FINAL PROJECT REPORT
WTFRC Project Number: AP-13-104

Project Title: Glyphosate fate in inland pacific northwest apple orchards

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Total Project Request: **Year 1:** $37,787 **Year 2:** $40,536 **Year 3:** $15,000

Other funding sources: None

Budget 1
Organization Name: CSS **Contract Administrator:**
Telephone: 509.335.2562 **Email address:**

Item	2013	2014	2015
Salaries	$25,587	$28,1496	
Benefits	$ 2,200	$ 2,387	
Wages			
Benefits			
Equipment			
Supplies	$10,000	$10,000	$20,000
Travel			
Plot Fees			
Miscellaneous			
Total	$37,787	$40,536	$20,000

Footnotes:

OBJECTIVES:
Objective 1: Recap of Objective 1: Experiment 1.1 and 1.2 will determine the fate of the glyphosate after application without a significant recent glyphosate use history in apple production systems, including fate of glyphosate absorbed through the bark.

Experiment 1.1: Fate of glyphosate in an orchard without a recent glyphosate use history.
Assessment of first and second year data from field experiment 1 (Sunrise), field experiment 2 (Quincy NE), and field experiment 3 (Quincy SW) are completed.

Experiment 1.2: Absorption and translocation of basal-applied and soil-applied glyphosate.
The greenhouse experiment for the absorption and translocation experiment 1 is completed, providing glyphosate absorption and translocation data.

Objective 2: Recap of Objective 2: Identify optimum conditions for microbial degradation to mitigate soil adsorption (and potential persistence) of glyphosate in inland Pacific Northwest orchards, and characterize shifts in bacterial and fungal communities in the soil.

Experiment 2.1: Genetic analysis of microbial communities.
Knowledge of the fungal and bacterial community composition within the nontreated control and the plots treated with glyphosate at 1920 g ae/ha at Sunrise and Quincy SW has been obtained.

SIGNIFICANT FINDINGS:
- No visual injury has been observed following the applications of glyphosate at Sunrise, Quincy NE, and Quincy SW.
- Tree growth was similar among treatments at Sunrise, Quincy NE, or Quincy SW regardless of glyphosate treatment - 80 trees per treatment were measured over the course of two consecutive seasons.
- Glyphosate absorption by bark treatments to juvenile trees in absorption and translocation experiment 1 was surprisingly higher than glyphosate absorption by the leaf treatment.
- Translocation of absorbed glyphosate from a basal application appears to result in translocation to the roots.
- Translocation of absorbed glyphosate from a foliar application appears to result in comparable accumulation of glyphosate above and below treated section.
- Relative to the 2013 growing season samples, there appears to be a modest partitioning of both microbial communities among the two soil treatments at Sunrise.
- Analysis for shifts in microbial communities at Quincy SW from root/rhizosphere samples were comparable to Sunrise.

METHODS:
Experiment 1.1: Fate of glyphosate in an orchard without a recent glyphosate use history.
Sunrise was established on April 24, 2013 in block 3C at the WSU Sunrise Orchard. Trunk diameter measurements as well as notes on trunk, graft, and overall bark condition were recorded for each tree. Sunrise was established with a randomized complete block design with a split-plot treatment arrangement and four replications. Main plots were 2.1 m wide by 24 trees, or ~24 m, in length and consisted of three treatments; 1) no postemergence glyphosate and maintained weed free by hand weeding or with a paraquat application at 140 g/ha, 2) glyphosate at 840 g ae/ha, and 3) glyphosate applied at 1920 g ae/ha. Split-plots were 2.1 m wide by 12 trees, or ~12 m, in length and were either 1) no vegetation facilitated by hand weeding or a directed application of paraquat or 2) a uniform stand of volunteer weeds. The trunk diameter measurements were converted to cross-section measurements of area. Quincy NE and Quincy SW were established on May 13, 2014 in two separate

Fuji blocks planted in 2013. Initial trunk measurements were recorded for each tree and the two field experiments were established with a randomized complete block design with four replications. Plots are 2.1 m wide by 24 trees in length. Each study includes three treatments; 1) no post emergence glyphosate and maintained weed free with applications of paraquat at 140 g/ha and hand weeding, 2) glyphosate at 840 g ae/ha, and 3) glyphosate applied at 1920 g ae/ha.

Prior to each glyphosate application at Sunrise, the no vegetation split-plots were hand weeded and the low hanging branches along with any suckers were trimmed. Glyphosate applications were applied to the whole plot and directed at the base of the tree. Glyphosate was applied on May 16, 2013, July 11, 2013, May 22, 2014, and July 31, 2014. To supplement the soil residue analysis as well as eliminate any concerns of glyphosate drift into the canopy during application, spray targets were placed systematically throughout the tree canopy and on the ground to document were the spray droplets were landing. Prior to each glyphosate application at Quincy NE and Quincy SW, the low hanging branches as well as any suckers were trimmed and the plots were hand weeded. Glyphosate applications were applied to the whole plot and directed at the base of the tree. Glyphosate was applied on May 22, 2014, July 31, 2014, April 28, 2015, and August 5, 2015.

