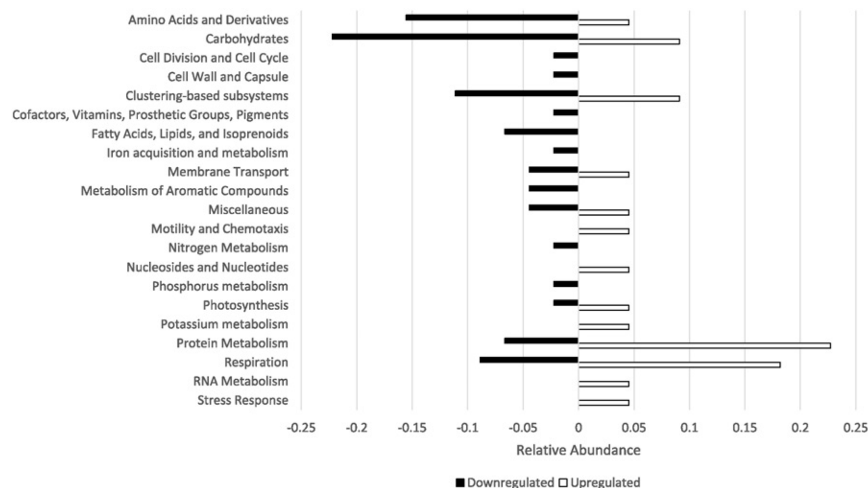

Fig. 3. Relative abundance of SEED functional roles down- and up-regulated in response to glyphosate treatment sorted by SEED Level 1 subsystem classifications. Relative abundance was estimated as the proportion of functional roles within a given Level 1 subsystem relative to the total number of functional roles down- or up-regulated.

Table 3
Most significantly affected transcripts ($p < 0.05$) obtained from rhizosphere soil following glyphosate treatment compared to non-treated controls. EdgeR-generated p-values and mean number of hits per treatment are given (N = 4 per treatment).

