Environmental Fate of Animal Manure-associated Antibiotics and Seed-coated Pesticide in Soils

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ACADEMIC ABSTRACT

There is growing concern over the environmental and human health impacts of chemical contaminants in agricultural systems. The environmental persistence of veterinary antibiotics applied to agricultural fields during manure fertilization could lead to increased antibiotic resistance. New generation, neonicotinoid pesticides pose a threat to aquatic ecosystem health due high water mobility and increased potential for non-target exposure. The objectives of this research were to develop a sensitive, analytical method for quantification of pirlimycin (PLY) in soils to be used in field research and determine the ability of second-generation neonicotinoids to move through soil when applied as a seed coating using a greenhouse study. Liquid-solid extraction of PLY from soil using (1:6, v/v) ammonium hydroxide/methylene chloride produced good PLY recovery (67-140%). Liquid-chromatography coupled with tandem mass-spectrometry for instrumental analysis provided good sensitivity with minimal matrix interferences. The mass balance distribution of neonicotinoid treatment coated onto corn seeds was determined in plant and soil samples for a single pot after 3 weeks of growth. A large percent (83-87%) of initial pesticide coating applied to seed was un-detected in plant in soil. Of the detected portion of neonicotinoid treatment, between 96-98% was observed to move out into the soil. This suggests the potential for long-range transport of seed-coated neonicotinoids.

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GENERAL AUDIENCE ABSTRACT

There is growing concern over the environmental and human health impacts of chemical contaminants in agricultural systems. The environmental persistence of veterinary antibiotics applied to agricultural fields during manure fertilization could lead to increased antibiotic resistance. New generation, neonicotinoid pesticides pose a threat to aquatic ecosystem health due high water mobility and increased potential for non-target exposure. The objectives of this research were to develop a sensitive, analytical method for quantification of pirlimycin (PLY) in soils to be used in field research and determine the ability of second-generation neonicotinoids to move through soil when applied as a seed coating using a greenhouse study. Liquid-solid extraction of PLY from soil using (1:6, v/v) ammonium hydroxide/methylene chloride produced good PLY recovery (67-140%). Liquid-chromatography coupled with tandem mass-spectrometry for instrumental analysis provided good sensitivity with minimal matrix interferences. The mass balance distribution of neonicotinoid treatment coated onto corn seeds was determined in plant and soil samples for a single pot after 3 weeks of growth. A large percent (83-87%) of initial pesticide coating applied to seed was un-detected in plant in soil. Of the detected portion of neonicotinoid treatment, between 96-98% was observed to move out into the soil. This suggests the potential for long-range transport of seed-coated neonicotinoids.

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Chapter 1: Literature Review

1.1 Background on Manure-Associated Antibiotics

Concentrated animal feeding operations (CAFOs) routinely administer sub-therapeutic antibiotic treatments for preventative purposes or to promote growth in animals. Depending on the species and chemical, up to 90% of antibiotic doses administered to livestock (swine, cattle, poultry) can be excreted via animal manure as the parent compound or a bioactive metabolite (Kumar et al., 2005; Song and Guo, 2014). Large portions of the antibiotics treated to livestock can enter soils through manure fertilization of agricultural fields. Evidence of antibiotic transport from agricultural lands was observed in a study monitoring for antibiotics at different sites along a river flowing directly from pristine mountain regions through agricultural lands. Antibiotics were not detected in water flowing through the mountains, however multiple tetracycline antibiotic compounds were detected in river water down stream to agricultural land receiving animal manure application (Yang et al., 2003). The potential transport of manure-applied antibiotics to surrounding water bodies via surface runoff and/or leaching may have deleterious biological effects to aquatic ecosystems. Little is understood about the environmental fate of manure-applied antibiotics, however their persistence in agricultural soils could promote the selection and transfer of antibiotic resistant genes in native microbial communities (Heuer et al., 2011). The abundance and spread of antibiotic resistant bacteria is of critical concern to human health as it relates to the efficiency of antibiotics used to treat human diseases (Davis et al., 2006). Pirlimycin (PLY) is a lincosamide antibiotic commonly used to treat mastitis in dairy cattle. Previous research on PLY is limited to biological and food matrices. An analytical method for pirlimycin in soils is needed to better assess the overall impact that could arise from extensive antibiotic use in the dairy industry.

1.2 Sample Preparation Used in Existing Methods

Analytical methods for PLY analysis in existing literature have typically employed acetonitrile (ACN)-based extraction techniques, such as ultrasonic extraction (USE), mechanical shaking, vortex mixing, and pressurized liquid extraction (PLE) during sample preparation. Samples extracted with 10 mL ethylenediaminetriacetic acid (EDTA) McIlvaine buffer solution (pH adjusted to 4) extraction via sonication, vortexing, and mechanical shaking steps produced 98% and 106% recovery of PLY in honey and animal muscle (Bohm et al., 2011, 2012). Animal tissues are often extracted using acidified solutions in order to denature proteins; promoting the release of matrix-bound analyte concentrations into extract solution (Chiaochan et al., 2010). Ultrasound treatment promotes the break down of sample particles and release of analytes into solution, however this technique can be time consuming and involve large volumes of organic solvents (Moreno-Bondi et al., 2009).

Pressurized liquid extraction (PLE) has been also employed for the analysis of PLY in bovine meat and milk. This technique is largely automated and enhances extraction efficiency by increasing solvent penetration into matrix at relatively high pressure and temperature. Samples were blended with 0.5 g EDTA chelating agent and 11-15 g of sea sand to aid in dispersion. Homogenous sample mixture was packed into extraction cells and extracted with ACN at a pressure and temperature setting of 1500 psi and 40^{o}C, respectively. Results from LC analysis reported recoveries between 70-77% with 4.1-5.1% RSD for PLY (Juan et al., 2010).

Dickson et al., 2014 added three 9.5 mm steel ball bearings to shrimp, salmon, and tilapia extraction vials for increased homogenization during extraction with ACN/phosphate buffer (pH=8). Results from LC/MS/MS analysis determined 85-92% PLY recovery with 7-10% RSD using this method. Milk, egg, and meat samples were mechanically shaken with 8:1 ACN/water

solution then frozen at -80°C for 30 minutes before thawing and centrifuging to induce phase separation using low temperature partition. Supernatant from ACN layer was transferred to a new vial containing 1 g C18 for d-SPE clean-up and 4 g $MgSO_4$ drying agent and LC analysis determine 97-106% recovery of PLY with 10-14% RSD (Chung et al., 2015). The addition of $MgSO_4$ was effective at removing all residual water from ACN layer prior to and provided much faster evaporation of ACN phase during concentration step.

Dispersive SPE provides a simple, effective method for purifying organic extracts compared to more traditional SPE methods. Other multi-residue analyses of animal tissues have used 4:1 ACN/water extraction followed by C18 dispersive SPE clean-up and LC-MS/MS analysis reported 70-94% PLY recovery with 5-19% RSD (Geis-Asteggiante et al., 2012; Lehotay et al., 2012; Schnieder et al., 2014; Yamaguchi et al., 2015). Solid-phase extraction with C18 (Dickson, 2014) and HLB (Turnipseed et al., 2008; Bohm et al., 2009) cartridge sorbent has been used for cleaning and/or concentration milk and tissue samples prior to LC analysis. Typical steps of SPE procedures include cartridge conditioning, sample loading, rinsing, washing, and elution. The sorbent material of HLB cartridges contains dipole-dipole and H-bonding interaction sites that allow for better retention of polar analytes compared to C18 cartridges. Advantages of cartridge SPE methods include automation capabilities and reduced solvent consumption.

1.3 Existing Instrumental Analysis

High performance liquid chromatography tandem mass spectrometry (LC/MS/MS) is the preferred analytical tool for quantifying antibiotics in environmental. Reverse-phase (RP) separation isolates compounds in a sample based on differences in affinity to nonpolar, C18 stationary phase and polar mobile phase. Mobile phase often flows at a gradient between

aqueous and polar organic solvent composition. Buffers are used to stabilize ionic species and mobile phases used in PLY methods frequently consist of (A) 0.1% formic acid in water and (B) 0.1% formic acid in ACN (Yamaguchi et al., 2015; Turnipseed et al., 2008). Analysis of PLY in chicken muscle employed hydrophilic interaction (HILIC) stationary phase with (A) 50 mM ammonium formate in water at pH 2.5 and (B) ACN mobile phases in an effort to improve LC retention and separation of more polar compounds compared to reverse-phase techniques (Chiaochan et al., 2010). Both RP and HILIC separation methods have reported good peak shape and resolution for PLY, however RP methods are more common in PLY analysis.

Electrospray ionization (ESI) is frequently used at the ion source that delivers charge to compounds before mass analyzer. The higher resolution achieved with time-of-flight (TOF)-MS was used instead of MS/MS techniques due to the degree of flexibility needed for analyzing unknowns, metabolites, or compounds without a reference standard (Kaufmann et al., 2008). Triple quadrupole (QqQ) MS monitored a compound and its multiple fragments based on their specific mass-to-charge (m/z) ratio, which provides increased selectivity and sensitivity in complex matrices. The LC/QqQ is ideal for trace analysis of antibiotics and most commonly used for PLY quantification in existing methods. Mass-to-charge ratios of PLY parent and daughter ions are 411 (m/z) and 112, 363 (m/z), respectively (Yamaguchi et al., 2015).

1.4 Method Development

Developing an effective strategy for the extraction and analysis of PLY in agricultural soils requires a strong understanding of the physiochemical properties of the compound structure and the potential matrix interactions. Due to the increased sensitivity and selectivity achieved with advance in LC/MS/MS analysis, it has become the preferred analytical technique employed for quantifying trace antibiotic in soil (Diaz-Cruz et al., 2007). Initial MS tuning determines best

ionization parameters (voltage, mode, etc.) and most informative fragments for optimal sensitivity. Positive ionization mode is common for organic base cations. Previous MS/MS parameters used for PLY analysis in biological and food samples can be applied for soil sample analysis. However, this is not the case for sample preparation methods due to differences in matrix properties. Strong adsorption forces can occur between negative charges on clay mineral surfaces and positive charge of PLY cations, making extraction difficult. Soil organic matter (SOM) can also interact with PLY cations through inorganic replacement at cation exchange sites and neutral species via partitioning with non-polar moieties (Droge and Goss, 2013). Previous studies of other organic base cation such as aromatic amines and quinolines suggested cation exchange to be the primary sorption mechanism to mineral surfaces. This mechanism was also evident for lincomycin ($pK_a = 7.6$) sorption from aqueous suspension to smectite clays saturated with exchangeable cations (K, Ca, or Cs). The increased concentration of lincomycin cation present when $pH < pK_a$ resulted in greater release of inorganic cations into aqueous solution compared to solutions with $pH > pK_a$ (Wang et al., 2009). A strong base solution can be used to readily accept protons and shift equilibrium of organic cations to neutral species. Simultaneous extraction with an organic solvent could promote PLY isolation from matrix impurities co-extracted in strong base layer (Hamscher et al., 2002). The low solubility of humic and fulvic acids in non-polar, organic solvents, such as ethyl acetate, could simplify or even omit the need for additional purification steps. Drying agents such as $MgSO_4$ and Na_2SO_4 are common in liquid-liquid extraction procedures to ensure removal of all residual water that may still be dissolved in the organic phase. Enhanced separation of phases can save time and improve recovery during evaporation of organic solvent in the concentration steps (Chung and Lam, 2015).

1.5 Use of Thiamethoxam and Clothianidin Insecticides

Thiamethoxam (TMX), and clothianidin (CLO) are second-generation neonicotinoid insecticides. They are the most commonly used pesticide treatments in corn and soybean fields (Hladik et al., 2014), two of the largest crop productions in the country. Chemical treatments are readily taken up by young roots and spread throughout the plant tissue, allowing for a more precise targeting of harmful insects. Once ingested, these compounds act selectively on neural receptor binding sites specific to the insect central nervous system, providing protection against a wide range of insects while having minimal toxicity towards vertebrates (Simon-Delso et al., 2015). Due to their particular mode of action, neonicotinoids used in agriculture is believed not to result in cross-resistance to other long-established insecticide classes (Jeschke and Nauen, 2008). The partial positive charge located on the amino nitrogen atom is the distinguishing feature that enables neonicotinoids to act systematically and selectively against insects. They demonstrate a high selectivity for electronegative interaction with specific sub-sites unique to insect nicotine acetylchonine receptors (nAchRs) found in the central nervous system. Neonicotinoid toxicity to mammals is low because they lack the full positive charge needed for interaction with the nAChR present in vertebrates (Yamamoto et al., 1998). Insecticidal activity and field efficiency vary somewhat between the 7 commercial neonicotinoids available because of slight differences in their chemical structure.

