Assessing Potential Solutions to Mitigate Pollution from Neonicotinoid Seed Coatings

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Thesis submitted to the faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of

Master of Science
In
Crop and Soil Environmental Sciences

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September 15, 2020
Blacksburg, Virginia

Keywords: thiamethoxam, clothianidin, leaching, persistence, corn, soybeans, organic carbon, buffer

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SCIENTIFIC ABSTRACT

Thiamethoxam and clothianidin are two neonicotinoids used in seed coatings for crops such as corn and soybeans. Both neonicotinoids have high solubility in water, so they are prone to transport via leaching and runoff. This thesis is comprised of two studies that evaluated potential solutions to mitigate neonicotinoid transport from fields. The first study examined the relationship between soil organic carbon content and neonicotinoid transport in a field planted in soybeans. Soils with increased organic carbon leached less thiamethoxam and clothianidin during early growing season leaching peaks; however, at the end of the season, higher organic carbon content only decreased leached mass of clothianidin. The second study was to determine neonicotinoid uptake of different ground covers used as cover crops or edge-of-field buffer strips, as well as the partitioning of thiamethoxam and clothianidin throughout the plants. Ground covers, such as crimson clover, had the highest recovery of applied thiamethoxam, meaning that it may be a good candidate to retain this pesticide in fields. Thiamethoxam and clothianidin concentrations were higher in leaf tissues than in stems or roots, indicating that above-ground biomass removal may be an effective way to reduce neonicotinoid loading in the environment. From these studies, I concluded 1) practices that raise the amount of organic carbon in the soil may help decrease early-season neonicotinoid transport, resulting in lower concentrations in surrounding waterways, and 2) careful selection of plant species, such as

crimson clover, may help reduce neonicotinoid transport in the environment, while potentially reducing exposure to beneficial insects.

PUBLIC ABSTRACT

Pesticides called neonicotinoids are commonly applied to seeds in row crops, such as corn and soybeans, before they are planted. These pesticides are highly soluble in water, which can lead to them exiting fields through runoff or leaching. This thesis is comprised of two studies that examined several potential solutions for decreasing the amount of neonicotinoids available for transport. The first study examined the relationship between organic carbon in the soil and neonicotinoids, and whether this relationship helps to retain neonicotinoids in a soybean field. Soils with high organic carbon content decreased the amount of neonicotinoids exiting the field during early growing season storms; however, at the end of the season, high organic carbon content only decreased losses for one of the pesticides studied. The second study was to determine which of six plant species and two mixes used as cover crops or buffers were the most effective at removing neonicotinoids from soil, as well as where in the plant these neonicotinoids go after uptake. Ground covers, such as crimson clover, had the highest recovery of applied neonicotinoids, meaning they would be good candidates for planting around fields. Ultimately, neonicotinoid accumulation was higher in leaves than in stems or roots, meaning that removing and disposing of leaves in an environmentally safe way could be an effective way to decrease neonicotinoid pollution. From these two studies, I found that 1) increasing organic matter in the soil can stop neonicotinoids from exiting the area it was applied in, and 2) careful consideration of plant species in or around the field may help intercept neonicotinoids before they exit the field.

ACKNOWLEDGEMENT

This thesis would not have been possible if not for the great deal of assistance I have received from my supervisors and my colleagues.

First and foremost, I would like to thank my advisor, Dr. Ryan Stewart, for always offering his guidance and expertise to keep me on track towards my research and academic goals. His teachings have helped to refine and sharpen my investigative process, as well as my communication through writing, to bring my work up to a much higher level.

I would also like to thank Dr. Kang Xia for providing her expertise on the environmental and analytical chemistry facets of this project. The direction she gave me in the lab helped me find more efficient and effective methods of data collection, which continually increased the quality of work that I produced. Thanks also to Dr. Thomas Thompson for serving on my graduate committee.

I cannot write this without a sending thanks to Dr. Jesse Radolinski, without whom I would not have had such a great base of neonicotinoid knowledge to build from. Along with this knowledge, Jesse also taught me the value of bringing enthusiasm to my work, which made this process much more fun.

To the lab groups I have been a part of, both Stewart and Xia, thank you for your help in both the lab and field. The knowledge that we shared in helping each other was invaluable in helping me succeed during my time working on this project.

Last, but certainly not least, I must thank my family for standing by my side and supporting me through all my endeavors, of which this was no exception.

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1 Introduction

After the introduction of imidacloprid (IMD) in 1991, the class of insecticides known as neonicotinoids quickly became the most used pesticides in the world (Jeschke, 2008). One of the main agricultural uses of neonicotinoids is in seed coatings for row crops, such as corn, soybean, and oilseed rape. Since 2011, over 80% of corn hectares and approximately 30-40% of soybean hectares planted in the U.S. have used neonicotinoid-treated seeds (Douglas & Tooker, 2015). Concerns about unintended environmental effects prompted the European Union to ban neonicotinoid use in crops that attract bees (Commission, 2013) and the U.S. to ban 12 of 59 registered pesticides containing neonicotinoids (Register, 2019), yet neonicotinoid seed coatings remain widely used.

Neonicotinoids work by disrupting nicotinic acetylcholine receptors (nAChR) in insects and other macro-invertebrates. Insect exposure to neonicotinoids can cause both lethal (i.e., acute) and sublethal (i.e., chronic) effects, even at relatively low doses. For example, the U.S. Environmental Protection Agency has determined that the oral LD_{50} of clothianidin (CLO) is 0.044 µg/bee and contact LD_{50} is 0.0037 µg/bee (EPA, 2010). Transformations between neonicotinoid compounds can also alter toxicity: the commonly used thiamethoxam (TMX) has a much lower binding affinity to the nicotinic receptors in insects as compared to other neonicotinoids, so the transformation to CLO makes the pesticide more effective at controlling pests, especially sucking pests such as aphids (Nauen et al., 2003). By contrast, neonicotinoids have a low binding efficiency in vertebrates, conferring relatively low mammalian toxicity (Simon-Delso et al., 2015). This factor has been one reason leading to the widespread adoption of neonicotinoid compounds.

Another reason for the popularity of neonicotinoids is their high solubility in water. For instance, TMX has a solubility of 4.1 g/L, making it suitable for systemic applications, in which it becomes dispersed relatively evenly throughout the plant that takes it up (Maienfisch et al., 2001). However, the high solubility of neonicotinoids also provides the opportunity for those compounds to migrate beyond the intended plant target (Sur & Stork, 2003). Previous studies have detected the presence of neonicotinoids in soils and water bodies surrounding fields planted with coated seeds (Hladik et al., 2014). The highest pesticide concentrations in waterways are often observed in late spring through early summer, coinciding with the "spring flush" (Thurman et al., 1991). In Japan, neonicotinoid use in rice paddies has led to detectible and persistent concentrations of the pesticide in freshwater bodies such as ponds and wetlands (Yamamuro et al., 2019). Similarly, in agricultural areas of California, 90% of sampled waterways tested positive for imidacloprid, and 20% of samples included concentrations that exceeded EPA limits (Starner et al., 2012). Other small-scale studies (e.g. soil columns and field plots) have determined TMX concentrations can mobilize through all runoff and leaching pathways, at times reaching concentrations of concern for non-target organisms (Radolinski et al., 2019; Radolinski et al., 2018).

Originally, neonicotinoid seed coatings were deemed to be an environmentally safe way to protect young plants from insects (Elbert et al., 2008). However, recent studies have detailed widespread effects of neonicotinoid pesticides on non-target organisms such as bees and butterflies. In particular, the Western Honeybee (*Apis mellifera*) has exhibited high sensitivity to neonicotinoids (Arena & Sgolastra, 2014). Exposure studies conducted on Western Honeybee populations have shown that IMD concentrations of 0.04 µg/bee are sufficient to kill 50% of test subjects (LD_{50}) within 24 hours (J. D. Stark et al., 1995). Sublethal effects such as decreased

associative learning (Stanley et al., 2015), decreased foraging and feeding activity (Decourtye & Devillers, 2010), and lower honeycomb production (Nauen et al., 2003) have been observed, even when exposed to low concentrations. These factors are not direct causes of bee mortality, but when compounded with other natural bee illnesses (e.g. varroa mites), higher rates of die off can result.

Other species may also experience negative effects from neonicotinoids. Monarch caterpillars that consume milkweed contaminated with environmentally realistic neonicotinoid concentrations have reduced size and growth rate through various stages of early development (Pecenka & Lundgren, 2015). At the same time, acute effects have been observed in higher levels of the food chain. Low doses of IMD (i.e., 10% and 25% of LD_{50}) resulted in both significant weight loss and temporary loss of directional orientation in the migratory white-crowned sparrow (Eng et al., 2017). During its spring migration, the consumption of contaminated or coated seeds by birds can result in migration delay, which can later affect survival and reproduction (Eng et al., 2019). Transport through the food chain has also been shown as the cause for smelt harvest collapse in Japan, due to the elimination of the zooplankton through neonicotinoid exposure (Yamamuro et al., 2019).

While water-facilitated transport remains an important avenue of neonicotinoid exposure, pollinators can be subjected to many other forms of direct and incidental contact to neonicotinoids, including dust expelled from machines during planting (Tapparo et al., 2012), trophallaxis, i.e., food sharing between bees (Decourtye & Devillers, 2010), and guttation drops in vascular plants (Girolami et al., 2009). Guttation typically occurs at (but is not exclusive to) night, due to an increase in water potential of water in the plant. As the water enters the plant, a slight rise in pressure causes some of the xylem fluid to be excreted through hydathodes in the

leaf margins (Figure 1.1). Guttation fluid can include toxins and other dissolved material such as pesticides or fungal pathogens (Koulman et al., 2007). As bees fulfill their need for water, they can consume theses guttation drops or carry them back to the hive for other uses. The result of this process is exposure to the contaminants at almost all stages of development (Shawki et al., 2006). Neonicotinoid accumulation in pollen represents another potential exposure pathway, though studies have shown mixed results, with some documenting concentrations exceeding 4 µg/kg dry mass (López-Fernández et al., 2015) and others showing no effects on concentrations in pollen or nectar (Wood & Goulson, 2017).

Figure 1.1 - Guttation drops on a Maximillian Sunflower

Having high quality habitat around agricultural fields may help to mitigate negative effects of neonicotinoids. For instance, areas adjacent to farm fields with diverse vegetation often have greater abundance and richness of bees compared to less biodiverse areas (Kennedy et al., 2013). This increase in biological diversity is also reflected in insect and bird populations (Schulte et al., 2017). Prairie strips placed on the downslope side of fields have also been shown to provide valuable ecosystem services, by decreasing the amounts of runoff and pollutants exiting the field, without compromising weed pressure or productivity (Hernandez-Santana et al., 2013; Hirsh et al., 2013). Although largely beneficial, the use of vegetated buffers could result in an unforeseen issue: since neonicotinoids are highly water soluble, the areas surrounding these fields could become contaminated by neonicotinoid-laden runoff and leaching (Goulson, 2013). This process could potentially expose these non-target populations to neonicotinoids through feeding and

pollen harvest plants contained in these strips (Botias, 2016). To date, however, no studies have examined neonicotinoid uptake from soils into plants commonly found surrounding agricultural fields.

Factors that influence the persistence of neonicotinoids in soil are also still largely unknown. The half-life of TMX, IMD, and CLO in soil can range from a few weeks to a few years (Fernández-Bayo et al., 2009; Gupta et al., 2008; Rexrode et al., 2003). Johnson et al. (1997) showed that incorporations of organic matter into the soil could be used to decrease the occurrence of pesticide leaching, as soil organic carbon (SOC) content can strongly influence pesticide sorption. Similarly, the sorption of IMD has a strong correlation with organic matter (Papiernik et al., 2006). When organic matter was removed from a clay soil, the sorption of IMD went down as well, demonstrating the importance of the organic fraction of the soil in the sorption of neonicotinoids (Cox et al., 1998). This finding is further supported by strong correlations between SOC and the sorption coefficient for IMD (Liu et al., 2006) and CLO (Wu et al., 2012). Given that TMX, CLO, and IMD are similar chemicals, the assumption is that TMX will behave similarly. However, these mechanisms have not been tested under field conditions.

Given the many concerns and uncertainties regarding non-target insect exposure and transport of neonicotinoid seed coatings, the goal of this project is to evaluate management practices that may reduce non-target exposure to neonicotinoid compounds. The project consists of two studies. The objective of the first study is to identify effects of biosolid additions on leaching and persistence of TMX in the soil. The objective of the second study is to quantify neonicotinoid uptake into plant species used in vegetated buffer or cover crop systems near agricultural fields. These studies are necessary to ensure that neonicotinoid use includes minimal losses to the environment and while protecting bees and other pollinators from adverse effects.

2 Increased Soil Organic Carbon Can Decrease Early-Season Neonicotinoid Pesticide Leaching

Abstract

Soil organic matter can sorb and attenuate many contaminants; however, sorption of neonicotinoid pesticides to soil organic matter remains poorly understood. The goal of this study was to determine if soil organic carbon influences persistence and leaching of the neonicotinoids thiamethoxam and clothianidin. Thiamethoxam-coated soybeans were planted into a clay soil containing a control treatment versus biosolid amendments applied with and without mulch at an agronomic rate (1x) or five times agronomic rate (5x). Beginning at planting, repeated leachate and soil samples were collected for ten weeks and were analyzed for pesticide concentrations using liquid chromatography/tandem mass spectrometry. During an early-season rainfall event, the highest organic carbon treatment (biosolids with mulch at 5x) leached significantly less thiamethoxam than all other treatments, and significantly less clothianidin than all treatments except biosolids at 1x ($p < 0.1$). By the end of the growing season, however, all treatments had leached similar cumulative masses of thiamethoxam and clothianidin. Also, there was no significant differences in thiamethoxam mass remaining in the soil at the end of the growing season, yet all four biosolids treatments had lower clothianidin concentrations than the control. Thus, higher soil organic carbon concentrations may reduce neonicotinoid leaching at times of peak loading but may not alter cumulative leaching over the entire growing season.

Keywords

Thiamethoxam, clothianidin, spring flush, contaminant transport, sorption

Highlights

Higher soil organic carbon content decreased mass of thiamethoxam and clothianidin leached during early-growing season storm event.

All treatments had similar cumulative mass of leached thiamethoxam and clothianidin at the end of the growing season.

Residual mass of thiamethoxam was not affected by soil organic carbon content, but all biosolid treatments retained less clothianidin than control.

2.1 Introduction

Row crop seeds used in production agriculture typically come with an insecticide coating and a fungicidal component. Fungicides are meant to control soil-borne fungal pathogens, while the insecticides are systemic in nature, and are used to kill chewing and sucking pests in early stages of plant development (Nettles et al., 2016). Neonicotinoid seed coatings begin to safeguard crops soon after their emergence, with continued uptake into plant tissues acting as a persistent source of protection (Elbert et al., 2008). However, less than 20% of the neonicotinoids applied as seed coatings are typically taken up by target crops (Goulson, 2013; Sur & Stork, 2003). Although some of the residual chemicals are rapidly degraded (Baskaran et al., 1999), the remainder can move into the surrounding environment (Gupta et al., 2008; Radolinski et al., 2019). For example, pesticide-laden runoff can lead to high concentrations of neonicotinoids in waterways shortly after planting due to a "spring flush" (Hladik et al., 2014) caused by high rainfall and low plant uptake rates early in the season. As neonicotinoids leach into waterways, they can reduce aquatic insect populations and lead to the collapse of fisheries (Yamamuro et al., 2019).

Clay and organic particles can increase the persistence of pesticides such as neonicotinoids in the soil (Flury et al., 1995). In particular, organic matter increases persistence of many contaminants, such as imidacloprid (IMD), even when present in small proportions (Capri et al., 2001; Sheng et al., 2001). Sorption of neonicotinoids into the soil can increase their residence times (Nettles et al., 2016) and half-lives (Mahapatra et al., 2017). At the same time, sorption to soil organic carbon (SOC) can decrease pesticide bioavailability and slow microbial degradation (Rouchaud et al., 1996). Therefore, SOC may restrict leaching losses of pesticides, but may also permit higher concentrations to persist in the near-surface environment, where they

could possibly be mobilized as dissolved in surface runoff (Ahuja et al., 1981; Radolinski et al., 2019). Thus, the dynamics by which SOC affects pesticide persistence and transport remain poorly understood, particularly under field conditions.

Many farmers are adopting practices that can increase SOC and increase productivity, such as amending soils with biosolids and other composts (Alvarez-Campos & Evanylo, 2019; Singh & Agrawal, 2008) or implementing conservation tillage and cover crops (Jian et al., 2020a; Stewart et al., 2018). Further, organic inputs with easily decomposable carbon can increase soil microbial activity relative to inputs with carbon that is not easily decomposable (Tu et al., 2006), thus potentially influencing sorption characteristics of the soil. These practices can also lead to increased nitrogen and phosphorus availability (Jian et al., 2020b), and decreased runoff (Ojeda et al., 2003). However, it is not known if different types and concentrations of SOC affect the persistence in soil versus migration of neonicotinoids applied as seed coatings.