SEED Level 1 subsystem	Function	p-Value	Control	Glyphosate-treated
Amino acids and derivatives	Branched-chain amino acid aminotransferase (EC 2.6.1.42)	0.0017	23.25	10.75
Amino acids and derivatives	Aminomethyltransferase (glycine cleavage system T protein) (EC 2.1.2.10)	0.0144	9.5	4
Amino acids and derivatives	N-methylhydantoinase B (EC 3.5.2.14)	0.0157	1.75	0
Amino acids and derivatives	Homoserine kinase (EC 2.7.1.39)	0.0170	2.75	0.25
Amino acids and derivatives	Glutamine synthetase family protein	0.0211	1.75	0
Amino acids and derivatives	Phosphoribosylanthranilate isomerase (EC 5.3.1.24)	0.0253	0.75	3
Amino acids and derivatives	Hydroxymethylglutaryl-CoA synthase (EC 2.3.3.10)	0.0289	5.5	2
Amino acids and derivatives	3-Dehydroquinate dehydratase II (EC 4.2.1.10)	0.0453	6	2.25
Carbohydrates	Acetoacetate metabolism regulatory protein AtoC	0.0009	4.5	0.5
Carbohydrates	Ribose 5-phosphate isomerase B (EC 5.3.1.6)	0.0149	3	0.25
Carbohydrates	Aldehyde dehydrogenase B (EC 1.2.1.22)	0.0206	9.75	4
Carbohydrates	Gluconate dehydratase (EC 4.2.1.39)	0.0229	2.75	0.5
Carbohydrates	Inositol transport system permease protein	0.0245	2.75	0.5
Carbohydrates	Fructose-6-phosphate phosphoketolase (EC 4.1.2.22)/xylulose-5-phosphate phosphoketolase (EC 4.1.2.9)	0.0264	30	18.5
Carbohydrates	HPr kinase/phosphorylase (EC 2.7.1.-) (EC 2.7.4.-)	0.0265	0	1.75
Carbohydrates	Phosphoenolpyruvate carboxykinase [GTP] (EC 4.1.1.32)	0.0316	14.5	6
Carbohydrates	Xylose ABC transporter, periplasmic xylose-binding protein XylF	0.0340	5.5	1.75
Carbohydrates	Mannonate dehydratase (EC 4.2.1.8)	0.0352	1.5	0
Carbohydrates	Glucose dehydrogenase, PQQ-dependent (EC 1.1.5.2)	0.0438	3	0.5
Carbohydrates	COG2152 predicted glycoside hydrolase	0.0498	2.75	5.5
Cell division and cell cycle	Stage 0 sporulation protein J	0.0118	2.75	0.25
Cell wall and capsule	Capsular polysaccharide biosynthesis protein	0.0157	1.75	0
Clustering-based subsystems	KH domain RNA binding protein YlqC	0.0006	2	7.25
Clustering-based subsystems	Putative Holliday junction resolvase (EC 3.1.-.-)	0.0124	1	4
Clustering-based subsystems	Sulfatase	0.0218	6	1.75
Clustering-based subsystems	Molybdenum transport protein, putative/ABC-type $Fe3+$ transport system protein	0.0273	1.75	0
Clustering-based subsystems	Competence/damage-inducible protein CinA	0.0336	4.75	1.5
Clustering-based subsystems	FIG123464: polysaccharide export protein	0.0352	1.5	0
Clustering-based subsystems	Peptidyl-tRNA hydrolase (EC 3.1.1.29)	0.0401	4.75	1.5
Cofactors, vitamins, prosthetic groups, pigments	Thymidylate synthase thyX (EC 2.1.1.-)	0.0001	4.75	0.25
Fatty acids, lipids, and isoprenoids	Phytoene dehydrogenase (EC 1.14.99.-)	0.0084	4.5	0.75
Fatty acids, lipids, and isoprenoids	Enoyl-[acyl-carrier-protein] reductase [FMN] (EC 1.3.1.9), inferred for PFA pathway	0.0263	2.25	0.25
Fatty acids, lipids, and isoprenoids	(3*R*)-hydroxymyristoyl-[acyl carrier protein] dehydratase (EC 4.2.1.-)	0.0488	7.75	3.5
Iron acquisition and metabolism	Ferric iron ABC transporter, ATP-binding protein	0.0469	2.75	0.5
Membrane transport	VgrG protein	0.0454	9.25	4.25
Membrane transport	Uncharacterized protein ImpB	0.0461	5.75	2
Membrane transport	Protein export cytoplasm protein SecA ATPase RNA helicase (TC 3.A.5.1.1)	0.0495	38.75	44.5
Metabolism of aromatic compounds	Benzoylformate decarboxylase (EC 4.1.1.7)	0.0138	3	0.5
Metabolism of aromatic compounds	Isoquinoline 1-oxidoreductase alpha subunit (EC 1.3.99.16)	0.0252	6.25	2.25
Miscellaneous	FIG000875: Thioredoxin domain-containing protein EC-YbbN	0.0021	2.5	0
Miscellaneous	Transcription regulator [contains diacylglycerol kinase catalytic domain]	0.0071	3.75	0.5
Miscellaneous	Putative lumenal protein, contains 8 pentapeptide repeats, sll0577 homolog	0.0392	0.5	2.5
Motility and chemotaxis	Flagellar hook-length control protein FliK	0.0465	3	6.25
Nitrogen metabolism	Nitrogen regulatory protein P-II	0.0408	22.75	13.5
Nucleosides and nucleotides	Ribonucleotide reductase of class Ib (aerobic), alpha subunit (EC 1.17.4.1)	0.0267	0	1.75
Phosphorus metabolism	Pyrophosphate-energized proton pump (EC 3.6.1.1)	0.0445	13	6.75
Photosynthesis	Photosystem II protein PsbK	0.0214	0.25	2.5
Photosynthesis	Photosystem II 13 kDa protein Psb28 (PsbW)	0.0436	1.5	0
Potassium metabolism	Potassium efflux system KefA	0.0182	1	3.5
Protein metabolism	Firmicutes ribosomal L7Ae family protein	0.0132	0	2
Protein metabolism	Translation elongation factor P	0.0177	21.25	11.75
Protein metabolism	LSU ribosomal protein L16p (L10e)	0.0277	20.75	28.5
Protein metabolism	SSU ribosomal protein S11p (S14e), chloroplast	0.0313	0	1.5
Protein metabolism	Hypothetical protein YaeJ with similarity to translation release factor	0.0353	1.5	0
Protein metabolism	SSU ribosomal protein S8e	0.0387	4.75	1.5
Protein metabolism	Proteasome subunit beta2 (EC 3.4.25.1)	0.0441	0.75	3.25
Protein metabolism	Aminopeptidase YpdF (MP-, MA-, MS-, AP-, NP- specific)	0.0477	1.5	4.5
Respiration	ATP synthase gamma chain (EC 3.6.3.14)	0.0101	8.25	16.5
Respiration	Putative formate dehydrogenase oxidoreductase protein	0.0225	5	1.5
Respiration	NADH-ubiquinone oxidoreductase chain N (EC 1.6.5.3)	0.0231	2.5	0.25
Respiration	NAD-dependent formate dehydrogenase alpha subunit	0.0234	3.5	0.75
Respiration	Arsenate reductase (EC 1.20.4.1)	0.0272	0.5	2.75
Respiration	Putative diheme cytochrome c-553	0.0363	0	1.75
Respiration	Transport ATP-binding protein CydC	0.0416	1.5	0
Respiration	ATP synthase delta chain (EC 3.6.3.14)	0.0466	3.25	6.75
RNA metabolism	23S rRNA (guanosine-2′-O-) -methyltransferase rlmB (EC 2.1.1.-)	0.0400	2.25	5
Stress response	Choline dehydrogenase (EC 1.1.99.1)	0.0219	4.75	9.75