1.6 Non-Target Organisms and Ecosystem Function

A number of aquatic species have demonstrated signs of immobilization or mortality when exposed to certain TMX and CLO levels over time. Crayfish and shrimp toxicity to CLO reported in 2003 by the USEPA observed 48 h EC_{50} values of 59 and 51 μg/L, respectively (Anderson et al., 2015). Many terrestrial non-target species have displayed varying degrees of

biological sensitivity. Several studies observed overall suppression of activity and diversity in soil microbial communities exposed to these treatments (Pisa et al., 2014; Schaafsma et al., 2015). Neonicotinoid toxicity has been observed in many pest-predator species that primarily feed on the targeted insects. Predaceous species sometimes feed on plant material during larvae growth stage or when prey availability is low. Ingestion of treated plant material or contaminated prey showed adverse effects to predator populations in a study comparing neurotoxic symptoms of larvae feeding on young plants grown from CLO and TMX-treated corn seeds (Moser and Obrycki, 2009).

Consumption of guttation droplets exuded from the plant xylem is common route of exposure to pollinators and it is speculated that the introduction of neonicotinoid insecticides has been the driving force responsible for the recent collapse of honeybee colonies all over the world (Goulson, 2013). Adverse biological effects have been observed in birds from direct ingestion of neonicotinoid-coated seeds (Uneme, 2011). Mice showed adverse biological health effects from high levels of neonicotinoid exposure. These concentrations, however, are not likely to be naturally occurring in the environment; these studies show the potential risk and health detriment associated with neonicotinoid exposure to mammals (Uneme, 2011). One study looking at rat toxicity to CLO determined LD_{50} values for both male and female rats to be > 5000 mg/kg. The same article reported LD_{50} values to be > 2000 mg/kg and > 105.8 mg/kg for the bobwhite quail and rainbow trout species, respectively.

1.7 Chemical Structure and Transformation in Plant and Soil Matrices

The chemical structure of neonicotinoid insecticides typically consists of 3 parts: the pharmacophore, a methylene group bridging chain, and the heterocyclic group (Jeschke et al. 2011). Some neonicotinoid metabolites and transformation products have been found to be more

potent than neonicotinoid parent compounds. This is evident with the studied transformation of TMX to CLO. These compound structures are nearly identical; however, hydroxylation of TMX converts the former, 6-membered methylene ring structure into CLO, a more potent, open-chained derivative. Rapid transformation of TMX to CLO has been observed in cotton plants and CLO concentration was reported to be nearly double the concentration of TMX in cotton plant leaves three days after TMX treatment of soil (Nauen et al., 2003). Characteristics of TMX and CLO chemical structure make them not easily degraded by microorganisms; however, a number of microbial strains isolated from treated soil have been found capable of degrading these recalcitrant compounds. *Ensifer adhaerens* strain TMX-23 is a nitrogen-fixing, plant-growth-promoting rhizobacterium and was identified in a previous study to be capable of degrading thiamethoxam, a second-generation neonicotinoid insecticide (Guang-can Zhou et al. 2013). Another study identified the highest rates performed by *Bacillus aerophilus* strain IMBL 4.1 and *Pseudomonas putida* strain IMBL 5.2 of the microbial strains tested (Rana et al. 2015).

1.8 Environmental Persistence and Transport

The environmental stability of neonicotinoids has been found highly variable under a broad spectrum of different conditions. Reported half-lives for TMX range from 1 to 3001 days, 6 to 1250 days for IMI, and 17 to 6931 days for CLO (Goulson, 2013). Temperature, soil type, soil organic content, moisture content, concentration have all been observed to influence neonicotinoid sorption, persistence, and rate of degradation. After 30 days, results showed 82%, 79%, 69%, and 56% TMX dissipation in soils with 0.84, 0.70, 0.50, and 0.40% organic carbon, respectively (Karmakar et al., 2006). TMX demonstrated longer persistence under dry conditions with reported half-lives ranging from 200.7-301 days (Gupta et al., 2008). Concentrations of TMX appeared to decrease with each level of increased moisture content. At field capacity

moisture, half-lives ranged between 91.2 and 94.1 days and decreased to 46.3-75.3 days under submerged conditions. The inverse relationship between TMX half-life and soil moisture content could be attributed to changes in the microbial activity characteristic of each moisture regime, suggesting microbial degradation as a major route of TMX loss in soil (Gupta et al., 2008). The faster dissipation of TMX in submerged soils observed in a study by Gupta et al. 2008, could suggest that anaerobic microbial communities are more efficient in TMX degradation (Gupta et al., 2008). Two concentration levels were also examined at each moisture level, showing faster dissipation at 10 mg/kg versus 1 mg/kg. Loss of TMX from soil has been found greater when runoff or leaching occurs shortly after application, and before there is an opportunity of binding to the soil (Gupta et al., 2008). The objectives of this research were to develop an effective strategy for the analysis of manure-borne antibiotics in soil and determine the possible redistribution of seed-coated neonicotinoids in soil.

1.9 References

Anderson, J.C., C. Dubetz and V.P. Palace. 2015. Neonicotinoids in the Canadian aquatic environment: A literature review on current use products with a focus on fate, exposure, and biological effects. Science of The Total Environment 505: 409-422.

Bohm, D.A., C.S. Stachel and P. Gowik. 2009. Multi-method for the determination of antibiotics of different substance groups in milk and validation in accordance with Commission Decision 2002/657/EC. Journal of Chromatography A 1216: 8217-8223.

Bohm, D.A., C.S. Stachel and P. Gowik. 2011. Validated Determination of Eight Antibiotic Substance Groups in Cattle and Pig Muscle by HPLC/MS/MS. Journal of AOAC International 94: 407-419.

Bohm, D., C. Stachel and P. Gowik. 2012. Validation of a multi-residue method for the determination of several antibiotic groups in honey by LC-MS/MS. Analytical and Bioanalytical Chemistry 403: 2943-2953.

Casida, J.E. 2011. Neonicotinoid metabolism: compounds, substituents, pathways, enzymes, organisms, and relevance. Journal of agricultural and food chemistry 59: 2923-2931.

Chiaochan, C., U. Koesukwiwat, S. Yudthavorasit and N. Leepipatpiboon. 2010. Efficient hydrophilic interaction liquid chromatography–tandem mass spectrometry for the multiclass analysis of veterinary drugs in chicken muscle. Analytica Chimica Acta 682: 117-129.

Chung, S.W.C. and C.-H. Lam. 2015. Development of a 15-class multiresidue method for analyzing 78 hydrophilic and hydrophobic veterinary drugs in milk, egg and meat by liquid chromatography-tandem mass spectrometry. Analytical Methods 7: 6764-6776.

Davis, J.G., C.C. Truman, S.C. Kim, J.C. Ascough, II and K. Carlson. 2006. Antibiotic Transport via Runoff and Soil Loss. Journal of Environmental Quality 35: 2250-2260.

Díaz-Cruz, M.S. and D. Barceló. 2007. Recent advances in LC-MS residue analysis of veterinary medicines in the terrestrial environment. TrAC Trends in Analytical Chemistry 26: 637-646.

Dickson, L.C. 2014. Performance characterization of a quantitative liquid chromatography–tandem mass spectrometric method for 12 macrolide and lincosamide antibiotics in salmon, shrimp and tilapia. Journal of Chromatography B 967: 203-210.

Droge, S.T.J. and K.-U. Goss. 2013. Sorption of Organic Cations to Phyllosilicate Clay Minerals: CEC-Normalization, Salt Dependency, and the Role of Electrostatic and Hydrophobic Effects. Environmental Science & Technology 47: 14224-14232.

Geis-Asteggiante, L., S.J. Lehotay, A.R. Lightfield, T. Dutko, C. Ng and L. Bluhm. 2012. Ruggedness testing and validation of a practical analytical method for >100 veterinary drug residues in bovine muscle by ultrahigh performance liquid chromatography–tandem mass spectrometry. Journal of Chromatography A 1258: 43-54.

Goulson, D. 2013. An overview of the environmental risks posed by neonicotinoid insecticides. Journal of Applied Ecology 50: 977-987.

Guang-can Zhou, Y.W., S. Zhai, F. Ge, Z. Liu, Y. Dai, S. Yuan, & J. Hou. 2013. Biodegradation of the neonicotinoid insecticide thiamethoxam by the nitrogen-fixing and plant-growth-promoting rhizobacterium Ensifer adhaerens strain TMX-23. Applied Microbiology and Biotechnology. 97: 4065–4074.

Gupta, S.,V.T. Gajbhiye and R.K. Gupta. 2008. Soil Dissipation and Leaching Behavior of a Neonicotinoid Insecticide Thiamethoxam. Bull. Environ. Contam. Toxicol. 80: 431-437.

Hladik, M.L., D.W. Kolpin and K.M. Kuivila. 2014. Widespread occurrence of neonicotinoid insecticides in streams in a high corn and soybean producing region, USA. Environmental Pollution 193: 189-196.

Heuer, H., H. Schmitt and K. Smalla. 2011. Antibiotic resistance gene spread due to manure application on agricultural fields. Current Opinion in Microbiology 14: 236-243.

Jeschke, P. and R. Nauen. 2008. Neonicotinoids—from zero to hero in insecticide chemistry. Pest Management Science 64: 1084-1098.

Jeschke, P., R. Nauen, M. Schindler and A. Elbert. 2011. Overview of the Status and Global Strategy for Neonicotinoids. Journal of Agriculture and Food Chemistry 59: 2897-2908.

Juan, C., J.C. Moltó, J. Mañes and G. Font. 2010. Determination of macrolide and lincosamide antibiotics by pressurised liquid extraction and liquid chromatography-tandem mass spectrometry in meat and milk. Food Control 21: 1703-1709.

Karmakar, R., B.S. Singh and G. Kulshrestha. Persistence and Transformation of Thiamethoxam, a Neonicotinoid Insecticide, in Soil of Different Agroclimatic Zones of India. Bulletin of Environmental Contamination and Toxicology 76: 400-406.

Kaufmann, A., P. Butcher, K. Maden and M. Widmer. 2008. Quantitative multiresidue method for about 100 veterinary drugs in different meat matrices by sub 2-µm particulate high-performance liquid chromatography coupled to time of flight mass spectrometry. Journal of Chromatography A 1194: 66-79.

Kim, S.-C. and K. Carlson. 2007. Quantification of human and veterinary antibiotics in water and sediment using SPE/LC/MS/MS. Analytical and Bioanalytical Chemistry 387: 1301-1315.

Kumar, K., S. C. Gupta, Y. Chander and A.K. Singh. 2005. Antibiotic Use in Agriculture and Its Impact on the Terrestrial Environment. Advances in Agronomy. Academic Press. p. 1-54.

Lehotay, S.J., A.R. Lightfield, L. Geis-Asteggiante, M.J. Schneider, T. Dutko, C. Ng, et al. 2012. Development and validation of a streamlined method designed to detect residues of 62 veterinary drugs in bovine kidney using ultra-high performance liquid chromatography – tandem mass spectrometry. Drug Testing and Analysis 4: 75-90.

MacDonald, J.M. and W.D. McBride. 2009. The transformation of US livestock agriculture scale, efficiency, and risks. Economic Information Bulletin.

Martos, P.A., F. Jayasundara, J. Dolbeer, W. Jin, L. Spilsbury, M. Mitchell, et al. 2010. Multiclass, Multiresidue Drug Analysis, Including Aminoglycosides, in Animal Tissue Using Liquid Chromatography Coupled to Tandem Mass Spectrometry. Journal of Agricultural and Food Chemistry 58: 5932-5944.

Moreno-Bondi, M., M. Marazuela, S. Herranz and E. Rodriguez. 2009. An overview of sample preparation procedures for LC-MS multiclass antibiotic determination in environmental and food samples. Analytical and Bioanalytical Chemistry 395: 921-946.

Moser, S.E. and J.J. Obrycki. 2009. Non-target effects of neonicotinoid seed treatments; mortality of coccinellid larvae related to zoophytophagy. Biological Control 51: 487-492.

Schaafsma, A., V. Limay-Rios, T. Baute, J. Smith and Y. Xue. 2015. Neonicotinoid Insecticide Residues in Surface Water and Soil Associated with Commercial Maize (Corn) Fields in Southwestern Ontario. PLoS One 10.