My objectives in this study were to evaluate if varying sources and concentrations of SOC 1) influence the persistence of thiamethoxam (TMX) and clothianidin (CLO) in soil; and 2) alter the ability of TMX and CLO to leach down through soil profiles. I hypothesized that higher SOC concentrations will increase the concentrations of TMX and CLO persisting in the upper soil profile, while decreasing amounts of TMX and CLO lost in leachate. Understanding these relationships is important for mitigating the amount of TMX and CLO that can exit the field, both by increasing residence time for degradation and by reducing concentrations present in leachate.

2.2 Materials and Methods

2.2.1 Experimental Procedures

The field site was located at the Virginia Tech Turfgrass Research Center in Blacksburg, VA. The site was previously used for a study by Alvarez-Campos and Evanylo (2019), in which they examined differences in organic carbon sequestration through applying different biosolid amendments. For the previous study, a 22 m long x 11.5 m wide x 0.6 m deep field was filled with Berks-Groseclose clay soil from a nearby location. Alvarez-Campos and Evanylo graded the site to a horizontal plane (slope of 0%), so I assumed subsurface lateral flow was negligible in my study. The original experiment was conducted in four replicated blocks, with fertility treatments laid out by randomized complete block design. Each plot was 1.8 m long x 2.5 m wide and was separated by a 0.6 m alley between blocks.

During the original study, the treatment plots received biosolid amendments in fall 2016, summer 2017, fall 2017, and summer 2018, while at those same times the control received urea (46-0-0) applied at 112 kg/ha. The biosolid treatments used two products obtained from Blue Plains Advanced Wastewater Treatment Plant (DC Water) in Washington, DC: biosolids (B) at agronomic N rate (1x) and five times agronomic N rate (5x), versus biosolids + mulch (BM) at agronomic N rate (1x) and five times agronomic N rate (5x). As a result of these treatments, individual plots had varying organic carbon concentrations at the beginning of my experiment (Table 2.1). No neonicotinoids were applied in the previous study.

Table 2.1 – Cumulative biosolids applied in the previous experiment, and resulting soil organic carbon (SOC) concentrations (mean ± standard deviation) for each treatment. B – biosolids, BM – biosolids + mulch.

Treatment	**Cumulative Applied (Mg/ha)**	**SOC (g/kg)**
Control	-	8.5 ± 1.5
BM– 1x	65	17 ± 1.3
B – 1x	52	18 ± 1.8
B – 5x	116	24 ± 3.0
BM – 5x	144	28 ± 1.0

I prepared for the present study in May 2019 by spraying a 2% glyphosate solution to eliminate all vegetation growing in the plots. In mid-June of 2019, soybeans (Variety No.: CL1561364, Syngenta Corp.; Basel, Switzerland) were planted lengthwise across treatment blocks. The row spacing of the beans was approximately 0.19 m, and within-row spacing was approximately 0.13 m, resulting in a plant density of 407,000 plants/ha (~180 plants/plot). Planting depth was 0.03 m. The soybeans were coated with Clariva Complete and Mertect 340-F seed treatments (Syngenta Corp.; Basel, Switzerland) at labelled rate, resulting in ~75 µg of TMX per seed. Excessive weed and rodent pressure led me to mechanically terminate remaining soybean plants after six weeks. Weeds were also terminated with glyphosate at the same time to eliminate the potential confounding effects presented by different levels of plant growth between replications.

Rainfall was supplemented with irrigation whenever < 10 mm of rainfall fell during a one-week period. Whenever this threshold was not achieved, 20 mm of irrigation water was applied using three pop-up sprinklers mounted inline on 50 mm (2”) PVC pipes. The sprinklers were mounted 3 m apart and all heads turned a full 360 degrees. For each irrigation event, I

placed this apparatus back in the center of the experimental site. I calibrated the system by placing 16 small cups randomly around the plot while irrigation was applied. After irrigation ended, the volume of water collected in each cup was measured. From this, I calculated that irrigation was applied at a rate of approximately 10 mm/hr evenly across each plot. Irrigation uniformity was periodically checked through the experiment to ensure consistency.

2.2.2 Leachate Sampling and Analysis

I sampled leachate throughout the experiment using zero-tension lysimeters to capture and track the amount of TMX that was moving downward through the soil. Lysimeters were constructed from a plastic snap end drainage pipe cap (Product #0867AA N-12, Advanced Drainage Systems, Hilliard, OH), and were installed in the center of each plot. These caps were 25 cm tall and 25 cm in diameter and were installed by boring 30 cm diameter by 40 cm deep holes and then placing the cap open-end up on the bottom of the hole. To prevent clogging by fine particles, the lysimeters were filled to 12.7 cm with acid-washed well gravel pack (Drillers Service, Inc., Roanoke, VA).

Throughout the experiment, I collected samples following storm events with at least 10 mm of accumulated precipitation, samples were taken 5, 15, 19, 24, 36, 38, 47, 53, 55, and 67 days after planting. Each lysimeter was completely cleared and the volume of collected water was recorded. Only 15 mL of the total volume was retained, and the remainder was discarded away from the site.

In the laboratory, leachate samples were vortexed (VWR VM-3000 Mini Vortexer; Radnor, PA, U.S.) at approximately 2,700 rpm for 15 seconds. Using a syringe, 3 mL of leachate was collected and then pressed through a series of 0.45 and 0.20 PTFE µm filters (Thermo

Scientific, Part Numbers F2513-3 & -4) into a labeled 2 mL amber High-Performance Liquid Chromatography (HPLC) vial (Agilent Technologies, Part Numbers 5182-0716 & -0717), capped, and stored at 0° C if not immediately analyzed on a ultra-performance liquid chromatograph/tandem mass spectrometry (UPLC/MS/MS).

2.2.3 Soil Sampling and Analysis

For sampling, I used a push probe to take three cores that were homogenized to form one aggregated soil sample per plot. I took soil samples from between rows at 0.05 m from the row center, and to the depth of the lysimeter tops (0.15 m). Soil samples were taken on the same schedule as water samples for the first six samples, and then during every other leachate collection for the remainder of the experiment.

For analysis, approximately 40 g of wet soil from each sample was placed in a 50 mL centrifuge tube. The exact mass of added soil was recorded (wet mass). Without capping, I covered the tube with a paper towel folded in half and secured with a rubber band and placed them in a freeze drier for 48 hours. At the end of that period, the samples were removed from the freeze drier and weighed immediately (dry mass). I calculated gravimetric water content at time of sampling as difference between wet and dry mass divided by dry mass.

The final step in preparing the soil samples was grinding. One at a time, the soil was poured from the vial into a large plastic weigh boat and any hard material (rocks, sticks, etc.) were removed before the remaining material was added the grinder. I ground the samples for 15 seconds using a coffee grinder. The resulting powder was poured back into the weigh boat and any large, hard particles that were missed initially were removed. The process repeated until all

small aggregates were broken apart. The soil was then poured back into the original tube, capped, and stored in the freezer at 0° C.

For extraction, 1.0 ± 0.1 gram of prepared soil was weighed into a 60 mL straight wall centrifuge tube. The soil was spiked with deuterium-labeled TMX (TMX-d3) and CLO (CLO-d3) stock solution (539 ng/g TMX-d3 and 518.5 ng/g CLO-d3) as internal standards and left uncapped to allow the carrier solvent to evaporate. Internal standards were used to correct for variability due to TMX/CLO loss in extraction and cleanup and matrix effect during instrumental analysis. Fifteen mL of HPLC-grade acetonitrile were then added to the solution with a pipette and the tube was capped immediately. The tube containing the sample was then vortexed for 2 minutes at 2,300 rpm using a VM-3000 Mini Vortexer (VWR, Inc.; Radnor, PA, U.S.). After vortexing, the solution was placed in a centrifuge for 15 minutes at 5000 rpm. With a pipette, 5.0 mL of the solution was taken from the 60 mL tube and transferred to a 12 mL screw top glass tube without a cap and placed in a vacuum evaporator (RapidVap, Labconco Corporation, Catalog No. 7900002). The vacuum evaporator was set to run at 75 °C, 330 mbar, and 95% speed for 180 minutes, or until a sample was completely dried. After the test tubes were cooled to room temperature, the dried residue in each glass tube was re-constituted using 1.0 mL of UPLC mobile phase (5mM NH_4Ac in H_2O: 5mM NH_4Ac in MeOH, v:v = 1:9) and vortexed for 10 seconds. This solution was then extracted with a 1 mL syringe and filtered through a 0.2 µm PTFE filter, and into a 2 mL amber HPLC vial with cap.

2.2.4 UPPLC/MS/MS Analysis

Analyses of the processed leachate samples and the soil extract samples for CLO and TMX and CLO-d3 and TMX-d2 were performed on a UPLC (1290 Infinity, Agilent Technologies; Santa Clara, CA, U.S.) coupled with a tandem mass spectrometer (6490 triple-

quadrupole, Agilent Technologies; Santa Clara, CA, U.S.). The UPLC included a guard column (Zorbax Extend, C18, 5 µm, 4.6 × 12.5 mm, Agilent Technologies; Santa Clara, CA, U.S.), a reversed phase analytical column (Zorbax Extend, C18, 5 µm, 4.6 × 50 mm, Agilent Technologies; Santa Clara, CA, U.S.). The mobile phase contained: (A) acetonitrile containing 5 mM ammonium acetate in water and (B) 5 mM ammonium acetate in methanol. The mobile phase was operated at 0.5 mL/min in the gradient mode as shown in Table 2.2.

Table 2.2 – Gradient conditions used for UPLC analysis.

Time (min)	A%	B%
0.00	60.0	40.0
3.00	15.0	85.0
3.10	5.0	95.0
5.00	5.0	95.0
5.50	60.0	40.0

The sample injection volume was 5.0 µL. The mass spectrometer was operated in the positive ion electrospray ionization mode and set up to monitor the ion transition of the precursor ion to the product ion in multiple reaction monitoring (MRM) mode. The mass spectrometer settings are shown in Table 2.3, and mass spectral conditions are shown in Table 2.4.

Table 2.3 – Mass spectrometer settings for CLO/CLO-d3 and TMX/TMX-d3 Quantification.

Parameter	Value
Capillary Voltage	3.5 kV
Cone Voltage	8 V
Collision Energy	14-34 V
Source Temperature	200 °C
Desolvation Temperature	250 °C
Cone Gas Flow	480 L/h
Desolvation Gas Flow Rate	840 L/h

Table 2.4 - Parent ion and product ions monitored for CLO/CLO-d3 and TMX/TMX-d3.

	CLO	CLO-d3	TMX	TMX-d3
Parent ion (*m*/*z*)	250	253	292	295
Product ion (*m*/*z*)				
Quantifier	169	172	211	214
Qualifier	132	135	181	184

Standards, Calibration, and Quantification

Concentrations (μg/L) of CLO and TMX in the cleaned up extractants from the leachate and soil extraction were quantified using 6-points external standards consisting of CLO and TMX dissolved in the UPLC mobile phase (5mM NH_4Ac in MeOH:part 5mM NH_4Ac in H_2O, v:v=9:1). Calibration standard concentration range for CLO and TMX was 0.076-13.6 μg/L. To each external standard, CLO-d3 and TMX-d3 were spiked at 3.9 μg/L and 4.0 μg/L, respectively. The peak area ratio for CLO/CLO-d3 and TMX/TMX-d3 for each external standard was used to construct the final calibration curve for quantification of CLO and TMX based on the peak ratios of CLO/CLO-d3 and TMX/TMX-d3 in each of the final sample extract.

2.2.5 Statistical Analysis

Equivalent TMX and CLO masses were calculated by multiplying soil concentrations by soil mass in each replication (soil samples) and leaching concentrations by collected volumes (leaching samples). Using this approach, I calculated cumulative TMX/CLO mass lost through leaching and total persistent mass in the soil throughout the experiment. Analysis on cumulative mass leached was only done for two sampling dates out of ten, the second and final sampling dates (15 and 67 days after planting). We chose these dates to represent the leached mass for the season (67), and to correspond with the spring flush (15). While there might be significant differences in single event leached mass, these may not be reflected in long-term totals. Although leached masses may differ between sample dates, they may not represent differences over time, so the analysis performed for these dates was specifically for the cumulative leached mass of TMX and CLO. While performing this analysis, all treatments were compared to the control.

Cumulative leached and retained masses of TMX/CLO had non-normal distributions for each treatment. Therefore, treatment differences were evaluated using a Wilcoxon/ Kruskal-Wallis non-parametric analysis, with post-hoc Wilcoxon test ($\alpha = 0.1$). Statistical analyses were performed using JMP 15 (SAS Institute). Note that the lysimeter installed in Plot 1 did not provide consistent leachate samples, and therefore was omitted from the leachate analysis.

2.3 Results

2.3.1 Weather

During this study, several days approached or exceeded 32º C (Figure 2.1). A total of 318 mm of precipitation fell at the soil surface, of which 199 mm came from irrigation (Figure 2.2). The smallest sampling event occurred following 14.2 mm of rainfall, and the largest came after 66.8 mm of combined rain and irrigation. Supplemental irrigation was necessary to achieve the minimum sampling benchmark for 7 out of 10 samples taken during the experiment.

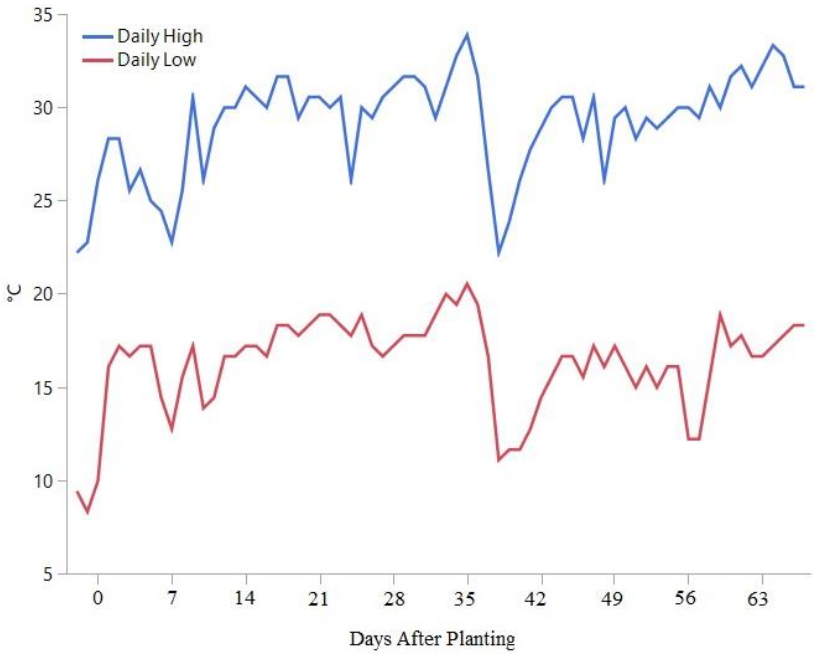

Figure 2.1 - Daily high and low temperatures (°C) for the duration of the experiment.

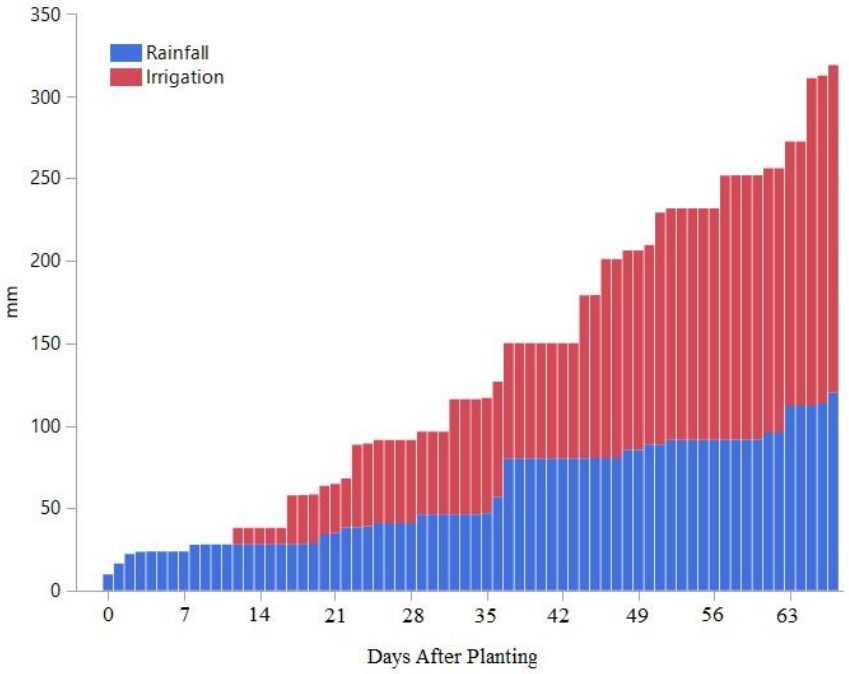

Figure 2.2 – Cumulative precipitation throughout the experiment, broken down by rainfall and manual irrigation.