The RNA-Seq approach used in this study provides information on bacterial activities within the rhizosphere metatranscriptome, and it also produces a more detailed view of the effects of glyphosate on rhizosphere bacterial community gene expression. Regarding general bacterial activities within the rhizosphere, the results of this study indicate that bacteria within the rhizosphere invest the majority of their energy in transcription (RNA metabolism) and carbohydrate metabolism. Specifically with respect to the effects of glyphosate on rhizosphere bacterial community gene expression, a 2-fold increase in expression was observed for subunits of the F_0F_1 ATP synthase. The observed increase in F_0F_1 ATP synthase subunit production, along with the increased expression of a cytochrome c-553 related transcript indicates a moderate increase in respiration due to glyphosate treatment, which agrees with previous studies examining the effects of glyphosate on microbial activity (Araújo et al., 2003; Zabaloy et al., 2008).

In addition, transcripts involved in protein metabolism dominated the transcripts with increased abundance following glyphosate treatment, while amino acid synthesis transcripts declined overall in relative abundance following glyphosate treatment. This observed increase in protein metabolism, particularly for transcripts involved in protein degradation, and concomitant decrease in amino acid synthesis is commonly observed when organisms experience a period of poor nutritional conditions (Goldberg and St. John, 1976). Taken together with the above-mentioned increase in respiration, these results may suggest a differential response of the rhizosphere bacterial community to glyphosate application. Glyphosate may induce a resting state in some bacteria, leading to decreased amino acid synthesis and increased protein metabolism due to sub-optimal environmental conditions; whereas, other bacterial taxa may be stimulated within the glyphosate-treated rhizosphere. These data agree with previous studies, such as Zabaloy et al. (2012) who observed enhanced respiration in pristine soils but no respiratory stress in agricultural soils with a history of glyphosate use. This observation led to the hypothesis that this difference may reflect selection for glyphosate-tolerant microorganisms and gradual elimination of glyphosate-sensitive taxa. In addition, reviews by Imfeld and Vuilleumier (2012) and Jacobsen and Hjelmsø (2014) cite observations indicating an adaptive shift in bacterial community composition towards more glyphosate-tolerant species over time, suggesting a differential response by bacteria to glyphosate treatment such as that observed in this study.

In addition to effects on amino acid and protein metabolism, rhizosphere samples from glyphosate-treated plants also showed downregulation of transcripts involved in several components of central carbohydrate metabolism, namely the pentose phosphate pathway and the Entner-Doudoroff pathway. The down-regulated pentose phosphate pathway transcripts included a ribose 5-phosphate isomerase B (EC 5.3.1.6), which catalyzes the conversion from ribulose-5-phosphate to ribose-5-phosphate (R.-g. Zhang et al., 2003), and a xylulose-5-phosphate/fructose-6-phosphate phosphoketolase (EC 4.1.2.9/EC 4.1.2.22) which reversibly acts on xylulose-5-phosphate/fructose-6-phosphate to produce glyceraldehyde 3-phosphate/erythrose 4-phosphate (Yin et al., 2005). The function of the pentose phosphate pathway is to yield reducing power in the form of NADPH, as well as to yield key intermediates involved in anabolic processes, such as amino acid and nucleotide synthesis. Downregulation of these enzymes would lead to decreased production of ribose-5-phosphate and glyceraldehydrate 3-phosphate, which both are eventually converted to erythrose-4-phosphate in the pentose phosphate pathway. This would cause an overall decrease in erythrose-4-phosphate production, and given the role of erythrose-4-phosphate as a precursor for aromatic amino acids would also lead to decreased aromatic amino acid synthesis. Decreased expression was also observed for a 3-dehydroquinate dehydratase II (EC 4.2.1.10) involved in catalyzing the conversion of dehydroquinate to 3-dehydroshikimate within the Shikimate pathway during aromatic amino acid synthesis (Herrmann and Weaver, 1999). Hoagland and Duke (1982) also found that glyphosate significantly interferes with the synthesis or non-aromatic and aromatic amino acids. A study by Bode et al. (1984) also observed glyphosate inhibition of dehydroquinate synthase, as well as two other enzymes involved in the Shikimate pathway, in the yeast *Candida maltosa*. This is particularly interesting given glyphosate's known role in inhibition of aromatic amino acid synthesis in plants via inhibition of the 5-enolpyruvylshikimate-3-phosphate synthase also present within the Shikimate pathway.