To quantify non-adsorbed and adsorbed glyphosate residue, soil samples were collected after each glyphosate application using a zero-contamination system (core diameter of 5 cm) set for 10 cm depth. Following each application at each field experiment site, two soil samples were systematically collected from within the plots at 0, 1, 8, and 15 days after application. After sampling was completed, samples were stored at -20 °C (-4 °F). In collaboration with Mark Mazzola and objective 2, the soil samples were removed from the freezer and split in half. One half of the soil was delivered to Mark Mazzola and the remaining half of the soil sample was returned to the -20 °C (-4 °F) storage until further analysis for free and adsorbed glyphosate and AMPA residues.

At Sunrise, tissue samples were collected 22 days after each application. Tissue samples were stored at -20 °C (-4 °F) until further analysis.

The harvest of Sunrise took place on August 28th 2013 (2013 report), but a whole plot harvest did not occur in 2014. A subsample of 20-40 apples, sized between 80 and 88, was saved from each split-plot for quality analysis and juice analysis in 2013 and for only juice analysis in 2014. No harvest or subsamples were collected from Quincy NE and Quincy SW.

Experiment 1.2: Absorption and translocation of basal-applied and soil-applied glyphosate.
Brookfield gala on M9 rootstock (no larger than 3/8'') were purchased from Willow Drive Nursery, Inc. and planted in tall tree pots in the greenhouse. Trees were allowed to grow until leaves were mature. Trees were arranged by height to utilize a randomized complete block design. Treatments included an application of 60 kBq of radiolabeled glyphosate, mixed in water and non-ionic surfactant, to either 1) a leaf, 2) bark above graft, or 3) bark below graft. After treatment, plants were allowed to grow in a greenhouse and destructively harvested at 1, 7, 14 and 28 days after treatment. Each harvest consisted of 4 replicates of each treatment. Each plant was divided into sections 30 cm in length, starting from the graft, and the soil and roots were allowed to dry and collected as well. The treated areas were rinsed with a mixture of water, methanol, and nonionic surfactant to obtain glyphosate not absorbed. Tree parts were dried at 40 C, weighed, and larger samples were ground and subsampled. The sub-samples were oxidized and the evolved ^{14}C-CO_2 was captured and quantified. Translocation of glyphosate was determined from the recovered radioactivity in the oxidized samples.

Experiment 2.1: Genetic analysis of microbial communities.
A composite apple root sample with adhering rhizosphere soil was collected from two trees in each treatment plot from a depth of 5-15 cm. DNA was extracted from duplicate sub-samples (5 g) for each plot using the MoBio PowerMax Soil DNA extraction kits and resulting DNA was pooled. Initial examination of microbial communities utilized a genetic approach to identify quantitative

shifts in populations. Bacteria were quantified from the duplicate soil extracts by real-time quantitative PCR (qPCR) by targeting the 16S gene with the primer set 338F and 518R, and fungi using the primer set NSI1 and 5.8S. Quantification was achieved using the StepOne Plus Real Time PCR thermocycler. All reactions were performed using three technical replicates. The standard curves for PCR quantification were generated by diluting DNA plasmid containing cloned amplification product. The plasmid used for the bacterial 16S standard curve was constructed with the 16S gene from *Methylobacterium* sp. amplified from soil using the primers 8F (50-AGA GTT TGA TCC TGG CTC AG-30) and 1406R (50-ACG GGC GGT GTG TRC-30). The fungal standard curve was prepared from the ITS region of *Mortierella alpina* amplified from soil using the qPCR primers in which the complete and correct plasmid insert was previously verified by DNA sequencing. Qualitative changes in microbial community structure were initially examined using a coarse genetic approach by employing terminal restriction fragment length polymorphism (T-RFLP) analysis. This method was used as a cost savings approach and served to identify the most appropriate community to target for examination by pyrosequencing. T-RFLP analysis of bacterial and fungal communities was conducted using methods previously described and commonly employed by the collaborators (Weerakoon et al., 2012). These data were utilized to determine what, if any, microbial populations should be targeted for analysis by pyrosequencing. The bacterial 16S gene was targeted for amplification using the universal primer pair 8f and 907R. The fungal intergenic transcribed spacer region was amplified using the universal fungal primer pair ITS1F and ITS4.

RESULTS AND DISCUSSION:

Experiment 1.1: Fate of glyphosate in an orchard without a recent glyphosate use history.

No injury was present following an application of glyphosate at Sunrise, Quincy NE, or Quincy SW and no injury was observed as Sunrise, Quincy NE, and Quincy SW trees began to break dormancy in the spring.

After obtaining tree growth data from Sunrise, Quincy NE, and Quincy SW and further investigation of the yield and fruit quality data (2013 report), it is likely that any yield or fruit quality differences reported in 2013 were not a result of glyphosate treatment or the presence of vegetation, but were rather a result of variable fruit thinning practices. Measuring tree growth will more accurately provide the data necessary to determine if treatment effects are present within the study. The tree growth (Table 1) at Sunrise, Quincy NE, and Quincy SW was not affected by the application of glyphosate and the presence of vegetation had no effect on tree growth at Sunrise.