Schneider, M.J., S.J. Lehotay and A.R. Lightfield. 2014. Validation of a streamlined multiclass, multiresidue method for determination of veterinary drug residues in bovine muscle by

liquid chromatography–tandem mass spectrometry. Analytical and Bioanalytical Chemistry 407: 4423-4435.

Simon-Delso, N., V. Amaral-Rogers, L.P. Belzunces, J.M. Bonmatin, M. Chagnon, C. Downs, et al. 2015. Systemic insecticides (neonicotinoids and fipronil): trends, uses, mode of action and metabolites. Environmental Science and Pollution Research 22: 5-34.

Turnipseed, S.B., W.C. Andersen, C.M. Karbiwnyk, M.R. Madson and K.E. Miller. 2008. Multi-class, multi-residue liquid chromatography/tandem mass spectrometry screening and confirmation methods for drug residues in milk. Rapid Communications in Mass Spectrometry 22: 1467-1480.

USEPA. 2013. Literature Review of Contaminants in Livestock and Poultry Manure and Implications for Water Quality. In: U. S. D. o. Agriculture, editor.

Wang, C., Y. Ding, B.J. Teppen, S.A. Boyd, C. Song and H. Li. 2009. Role of Interlayer Hydration in Lincomycin Sorption by Smectite Clays. Environmental Science & Technology 43: 6171-6176.

Yamaguchi, T., M. Okihashi, K. Harada, K. Uchida, Y. Konishi, K. Kajimura, et al. 2015. Rapid and Easy Multiresidue Method for the Analysis of Antibiotics in Meats by Ultrahigh-Performance Liquid Chromatography–Tandem Mass Spectrometry. Journal of Agricultural and Food Chemistry 63: 5133-5140.

Yamamoto, I., M. Tomizawa, T. Saito, T. Miyamoto, E.C. Walcott and K. Sumikawa. 1998. Structural factors contributing to insecticidal and selective actions of neonicotinoids. Archives of Insect Biochemistry and Physiology 37: 24-32.

Yang, S. and K. Carlson. 2003. Evolution of antibiotic occurrence in a river through pristine, urban and agricultural landscapes. Water Research 37: 4645-4656.

Chapter 2: Analysis of Pirlimycin in Soils

2.1 Abstract

Due to the growing concern of antibiotic resistance, development of an advanced, sensitive analytical method would allow better monitoring of pirlimycin in the environment. In this method, a combination of 1:6 ammonium hydroxide/methylene chloride with 1 g anhydrous sodium sulfate was used to extract PLY spiked into agricultural soils via ultra-sound bath treatment. Identification and quantification of PLY in final extracts was performed with ultra-performance liquid chromatography (UPLC) coupled with tandem mass spectrometry (MS/MS). Reverse-phase LC separation on a Zorbax C_{18} column using (A) 0.1% formic acid in water and (B) 0.1% formic acid in methanol mobile phases produced good peak resolution of PLY in sample extract. Positive mode electrospray ionization combined and multiple reactions monitoring (MRM) of precursor ion, 411 m/z, and fragment ions, 363,112 m/z, were selected for MS analysis. Method testing determined little matrix effect (8-15%) and a low method detection limit (MDL) of 1.31 μg/kg. Intra-day and inter-day testing for three spiking levels achieved recovery ranging from 92-128% with 20-46% RSD and 67-140% with 11-14% RSD, respectively. Liquid-solid extraction provides a quick and inexpensive method for effectively recovering PLY in complex soil matrices. The method developed in this study can be used in future research studies to better understand the environmental fate of PLY applied to agricultural soils in field via manure fertilization.

2.2 Introduction

Pirlimycin (PLY) is a lincosamide antibiotic commonly used for the treatment of mastitis in dairy cattle. Previous research has investigated the distribution and metabolism of PLY in the PLY-treated cows and reported 34% of the administered dose was excreted via animal manure as

parent compound or an active metabolite (Huston et al., 1992). Once excreted, PLY can enter the environment through land-applied dairy manure fertilization of crop fields, manure storage leaks, or directly from grazing animals. The persistence and spread of manure applied-PLY in terrestrial environments could promote the selection and transfer of antibiotic resistant genes in native microbial communities (MacDonald and McBride, 2009). Field studies of PLY are needed to better assess the overall human impact associated with the extensive clinical and sub-clinical use of animal antibiotics like PLY in livestock production. However there are currently no existing methods for analysis of PLY in soils.

In general, PLY is a relatively polar, ionizable antibiotic compound with $pK_a = 8.5$, primarily due to presence of N-amine functional groups in the PLY chemical structure (Figure 2-1). Nitrogen atoms each carry a set of lone-pair electrons that will often attract positively charged protons, increasing PLY solubility in water and polar organic solvents. When pH = 8.5, there is equal portion of the molecules in the neutral and positively ionized form.

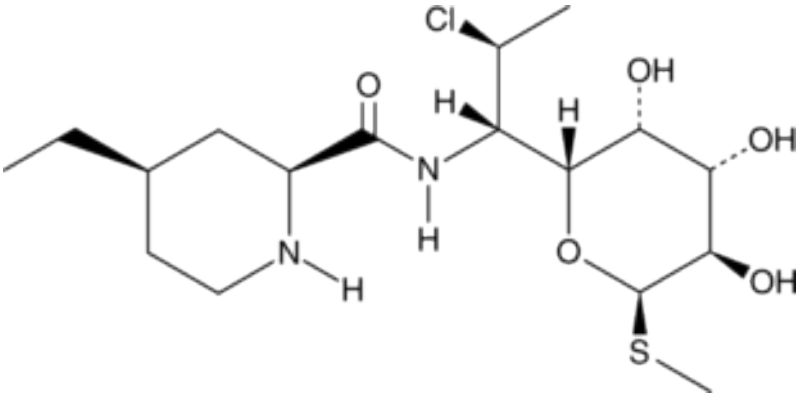

Figure 2-1 Chemical structure of pirlimycin

Extraction with a McIlvaine buffer solution adjusted to a pH of 4 followed by SPE clean-up has been employed in a multi-residue LC/MS/MS analysis of antibiotics in honey (Bohm et al., 2012), milk (Bohmn et al., 2009) and muscle (Bohm et al., 2012) samples, achieving PLY recoveries of 98%, 100%, and 106%, respectively, with RSD < 15%. Pressurized liquid

extraction (PLE) methods for multi-residue LC/MS/MS analysis of macrolides and lincosamide antibiotics in bovine meat and milk achieved 71-90% pirlimycin recovery with RSD < 6 %. During the PLE extraction, samples were mixed with EDTA and sea sand before extraction with 100% acetonitrile at pressure and temperature settings of 1500 psi and 40°C, respectively (Juan et al., 2010).

Using polar solvents to extract PLY out of a sample matrix may not be applicable for soil samples because of strong adsorption that tightly binds protonated PLY molecules to negatively charged soil particles. Sorption processes between smectite clays and other organic bases, such as lincomycin, have been found to decrease with increasing pH suggesting cation exchange to be the primary sorption mechanism. In addition, lincomycin sorption also decreased with increased presence of inorganic cations, resulting in competition for negatively charged clay binding sites (Wang et al., 2008; Tolls, 2001).

To overcome these analytical challenges involving strong adsorption to complex soil matrices, implementing a strong base such as aqueous ammonia will drive up the pH, remove excess protons in soil solution and thus modify the speciation of PLY. This pH manipulation will result in a shift of PLY molecules to their deprotonated, uncharged form. Polarity of this species decreases becoming more soluble in a less polar organic solvent such as methylene chloride (Tolls, 2001). Using organic solvents has also been beneficial in previous analytical methods in avoiding addition extraction clean-up or purification stages (Kaufmann et al., 2008). The objective of this study was to develop a sensitive analytical method for the analysis of pirlimycin in soils that can be used in future research on environmental fate of PLY in manure-applied fields.

2.3 Sample Preparation

A pressurized liquid extraction system (Buchi Speed Extractor E-916, East Anglia, U.K.) was used for initial PLY soil extraction method development. Five grams of control soil was weighed out and blended with 0.5 g disodium EDTA and 3 g sand before transferring into 11 mL Speed Extractor cell for pressurized liquid extraction. Extraction method consisted of two extraction cycles with a methanol/0.1 M aqueous ammonia solution (v/v, 1:1) at 100 bar (1500 psi) pressure. Two temperatures, 80°C and 30°C, were compared for optimizing PLY recovery.

Extracts were cleaned-up with OASIS HLB cartridges using a SPE vacuum manifold. Cartridges were conditioned sequentially with 2 mL ACN followed by 2 mL water. Sample extracts were then loaded into SPE cartridges at flow rate of approximately 2-3 drops per second. Each sample vial was then rinsed with 2 mL methanol/water (v/v, 1:1) and the rinsate was then loaded on to the corresponding cartridge. Each cartridge was washed with 3 mL water followed by 3 mL ACN/water (v/v, 1:10). After washing solutions drained through, each cartridge was left to dry for 15-20 minutes before 5 mL of elution solvent was passed through each cartridge and collected in a glass test tube. The collected eluates were then dried in the glass collection tubes on a vacuum evaporator (RapidVap, Labconco, Kansas City, MO). Different eluting solvents listed in Figure 2-4 were tested for optimal PLY recovery during the SPE clean-up. Each final completely dried sample was reconstituted in 1 mL methanol/water (30:70, v/v) containing 0.1% formic acid LC mobile phase solution before UPLC/MS/MS analysis.

For comparison, a liquid-solid extraction procedure was also investigated. One gram of control soil was transferred and spiked with appropriate level of PLY stock, air-dried for several minutes. Samples were then mixed with 1 g anhydrous sodium sulfate and transferred to 35-mL glass centrifuge tubes. Exactly 1 mL of concentrated ammonia hydroxide and 6 mL methylene chloride were added to each glass vial and screw-top lids were securely fastened. Samples were

sonicated for 20 minutes and then placed in centrifuge for 5 minutes at 1200 g. All supernatant was transferred to a new glass centrifuge tube and covered with screw caps. The extraction was repeated as previously mentioned. The supernatants from two extractions were combined and centrifuged again at 1200 g for 5 minutes. Exactly 5 mL of methlyene chloride was collected and evaporated to dryness on a vacuum evaporator (RapidVap, Labconco, Kansas City, MO). Dried samples were reconstituted in 1 mL 0.1% formic acid in MeOH/0.1% formic acid in water (3:7, v/v) mobile phase and analyzed using UPLC/MS/MS.

2.4 Instrumental Analysis

An Agilent 1290 Ultra-high pressure liquid with Agilent 6490 Triple Quad tandem mass spectrometry (Agilent, Santa Clara, CA) was used for the qualification and quantification of PLY in the reconstituted samples. A Zorbax Extend C_{18} guard column (4.6 x 12 mm, 5 μm particle size, Agilent, Santa Clara, CA) and a Zorbax Extend C_{18} analytical column (4.6 x 50 mm, 5 μm particle size, Agilent, Santa Clara, CA) were used in tandem for chromatographic separation on the UPLC (Agilent 1290, Santa Clara, CA). Gradient elution at a flow rate of 0.5 mL/min was used with mobile phase consisting of 0.1% formic acid in water (A) and 0.1% formic acid in methanol (B). Positive electrospray ionization combined with multiple reactions monitoring (MRM) of parent ion, 411 m/z, and two fragment ions, 363 and 112 m/z were used. The fragment ion with the highest peak intensity was used for quantification and the fragment ion with the next highest peak intensity was used for PLY qualitative confirmation. A PLY standard was compared with that in the test samples for confirmation and a PLY standard curve was used for PLY quantification in the tested samples.

2.5 Method Validation

Control soils were used for validation of the PLY method. The linearity and precision of

the instrumental detection were evaluated by analyzing 5 concentrations (2, 5, 10, 20, 50 ng/mL) of PLY standards in mobile phase in triplicate and plotting the corresponding peak areas detected for the standards against the standard concentrations. The resulting regression equation and R^2 coefficient produced from this calibration curve were used to validate instrument linearity. Matrix effect (ME%) was evaluated by comparing the PLY response in a solvent standard to the response of the same PLY concentration spiked in the soil sample matrix. Matrix-matched PLY calibration standards (2, 5, 10, 50 ng/mL) were analyzed in triplicate.