2.3.2 Leachate

The cumulative volume of leachate collected from the lysimeters through all sample dates was 6,407 ± 3,213 mL (mean ± standard deviation) (Appendix A). There were no significant differences in the cumulative volume leached between any treatments ($p \geq 0.1$). The largest increase in leached mass of TMX in all treatments occurred on 5 days after planting, reaching a maximum of 345 μg/m^2 in the B-1x treatment (Figure 2.3a). By the next sampling date (15 days after planting), the highest SOC treatment (BM-5x) lost significantly less TMX in leachate compared to the control ($p = 0.0606$; Figure 2.3b). The other treatments had similar leached mass as one another ($p \geq 0.1$). After that initial spike, leached mass was always less than 109.6 μg/m^2 per event. The cumulative mass of TMX leached at the end of the experiment did not differ significantly between treatments ($p > 0.1$; Figure 2.3c).

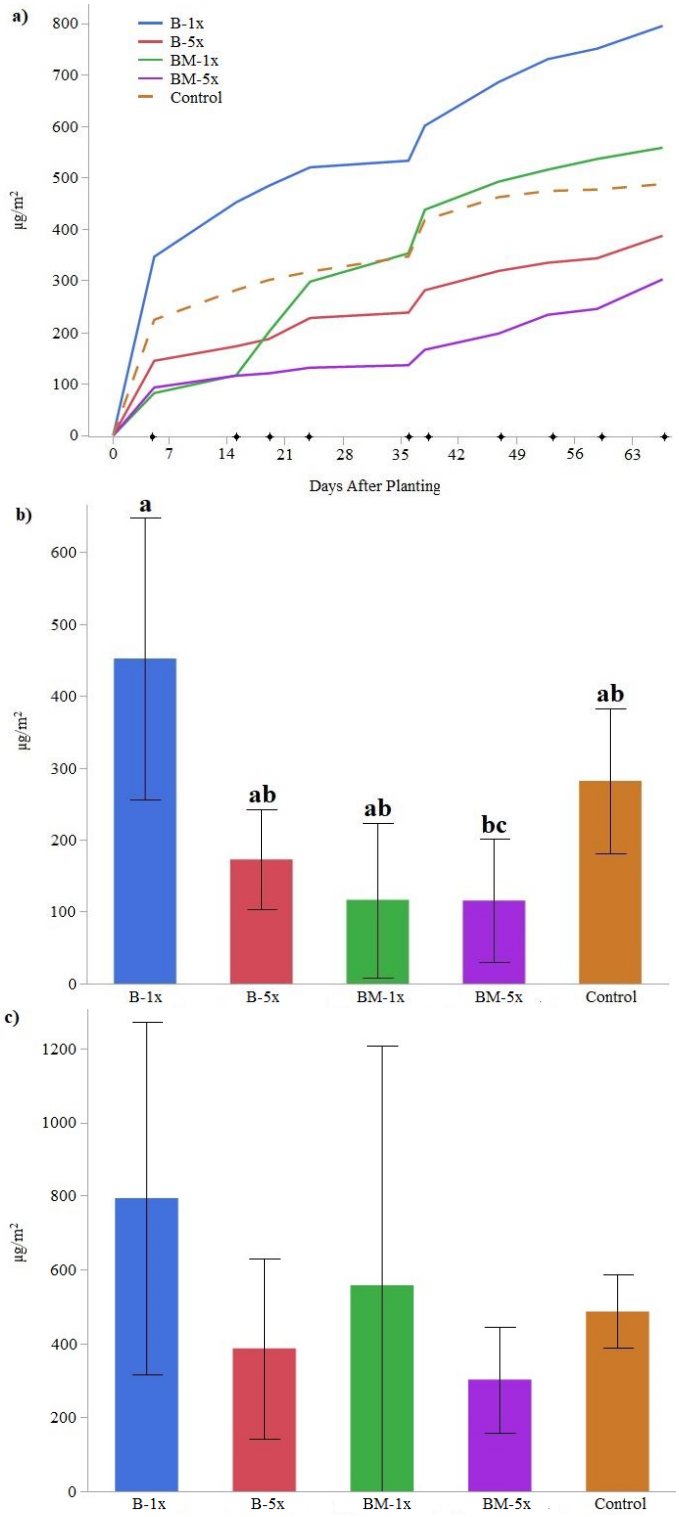

Figure 2.3 – Cumulative TMX Mass (µg) collected in leachate a) throughout the season, b) by the second sampling date (15 days after planting), and c) by the end of the study period (67 days after planting).

By day 15, the mass of leached CLO was significantly less in both the B-1x and BM-5x treatments compared to the control ($p = 0.0211$ and $p = 0.0304$; Figure 2.5a). However, by the end of the experiment the median mass of CLO leached from the control was 2-4x higher than all other treatments. Despite this, only the highest SOC treatment (BM-5x) leached significantly less CLO ($p = 0.0606$; Figure 2.5b).

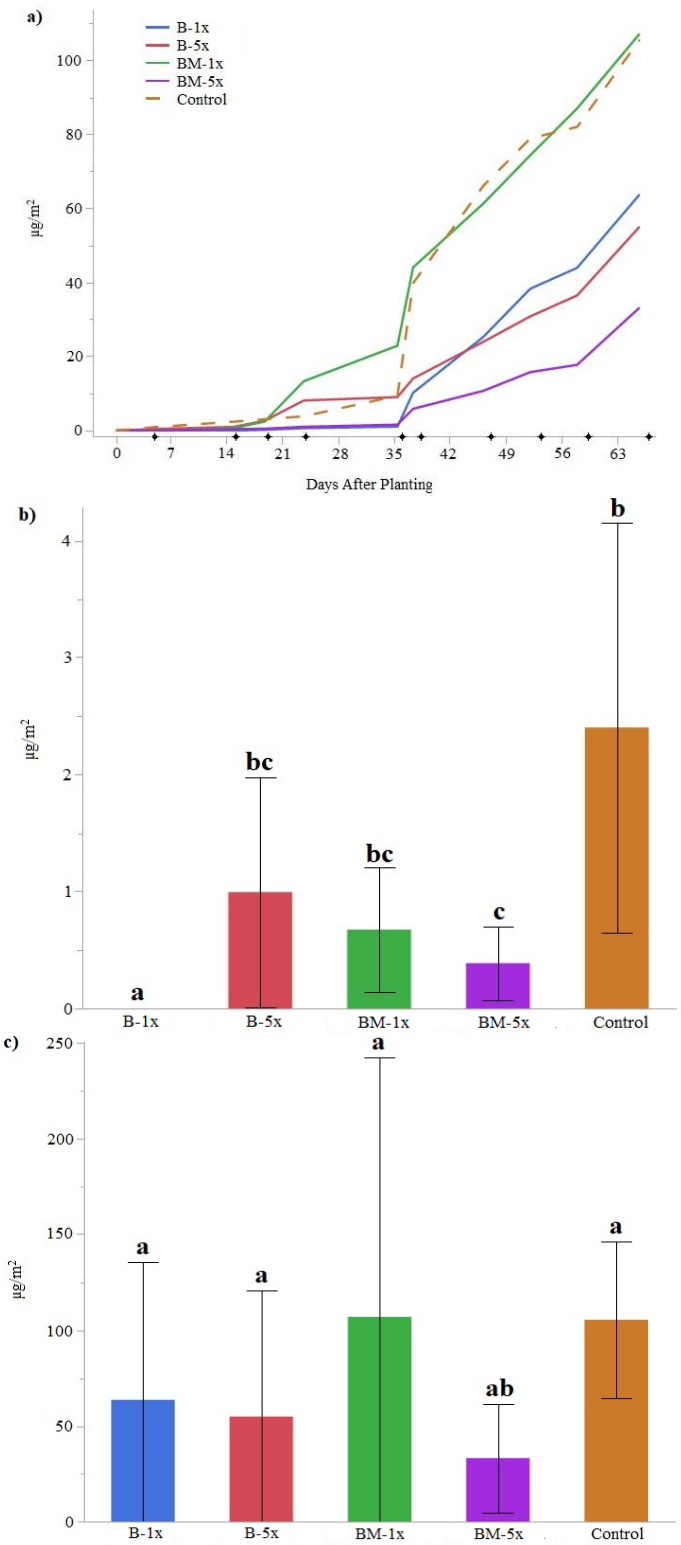

Figure 2.4 – Cumulative CLO (µg) collected in leachate a) throughout the season, b) by the second sampling date (15 days after planting), and c) by the end of the season (67 days after planting).

2.3.3 Soil

At the final sampling date, all treatments had the same concentration (Figure 2.5a) and residual mass (Figure 2.5b) of TMX as the control ($p \geq 0.1$). In contrast, three of four biosolid treatments, excluding the highest SOC (BM-5x), had lower concentrations (Figure 2.6a) and significantly less residual CLO mass (Figure 2.5b) than the control ($p < 0.1$).

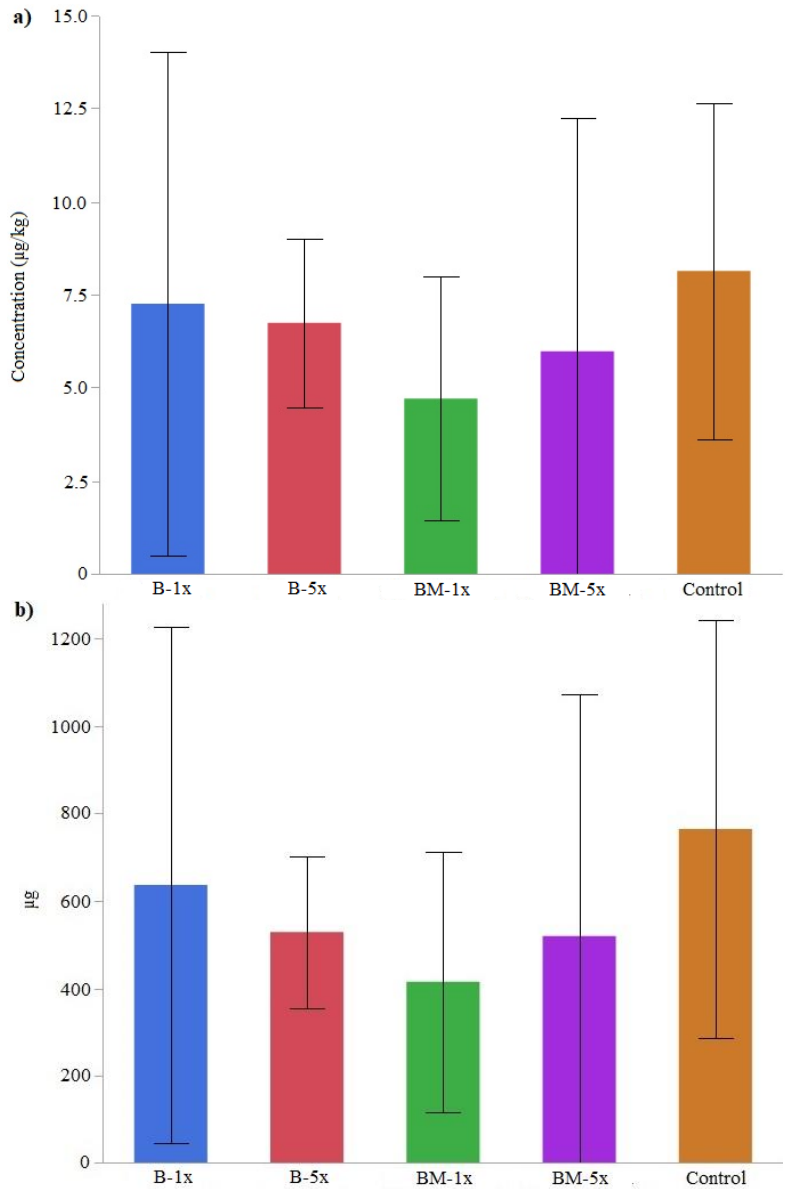

Figure 2.5 – a) Residual TMX concentration, and b) residual TMX mass in the soil at the end of the season.

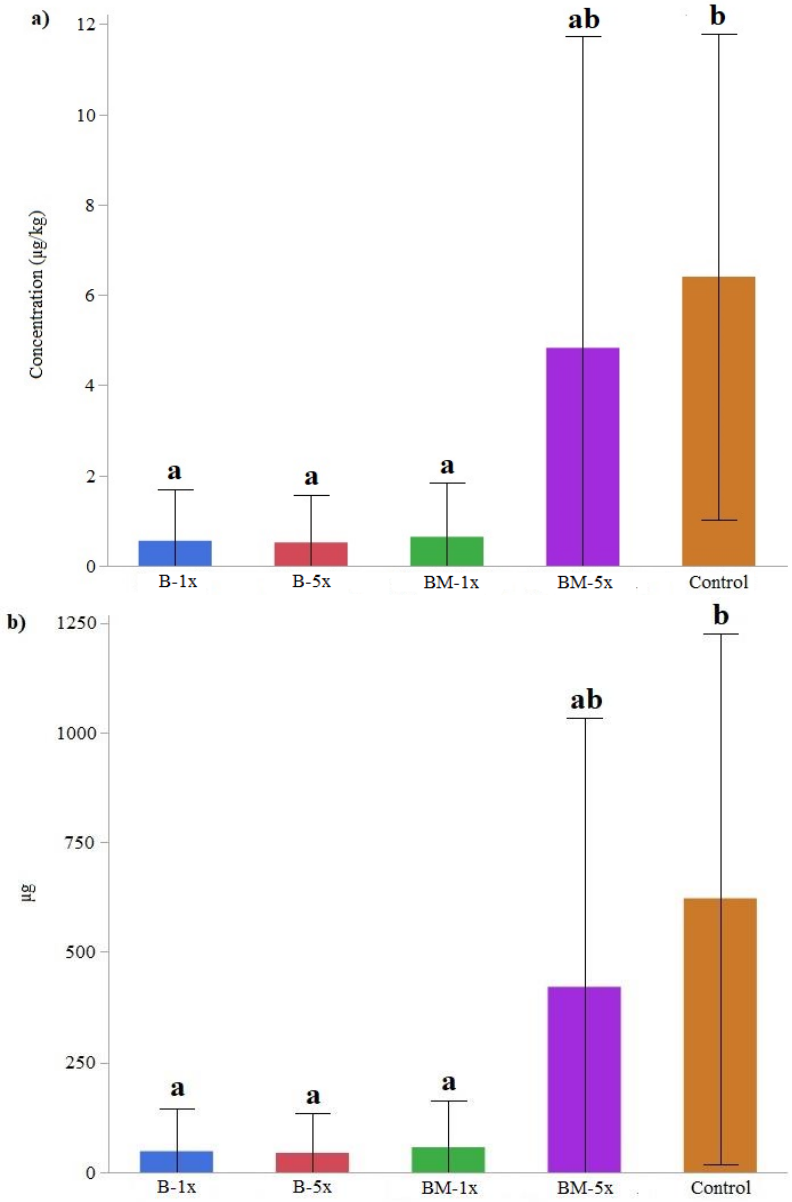

Figure 2.6 – a) Residual CLO concentration, and b) residual CLO mass in the soil at the end of the season.

For all replications, a mass balance was calculated accounting for applied TMX recovered in both soil and leachates. This included applied TMX recovered as CLO (hereafter referred to as TAC). There were no significant differences in recovered mass between treatments ($p \geq 0.1$; Figure 2.7).

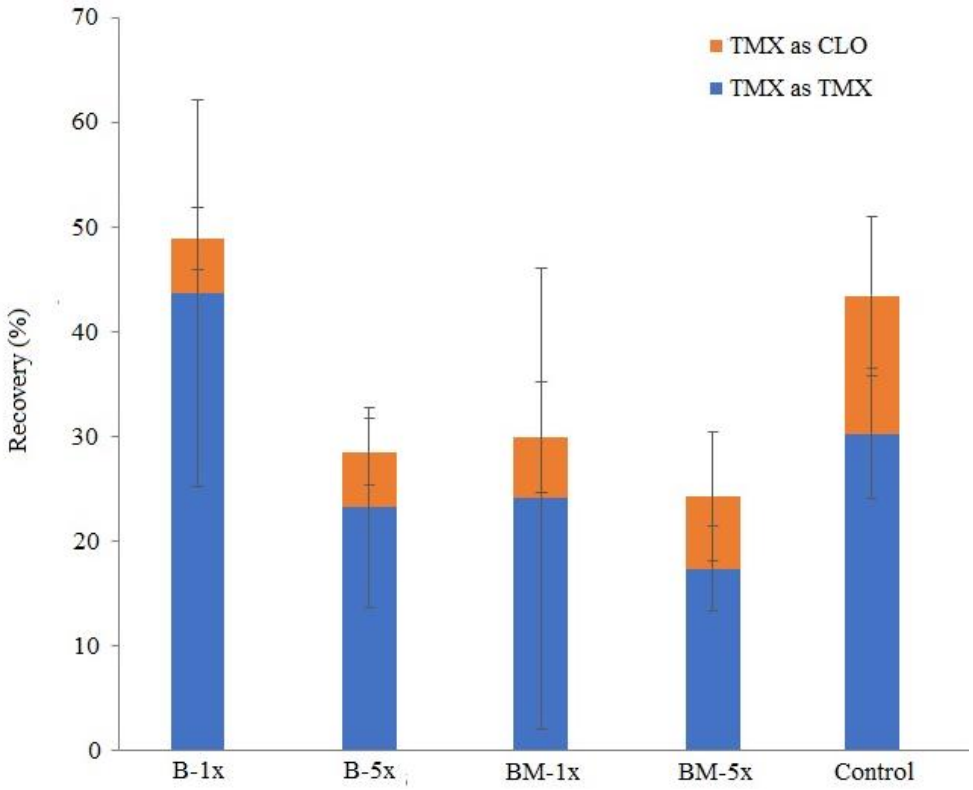

Figure 2.7 – Mass balance of applied TMX.