Although the glyphosate treatments at Sunrise, Quincy NE, and Quincy SW did not have a significant effect on tree growth, there was a trend present at Quincy SW in year 1. As the rate of glyphosate increased, the tree growth in year 1 decreased. The trees at Quincy NE and Quincy SW were planted in 2013 and the trees at Quincy SW are smaller caliper trees than the trees at Quincy NE. Therefore, in the smaller and less mature trees, absorption and translocation of glyphosate may have occurred and resulted in reduced tree growth. The decreasing tree growth with increasing glyphosate at Quincy SW was not observed in year 2. Glyphosate applied at 1920 g ae/ha did result in the lowest total growth over two consecutive years of two applications per season at Quincy SW.

Table 1. Glyphosate treatment effects on the growth of 80 trees per treatment from Experiment 1.1.

	Sunrise		
	Year 1 tree growth (mm^2 $tree^{-1}$)	**Year 2 tree growth (mm^2 $tree^{-1}$)**	**Total tree growth (mm^2 $tree^{-1}$)**
Treatment	**Mean**	**Mean**	**Mean**
Nontreated	5.6 ± 1.1	3.3 ± 0.7	8.8 ± 1.2
Glyphosate 840 g ae/ha	10.1 ± 0.8	4.2 ± 1.1	14.3 ± 1.4
Glyphosate 1920 g ae/ha	6.2 ± 1.3	8.6 ± 2.4	14.8 ± 2.7
Split-plot	**Mean**	**Mean**	**Mean**
Vegetation	6.7 ± 1.1	6.4 ± 1.3	13.0 ± 1.7
No Vegetation	8.0 ± 0.9	4.4 ± 1.5	12.4 ± 1.7
	Quincy NE		
	Year 1	**Year 2**	**Total**
Treatment	**Mean**	**Mean**	**Mean**
Nontreated	41.7 ± 4.6	33.9 ± 3.0	75.6 ± 4.3
Glyphosate 840 g ae/ha	49.6 ± 3.9	18.9 ± 3.2	68.4 ± 6.2
Glyphosate 1920 g ae/ha	43.7 ± 5.8	26.9 ± 5.4	70.6 ± 7.1
	Quincy SW		
	Year 1	**Year 2**	**Total**
Treatment	**Mean**	**Mean**	**Mean**
Nontreated	100.8 ± 5.8	63.9 ± 3.6	164.7 ± 4.7
Glyphosate 840 g ae/ha	97.2 ± 7.6	73.0 ± 7.4	170.2 ± 12.0
Glyphosate 1920 g ae/ha	83.9 ± 8.7	74.5 ± 15.3	158.4 ± 22.8

Experiment 1.2: Absorption and translocation of basal-applied and soil-applied glyphosate. The absorption of glyphosate by the bark of young gala/M9 trees compared to the leaf was not an expected result (Figure 1). Overall translocation of absorbed glyphosate was less than 2% for treatments made to above graft basal (AGB), below graft basal (BGB), and to a leaf (Foliar). The total amount translocated was ~0.0051 ug of glyphosate, an exceedingly small amount.

Absolute translocation of glyphosate after 28 DAT was similar among applications (Figure 2). Increasing glyphosate per gram of plant material vs time was observed below treated section in both the AGB (Figure 3) and BGB (Figure 4) applications, whereas, comparable glyphosate per gram of plant material was observed in both above and below treated sections in foliar applications (Figure 5). Interestingly, once all plant material above the graft was harvested, the rootstocks were allowed to continue to grow and produce suckers and glyphosate was detected in the suckers (Figure 6). The detection of glyphosate in the suckers indicates that glyphosate was translocated to the rootstock and then remobilized into the suckers.

In summary, absorption was observed following basal applications. Observations of absorption following basal application would corroborate that glyphosate should not be used as a 'desuckering' treatment, and care should be exercised when applying glyphosate to juvenile trees. Although translocation was a very low percentage of absorbed, translocation following basal and foliar treatments *was* observed. Most importantly, basal applications appear to result in translocation to the roots. Future work is needed to determine if absorption and translocation in field conditions is similar to what we have observed in the lab. If it is, then we need to know if glyphosate accumulates in the tree after repeated applications to better understand whether or not injury from basal applications of glyphosate is possible or occurring. Additionally, absorption and translocation of glyphosate may differ by variety, rootstock, or timing of application.