The method detection limit (MDL) was evaluated by extracting 7 replicates of soil each spiked with 3-5 x the estimated detection limit. The standard deviation of these samples was multiplied by the Student's t value for 98% confidence to calculate the MDL. Limit of detection (LOD) and limit of quantification (LOQ) were also calculated by multiply the standard deviation from the MDL test by 3.3 and 10, respectively. Three spiking levels (2.5, 5, 10 x LOQ) were used for testing method accuracy and precision. Intra-day and inter-day precision were determined from analyzing 7 soil replicates spiked at 2.5, 5, and 10 x LOQ concentrations and prepared in same day for each spike level and 1 replicate of each spike level prepared on 7 different days, respectively.

2.6 Results and Discussion

Little variation was observed in PLY recovery between the three different drying temperatures tested (30, 40, and 50 ˚C) and therefore 50 ˚C was selected to minimize drying time (Figure 2). Vacuum evaporation parameters were optimized to minimize PLY loss and time required for complete evaporation of 5 mL ACN during concentration step of procedure. Temperature, vacuum pressure, and vortex speed settings were tested for optimal PLY recovery and drying time. Influence of evaporation temperature on PLY recovery was tested using fixed

vacuum pressure and vortex speed parameters of 100 mbar and 45%, respectively.

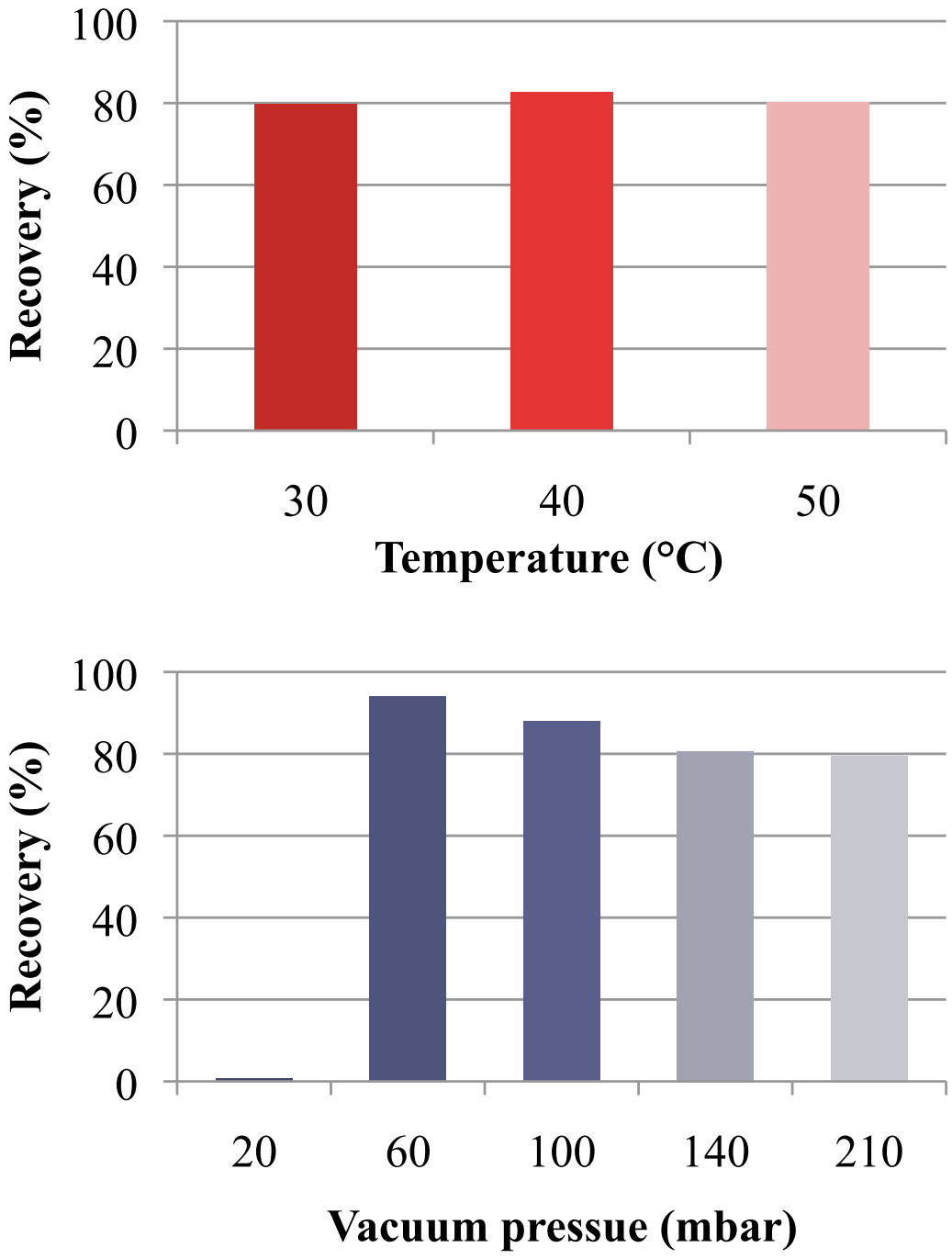

Figure 2-2 Optimization of vacuum evaporator settings to completely dry 5 mL ACN sample eluents during concentration step of the procedure.

Similar to the results from optimization testing by Hornish et al. (1995), who observed little to no deleterious loss of PLY when left in 60°C, under nitrogen stream for 10, 30, and 60 minutes after complete evaporation during concentration step. Five vacuum strengths (20, 60, 100, 140, 210 mbar) were compared with temperature and vortex speed parameters fixed at 50˚C and 45%, respectively. Optimal drying time and PLY recovery of 94% were observed for samples evaporated with 60 mbar vacuum setting. The weaker vacuum evaporation settings (100, 140, 210 mbar) produced slightly lower recoveries, between 79-88%, and took longer to completely dry samples. Prolonged exposure to the harsh vacuum evaporation conditions due to longer drying times could explain the decrease in PLY recovery for 100-210 mbar. The 20 mbar setting was too strong and resulted in severe spills of sample extracts.

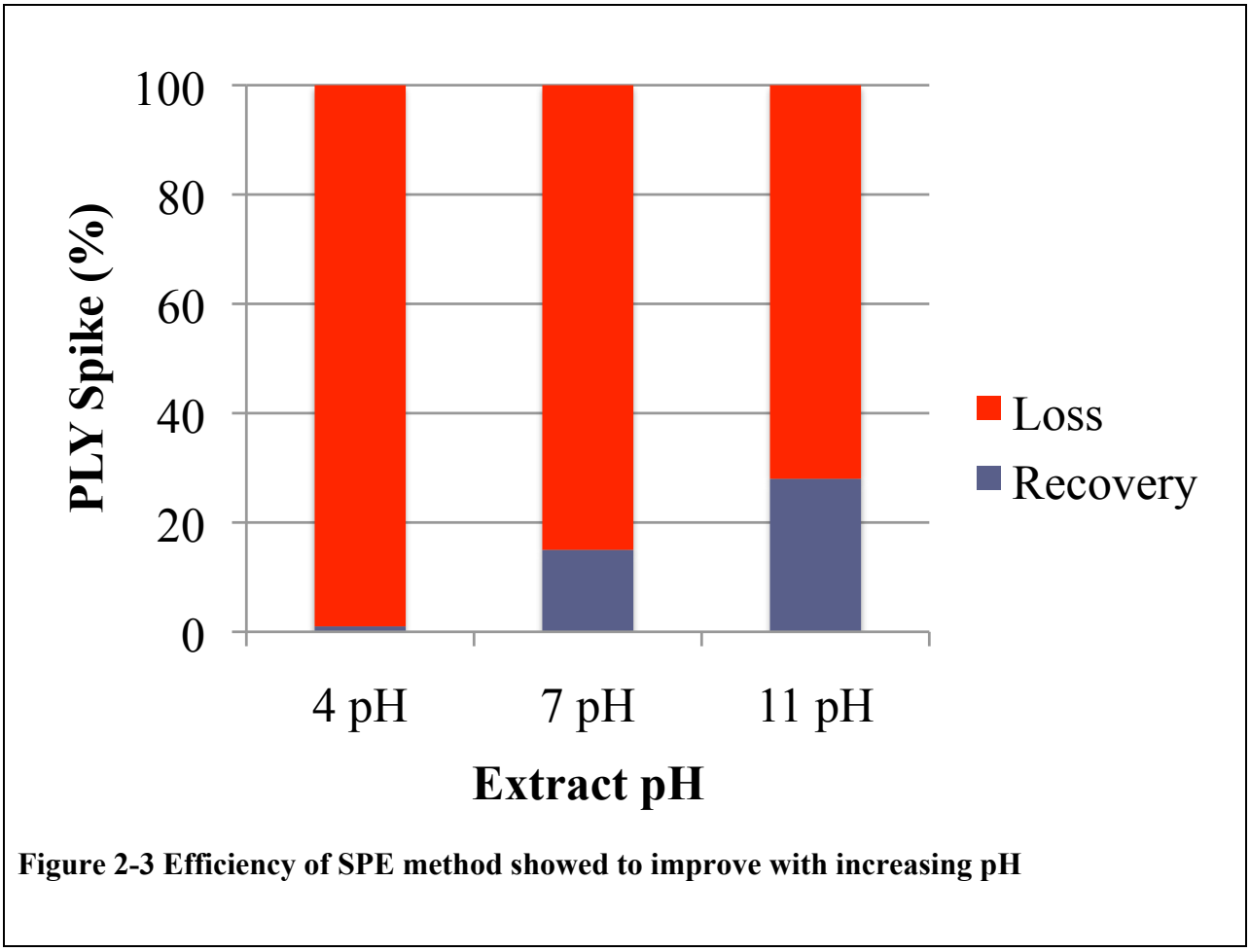

Figure 2-3 Efficiency of SPE method showed to improve with increasing pH

The SPE clean-up efficiency was tested by spiking PLY into 10 mL aliquots of 0.1 M aqueous ammonia/methanol (1:1, v/v) extract solution and calculating the recovery. Variations in SPE method parameters, such as extract pH and elution solvent, were tested for optimal SPE efficiency. The influence of extract pH on SPE method was most significant in recovering PLY from basified extracts; however, recovery was still very poor overall (Figure 2-3). Only 1%, 15%, and 28% recoveries were observed for acidified, neutral, and basified extracts, respectively. Previous PLY methods employing SPE clean-up have typically acidified polar aqueous/organic extracts prior to loading onto Oasis HLB cartridges. These findings suggest the de-protonated, neutral form of PLY present in the basified extract may have adsorbed strongly to the HLB cartridge via hydrophobic so that the compound cannot elute well using a solvent like ACN. This finding is not consistent with a previous method for PLY analysis in milk that used HLB purification combined with ACN elution (Turnipseed et al. 2008).

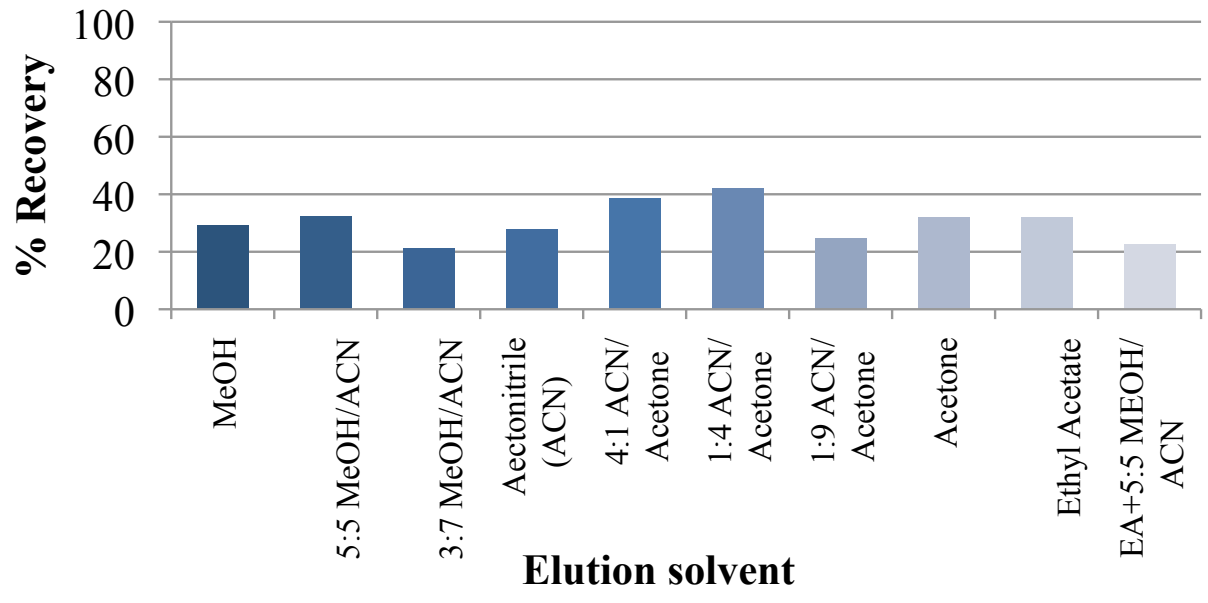

Figure 2-4 Elution solvents, listed in order of descending polar strength (left to right), tested for optimal recovery of PLY during SPE steps

There was no detection of PLY in leachate passed through HLB cartridges for each step of SPE operation, suggesting PLY spiked into the extract was likely being retained in the HLB cartridge. Elution using less polar solvents were tested and showed slight improvement in PLY recovery during purification compared to ACN (Figure 2-4). The best recovery was achieved when eluting with 5 mL acetonitrile/acetone (v/v, 1:4). Due to little improvement seen with parameters tested during SPE optimization and clogging issues with the pressurized liquid extractor during the preliminary testing, an alternative liquid-soil extraction method was investigated for PLY extraction from soils.