2.4 Discussion

The two main objectives of this study were to evaluate if varying sources and concentrations of SOC: 1) influence the persistence TMX and CLO in the soil; and 2) alter the ability of TMX and CLO to leach down through the soil profile.

I hypothesized that higher SOC concentrations would be associated with increased concentrations of TMX and CLO persisting in the upper soil profile. The experimental results, however, did not support my hypothesis. Specifically, there were no differences in TMX concentrations in soils, while the treatment with the lowest SOC concentration (i.e., the urea control) retained more CLO than all four biosolid treatments with increased SOC. This finding is also in contrast with Mörtl et al. (2016), who found that the higher organic matter content of a loam soil (compared to the sand and clay soils) contributed to higher persistence of both TMX

and CLO. Likewise, K_d values for both TMX and CLO can increase with increasing SOC (Dankyi et al., 2018). It is possible, however, that a high prevalence of dissolved organic carbon in the soil solution could cause competition between dissolved carbon and TMX or CLO for binding sites in the soil, which has been demonstrated for other neonicotinoids (Flores-Céspedes et al., 2002). While competition is a possibility, the likelihood of large amounts of competition for sorption sites is low in the studied soil.

Given that the control both leached more and had more persisting CLO than biosolid treatments, there is a possibility that biosolid additions decrease the transformation of TMX to CLO; however, this effect has not been studied. While the TMX to CLO pathway is common in plants and animals (Casida, 2011; Karmakar et al., 2009), its kinetics in soil are not well understood. Organic amendments can increase microbial biomass in soil (C. Stark et al., 2007), thus, it is possible that an increase in microbial diversity or abundance played a role in the degradation of TMX to compounds, such as urea, nitrosoguanidine, and desnitro compounds, rather than CLO (Rana et al., 2015) in biosolid treatments. It is also possible that organic matter may have stimulated CLO conversion to other compounds.

In a prior study, Radolinski et al. (2018) observed TMX concentrations in leachate of approximately 20 µg/L, which exceeded the EPA aquatic benchmark for acute invertebrate toxicity of 17.5 µg/L (Anderson et al., 2013). The TMX concentrations measured in this study were lower, reaching a maximum of 5.4 µg/L. Thus, the peak TMX concentrations in my study were less than one-third of those measured by Radolinski et al. (2018). At the same time, the TMX loading per square meter used in this study was approximately one-third of that of a typical corn planting due to differences in active ingredient applied to soybeans versus corn. These

results suggest that the mass of neonicotinoids applied to seed coatings may influence the maximum concentrations of those pesticides in leachate.

Although there were no significant differences in the amount of TMX leached at the end of the experiment, the soil with the highest SOC content leached significantly less TMX and CLO early in the experiment. These differences occurred in late spring, and therefore may correspond to the "spring flush" often observed in the literature (Hladik et al., 2014; Thurman et al., 1992). In this case, the flush occurred over a (< 1-week) period, after which time TMX mass became consistently low. Altogether, this spring flush in this experiment leached more than 50% or more of the season total in the first week alone. Although increased organic carbon may not decrease season long TMX leaching totals, we can see that high SOC may be a useful tool in decreasing the leached mass of TMX during periods when TMX loading to the environment is high.

CLO leaching was minimal for the first 2-3 weeks of the experiment, because degradation from TMX to CLO takes some time. The highest SOC treatment leached significantly less CLO than the control during the "spring flush" event and cumulatively at the end of the experiment. It is worth noting that CLO concentrations in leachate were minimal or zero during the spring flush. Apart from a spike late in the experiment, leached CLO mass per event in all treatments stayed approximately 8x lower than leached TMX mass (Figure 2.8). Since CLO is more toxic to invertebrates than TMX (Nauen et al., 2003), the delay in leaching and the absence of a large spike in CLO mass leached in the soil could reduce the downstream impacts. I also note that the mass balance used in this study showed that an average of 25-50% of the applied pesticide was recovered in the soils and leachate. This range is approaching the recovery seen in controlled laboratory studies (Gupta et al., 2008; Smalling et al., 2018), and

shows that the zero-tension lysimeters used in this study were effective in capturing the leaching dynamics of TMX.

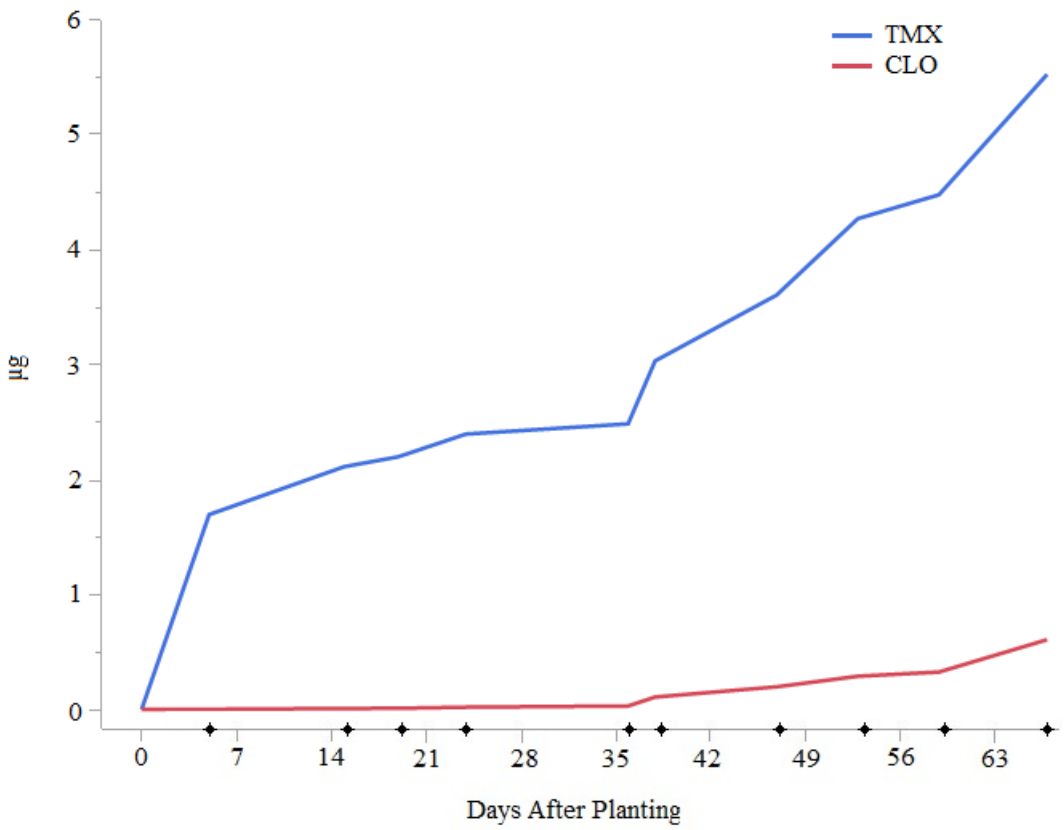

Figure 2.8 – Mass of TMX and CLO in leachates for the BM-5x treatment.

2.5 Conclusions

The goal of this study was to examine the relationship between the leaching and sorption of TMX and CLO, and the concentration of organic carbon in the soil. Using TMX-coated soybeans, I found that higher concentrations of SOC did not reduce the mass of either chemical leached out in over time. However, higher SOC content reduced the mass of TMX and CLO that leached early in the season, corresponding to a spring flush. At the end of the experiment, there were no significant differences in the mass of TMX retained in the soil, yet all biosolid amendments had significantly less CLO retained in the soil than the urea control. According to

my findings, adding biosolids is an adequate way to reduce thiamethoxam and clothianidin leaching during early season peaks, but not season totals. Further, biosolid additions may decrease the rate at which TMX is converted to CLO in the soil, compared to untreated control.

3 Neonicotinoid Pesticide Uptake by Plants Used as Buffers and Cover Crops

Abstract

Runoff and drainage from fields planted with neonicotinoid-coated seeds often contain pesticide concentrations considered toxic to aquatic life and other non-target organisms. Management strategies such as in-field cover cropping and edge-of-field buffer strips may reduce pesticide mobility, yet it is not known if and how well different plants used in these interventions can sequester neonicotinoids. In this greenhouse study we evaluated uptake of thiamethoxam, a commonly used neonicotinoid, in six species – crimson clover, fescue, oxeye sunflower, Maximillian sunflower, common milkweed, and butterfly milkweed – along with a forb mixture and a native grass plus forb mix. We also examined thiamethoxam partitioning within plant tissues. All plants were irrigated with water containing 100 or 500 µg/L of thiamethoxam for 60 days, after which time tissues and soils were sampled and analyzed for thiamethoxam and its metabolite clothianidin. The results showed that crimson clover absorbed significantly more of the applied thiamethoxam than all other plants, suggesting that it has good potential for sequestering neonicotinoids from soil. In all plants, recovered masses of thiamethoxam and clothianidin were higher in above-ground tissues (leaves and stems) than below-ground roots, with leaves having higher concentrations and more mass than stems. Because thiamethoxam tends to accumulate in above-ground tissues, management strategies that include biomass removal may help reduce the input of pesticides like TMX from agricultural operations to the adjacent environment. At the same time, high pesticide concentrations in leaf tissues indicate that special consideration should be taken to ensure that species chosen will not put beneficial insects at elevated risk of exposure.

Keywords

thiamethoxam, clothianidin, runoff, leaching, edge-of-field buffer, wildflowers, prairie strips, crimson clover

3.1 Introduction

Since the introduction of imidacloprid (IMD) in 1991, neonicotinoids have become the most widely used class of insecticides in the world (Jeschke, 2008). In 2018, approximately 36 million hectares of neonicotinoid-coated corn seed were planted in the U.S. (Honig, 2018). Besides IMD, neonicotinoid seed coatings also include thiamethoxam (TMX) or clothianidin (CLO). As of 2014, farmers were applying more than 100 g of TMX per square kilometer per year in many areas of the United States (USGS, 2018). The high prevalence of neonicotinoids in the soil in these areas could potentially make them a non-point source for pollution in adjacent areas and waterways.

Thiamethoxam has an aqueous solubility of 4.1 g/L and CLO has a solubility of 0.33 g/L. High solubility means that any pesticide mass not taken into the target plant or quickly degraded can be transported by moving water. For instance, previous studies have detected the presence of neonicotinoids in soils and water bodies surrounding fields planted with coated seeds (Hladik et al., 2014). The highest concentrations often come in late spring and early summer during a period known as the “spring flush” (Thurman et al., 1991). Spring flushes occur when wet soils and high rainfall coincide with planting of neonicotinoid coated seeds, creating ideal conditions for the pesticides to be washed out of the fields and into waterways. Aside from the spring flush, neonicotinoids have been detected in both rural and urban water bodies year-round (Starner et al., 2012; Yamamuro et al., 2019).

Solubility also aids in uptake and translocation within plants (Maienfisch et al., 2001). Neonicotinoids have high xylem mobility, allowing the chemical to be rapidly transported from roots to above ground tissues (Ge et al., 2017; Li et al., 2018; Sur & Stork, 2003). Under typical growing conditions, however, target crops may absorb less than 5% of the neonicotinoid mass applied within seed coatings (Sur & Stork, 2003). Depending on environmental conditions the remaining mass can accumulate within fields (Gupta et al., 2008), or can accumulate in plants growing in field margins. A study on wild plants around oilseed rape fields found that 52% of samples tested positive for one of three neonicotinoid insecticides (IMD, CLO, and TMX) at concentrations ranging from 8.71 ± 21.13 µg / kg of TMX, 0.51 ± 1.67 µg / kg of CLO, and 1.19 ± 4.28 µg / kg of IMD, while 46% tested positive for two or more (Botias, 2016) at similar concentrations. These findings point to the need to develop better strategies to prevent neonicotinoids from exiting fields.

Cover cropping and edge-of-field buffer strips have been used to solve other water quality issues caused by excess soil erosion (Battany & Grismer, 2000), nutrient leaching (Wyland et al., 1996), and pollutant runoff (Dabney, 2006). Perennial grasses such as fescue [*Festuca arundinacea*] are commonly used in vegetated buffer strips, while annual grasses such as cereal rye [*Secale cereale*] and legume such as crimson clover [*Trifolium incarnatum*] are widely used as cover crops. These plants tend to be highly effective in slowing surface water runoff (Carluer, 2017) and decreasing pollution (Fox, 2010), yet provide little additional biodiversity within agroecosystems. For this reason, some farmers and agencies have begun planting buffers with pollinator-friendly species such as butterfly milkweed [*Asclepias tuberosa*], lance leaf coreopsis [*Coreopsis lanceolate*], and purple coneflower [*Echinacea purpurea*] (Dively et al., 2020).

Careful selection of pollinator-friendly species can increase pollinator traffic to an area, and perennial prairie strips can also increase the abundance and diversity of native bird species (Schulte et al., 2016). At the same time, species-rich wildflower strips can increase crop yield and decrease insect pest presence more effectively than sown grass strips (Carvell et al., 2006; Haaland et al., 2011). Sunflowers can be used to take up contaminants from the soil (Indelicato, 2014) and also provide a pleasing aesthetic, making them a good candidate for use in buffer strips. For these reasons, wildflower strips adjacent to fields may be both desirable and effective solutions. However, few or no studies to date have evaluated the efficacy with which different non-target plant species take up neonicotinoids from soils. Further, there is limited knowledge of how neonicotinoids become partitioned within different plant tissues once taken up, which can be important for optimizing management strategies and reducing possible pesticide exposure by pollinators and insects that may rely on these plants.

My objectives were to: 1) to measure TMX uptake and accumulation in plants that may be used as cover crops or in edge-of-field buffers; and 2) to measure the relative distribution of TMX and CLO within plant tissues. These results can be used to make recommendations for vegetated buffer plantings and cover crops that may help to reduce neonicotinoid migration from agricultural fields.

3.2 Materials and Methods

3.2.1 Soil characterization

This study took place in a greenhouse in Blacksburg, VA, from October 2018 to January 2019. Soil was obtained from the Northern Piedmont Research Center in Orange, VA, and was classified as a Starr silt loam (*Fluventic Dystrudept*). Textural class was determined using a laser-diffraction particle size analyzer (CILAS 1190; CILAS Inc.; Orleans, France). The soil was

screened to eliminate larger aggregates (>2.5 cm) and was then packed in 2.5 cm increments into pots until reaching the measured field bulk density (1.28 g/cm^3). An initial analysis showed background TMX concentrations in the soil were 0.05 ng/g. No nutrient amendments were made during the experiment.

Cation exchange capacity (CEC) was determined using the summation of cations method (Maguire & Heckendorn, 2005). Approximately 5 g of soil was added to a 60 mL straight-walled plastic test tube along with 20 mL of Mehlich I extract solution. Tubes were capped and shaken for 5 minutes and then the solution was filtered through a Whatman No. 2 filter. pH was obtained from the filtered solution using a benchtop analyzer (Orion Star A211; Thermo Scientific; Waltham, MA; U.S.), and the remaining solution was analyzed for elemental composition using a Spectro ARCOS II Multiview ICP-AES (Model: FHM22 with CETAC Autosampler; Spectro Scientific – Ametek; Chelmsford, MA; U.S.). Soil CEC was calculated using:

$$\text{CEC} = \text{Acidity} + \text{Ca} + \text{Mg} + \text{K} \tag{3.1}$$

where Acidity (meq/100 g of soil) is calculated as:

$$\text{Acidity} = 37.94 - (5.928 \times \text{BpH}) \tag{3.2}$$

and BpH is equal to the pH reading taken from the filtered solution.

3.2.2 Experimental Design

A factorial design was used with the following factors (n = 3): plant type (8 species and mixtures, plus a no plant control; Table 3.1), and TMX application rate (100 versus 500 µg/L TMX in irrigation). Note that TMX concentrations were calculated using peak concentrations of approximately 20 µg/L measured during a preliminary field study (Radolinski et al., 2019; Radolinski et al., 2018). This observed concentration was multiplied by five (100 µg/L) and

twenty-five (500 µg/L) to account for the relatively low planting density in the preliminary study, and also to ensure that concentrations at the end of the study would be detectable. We still feel that these concentrations are possible for corn and soybean plantings that encompass large land areas, such as the American Midwest.