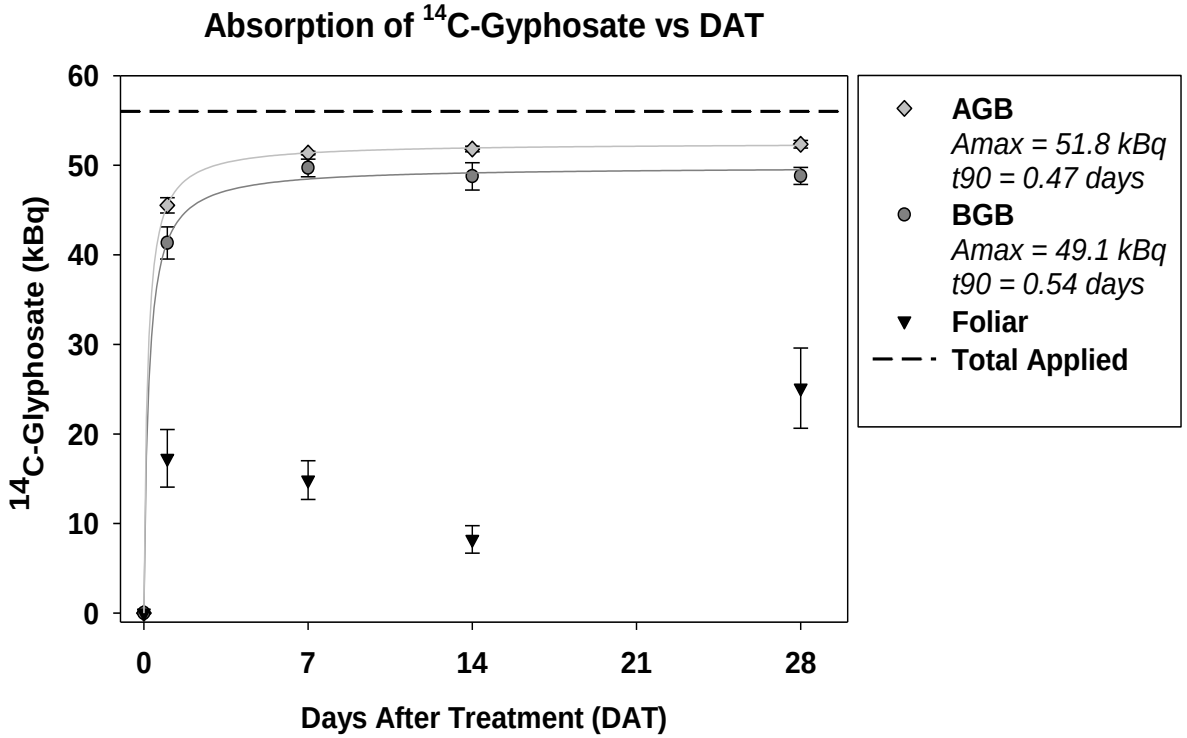

Figure 1. Percent of radiolabeled glyphosate absorbed by above graft basal, below graft basal, and foliar applications. Amax represents the maximum amount of glyphosate absorbed. The time for 90% of total glyphosate applied to absorb is represented by t90.

