DISSERTATION

DETERMINATION OF SPATIAL DISTRIBUTION, DISSIPATION, AND EFFICACY OF INSECTICIDES USED FOR CONTROL OF CITRUS GREENING DISEASE

Submitted by

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ABSTRACT

DETERMINATION OF SPATIAL DISTRIBUTION, DISSIPATION, AND EFFICACY OF INSECTICIDES USED FOR CONTROL OF CITRUS GREENING DISEASE

Citrus greening disease has devastated citrus production globally. While Florida growers explore management strategies, Asian citrus psyllids (ACP) continue spreading this detrimental disease. Determining the efficacy of insecticides applied in citrus groves is a necessity. In these field studies, the efficacies of foliar insecticide treatments to citrus trees were investigated with liquid chromatography tandem mass spectrometry. Insecticide spatial distribution, dissipation, degradation, and effectiveness at reducing ACP were quantified over time after commercial application at a field site in Florida. Citrus leaves, and sample discs attached to leaves, were collected at specific times and locations within individual citrus trees. ACP were inspected before and after treatments to quantify reductions associated with insecticide concentrations over time.

We investigated several insecticides commonly used against ACP including malathion, imidacloprid, dimethoate, and one newer insecticide, afidopyropen. Our findings showed highly variable spatial distribution of insecticides throughout individual trees and rapid dissipation within 24 hours after application. Inadequate distribution to different sides of the leaf and tree canopy areas was observed for all aerial and ground spraying methods tested. Fast degradation rates were observed in sampling discs and citrus leaves with half-lives ranging from 0.6 to 4.0 hours while metabolite concentrations increased. Results showed faster dissipation rates during warmer months (July) and in younger-aged trees ground sprayed with the speed-sprayer. A wide range of insecticide efficacy was observed, with ACP reductions of 63 to 100%. When ACP remained after

treatment, effectiveness decreased over time and ACP increased (e.g. from 6 to 172% after afidopyropen treatment).

The observed variable spatial distribution, rapid insecticide dissipation, and inadequate efficacy allow remaining ACP or ACP from surrounding groves to continue spreading citrus greening disease, leaving citrus trees unprotected. For contact, or semi-systemic insecticides like afidopyropen, full coverage to both sides of the leaves and tree canopy is crucial to effectively manage ACP populations. ACP regeneration suggests lower metabolite toxicity or pest resistance development and reveals ineffective pest management.

This research not only helps inform citrus growers of actual insecticide efficacy in the field, which may influence their pest and disease management strategies, but also provides better understanding of insecticide dissipation from citrus leaves, which assists those advancing predictive models for agricultural applications. Additionally, these results help inform insecticide manufacturers of their products' performance in field conditions which can be compared to laboratory studies. Lastly, this work reveals information on the fate of insecticides in the field which could be used to evaluate its impact on other species and the environment.

ACKNOWLEDGEMENTS

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I would also like to thank some family and friends who supported me emotionally while in graduate school. I am thankful for my parents and twin sister for their constant encouragement and listening to my exciting news, concerns, field experiment and travel stories, and data explanations. I am thankful for the Fort Collins Tri Delta Alumnae who really served as a second family for me while in Colorado. They provided a "home away from home" for me and helped me make friends and get involved. Although I am sad to be leaving, I will cherish the lifelong friendships I formed while active in the local alumnae chapter.

Lastly, several people supported this research. I would like to thank Cutrale Citrus Juices USA, Inc for funding this project and thank the entire team at Cutrale Farms in Venus, Florida for their assistance while conducting field experiments. I am thankful for my outstanding collaborators who have not only contributed to this work but have supported my development as a scientist. This includes Pankaj Trivedi, Kim Hageman, Greg Dooley, and Sean Stokes. I am grateful for Julia Sharp with the Graybill Statistics and Data Science Laboratory and William Bahureksa—collaborators that assisted with statistics for field sampling and data analysis. I would like to thank those that have helped with field experiments and sample preparation including Bianca Hodge-Pardo, Sean Stokes, Hannah Miller, Ruth Menger, and Paul Ode. Samples from this work were analyzed at two facilities at Colorado State University (CSU). I'd like to thank Greg Dooley with the Analytical Toxicology Laboratory and Claudia DeBoot with the Analytical Resources Core-Materials and Molecular Analysis (ARC-MMA) Laboratory for their help with instrumental method development and analysis.

This work may be described with "I" and "my" throughout this dissertation. This is simply a formality and the credit for this work is shared among me, Thomas Borch, and the collaborators listed above.

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CHAPTER 1: INTRODUCTION

1. CITRUS GREENING DISEASE

Citrus greening disease, or Huanglongbing (HLB), continues to spread throughout citrus groves globally, plummeting citrus production and profits. Brazil, China, and the United States (US), the largest citrus producers worldwide, are struggling the most with devastation to the citrus industry due to HLB.[1,2] Since detection in Florida in 2005, citrus production has decreased by 74%.[3,4] As one of the largest citrus producing states in the US, Florida has experienced a decline of about 5,000 jobs and $1 billion annually since 2015.[2,5,6] HLB causes citrus trees to develop weakened root systems, discolored leaves, and greener fruit that prematurely falls off the tree, leading to lower crop yields.[7,8] Chemical changes in fruit due to HLB infection results in distinctly bitter juice that lacks sweetness and fruity/orange flavor.[6] The causative agent of HLB in the US, *Candidatus* Liberibacter asiaticus (*C*Las) is vectored by the Asian citrus psyllid (ACP) *Diaphorina citri* Kuwayama as it feeds on the citrus phloem.[8] Currently no cure exists for HLB despite several research efforts of potential treatments and management strategies.[2]

2. INSECTICIDE APPLICATION METHODS

Insecticides are widely used to control ACP populations and prevent further spread of the disease. [7,9,10] In order to halt transmission of HLB, effective insecticides must quickly kill ACP or interrupt the feeding processes by which they infect the phloem.[11] Since ACP prefer new flush,[11] or new foliar growth, effort is made to have full coverage of insecticides to the outer-most parts of the tree; and spraying prior to new flush growth is critical in managing ACP populations to prevent reproduction.[7,10] The use of chemical insecticides to control ACP populations remains the primary HLB management strategy.[7,9,10] Many commercial groves implement complex integrated pest management strategies that require the rotation of insecticide types based on

varying classifications, modes of action, and application methods in order to prevent pest resistance and optimize efficacy.[7,12–14]

Common insecticides used to combat sucking insects in citrus crops include selective or broad-spectrum organophosphates (e.g. malathion, dimethoate), neonicotinoids (e.g. imidacloprid, thiamethoxam), pyrethroids/pyrethrins (e.g. cypermethrin) and newer pyropenes (i.e. afidopyropen).[15–18] These insecticides have distinctive chemical classes and modes of action, thus they impact ACP of various life stages (egg, nymph, adult). For our studies, we chose to investigate imidacloprid (IMI), malathion (MAL), dimethoate (DIM), and afidopyropen (AFI) insecticides to assess both contact and systemic types of insecticides and different application methods, but also because these insecticides were scheduled for treatment during our selected field sampling months with higher ACP presence. Additionally, our intensely managed grove partners employ specifically coordinated aerial applications to the grove only once or twice per month and rotate insecticides used throughout the entire grove which also influenced our chosen insecticides.

Insecticides are classified into various modes of action based on the method by which its active ingredient (ai) kills the target insect. This process is often related to the insecticide's chemical class and type (contact or systemic).[14,19] Contact insecticides, like MAL, are often sprayed via ground or aerial application and require direct contact in order to kill the target pest.[16] In comparison, systemic insecticides can be applied via ground or aerial spray, as well as incorporated with irrigation drenching. Systemic insecticides are absorbed into the plant through the leaf surface or roots, depending on application method, then distributed throughout the tree and kill the target pest through ingestion while feeding on plant juices, which may offer protection over longer periods of time.[20] Some insecticides classified as contact and systemic can harm insects by both direct contact and from exposure during feeding.[7,14,21] IMI and DIM are both

contact and systemic insecticides which offers an initial quick knockdown of ACP populations from contact and better ACP control over time.[17,18] AFI is a contact and semi-systemic insecticide.

The most practiced management techniques involve combining systemic drenching on younger trees with foliar applications to quickly kill ACP. Newer management strategies include removing infected trees and implementing area-wide management, coordinating spraying 10-50 thousand-acre areas in order to combat the spread of ACP from "bad neighbors" with less frequent management practices.[9,22] Overall, insecticide application is an expensive HLB management strategy that has significantly increased in cost due to HLB.[23] Additionally, with insecticide costs around 25% of total citrus production, and increasing with current ACP infestation rates, the area of insecticide application has a lot of potential for optimization.[24]

3. Insecticide Dissipation

Insecticides may experience dissipation, or other loss processes during or after application in the field. This can be due to drift, volatilization, run-off or wash-off, plant metabolism, or degradation by photolysis. Various application parameters (e.g. spray droplet size, temperature) have been optimized to reduce product loss to drift. Additionally, adjuvants are often added to insecticide products or mixtures to help reduce loss. For instance, most adjuvants help the chemical product "stick" to the surface of a leaf and reduce run-off. Therefore, dissipation rates on leaves can be affected by varying field and meteorological conditions, pesticide physiochemical properties, application parameters, plant characteristics, and chemical additives such as adjuvants.[25–27] If an insecticide's active ingredient concentration is decreased, it may become too low to effectively eradicate ACP. [7,28] Many have investigated insecticide degradation in water or solvent samples in laboratory studies and in soil and groundwater samples in the field.[29–31] Little is known about the dissipation, or degradation kinetics of many insecticides from leaf samples in

the field; however, this is important to better understand insecticide efficacy and environmental fate.

4. Publications and Presentations

Most of this dissertation work is either submitted to or already published in peer-reviewed journals. Chapter 2 (Rehberg et al., 2021) was published in *Pest Management Science*.[32] Chapter 3 (Rehberg et al.) was submitted to *Pest Management Science* last winter.[33] Chapter 4 (Rehberg et al.) was submitted to *ACS Agricultural Science and Technology* this spring. My collaborations with other researchers involved two co-authored publications (Appendices C-D). First, the work in Appendix C was published in *Phytopathology* (Menger et al., 2022). Second, the work in Appendix D was published in *Environmental Pollution* (Shariq et al., 2021).[34]

Parts of this research have also been presented at several events including a departmental 3-Minute Lightening Talk (Rehberg 2019), CSU Speaks (Rehberg et al., 2020), a department research poster show (Rehberg et al., 2020), American Chemical Society conferences (Miller et al., 2018; Rehberg et al., 2020), CSU GradShow (Rehberg et al., 2021), and three invited seminar presentations (2020, 2021, and 2022). While presenting our research, I won first place at the Soil and Crop Science and Agricultural Biology departments' 3-Minute Lightening Talk competition. I also won first place in the Great Minds in Research Award at the CSU GradShow.

CHAPTER 2: QUANTIFICATION OF INSECTICIDE SPATIAL DISTRIBUTION WITHIN INDIVIDUAL CITRUS TREES AND EFFICACY THROUGH ASIAN CITRUS PSYLLID REDUCTIONS UNDER DIFFERENT APPLICATION METHODS[1]

1. INTRODUCTION

Previous studies have evaluated the spatial distribution of insecticides when applied at different application rates, sprayer types, spray volumes and droplet sizes, ground speed, and weather conditions in the laboratory and field, often using water sensitive papers or fluorescent dyes.[24,35–37] Currently, the most common ways to assess insecticide coverage to crops in the field is to implement fluorescent dyes or water sensitive papers that change color when contacted with water. Water sensitive papers and dyes allow growers to visually see the sprayed droplets on a leaf.[38] This presence of dye or color change is assumed to translate to the presence of insecticide.[24,39–41] Citrus trees have high total foliar surface areas[40] and leaves are often wet in humid environments, like Florida's conditions. Therefore, distributing large water sensitive papers throughout a wet citrus tree often cause misrepresentations of insecticide presence.[38] Some studies investigated spatial distribution in more depth, exploring canopy penetration to a variety of crops including wheat, peppers, onion, tomatoes, oat, and bay laurel. These spatial distribution results of insecticides may be especially inadequate for citrus trees due to their larger canopy and total foliar surface area.[42–46] Few studies have investigated canopy penetration in citrus with metal or fluorescent tracers and have reported outer canopy receives more spray deposition than inner

[1] Reproduced with permissions from Rehberg, R., Trivedi, P., Bahureksa, W., Sharp, J., Stokes, S., Menger, R., Borch, T. Quantification of insecticide spatial distribution within individual citrus trees and efficacy through Asian citrus psyllid reductions under different application methods. Pest Management Science 2021, 77 (4): 1748-1756. Copyright 2020, Society of Chemical Industry.

canopy regions.[24,40,47,48] Therefore, inner canopy leaves could risk an inadequate amount of insecticide necessary to target ACP populations.[24] These studies have not fully investigated all aspects of a citrus tree, including the side of the leaf, which is critical to consider when investigating different kinds of insecticides (contact or systemic) and their application approach. Most studies have not quantified insecticide active ingredient concentrations in sprayer tank mixtures and on leaves while simultaneously inspecting ACP population responses by for instance mass spectrometry, which provides more accurate quantification.[24,39–4113-16] Due to volatilization or degradation, the insecticide active ingredient concentration could become too low to kill ACP or prevent pest resistance.

Previous studies investigating ACP response to insecticides in the laboratory and field conclude that insecticides are effective at killing psyllids.[11,20,21,49–52] The few studies assessing both insecticide application efficacy with ACP inspections either lacked the variety of application methods commonly used in high management groves or an effective ACP inspection method. [11,20,21,49] To date, no studies have thoroughly examined insecticide efficacy by quantifying insecticide concentrations and spatial distribution, extensively sampling entire citrus trees while simultaneously quantifying ACP population response, from multiple insecticide spray methods in a high-management commercial field.

It is clear how insecticides should be applied, kill target pests, and impact ACP at specific concentrations in lab studies.[24,35–37] However, it is not yet known how well insecticides actually distribute and kill ACP among multiple application methods and insecticides when applied to citrus trees in the field. Our objective was to evaluate insecticide application effectiveness by quantifying the concentration and distribution of insecticides applied and their resulting ACP population counts before and after various foliar application methods. We hypothesized that the

concentrations of insecticides applied would be high enough to kill ACP, but the spatial distribution would be inconsistent due to larger citrus canopies.

2. Materials and Methods

The following methods were used in order to collect and analyze field samples. Our goal was to effectively sample trees and quantify the concentration of insecticide active ingredients and ACP present throughout citrus trees after application to better understand the coverage and effectiveness of the insecticides applied.

2.1 Sample Site and Collection

Two field studies were conducted at a citrus grove in Venus, Florida in October 2018 and July 2019. This large-scale and high management grove is 8567 acres and consists of about 60 blocks of citrus trees (Figure 2.1). The weather typically ranges from 85 to 91° F with an average of 81-185 mm of rain in October and July, respectively. At the grove, the wind typically blows from East to West. Weather data was provided by the grove from a weather station on site.

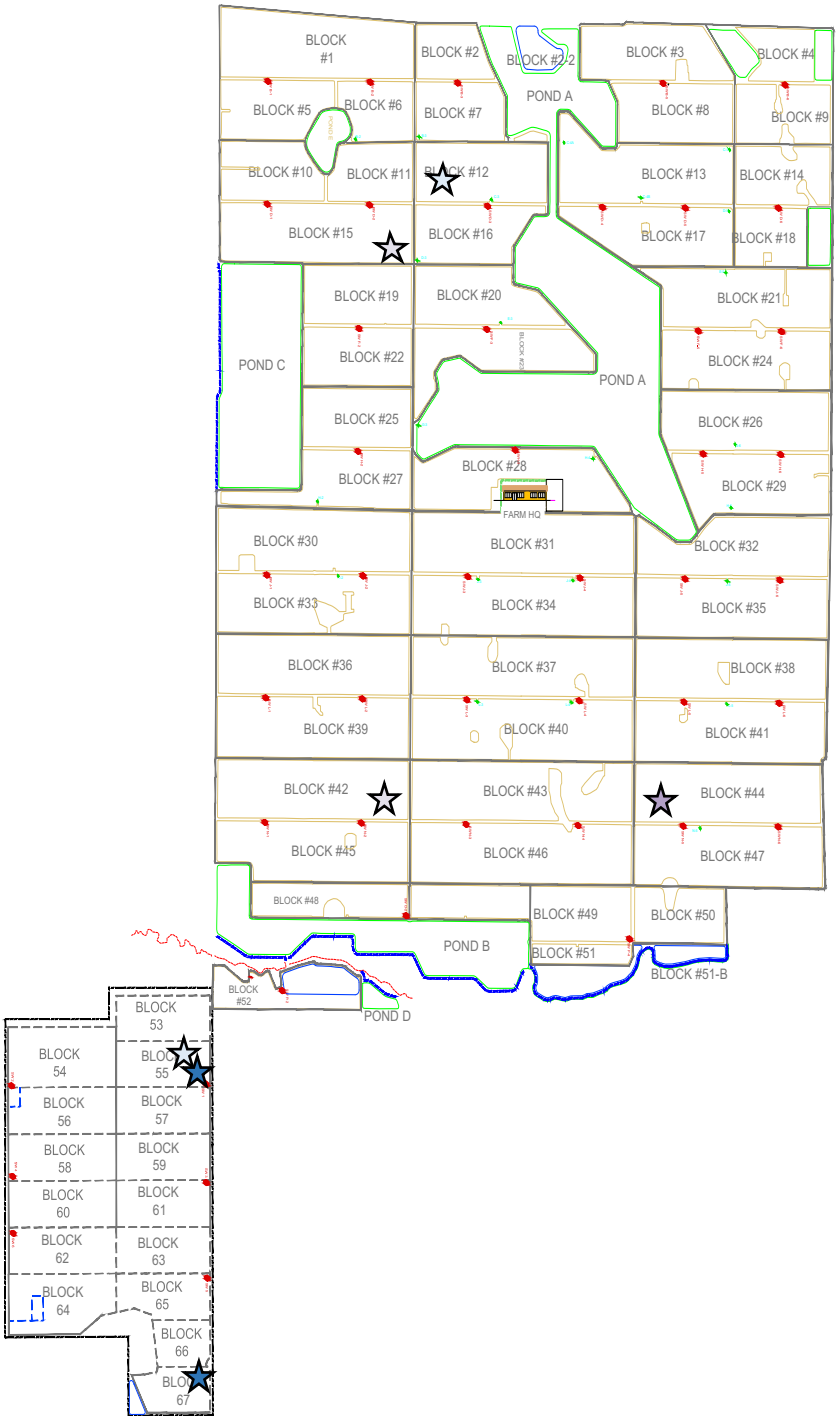


Figure 2.1. Citrus grove field site and experimental locations for various block tests executed in field study #1 and #2. Color coded stars represent block test sampling locations and correlate with Table S1 color coding.

Pilot experiments were performed to test various chemical and inexpensive sorbent materials for samplers as well as methods of attaching samplers to the citrus leaves and trees. After optimizing sampling materials and methods, it was observed that laser cut Whatman filter (WF) discs clipped to the tops and bottoms of citrus leaf surfaces with mini binder clips (9/16") was the most effective and representative method for field sampling of insecticide residues. The WF sampling discs were precut from Whatman filter paper #1 with an Epilog Zing CO^2 laser cutter into 47 mm diameter circles, including sample number labels. The cardinal-directional side of the tree, canopy depth and height, and side of the leaf were examined. The WF discs were labeled and attached in specific sampling locations to best encompass the entire tree equally. The labeling scheme included four letters for the four location identifiers (Figure 2.2).

1) Cardinal direction (North (N), South (S), East (E), or West (W))
2) Canopy depth (Outer (O) or Inner (I))
3) Canopy height (Upper (U), Middle (M), or Lower (L))
4) Side of leaf (Top (T) or Bottom (B))

For example, the notation NOMT describes a sample from the North side of the tree, Outer-Middle canopy, and Top side of the leaf.

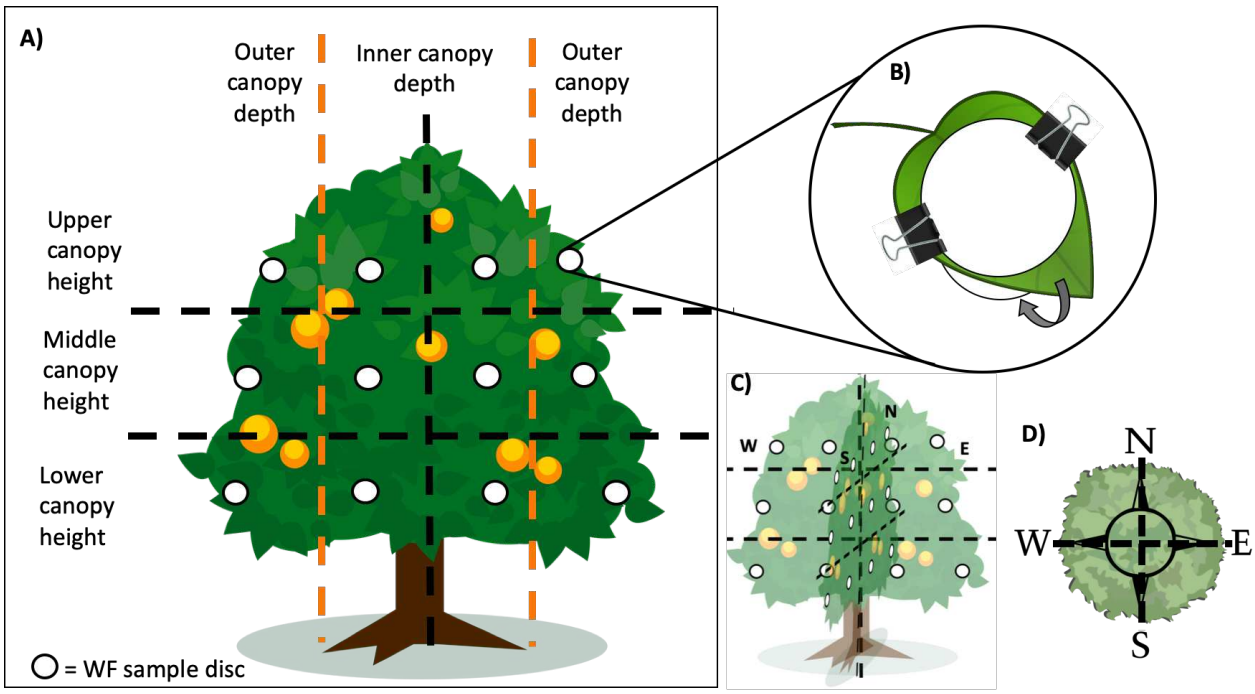


Figure 2.2. Tree location labeling scheme for field sampling. Trees were divided to investigate canopy depth (inner and outer) and height (upper, middle, and lower) (A). Each sample location had a Whatman filter paper disc attached on the top and bottom of a leaf in each canopy height and depth section (B), along with this scheme repeated on each cardinal side of the tree (North, South, East, and West) (C). The top view looking down upon the tree is shown (D) to better understand where samples were located within the tree. This scheme generated 48 samples plus 3 field blank samples per tree (n=255 samples per block test). Example labeling: NUOT=North side, Upper-Outer canopy, Top side of leaf.

Field study #1: In October 2018, five trees were sampled for both ages of trees (old vs young) and application type (aerial vs ground spray) for a total of 20 trees (Figure 2.1, 2.3 Table 2.1). Tree age was provided by our industry partner and identified by sprayer type; ground sprayed with a speed (older) or side (young) sprayer (Figure 2.4). Trees around 2 years and younger are typically small enough to be sprayed with the side sprayer (Figure 2.5).

Table 2.1. Field studies #1 and #2 experimental details including date, block number tested, tree age, application method, sprayer type, and commercial insecticide product applied. Color coded rows correlate with colored stars in Figure S1. *At time of experiment.

Date	Block #	Tree Age (yr)*	Tree Age Classification	Application Method	Sprayer Type	Insecticide Common Name
10/16/18	55	~1	Younger	Ground	Side	Admire
10/16/18	12	~3-4	Older	Ground	Speed	Admire
10/18/18	55	~1	Younger	Aerial	Airplane	Malathion
10/18/18	67	~3	Older	Aerial	Airplane	Malathion
7/10/19	42	~2-3	Younger	Ground	Side	Admire
7/10/19	15	~4	Older	Ground	Speed	Admire
7/11/19	44	~3	Older	Aerial	Airplane	Malathion

Figure 2.3. Aerial application of insecticides to citrus trees during field experiments.

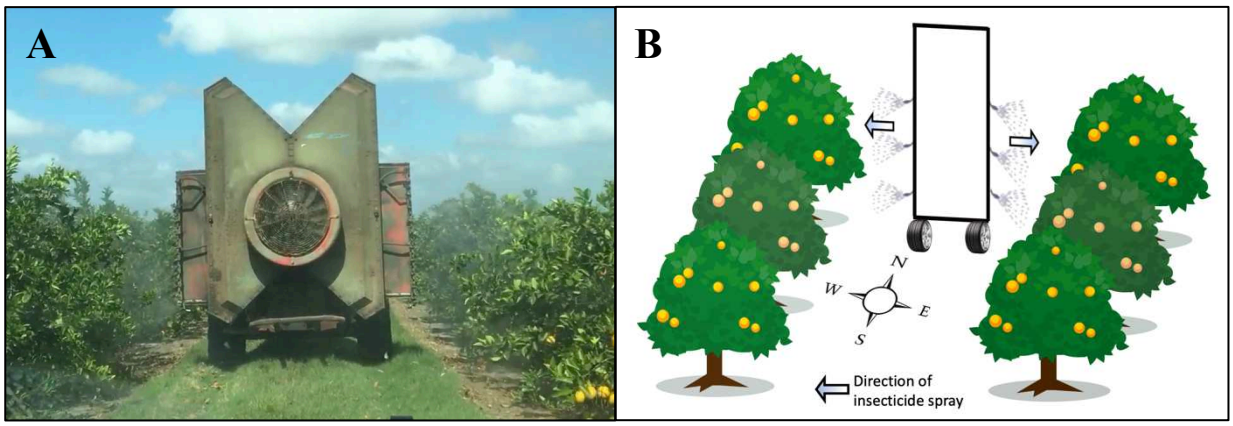


Figure 2.4. Photo (A) and schematic (B) of ground speed sprayer applying insecticides to the East and West cardinal sides of the tree. The tractor drives up and down rows (North to South) in between the trees to apply to both sides of the trees.