Both species of sunflower (*Helianthus maximillianii* and *Heliopsis helianthoides*) and milkweed (*Asclepias syriaca* and *Asclepias tuberosa*) were potted in 11.4 L pots so they would not become root bound; all other species, the mixes, and the no plant control were potted in 3.8 L containers. The composition of the forb and native grass + forb mixtures are detailed in the Appendix (Tables B and C). Note that milkweed was included in both forb mixes; however, we also grew individual common milkweed and butterfly milkweed plants in 11.4 L pots to isolate plant uptake, given their importance as the food source for the monarch butterfly (Wassenaar & Hobson, 1998).

Table 3.1 – Plant species and mixtures used as experimental treatments, separated by container size (3.8 L versus 11.4 L).

3.8 L	**11.4 L**
Crimson clover [*Trifolium incarnatum ssp. Dixie*]	Maximillian sunflower [*Helianthus maximillianii*]
Fescue [*Festuca arundinacea*]	Oxeye sunflower [*Heliopsis helianthoides*]
Forb Mix[1]	Common milkweed [*Asclepias syriaca*]
Forb + Native Grass Mix[2]	Butterfly milkweed [*Asclepias tuberosa*]

[1]Wildflower mix containing wildflowers native to the northeast U.S., provided by Ernst Seeds. Showy Northeast Native Wildflower Mix - ERNMX-153-1 (Appendix Table A)
[2]Wildflower mix containing wildflowers native to the northeast U.S. with the addition of native grasses, provided by Ernst Seeds. Showy Northeast Native Wildflower & Grass Mix - ERNMX-153 (Appendix Table B)

Seeds were planted and allowed 45 days to germinate and become established. This period replicated the approximate species growth stage at the time of corn planting. During this

time, the samples were irrigated with tap water. After the 45-day establishment period, the samples were irrigated with TMX-spiked water at 100 µg/L or 500 µg/L. The concentration of TMX in the irrigation water was remained constant throughout the experiment for 60 days. We used repeated applications of TMX as opposed to a single application to replicate the continued delivery of TMX from seed coatings throughout the early growing season.

Pots with soil but no plant were included in the experiment as a control. These pots were watered with TMX-spiked water for 60 days (n = 3 per TMX concentration). We also grew a set of all plant species and mixtures that were irrigated using tap water without TMX (n = 1) to verify that there were no unknown sources of TMX in the study. In total we had 62 pots: 34 of the smaller size (3.8 L) and 28 of the larger size (11.4 L).

Plants were given a 14-hour photoperiod using a 90-watt white LED source to simulate day length in the field during the growing season. The 3.8 L pots were irrigated every 3 days and the 11.4 L pots every 5 days. All containers were weighed prior to irrigation, at which point we added enough water to return each one to field capacity. Field capacity was determined by analyzing cores from the field site using pressure pots set to 33 kPa, as well as by performing small-scale drainage tests on the pots prior to the beginning of the experiment. Note that irrigation to field capacity was designed to eliminate loss of TMX and nutrients due to runoff or leaching, and to distribute the TMX evenly throughout the soil in each pot. As an additional precaution against water losses, saucers were placed under the pots during watering; any drainage and runoff was collected and re-applied immediately following irrigation.

Temperature, humidity, and dew point were recorded by a miniature weather station (HOBO MX 1101 Bluetooth data logger; Onset Corporation; Bourne, MA; U.S.).

3.2.3 Troubleshooting

During the experiment, two remedial interventions were necessary. While the plants were still in the establishment stage (no TMX irrigation) some plants, mainly common milkweed, and crimson clover, began to develop significant populations of aphids. This infestation was accompanied in its later stages by ants farming the aphids for use of their honeydew. To resolve this issue, a light application of oak wood ash was sprinkled on all plant species affected by the aphids. After this step, aphid presence was minimal and local to plants with no TMX irrigation.

The second remedial application was a foliar spray of myclobutanil (Systhane), at a rate of 0.4 mg/L, to eliminate powdery mildew on Maximillian sunflowers. The sunflowers were sprayed on Day 64, and then all other 11.4 L plants were sprayed as a preventative measure on Day 66. Plants were sprayed until they were wet, and a total of 0.5 L of fungicide was applied. The application successfully eliminated the powdery mildew; however, one of the sunflowers remained stunted for the rest of the experiment.

3.2.4 Harvest

At the end of the experiment (105 days after planting), all plants were harvested by destructive sampling. Any excess soil adhered to the plant samples was washed off with deionized water and discarded. Plants in the 11.4 L pots were then separated by tissue type as roots, stems, or leaves. Plants in the 3.8 L pots were separated into below-ground (i.e., roots) versus above-ground (i.e., stems and leaves) tissue samples, due to the negligible mass of stems in those treatments. All samples were frozen after harvesting until time of analysis.

Soil samples were also collected during this process, by homogenizing the unconsolidated soil that remained after the root harvest. Approximately 100 g of soil was taken from each pot.

3.2.5 Sample Preparation and Extraction

All plant tissue samples were first ground with liquid nitrogen. Samples were frozen after harvest and stored in a freezer until grinding. To grind the samples, plant tissue was taken from the freezer and placed into a ceramic mortar. Large samples were cut into smaller pieces with clean scissors before grinding. Sufficient liquid nitrogen was applied to the mortar to submerge 75% of the sample. The samples were then ground to the consistency of sand/powder with the pestle. If the sample began to appear wet or start to smear in the mortar, then more liquid nitrogen was applied until the desired consistency was met. Each sample was then left for 10 minutes to allow any remaining nitrogen to evaporate.

After evaporating, the sample was weighed (wet mass) and transferred to a 25 mL polystyrene dilution vial (Item #201-5266-050; Evergreen Scientific; Buffalo, NY, U.S.). Samples were left in the freeze drier for 48 hours, after which time the sample was reweighed (dry mass). Water content of the samples at the time of collection was calculated on a gravimetric basis as the difference between the wet and dry masses divided by the dry mass. After samples were ground, I scrubbed the mortar and pestles with a brush and rinsed them out with water. Then they were submerged in 0.1 M HCl for 12 hours.

For plant tissue extraction, 1.0 ± 0.1 gram of prepared soil was weighed into a 60 mL straight wall centrifuge tube. 750 µL of CLO-d3/TMX-d3 was spiked with deuterium-labeled TMX (TMX-d3) and CLO (CLO-d3) stock solution (539 ng/g TMX-d3 and 518.5 ng/g CLO-d3) as internal standards and left uncapped to allow the carrier solvent to evaporate. After evaporation, 0.5 ± 0.05 grams of dried $MgSO_4$ and NaCl were added to each tube, before adding 3 mL of Milli-Q water and exactly 10 mL of acetonitrile. Tubes were then capped and vortexed for 1.5 minutes at 2,200 rpm using a VM-3000 Mini Vortexer (VWR, Inc.; Radnor, PA, U.S.).

Using a swinging bucket centrifuge, tubes were then centrifuged for 10 minutes at 4,000 rpm. After the centrifuge, a plug consisting of plant material, water, and reagents had formed in the bottom, and the acetonitrile on top was transferred to a new 60 mL straight wall tube containing: 0.1 ± 0.01 g of primary secondary amine, 0.1 ± 0.01 g of end capped C18, and 0.05 ± 0.001 g of graphitized carbon black to further clean the plant tissues. Tubes were then capped and vortexed again at 2,200 rpm. This was followed by the centrifuge again, at 4,000 rpm for 15 minutes. At this point supernatant was filtered through a 0.2 µm PTFE filter and diluted into a UPLC mobile phase solution consisting of 5 mM NH_4AC in MeOH: 5 mM NH_4AC in H_2O, 9:1 v=v, and stored in a capped, 2 mL amber HPLC vial until analysis. Dilution rate was dependent on the treatment group, 100:900 (supernatant: mobile phase, v/v) for the plant tissue from the 500 µg/L irrigation treatment, and 200:800 (supernatant: mobile phase, v/v) for the plant tissue from the 100 µg/L irrigation treatment. 1.5 mL of filtered supernatant was saved in in a 2 mL amber HPLC vial and preserved at 0° C in case re-dilution was necessary.

For analysis, approximately 40 g of wet soil from each sample was placed in a 50 mL centrifuge tube. The exact mass of added soil was recorded (wet mass). Without capping, I covered the tube with a paper towel folded in half and secured with a rubber band and placed them in a freeze drier for 48 hours. At the end of that period, the samples were removed from the freeze drier and weighed immediately (dry mass). I calculated gravimetric water content at time of sampling as difference between wet and dry mass divided by dry mass.

The final step in preparing the soil samples was grinding. One at a time, the soil was poured from the vial into a large plastic weigh boat and any hard material (rocks, sticks, etc.) were removed before the remaining material was added the grinder. I ground the samples for 15 seconds using a coffee grinder. The resulting powder was poured back into the weigh boat and

any large, hard particles that were missed initially were removed. The process repeated until all small aggregates were broken apart. The soil was then poured back into the original tube, capped, and stored in the freezer at 0° C.

For extraction, 1.0 ± 0.1 gram of prepared soil was weighed into a 60 mL straight wall centrifuge tube. The soil was spiked with deuterium-labeled TMX (TMX-d3) and CLO (CLO-d3) stock solution (539 ng/g TMX-d3 and 518.5 ng/g CLO-d3) as internal standards and left uncapped to allow the carrier solvent to evaporate. Internal standards were used to correct for variability due to TMX/CLO loss in extraction and cleanup and matrix effect during instrumental analysis. Fifteen mL of HPLC-grade acetonitrile were then added to the solution with a pipette and the tube was capped immediately. The tube containing the sample was then vortexed for 2 minutes at 2,300 rpm using a VM-3000 Mini Vortexer (VWR, Inc.; Radnor, PA, U.S.). After vortexing, the solution was placed in a centrifuge for 15 minutes at 5000 rpm. With a pipette, 5.0 mL of the solution was taken from the 60 mL tube and transferred to a 12 mL screw top glass tube without a cap and placed in a vacuum evaporator (RapidVap, Labconco Corporation, Catalog No. 7900002). The vacuum evaporator was set to run at 75 °C, 330 mbar, and 95% speed for 180 minutes, or until a sample was completely dried. After the test tubes were cooled to room temperature, the dried residue in each glass tube was re-constituted using 1.0 mL of UPLC mobile phase (5mM NH_4Ac in H_2O: 5mM NH_4Ac in MeOH, v:v = 1:9) and vortexed for 10 seconds. This solution was then extracted with a 1 mL syringe and filtered through a 0.2 µm PTFE filter, and into a 2 mL amber HPLC vial with cap.

For UPLC/MS/MS analysis procedures and settings, refer to Chapter 2.2.4.

3.2.6 Data analysis

Unit Conversions

To determine total recovered mass of TMX or CLO (µg) in a particular plant tissue, concentrations identified in the tissue using the UPLC/MS/MS (in µg/kg dry weight) were multiplied by the dry mass of the plant tissue m_{pool} (in kg:

$$m_{TMX,\,i} = [TMX]_{pool,\,i} \times m_{pool,\,i} \quad (3.3a)$$

$$m_{CLO,\,i} = [CLO]_{pool,\,i} \times m_{pool,\,i} \quad (3.3b)$$

where *i* represents the individual pools in which TMX and CLO were sampled (i.e., roots, stems, leaves, soil).

Applied TMX recovered as CLO (hereafter referred to as TAC) was also calculated by assuming a 1:1 molar conversion of TMX (291.7 g/mol) to CLO (249.7 g/mol):

$$m_{TAC,\,i} = m_{CLO,\,i} \times \textit{TMX molecular mass / CLO molecular mass} \quad (3.4)$$

where m_{TAC} is the equivalent mass of TMX that was detected as CLO (m_{CLO}) in each pool *i*.

The masses of TMX and TAC within a plant, along with the total recovered thiamethoxam ($m_{total,\,plant}$), were calculated as:

$$m_{TMX,\,plant} = m_{TMX,\,stems} + m_{TMX,\,leaves} + m_{TMX,\,roots} \quad (3.5a)$$

$$m_{TAC,\,plant} = m_{TAC,\,stems} + m_{TAC,\,leaves} + m_{TAC,\,roots} \quad (3.5b)$$

$$m_{total,\,plant} = m_{TMX,\,plant} + m_{TAC,\,plant} \quad (3.5c).$$

Percent recovery of TMX was then calculated for plants plus soils (*% Recovery, total*) and for plant tissues only (*% Recovery, plant*) by:

$$\% \ Recovery, total = 100 * (m_{total, plant} + m_{TMX, soil} + m_{TAC, soil}) / m_{applied, TMX} \quad (3.6).$$

$$\% \ Recovery, plant = 100 * m_{total, plant} / m_{applied, TMX} \quad (3.7)$$

TMX masses were also used to calculate relative distribution within the plant:

$$\% \ Distribution, i = m_{pool, i} / m_{total, plant} \quad (3.8).$$

Statistical Analysis

All analysis matrices were transformed using cube root or log functions to fit a normal distribution. After transformation, one-way ANOVA was performed, and post-hoc Tukey HSD determined if significant differences between treatments exist. If the data were not normally distributed after transformation, a Wilcoxon/Kruskal-Wallis nonparametric analysis was performed. For all tests, $\alpha = 0.05$. Statistical analyses were performed using JMP 15 software (SAS Institute; Cary, NC, U.S.)

3.3 Results

3.3.1 Environmental conditions

The CEC for this soil was 36.7 $cmol_c$ / kg soil and the pH = 6.1. Greenhouse temperatures never fell below 15 °C or exceeded 25 °C, and relative humidity remained between 30-50% for most of the experiment (Figure 3.1).

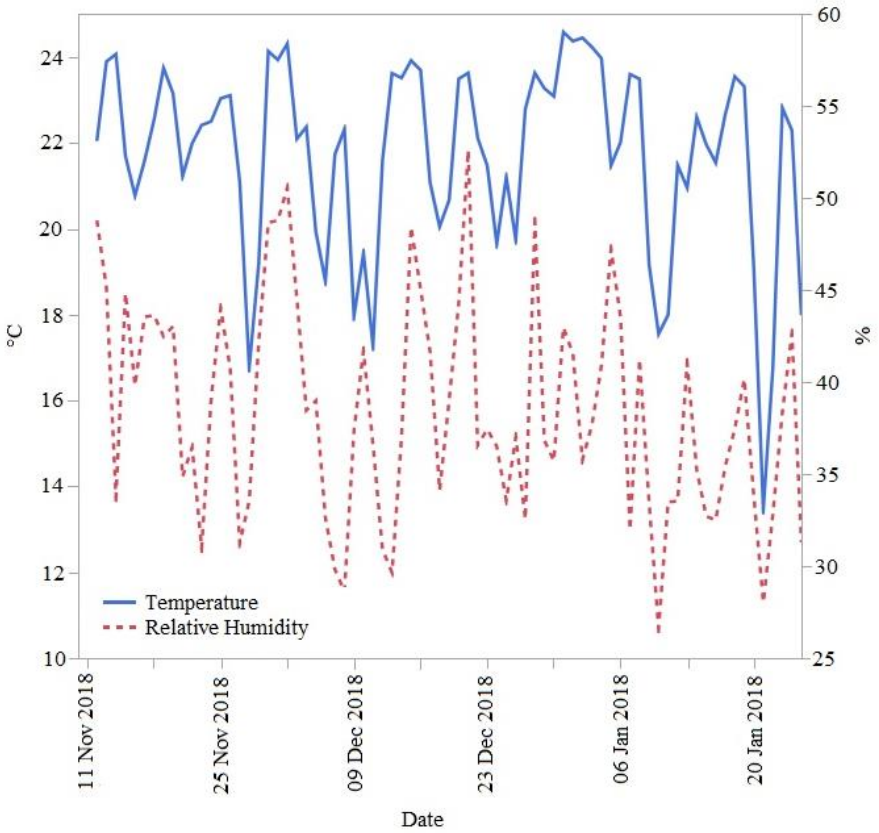

Figure 3.1 – Daily temperature and humidity during the study period.

3.3.2 Irrigation and TMX Applied

Crimson clover and fescue received significantly higher cumulative irrigation than all other species and the no-plant control ($p < 0.05$; Figure 3.2). The amount of water applied to butterfly milkweed and common milkweed did not significantly differ from the no-plant control, probably because the plants were grown individually and therefore had relatively low transpiration per pot.