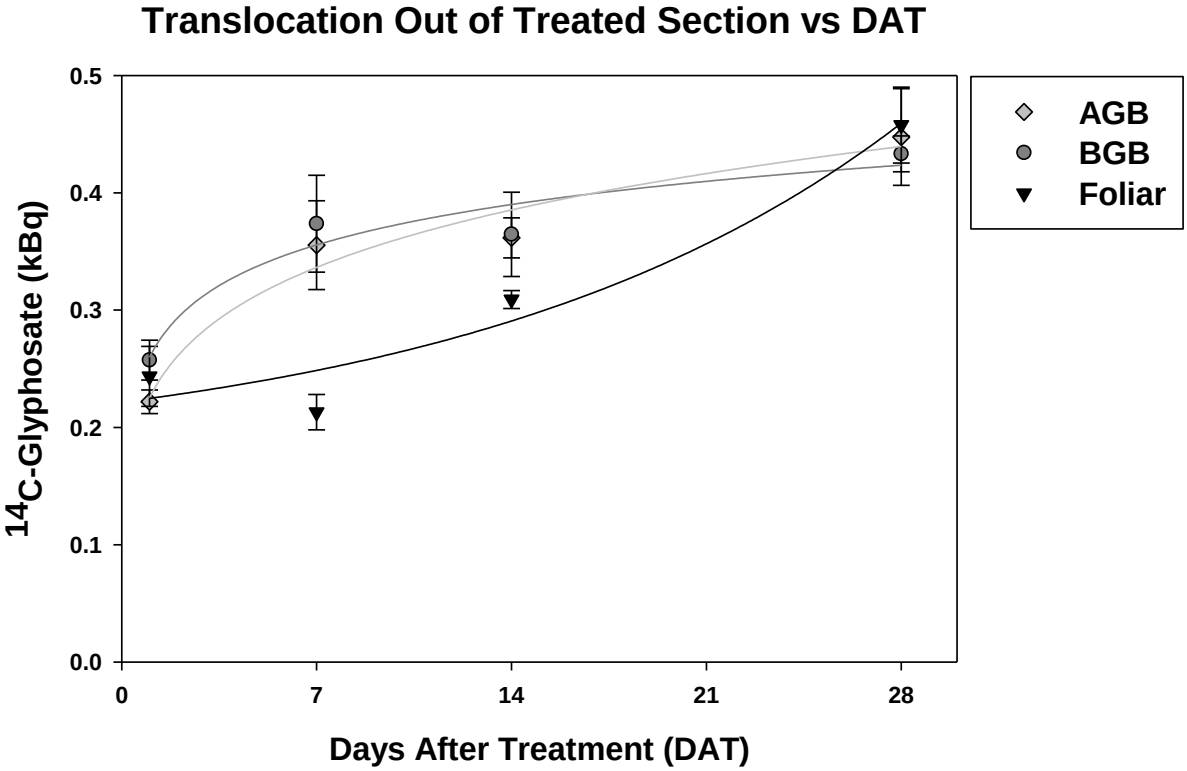

Figure 2. Translocation of glyphosate at 28 days after treatment.

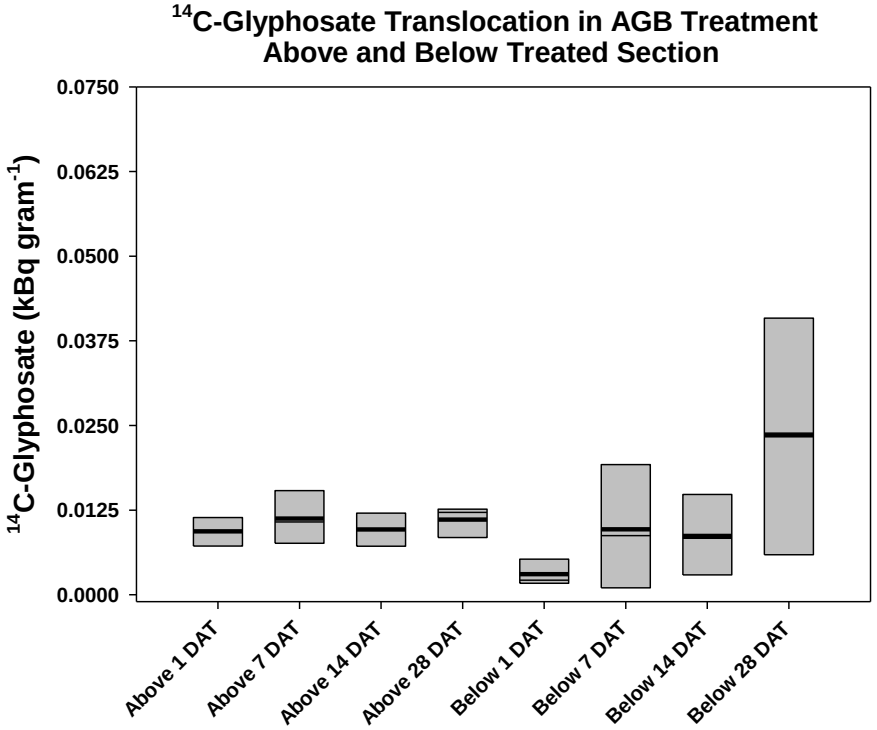

Figure 3. Glyphosate per gram of plant material vs time that was observed above treated section and below treated section in AGB treatment.

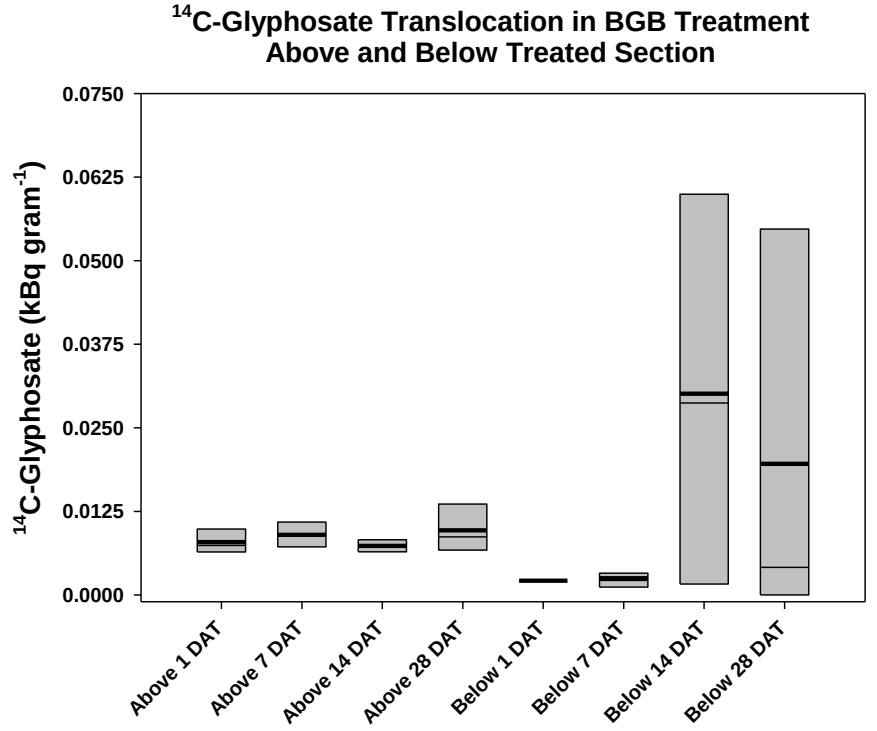

Figure 4. Glyphosate per gram of plant material vs time that was observed above treated section and below treated section in BGB treatment.