Methylene chloride and hexane were compared during initial liquid-liquid extraction of PLY spiked into 5 mL of aqueous ammonia/methanol (1:1, v/v) mixed with 50 µL of concentrated ammonium hydroxide to determine optimal solvent for extracting unionized PLY molecules from strong polar extract phase. Lack of PLY detected in hexane extracts during LC-MS/MS analysis suggest that unionized PLY molecules maintain intermediate polarity from functional groups that prevent hydrophobic partitioning into strong non-polar solvents. About 55% of PLY was recovered from liquid-liquid extraction (LLE) of 5 mL of aqueous ammonia/methanol (1:1, v/v) mixed with 50 µL of concentrated ammonium hydroxide with 3 mL of methylene chloride. Aqueous solutions were then basified with 50 µL ammonium hydroxide to deprotonate PLY molecules so that they could be transferred to nonpolar phase when extracting aqueous layer with methylene chloride (Turnipseed et al., 2008). This is an example that raising the pH of a polar extract can manipulate PLY solubility and transfer PLY molecules from polar organic phase to nonpolar, methylene chloride phase.

The incorporation of 1 g anhydrous sodium sulfate drying agent to 1:6 aqueous ammonia/methylene chloride (v/v) and 20 minute interval sonication steps in extraction

procedure was found to be effective in the extraction of PLY from soil samples. The matrix effect (%ME) in soil was between 8-15% (Figure 2-5). Matrix matched calibration curve was used for analyte quantification during method testing. Linearity of standard curves consistently had an $r^2 > 0.990$. The limit of detection (LOD) and limit of quantification (LOQ) were determined by multiplying the standard deviation used to find the MDL by 3.3 and 10, respectively, resulting in an LOD of 1.38 ng/g and LOQ of 4.17 ng/g. The LOQ x 2.5, 5, and 10 were the spike levels selected for method validation testing of inter-day and intra-day precision.

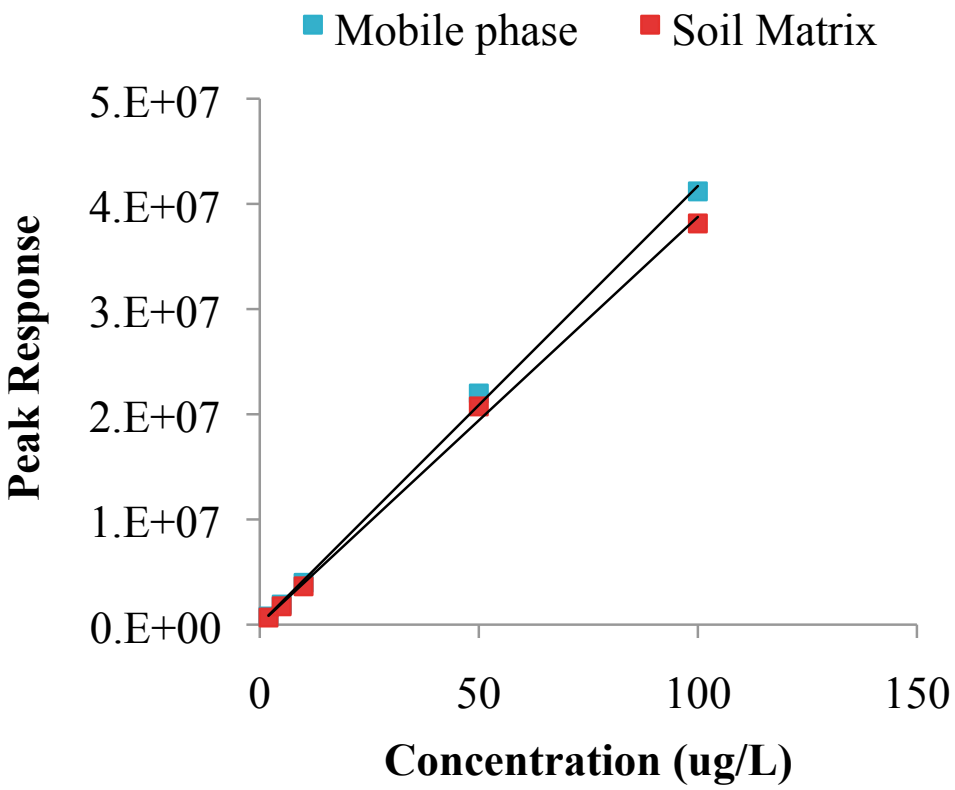

Figure 2-5 Comparison of five-point PLY calibration curve (2, 5, 10, 50, 100 ng/g) prepared in control matrix extract (red) and pure mobile phase (blue)

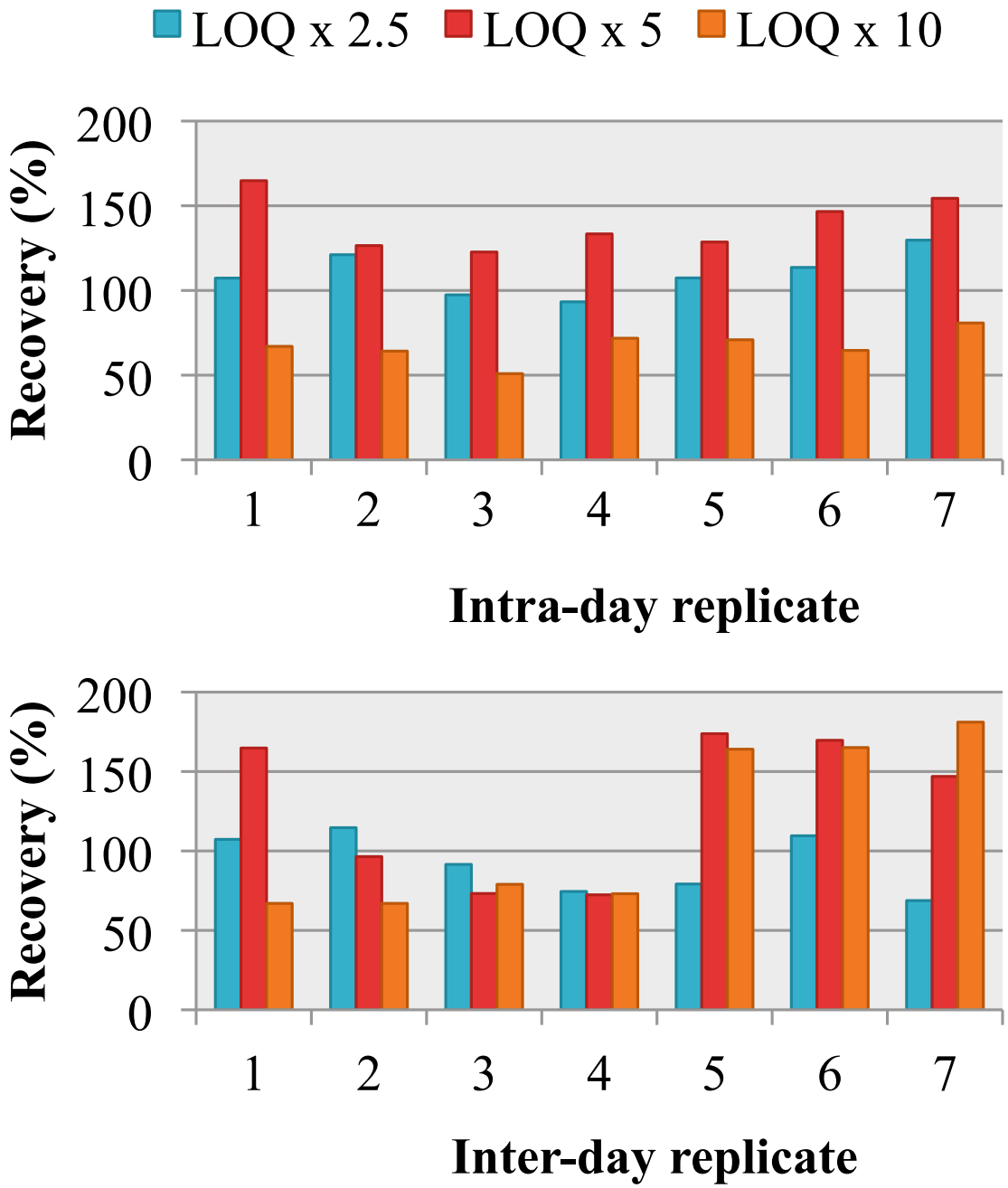

Figure 2-6 Method accuracy and precision of soil spike replicates prepared on the same day and between different days for three spiking levels

Average recoveries for inter-day precision of the three spike levels ranged between 92-128% with % RSD between 20-47% (Figure 2-6). Intra-day precision % recoveries and % RSD ranged between 67-140% and 11-13%, respectively. The variation in the spike recovery within each spike level was similar to that of existing analytical methods for organic base compounds in soil. Saliva et al. (2012) suggested that the high % RSD for several antibiotic compounds is a result of the complexity of the matrix and adsorption strength of interactions with trace levels of antibiotics in soil. Variation is also likely due to the trace concentrations in the soil. These results demonstrate strong feasibility for the proposed analytical method for PLY in soils; however, all average recoveries, repeatability, and reproducibility parameters do not fall within all validation criteria according to USEPA C.F.R. 40, which requires 70-130% recovery and % RSD < 20%.

2.7 Conclusions

The liquid-solid extraction procedure developed in this study provides a quick and cost-effective strategy for efficient recovery of PLY from soil. The selectively and sensitivity achieved with LC/MS/MS analysis enables efficient, reliable detection of PLY in soil at trace levels. This method could be used for the analysis of environmental soil samples from the agricultural fields where dairy-manure has been applied to monitor the extent of long-term environmental persistence and movement of pirlimycin through soils via leaching or surface runoff. Controlled field experiments such as rainfall simulation studies with dairy-manure applied to field plots could make use of this efficient method to investigate leaching risk of PLY with different manure application practices in agriculture.

2.8 References

Bohm, D.A., C.S. Stachel and P. Gowik. 2011. Validated Determination of Eight Antibiotic Substance Groups in Cattle and Pig Muscle by HPLC/MS/MS. Journal of AOAC International 94: 407-419.

Bohm, D., C. Stachel and P. Gowik. 2012. Validation of a multi-residue method for the determination of several antibiotic groups in honey by LC-MS/MS. Analitical and Bioanalytical Chemistry 403: 2943-2953.

Heller, D.N. 1996. Determination and Confirmation of PIrlimycin Residue in Bovine Milk and Liver by LIquid Chromatography/Thermospray Mass Spectrometry: Interlaboratory Study Journal of AOAC International 76.

Hornish, R.E., A.R. Cazers, S. Theodore Chester Jr and R.D. Roof. 1995. Identification and determination of pirlimycin residue in bovine milk and liver by high-performance liquid chromatography-thermospray mass spectrometry. Journal of Chromatography B: Biomedical Sciences and Applications 674: 219-235.

Hutson, D.H., A. American Chemical Society. Division of and N.Y. American Chemical Society. Meeting New York. 1992. Xenobiotics and food-producing animals: metabolism and residues. American Chemical Society, Washington, D.C.

Juan, C., J.C. Moltó, J. Mañes and G. Font. 2010. Determination of macrolide and lincosamide antibiotics by pressurised liquid extraction and liquid chromatography-tandem mass spectrometry in meat and milk. Food Control 21: 1703-1709.