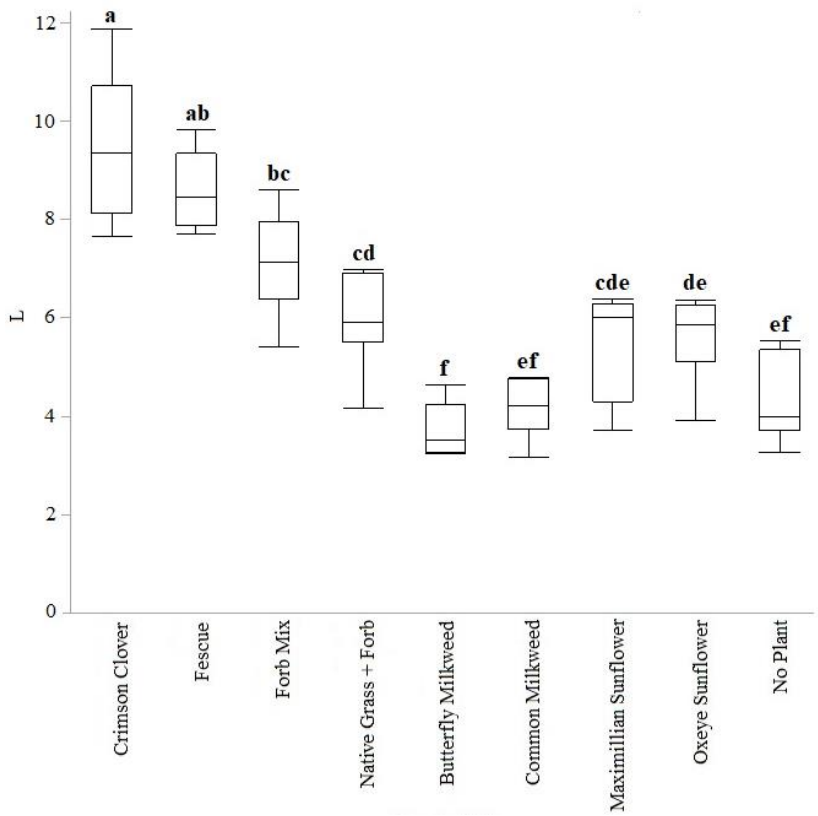

Figure 3.2 – Cumulative irrigation added for each plant type during the 45-day period of TMX additions. Different lower-case letters indicate significant differences ($p < 0.05$).

After adjusting for differences in soil surface area between pots, applied TMX followed the same trend as applied irrigation for both the 100 and 500 µg/L treatment groups. In both treatment groups, crimson clover and fescue were subject to more total TMX input than all other species except the forb mix ($p < 0.05$; Figure 3.3a and 3.3b).

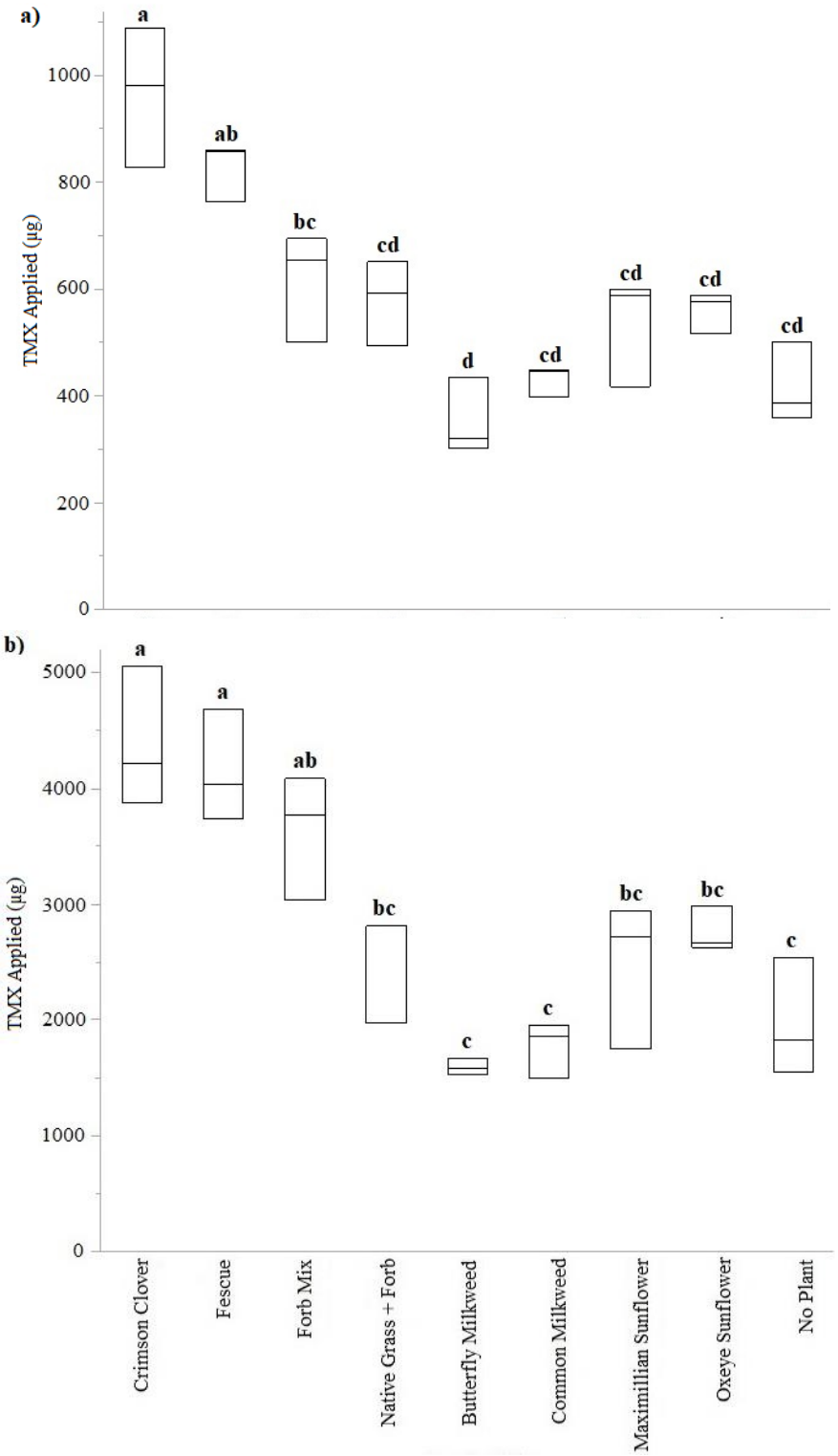

Figure 3.3 – Cumulative TMX mass applied over the 60-day water period in the a) 100 µg/L treatment group, and b) 500 µg/L treatment group. Different lower-case letters indicate significant differences ($p < 0.05$).

3.3.3 Concentrations

Crimson clover, fescue, and the native grass + forb mix had significantly higher TMX concentrations in stem and leaf tissues (Figure 3.4a) and root tissues (Figure 3.4b) than both milkweed species ($p < 0.05$). Butterfly milkweed had significantly lower TMX concentrations in leaves and stems that all other plants (Figure 3.4a) and had significantly lower root tissue concentrations than all species except common milkweed (Figure 3.4b). The CLO concentrations in stem and leaf were significantly higher in fescue, oxeye sunflower, and the native grass + forb mixture compared to both milkweed species ($p < 0.05$), and the forb mix also had significantly higher CLO concentrations than butterfly milkweed ($p = 0.0306$; Figure 3.5a). Fescue also had significantly higher CLO concentrations in root tissues than butterfly milkweed, common milkweed, fescue, and the forb mix ($p < 0.05$; Figure 3.5b). No differences were observed in soil TMX concentrations (Figure 3.6a), whereas soil CLO concentrations differed between plants, with fescue having higher concentrations than all plants except the forb mix (Figure 3.6b).

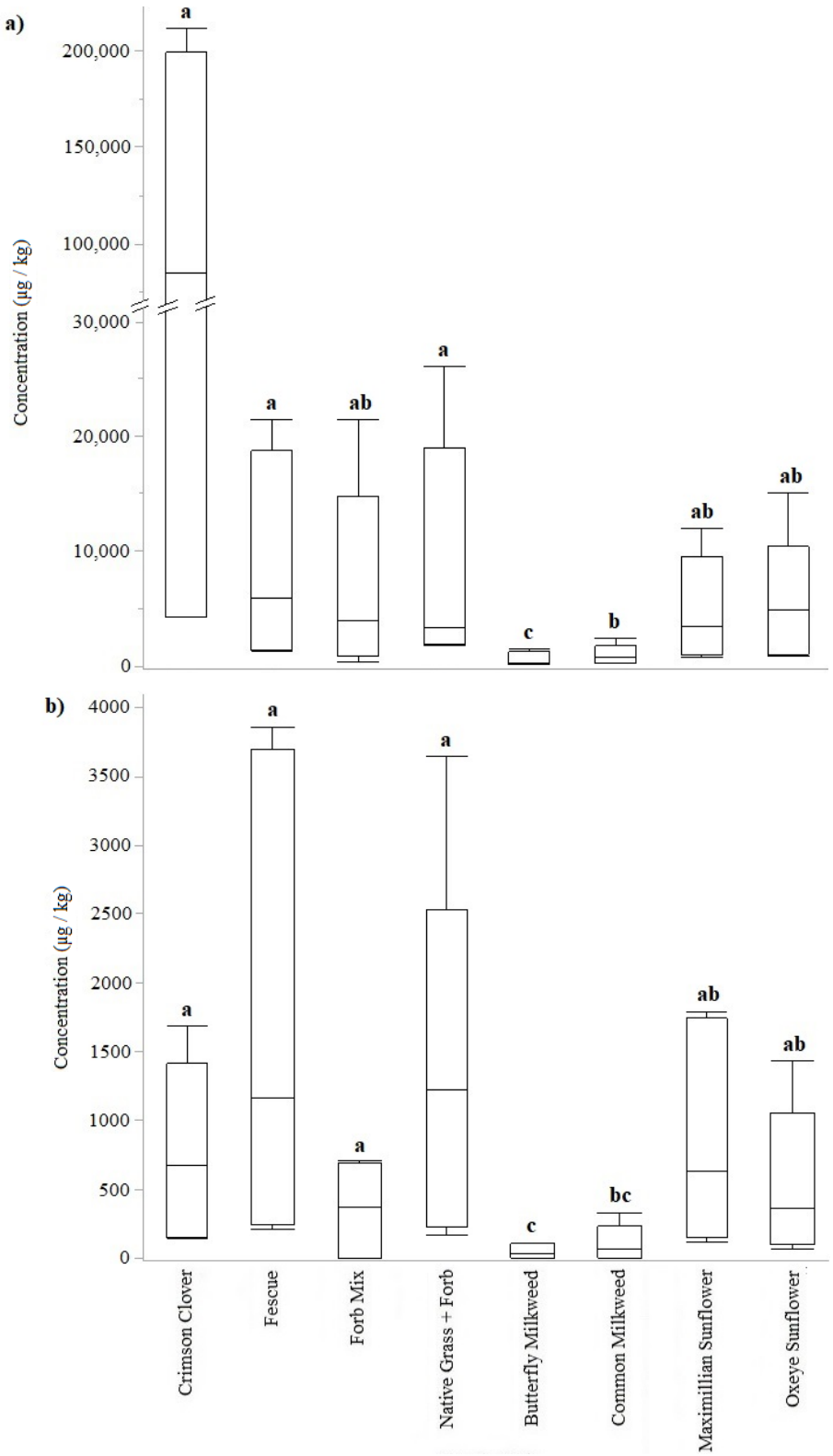

Figure 3.4 – TMX concentrations in a) above-ground stem and leaf tissues and b) below-ground root tissues. Different lower-case letters indicate significant differences ($p < 0.05$).

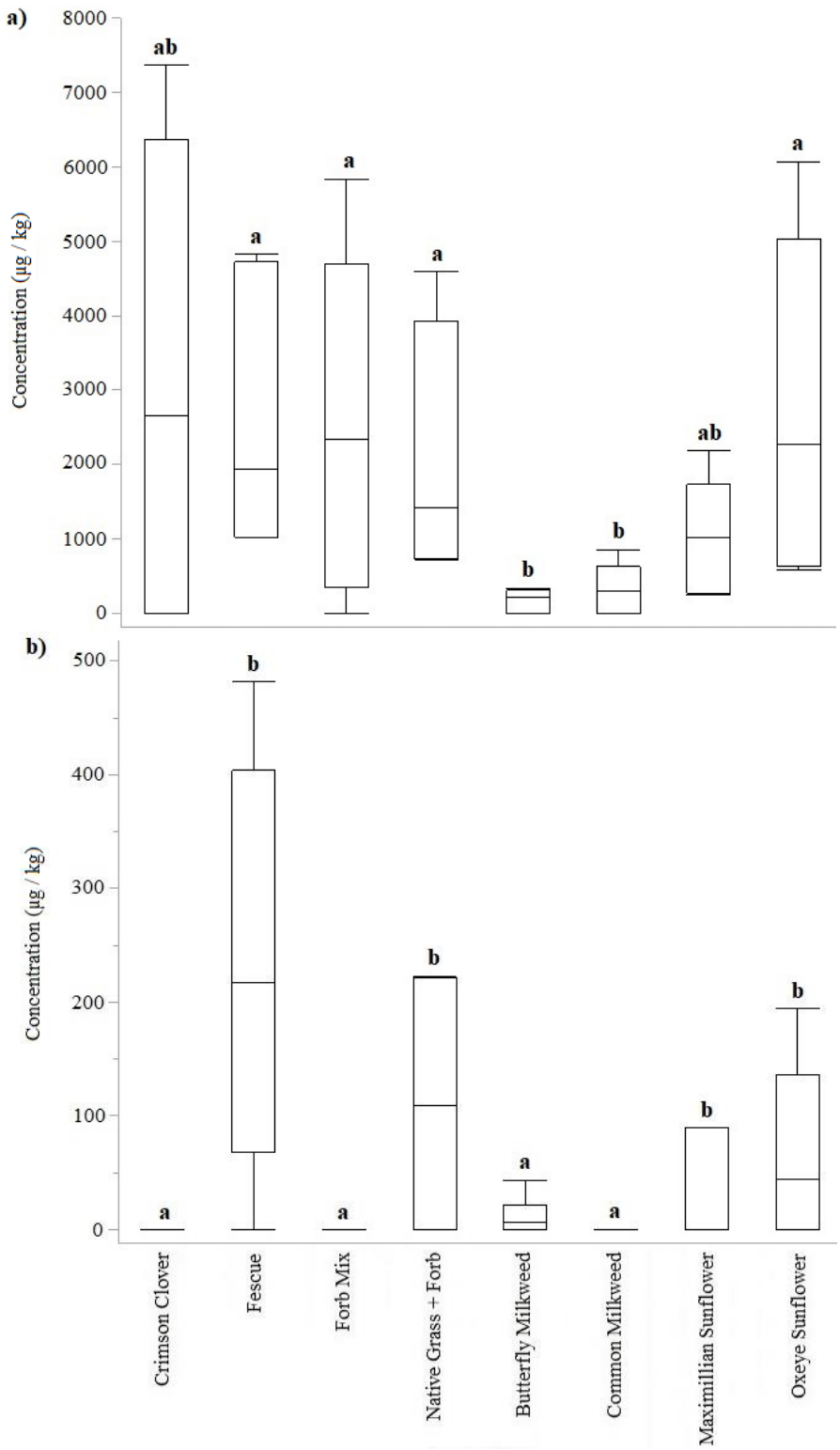

Figure 3.5 – CLO concentrations in a) above-ground stem and leaf tissues and b) below-ground root tissues. Different lower-case letters indicate significant differences ($p < 0.05$).