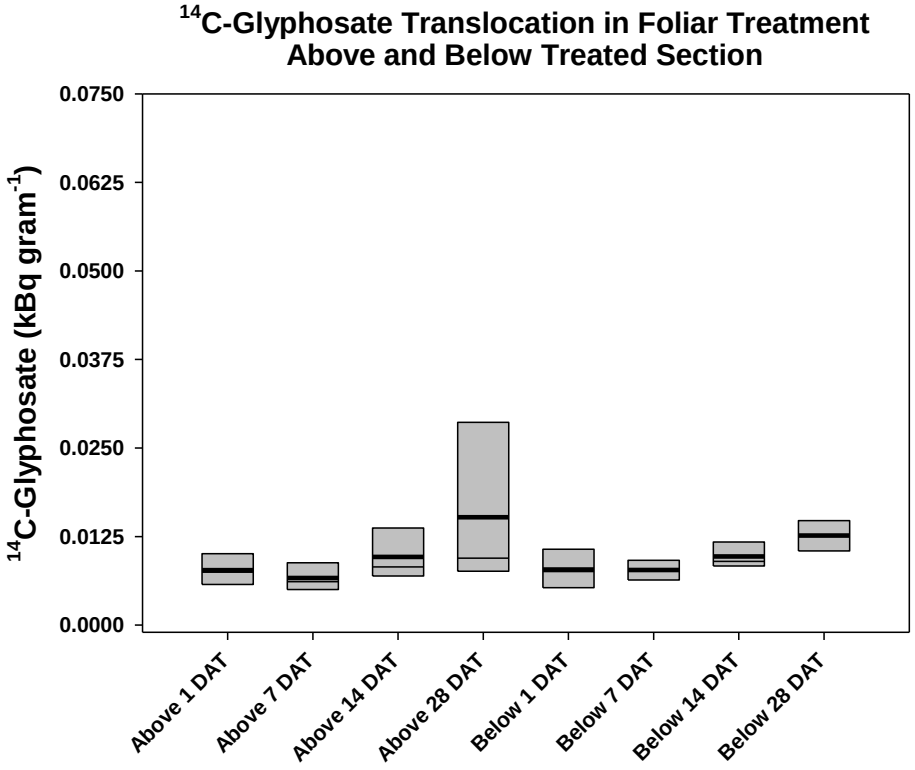

Figure 5. Glyphosate per gram of plant material vs time that was observed above treated section and below treated section in foliar treatment.

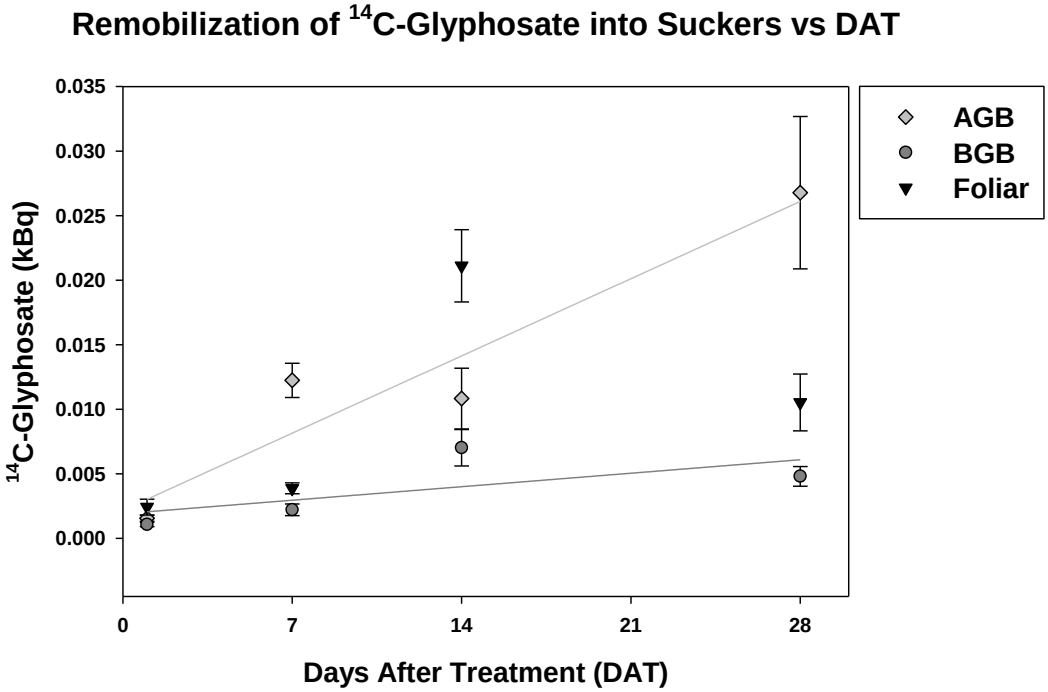

Figure 6. The detection of glyphosate in rootstock suckers vs time. More glyphosate was recovered from suckers arising from AGB treatments than from BGB or foliar treatments.