Kaufmann, A., P. Butcher, K. Maden and M. Widmer. 2008. Quantitative multiresidue method for about 100 veterinary drugs in different meat matrices by sub 2-µm particulate high-

performance liquid chromatography coupled to time of flight mass spectrometry. Journal of Chromatography A 1194: 66-79.

Kim, S.-C. and K. Carlson. 2007. Quantification of human and veterinary antibiotics in water and sediment using SPE/LC/MS/MS. Analitical and Bioanalytical Chemistry. 387: 1301-1315.

Kumar, K., S. C. Gupta, Y. Chander and A.K. Singh. 2005. Antibiotic Use in Agriculture and Its Impact on the Terrestrial Environment. Advances in Agronomy. Academic Press. p. 1-54.

MacDonald, J.M. and W.D. McBride. 2009. The transformation of US livestock agriculture scale, efficiency, and risks. Economic Information Bulletin.

Tolls, J. 2001. Sorption of Veterinary Pharmaceuticals in Soils: A Review. Environmental Science & Technology 35: 3397-3406.

Turnipseed, S.B., W.C. Andersen, C.M. Karbiwnyk, M.R. Madson and K.E. Miller. 2008. Multi-class, multi-residue liquid chromatography/tandem mass spectrometry screening and confirmation methods for drug residues in milk. Rapid Communications in Mass Spectrometry 22: 1467-1480.

USEPA. Guidelines establishing test procedures for the analysis of pollutants. 40 C.F.R. United Stated Environmental Protection Agency

USEPA. 2013. Literature Review of Contaminants in Livestock and Poultry Manure and Implications for Water Quality. In: U. S. D. o. Agriculture, editor.

Wang, C., Y. Ding, B.J. Teppen, S.A. Boyd, C. Song and H. Li. 2009. Role of Interlayer Hydration in Lincomycin Sorption by Smectite Clays. Environmental Science & Technology 43: 6171-6176.

Chapter 3: Fate of Thiamethoxam (TMX) and Clothianidin (CLO) Coated on Corn Seeds

3.1 Abstract

Thiamethoxam (TMX) and clothianidin (CLO) are highly soluble, neonicotinoid insecticides commonly used as seed coatings to provide protection for seeds and young plants from insect damage. Neonicotinoids have been found to be toxic to certain non-target aquatic organisms even at concentrations as low as 0.01 μg/L. Therefore, the objective of this research was to determine if TMX and CLO can move from coated corn seeds to the surrounding soil environment. Commercially produced corn seeds coated with TMX and CLO were planted in pots in a greenhouse and the plants were harvested at V4 growth stage. The levels of TMX and CLO in bulk soil, root soil, and rhizosphere soil, and in plant roots and shoots were analyzed using liquid chromatography/tandem mass spectrometry (LC/MS/MS). Results from this study demonstrated that both TMX and CLO coated on corns seeds moved, within 3 weeks of planting, into the surrounding soil in addition to being taken up by the plants. After 3 weeks, 11-16% of TMX and CLO initially coated on the seeds could be detected within all sample matrices. Of the detectable TMX and CLO, < 1% and 2% remained on the seed casing and were taken up by the plant, respectively. About 97% of the detectable TMX and CLO remained in the soil, with evidence of outward movement from the seeds to the rhizosphere soil and to bulk soil. This data suggested that seed coating neonicotinoids have the potential to migrate to the surrounding soil and aquatic environment during the growing season.

3.2 Introduction

Since their introduction to the market in the early 1990s, neonicotinoids have become the most widely used class of insecticides. They are registered in over 120 countries and make up almost a quarter of the global market today (Jeschke et al., 2011). Chemical treatments are

readily taken up by young roots and spread throughout the plant tissue, allowing for a more precise targeting of harmful insects. Due to their particular mode of action, neonicotinoids used in agriculture are believed not to result in cross-resistance to other long-established insecticide classes (Jeschke and Nauen, 2008).

Neonicotinoid application methods include surface spraying, granules, in-furrow, surface injection, irrigation water treatment, seed coating, etc. (Simon-Delso et al., 2015). Surface-application of systemic insecticides have a high risk of reaching surface and groundwater bodies via surface runoff and/or infiltration following a watering/precipitation event (Gupta et al., 2008). In an effort to minimize the spread of surface-applied pesticides in water runoff, neonicotinoids are commonly applied as a seed coating, making up 80% of the global seed treatment market. Studies suggest that only 2-20% of the neonicotinoid seed treatment was expected to be taken up by the plant (Sur and Stork, 2003), leaving 80-98% of the original neonicotinoid coating remaining behind in the soil. The environmental fate of neonicotinoids has become a growing concern over the last several decades due to the possible biological impacts on non-target organisms and ecosystem functions (Schaafsma et al., 2015). Aquatic invertebrate species have demonstrated sensitivity to neonicotinoid levels that have been reported in these water contamination studies (Pisa et al., 2014).

The fate of TMX and CLO are of particular interest because of their extensive use in two of this country's largest crop productions, corn and soybeans, both of which use neonicotinoid-coated seeds extensively (Hladik et al., 2014). Contaminated surface waters have been reported in the U.S., Canada, Sweden, Australia, Switzerland, and Japan with maximum concentrations up to 55.7 ug/L CLO and 63.4 ug/L TMX (Morrissey et al., 2015). A study in Iowa detected TMX and CLO in 75% and 47%, respectively, of the 79 water samples collected from 9 stream

sites in a region of high corn and soybean production (Hladik et al., 2014). In Wisconsin, maximum concentrations of TMX and CLO up to 8.93 ug/L and 3.43 ug/L, respectively, were detected in groundwater used to irrigate potato agroecosystems where neonicotinoids are regularly used (Huseth and Groves, 2014).

Given the extensive rate at which neonicotinoids are being used worldwide, further research is needed to better understand the fate of neonicotinoids used with different agricultural practices and identify the mechanisms and pathways responsible for exposure to non-target aquatic ecosystems. Therefore, the objective of this research was to determine if TMX and CLO coated onto corn seed can move away from the seed, out into the soil during the early stages of plant development.

3.3 Materials and Methods

3.3.1 Greenhouse Setup

CLO-treated corn seeds (Poncho 250, Bayer), TMX-treated corn seeds (Cruiser 1250, Syngenta), and TMX and CLO-free control corn seeds were used during a three-week greenhouse study to investigate TMX and CLO distribution in plants and soil. Control corn seeds were obtained by thoroughly rinsing CLO off Poncho 250 corn seeds with 10 mL of fresh water for three consecutive times, three seeds at a time. Analysis of the rinsed corn seeds showed complete removal of CLO from the seeds. Three seeds/treatment were planted in 2 kg of soil in an 11.3 L plastic pot. Groseclose (fine, mixed, semiactive, mesic, Typic, Hapludults) and poplimento (fine, mixed, subactive, mesic, Ultic, Hapludulfs) soils collected from Montgomery County, VA (37.21757^{o}, -80.46301^{o}) were used.

3.3.2 Sample Collection

Once corn plants reached V4 growth stage, approximately 3 weeks after germination, samples were collected for analysis. During sample collection, all soil and plant material from each pot was divided into six sample matrices: corn above ground vegetation, corn roots, treated seed casing, rhizosphere soil, root soil and bulk soil (Figure 3-1). Entire soil and root system for each pot was transferred to a large plastic container and soil was carefully loosened with hands in order to free plant roots from the bulk soil. Roots and attached root soil were transferred into a large, clean beaker. The soil left behind in the plastic bucket was sieved (0.2 cm) to remove small root material, transferred into pre-weighed plastic bag, and weighed.

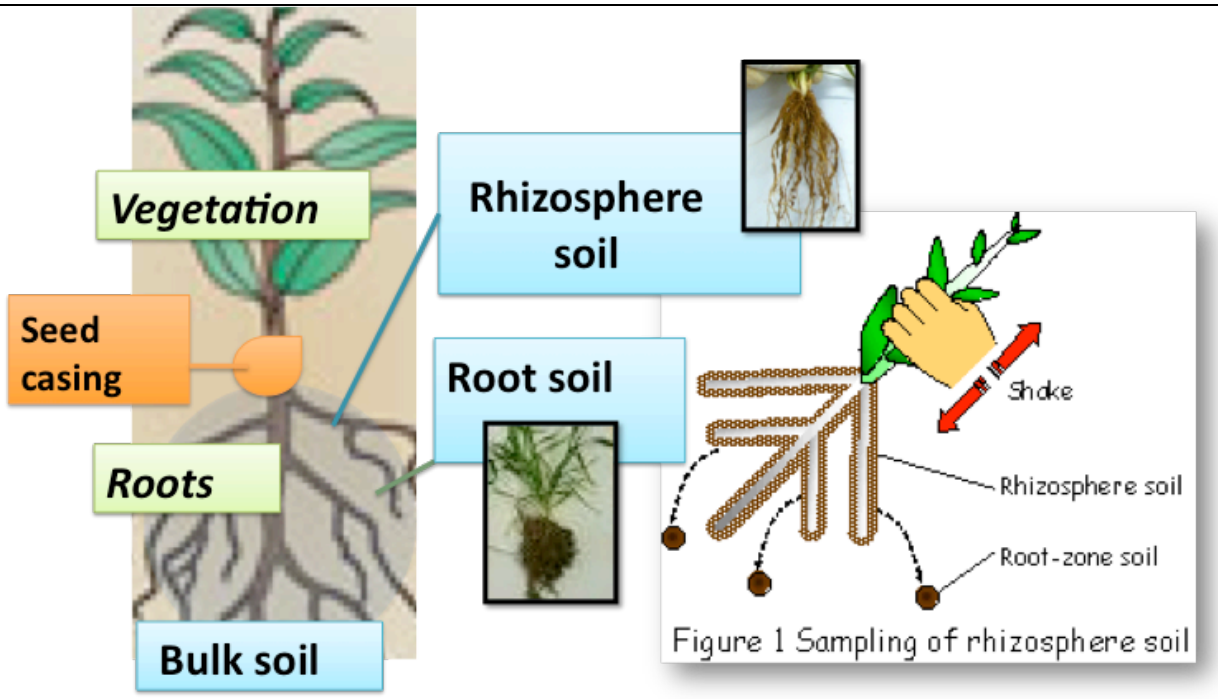

Figure 3-1 Diagram of the 6 difference sample matrices collected during harvest

Root soil clumped onto root system was thoroughly shaken off from the roots into a large beaker, sieved, collected in a pre-weighed plastic bag, and weighed. The three seed casings on each plant were visible at this time and were carefully collected into plastic sample bag. In

order to collect the rhizosphere soil sample, root systems of all three plants were rinsed carefully in a clean glass beaker containing approximately 150-200 mL de-ionized water until all residual soil was rinsed off into the water. The soil-water solution was mixed vigorously and divided evenly between two small, pre-weighed plastic cups with snap on lids. Corn roots were then separated from the stem using scissors and both root and vegetation samples were transferred into their own pre-weighed plastic bag and weighed. All samples were stored in – 10^{o}C until analysis.

3.3.3 Sample Extraction and Analysis

Seed casings collected from a single pot were placed in a clean, glass centrifuge tube. The seed casings from each sample pot were mixed with 5 mL acetonitrile, sonicated for 20 minutes, and centrifuged at 1200 x g for 3 minutes to settle soil residue left on the seed casings. Exactly 2 mL of the acetonitrile sample extract was placed into a clean test tube and evaporated to dryness in a RapidVap (Labconco, Kansas City, MO) using the following parameters: 60% vortex, 40^{o}C, and 100 mbar for 30-40 minutes. Dried samples were reconstituted in 1 mL LC mobile phase, 5 mM NH_4-Ac (1:9, v/v) MeOH/H_2O. Reconstituted samples were passed through a 0.2 mm filter before transferring to a clean HPLC vial. Final 1 mL seed extracts were diluted 10 and 20 times for Poncho 250/Control and Cruiser 1250 treatments, respectively, before UPLC-MS/MS analysis.