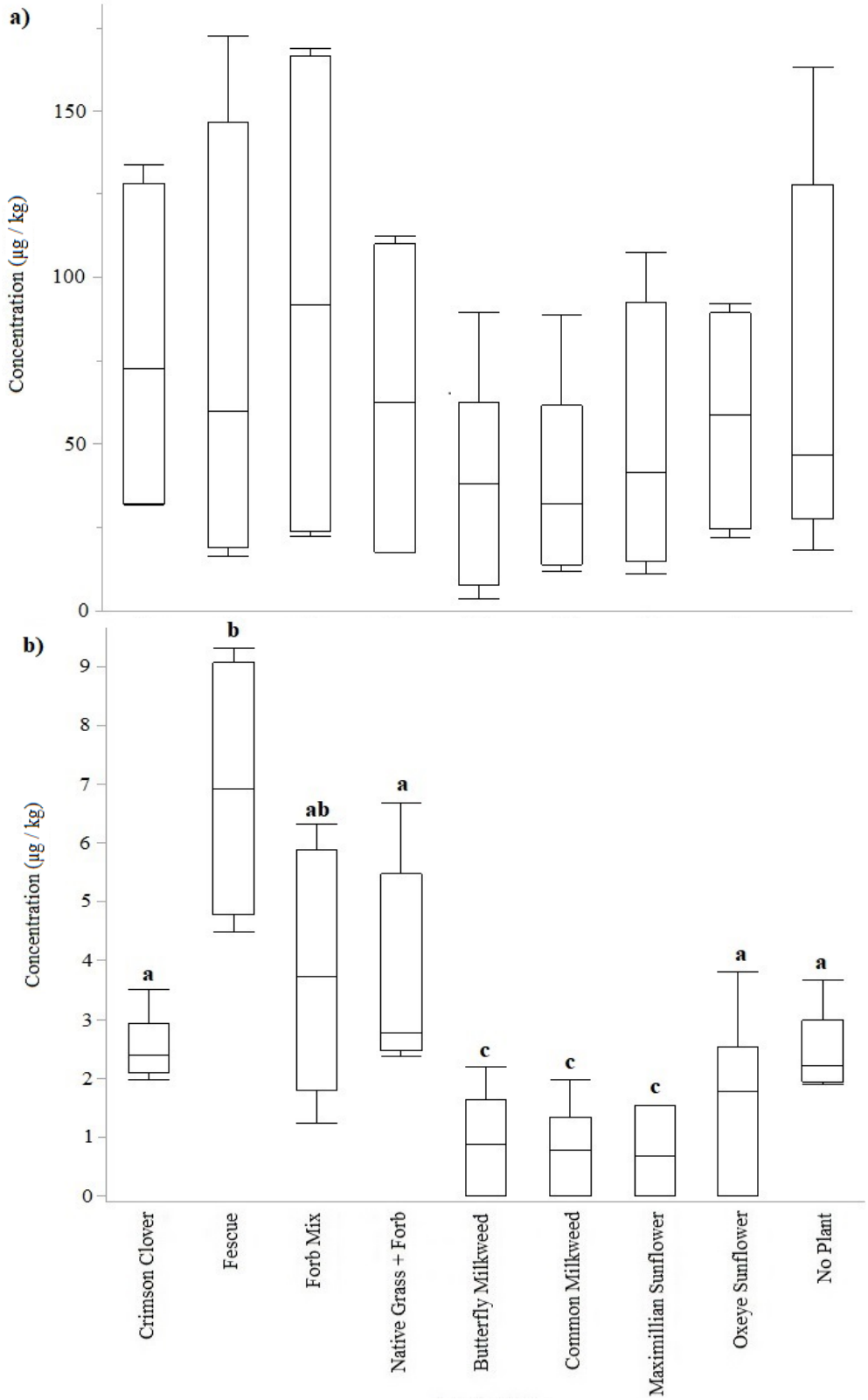

Figure 3.6 – Soil concentrations at the end of the 105-day growing period of a) TMX and b) CLO. Different lower-case letters indicate significant differences ($p < 0.05$).

The above-ground tissue concentrations were also separated between leaves and stems for species planted in the 11.4 L species. Oxeye sunflower had significantly higher TMX concentrations in leaf tissues than both butterfly and common milkweed ($p = 0.0131, 0.0453$), while maximillian sunflower also had significantly higher concentration than butterfly milkweed ($p = 0.0306$; Figure 3.7a). CLO concentration was only different between species in leaves and followed the same trend as leaf TMX. Oxeye sunflower leaf concentration was significantly more than both butterfly and common milkweed ($p = 0.0051, 0.0131$) and maximillian sunflower leaves had significantly higher concentrations than butterfly milkweed ($p = 0.0453$; Figure 3.7b). Stem tissues did not significantly vary between plants for TMX or CLO.

When parts of the same plant were compared to each other, butterfly milkweed, common milkweed, and oxeye sunflower all had significantly higher TMX concentrations in leaves than in stems and roots ($p < 0.05$; Figure 3.7a). Maximillian sunflower leaves had significantly higher TMX concentration than stems, but not roots. Similarly, CLO concentrations in the leaves of common milkweed, maximillian sunflower, and oxeye sunflower were significantly higher than in stems or roots ($p < 0.05$; Figure 3.7b). The leaves of butterfly milkweed had significantly higher CLO concentrations than the stems but not the roots.

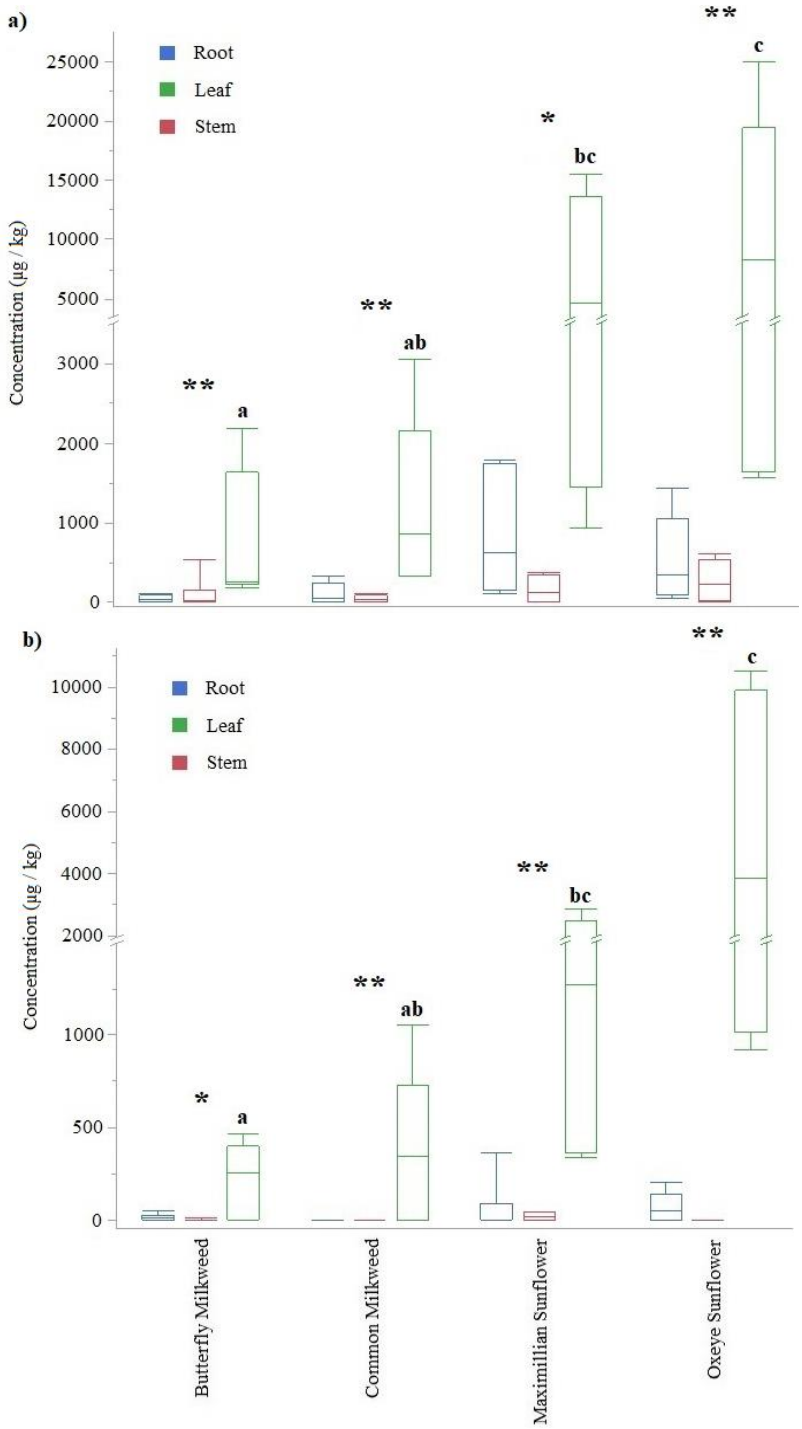

*Figure 3.7 – Root, stem, and leaf concentrations of a) TMX and b) CLO for plant species grown in the 11.4 L pots. * = Higher concentration in leaves than stems (but not roots). ** = Higher concentration in leaves than both stems and roots. Different lower-case letters indicate significant differences between leaf tissue concentrations in the four plant types ($p < 0.05$).*

3.3.4 Recovery and Distribution

Total TMX recovered (*% Recovery, total*; Eq. 3.6) was only significantly different between crimson clover and fescue (p = 0.0075) and between crimson clover and butterfly milkweed (p = 0.0391; Figure 3.8).

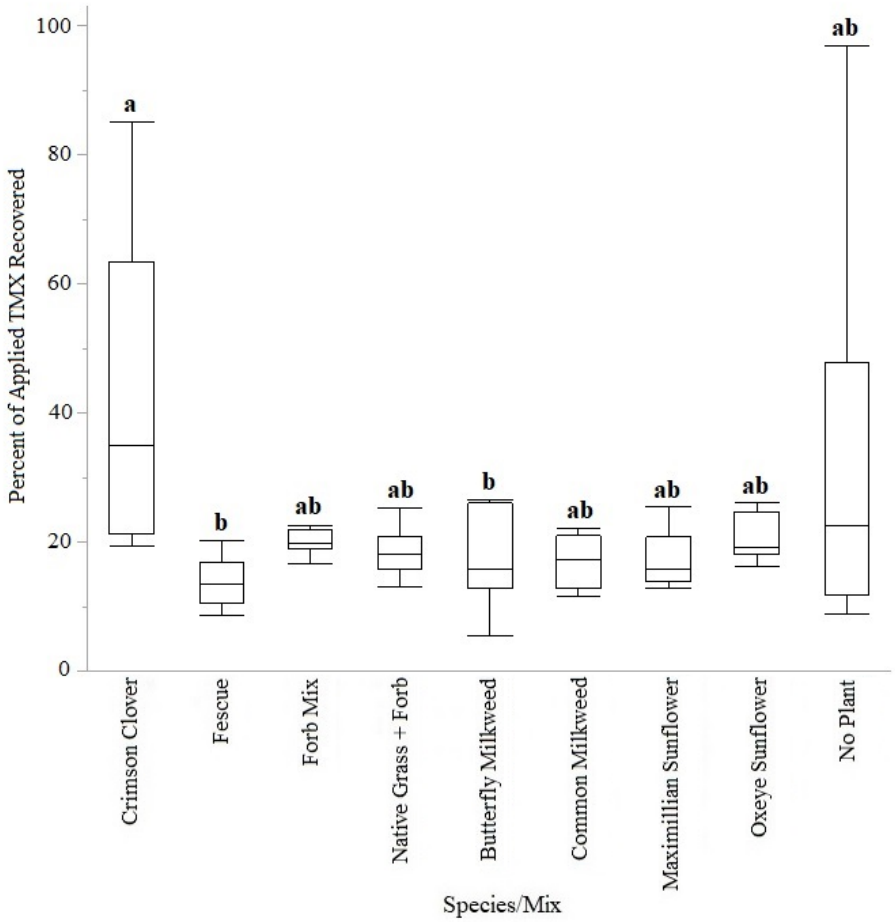

Figure 3.8 – Cumulative TMX recovered (soil + plant) in all species and mixes. Different lower-case letters indicate significant differences (p < 0.05).

In terms of uptake into plant tissue (*% Recovery, plant*; Eq. 3.7), crimson clover absorbed significantly more TMX mass on a relative basis than all other species and mixes (Figure 3.9). Behind crimson clover, the plants with the next highest TMX uptake were (in descending order): fescue, oxeye sunflower, the native grass + forb mix, and maximillian sunflower. While these

four plants exhibited no significant differences from each other, they all absorbed significantly more TMX than common milkweed and butterfly milkweed.

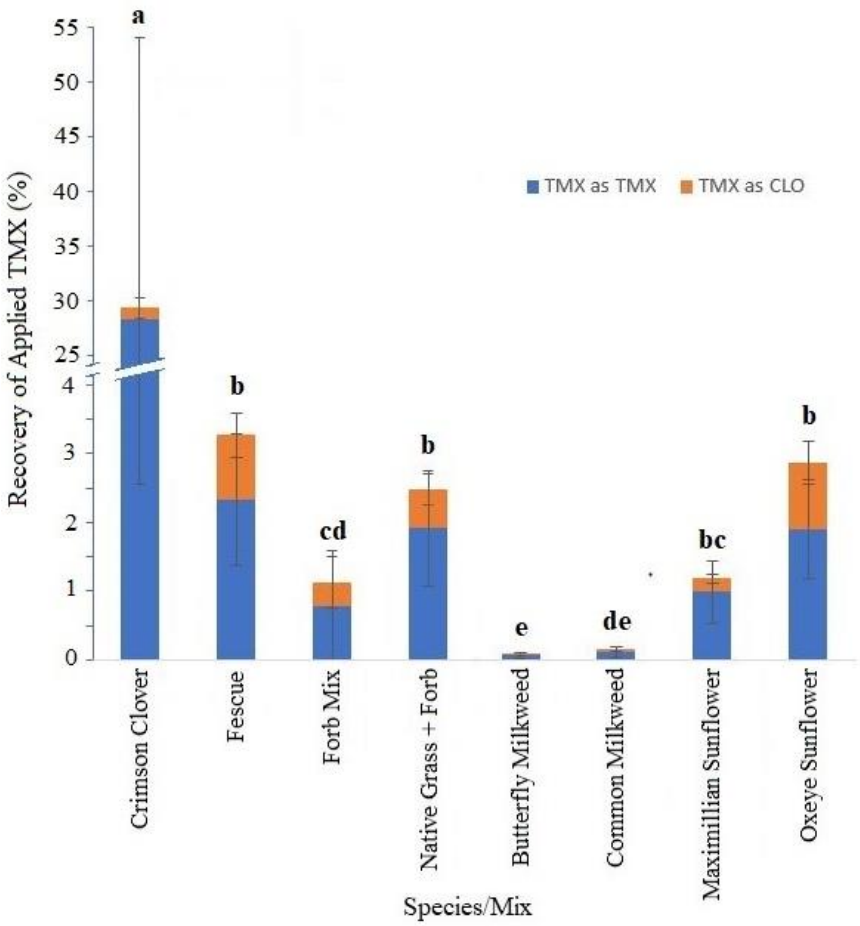

Figure 3.9 – Total TMX recovered in plant tissues for each plant type. Note the scale break on the y-axis. Different lower-case letters indicate significant differences ($p < 0.05$).

In all species above-ground tissues had significantly higher recovery of TMX and CLO than below-ground roots ($p < 0.0001$; Figure 3.10a). The relative distribution of TMX in stem and leaf tissues versus root tissues were statistically similar for all plant types ($p \geq 0.05$). However, common milkweed and the forb mix had significantly higher distributions of CLO in stem and leaf tissue, and significantly lower CLO in roots, than butterfly milkweed. Otherwise, there were no distribution differences within tissue types across all species.

The above-ground tissue from plants in the 11.4 L pots were further divided into leaf versus stems. In all instances, relative distributions of TMX and CLO were significantly higher in leaves than in either stems or roots ($p < 0.0001$; Figure 3.11b). No difference was observed between stems and roots ($p \geq 0.05$). CLO distribution in stems was mostly negligible, while distribution in roots was <10%.

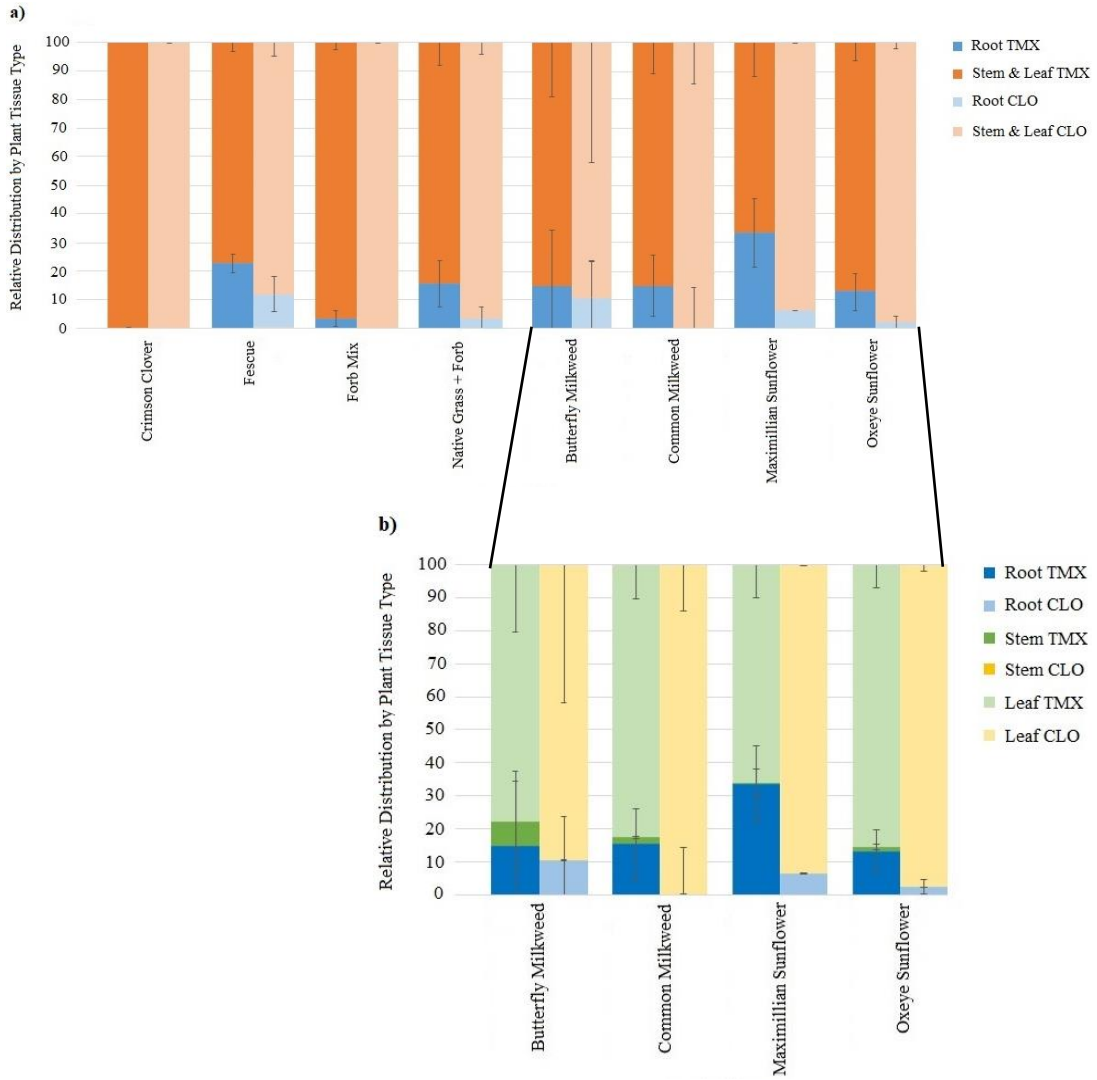

Figure 3.10 – Relative Distribution of TMX and CLO in a) Above and Below Ground Tissue, and b) Root, Stem, and Leaf Tissue of plants grown in 11.4 L pots.