Experiment 2.1 Genetic analysis of microbial communities.

In year one, there were clearly no treatment effects of glyphosate on fungal or bacterial community composition between the nontreated plots and the plots treated with glyphosate at 1920 g ae/ha (2013 report). Principal coordinate analysis was conducted on bacterial and fungal community derived T-RFLP data for samples collected after the first glyphosate application at 1920 g ae/ha at Sunrise in May 2014. Statistically, there were no significant differences in composition of either the bacterial (Figure 7A) or fungal community (Figure 7B) between the nontreated and glyphosate treated plots. However, relative to the 2013 growing season samples, there appeared to be a modest partitioning of both microbial communities among the two soil treatments. In addition to principal coordinate analysis conducted on bacterial and fungal community at Sunrise, analysis was performed on root/rhizosphere samples at Quincy SW. Observations observed at Quincy SW (Figure 8) were comparable to previous year's results at Sunrise – modest clustering of treated and nontreated microbial communities.

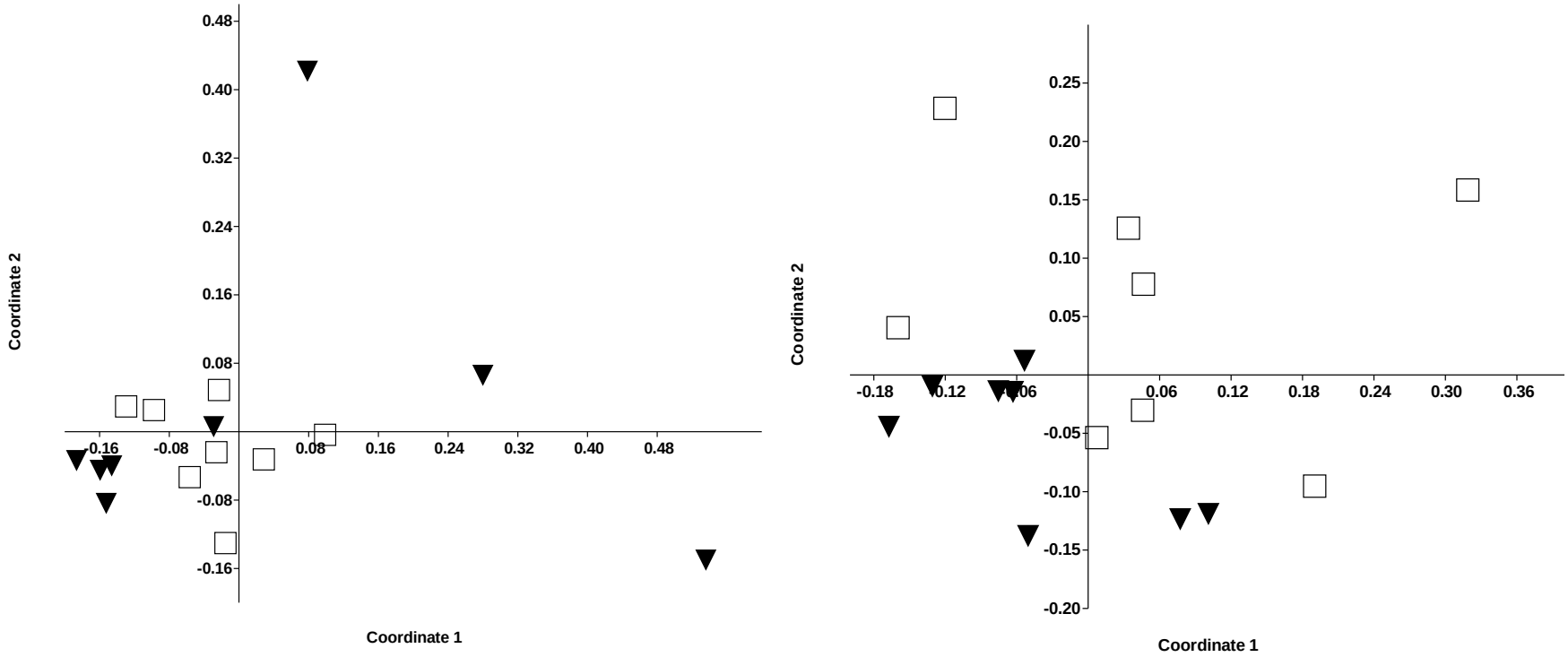

Figure 7. (A) The effect of glyphosate on bacterial community composition based upon principal coordinate analysis of T-RFLP data. Open squares represent nontreated control and inverted triangles represent data points from plots treated with glyphosate at 1920 g ae/ha. **(B)** The effect of glyphosate on fungal community composition based upon principal coordinate analysis of T-RFLP data. Open squares represent nontreated control and inverted triangles represent data points from plots treated with glyphosate at 1920 g ae/ha.