Figure 2.5. Photo (A) and schematic (B) of ground side sprayer applying insecticides to the top, East and West cardinal sides of the tree. The schematic above shows only one side of the spraying design. Since the ground side sprayer applies to both sides of the tree simultaneously, it only needs to pass by the tree one time.

Field blanks were also collected for each tree by exposing the WF discs to the air and leaves on their respective tree and storing in the freezer prior to insecticide application. Three field blanks and 48 samples were collected per tree, totaling 1020 samples collected altogether for the four age (old vs young) and application type (ground vs aerial) combination block tests (Table 2.1). All samples were collected within 30 minutes after application, stored separately in Ziploc bags and foil, and stored in the freezer. Samples were shipped cold overnight from Florida to Colorado State University.

The insecticides, Admire (ai: imidacloprid) and Malathion (ai: malathion), were prepared per label instructions and applied via ground and aerial applications respectively. The airplane used to apply insecticides aerially was an air tractor consisting of 86 flat fan #15 nozzles (Figure 2.3). Ground applications were carried out with side and speed sprayers to young (1 yr) and old (3 yrs) citrus trees respectively (Table 2.1). The speed sprayer (FM Copling) has two vertical nozzle booms on each side of the sprayer consisting of 8 D3-C25 nozzles (Albuz/Teejet) on each side. This sprayer is used to apply pesticides to older, larger-canopy trees that no longer fit under the side sprayer apparatus. The speed sprayer applies pesticides at a higher pressure and application

rate (200 psi and 35 gallons per acre) (Figure 2.4). The side sprayer (Newton Crounch) has three nozzle booms on each side of the sprayer, covering three sides of the trees and applying to 2 rows at a time. For each side, the two vertical booms each consist of 4 TXR80017VK nozzles (Teejet) that spray inward toward the east and west side of the tree. The horizontal boom consists of 2 TCR80049VK nozzles (Teejet) that spray down onto the top of the tree (Figure 2.5).

Tank mix samples from each insecticide mixture applied were collected to quantify the actual concentration of insecticide active ingredient applied to the samples collected. The tank mix solution was mechanically agitated for at least 10 minutes prior to sample collection to ensure proper mixing and homogeneity. Samples were collected by the certified pesticide handler in 40 mL amber vials with Teflon cap and stored at 4°C.

Field Study #2: In July 2019, Admire and Malathion were applied via ground and aerial spraying along with ACP counting practices before- and after-treatment to compare insecticide applications with ACP response. Select sample locations from field study #1 were repeated to determine reproducibility. WF samples included 21 samples per block test (3 field blanks and 18 samples). Tank mix samples were again collected by the aforementioned method (Table 2.1).

2.2 ACP COUNTING

ACP data was obtained by a professional psyllid inspector in the grove. For each block test, 30 rows were inspected for ACP and sprayed with insecticide. ACP were inspected in 3 trees in each row (the north border, middle, and south border) totaling n=90 trees inspected for each block test (Figure 2.6). The inspector surveyed the entire tree, thoroughly counting ACP adults and nymphs. During the aerial application of malathion in field study #2, the pilot only sprayed rows 1-15, therefore only ACP data for rows #1-15 (n=45 trees) were included for analysis. Psyllid

inspectors counted ACP the day before and day after insecticide application. All ACP counts post-application were confirmed alive.

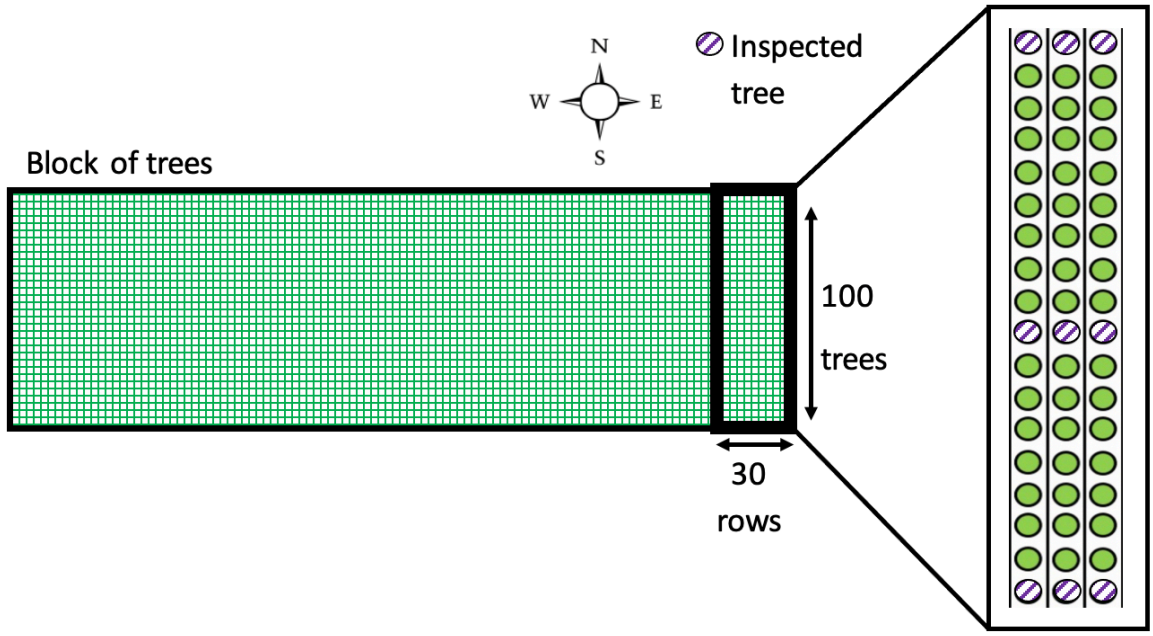


Figure 2.6. Psyllid inspection schematic. For each block test, 3 trees were inspected in each row (south, middle, north) for 30 rows total (n=90 trees sampled)

Along with ACP counting methods performed prior to and post insecticide application, sticky traps (AlphaScents ACP Traps, 46.75 in^2 area) were implemented in order to benchmark our counting method (developed with our industry partner) with traditional sticky trap surveying methods. The sticky traps were attached with wire to the first, middle, and last trees in the 1^{st}, 15^{th}, and 30^{th} rows each (n=9 per block test). Sticky traps were exposed for four hours then collected and inspected.

2.3 Chemicals and Standards

Acetonitrile (ACN), and acetone (Thermo Fischer Scientific Waltham, MA, USA) were used for sample preparation. The following insecticide standards were used for quantification of

extraction recovery rates and field samples. Imidacloprid and malathion were purchased as neat materials (purity >98%) from Sigma-Aldrich (St. Louis, MO, USA). Individual standard solutions containing 100 µg/ml imidacloprid or malathion in ACN were prepared for calibration standards and recovery tests. Eight calibration levels were prepared ranging from 0.001 to 20 µg/ml for each set of standards.

2.4 Sample Preparation

Extraction recovery tests and methods were modified for Whatman filter discs from EPA extraction disc methods (Empore Solid Phase Extraction Discs, C8, C18, and SDB-RPS). Extraction recovery tests were performed by pipetting 50 µL of 100 µg/ml standard droplets onto 5 replicates of each sorbent material with a micro-syringe. Extraction recovery test samples were extracted and analyzed with LC-MS/MS. Whatman filter paper discs were chosen as a inexpensive sorbent material with good extraction recoveries. The sample discs (Whatman filter papers; WF) were rolled and inserted into 12 ml amber vials (Teflon cap liner), extracted with 10 ml ACN, and shaken at 170 rpm, 5°C, for 20 minutes. Sample discs were removed from solution and 0.5 mL aliquots prepared in autosampler vials for instrumental analysis. Each tank mix sample was diluted with ACN in 10 mL volumetric flasks and prepared for LC-MS/MS analysis. Hamilton glass syringes were used for preparation of standards and handling of tank mix samples. Syringes were fully rinsed 3 times with acetone, 3 times with ACN, then conditioned with the standard or sample being measured.

2.5 LC-MS/MS Analysis

Methods were optimized for Imidacloprid and Malathion with LC-MS/MS (USGS, EPA). Sample analysis was carried out with a Waters Xevo UPLC-MS/MS triple quadrupole with Mass Lynx software for instrumental control and data acquisition. The instrument was operated in the

positive ion electrospray mode. An Aquity UPLC BEH C18 column (1.7 µm), maintained at 40°C, was used for chromatic separation. Mobile phase A consisted of LC-MS grade Optima water with 5% formic acid and mobile phase B was ACN. An elution was applied at a flow rate of 0.4 ml/min with a cycle time of 4 minutes. The sample injection volume was 1 µL. The electron spray ionization (ESI) source settings were as follows: desolvation temperature 300°C; gas flow desolvation 800 L H^{-1} and cone 1 L H^{-1}; source temperature 150°C; extractor 3 V; RF lens 2.5 V. Mass spectra were recorded in the m/z range of 50 to 1200. For imidacloprid: capillary 3.5 kV; cone 15 V; transition masses were 256 m/z for the parent ion and 175 and 209 m/z for the quantifier and qualifier daughter product ions respectively. Dwell time was 0.4 sec with a collision energy of 12 V for both. For malathion: capillary 3 kV; cone 25 V; transition masses were 331 m/z for the parent ion and 99 and 126.9 m/z for the quantifier and qualifier daughter product ions respectively. Dwell time was 0.083 sec with a collision energy of 10 V and 5 V for the 99 and 126.9 masses respectively. Insecticide active ingredient concentrations were calculated using the Mass linx software and considering the calibration curve and extraction method.

2.6 METHOD VALIDATION

Correlation coefficients, limits of detection (LOD) and quantification (LOQ) are shown in Table 3. Extraction recovery tests were performed by pipetting 50 µL droplets of 100 µg/ml standard onto 5 replicates of WF sample discs. The extraction recovery test samples were extracted and analyzed by the aforementioned method. Percent recoveries were calculated to be 73.6 and 94.5% for imidacloprid and malathion respectively (Table 2.3). Ten ACN blank samples were analyzed to determine limits of detection (LOD) and quantification (LOQ). For imidacloprid, the LOD and LOQ were both determined to be 0.001µg/mL and the correlation coefficient was 0.999.

For malathion, the LOD and LOQ were 0.007 and 0.01 µg/mL respectively with a correlation coefficient of 0.998 (Table 2.3).

Table 2.3. Instrumental analysis parameters for insecticides studied, including correlation coefficients, limits of detection (LOD) and quantification (LOQ), and percent recoveries.

Analyte	Coefficient of Correlation	LOD (µg/ml)	LOQ (µg/ml)	Extraction Recovery (%)
Imidacloprid	0.999	0.001	0.001	73.6
Malathion	0.998	0.007	0.01	94.5

2.7 STATISTICAL ANALYSIS

Calibration curves, extraction recovery methods, and the sample area (17.35 cm^2) were considered to determine the concentration of insecticide present on each sample disc. Statistical analysis was conducted in R using the lmer function in the lmer4 package. Observations that were <LOQ and field blanks were removed prior to statistical analysis. Due to violation of the normality assumption, the log concentration was used for analysis. Concentrations of zero value were replaced with 0.0000001 prior to the log transformation. Summary statistics were calculated for the log concentration of each insecticide by the sampling location and application spray method. (Table 2.1). A mixed model analysis was utilized to compare canopy height, canopy depth, cardinal side of the tree, side of leaf, and application method and all two-way interactions (fixed effects) with individual leaf within tree as random effect for individual leaf within tree and tree to account for filters being attached to the top and bottom of the same leaf (i.e., measurements within a tree and on a leaf are correlated). Tukey's multiple comparison adjustment was used for all follow up comparisons. A significance level of 0.05 was used for statistical significance in all analyses.

3. Results and Discussion

3.1 Insecticide Concentrations

All insecticide tank mixture samples collected during field experiments were confirmed to have high enough (160-56,900 µg/ml range) concentrations to kill ACP based on recommended mixing instructions provided by the manufacturer's product labels (Table 2.4).

Table 2.4. Insecticide properties and tank mix concentrations

Date	Block #	Insecticide Common Name	Active Ingredient	Insecticide concentraction (ug/mL)	Mode of Action
10/16/18	55	Admire	Imidacloprid	165	Systemic and Contact
10/16/18	12	Admire	Imidacloprid	159	Systemic and Contact
10/18/18	55	Malathion	Malathion	39,911	Contact
10/18/18	67	Malathion	Malathion	39,911	Contact
7/10/19	42	Admire	Imidacloprid		Systemic and Contact
7/10/19	15	Admire	Imidacloprid		Systemic and Contact
7/11/19	44	Malathion	Malathion	56,900	Contact

3.2 Spatial Distribution and Application Method Impacts

3.2.1 Side of Citrus Leaf

Results of active ingredient measured on the WF sample discs from various locations throughout the citrus trees and multiple insecticide applications reveal large variability in coverage within citrus trees of different canopy size and application method. The data especially show a large range in the amount of insecticide that contacts the top vs. bottom side of each leaf (Figures 2.7-2.10).

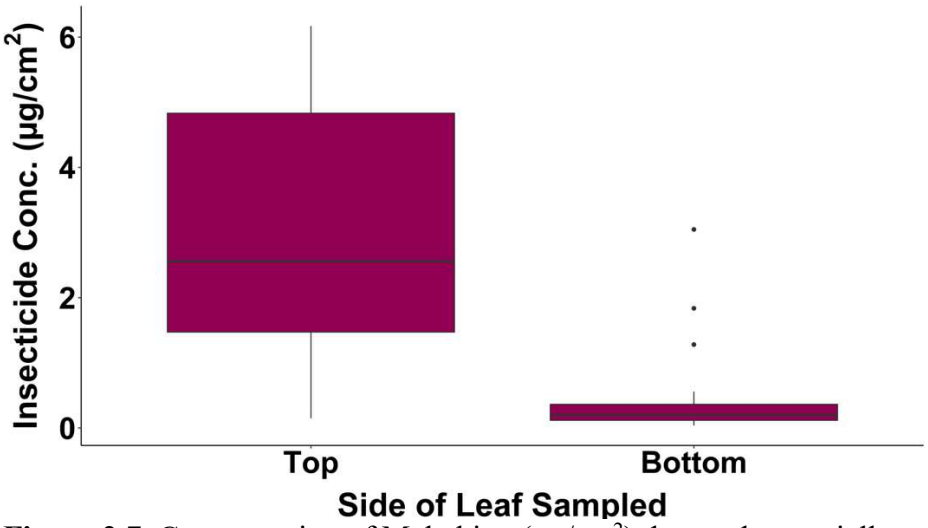


Figure 2.7. Concentration of Malathion (µg/cm^2) detected on aerially sprayed Whatman filter sample discs from the top and bottom sides of the leaf collected from various locations within a citrus tree. Data comprised of 5 older-aged tree replicates during the October 2018 field experiment (n=255 samples).

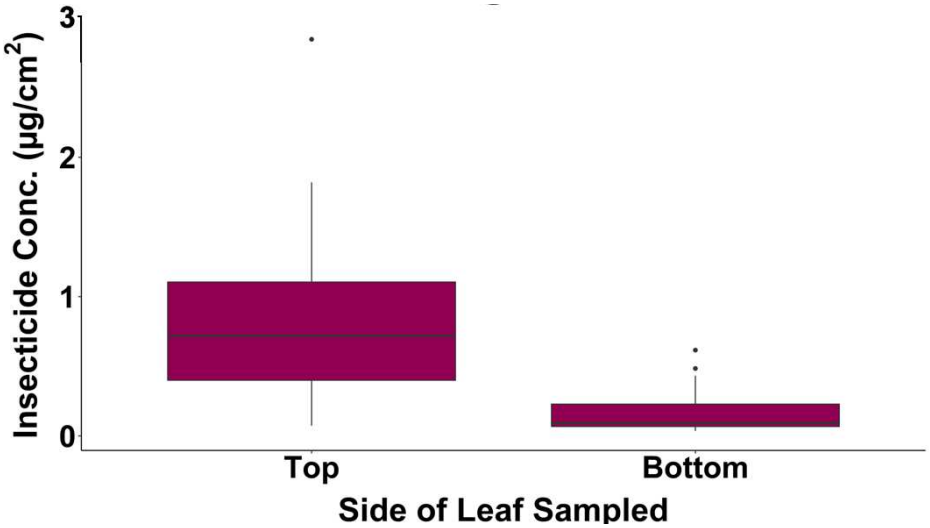


Figure 2.8. Concentration of malathion (µg/cm^2) on Whatman filter sample discs from top and bottom sides of the leaf collected from various locations within a citrus tree. Samples were aerially sprayed with the airplane during FS-1 and collected from younger-aged trees in block 67. Data comprised of 5 younger-aged tree replicates (n=255 samples).

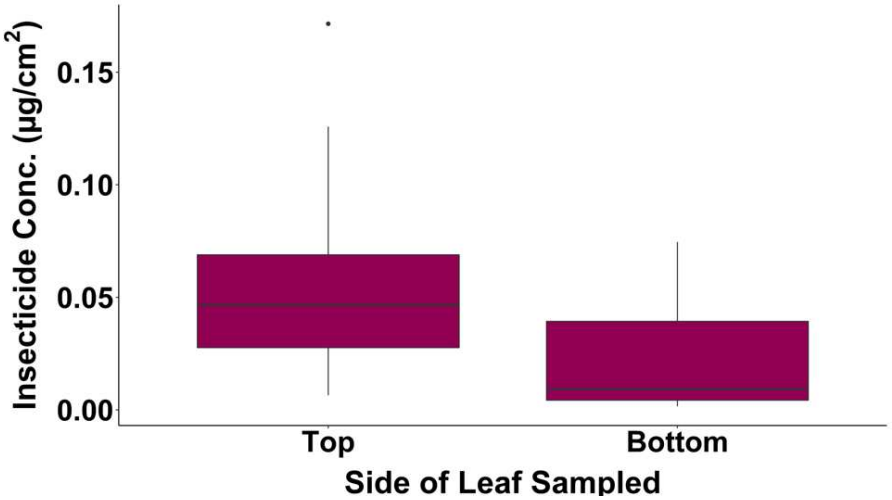


Figure 2.9. Concentration of imidacloprid (µg/cm^2) on Whatman filter sample discs from top and bottom sides of leaf samples collected from various locations within a citrus tree. Samples were ground sprayed with the speed-sprayer during FS-1 and collected from older-aged trees in block 12. Data comprised of 5 older-aged tree replicates (n=255 samples).

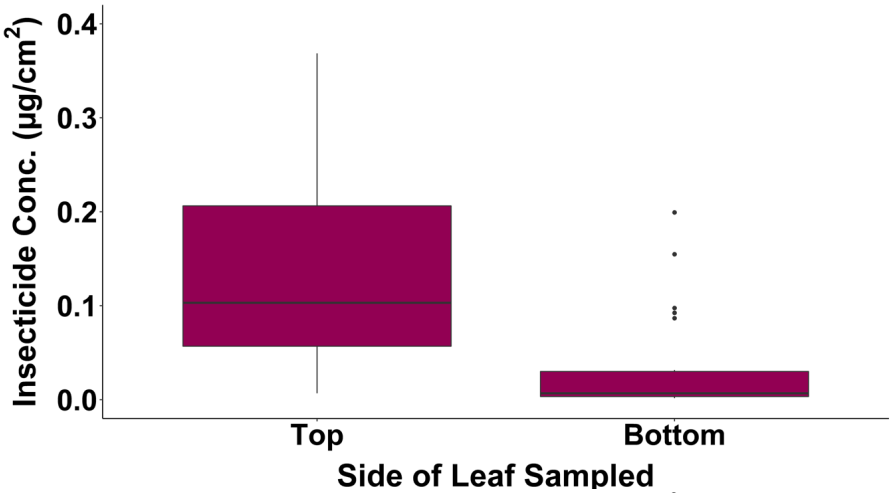


Figure 2.10. Concentration of imidacloprid (µg/cm^2) on Whatman filter sample discs from top and bottom sides of leaf samples collected from various locations within a citrus tree. Samples were ground sprayed with the side-sprayer during FS-1 and collected from younger-aged trees in block 55. Data comprised of 5 younger-aged tree replicates (n=255 samples).

The top side of the leaf receives significantly more insecticide than the underside regardless of application method, canopy height, canopy depth, or cardinal side (Figures 2.7-2.10, Table 2.4). This variation has a substantial impact on the insecticide's ability to effectively kill ACP, especially with contact insecticides.[7,14,53] Therefore, the ACP, which are primarily located on the underside of leaves, are less likely to be exposed to contact insecticides. However, the use of

systemic insecticides that absorb into and throughout the entire leaf would expose ACP feeding anywhere on the leaf to the insecticide.[14,19] This may help control ACP populations and limit reproduction over time, but still allows for ACP to transfer *C*Las and infect the tree with HLB in the short term because these modes of action are not immediately lethal to the ACP.[49]

Additionally, comparing imidacloprid concentrations on both sides of leaf between ground (side and speed sprayer) application methods revealed that bottom sides of leaves were not statistically different ($diff_{conc}$=0.01 µg/cm^2, diff=0.39, p=0.162), while top sides of leaves were statistically different ($diff_{conc}$=0.14 µg/cm^2, diff=1, p<0.001). Results also showed a greater difference between top and bottom leaf samples for the side sprayer than the speed sprayer ground application methods. The side sprayer resulted in higher concentrations on top side of leaf samples (conc=0.19 µg/cm^2) than the speed sprayer (conc=0.05 µg/cm^2) whereas the speed sprayer produced higher concentrations on bottom side of leaf samples (conc=0.03 µg/cm^2) than the side sprayer (conc=0.02 µg/cm^2). The speed sprayer, with potentially lower nozzle alignment (Figure 2.4) and a higher application pressure, may allow for a more direct spray angle and disturbance of leaves during application to produce better coverage to both top and bottom sides of the leaf. Moreover, the design of each sprayer does impact the spray deposition onto citrus trees, with the side sprayer better covering the tops of leaves and the speed sprayer producing better coverage to the bottoms. This ultimately impacts the ability of insecticide applications to effectively control ACP populations and manage HLB disease.

3.2.2 Citrus Tree Canopy Depth and Height

There is large variability in the insecticide distribution with varying canopy depth, canopy height, and cardinal side of the tree (Figures 2.11-2.18). Our results showed that outer canopy depth, and middle and upper canopy height regions of the tree receive more imidacloprid insecticide than inner and lower areas (Figure 2.11).

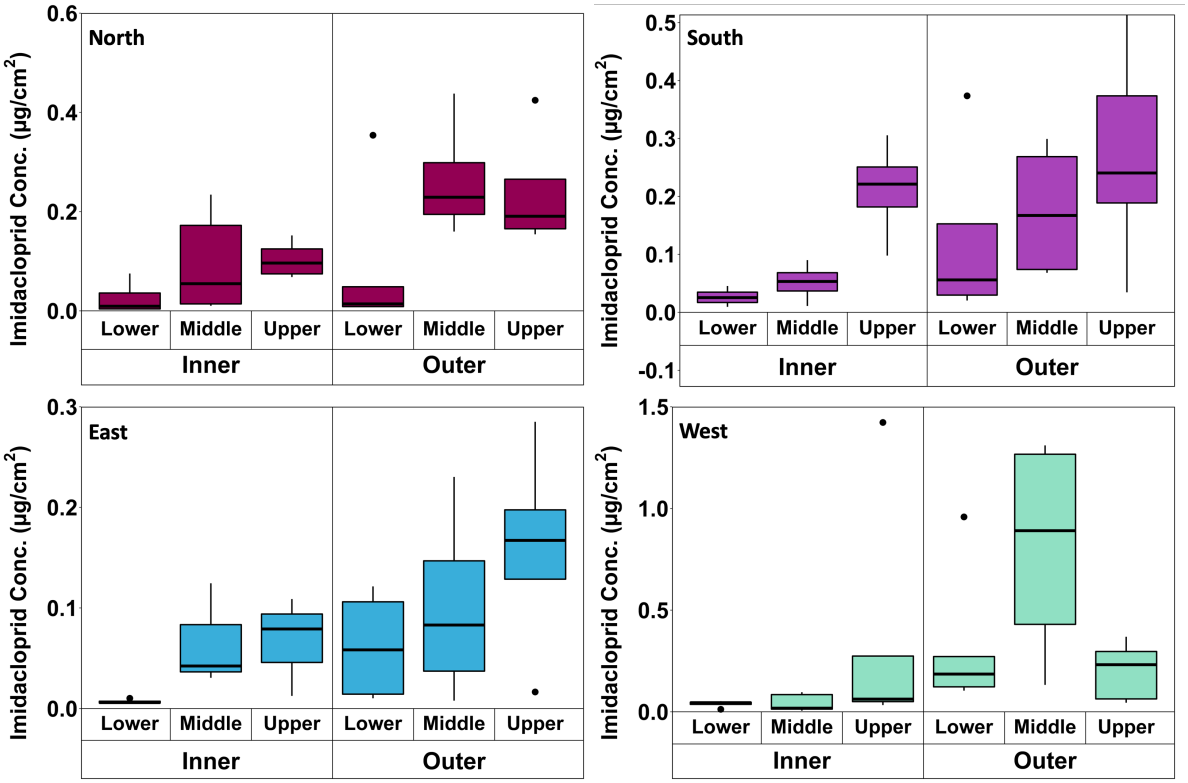


Figure 2.11. Concentration of Imidacloprid (µg/cm^2) detected on ground sprayed Whatman filter sample discs from various locations within a citrus tree. Data comprised of 5 younger-aged tree replicates (top of leaf samples only) during the October 2018 field experiment. Each cardinal side of the tree is shown in separate colors: North (red), South (purple), East (blue), and West (green). The North and South sides of the tree touch its neighboring trees, while the East and West sides have about 10 feet between the rows of trees. Trees were sprayed with the side sprayer.