3.4 Discussion

My objectives in this study were to: 1) determine TMX uptake and accumulation in plants that may be used as cover crops or in edge-of-field buffers, including milkweed and sunflower species favored by pollinators; and 2) examine the relative distribution of TMX and CLO within plant tissues.

For the first objective, I found TMX recovery was highest in crimson clover, with an average of nearly 30% of applied neonicotinoids becoming absorbed in plant tissues. This amount was an order of magnitude higher than the other species and mixes tested in this study, and also much greater than neonicotinoid plant uptake measured in previous field studies (Botias, 2016; Radolinski et al., 2019). Crimson clover is already used as a cover crop in many agricultural fields, where it may provide additional benefits by sequestering neonicotinoid pesticide compounds. However, cropping systems in the United States often terminate cover crops in the spring before planting cash crops seeds that contain neonicotinoids, meaning that the timing may preclude nearly as much uptake as I measured in this study. Also, crimson clover can attract pollinators when flowering, which could create a pathway for neonicotinoid exposure if chemicals such as TMX and CLO accumulate in flowers or pollen at concentrations like those that I measured in leaves.

The fescue, the native grass + forb mix, oxeye sunflower, and maximillian sunflower all recovered 1-3% of applied TMX. Thus, while not nearly as effective as crimson clover, these plants have potential to sequester TMX within and around agricultural fields. These other plants also offer some other benefits compared to crimson clover. For one, they are perennial plants, meaning that they can be used in edge-of-field situations that do not receive continued maintenance (e.g., annual harvesting and planting). Perennials also are more likely to be actively

growing during the critical spring flush period when neonicotinoid transport may be most acute. Since forb mixes and fescue are low maintenance, both may be desirable choices. Fescue is already a common planting on the edges of many agricultural fields, although this could easily be switched out with a forb and native grass mix, which performed similarly to fescue. The deciding factor between these options may be the ease of which one could implement a removal-and-disposal management plan for fescue, as it can be mowed, and the clippings can be collected and taken offsite.

The tested milkweed species (common milkweed and butterfly milkweed) had lower TMX and CLO concentrations than other plants. Therefore, these plants may pose little risk to pollinators that rely on them for food and habitat (e.g., monarch butterflies and their larvae). Edge-of-field buffers that feature native forbs (including milkweeds) and native grasses have the potential to retain neonicotinoids, raise biodiversity, and increase pollinator habitat around agricultural fields. Note that both forb mixes included milkweed species, though the uptake of the forb mix was significantly greater than the milkweed. This contrast suggests that other forb species in the mix take up more TMX than the milkweed plants. At the same time, the native grasses, whose uptake is similar to fescue, may be a driver in higher retention rates seen in forbs mixes with native grasses than forbs alone. However, future work should still consider whether neonicotinoids accumulation in pollen and leaves of these plants imparts elevated ecological risk to non-target insect species.

Results associated with the first study objective also indicated that plant water use efficiency may affect neonicotinoid uptake. Specifically, plants with the highest irrigation requirements (i.e., crimson clover) also had the highest relative concentrations of TMX. On the other hand, drier soil conditions can limit pesticide degradation, with the half-lives of some

neonicotinoids being up to ten times longer when soils were dry, as opposed to field capacity or saturated (Gupta et al., 2008).

For the second study objective, we found that TMX was easily mobilized from roots into above-ground tissues, in agreement with Ge et al. (2017) and Li et al. (2018). We expanded upon these earlier findings by showing that TMX and CLO both tend to accumulate more in leaves than in stems. Further, TMX was detected in all stem tissues tested, yet CLO was rarely detected in those same samples, suggesting that the transformation from TMX to CLO primarily occurs in leaf tissues. Because CLO is more toxic to chewing and sucking insects than TMX (Nauen et al., 2003), these processes may help illustrate why seed coatings can effectively control insects that feed on leaves. Given that both TMX and CLO tend to accumulate in leaves more than other plant parts, removal of above-ground biomass may be a feasible method of removing excess TMX and CLO from the field.

3.5 Conclusions

This study was conducted to determine which of six plant species and two plant mixes could be used to decrease the amount of neonicotinoid pollution exiting agricultural fields planted with neonicotinoid coated seeds. Using TMX-spiked irrigation, we determined that crimson clover had the highest concentrations of TMX and CLO, making this species a good candidate to sequester TMX when used as a cover crop. This recommendation should be used with caution, because when crimson clover is flowering, it still may pose risk to pollinators. We also showed that fescue and native grass mixtures were capable of adsorbing TMX at higher concentrations than other plants. These grasses therefore represent valid, low maintenance options for use in edge-of-field buffer strips.

The results showed that pesticides primarily accumulated in leaf tissues of all plants, including native forbs such as common and butterfly milkweed. These plants can provide important habitat for pollinators such as monarch butterfly larvae and increase overall biodiversity in cropping system. Therefore, it is imperative to verify that their usage does not create “ecological traps”, in which pollinators are lured into buffer habitats but then become subjected to neonicotinoid contact due to buffer plant uptake. In this study the two milkweed species accumulated neonicotinoids at much lower concentrations than other plants, suggesting that despite their low TMX recovery, these plants may be safe to use in agroecosystems that contain neonicotinoids. Nonetheless, future work should examine accumulation of TMX and CLO in native forbs under field conditions, and verify that concentrations in tissues such as leaves, flowers, and pollen do not pose risk to non-target pollinators.

4 Conclusion

Neonicotinoid pesticides can be hazardous to non-target organisms such as pollinators or aquatic insects when transported through the environment in runoff and leachate. These studies were conducted to evaluate the ability of plants and soils to retain thiamethoxam (TMX) and its metabolite, clothianidin (CLO), and thereby mitigate the environmental pollution from these two neonicotinoid chemicals. More specifically, the studies aimed to 1) determine whether organic carbon content in the soil had an influence on the leaching and persistence of thiamethoxam and clothianidin, and 2) evaluate species used in edge-of-field buffers and cover crops for efficacy in sequestering thiamethoxam and clothianidin in from the soil.

Through the first study, I demonstrated that increasing soil organic carbon content may be a useful strategy for reducing peak neonicotinoid loads in leachate. Soils with higher organic carbon content leached lower cumulative masses of TMX through early season events that typically generate a high degree of neonicotinoid runoff. Also, the soil with the highest organic carbon content leached significantly less CLO over the course of the growing season, even though cumulative TMX losses were not different between treatments. I also showed that organic amendments (which result in higher organic carbon content), can decrease the mass of CLO remaining in the soil at the end of the season, thus suggesting that higher organic carbon may inhibit the conversion of TMX to CLO. Further, degradation of CLO may be stimulated by the organic matter in the soil. From these findings, I conclude that agricultural practices that increase the carbon content of a soil can help decrease neonicotinoid transport from fields.

Through the second study, I demonstrated that species selection is important in finding a vegetative solution that can take up excess neonicotinoids in the field or the field margins. Specifically, crimson clover, a widely used cover crop appears to be a promising option for sequestering neonicotinoids. Fescue, which is already common around many agricultural fields, is an acceptable accumulator of TMX in soil as well, along with forb + native grass mix and both maximillian and oxeye sunflowers. Further, forb and native grass mixtures can also sequester large amounts of neonicotinoids while offering the bonus of increasing pollinator diversity and traffic; however, more research is necessary to ensure that neonicotinoids do not accumulate in pollen, posing risk to pollinators. I also found that accumulation of both TMX and CLO is much higher in leaf tissue than stems and roots. This finding indicates that management strategies that remove and dispose of the contaminated leaves may aid in removal of both pesticides from the system all together. With a planting like fescue, this could be very simple, as the grass can be mowed, and the clippings collected and disposed.

The findings from both studies could be combined to prescribe a management plan that favors the uptake and removal of neonicotinoids and decreases risk of pollinator exposure, while also increasing organic carbon in the soil. For example, a fescue planting installed on downslope field margins could encourage the accumulation of soil organic matter while simultaneously sequestering thiamethoxam from runoff. Forb and native grass mixes that feature milkweed species can have similar neonicotinoid retention as fescue while also providing better biodiversity and pollinator habitat. At the same time, no-till practices combined with a cover crop like crimson clover can increase soil organic matter over time. While the removal and disposal of clover is not as viable as fescue, it could be planted after the harvest of the cash crop, where it could take up residual pesticides in the field after the growing season; although, its

propensity for high concentrations may be a concern for insects. This may suggest that a balance of annual and perennial plants around the field may be an important consideration.

While these findings further the collective understanding of the factors that influence the movement of neonicotinoids in the environment, there is still much to be learned. For one, it is still important to understand the accumulation of neonicotinoids in key plant parts (e.g., flowers and pollen) under field conditions, to protect non-target species. For another, it would also be worthwhile to examine how degradation pathways of TMX and CLO are influenced by soil organic carbon concentration and organic carbon source, which could better guide selection of organic input types. By further refining and encouraging adoption of management practices like those recommended here, these studies could be important factors in assisting agricultural land managers in the reduction of neonicotinoid pollution.

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Appendix

Table A. Lysimeter Volume Collected for Each Sampling Date (Days After Planting).

Treatment	5	15	19	24	36	38	47	53	55	67	Cum. Volume
BM-1x	-	270	270	-	-	450	50	45	60	50	1195
B-5x	1975	250	100	50	80	1000	500	320	200	1360	5835
Control	1850	290	100	50	30	1500	1480	700	210	1200	7410
BM-5x	1800	350	100	320	150	640	820	380	150	1240	5950
B-1x	2200	560	100	60	20	540	180	70	30	20	3780
BM-5x	1975	310	80	250	108	880	640	240	220	1640	6343
B-1x	1900	970	320	680	200	1740	1460	1420	960	1440	11090
BM-1x	1700	400	850	1400	1300	1680	1640	1420	1520	1360	13270
B-5x	1950	400	240	1260	340	580	1740	1620	1460	1700	11290
Control	2000	440	100	80	850	1940	1680	680	220	220	8210
B-5x	1775	180	50	40	15	240	320	160	100	180	3060
Control	1850	240	100	100	20	720	520	130	110	1500	5290
B-1x	1900	200	180	220	140	1200	1060	540	210	1380	7030
BM-1x	1800	180	120	300	280	1200	840	-	260	500	5480
BM-5x	2400	360	200	600	860	1980	1720	1720	600	1560	12000
B-5x	1300	200	100	70	80	860	500	180	80	380	3750
Control	1425	200	120	100	186	1300	960	580	70	1140	6081
BM-1x	1800	340	160	80	110	1140	600	180	130	640	5180
B-1x	1900	440	100	30	60	150	1620	360	160	90	4910
BM-5x	1875	340	60	100	50	460	140	40	50	25	3140

Table B. Forb Mix (ERNMX-153-1; Ernst Conservation Seeds, Meadville, PA, USA)

%	Latin Name	Common Name
20.1	*Echinacea purpurea*	Purple Coneflower
12.0	*Coreopsis lanceolata*	Lance Leaf Coreopsis
12.0	*Rudbeckia hirta*	Black-eyed Susan, Coastal Plain NC Ecotype
10.0	*Chamaecrista fasciculata*	Partridge Pea, PA Ecotype
7.0	*Heliopsis helianthoides*	Oxeye Sunflower, PA Ecotype
6.4	*Tradescantia ohiensis*	Ohio Spiderwort, PA Ecotype
5.0	*Liatris spicata*	Marsh Blazing Star
3.5	*Aster oblongifolius*	Aromatic Aster, PA Ecotype
3.5	*Aster prenanthoides*	Zigzag Aster, PA Ecotype
3.0	*Zizia aurea*	Golden Alexanders, PA Ecotype
2.0	*Aster laevis*	Smooth Blue Aster, NY Ecotype
2.0	*Aster novae-angliae*	New England Aster, PA Ecotype
2.0	*Baptisia australis*	Blue False Indigo, Southern WV Ecotype
1.5	*Asclepias tuberosa*	Butterfly Milkweed
1.5	*Pycnanthemum tenuifolium*	Narrowleaf Mountain Mint
1.5	*Senna hebecarpa*	Wild Senna, VA & WV Ecotype
1.2	*Monarda fistulosa*	Wild Bergamot, Fort Indiantown Gap-PA Ecotype
1.1	*Solidago nemoralis*	Gray Goldenrod, PA Ecotype
1.0	*Eupatorium coelestinum*	Mistflower, VA Ecotype
1.0	*Geum canadense*	White Avens, PA Ecotype
1.0	*Penstemon digitalis*	Tall White Beardtongue, PA Ecotype
0.6	*Coreopsis tripteris*	Tall Coreopsis, PA Ecotype
0.5	*Senna marilandica*	Maryland Senna
0.2	*Oenothera fruticosa var. fruticosa*	Sundrops
0.2	*Solidago odora*	Licorice Scented Goldenrod, PA Ecotype
0.1	*Penstemon hirsutus*	Hairy Beardtongue
0.1	*Rudbeckia fulgida var. fulgida*	Orange Coneflower, Northern VA Ecotype

Table C. Native grass + forb mix (ERNMX-153; Ernst Conservation Seeds, Meadville, PA, USA)

%	Latin Name	Common Name
30.5	*Schizachyrium scoparium*	Little Bluestem, 'Camper'
30.0	*Bouteloua curtipendula*	Sideoats Grama, Butte
15.0	*Elymus virginicus*	Virginia Wildrye, PA Ecotype
4.0	*Echinacea purpurea*	Purple Coneflower
3.5	*Chamaecrista fasciculata*	Partridge Pea, PA Ecotype
3.0	*Coreopsis lanceolata*	Lance Leaf Coreopsis
3.0	*Rudbeckia hirta*	Black-eyed Susan, Coastal Plain NC Ecotype
2.0	*Heliopsis helianthoides*	Oxeye Sunflower, PA Ecotype
1.2	*Liatris spicata*	Marsh Blazing Star
1.0	*Tradescantia ohiensis*	Ohio Spiderwort, PA Ecotype
0.7	*Pycnanthemum tenuifolium*	Narrowleaf Mountain Mint
0.7	*Senna hebecarpa*	Wild Senna, VA & WV Ecotype
0.5	*Asclepias tuberosa*	Butterfly Milkweed
0.5	*Baptisia australis*	Blue False Indigo, Southern WV Ecotype
0.5	*Zizia aurea*	Golden Alexanders, PA Ecotype
0.4	*Aster laevis*	Smooth Blue Aster, NY Ecotype
0.4	*Aster novae-angliae*	New England Aster, PA Ecotype
0.4	*Aster oblongifolius*	Aromatic Aster, PA Ecotype
0.4	*Aster prenanthoides*	Zigzag Aster, PA Ecotype
0.4	*Eupatorium coelestinum*	Mistflower, VA Ecotype
0.4	*Monarda fistulosa*	Wild Bergamot, Fort Indiantown Gap-PA Ecotype
0.4	*Solidago nemoralis*	Gray Goldenrod, PA Ecotype
0.3	*Penstemon digitalis*	Tall White Beardtongue, PA Ecotype
0.2	*Coreopsis tripteris*	Tall Coreopsis, PA Ecotype
0.1	*Geum canadense*	White Avens, PA Ecotype
0.1	*Oenothera fruticosa var. fruticosa*	Sundrops
0.1	*Penstemon hirsutus*	Hairy Beardtongue
0.1	*Rudbeckia fulgida var. fulgida*	Orange Coneflower, Northern VA Ecotype
0.1	*Senna marilandica*	Maryland Senna
0.1	*Solidago odora*	Licorice Scented Goldenrod, PA Ecotype