Soil samples (bulk, root, rhizosphere) were freeze-dried and ground with pestle. Approximately 1 g freeze-dried soil was weighed into a 35 mL-glass centrifuge tubes. Exactly 10 mL of acetonitrile was transferred to each tube and foil lined-screw top lids were securely fastened. Samples were mixed on a vortex mixer at maximum speed for about 15-20 seconds before adding 2 g anhydrous $MgSO_4$ and 0.5 g NaCl to each tube. Lids were secured and

samples were mixed vigorously on vortex for another 2 minutes before placing in centrifuge. Further separation was achieved via centrifugation of samples at 1,782 x g for 10 minutes. Without disturbing any of the soil slurry, entire acetonitrile extract was transferred to another 35 mL-centrifuge tube. Screw tops were placed on new group of centrifuge tubes containing ~10 mL of ACN extract only. Another 5 mL of pure acetonitrile was added to the first group of sample tubes containing soil. Samples were vortexed again for 2 minutes and centrifuged as previously mentioned. Acetonitrile extract from the second extraction cycle was combined with the first ~10 mL acetonitrile extract collected. A 5 mL aliquot of the combined supernatant was transferred to a new glass centrifuge tube containing 0.05 g PSA and 0.25 g $MgSO_4$ for further sample clean-up. These tubes were also vortexed and centrifuged following the same steps stated previously (Anastassiades et al., 2003). Depending on the sample type and treatment, a range between 0.01-0.50 mL of the 5 mL cleaned up sample extract was transferred into a clean 10 mL test tube and dried down in RapidVap using the same sample drying parameters previously described for the seed extraction procedure.

Plant material samples were mixed with liquid N_2 and finely ground with a mortar and pestle and then freeze dried. Approximately 0.2 g freeze-dried root material and 0.1 g freeze-dried vegetation were weighed out and transferred to 35 mL-glass centrifuge tubes. Exactly 15 mL acetonitrile was added to each sample tube containing plant material and foil-lined screw top lids were securely fastened. Samples were vortexed for 10-15 seconds before adding 2 g of anhydrous $MgSO_4$ and 0.5 g of NaCl to each tube and vigorously mixing again on vortex for 2 minute. Tube caps were unscrewed to release built up pressure before samples were centrifuged for 10 minutes at 1,782 x g. A 5 mL aliquot of acetonitrile sample extract was transferred to a new, clean 12 mL glass centrifuge tube containing 0.05 g PSA and 0.25 g anhydrous $MgSO_4$ for

sample clean-up. These new 12-mL tubes were vortexed for 1 minute and centrifuged for 10 minutes at 1200 x g force. Depending on the sample type and treatment, a range between 0.01-0.50 mL of the 5 mL cleaned up sample extract was transferred into clean 10 mL test tube and dried down in RapidVap using the same sample drying parameters previously described for the seed extraction procedure.

3.3.4 Ultra-pressure liquid chromatography tandem mass spectroscopy analysis

Analysis and quantification of TMX and CLO levels in plant and soil samples were determined using an Agilent 1290 Ultra-pressure liquid chromatography with Agilent 6490 Triple Quad tandem mass spectrometry (Agilent, Santa Clara, CA). A 4.6 x 12 mm Zorbax Extend C_{18} guard column with 5 μm particle size and 4.6 x 50 mm Zorbax Extend C_{18} analytical column with 5 μm particle size were used in tandem for chromatographic separation (Agilent, Santa Clara, CA). Using positive ionization mode, TMX parent ion, 292 m/z, and daughter ions, 211 and 181 m/z, were the parameters used for MS analysis. Parent ion, 250 m/z, and daughter ions, 132 and 169 m/z, were the parameters used for MS analysis of CLO.

3.4 Results and Discussion

Three weeks after germination, 16% and 11% of the initial 1.25 mg TMX and 0.25 mg CLO per seed as seed coating could be detected from Cruiser 1250 and Poncho 250 treatments, respectively (Figure 2). Figure 2 also shows the 1% of the initial TMX coated on the seeds was in the form of CLO, a metabolite of TMX transformation (Casida, 2011). Large percentages of the initial TMX and CLO treatments coated onto seeds, 83% and 89% respectively, were unaccounted for in plant and soil samples at the end of 3-weeks (Figure 3-2). The undetected TMX and CLO on seed treatments are likely attributed to transformational losses of the parent TMX and CLO compounds to unknown metabolite compounds. Very little of the TMX treatment

was detected in the form of CLO metabolite, suggesting that CLO may not be a major metabolite of TMX or that the CLO metabolite of TMX is being further transformed to unknown final products. Significant transformation of initial CLO coated onto corn seeds in this study demonstrates the potential for further transformation of CLO metabolites.

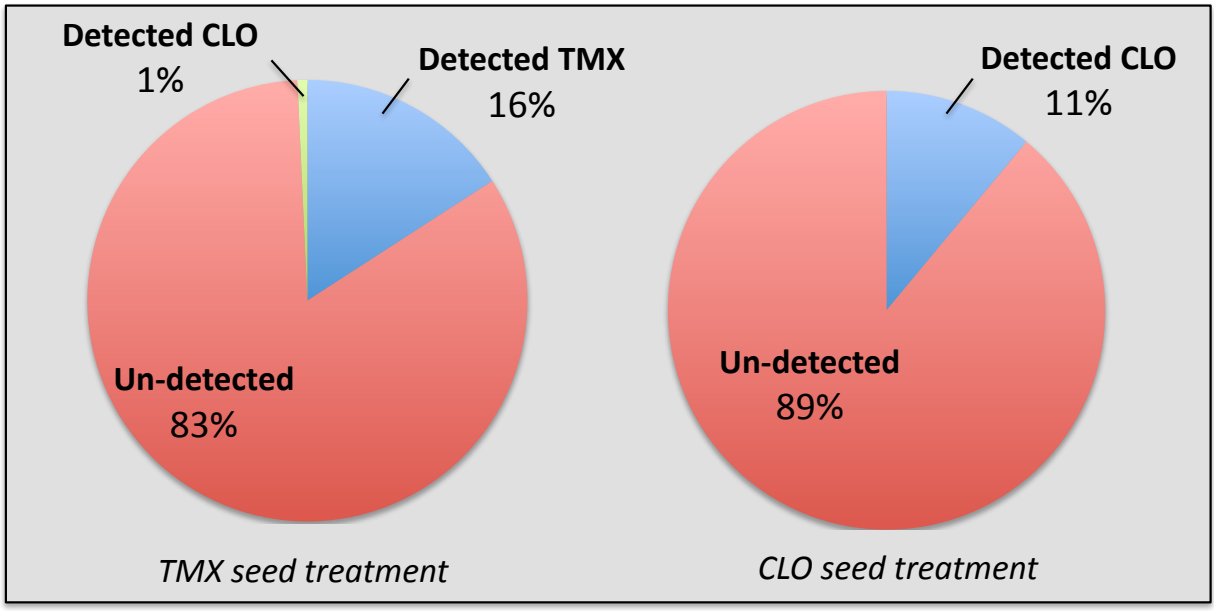

Figure 3-2 Total percentage of the initial TMX (left) and CLO (right) seed treatment that could be detected from all sample types at time of harvest.

Similar rates of CLO loss have also been reported in other soils collected from different geographical locations in China. Li et al. (2012) study reported 67-94% dissipation of CLO in soils 21 days after CLO spraying. In sandy loam soil, a separate study reported 79% dissipation of CLO was observed after 21 days (Ramasurbramanian, 2013). These studies support the CLO transformation rate of CLO treated as seed coat as well as the possibility of further transformation of CLO generated from transformation of TMX coated on the seeds. Full scan LC/MS analysis of sample extracts could be used as a possible method for the identification of unknown TMX and CLO transformation products in plant and soil samples. Interpretation of MS

data reveals structural information about transformation products based of specific mass to charge ratio present at particular retention times (Xiao et al., 2012).

Table 1 Concentrations and TMX and CLO detected in plant and soil samples for each seed treatment

Sample matrix	Avg Sample Weight, g	TMX seed treatment, ng/g		CLO seed treatment, ng/g
		TMX	CLO	CLO
Vegetation	**25**	9744	--	496
Roots	**13**	1221	--	91
Seed[a]	**--**	722	20	453
Rhizosphere soil	**45**	584	31	134
Root soil	**148**	561	27	124
Bulk soil	**1908**	248	12	35

[a] units in ng/seed

The concentrations of TMX and CLO treatments measured in plant and soil samples are shown in Table 1. The highest concentrations of TMX and CLO were measured in vegetation samples at 9,744 and 496 ng/g, respectfully. These concentrations only accounted for 4.13% and 1.20% of total TMX and CLO seed treatments detected 3 weeks after germination, respectfully. In contrast to a study that observed CLO metabolite concentrations approximately twice as high as TMX in cotton leaves 3 days after TMX soil drenching, transformed CLO was not observed in any plant tissue of the TMX treatment (Nauen et al., 2003). The concentration of TMX was lowest in bulk soil samples. However, due to its large sample mass, bulk soil samples accounted

for the highest percentages of detected TMX and CLO treatments ranging between 69.26-83.20%. In bulk soil, 248, 12, and 35 ng/g were measured for TMX, CLO metabolite, and CLO, respectively. The TMX was the only compound detected in root material, accounting for 0.44% of TMX seed treatment (Figure 3-3). Virtually all of the residual seed-applied pesticide that was not taken up by the plant and remain in parent form after 3 weeks appeared to move away from the seed and out into the soil. Only 0.34%, 0.21%, and 1.51% of total TMX, CLO metabolite, and CLO detected was found remaining on the original seed casing of TMX and CLO treatments (Figure 3-3 and 3-4).

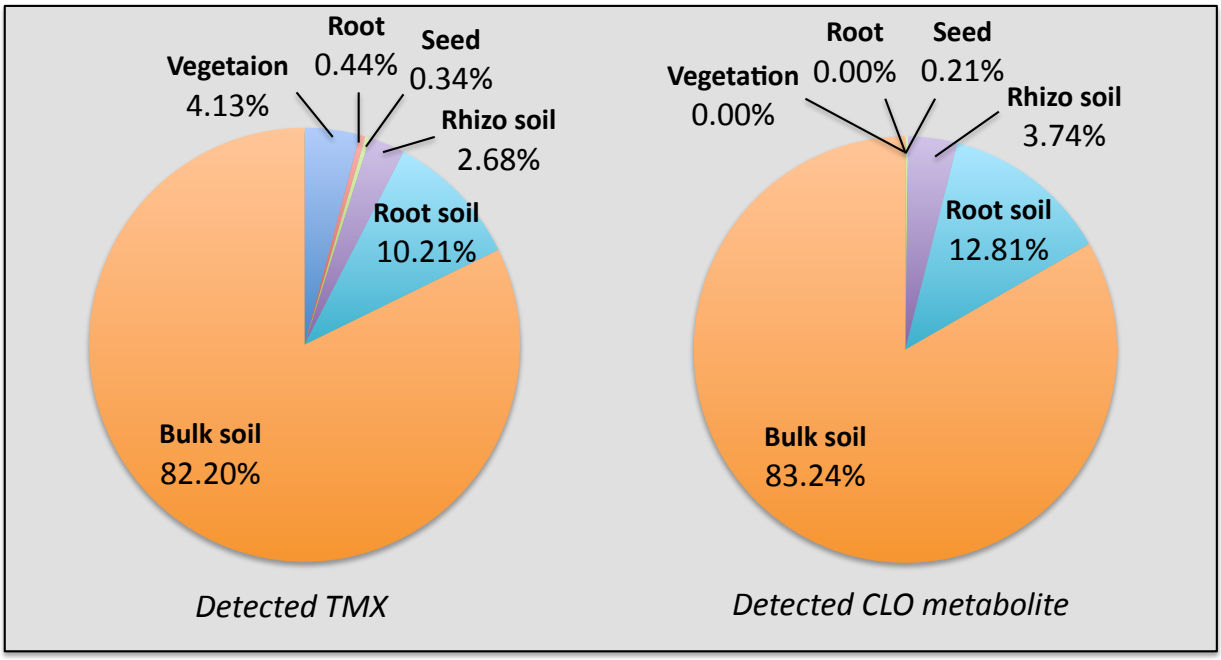

Figure 3-3 Distribution of initial TMX seed treatment detected at V4 growth stage in vegetation and soil as parent TMX compound (left) and CLO transformation product (right)

Due to high water solubility, the movement of TMX and CLO from coated seeds out into soil could have occurred from daily watering events. Soil column leaching studies have observed

66-79% of TMX recovered in leachate, demonstrating the ability of TMX to move through percolating soil water (Gupta et al., 2008). The majority of the remaining neonicotinoid detected after 3 weeks was found in the bulk soil, 82.20% of the detected TMX (Figure 3-3) and 69.26% of the detected CLO (Figure 3-4). The next highest levels of detected TMX and CLO were present in root soil with 10.21% and 24.09%, respectively, followed by rhizosphere soil, 2.68% and 3.76%, respectively (Figure 3-3 and 3-4). This initial movement away from seed to the outer most zone of soil in a 11.3 L pot suggests the potential for further long-range movement of seed coated neonicotinoids through soil and possible entry into nearby aquatic ecosystems.