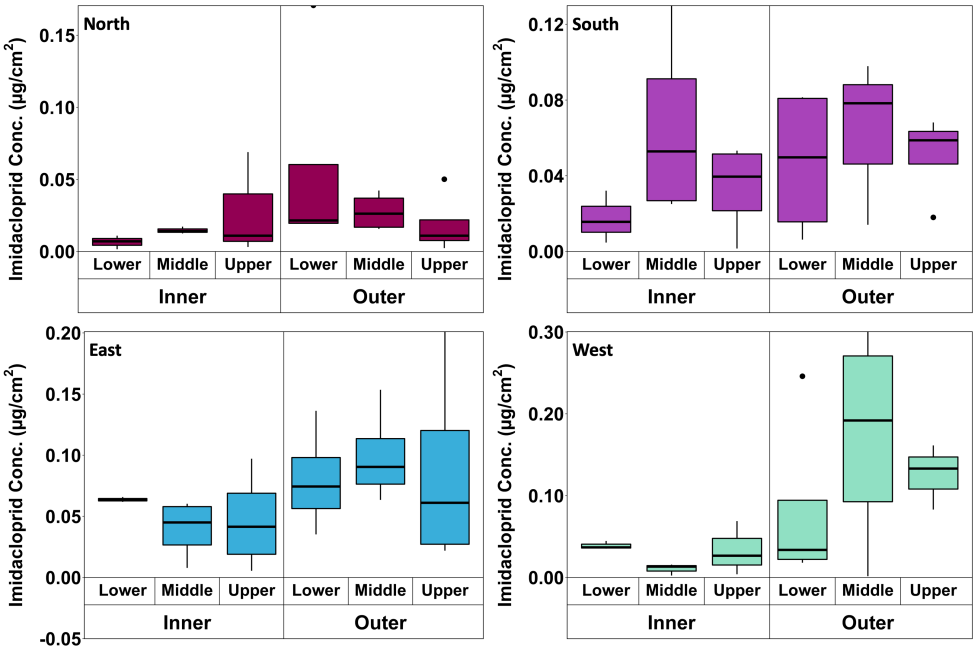


Figure 2.12. Concentration of imidacloprid on samples from various locations throughout the citrus tree (See Figure 1). Samples were ground sprayed with the side-sprayer during field study #1 and collected from older trees in block 12. The x-axis sorts data by canopy depth (inner and outer) and height (lower, middle, and upper). Each colored box represents a cardinal side of the tree (North-red, South-purple, East-blue and West-green).

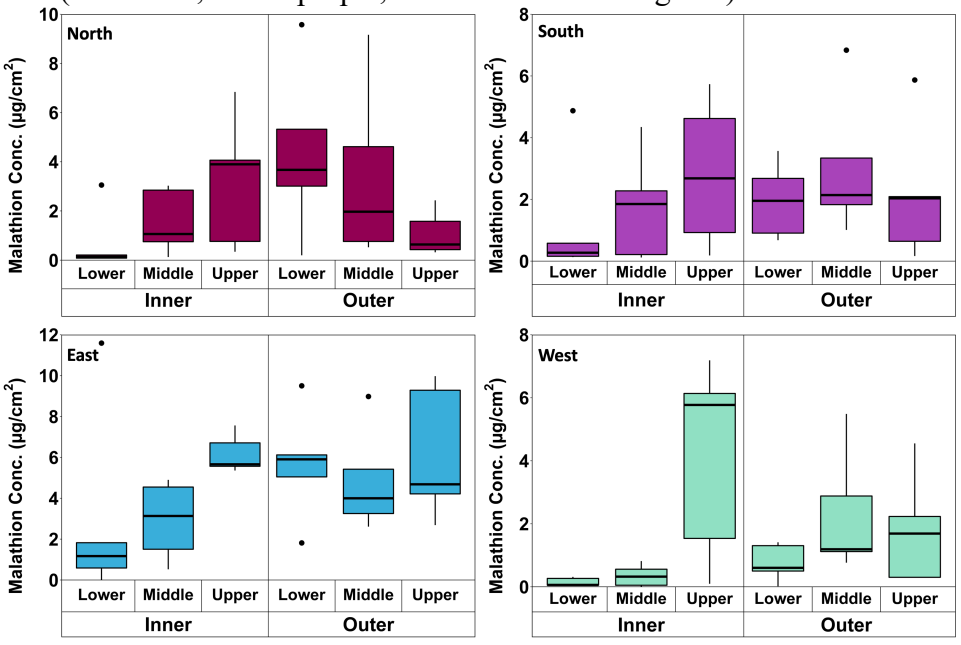


Figure 2.13. Concentration of malathion on samples from various locations throughout the citrus tree (See Figure 1). Samples were aerially sprayed during field study #1 and collected from older trees in block 55. The x-axis sorts data by canopy depth (inner and outer) and height (lower, middle, and upper). Each colored box represents a cardinal side of the tree (North-red, South-purple, East-blue and West-green).

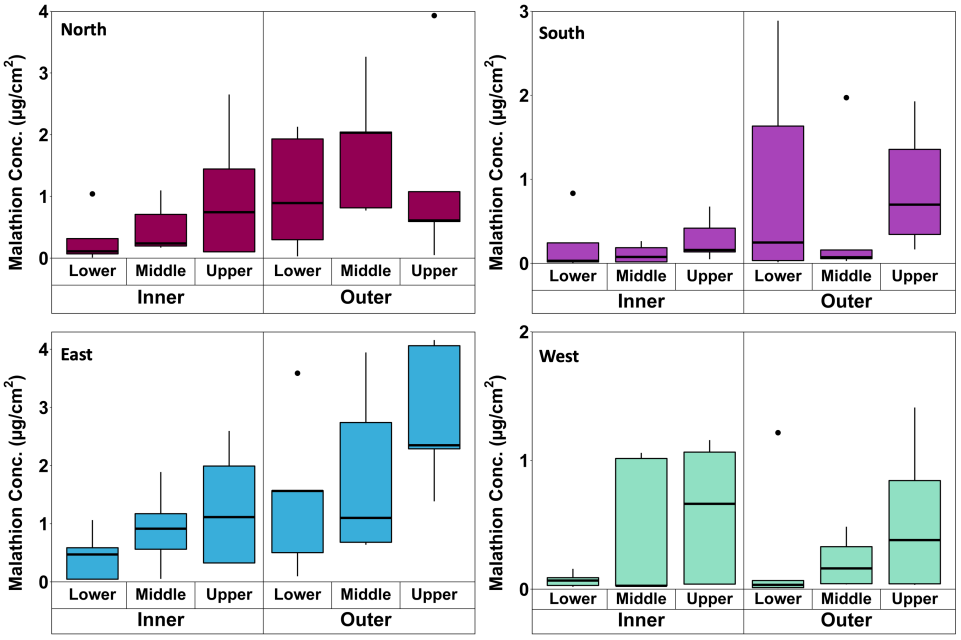


Figure 2.14. Concentration of malathion on samples from various locations throughout the citrus tree (See Figure 1). Samples were aerially sprayed during field study #1 and collected from younger trees in block 67. The x-axis sorts data by canopy depth (inner and outer) and height (lower, middle, and upper). Each colored box represents a cardinal side of the tree (North-red, South-purple, East-blue and West-green).

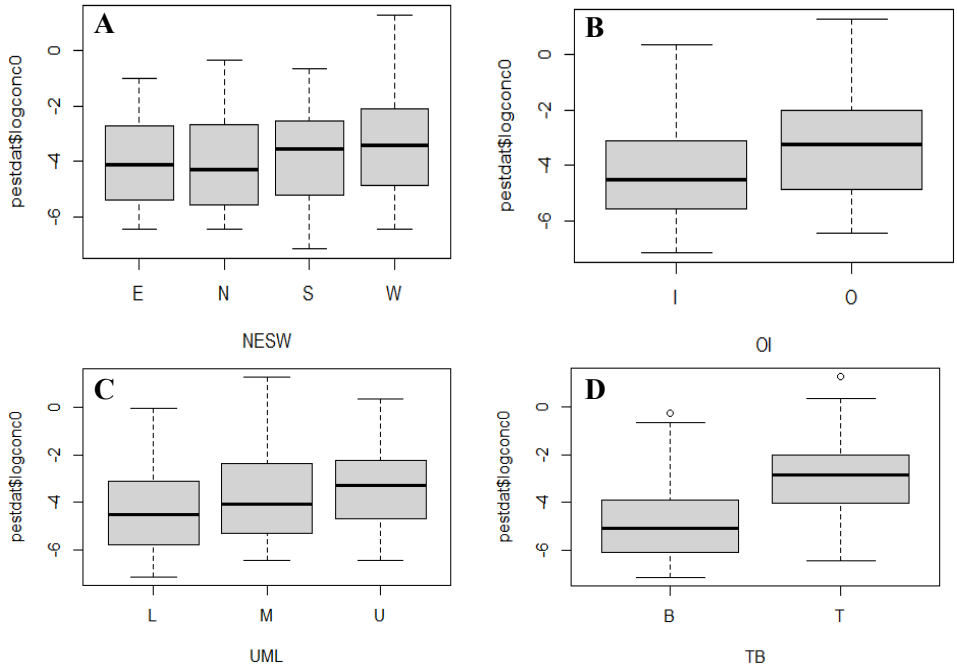


Figure 2.15. Box Plots of imidacloprid log-concentration values for all comparisons of A) cardinal sides North, East, South, and West, B) inner (I) and outer (O) canopy depth, C) lower (L), middle (M) and upper (U) canopy heights, and D) bottom (B) and top (T) side of leaf samples.

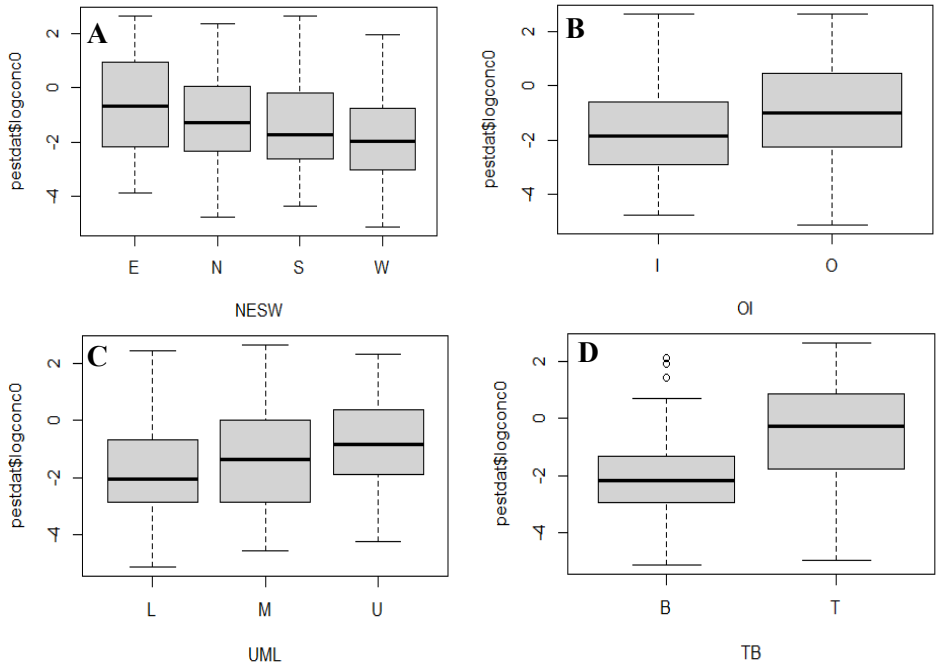


Figure 2.16. Box Plots of malathion log-concentration values for all comparisons of A) cardinal sides North, East, South, and West, B) inner (I) and outer (O) canopy depth, C) lower (L), middle (M) and upper (U) canopy heights, and D) bottom (B) and top (T) side of leaf samples.

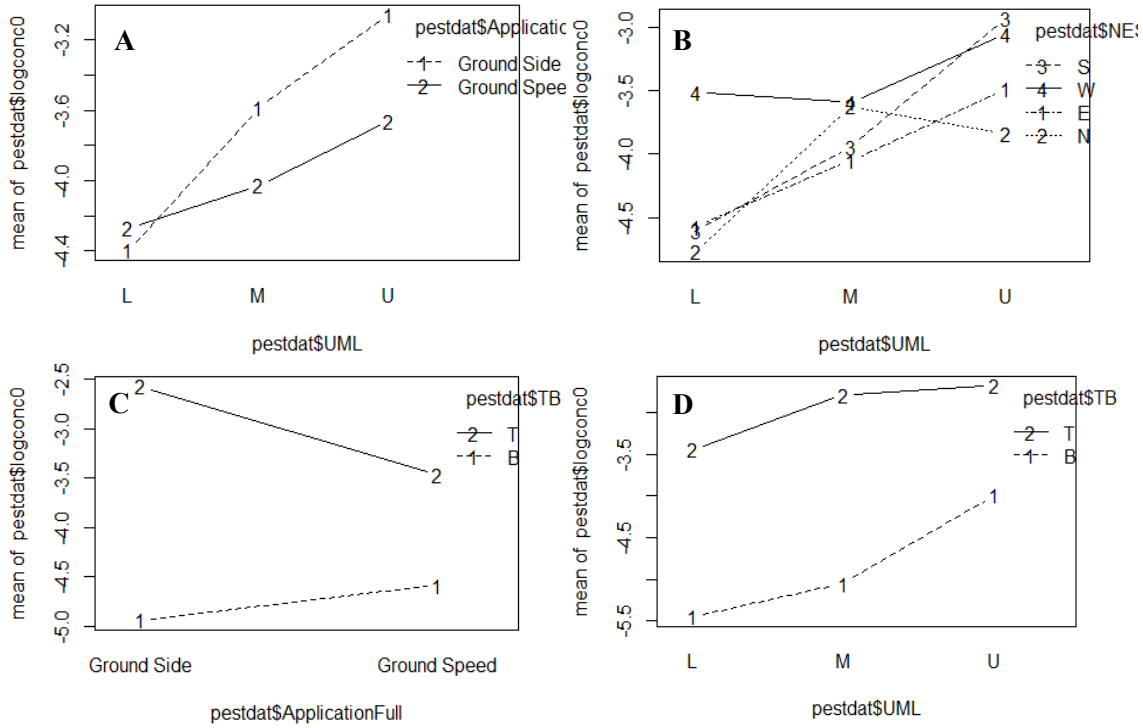


Figure 2.17. Interaction plots of imidacloprid log-concentration values for interactions between A) ground application method and lower (L), middle (M), and upper (U) canopy heights, B) cardinal sides North, East, South, and West, and canopy height, C) side of leaf (top (T) and bottom (B)) and ground application method, and D) side of leaf and canopy height.

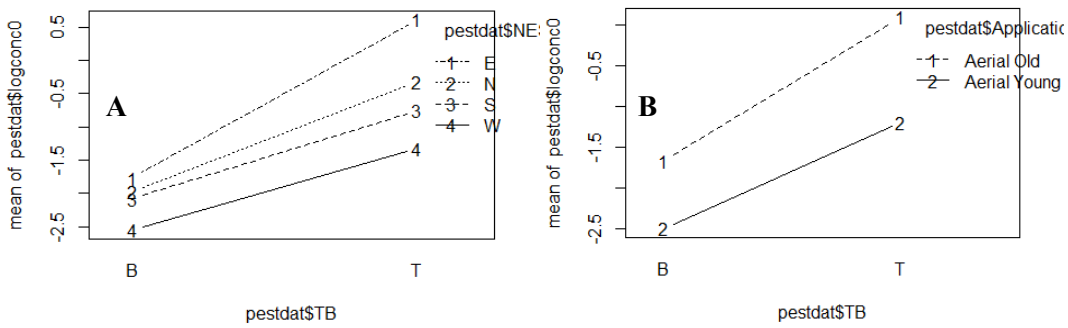


Figure 2.18. Interaction plots of malathion log-concentration values for interactions between A) cardinal sides North, East, South, and West, and side of leaf (Top and Bottom) and B) Aerial application to older and younger trees and side of leaf.

For instance, the middle canopy heights sampled contained about 7 times higher concentrations on the outer canopy region (0.26 µg/cm^2) compared to inner canopy (0.04 µg/cm^2) for all cardinal sides (median differences (µg/cm^2): N=2, S=1.7, E=0.3, W=0.9) (Figure 2.11). Statistically, outer canopy samples received 3x more insecticide than inner canopy samples regardless of application method (conc. ranges 0.02-0.17 µg/cm^2, diff=1.05, $p<0.001$). This supports that citrus tree foliage hinders insecticide spray from penetrating through to inner canopy regions.

Furthermore, interesting differences between canopy heights were observed with varying application method ($p=0.012$) and cardinal side of the tree ($p=0.017$). For instance, when comparing the ground sprayer application methods, the speed sprayer had higher concentrations in the lower canopy height (diff=0.03µg/cm^2), but the middle and upper heights had lower concentrations (diff=0.11and 0.09µg/cm^2 respectively) than the side sprayer (Figure 2.17A). There was no statistical difference in the insecticide concentrations between the lower heights and middle heights, but a statistical difference was reported between the upper canopy heights for varying spray method, with the side sprayer upper regions receiving 3x more insecticide than the speed sprayer upper regions. For the side sprayer, differences were reported between upper, middle, and

lower canopy heights. For the speed sprayer, although only a statistical difference was determined between the lower and upper canopy heights (diff=0.64, p=0.040), similar trends of the side sprayer were observed, with the middle greater than the lower regions. On average, insecticide concentrations increased with canopy height (Figure 2.17). After aerial application, canopy height results showed the lower height (0.77 μg/cm^2) statistically less than the middle (1.16 μg/cm^2, diff=0.41, p=0.005) and upper (1.40 μg/cm^2, diff=0.99, p<0.001) heights. In addition, ground-sprayed bottom side of leaf samples were similar at lower-middle canopy heights ($diff_{conc}$=0 μg/cm^2, diff=0.36, p=0.585) and statistically different for lower-upper ($diff_{conc}$=0.02 μg/cm^2, diff=1.45, p<0.001) and upper-middle comparisons ($diff_{conc}$=0.02 μg/cm^2, diff=1.09, p<0.001). Relatedly, top side of leaf samples had statistically similar concentrations at upper-middle canopy heights ($diff_{conc}$=0.04 μg/cm^2, p=0.999) with statistical differences at the lower-middle ($diff_{conc}$=0.1 μg/cm^2, p=0.021) and lower-upper comparisons ($diff_{conc}$=0.06 μg/cm^2, p=0.006). Castle et al.,[20] also investigated spatial distribution of imidacloprid to citrus trees via systemic drenching application and reported little to no differences in various canopy depths. This is important for HLB management to identify common ground spraying techniques that cause unequal insecticide distribution and supports the explanation of how ground sprayer designs of nozzle angles, placement, and application pressure can lead to uneven distribution throughout the entire citrus tree.

3.2.3 Cardinal Side of Citrus Trees

In order to better understand how application method impacts distribution throughout the entire citrus tree, comparisons between cardinal sides were investigated (Figure 2.2). Additional statistical analyses of ground sprayed samples from each cardinal side showed similar trends for canopy height previously reported, with the exception of the north side middle-height which

received slightly more insecticide than the upper region and the west side middle-height that received slightly less than the lower areas (Figure 2.11). There was no evidence of statistical difference between cardinal sides at the middle and upper heights, but there were statistical differences at the lower canopy height between the west and north, east, and south sides with much higher insecticide concentrations in the west (W-N: diff=1.40, $p<0.001$, W-E: diff=1.04, $p=0.02$, W-S: diff=1.21, $p=0.004$). As a whole, the west side (0.14 $\mu g/cm^2$) was statistically different from the east (0.05 $\mu g/cm^2$) ($diff_{conc}$=0.09 $\mu g/cm^2$, $p=0.003$) as well as the north (0.05 $\mu g/cm^2$, $diff_{conc}$=0.09 $\mu g/cm^2$, $p<0.001$). All other cardinal side (North, East, South) comparisons were statistically similar, including comparisons between the north and south. This suggests that the arrangement of citrus trees in the field, with trees planted in rows north to south, and the application motion of the sprayers directed down the rows (Figure 2.19), encourages lower spray deposition to both the north and south sides of trees that touch their neighboring trees, and could cause more hinderance due to foliage. The grove arrangement also instigates inconsistencies on the east and west tree sides. For the ground spraying methods, these differences between the east and west sides of the trees could be due to varied distance to the sprayer, larger 10 ft spaces between the east and west sides of trees across rows, or whether the tractor drives down a lower ditch or raised bed between the rows of trees. The sprayer nozzles align lower or higher in relation to the tree canopy when sprayed from a ditch or bed respectively (Figure 2.19).

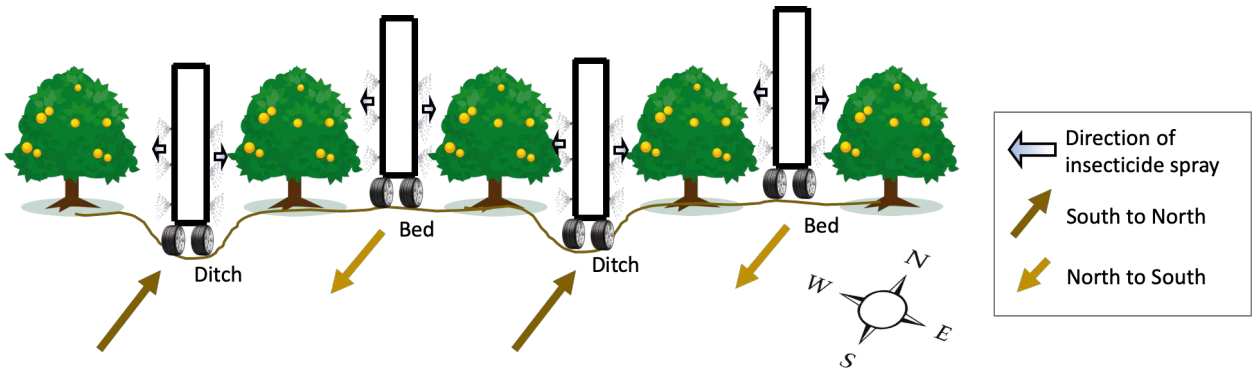


Figure 2.19. Schematic showing raised beds and irrigation draining ditches that tractor sprayers drive down while applying insecticides. Tractors drive North and South while applying to the East and West sides of the tree.

The ground side-sprayer design allows for more nozzles to spray at a closer distance (0-12 inches) on three sides of the tree (East, West, and top). The ground speed-sprayer used on older, larger foliage trees, only sprays insecticides from a further distance (1-2 ft) to the East and West sides of the trees, thus increasing distance to the sprayer and foliage that potentially block spray droplets. However, the opposite was observed between the east and west sides during aerial application, with the east cardinal side receiving more insecticide on average than the west (Figure 2.13-2.14). The north and south side concentrations (not significantly different from each other) fell between the range of east and west results. This could be due to application flying patterns (spraying in rows north to south, while starting east and working towards the west) or the wind direction during application (typically east to west wind). Increased distance to sprayer, nozzle alignments, and wind direction can all impact drift and effective insecticide application.[54]

3.3 ACP Inspections and Application Efficacy

In comparison to the traditional sticky trap method, our modified ACP counting protocol, adopted by our industry partner, allowed for better quantification of ACP before and after insecticide applications. For the purpose of our experiment, we discovered the sticky traps poorly

represented the actual amount of ACP in the citrus trees, as we counted only 3 ACP on the traps compared to hundreds counted by our psyllid inspector (Table 2.5).

Table 2.5. Sticky Trap nymph and adult psyllid count data collected pre and post insecticide application to benchmark with the psyllid counting method used in this study.

App. Method	Ground				Aerial	
Tree Age	Old		Young		Old	
Pre/Post App.	Pre	Post	Pre	Post	Pre	Post
# Nymphs	0	0	0	0	0	0
# Adults	0	1	0	0	2	0

For each block test, the ACP count decreased after initial insecticide application, but live ACP were always detected in the trees post-insecticide application (Figure 2.20). ACP population responses to the insecticide applications resulted in percent reductions of 85% for aerial application of malathion, 48% for imidacloprid ground applied with the side sprayer (smaller trees), and 80% imidacloprid ground applied with the speed sprayer (larger trees) (Figure 2.20). Although it was predicted the side sprayer, with more nozzles, a smaller distance between the nozzles and leaves, and application to more sides of the tree, would have better insecticide effectiveness than the speed sprayer, this was not the case. As demonstrated with spatial distribution results, the speed sprayer which had higher concentrations (Figure 2.21) and better coverage to undersides of leaves, was more effective at reducing the ACP population (Figure 2.20). Although, results varied compared to aerial application of malathion and ground application of imidacloprid during field study #1 (Figures 2.22-2.23).