The downregulated transcripts involved in the Entner-Doudoroff pathway included gluconate dehydratase (EC 4.2.1.39), which converts D-gluconic acid to 2-dehydro-3-deoxy-D-gluconate (White et al., 2007), and a PQQ-dependent glucose dehydrogenase (EC 1.1.5.2) responsible for converting D-glucose to D-glucono-1,5-lactone (Schomburg et al., 2009). Downregulation of the expression of these transcripts would result in an eventual decrease in the production of pyruvate, potentially influencing various aspects of bacterial metabolism. Not all bacteria possess the enzymes necessary to carry out the Entner-Doudoroff pathway; historically, it has been found more in Gram-negative bacteria, particularly *Pseudomonas* species, rather than Gram-positive bacteria (Conway, 1992). Decreased abundance of these transcripts involved in the Entner-Doudoroff pathway may suggest, as with the observed increased protein metabolism and concomitant decrease in amino acid metabolism noted above, that glyphosate treatment of a crop is resulting in an altered rhizosphere bacterial community. In a parallel study of the same samples used in this study (Newman et al., 2016), a decrease in the abundance of Gram-negative gammaproteobacteria was observed in the rhizosphere of glyphosate-treated plants. It is possible that the reduced expression of these Entner-Doudoroff pathway enzymes is due to a reduced abundance in the gammaproteobacteria which produce these enzymes.

Several transcripts involving nutrients, such as iron, nitrogen, phosphorus, and potassium, differed significantly in abundance between glyphosate-treated and untreated control rhizosphere soil samples. These results indicate an effect of glyphosate on nutrient uptake within the rhizosphere bacterial community. Transcripts involved in acquisition of iron, ammonia assimilation, and phosphate metabolism were all downregulated in the rhizosphere of glyphosate-treated crops; whereas the transcript for the KefA protein involved in a potassium efflux system was upregulated following glyphosate treatment. Reduced expression of an iron acquisition transcript suggests reduced abundance or availability of iron in the rhizosphere of glyphosate-treated crops. Iron is a divalent cation, and previous studies have shown an ability of glyphosate to strongly complex divalent and trivalent cations (Cakmak et al., 2009). Therefore, it is possible that the decreased expression of this transcript is due to decreased availability of these cations within the rhizosphere, having been strongly complexed to glyphosate and essentially unavailable. The increased expression of a transcript for the protein KefA, which is a component of a potassium efflux system, indicates increased transport of potassium out of bacterial cells and into the soil environment. Simultaneous decreases in the expression of nitrogen and phosphorus metabolism transcripts would suggest decreased availability of these nutrients in the rhizosphere following glyphosate exposure. Interestingly, a study by Zobiole et al. (2010) found that glyphosate decreased the total amount of macro and micronutrients absorbed in glyphosate-resistant soybeans. In their study the decreased uptake of nutrients in the plant was attributed to glyphosate's strong chelating effect resulting in nutrient immobilization. This nutrient immobilization would also affect soil microbial community function, possibly manifesting in decreased expression of genes involved in nutrient metabolism, such as that observed in this study.

The metatranscriptomic approach used in this study allowed for a holistic assessment of the gene expression response of the rhizosphere bacterial community to glyphosate treatment. The results of this study indicate that glyphosate affects various components of overall rhizosphere bacterial community metabolism, affecting bacterial nutrient cycling as well as aspects of amino acid synthesis and carbohydrate and protein metabolism within the rhizosphere bacterial community.

These results provide a starting point for subsequent studies to investigate glyphosate effects on rhizosphere microbial community function. Continued advancement in this area will allow for a more detailed understanding of glyphosate's effects on the rhizosphere microbial community both in terms of its composition as well as its function, which will aid in understanding glyphosate's effects on overall soil ecosystem health.

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