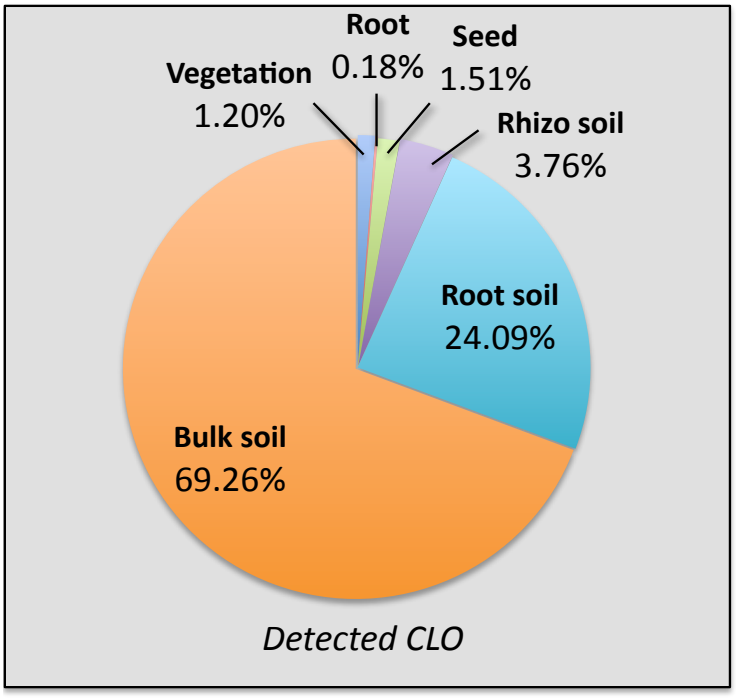

Figure 3-4 Distribution of initial CLO seed treatment detected at V4 growth stage in vegetation and soil as parent compound

A number of microbial strains isolated from neonicotinoid-treated soil have been found capable of degrading these recalcitrant compounds. *Ensifer adhaerens* strain TMX-23 is a nitrogen-fixing, plant-growth-promoting rhizobacterium and was identified in a previous study to be capable of degrading TMX (Guang-can Zhou, 2014). The low levels of TMX and CLO detected in sample matrices occupying the rhizosphere area (seed casing, root material, and rhizosphere soil) could be attributed to the microbial communities in this region

(Guang-can Zhou, 2014).

3.5 Conclusions

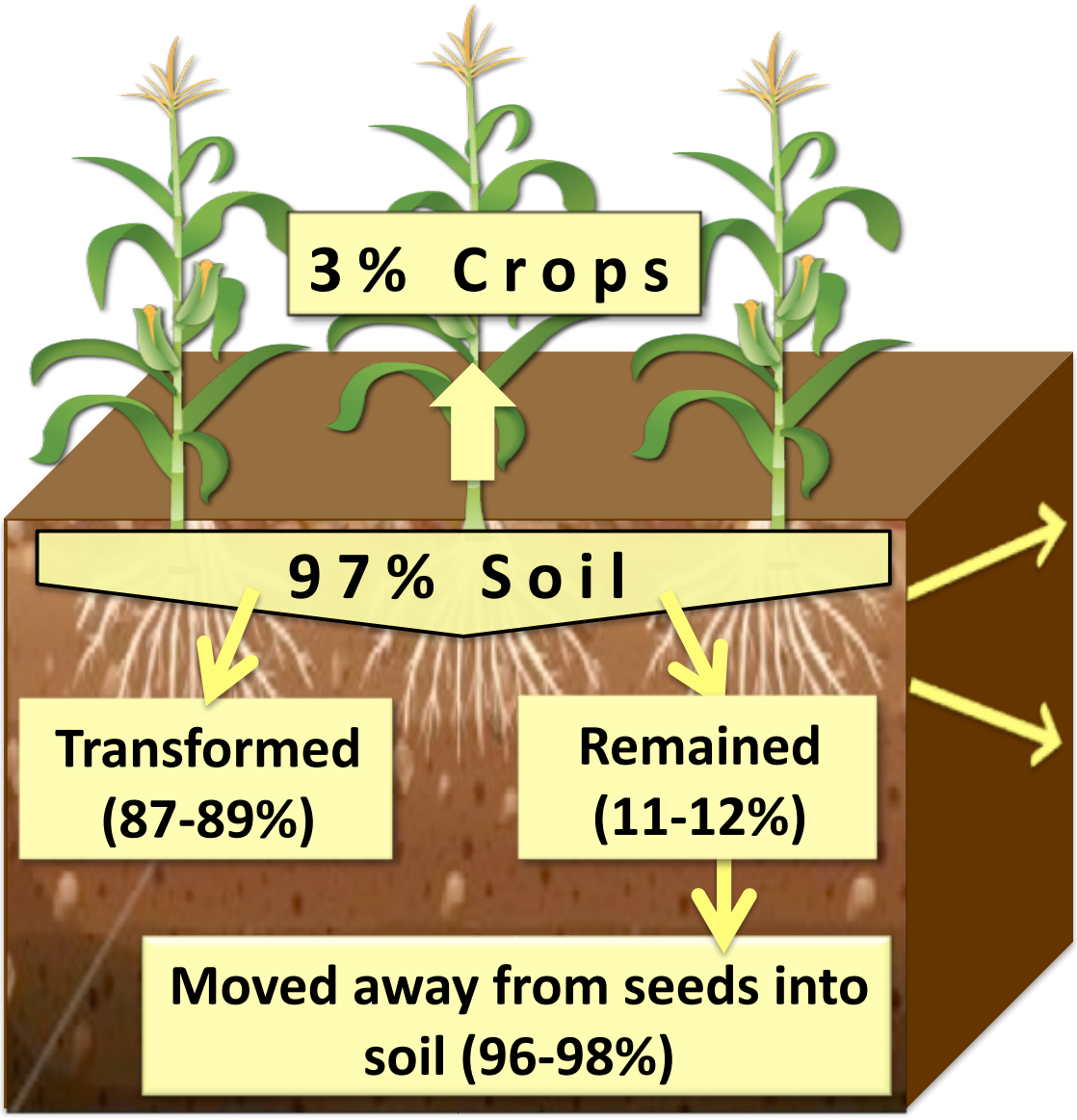

Figure 3-5 Overall summary of conclusions made from results of this study about the distribution and fate of TMX and CLO seed treatments

To put this small greenhouse study into perspective on a larger, field scale, we are to consider using a 67,000 corn seeds/hectare seeding rate (average in Virginia). Assuming each

seed is pre-treated with 1.25 mg TMX, results from this study estimate about 97% of the seed coat would not be taken up by plant and therefore remain in the soil. If 11% of the pesticide coating in soil remains after 3 weeks, roughly 9 g TMX will be present in the soil per hectare of corn. There are about 36 million hectares of corn grown in the U.S. every year. This translates to a grand total of 322,000 kg TMX/year moving from the seed and into the soil from cornfields alone. Neonicotinoids are commonly applied as seed coats in many other major U.S. crop production including soybeans, rice, cotton, and wheat to name a few. Persistence and spread of these chemicals is evident in studies detecting trace levels in a variety of aquatic ecosystems (Figure 3-5). Environmental accumulation of these toxic compounds is possible given the range of half-life values and growing magnitude of use worldwide. Further research is needed to gain better understanding of the overall threat and possible consequences posed by neonicotinoid extensive use to ecosystem function.

3.6 References

Anastassiades, M., S.J. Lehotay, D. Štajnbaher and F.J. Schenck. 2003. Fast and easy multiresidue method employing acetonitrile extraction/partitioning and "dispersive solid-phase extraction" for the determination of pesticide residues in produce. Journal of AOAC international 86: 412-431.

Casida, J.E. 2011. Neonicotinoid metabolism: compounds, substituents, pathways, enzymes, organisms, and relevance. Journal of agricultural and food chemistry 59: 2923-2931.

Guang-can Zhou, Y.W., S. Zhai, F. Ge, Z. Liu, Y. Dai, S. Yuan, & J. Hou. 2013. Biodegradation of the neonicotinoid insecticide thiamethoxam by the nitrogen-fixing and plant-growth-promoting rhizobacterium Ensifer adhaerens strain TMX-23. Appl. Microbiol. Biotechnol. 97: 4065–4074.

Gupta, S.,V.T. Gajbhiye and R.K. Gupta. 2008. Soil Dissipation and Leaching Behavior of a Neonicotinoid Insecticide Thiamethoxam. Bull. Environ. Contam. Toxicol. 80: 431-437.

Hladik, M.L., D.W. Kolpin and K.M. Kuivila. 2014. Widespread occurrence of neonicotinoid insecticides in streams in a high corn and soybean producing region, USA. Environmental Pollution. 193: 189-196.

Huseth, A.S. and R.L. Groves. 2014. Environmental Fate of Soil Applied Neonicotinoid

Jeschke, P. and R. Nauen. 2008. Neonicotinoids—from zero to hero in insecticide chemistry. Pest Management. Science. 64: 1084-1098.

Jeschke, P., R. Nauen, M. Schindler and A. Elbert. 2011. Overview of the Status and Global Strategy for Neonicotinoids. J.Agri. Food Chemistry. 59: 2897-2908.

Li, L., G. Jiang, C. Liu, H. Liang, D. Sun & W. Li. 2012. Clothianidin dissipation in tomato and soil, and distribution in tomato peel and flesh. Food Control. 25: 265-269.

Morrissey, C.A., P. Mineau, J.H. Devries, F. Sanchez-Bayo, M. Liess, M.C. Cavallaro, et al. 2015. Neonicotinoid contamination of global surface waters and associated risk to aquatic invertebrates: A review. Environment International. 74: 291-303.

Nauen, R., U. Ebbinghaus-Kintscher, V.L. Salgado and M. Kaussmann. 2003. Thiamethoxam is a neonicotinoid precursor converted to clothianidin in insects and plants. Pesticide Biochemistry and Physiology 76: 55-69.

Pisa, L.W., V. Amaral-Rogers, L.P. Belzunces, J.M. Bonmatin, C.A. Downs, D. Goulson, et al. 2014. Effects of neonicotinoids and fipronil on non-target invertebrates. Environ. Sci. Pollut. Res 22: 68-102.

Ramasubramanian, T. 2013. Persistence and Dissipation Kinetics of Clothianidin in the Soil of Tropical Sugarcane Ecosystem. Water, Air, & Soil Pollution 224: 1-5.

Schaafsma, A., V. Limay-Rios, T. Baute, J. Smith and Y. Xue. 2015. Neonicotinoid Insecticide Residues in Surface Water and Soil Associated with Commercial Maize (Corn) Fields in Southwestern Ontario. PLoS One 10.

Simon-Delso, N., V. Amaral-Rogers, L.P. Belzunces, J.M. Bonmatin, M. Chagnon, C. Downs, et al. 2015. Systemic insecticides (neonicotinoids and fipronil): trends, uses, mode of action and metabolites. Environ Sci Pollut Res 22: 5-34.

Sur, R. and A. Stork. 2003. Uptake, translocation and metabolism of imidacloprid in plants. Bulletin of Insectology 56: 35-40.

Xiao, J.F., B. Zhou and H.W. Ressom. 2012. Metabolite identification and quantitation in LC-MS/MS-based metabolomics. Trends in analytical chemistry : TRAC 32: 1-14.

Conclusions and Future Research

Initial validation tests for the PLY-soil method developed in this study demonstrate strong feasibility for use in future field research which is critical to better understanding the factors and mechanisms influencing environmental persistence, transport, and fate of manure-applied PLY in agricultural systems. A sensitive reliable method is also important to accurate environmental monitoring and risk assessment. The continued use of antibiotics in animal agricultural is essential to economic production of livestock. Implications from field studies could provide insight to improving agricultural practices to reduce environmental risk.

Results obtained from the greenhouse study investigating the distribution of corn seed-coated neonicotinoids and their potential for movement away from seed, out into soil. Increasing concentration of TMX and CLO was observed in soil zones of increasing distance away from seed. Dust produced from handling neonicotinoid treated seeds has been speculated as the major pathway of non-target exposure in surrounding aquatic habitats. Results from this study demonstrate the potential for long-range transport of seed treatments through the soil into nearby surface and groundwater via subsurface leaching. Further research is needed to assess this hypothesized transport pathway in an agricultural field setting.