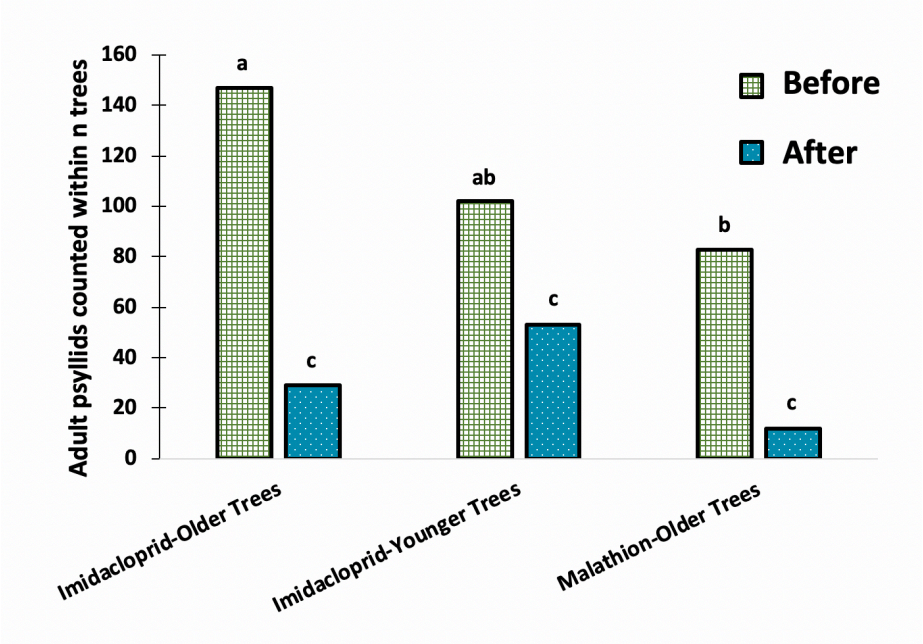


Figure 2.20. The total number of adult psyllids counted in all trees before- and after-insecticide application during field study #2. Imidacloprid was ground sprayed with the speed and side sprayers to old and young trees respectively (n=90 trees included). Malathion was applied aerially to older trees. Only data from the first 15 rows were included (n=45 trees). Bar plots with the same letter are not statistically different.

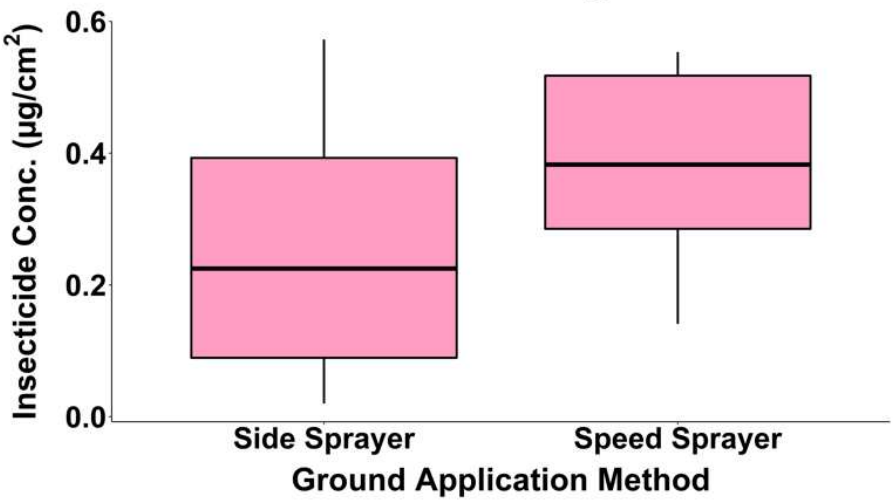


Figure 2.21. Concentration of imidacloprid (µg/cm^2) in Whatman filter paper sample discs from older and younger aged trees ground sprayed with the speed- and side-sprayers respectively during FS-2. Data comprised of 5 older-aged tree replicates and 5 younger-aged tree replicates (n=240 samples).

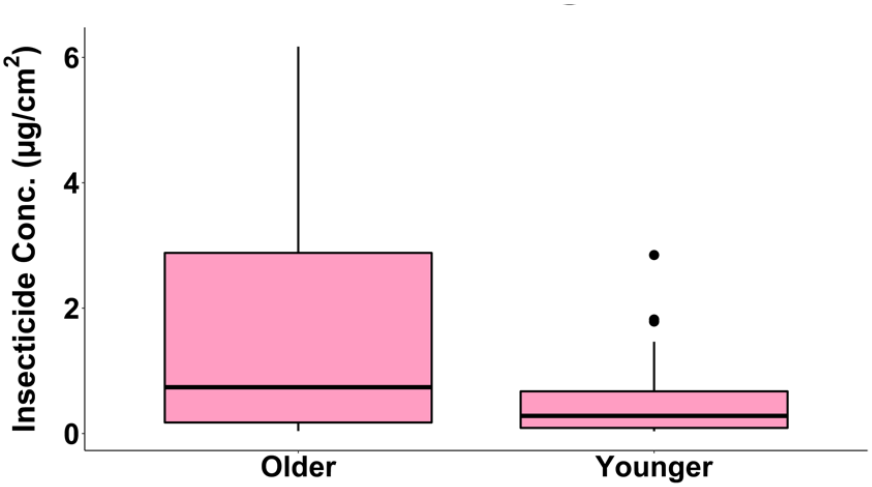


Figure 2.22. Concentration of malathion (µg/cm^2) in Whatman filter paper sample discs from older and younger trees (blocks 67 and 55 respectively) aerially sprayed simultaneously during FS-1. Data comprised of 5 older-aged tree replicates and 5 younger-aged tree replicates (n=240 samples).

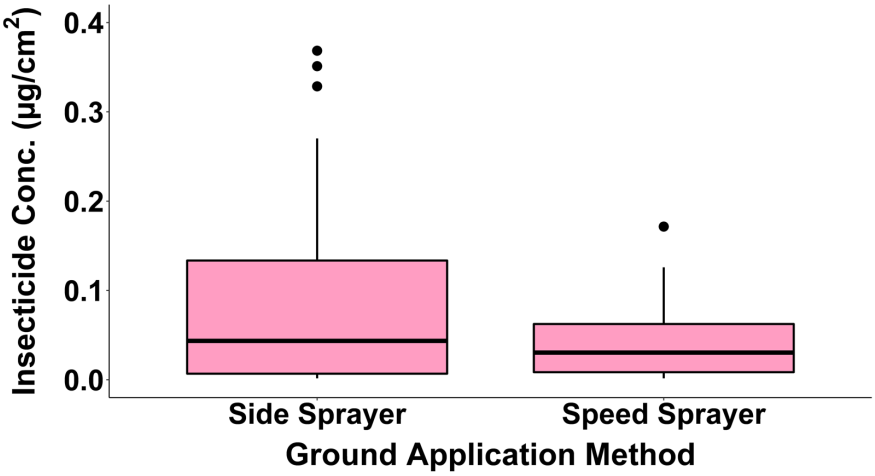


Figure 2.23. Concentration of imidacloprid (µg/cm^2) in Whatman filter paper sample discs from older (block 12) and younger (block 55) trees ground sprayed with the speed-and side-sprayers respectively during FS-1. Data comprised of 5 older-aged tree replicates and 5 younger-aged tree replicates (n=240 samples).

It is possible that new ACP migrated into the blocks within 24 hours after application, however we suspect that the ACP observed post-application in all block tests either had not come in contact with the insecticide due to poor coverage or had not yet experienced the full effect of the insecticide and thus still alive.[49,50,55,56] The high management commercial citrus groves that implement largescale insecticide spray applications resembling those investigated in this field

study could witness a rapid regeneration of ACP from the ~ 50% population remaining post-application.[7] According to Boina et al., ACP populations at higher levels or with higher resistance in the field are more likely to repopulate. In addition, infected adult ACP have increased pathogen transmission when they contracted *C*Las pathogen as nymphs rather than as adults, thus improved control and reduction of ACP populations is critical. Our results agree with imidacloprid control of adult ACP populations (reductions of 50-90%).[14] These results also agree with recent findings for imidacloprid (44%) applied to Kinnow mandarin plants via a knapsack sprayer.[49] However, several studies recommend repeated insecticide applications within the week in order to reach effective percent reductions (73% imidacloprid).[49,57,58] Although these findings reaffirm the percent reductions obtained during our field studies and align with previous studies showing initial ACP response to the active ingredient, they do not all fall within the effective percent reductions limit (73%) or offer realistic pest management improvements for a large commercial grove located in regions with specific EPA regulations.[59,60] High management commercial citrus groves cannot use fitted knapsack sprayers, which may offer a more targeted application to individual trees in smaller farms or research facilities.[49] In addition, recommended rotations of insecticide application types to prevent ACP insecticide resistance development and meet EPA regulations inhibit high management groves from repeated applications of the same insecticide within a week of the previous application.[61–63] Therefore, suggestions to increase application frequency or concentration do not offer realistic options for some of the large citrus producers. In addition, lab studies show a 94-100% mortality rate of ACP with direct spray of imidacloprid, even though the same percent reductions are not generated in the field.[49,50,64] Thus, exploring ways to enhance current application sprayer methods with increased agitation to canopy foliage or additional nozzles to spray upward from lower angles and increase deposition onto undersides of leaves,

could provide improved distribution and efficacy of insecticide applications to citrus crops. Additionally, limitations in this study include variability of field conditions, seasonal impacts, and short-term psyllid population responses. More research is needed to understand psyllid response over time as a result of seasonal impacts, field conditions, insecticide mode of action and degradation.

4. CONCLUSION

The results presented in this paper clearly show unequal spatial distribution of insecticides applied to citrus trees with varying application methods. On average, outer canopy depths and middle-upper canopy heights received more insecticides than inner and lower canopy locations, while the top of leaves received significantly more insecticide than the bottom. This lack of insecticide coverage to inner canopy regions and undersides of leaves could greatly impact the insecticide's effectiveness at killing ACP, especially if ACP are primarily found on undersides of leaves or on interior leaves to stay cooler when not feeding. The statistical interactions observed between side of leaf, cardinal side, and application method demonstrate the need for optimization of current insecticide application methods in citrus in order to more effectively control the spread of pests. This insufficient spatial distribution is of even greater importance in citrus groves combatting the spread of HLB, because ACP population reductions of less than 100% leave trees vulnerable to infection since it only takes one ACP to permanently infect a tree with HLB. This greatly impacts the ability to control ACP populations, protect crops, and slow the spread of citrus greening disease.

CHAPTER 3: AFIDOPYROPEN EFFICACY AND DEGRADATION WITHIN A CITRUS GREENING DISEASE-INFECTED GROVE

1 INTRODUCTION

For an insecticide application to be effective, it must contain the proper active ingredient concentration and have adequate coverage to the leaves and areas of the tree canopy where ACP reside to protect the trees until their next treatment. The mechanisms of action and effects of insecticides on pests, including on ACP, have been investigated extensively in lab studies;[13,50] however, field studies are also needed to gain a full understanding of insecticide effectiveness. Previous field studies have assessed insecticide spray distribution on numerous crops (e.g. wheat, peppers, onion, tomatoes, oat, and bay laurel)[42–46] with varying parameters (i.e. sprayer types, rates, spray volumes, droplet sizes, and ground speed)[24,35–37] and techniques (i.e. water sensitive papers, metal or fluorescent tracers and dyes).[24,35–38,42–46] Citrus field studies have shown that the outer canopy receives more spray deposition than inner canopy regions.[24,40,47,48] Therefore, weak effectiveness of insecticides against ACP may be due to the inner canopy leaves of citrus trees not receiving an adequate amount of insecticide.[24] However, several other factors also need further investigation; for example, there is limited knowledge about insecticide coverage on the underside of the leaf or active ingredient degradation within leaves. Both of these factors are critical to consider when investigating different types of insecticides (contact or systemic) and their efficacy at controlling ACP.[35–41,48] Although these methods allow growers to visually see spray deposition via a color change or measured dye,[38] which is assumed to translate to presence of insecticide,[24,39–41] they do not provide information about degradation whereas analytical techniques, like liquid

chromatography mass spectrometry, provide more accurate quantification of active ingredient concentrations responsible for killing the target pests.[32,38,65]

Oftentimes, intensive-management commercial groves spend more than 25% of total citrus production costs on insecticide applications alone,[23] implementing >20 different insecticides in rotation to reduce pest resistance. Occasionally new insecticides are developed to prevent pest resistance and better target these trouble pests, and less is known about their performance in the field. Afidopyropen is a newer, semi-systemic insecticide that demonstrates translaminar activity, meaning it is somewhat absorbed into the plant through its leaves and distributed systemically throughout the plant tissues.[66,67] Systemic insecticides may provide prolonged protection against feeding insects over time. Afidopyropen, a chordotonal organ Transient Receptor Potential Vanilloid channel modulator (Group 9D) insecticide, was developed for use in crops in the US in 2018; thus, few studies have investigated its efficacy.[66,67] Insecticides are beneficial for use against their intended target but can be harmful when contaminating unintended water or food sources, or degrading into more toxic metabolites that pose risks to humans or other species.[68–70] For this reason, the first studies on newer insecticides, like afidopyropen, often focus on toxicity and environmental risks rather than efficacy after application in the field.

Previous studies have investigated the toxicity of afidopyropen on various species, including several aquatic species[68] and insects (e.g. white flies, aphids, mealy bugs, bark scales, lace bugs, leaf scorch mites) with various crops (e.g. cucumber, Japanese laurel, soybeans, pecans, rice, cotton, Chinese cabbage, tomatoes, grape, eggplant, and myrtle trees) in lab, greenhouse, or field studies.[69,71,80–84,72–79] The major findings of these studies concluded that afidopyropen was toxic to the target species tested in each study with similar control as other common broad-spectrum insecticides.[69] Afidopyropen treatments showed delayed killing of soybean aphids in

greenhouse conditions and was non-toxic to its natural enemies.[69] After application in the field, afidopyropen's effectiveness against cotton flea hopper decreased after 10 days.[84] Several additional afidopyropen studies have focused on its toxicity to ACP and other insect species (e.g. citrus thrips) in lab or greenhouse conditions, and to humans from consumption of residues in edible parts of the crop (cucumber, nectarine).[82,85–87] However, a few recent studies have reported effective ACP population reductions over time after afidopyropen application to oranges in the field (100% at 14 and 21 days).[88,89] However, they had low ACP population counts (avg 5.33 ACP) before application and their ACP counting method removed ACP from the trees.[88,89]

Very few studies of dissipation of afidopyropen and its metabolites in the field exist.[74,90] Known afidopyropen metabolites include M440I007, M440I001, M440I002, M440I003, M440I004, M440I005, M440I006, M440I024, M440I046, M440I047, M440I057, M440I014, M440I015, M440I0416, M440I046.[74,90] Laboratory studies of afidopyropen and metabolite standards spiked into various samples (soils, tomato, watermelon, pepper, cucumber, pear, grape, and cabbage) resulted in recoveries of 80 to 100%.[74] Briefly, afidopyropen has been detected after 10 days in cucumber (0.004 mg/kg) and pepper (0.016 mg/kg)[74] and showed 90% dissipation after 5 days in cotton with a half-life of 1 to 3 days.[90] Metabolite half-lives were not reported; however, other studies presume them to be less mobile, or similar to afidopyropen.[91] Moreover, none of the current research on afidopyropen or its metabolites has assessed dissipation alongside efficacy in the field.

Degradation can decrease insecticides' active ingredient concentration in leaves to be too low to control ACP. If not adequately removed, ACP populations can regenerate or develop insecticide resistance.[7,28,62] Knowledge of insecticide degradation within different leaf layers is limited and especially scarce for field studies; however, this information is pertinent to better

understand insecticide efficacy in the field. Laboratory studies along with predictive models strive to estimate the fate of insecticides on leaves in the field.[25,92–94]

Recent studies reported faster dissipation rates in leaves than other media (e.g. soil) affected by varying field and meteorological conditions, insecticide physiochemical properties, application parameters, and plant characteristics.[25,95,96] These studies often spiked active ingredients onto wax layers or leaves and showed mixed results compared to predictive models and field samples, thus are potentially inapplicable to field trial observations.[27,93] Controlled laboratory environments may cause different dissipation rates compared to field studies due to varying meteorological conditions and insecticide mixtures applied with e.g. varying additives such as adjuvants.[95,97] Therefore, there is a greater need to analyze insecticide active ingredient degradation on leaves after application in the field to not only help provide additional data for those developing predictive models,[93] but to help growers understand the efficacy of insecticides in the field. Because insecticide application costs are increasing,[23] growers need optimized foliar application methods for citrus to better fight HLB spread while reducing product loss to the environment.[24]

To date, no study has *simultaneously quantified* afidopyropen application concentrations, degradation rates in leaves, and spatial distribution throughout the entire tree canopy along with ACP population reductions in citrus field conditions with different application methods. Our objectives were to effectively quantify afidopyropen spatial distribution, degradation, and efficacy after application in a commercial citrus grove. We assessed the spatial distribution to entire citrus canopies and both top and bottom sides of leaves with different application methods and rates. Afidopyropen degradation was quantified in citrus leaves and Whatman filter paper (WF) sampling discs over time after application. ACP population reductions were recorded before and

after afidopyropen applications in the field. Our novel approach assessed afidopyropen efficacy from a unique perspective, combining analytical techniques to quantify active ingredient spatial distribution and concentrations in leaves over time, along with a thorough ACP inspection method. Our results have broader impacts on growers by revealing areas of improvement in pest and disease management strategies including methods for application and ACP inspections.

2. Materials and Methods

2.1 Field Site Description, Pesticide Application, and Sample Collection

Two field studies were conducted at a citrus grove in Venus, Florida in April and October of 2019. This large-scale and intensively managed grove is 8,567 acres and consists of about 60 blocks of citrus trees (Figure 3.1, Table 3.1). Trees are planted in rows with ground spraying applications to the East and West sides. The weather is typically 30° C with an average of 57 mm rain in April and 29° C with 81 mm of rain in October. At the grove, the wind typically blows from East to West. Meteorological data was provided by the grove from an on-site weather station (Figure 3.2-3.3).

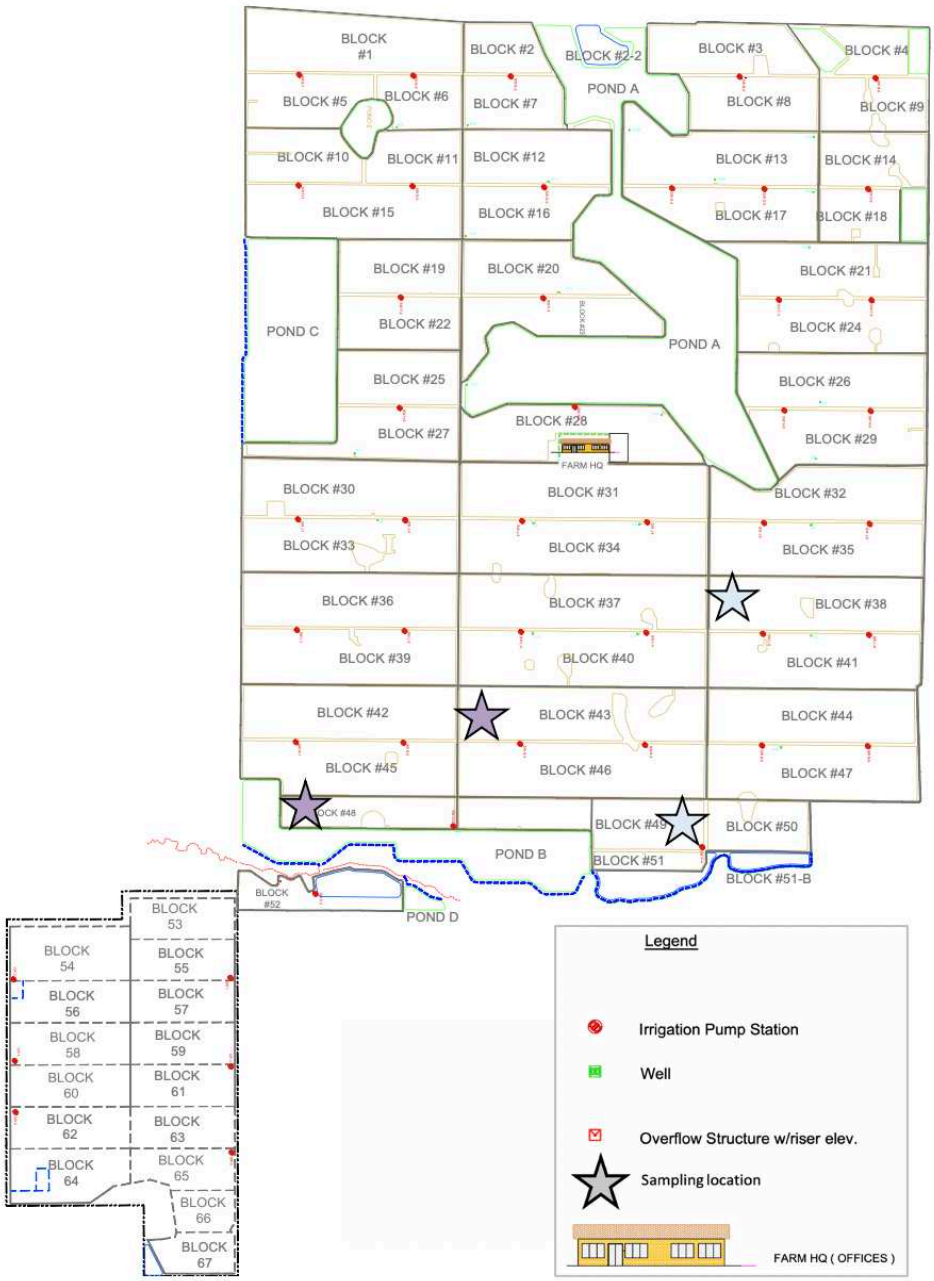


Figure 3.1. Citrus grove field site and experimental locations for various block tests executed in field study #1 and #2. Color coded stars represent block test sampling locations and correlate with Table S1 color coding.

Table 3.1. Field studies #1 and #2 experimental details including date, block number tested, tree age, application method, sprayer type, commercial insecticide product applied, and LC measured concentration of tank mix samples. Color coded rows correlate with colored stars in Figure 1. *At time of experiment.

Date	Block #	Tree Age (yr)*	Tree Age Classification	Application Method	Sprayer Type	Insecticide Common Name	Active Ingredient	Insecticide concentraction (ug/mL)	Mode of Action
4/17/19	49	~1	Younger	Ground	Side	Sefina Inscalis	Afidopyropen	100	Not fully systemic
4/17/19	38	~2	Older	Ground	Speed	Sefina Inscalis	Afidopyropen	100	Not fully systemic
10/15/19	48	~1	Younger	Ground	Side	Sefina Inscalis	Afidopyropen	100	Not fully systemic
10/15/19	43	~2	Older	Ground	Speed	Sefina Inscalis	Afidopyropen	100	Not fully systemic

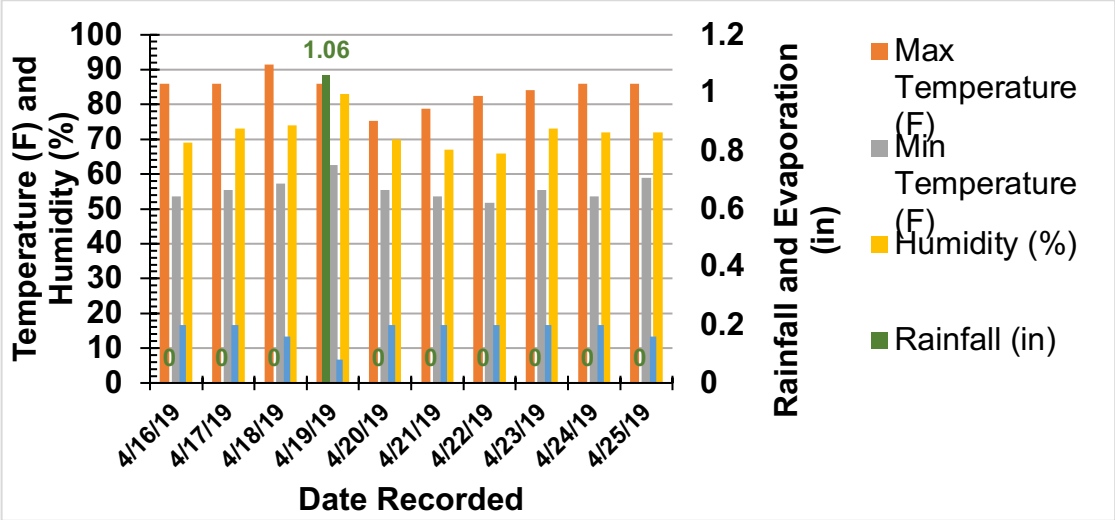


Figure 3.2. FS-1: April weather data recorded during field work from a weather station at the grove.