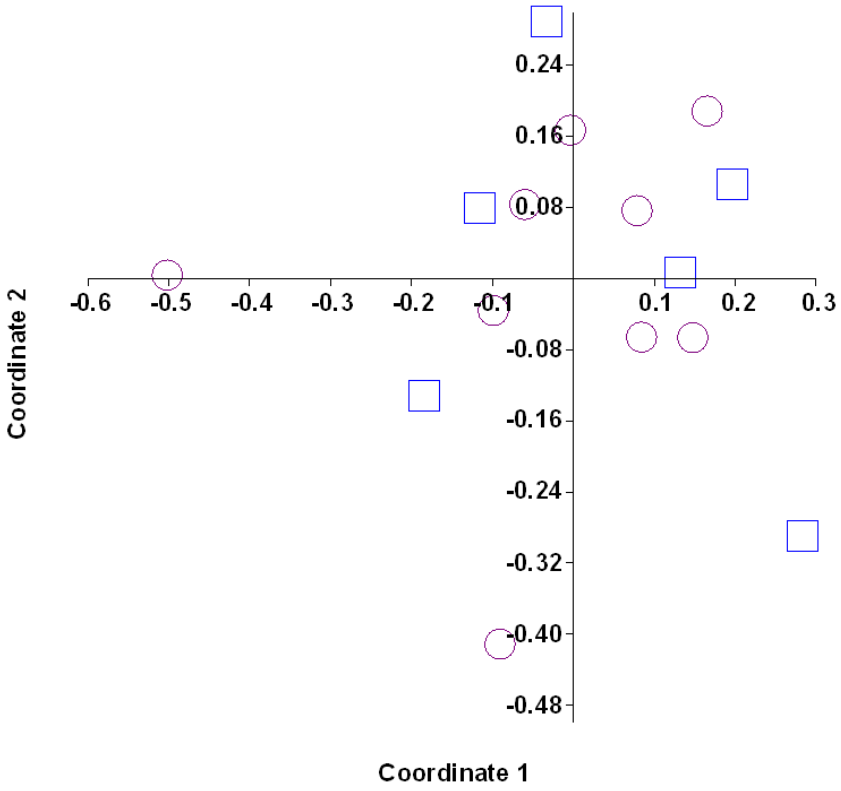

Figure 8. Nonmetric multidimensional scaling plot of the bacterial T-RFLP data from root/rhizosphere samples collected from Quincy SW in 2015 growing season. Open squares represent nontreated control and open circles represent plots treated with glyphosate at 1920 g ae/ha.

EXECUTIVE SUMMARY:

No visual injury has been observed in applications of glyphosate to apple orchards during the course of this research. Glyphosate did not cause injury following the applications at Sunrise, Quincy NE, and Quincy SW orchard experiments. Tree growth was similar among treatments at Sunrise, Quincy NE, or Quincy SW regardless of glyphosate treatment - 80 trees per treatment were measured over the course of two consecutive seasons per treatment. In a single year, 2014, there was a decrease in tree growth with increasing glyphosate rate at Quincy SW – tree growth was also the greatest that year at that location. The trees at Quincy SW were transplanted in 2013 as saplings, and thus were rapidly growing at the time of application.

Relative to the 2013 growing season samples, there appears to be a modest partitioning of both microbial communities among the two soil treatments at Sunrise. Analysis for shifts in microbial communities at Quincy SW from root/rhizosphere samples were comparable to Sunrise - statistically, there were no significant differences in composition of either the bacterial or fungal community between the nontreated and glyphosate treated plots. However, relative to the 2013 growing season samples, there appeared to be a modest partitioning of both microbial communities among the treated and nontreated soils. We do not know the functional consequence of the changes in microbial community composition as a consequence of glyphosate application.

In absorption and translocation greenhouse experiments, absorption was observed following basal applications – glyphosate entered trees in basal applications. In contrast to previous research, observations of absorption following basal application would suggest that glyphosate should not be used as a 'desuckering' treatment, and care should be exercised when applying glyphosate to juvenile trees. Although translocation was a very low percentage of absorbed (less than 2% of the applied material), translocation following basal and foliar treatments was observed, and more importantly, basal applications appear to result in translocation to the roots. Glyphosate was detected in suckers following basal treatments.

Future work is needed to determine if absorption and translocation in field conditions is similar to what we have observed in the greenhouse, and if glyphosate accumulation occurs following repeated applications. If accumulation occurs, then we need to know how much glyphosate can be applied basally before tree injury occurs. Additionally, absorption and translocation of glyphosate may differ by variety, rootstock, or timing of application. Finally, if glyphosate is accumulating in the roots, then it is likely leaking into the adjacent rhizosphere.

Glyphosate is an important labor saving tool for the tree fruit industry, and we encourage both continued research to understand the physiological and microbial consequences of its use as well as grower-focused training on minimizing glyphosate-bark contact in juvenile or injured trees.