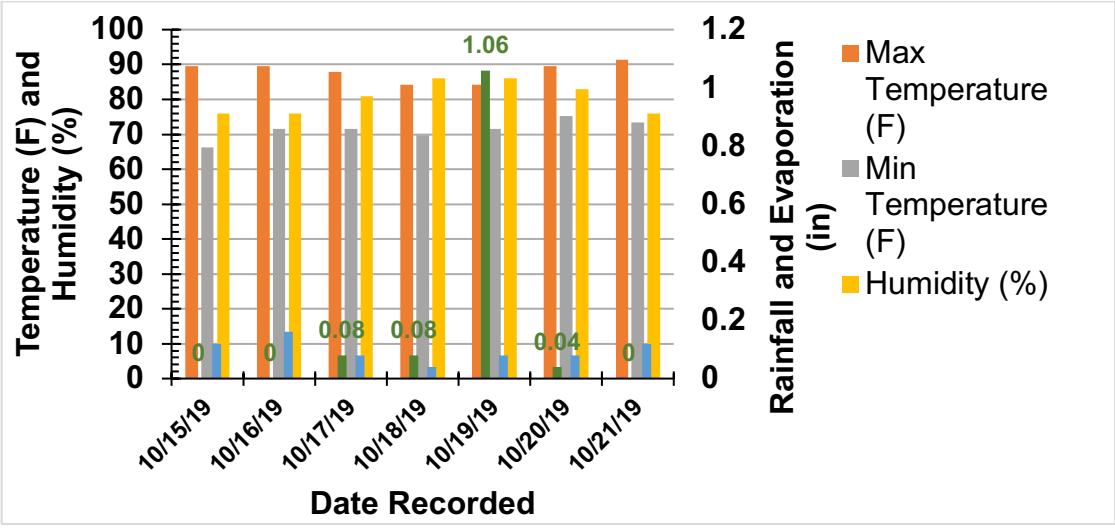


Figure 3.3. FS-2: October weather data recorded during field work from a weather station at the grove.

The BASF product, Sefina Inscalis (ai: afidopyropen), was prepared following label instructions and applied by our industry partners at the grove.[66,67] According to the BASF Sefina Inscalis product label and MSD sheet, the composition includes 4.89% afidopyropen (CAS #: 915972-17-7) active ingredient and 5-15% propylene carbonate (108-32-7) adjuvant. The product can be mixed with most recommended fungicides, insecticides, liquid fertilizers, adjuvants, and additives. Sprayer tank mix samples from each pesticide mixture applied were collected to quantify the initial concentration of insecticide active ingredient applied to the samples collected. The tank mix solution was mechanically agitated for at least 10 minutes prior to sample collection to ensure proper mixing and homogeneity. Samples were collected by the certified pesticide handler in 40 ml amber vials with Teflon cap and stored at 4°C.

Two ground-spraying application methods were tested: the side and speed sprayers to younger (1 yr: 1.1m x 1.4m) and older (3 yr: 1.4m x 1.7m) citrus trees respectively. Trees around 2 years and younger are typically small enough to be sprayed with the side sprayer. Therefore, in this study, older trees were sprayed with the speed-sprayer and younger trees sprayed with the side-sprayer (Table 3.2). These application methods were used in our study simply because they are the methods currently used in the commercial grove based on tree age and the insecticide product label recommendations. The application parameters (i.e. application rate) used for the two different ground spraying techniques varied; however, in this study afidopyropen's efficacy was investigated under relevant field conditions used by growers in a commercial grove.

Field study #1 (FS-1): In April 2019, WF sampling discs were implemented to assess spatial distribution of afidopyropen applied in the field by the aforementioned ground-spraying methods.[32] Briefly, to assess differences in cardinal side of the tree, canopy height, canopy depth, and side of leaf (top vs. bottom), WFs were attached to leaves throughout the entire citrus tree and

collected within 30 minutes after pesticide application. The WF drying time was about 5 minutes, therefore, WF samples were dry prior to collection. Five tree replicates from each age group (old and young) and ground sprayer application type (speed and side) were tested (n=10 trees total). Tank mix samples (n=2), field blanks (n=30), and WFs (n=480) were collected and stored properly following protocols described in Rehberg et al.[32] The grove psyllid inspector counted adult ACP the day before and after application. Further ACP counting details are described in section 2.2.

Field Study #2 (FS-2): In October 2019, leaf samples were collected at specific times before and after afidopyropen applications to quantify degradation in leaves under field conditions. A total of 240 leaf samples were collected from 22 trees sampled in the grove. A Fiskars 1-inch diameter Circle Squeeze Punch was used to obtain consistent leaf punch samples with an area of 0.79 in^2. Field blanks were collected for each tree prior to insecticide application by randomly collecting leaf samples from the outer canopy area (n=22 total). Ten leaf samples were collected at each time interval for the two foliar application tests (speed- and side-sprayers) (Table 3.2). Leaf samples were visibly dry by 10 to 15 minutes after application; thus, the first collection time (time-zero) was 10 minutes after application. All field blanks and leaf samples were collected at the specified times before or after insecticide application, stored separately in Ziploc bags and foil, and stored in the freezer. Samples were shipped cold overnight from Florida to Colorado State University. Again, samples of the sprayer tank mixture were collected and stored properly prior to analysis.[32] Proper personal protective equipment including closed-toed shoes, head and eye protection, Tyvek suits, and chemical resistant gloves were used during sample collection and handling. ACP data included adult and nymph counts from the day before, the day after, and 6 or 8 days after insecticide application.

Table 3.2. Details for each field application test.

Date	Block #	Tree Age (yr)*	Tree Age Classification	Application Method	Sprayer Type	Insecticide Common Name	Application Rate (gal/acre)
4/17/19	49	~1	Younger	Ground	Side	Sefina Inscalis	20
4/17/19	38	~2	Older	Ground	Speed	Sefina Inscalis	90
10/15/19	48-W	~1	Younger	Ground	Side	Sefina Inscalis	35
10/15/19	43	~2	Older	Ground	Speed	Sefina Inscalis	50

2.2 ACP Counting Protocol

ACP data was acquired by a professional psyllid inspector in the grove before- and after-foliar spray to compare insecticide applications with ACP reduction.[32] For each insecticide treatment, 30 rows of trees were sprayed with insecticide and inspected for ACP. ACP were counted in 3 trees in each row (the north border, middle, and south border) totaling n=90 trees inspected for each treatment. The inspector visually surveyed the entire tree foliage, thoroughly counting ACP adults and nymphs within 5 minutes consistently for each tree. All ACP counts after treatment were confirmed alive. During FS-1, the psyllid inspector only counted adult ACP the day before and after application. During FS-2, the psyllid inspector recorded adult and nymph ACP the day before, the day after, and 6 or 8 days after application. ACP population percent changes were calculated using only trees that contained ACP before treatment. Averaged percent change was calculated for each tree then averaged over total trees with ACP data. The calculated total percent change shows a more accurate depiction of the efficacy of afidopyropen across many trees, which is more scalable for a larger grove.

2.3 Chemicals and Standards

Liquid chromatography mass spectrometry (LC-MS) grade acetonitrile (ACN) and acetone (Thermo Fischer Scientific Waltham, MA, USA) were used for standards and sample preparation. The following insecticide standards were used for quantification of residues in field samples and extraction recoveries from WFs. Afidopyropen (purity >98%) was purchased from HPC Standards (Alabama, GA, USA) and malathion (purity >98%) was purchased from Sigma-Aldrich (St. Louis,

MO, USA). A standard solution containing 100 µg/ml afidopyropen in ACN was prepared for solvent calibration standards and recovery tests. Ten calibration levels were prepared (ranging from 0.001 to 100 µg/ml) for WF and tank mix samples analysis. Since afidopyropen metabolite and isotopically labeled standards could not be obtained, matrix matched standards were prepared for analysis of leaf sample extracts by spiking standards onto field blank leaf samples (ranging from 0.0003-1 µg/ml). Malathion insecticide behaved chemically similar to afidopyropen with the same retention time; therefore, it was utilized as a surrogate standard to account for loss during instrumental analysis. QuEChERS salt mixtures (4,000 mg $MgSO_4$ and 1,000 mg NaCl) and SpinFiltr dSPE-microcentrifuges (150mg $MgSO_4$, 50 mg primary secondary amine (PSA), 50 mg C18, and 50 mg Chlorofiltr) (purchased from United Chemical Technologies (Bristol, PA, USA)) were used for extraction of afidopyropen from leaf samples.

2.4 Sample Preparation and LC-MS/MS Analysis

Methods were optimized for afidopyropen for LC-MS/MS analysis (USGS, EPA). A waters LC-QQQ was used for field study #1 spatial distribution samples and an Agilent LC-QQQ was used for analysis of field study #2 pesticide degradation in leaf samples. Each tank mix sample was diluted with ACN in 10 ml volumetric flasks and prepared for analysis with liquid chromatography tandem mass spectrometry (LC-MS/MS). Whatman filter paper sample discs were prepared following methods described in Rehberg et al.[32] and analyzed with the solvent calibration standards on a Waters Xevo UPLC-MS/MS triple quadrupole with Mass Linx software for instrumental control and data acquisition (Table S3). The instrument was operated in the positive ion electrospray mode. An Aquity UPLC BEH C18 column (1.7 µm), maintained at 40°C, was used for chromatographic separation. Mobile phase A consisted of LC-MS grade Optima water with 5% formic acid and mobile phase B was ACN. An elution was applied at a flow rate of

0.2 ml/min with a cycle time of 4 minutes. The sample injection volume was 1 µL. The electron spray ionization (ESI) source settings were as follows: desolvation temperature 500°C; gas flow desolvation 800 L H^{-1} and cone 50 L H^{-1}; source temperature 120°C; extractor 3 V; RF lens 2.5 V. Mass spectra were recorded in the m/z range of 50 to 1200.

Afidopyropen was extracted from leaves using the QuEChERS method.[98] Three leaf sample punches (1" diameter) from each collection time were combined in triplicate and freeze dried for 2 h. Leaves were crushed with a mortar and pestle and placed into pre-weighed 50-ml polypropylene centrifuge tubes with Teflon caps. Dry sample masses were recorded. MilliQ water (7.5 ml) was added to hydrate the samples (15 min), then ACN (10 ml) containing 0.01 µg/ml of malathion surrogate standard was added and the samples vortex shaken (1 min). A QuEChERS salt mixture was added and the samples shaken (1 min). The samples were centrifuged (3,000 rpm, 5 min). The upper ACN layer (1 ml) was transferred to another dSPE tube and vortexed (1 min). The samples were centrifuged (3,000 rpm, 5 min), then supernatant (1 ml) pipetted into an autosampler vial for storage and sample analysis. Matrix matched calibration standards were extracted following the aforementioned protocol. Matrix matched calibration standards, leaf sample extracts, and tank mix samples were analyzed on an Agilent 1290 LC with 6460 MS/MS triple quadrupole with Mass Hunter software for instrumental control and data acquisition. The instrument was operated in the positive ion electrospray mode. An Agilent Poroshell C18 column (2.1mm x 100mm x 2.7µm) maintained at 40°C, was used for chromagraphic separation. A sample volume of 3 µL was injected and a mixture of water with 5 mM ammonium formate/0.05% formic acid (A) and methanol with 5 mM ammonium formate/0.05% formic acid (B) at a flow rate of 0.4 mL/min. The gradient elution used was 20% B for 30 seconds, increasing to 100% B at 4 mins, and held at 100% B for 1 min. The ionization source conditions used were as follows: nebulizer

45 psi; gas flow of 12 L/min at 300°C; sheath gas flow of 12 L/min at 375°C. Further method details and operational parameters for both instrumental analyses are described in Tables 3.3-3.4. Due to an inability to obtain chemical standards, the metabolites of afidopyropen were not quantified in this study.

Table 3.3: Afidopyropen method parameters for the Waters LC-MS/MS. Quant=Quantifier and Qual=Qualifier product ions.

Analyte	Precursor ion (m/z)	Product ion (m/z)	Ion type	Dwell time (sec)	Collision Energy (V)	Cell voltage (V)	Retention time (min)
Afidopyropen	594.3	148.01	Quant	0.4	48	2	2.35
Afidopyropen	594.3	202.08	Qual	0.4	34	2	2.35

Table 3.4: LC-MS/MS method parameters for pesticide analysis. Quant=Quantifier and Qual=Qualifier product ions.

Analyte	Precursor ion (m/z)	Product ion (m/z)	Ion type	Dwell time (sec)	Fragmentor	Collision Energy (V)	Cell voltage (V)	Retention time (min)
Afidopyropen	594.3	148	Quant	20	185	60	2	4.996
Afidopyropen	594.3	202.0	Qual	20	185	36	2	4.996
Malathion	331.1	284.9	Quant	20	72	0	4	4.976
Malathion	331.1	127	Qual	20	72	8	4	4.976

2.5 Method Validation

WF extraction recovery tests were performed by pipetting 50 µL droplets of 100 µg/ml standard onto 5 replicates of WF sample discs. The afidopyropen extraction recovery from WFs was 72.3%. Ten ACN blank samples were analyzed to determine limits of detection (LOD) and quantification (LOQ) (Table 3.5). Due to limited resources, isotopically labeled standards could not be obtained, so matrix matched standards were used for analyzing leaf extract samples and malathion insecticide standard served as an internal standard. Matrix matched standards' accuracy averaged 100.4 ± 44.8 % with a range of 62 to 184.6 %.

Table 3.5. Instrumental analysis parameters for afidopyropen, including calibration curve correlation coefficients, limits of detection (LOD) and quantification (LOQ), and percent recoveries for WF samples (n=5).

Field Study - Sample Matrix: Study	LC-MS/MS	Coefficient of Correlation	LOD (ng/ml)	LOQ (ng/ml)	Extraction Recovery (%)
FS-1-WF: Spatial Distribution	Waters	0.9994	39.7	41.9	72.3
FS-2-Leaves: Degradation (matrix matched standards)	Agilent	0.9973	0.014	0.032	NA
FS-2- Tank Mixes: Concentration (solvent standards)	Agilent	1	0.014	0.032	NA

2.6 Data and Statistical Analysis

For WF sample analysis, the calibration curves, extraction recovery, and WF sample area (17.35 cm^2) were used to quantify insecticide residues present in each sample. The lmer function in the lmer4 package of R was used for statistical analysis of WF spatial distribution data.[32] Due to violation of the normality assumption, the log concentration was used for analysis. Concentrations of zero value were replaced with 0.0000001 ug/ml prior to the log transformation and values <LOQ were removed prior to statistical analysis. Summary statistics were calculated for the log concentration of afidopyropen by the sampling location and application spray method. A mixed model analysis was used to compare canopy height, canopy depth, cardinal side of the tree, side of leaf, and application method and all two-way interactions (fixed effects) with tree and individual leaf within tree as random effects to account for WFs being attached to the top and bottom of the same leaf (i.e., measurements within a tree and on a leaf are correlated). Tukey's multiple comparison adjustment was used for all follow up comparisons. A significance level of 0.05 was used for statistical significance in all analyses. Statistics reported include p-values and differences between logarithm concentrations (diff). For leaf sample analysis, the average and

standard deviation of afidopyropen concentrations from replicate samples are reported. The dissipation kinetics of afidopyropen from leaves were evaluated by comparing zero-, first-, and second-order models fitted to all data. Natural logarithm-transformed residues (mg/kg) were plotted versus time (h). Rate constants and half-lives were calculated across the entire time-period of dissipation; however, it was observed that this method gave a misleading result. Thus, we determined the rate constants from two phases in which separate linear regressions were performed for both the initial rapid dissipation (phase 1) and slower dissipation over time (phase 2). A first-order model of the initial dissipation phase was chosen as the best fit for our results. The first-order model and linear regression (Eqn 1) were used to calculate dissipation rate constants. The first-order integrated rate equation was determined as:

$$\ln[A] = -kt + \ln[A_0] \quad (1)$$

where A is the concentration (mg kg^{-1}), k is the rate constant (h^{-1}), and t is time (h).

Others have observed differences in dissipation rates and used two first-order models, or multiple linear regressions of logarithm transformed concentrations to describe data.[99,100] Dissipation half-lives for phase 1 (about 0 to 6 hours) were determined by:

$$t_{\frac{1}{2}} = \frac{\ln(2)}{k} \quad (2)$$

where $t_{½}$ is the half-life (h) and k is the rate constant (h^{-1}). Any data <LOQ was not included in half-life calculations. Additionally, the maximum peak concentration (at 0 to 1 HAT) was used as the initial insecticide concentration to determine the dissipation rate constants and half-lives in leaves after each insecticide treatment.

3. Results and Discussion

3.1 Pesticide Concentrations

All pesticide tank mixture samples collected during field experiments were confirmed to have high enough (100 µg/ml) concentrations to kill ACP based on recommended mixing instructions and application rates provided by the manufacturer's product label.[66]

3.2 Spatial Distribution in the Field

The spatial distribution of afidopyropen varied considerably when applied with the two different ground application methods. The top side of leaf samples received significantly more pesticide than the bottom side of leaves, regardless of application method (Old: diff=0.48, p=0.0002; Young: diff=1.03, p<0.0001) (Figures 3.4-3.8). The top side of leaf samples in the older-aged trees also received significantly more insecticide than the tops of leaves in the younger-aged trees (Figure 1). The afidopyropen concentrations on top side of leaf samples were 0.04 µg/cm^2 (old) and 0.02 µg/cm^2 (young) higher than on their undersides (Figure 4). This is critical for contact and semi-systemic insecticides, like afidopyropen, where full coverage is necessary to effectively target ACP that primarily reside on new flush and the underside of leaves.[7,9,10,32] Samples from the older trees sprayed with the speed-sprayer application method showed greater afidopyropen concentrations compared to those sprayed with the side-sprayer. This is likely due to a higher application rate (90 vs 20 gal/acre) during FS-1. This higher application rate with higher pressure nearly doubles the amount of active ingredient applied, increasing the concentration detected in WF samples and number of samples >LOQ to be included in statistical analysis. For instance, out of 255 total samples collected for each application method tested, the number of samples >LOQ included for statistical analysis was 87 (side-sprayer) compared to 237 (speed-sprayer) (Figures 3.5). Although increased application rates improve insecticide distribution,

coverage was still inadequate and unable to eradicate ACP. Therefore, increased application rate is not a viable long-term solution for growers who manage HLB with integrated pest management strategies and limit the amount of insecticide applied for environmental or pest resistance concerns.[32,65]

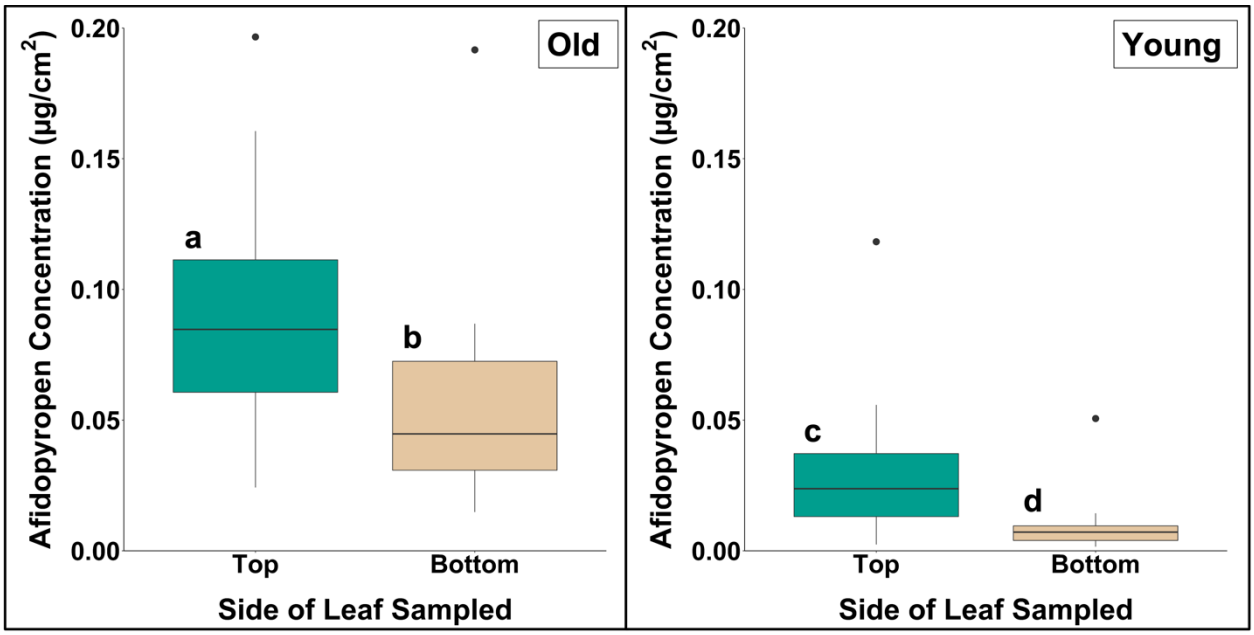


Figure 3.4. Concentration of afidopyropen on WF samples from the top and bottom sides of all leaf samples throughout the citrus trees. Samples were ground sprayed during FS-1 with the speed-sprayer to older trees and the side-sprayer to younger trees. The data are shown as a box plot of the afidopyropen concentration quantified from each location sampled. The observations indicated above the box plot represent outliers. Boxplots with different letters are statistically different.

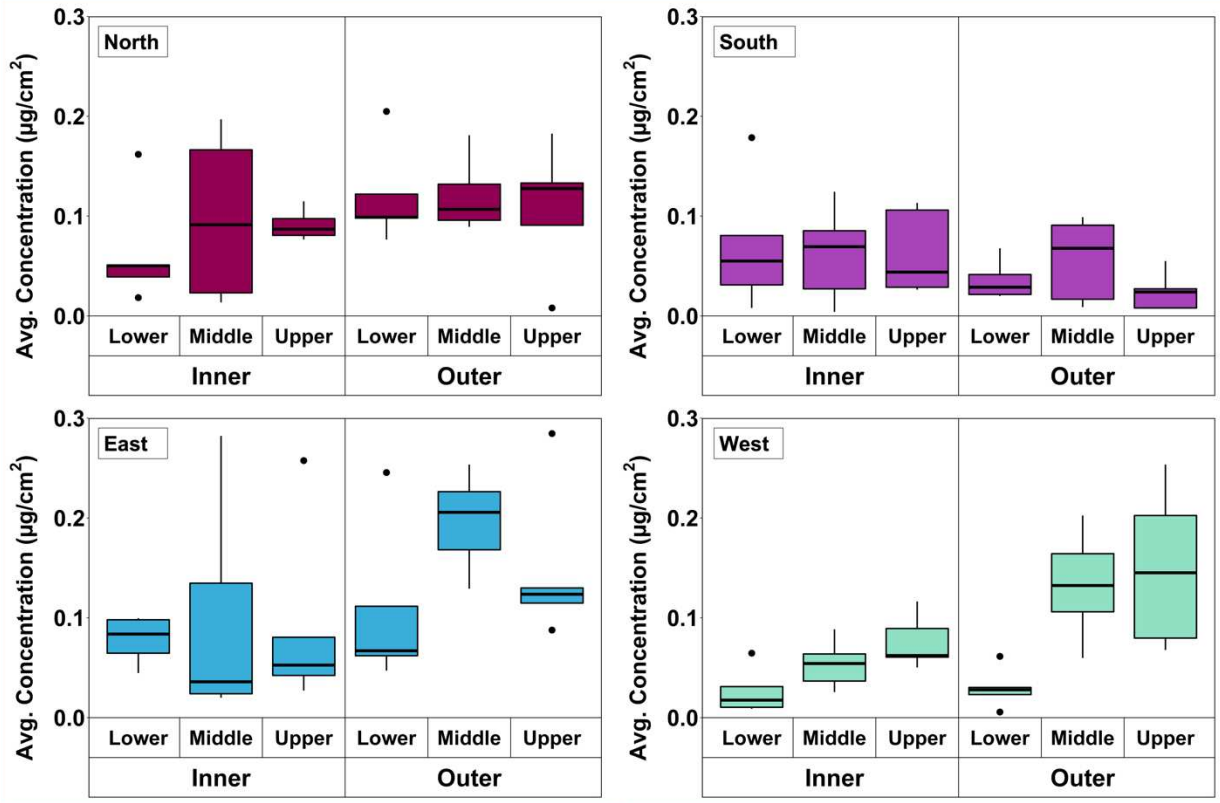


Figure 3.5. Concentration of afidopyropen on samples from various locations throughout the citrus tree. Samples were ground sprayed with the speed-sprayer during FS-1 and collected from older trees in block 38. The x-axis sorts data by canopy depth (inner and outer) and height (lower, middle, and upper). Each colored box represents a cardinal side of the tree (North-red, South-purple, East-blue and West-green). N=237 >LOQ.

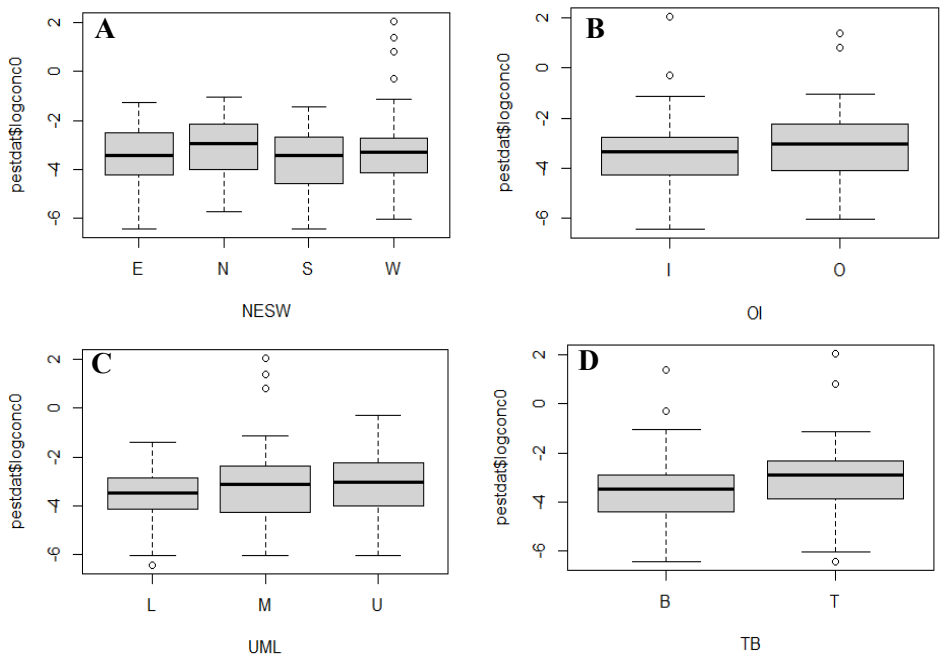


Figure 3.6. Box Plots of afidopyropen log-concentration values for all comparisons of A) cardinal sides North, East, South, and West, B) inner (I) and outer (O) canopy depth, C) lower (L), middle (M) and upper (U) canopy heights, and D) bottom (B) and top (T) side of leaf samples.

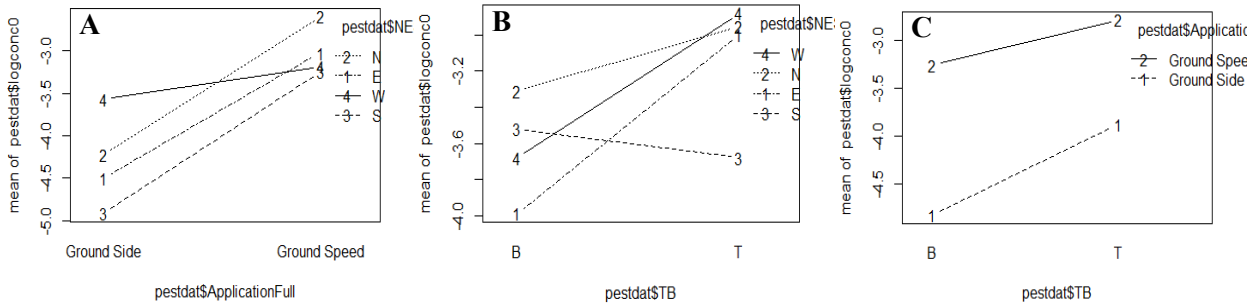


Figure 3.7. Interaction plots of afidopyropen log-concentration values for interactions between A) ground application method and cardinal sides North, East, West, and South, B) side of leaf (top (T) and bottom (B)) and cardinal sides North, East, South, and West, and canopy height, C) side of leaf and ground application method

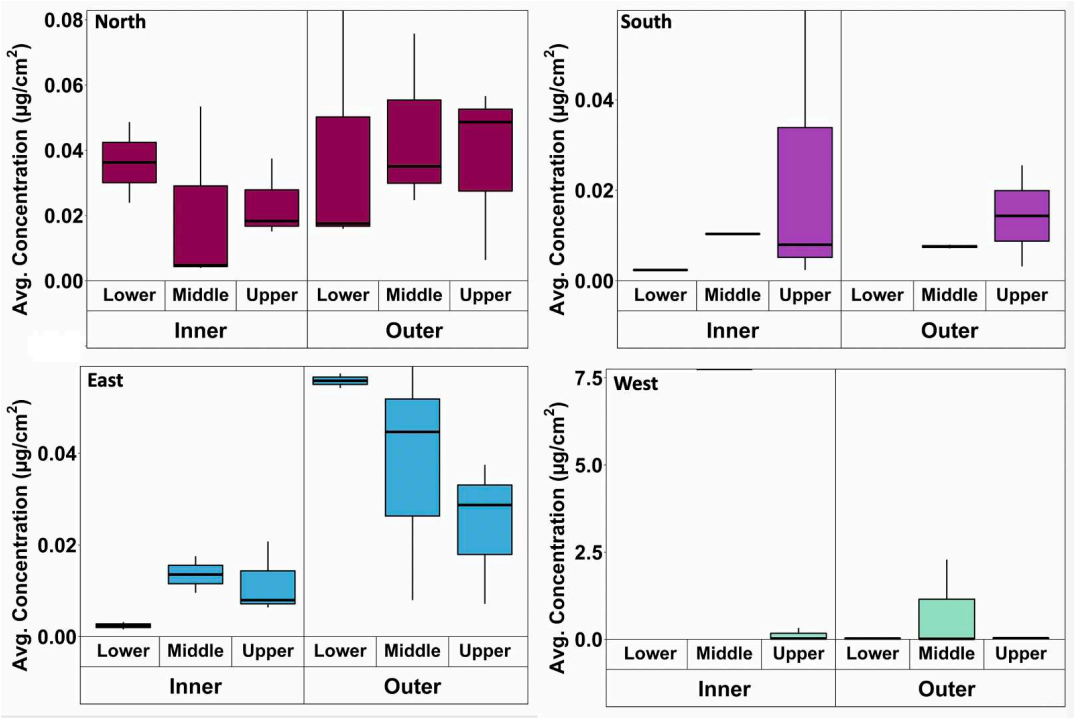


Figure 3.8 S10. Concentration of afidopyropen on samples from various locations throughout the citrus tree (See Figure 1). Samples were ground sprayed with the side sprayer during field study #1 and collected from younger trees in block 49. The x-axis sorts data by canopy depth (inner and outer) and height (lower, middle, and upper). Each colored box represents a cardinal side of the tree (North-red, South-purple, East-blue and West-green). N=87 >LOQ

Overall, there is statistical evidence of interactions between the side of leaf and application method ($p=0.020$) and cardinal side of tree ($p<0.001$), and between the cardinal side of tree and application method ($p=0.011$) (Figure 3.6-3.7). Pesticide variability with canopy depth, height, and cardinal sides of the tree was observed. The results showed statistical differences between inner and outer canopy depths (diff=0.372, $p=0.0115$) and the lower and upper canopy heights (diff=0.4815, $p=0.0241$). Therefore, the outer-upper canopy area received significantly more pesticide than the lower, inner canopy regions (Figure 3.5) which agrees with previous studies' findings.[24,32,40,47,48,65] For both sprayer types, the South side of trees had the lowest concentrations of all four cardinal sides. Higher concentrations on the East and West sides versus the North and South sides may have been due to more direct contact from the nozzles to these adjacent sides, as

well as increased canopy density between trees on the North and South sides.[24,32,40,47,48] However, the differences between cardinal sides for the speed-sprayer were not statistically different, thus the increased application rate (from 50 to 90 gal/acre) improved distribution to all sides of the citrus tree. These results agree with Menger et al. who showed increased application rates improved distribution of a dye applied with the same foliar methods.[65] One would expect better coverage from the side-sprayer's nozzle arrangement, dispersing to three sides of the citrus trees from a closer distance, however West-side samples sprayed with the side-sprayer had about 20 times higher concentration than the other cardinal sides. This may be due to nozzle adjustments or a closer distance between the nozzles and West-side of the trees during application. Overall, these results from specific locations within the citrus tree canopy informs grove managers of areas of lower pesticide protection due to application parameters or meteorological factors, and the need to develop better application methods for citrus. The variability in pesticide spatial distribution throughout citrus trees in the field greatly impacts the initial efficacy of afidopyropen to target and kill ACP. Although inadequate spatial distribution is likely the main culprit for poorer efficacy, degradation results can lead to a better understanding of afidopyropen efficacy over time after application.

3.3 DEGRADATION

Afidopyropen concentrations measured in samples collected at various times after multiple applications reveal rapid degradation in WFs (half-lives: 3.0 and 1.7 h) and leaves (half-lives: 3.4 and 2.3 h) regardless of canopy size and application method (Figures 3.9-3.11). From both older and younger trees, the concentrations in leaf samples initially increased from time zero (speed: 11:00 am; side: 11:40 am) to 0.5- and 1-hour collection times, with a quick decrease in concentration by 6 hours. As expected, previous studies have shown that less degradation occurred

from 6 to 24 hours after application during nighttime (5 pm-11 am) due to limited UV exposure.[91,92]

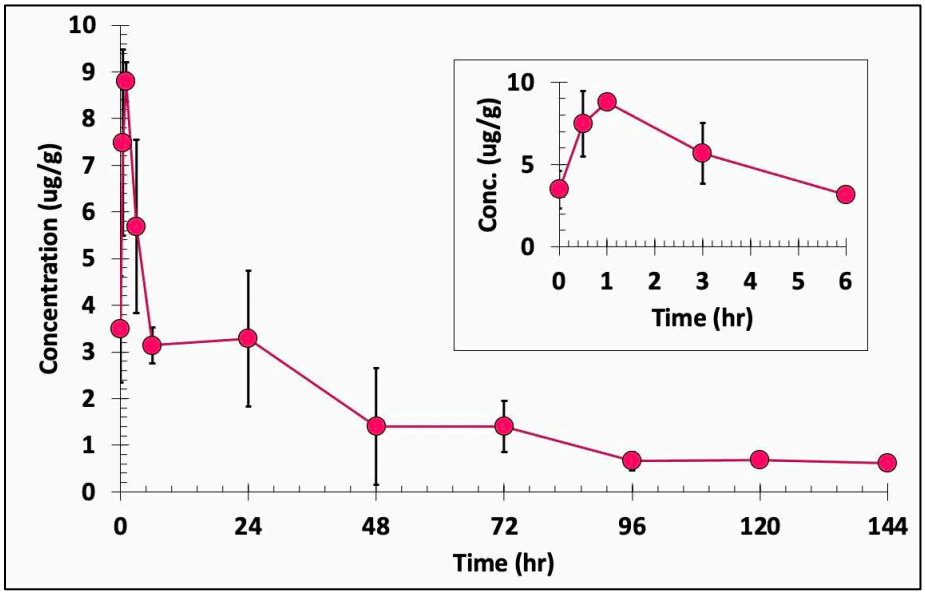


Figure 3.9. Concentration of afidopyropen in leaf samples (n=99) collected at various times after ground application (Time 0 =11:40 AM) with the speed sprayer to older-aged trees during field study #2 (October). Each observation is the average concentration of afidopyropen with standard deviation of the three composite samples for each collection time (n=9 leaf samples for each observation) as error bars. Error bars at some times are smaller than the point on the plot.

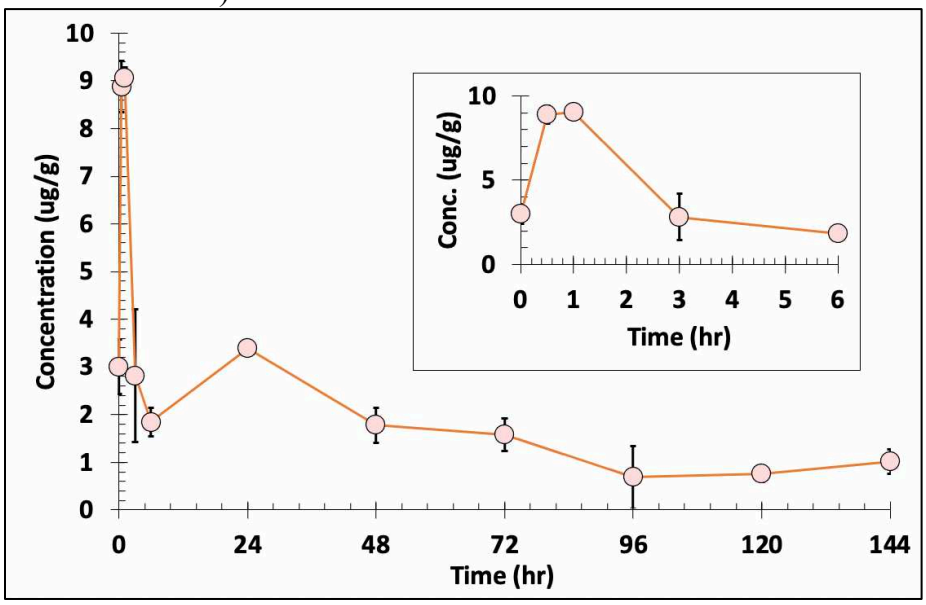


Figure 3.10. Concentration of afidopyropen in leaf samples (n=99) collected at various times after ground application (Time 0 =11:00 AM) with the side sprayer to younger-aged trees during field study #2 (October). Each observation is the average concentration of afidopyropen with standard deviation of the three composite samples for each collection time (n=9 leaf samples for each observation) as error bars. Error bars at some times are smaller than the point on the plot.

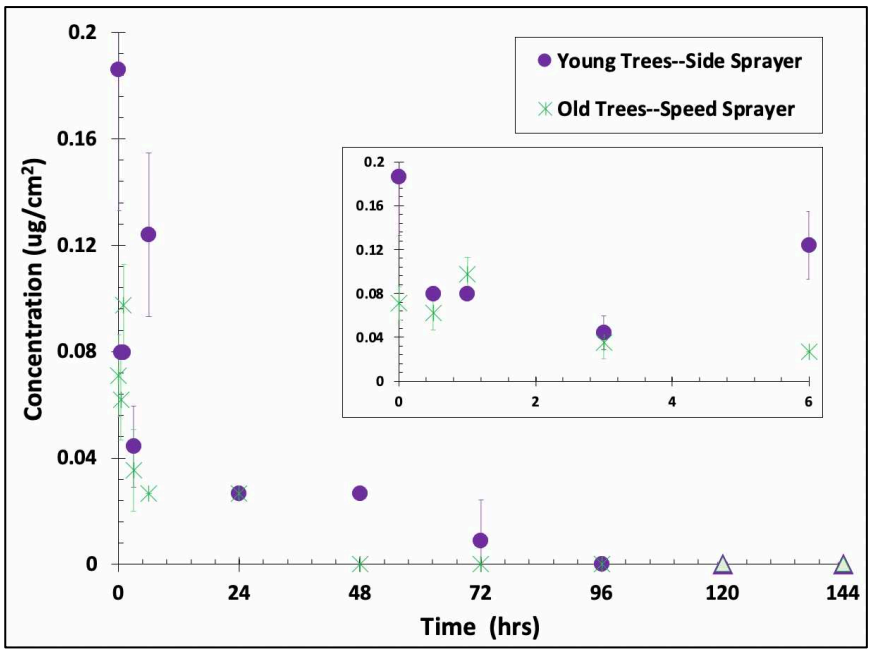


Figure 3.11: Afidopyropen degradation in Whatman filters collected during field study #2 in October. Each data point represents the concentration of afidopyropen detected in 3 composite WF samples (n=9) collected at various time intervals after pesticide application. Afidopyropen appeared to degrade more rapidly in the initial 6 hours for the younger-aged trees (purple squares), but was at a lower concentration in the older-aged trees (green Xs). It is possible that the sample collected at 6 h for the younger trees was in closer proximity to the sprayer nozzles and received a higher amount in pesticide. Overall, both applications showed afidopyropen nearly gone after 3 days and <LOQ at 5 days after application (triangle data markers). Total WF samples n=99.

Increasing afidopyropen concentration was observed within the first hour after application in leaf samples; however, this was not observed in WFs, which is likely related to the sampling method or semi-systemic type of pesticide applied to the trees. (Figure 3.11). With our sampling method, WFs dried faster than leaves. Therefore, if a leaf was wet during collection, then the pesticide residues could have rubbed off the leaf surface during sample collection within 0.5 h after application. Additionally, previous studies suggest that the binding strength of pesticides to leaf surfaces increases within the hours right after application.[93,101] This may explain the lower pesticide concentrations quantified on leaves from early sampling times. Therefore, the maximum peak concentration (between 0 to 1 h) (Figures 3.9-3.10) was used to determine the degradation

half-lives in leaves after afidopyropen application with the speed- (3.4 h) and side- (2.3 h) sprayers for the older-and younger-aged trees, respectively. The dissipation rate constant observed in the older aged trees (0.21 h^{-1}; R^2=0.999) was lower than in the younger-aged trees (0.31 h^{-1}; R^2=0.91).

Our results show faster degradation rates than reported ones for afidopyropen photolysis in other media i.e. soil (stable) and water (6-19.3 days).[91] This is likely due the type of pesticide and how the pesticide mixture interacts with the leaf sample matrix. Since afidopyropen is semi-systemic, the pesticide absorbs into the layers of the leaf as time progresses. Thus, more pesticide residues are present on the leaf's surface than within the leaf's internal layers at earlier sampling times. Inside the leaf layers, photolysis slows due to plant metabolism and reduced light exposure.[92,93] This supports our observations of faster degradation within the first 24 hours and slowed degradation after 48 hours after application. Other studies also observed faster pesticide dissipation rates in leaves (1-3 days) than other medium (water: 9.8 to 1261 days, soil: 1 to 44 days, air: nonvolatile)[81,91] The observed faster initial degradation followed by slower degradation over time suggests that afidopyropen may persist longer at lower concentrations, which could promote ACP resistance.

Additionally, the grove recorded 1.06 inches of rainfall on the third day after application during the October observations (FS-2), which could explain the decrease in pesticide concentration detected in leaf samples after 96 hours (Figure 3.9). Since afidopyropen is only semi-systemic, pesticide residues remaining on the leaf surface may have rinsed off during this rainstorm. Furthermore, studies suggest that a pesticide active ingredient degrades faster on the surface of a leaf than inside internal leaf layers due to available UV for photolysis.[92] Therefore, contact or semi-systemic pesticides like afidopyropen may degrade faster than other fully systemic insecticides, especially in field conditions with a greater UV index.[93] Other studies investigating

meteorological effects on pesticide efficacy suggest applying during nighttime, or times of day with cooler, drier temperatures. Varying meteorological conditions, like temperature, humidity, rainfall, UV exposure, and wind can all impact pesticide dissipation and efficacy in the field, and should be considered when assessing reported efficacies for afidopyropen applied under different field or meteorological conditions.[25,27,92,93,96]

3.3 ACP Inspections and Application Efficacy

ACP populations initially decreased (range 65 to 100 %) after each afidopyropen insecticide application, but live ACP were detected in the trees the day after application for three out of the four applications then increased (6 to172% higher) by 1 week after application in trees observed with remaining ACP (Figures 3.12-3.14, Tables 3.6-3.7). Applications of afidopyropen during FS-1 resulted in ACP population reductions of 87.1% (n=21 trees) and 66.7% (n=7 trees) for the speed and side sprayers, respectively (Tables 3.7, A.1-A.2, Figure 3.13). During FS-2, the older-aged trees contained less ACP (total=2) than the younger-aged trees (total=24). Thus, the ACP population reductions observed in the older trees sprayed with the speed-sprayer was 100% for both 1-day and 1-week after application (Table 3.6-3.7 A.3-A.4, Figure 3.14).

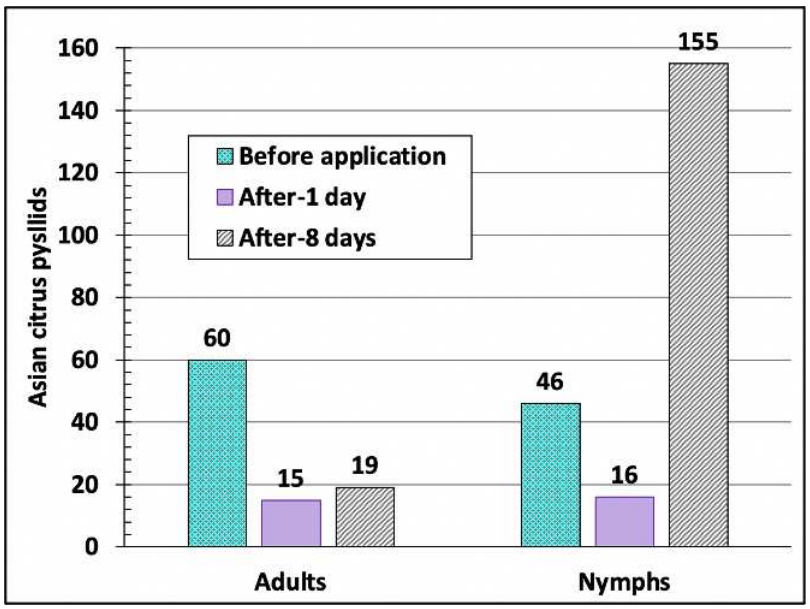


Figure 3.12. The total adult and nymph Asian citrus psyllids counted before and after ground application with the side-sprayer to younger-aged trees during field study #2 (October). ACP data was only included for trees that contained ACP before insecticide treatment. The x-axis shows adult and nymph ACP data recorded before (green), 1 day after (purple), and 8 days after (grey) application.

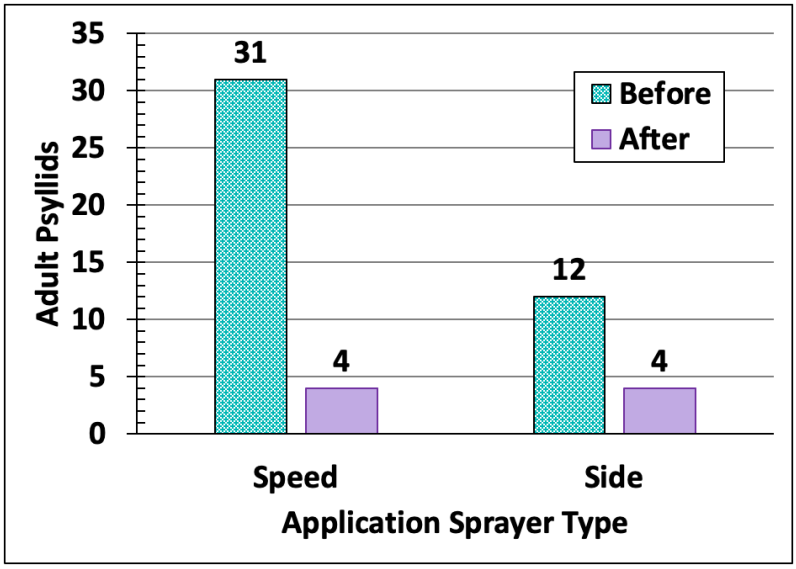


Figure 3.13. The total adult Asian citrus psyllids counted before and after ground application with the speed-sprayer to older-aged trees and the side-sprayer to younger-aged trees during field study #1 (April). ACP data was only included for trees that contained ACP before insecticide treatment. The y-axis shows the number of adult ACP counted and the x-axis shows ACP data recorded before (green), 1 day after (purple), and 8 days after (grey) application. A total of 90 trees were inspected, with n=21 (speed) and n=7 (side) trees containing ACP before application.

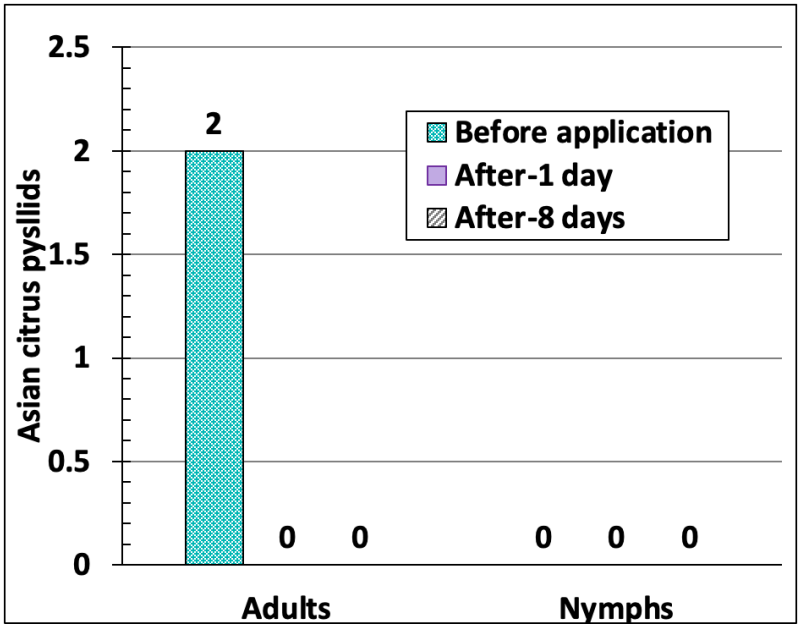


Figure 3.14. The total adult and nymph Asian citrus psyllids counted before and after ground application with the speed-sprayer to older-aged trees during field study #2 (October). ACP data was only included for trees that contained ACP before insecticide treatment. The x-axis shows adult and nymph ACP data recorded before (green), 1 day after (purple), and 8 days after (grey) application. A total of 90 trees were inspected, with only n=2 trees containing ACP before application.

Table 3.6. Total Asian citrus psyllid counts during ground foliar application of afidopyropen with the speed (older) and side (younger) sprayers in field study #1 and #2.

MONTH	PESTICIDE	APPLICATION METHOD	TREE AGE	ACP	# Trees w/ACP before pesticide application	TOTAL ACP POPULATION COUNTS (n)		
						BEFORE	AFTER	
							1-day	1-week
APRIL	Afidopyropen	Ground-Speed	Old	Adults	21	31	4	NA
APRIL	Afidopyropen	Ground-Speed	Old	Nymphs	NA	NA	NA	NA
APRIL	Afidopyropen	Ground-Side	Young	Adults	7	12	4	NA
APRIL	Afidopyropen	Ground-Side	Young	Nymphs	NA	NA	NA	NA
OCTOBER	Afidopyropen	Ground-Speed	Old	Adults	2	2	0	0
OCTOBER	Afidopyropen	Ground-Speed	Old	Nymphs	0	0	0	0
OCTOBER	Afidopyropen	Ground-Side	Young	Adults	21	60	15	19
OCTOBER	Afidopyropen	Ground-Side	Young	Nymphs	3	46	16	155

In the younger aged trees, adult ACP population responses to the afidopyropen applications with the side-sprayer resulted in total percent reductions of 75% (adults) and 65.2% (nymphs) the day after application (Table 3.7). However, when inspected 8 days after application, both adult and nymph ACP populations had increased. Although the total adult ACP observed after 8 days was still less than before treatment, afidopyropen was less effective after a week (68.3%) than

initially (75%). Furthermore, nymph ACP populations increased between 1 day- to 8 days-after application by 172%, a total ACP population regeneration of 237% compared to ACP counts (46 to 155 nymph ACP in n=3 trees) before application (Tables 3.6-3.7, Figure 3.12). Afidopyropen was 20% (FS-1) and 25% (FS-2) more effective in older-aged trees than younger-aged trees (Table 1), which is likely due to afidopyropen persisting longer in the older-aged trees. This may be due to greater canopy foliage which can affect light exposure, shade, temperature, and moisture on leaf samples. These are factors we know impact dissipation or degradation observed in the field.[26,102]

Table 3.7. Asian citrus psyllid (ACP) population percent change after ground application of afidopyropen with the speed (older) and side (younger) sprayers in both field studies (April and October). Changes are calculated only for trees that had ACP before treatment and are presented for the total ACP counts compared to the average ACP percent change per tree. ACP response was reported for 1 day after treatment as well 6 days (older) and 8 days (younger). N=90 trees inspected for each treatment. ND=no data, meaning 0 ACP so there was no population change observed. NA=not applicable, meaning we did not inspect ACP at that time.

MONTH	APPLICATION METHOD	TREE AGE	ACP	# Trees w/ACP before insecticide	ACP POPULATION RESPONSE (%) (-) = decrease, (+) = increase			
					1-DAY AFTER		1-WEEK AFTER	
					TOTAL	AVERAGE	TOTAL	AVERAGE
APR	Ground-Speed	Old	Adults	21	87.1 (-)	85.7 (-)	NA	NA
APR	Ground-Speed	Old	Nymphs	NA	NA	NA	NA	NA
APR	Ground-Side	Young	Adults	7	66.7 (-)	76.2 (-)	NA	NA
APR	Ground-Side	Young	Nymphs	NA	NA	NA	NA	NA
OCT	Ground-Speed	Old	Adults	2	100 (-)	100 (-)	100 (-)	100 (-)
OCT	Ground-Speed	Old	Nymphs	0	ND	ND	ND	ND
OCT	Ground-Side	Young	Adults	21	75.0 (-)	85.1 (-)	68.3 (-)	76.2 (-)
OCT	Ground-Side	Young	Nymphs	3	65.2 (-)	74.6 (-)	237 (+)	165.6 (+)

Because afidopyropen is semi-systemic, it continues to kill ACP as they feed on citrus phloem, however they may continue to spread HLB or reproduce until fully exterminated. One recent citrus field study of afidopyropen's efficacy against ACP reported insignificant population reduction until 14 days (nymphs) after treatment.[88] Another reported ACP reduced for 10 days (adults) and 7 days (nymphs) and increased between 10 to 27 days after application.[89] Similarly, our ACP initially decreased; however, we observed ACP increase sooner after application, likely

due to their lower initial ACP populations or ACP counting methods. We observed greater initial ACP counts (108 total ACP counted within 21 out of 180 trees inspected) and inspected more trees than other studies (i.e. 5.33 avg nymph ACP within 6 leaves in 3 trees;[88] and 0.3 avg ACP per tap with 14 total taps, 3 total ACP in 4 trees[89]) prior to each application. We deduce that higher ACP populations present before treatment allow more ACP to persist and remain on leaves after application, regenerating over time. Additionally, their counting "tap" method collects ACP that fall after tapping branches, thus removes remaining ACP from the tree and subsequently reduces ACP that may have otherwise continued to reproduce. This could improve their ACP population reductions reported over time and explain our observed increases at a faster rate. Furthermore, varying ACP populations or meteorological factors could affect ACP responses. Whether naturally occurring or purposefully placed, ACP populations develop differing resistance depending on varying insecticide exposure and behavioral movement between neighboring groves. Varying meteorological factors (e.g. humidity, wind, rainfall) may have altered application efficacy observed in these field studies in different regions or controlled greenhouse and lab environments.

Comparing our afidopyropen efficacy results to other pesticides applied under similar field conditions reveals afidopyropen is not as effective initially, nor up to one week after application. For instance, compared to other pesticides (malathion, dimethoate, imidacloprid) we tested under the same field conditions, on average, ACP reductions were less for afidopyropen (78.8 ± 14.7%) than malathion, dimethoate, and imidacloprid combined (87.6 ± 6.4 %) one day after application.[103] However, compared to another study of seven different pesticides against ACP applied with knapsack sprayers in the field, our observed efficacy was higher than theirs, which ranged from 24 to 51% at 3 days after initial applications up to 50 to 64% at 7 days after application. This could be due to differences in ACP inspection date after application, application

method, field conditions, or different pesticide physiochemical properties and interactions on the leaf surfaces.[93] Different ACP populations in the field with varying pest resistance and neighboring groves can also impact differences observed between pesticide efficacy at one field and another. For instance, "bad neighbors," or nearby groves implementing minimal ACP management can allow for infected ACP to migrate to inadequately protected trees, even in highly managed groves.[22] Iqbal et al. recommended reapplying the same insecticide for a second spray 2 weeks after the first application in order to reach effective pesticide applications (56 to 93% after 7 days). Again, increased applications may not be acceptable for groves in e.g. Florida due to EPA application limits and recommended rotations for reduced pest resistance.[62,66] Furthermore, limitations in this study include variability of seasonal impacts, field conditions, limited weather data, ACP populations, ACP movement, and short-term ACP population responses. More research with analytical methods for measuring active ingredient concentrations in the field (e.g. mass spectrometry) is needed to understand how larger ACP populations respond to afidopyropen applications in commercial field conditions over a longer time-period with varying field conditions and seasonal impacts, insecticide mode of action and pest resistance, and metabolite fate and toxicology.

4. Conclusion

In summary, the results from this field study clearly show inadequate spatial distribution of afidopyropen insecticide applied to citrus trees with two foliar, ground spraying methods. Overall, the top sides of leaves contained significantly more afidopyropen than the undersides and cardinal sides of trees received unequal distribution, with one of the sides non-adjacent to the sprayers (South-side) having the lowest concentration of afidopyropen. Increasing application rate did improve distribution throughout the citrus tree canopy, however increased rates may not be a

viable long-term option for high management groves. Rapid degradation of afidopyropen was observed in both WF and leaf samples, with half-lives < 3.4 h for the two application methods tested (speed- and side-sprayers). We conclude that afidopyropen degrades quicker on leaves (half-lives: 2.3 and 3.4 h) than in soil or water but degrades fastest on WFs (half-lives: 3.0 and 1.7 h) due to greater light exposure. Therefore, pesticide residues quantified from WFs or non-systemic pesticides would photodegrade faster to lower active ingredient concentrations. ACP populations quantified before and after afidopyropen applications resulted in initial decreases, then drastic increases in both adult and nymph ACP populations within the week after application. Altogether, this combination of high variability in distribution to different sides of the leaf and rapid degradation of afidopyropen active ingredient within leaves in the field creates an opportunity for remaining ACP in the grove, or ACP migrating from "bad neighbors," to continue spreading HLB. Further research is needed to better understand the fate of afidopyropen on crop leaf surfaces and in internal layers and the impact this semi-systemic pesticide has on ACP over extended periods of time in the field. Furthermore, information on metabolite formation and toxicity to ACP could help improve ACP management strategies. Lastly, there is great need for optimization of current foliar application methods for citrus to better protect trees and stop the spread of citrus greening disease

CHAPTER 4: DISSIPATION RATES AND EFFECTIVENESS OF MALATHION, IMIDACLOPRID, AND DIMETHOATE AT CONTROLLING ASIAN CITRUS PSYLLIDS UNDER FIELD CONDITIONS

1. INTRODUCTION

Citrus groves often implement complex integrated pest management strategies that require the rotation of insecticide types based on varying classifications, modes of action, and application methods in attempts to prevent pest resistance and optimize efficacy.[7,12–14,28] Insecticides commonly used to combat sucking insects in citrus crops include selective or broad-spectrum organophosphates (e.g. malathion, dimethoate), neonicotinoids (e.g. imidacloprid, thiamethoxam), pyrethroids/pyrethrins (e.g. cypermethrin) and newer pyropenes (i.e. afidopyropen).[15–18] Since these insecticides belong to distinct chemical classes and have different modes of action, they may impact ACP at various life stages (egg, nymph, adult). To halt transmission of HLB, insecticides must quickly kill ACP or interrupt the feeding processes by which they infect the phloem.[11] While contact insecticides must directly contact pest insects to be effective, systemic insecticides absorb into the plant and kill target pests through ingestion while they are feeding on plant juices, which may offer protection over longer periods of time. Insecticides classified as both contact and systemic insecticides, may provide an initial quick knockdown of ACP populations from contact as well as control over time.[17,18] Since ACP prefer new foliar growth,[11] growers strive to have full coverage of insecticides to the outer-canopy areas of the tree.[7,10] To combat ACP, insecticides are applied to citrus trees with different application methods including soil drenching and foliar sprays. Foliar spray application methods commonly used at commercial groves include aerial and ground sprayers. Newer management strategies involve spraying 10-50 thousand-acre areas to combat the spread of ACP within the grove from "bad neighbors" with less frequent management

practices.[14,22] Insecticide applications are expensive, currently accounting for 25% of total citrus production and increasing due to HLB and ACP infestation rates.[23] Due to the high cost of insecticides, field efficacy should be considered when assessing the benefits of using insecticides;[104] however most of this information has been obtained through greenhouse trials. Insecticide efficacy is influenced by field conditions, degradation kinetics, and ACP response and behavior. Parameters in the field include meteorological conditions (e.g. temperature, humidity, precipitation), tree canopy size and foliage, and commercial foliar spray application methods. Various meteorological conditions can impact how well insecticides are applied onto the target crop and protect crop leaves. Furthermore, dissipation of insecticides can reduce chemical control of ACP and promote pest resistance over time.[28] While much is known about insecticide behavior and efficacy in controlled environments, more observations from field studies are needed to better understand insecticide efficacy against ACP.

The degradation kinetics and efficacy of insecticides against pests have been investigated in lab studies and under controlled greenhouse conditions previously.[13,29,30,50,105–109] Previous studies have reported that insecticides (e.g. malathion, imidacloprid, and dimethoate) are effective at killing ACP[11,21,49–52] and revealed a broad range of half-lives ranging from 5.9 min to 1,250 days under various conditions and sample mediums (e.g. solvent, water, soil, crops) (Table B.1).[25,29,30,105,106,109–111] The most common, first-order degradation pathways of these insecticides include hydrolysis and photolysis, which could occur in the aqueous tank mixture or in the plant material in the field.[29–31] Insecticide dissipation in the field includes degradation as well as other loss processes like volatilization and wash-off.[93] Insecticides that have non- or semi-volatile physiochemical properties, may be more likely to dissipate via hydrolysis and photolysis rather than volatilization. Dissipation in plant samples may occur on the leaf surface or within inner leaf

layers. Pesticide measurements in plant materials from the field have shown that dissipation half-lives are 1.8 to 3.8 days (imidacloprid), 1.2 to 2.2 days (malathion), and 1.1 to 4.1 days (dimethoate) in various fruit and vegetable crops or flower plants (e.g. cotton, cherries, tomatoes, broccoli, figs, lettuce, kidney beans, tea, spinach, and chicory) (Table B.1).[105,111–117] However, observations from field studies are needed to understand the connection between insecticide dissipation and efficacy against ACP. While some have begun to investigate insecticide dissipation and their impact on controlling target pests,[115] it's important to determine active ingredient and metabolite residual kinetics and their impact on controlling ACP over time to better understand efficacy in citrus groves battling HLB. Previous field studies that have evaluated insecticide efficacy with ACP inspections either failed to assess commercial application methods commonly used in high-management groves, degradation within leaf samples, or implement an optimal ACP inspection method.[11,21,49–51] Thus, to the best of our knowledge, no studies have thoroughly quantified active ingredient and metabolite concentrations and degradation in citrus leaf samples over time while simultaneously quantifying ACP population response after various treatments in a high-management, commercial grove.

Therefore, we investigated malathion (MAL) (a contact insecticide)[16] as well as imidacloprid (IMI) and dimethoate (DIM) (which both act as contact and systemic insecticides)[17,18] and several application methods to different tree canopy sizes. These were chosen as model insecticides since they are commonly used to control ACP in citrus crops and were scheduled for treatment by the grove managers during our field sampling months, which were expected to have high ACP presence. The known major metabolites most likely to form under our field conditions were chosen and include IMI-urea and desnitro-IMI, malaoxon (MALX), and omethoate (OME) from IMI, MAL, and DIM parent insecticides, respectively.[29–31,96,106,109,116]

Our objective was to better understand insecticide efficacy against ACP in citrus groves by quantifying the concentration and dissipation kinetics of common insecticides (i.e. IMI, MAL, DIM) and major metabolites (i.e. IMI-urea, desnitro-IMI, MALX, DIM) in citrus leaf samples as well as determining ACP population reductions after various application methods under relevant field conditions. Our results provide pertinent information for citrus growers about how insecticide dissipation varies between different application methods, tree canopy sizes, and insecticide classes and impacts effectiveness at controlling ACP.

2. Materials and Methods

The following methods describe the field experiments, sample preparation and analysis methods used in this study. Additional information on insecticide parent and metabolite compound structures and likely dissipation pathways in our system can be found in Tables 4.1-4.2.

Table 4.1. Insecticide active ingredient details and properties

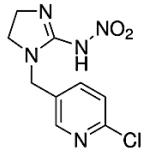
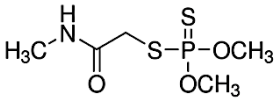
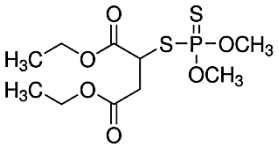
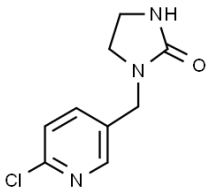
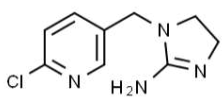
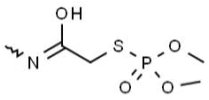
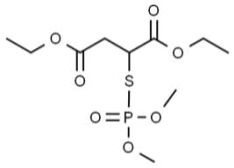
	Imidacloprid	**Dimethoate**	**Malathion**
CAS #	138261-41-3	60-51-5	121-75-5
Formula	$C_9H_{10}ClN_5O_2$	$C_5H_{12}NO_3PS_2$	$C_{10}H_{19}O_6PS_2$
Volatility	Non-volatile	Semi-volatile	Low volatility
Chemical Group	4A	1B	1B
Chemical Class	Neonicotinoid	Organophosphate	Organophosphate
Mode of Action	Nicotinic acetylcholine receptor (nAChR) competitive modulator	Acetylcholinesterase (AChE) inhibitor	Acetylcholinesterase (AChE) inhibitor
Degradation Mechanisms	Photolysis, hydrolysis	Photolysis, hydrolysis	Photolysis, hydrolysis, oxidation
Structure			

Table 4.2. Insecticide metabolite details

	Imidacloprid-urea	Desnitro-imidacloprid	Omethoate	Malaoxon
CAS #	120868-66-8	127202-53-3	1113-02-6	1634-78-2
Formula	$C_9H_{10}ClN_3O$	$C_9H_{11}ClN_4$	$C_5H_{12}NO_4PS$	$C_{10}H_{19}O_7PS$
Structure				

2.1 Field Site Description, Pesticide Applications, and Field Sampling

Field studies (FS) were conducted at a commercial citrus grove in Venus, Florida in July (FS-1) and October (FS-2) 2019. This large-scale, intensively-managed grove is 8,567 acres with ~70 blocks of Valencia orange trees (aged <4 years at the time of field sampling) (Figure B.1). The weather typically ranges from 29 to 33° C with an average of 81 and 185 mm of rain in October and July, respectively. At the grove, the wind typically blows from east to west. During FS-1, there was an average rainfall of 12.7 mm per day, humidity of 79.8% per day, a maximum temperature of 32.3° C, and a minimum temperature of 22.8° C. During FS-2, there was an average rainfall of 4.6 mm per day, humidity of 80.6% per day, a maximum temperature of 31.1° C, and a minimum temperature of 21.9° C.

All insecticides, Malathion 5EC (ai: malathion (MAL)), Admire 4.6F (ai: imidacloprid (IMI)), and Dimethoate 4EC (ai: dimethoate (DIM)), were prepared in water following label instructions and applied via aerial and ground methods (Table S4, Text S1).[16–18,118–120] According to the Bayer Admire Pro product label and MSD sheet, the composition includes 42.8% imidacloprid (CAS #: 138261-41-3) active ingredient and 14% glycerin (56-81-5) adjuvant. According to the Drexel Dimethoate 4EC product label and MSD sheet, the composition includes

43.5% dimethoate (CAS #: 60-51-5) active ingredient and no adjuvants listed. According to the Drexel Malathion 5EC product label and MSD sheet, the composition includes 57% malathion (CAS #: 121-75-5) active ingredient and no adjuvants listed. The products can be mixed with most recommended fungicides, insecticides, miticides, liquid fertilizers, adjuvants, and additives as long as they have been tested on crops prior to application.[16–18]

At the selected grove, ground application by two types of sprayers and specifically coordinated aerial applications are used once or twice per month. The three foliar application methods used in the grove include an aerial-spray, ground-speed sprayer, and ground-side sprayer. (Table 4.3). Two ages of trees, older (4-yr-old: 1.6m x 1.9m) and younger (3-yr-old: 1.4m x 1.7m), were sampled. Trees ~3 years and younger are typically small enough to be sprayed with the side sprayer. Therefore, ground spray applications were carried out to older trees with the speed-sprayer and younger trees with the side-sprayer (Table 4.3). These application methods were used in our study simply because they are the methods currently used in the commercial grove based on tree age and the insecticide product label recommendations. We have previously shown how application method can impact insecticide distribution and concentration,[32,33,65] and thus efficacy;[32,33] however, here we investigate the impact of application method on dissipation kinetics.

Table 4.3. FS-1 and FS-2 experimental application date, block number tested, tree age, application method, sprayer type, commercial insecticide product applied, active ingredient and insecticide type (contact or systemic insecticide). *At time of experiment.

Date	Block #	Tree Age (yr)*	Tree Age Classification	Application Method	Sprayer Type	Insecticide Common Name	Insecticide Active Ingredient	Type
7/10/19	42	~2-3	Young	Ground	Side	Admire 4.6F	Imidacloprid	Systemic and Contact
7/10/19	15	~4	Old	Ground	Speed	Admire 4.6F	Imidacloprid	Systemic and Contact
7/11/19	44	~3	Young	Aerial	Airplane	Malathion 5EC	Malathion	Contact
10/16/19	14	~3	Old	Aerial	Airplane	Malathion 5EC	Malathion	Contact
10/15/19	48	~3	Young	Ground	Side	Dimethoate 4EC	Dimethoate	Systemic and Contact
10/15/19	17	~4	Old	Ground	Speed	Dimethoate 4EC	Dimethoate	Systemic and Contact

Field study #1 (FS-1): In July 2019, IMI was applied to older- and younger-aged citrus trees with the ground speed- and side-sprayers, respectively, and ACP data collected. Eleven trees were sampled for each application type for a total of 22 trees and 240 leaf samples collected. Application occurred on July 10th at 11:30 AM and 11:55 AM for the younger- and older-aged trees, respectively (Tables 4.1-4.3, Figure B.1). While malathion was applied aerially in both field studies, only the FS-2 malathion residual results were included in analysis due to a miscommunication with the pilot. Leaf samples were collected from row 15, but we later learned that they only applied malathion to rows 1-15, instead of the intended rows 1-30. Since there was uncertainty if the samples were sprayed properly and the malathion residues quantified from these samples were much lower than expected (3.43 mg/kg (FS-1) vs. 34.3 mg/kg (FS-2)), this data was not included in analysis. However, this field trial does still offer interesting comparisons with ACP data from rows that did and did not receive malathion treatment (see Tables 4.11, B.4). Therefore, we chose to use the ACP data from untreated trees in rows 16-30 as a control.

Field Study #2 (FS-2): In October 2019, MAL was applied aerially to older-aged trees and DIM was applied to older- and younger-aged trees with the ground speed- and side-sprayers, respectively, and ACP data was obtained. A total of 350 leaf samples were collected from 33 trees sampled in the grove. DIM application occurred on October 15th at 11:15 AM and 11:45 AM for the younger- and older-aged trees, respectively. Aerial application of MAL occurred on October 16th at 10:45 AM. Additional details are provided in the supporting information (Tables 4.1-4.3, Figure B.1).

Proper personal protective equipment including Tyvek suits, closed-toed shoes, head and eye protection, and chemical resistant gloves were used during sample collection and handling. A Fiskars 1-inch diameter Circle Squeeze Punch was used to obtain consistent leaf punch samples

with an area of 0.79 in^2. Field blanks were collected for each tree prior to insecticide treatment by randomly collecting ten leaf samples from the outer canopy area. For each collection time after insecticide treatment, ten leaf samples were collected from the outer canopy area at eye level from an individual tree. Leaf samples were stored separately in Ziploc bags and foil and on ice immediately in the field, then stored in the freezer. Samples were shipped cold overnight from Florida to Colorado State University and stored in the freezer until extraction.

Tank mix samples from each insecticide mixture were collected to quantify the initial concentrations of insecticide active ingredient and metabolites applied to the trees. The tank mix solution was mechanically agitated for 10 minutes prior to sample collection to ensure proper mixing and homogeneity. Samples were collected by a certified pesticide handler in 40 mL amber vials with Teflon cap and stored at 4°C.

2.2 ACP Counting Protocol

ACP data was obtained by a professional ACP inspector in the grove before and after foliar spray to compare insecticide treatments with ACP population reductions.[32,33] For each insecticide treatment, 30 rows of trees were sprayed with insecticide and inspected for ACP. ACP were counted in 3 trees in each row (the north border, middle, and south border) totaling n=90 trees inspected for each treatment. The inspector visually surveyed the entire tree, thoroughly counting ACP adults and nymphs. All ACP counts after treatment were confirmed alive. The inspector recorded adult and nymph ACP 24 hours before treatment (HBT), 24 hours after treatment (HAT), and 192-216 HAT. ACP population percent changes were only calculated for trees that contained ACP before treatment. Averaged percent change was calculated for each tree then averaged over total trees with ACP data. The calculated total percent change shows a more accurate depiction of the efficacy of insecticides across many trees, which is more scalable for a larger grove.

2.3 Chemicals and Standards

Liquid chromatography mass spectrometry (LC-MS) grade acetonitrile (ACN) and acetone (Thermo Fischer Scientific Waltham, MA, USA), were used for standards and sample preparation. The following insecticide standards were used for quantification of insecticide residues in field samples. IMI, IMI-D4, MAL, DIM, and DIM-D6 were purchased as neat materials (purity >98%) from Sigma-Aldrich (St. Louis, MO, USA). IMI-urea (98.5%), MALX (100%), and OME (100%) were purchased from AccuStandard (New Haven, CT, USA). MAL-D6 (98%) and desnitro-IMI (98%) were purchased from Toronto Research Chemicals (North York, ON, Canada). Individual standard solutions containing 100 µg/ml insecticide in ACN were prepared for solvent calibration standards and recovery tests. Ten calibration levels were prepared (ranging from 0.001 to 20 µg/ml) for tank mix sample analysis. Since isotopically labeled standards could not be obtained for all insecticide metabolites, matrix-matched standards were prepared for leaf sample analysis by spiking standards onto field blank leaf samples (ranging from 0.0003-1 µg/mL). MAL-D6, DIM-D6, and IMI-D4 were used as surrogate standards to account for loss during instrumental analysis. QuEChERS salt mixtures (4,000 mg $MgSO_4$ and 1,000 mg NaCl) and SpinFiltr dSPE-microcentrifuges (150mg $MgSO_4$, 50 mg primary secondary amine (PSA), 50 mg C18, and 50 mg Chlorofiltr) were purchased from United Chemical Technologies (Bristol, PA, USA) and used for extraction of insecticides from leaf samples.

2.4 Sample Preparation

Each tank mix sample was diluted with ACN in 10 mL volumetric flasks and prepared for analysis with liquid chromatography-tandem mass spectrometry (LC-MS/MS). Insecticide residues were extracted from leaf samples with the QuEChERS method (Text S2).[33,98,112] From each collection time, three leaf sample punches (1-inch diameter) were combined in triplicate and

freeze dried for 6 h. Leaves were crushed with a mortar and pestle and placed into pre-weighed 50-mL polypropylene centrifuge tubes with Teflon caps. Sample dry masses were recorded. MilliQ water (7.5 mL) was added to hydrate the samples for 15 min, then 10 mL ACN containing 0.01 µg/ml of IMI-D4, DIM-D6, and MAL-D6 surrogate standards were added and the samples vortex shaken for 1 min. A QuEChERS salt mixture was added and the samples shaken again for 1 min. The samples were centrifuged at 3,000 rpm for 5 min. 1 mL of the upper ACN layer was transferred to the dSPE tube and vortexed for 1 min. The samples were centrifuged at 3,000 rpm for 5 min, then 1 mL supernatant pipetted into an autosampler vial for storage and sample analysis. Matrix-matched calibration standards were extracted following the aforementioned protocol.

Even though a modified QuEChERS method was to extract insecticide residues from entire leaf samples, another leaf sample preparation method was tested to determine if leaf samples from the field could be extracted to quantify insecticide concentrations present both on the surface of the leaf, as well as in internal leaf layers. We believed this could lead to interesting data especially for absorption of systemic insecticides into the leaf overtime after application. However, this tested method was not chosen because we were unable to determine accurate concentrations and dry sample masses of both the "rinsed" and "QuEChERS" extracts combined. Briefly, we spiked insecticide standards onto field blank leaf samples in a laboratory fume hood with light exposure and removed samples in triplicate at specific time intervals matching those from the field trials. Leaf samples were then rinsed in 10 ml acetonitrile and set aside for sample prep following the described QuEChERS method. The remaining solvent was evaporated to 1 ml with N_2 and anayzed with LC-MS/MS following the described methods. Since the residues in the solvent "rinsed" samples could not be translated equally to those obtained due to dry masses, and the tested method

was time consuming and impractical, we chose to obtain insecticide residues in total leaf samples via the modified QuEChERS methods.

2.5 LC-MS/MS ANALYSIS

All tank mix and leaf samples were analyzed on an Agilent 1290 UHPLC with 6460 MS/MS triple quadrupole with Mass Hunter software for instrumental control and data acquisition (Table 4.4). The instrument was operated in the positive ion electrospray mode. An Agilent Poroshell C18 column (2.1mm x 100mm x 2.7µm) maintained at 40°C, was used for chromatographic separation. A sample volume of 3 µL was injected and a mixture of water with 5 mM ammonium formate/0.05% formic acid (A) and methanol with 5 mM ammonium formate/0.05% formic acid (B) at a flow rate of 0.4 mL/min. The gradient elution used was 20% B for 30 seconds, increasing to 100% B at 4 mins, and held at 100% B for 1 min. The ionization source conditions used were as follows: nebulizer 45 psi; gas flow of 12 L/min at 300°C; sheath gas flow of 12 L/min at 375°C.

Table 4.4. LC-MS/MS method parameters for pesticide analysis. Abbreviations are as follows: DIM=dimethoate, OME=omethoate, IMI=imidacloprid, MAL=malathion, MALX=Malaoxon. Quant=Quantifier and Qual=Qualifier product ions.

Analyte	Precursor ion (m/z)	Product ion (m/z)	Ion type	Dwell time (sec)	Fragmentor	Collision Energy (V)	Cell voltage (V)	Retention time (min)
DIM	230	198.9	Qual	20	62	8	4	3.639
DIM	230	125	Quant	20	62	20	4	3.639
DIM-D6	236	205	Qual	20	67	8	4	3.619
DIM-D6	236	131	Quant	20	67	20	4	3.619
OME	214	182.9	Qual	30	72	4	7	2.539
OME	214	124.9	Quant	30	72	20	7	2.539
IMI	256.1	209	Qual	20	109	12	2	3.449
IMI	256.1	175.1	Quant	20	109	16	2	3.449
IMI-D4	260.1	213	Qual	20	109	12	2	3.444
IMI-D4	260.1	179.1	Quant	20	109	16	2	3.444
IMI-Urea	212.1	128	Quant	20	89	16	7	3.447
IMI-Urea	212.1	99	Qual	20	89	16	7	3.447
Desnitro-IMI	211.1	125.9	Quant	30	99	20	7	2.628
Desnitro-IMI	211.1	90	Qual	30	99	36	7	2.628
MAL	331.1	284.9	Quant	20	72	0	4	4.976
MAL	331.1	127	Qual	20	72	8	4	4.976
MAL-D6	337.08	127	Quant	20	70	12	4	4.969
MAL-D6	337.08	99	Qual	20	70	24	4	4.969
MALX	315.1	127	Qual	20	77	8	7	4.338
MALX	315.1	99	Quant	20	77	20	7	4.338

2.6 METHOD VALIDATION

Due to limited resources and availability, isotopically labeled standards could not be obtained for some metabolites, therefore matrix matched standards were used for analyzing leaf extract samples. The matrix matched standards' accuracy for all three insecticides averaged 113.2 ± 50.8 % with a range of 83.3 to 135.9 % (Table 4.5). Ten ACN blank samples were analyzed to determine limits of detection (LOD) and quantification (LOQ) at 3x S/N and 10x S/N, respectively. LODs and LOQs for each insecticide are reported in Table 4.6. Calibration curves, extraction recovery methods, and the sample dry weight were addressed when calculating the concentration of insecticide present in each sample. Insecticide active ingredient concentrations were calculated using LC-MS/MS Mass Linx and Mass Hunter softwares.

Table 4.5. Parameters for matrix-matched calibration curves and leaf sample analysis, including correlation coefficients and linear equations.

Analyte	Coefficient of Correlation	Linear Equation
DIM	0.9988	$y = (139.1)x - 0.6275$
OME	0.9992	$y = (8 \times 10^6)x - 38613$
IMI	0.9996	$y = (132.541)x - 0.4408$
IMI-Urea	0.9994	$y = (188)x - 1.3224$
Desnitro-IMI	0.9995	$y = (1 \times 10^6)x + 10957$
MAL	0.9972	$y = (110.44)x - 0.9584$
MALX	0.9926	$y = (212.44)x - 0.0854$

Table 4.6. Parameters for solvent calibration curves, including correlation coefficients, limits of detection (LOD) and quantification (LOQ), and linear equations.

Analyte	Coefficient of Correlation	LOD (ng/ml)	LOQ (ng/ml)	Linear Equation
DIM	0.9995	0.035	0.072	$y = (3 \times 10^7)x$
OME	0.9992	0.153	0.022	$y = (9 \times 10^6)x - 1088.9$
IMI	0.9984	0.151	1.41	$y = (7 \times 10^6)x + 8734.5$
IMI-Urea	0.9996	0.027	0.17	$y = (7 \times 10^7)x + 449.91$
Desnitro-IMI	0.9984	0.138	0.48	$y = (2 \times 10^6)x + 111.54$
MAL	0.9996	0.017	0.036	$y = (7 \times 10^7)x$
MALX	0.9997	0.010	0.020	$y = (1 \times 10^8)x$

2.3 DATA ANALYSIS

For citrus leaf sample analysis, the average and standard deviation of insecticide concentrations from triplicate samples are reported. The dissipation kinetics of insecticides from leaves were evaluated by comparing zero-, first-, and second-order models fitted to all data. We

chose to calculate dissipation kinetics by using the first-order model, linear regression (Eqn 4.1) of natural logarithm-transformed residues (mg/kg) plotted versus time (h). Rate constants were calculated across the entire time-period of dissipation, as well as two phases in which separate linear regressions were performed for both the initial rapid dissipation (phase 1) and slower dissipation over time (phase 2). Calculating the rate constant from the entire time-period would give a misleading result, therefore, this initial first-order phase method was chosen based on our results (Figure B.2). The first-order integrated rate equation was determined as:

$$\ln[A] = -kt + \ln[A_0] \tag{4.1}$$

where A is the concentration (mg kg^{-1}), k is the rate constant (h^{-1}), and t is time (h).

Others have observed faster initial dissipation and used two first-order models, or linear regressions to describe data.[99,100] Describing each insecticide with the first-order model allowed for comparisons between rate constants and half-lives calculated from similar factors. Dissipation half-lives for phase 1 (about 0 to 6 hours) were determined by:

$$t_{\frac{1}{2}} = \frac{\ln(2)}{k} \tag{4.2}$$

where $t_{½}$ is the half-life (h) and k is the rate constant (h^{-1}). Any data <LOQ was not included in half-life calculations. Additionally, the maximum peak concentration (at 0 to 1 HAT) was used as the initial insecticide concentration to determine the dissipation rate constants and half-lives in leaves after each insecticide treatment.

3. Results and Discussion

3.1 Insecticide Dissipation in the Field

The initial concentrations of insecticide active ingredients in the tank mixture samples collected from the aerial, speed- and side-sprayers during both field studies were quantified. Parents compounds (MAL, IMI, DIM) ranged from 56.9 to 82,853 µg/mL and metabolites (MALX, IMID-Urea, Desnitro-IMI, and OME) ranged from 0 to 4.19 µg/mL range (Table 4.7). Others have detected insecticide metabolites in spray tank mixtures at low concentrations as well.[113] This information confirms that the concentrations of insecticides initially prepared were high enough to kill psyllids, according to label instructions, and that degradation does occur in aqueous conditions in tank mix samples prior to treatment.

Table 4.7. Initial insecticide active ingredient parent and metabolite concentrations present in tank mix samples prior to treatment. Details for each tank mix include the field study month, sprayer tank mix, compound, analyte, and concentration. IMI=imidacloprid, MAL=malathion, MALX=Malaoxon, DIM=dimethoate, OME=omethoate.

Month	Tank Mix Sample	Compound	Analyte	Concentration (ug/mL)
JULY	Speed	Parent	IMI	56.98
JULY	Speed	Metabolite	IMI-Urea	1.30
JULY	Speed	Metabolite	Desnitro-IMI	1.33
JULY	Side	Parent	IMI	59.40
JULY	Side	Metabolite	IMI-Urea	1.34
JULY	Side	Metabolite	Desnitro-IMI	1.03
JULY	Aerial	Parent	MAL	219.92
JULY	Aerial	Metabolite	MALX	0.024
OCTOBER	Aerial	Parent	MAL	261.09
OCTOBER	Aerial	Metabolite	MALX	0.023
OCTOBER	Speed	Parent	DIM	24003.28
OCTOBER	Speed	Metabolite	OME	4.19
OCTOBER	Side	Parent	DIM	82852.97
OCTOBER	Side	Metabolite	OME	0

Quick dissipation of insecticide parent compounds were observed after all treatments in the field, regardless of tree canopy size and application method (Figures 4.1-4.3). We observed both IMI and DIM decrease 90% by 24 HAT with the side-sprayer (younger trees) and 95% by 48 HAT with the speed-sprayer (older trees) (Figures 4.1-4.2). Malathion persisted longer and

decreased 95% by 72 HAT aerially (Figure 4.3). Metabolite concentrations observed were much lower than the parent compounds (e.g. OME ranged from 10 to 60 times lower than DIM (Figure 4.2a)) and experienced small variations overtime (Figures 4.1-4.3). Overall, metabolite concentrations increased while parent compounds decreased as predicted, within the first 6 HAT. Minimal changes in concentrations were observed between 6 and 24 HAT, which was during nighttime. After 24 HAT, insecticide parent and metabolite residues slowly decreased over time. Desnitro-IMI metabolite did experience increases in concentration at 96 h (older trees) and 24 h (younger trees). However, these increases do not appear related to IMI or IMI-urea observations and may be due to other factors or metabolite transformations beyond the scope of this research. Some increases in insecticide concentrations were observed in leaf samples within 1 HAT to older-aged trees and varied between insecticides (Figures 4.1a, 4.2a, 4.3). Others have observed similar increases in insecticide concentrations initially and slowed dissipation rates over time.[27] This could be attributed to several factors, including reduced direct light exposure to leaves or a systemic insecticide after it has absorbed further into plant tissues, increased deposition from insecticides in the air, or increased loss of insecticide due to sample handling.[33] If a leaf was wet during collection, then the insecticide residues could have rubbed off the leaf surface during sample collection and handling within 0.5 HAT. Additionally, previous studies suggest that the binding strength of insecticides to leaf surfaces increases within the hours right after application, thus insecticide concentrations observed may have varied slightly.[93,101] The slower dissipation rates observed over time may promote pest resistance development with lower concentrations prolonging for longer periods of time and ineffective insecticide treatments in the field (Figures 4.1-4.3).

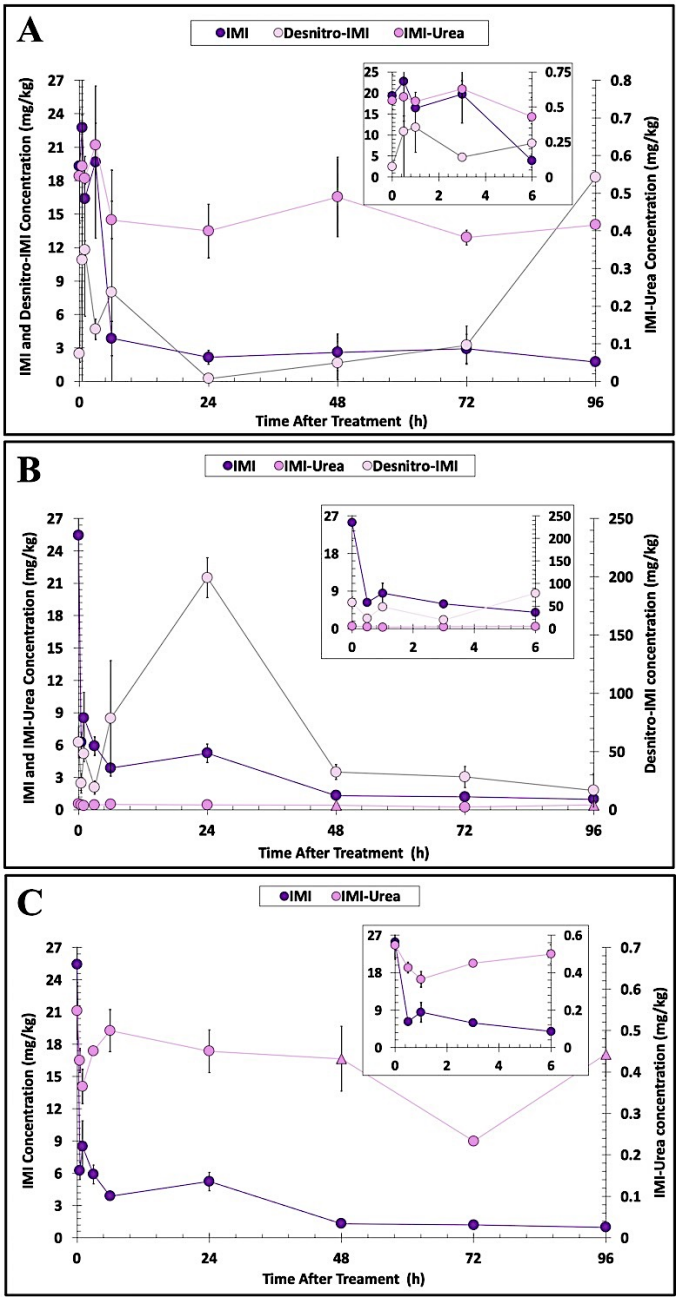


Figure 4.1. Concentration of imidacloprid insecticide parent compound (IMI) and its metabolites (IMI-Urea, Desnitro-IMI) in leaf samples collected at various hours after treatment (HAT) to citrus trees in the field. Data is shown for FS-1 in July and both ground application methods (speed- and side-sprayers). For all plots, the insert shows concentrations within the first 6 HAT, error bars show the standard deviation between n=3 composite samples (n=9 leaf samples total) for each collection time, and triangle data markers represent samples <LOQ and >LOD. In each plot we present A) IMI, desnitro-IMI, and IMI-urea after ground speed-sprayer application to older-aged trees, B) IMI, IMI-urea, and desnitro-IMI after ground side-sprayer application to younger-aged trees, and C) a closer look at IMI and IMI-urea concentrations after the ground side-sprayer application to younger-aged trees.

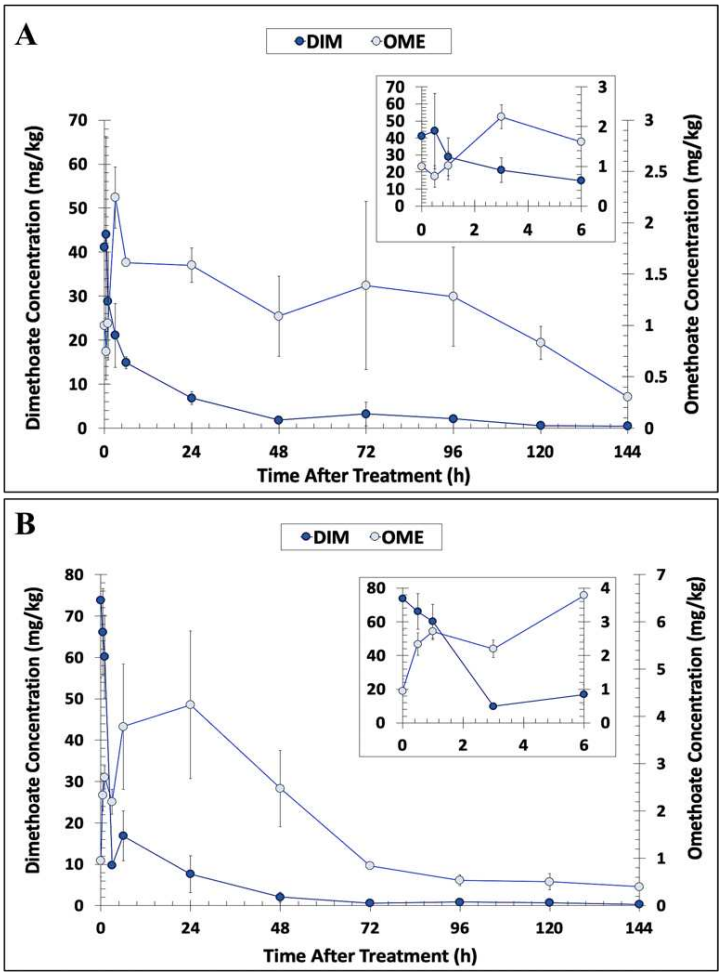


Figure 4.2. Concentration of dimethoate insecticide parent compound (DIM) and its metabolite (OME) in leaf samples collected at various hours after treatment (HAT) to citrus trees in the field. Data is shown from FS-2 in October and both ground application methods (speed- and side-sprayers). For all plots, the insert shows concentrations within the first 6 HAT, error bars show the standard deviation between n=3 composite samples (n=9 leaf samples total) for each collection time, and all data is >LOQ. We present A) DIM and OME concentrations after ground speed-sprayer application to older-aged trees and B) DIM and OME concentrations after ground side-sprayer application to younger-aged.

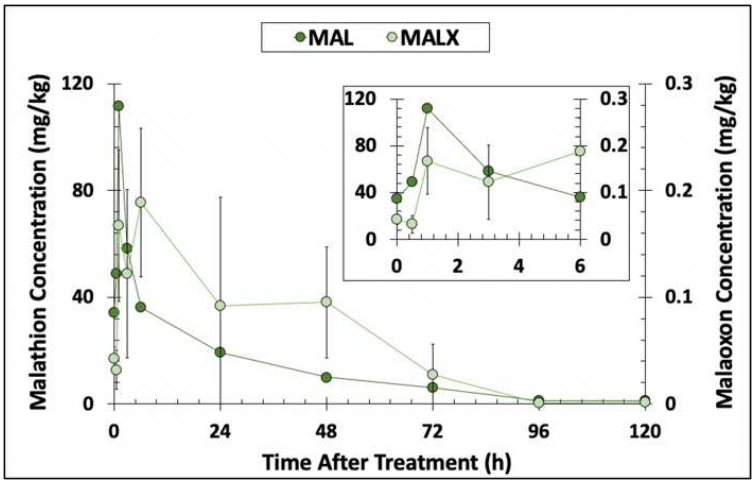


Figure 4.3. Concentration of malathion insecticide parent compound (MAL) and its metabolite (MALX) in leaf samples collected at various hours after treatment (HAT) to citrus trees in the field. Data is shown from aerial application to older-trees in October (FS-2). The insert shows concentrations within the first 6 HAT, error bars show the standard deviation between n=3 composite samples (n=9 leaf samples total) for each collection time, and all data is >LOQ.

Overall, residual insecticide concentrations in leaf samples initially decreased rapidly, with slowed decreases overtime. Malathion underwent rapid dissipation with a half-life of 3.1 h after aerial application to older-aged trees in FS-2 (Table 4.8). Both IMI and DIM experienced rapid dissipation with half-lives of 0.6 and 2.3 h (IMI, FS-1), and 1.0 and 4.0 h (DIM, FS-2) after treatment to younger and older trees with the ground side- and speed-sprayers, respectively (Table 4.8). Therefore, shorter half-lives were observed for insecticides applied to younger-aged trees with the side-sprayer, as well as for insecticides tested in July. Furthermore, the neonicotinoid, IMI, experienced a 0.8 and 1.7 h shorter half-life than both organophosphate insecticides (MAL and DIM) when applied to older-aged trees (Table 4.8).

Table 4.8. Residual dissipation kinetics of imidacloprid (IMI), dimethoate (DIM) and malathion (MAL) insecticides from leaf samples collected at various hours after treatment (HAT) to citrus trees in the field. Data includes both months (July and October), tree ages (young and old), and all application methods (aerial and ground speed- and side-sprayers) sampled. Data was fitted to a first-order model for phase 1 (0 to 6 HAT) and shows the integrated rate equation, correlation coefficient, rate constant, and half-life for each treatment tested.

Insecticide	Month	Application Method	Tree Age	Correlation Coefficient	Rate Constant (1/h)	Half-life (h)
IMI	JUL	Ground-Side	Young	0.55	1.1	0.6
IMI	JUL	Ground-Speed	Old	0.81	0.3	2.3
DIM	OCT	Ground-Side	Young	0.95	0.7	1.0
DIM	OCT	Ground-Speed	Old	0.88	0.2	4.0
MAL	OCT	Aerial	Old	0.96	0.2	3.1

Observed differences in reported half-lives and rate constants may be due to various factors including application method, insecticide physiochemical properties and type (contact or systemic), tree canopy size (older vs younger trees), or meteorological conditions like temperature, humidity, or rainfall. The spray application method impacts coverage,[32,33,65] and thus the insecticide concentration measured in leaf samples which could affect the rate constant when concentration-dependent (Table 4.8). Greater tree canopy size and foliage can affect light exposure to leaf samples.[25,26,102] Thus, smaller trees, like our younger-aged trees sampled, are more likely to have less shade and moisture on leaves, and higher temperatures due to increased light exposure. This could explain our higher dissipation rates observed when insecticides were applied to younger-aged trees.[26] Additionally, increased moisture on leaf surfaces, whether from rainfall, humidity, or dew, may decrease insecticide concentrations from dilution or wash-off in older-aged trees.[25] Contact insecticides like MAL that remain on the surface of the leaf rather than absorbing into the internal leaf layers may be more likely to experience loss from various meteorological conditions including Florida's typical heavy rainfall, however, we did not observe significant differences between MAL and the other systemic insecticides. However, less rainfall occurred during our field studies than the typical average rainfall during that time.

The insecticide active ingredient concentration present in citrus leaves may decrease over time due to dissipation and become too low to eradicate ACP. If not adequately removed, ACP populations can regenerate or develop pest resistance to insecticides.[7,28] Although some studies in laboratory, greenhouse, or field environments have reported IMI, DIM, and MAL degradation kinetics, they typically have been conducted in solvent, water, or soil samples, which may not translate to observations on leaves in a citrus grove and there is little information about insecticide degradation rates in plant tissues. Our observed IMI, DIM, and MAL dissipation half-lives from citrus leaves (ranging about 1 to 4 h) were lower than ones previously reported (ranging about 1 to 4 days) from other crop or plant materials (Table B.1).[26,105,109,111–115,117] Other recent studies reported higher insecticide dissipation rates on leaves than in other medium (water, soil, air) affected by varying field and meteorological conditions, pesticide physiochemical properties, application parameters, plant characteristics, and insecticide mixtures applied with e.g. varying additives such as adjuvants. [25–27] However, these studies often involved active ingredients spiked onto wax layers or leaves in a laboratory and have shown mixed results compared to predictive models and field samples, thus they may not be applicable to what is observed in field trials.[25–27,93] Initial metabolite formation and decreased concentrations over time could affect target pests if the metabolite is toxic toward ACP (e.g. MAX is more toxic toward ACP than MAL).[31] Therefore, the insecticide's mode of action and metabolite toxicity are important to consider. For instance, initially, IMI concentrations were lower than MAL concentrations, but IMI-urea and desnitro-IMI metabolite concentrations were higher than the MALX metabolite. A higher concentration does not necessarily compute to better pest control since the compound's mode of action and other factors may contribute to efficacy observed under field conditions.

3.3 INSECTICIDE EFFICACY AT ERADICATING ACP

Overall, ACP population responses resulted in decreases in adults ranging from 63-100% and nymphs ranging from 85-100% (Tables 4.9-4.11). Treatments of all three insecticides (IMI, DIM, and MAL) resulted in reductions of ACP adult populations, however only the treatment of MAL resulted in zero adult ACP inspected 24 HAT (Figures 4.4-4.5, Table 4.10).

Table 4.9: Asian citrus psyllid (ACP) population percent change after treatments of imidacloprid (IMI), malathion (MAL), and dimethoate (DIM) in both field studies (July and October). Changes are calculated only for trees that had ACP before treatment and are presented for the total ACP counts 24 h before treatment (HBT) and 24 h after treatment (HAT) compared to the average ACP percent change per tree. ACP response was reported for 24 HAT as well as 144 HAT (older) and 192 HAT (younger). N=90 trees inspected for each treatment**.** ND=no data, meaning 0 ACP so there was no population change observed. NA=not applicable, meaning we did not inspect ACP at that time.

Month	Insecticide	Application Method	Tree Age	ACP	# Trees w/ACP before insecticide	ACP Population Response (%) (-) = decrease, (+) = increase			
						24 HAT		192 to 216 HAT	
						Total	Average	Total	Average
JUL	IMI	Ground-Speed	Old	Adults	47	87.8 (-)	80.4 (-)	NA	NA
JUL	IMI	Ground-Speed	Old	Nymphs	8	91.5 (-)	89.1 (-)	NA	NA
JUL	IMI	Ground-Side	Young	Adults	44	62.7 (-)	70.5 (-)	NA	NA
JUL	IMI	Ground-Side	Young	Nymphs	1	100 (-)	100 (-)	NA	NA
OCT	MAL	Aerial	Old	Adults	5	100 (-)	100 (-)	NA	NA
OCT	MAL	Aerial	Old	Nymphs	1	100 (-)	100 (-)	NA	NA
OCT	DIM	Ground-Speed	Old	Adults	2	80.0 (-)	87.5 (-)	100 (-)	100 (-)
OCT	DIM	Ground-Speed	Old	Nymphs	0	ND	ND	ND	ND
OCT	DIM	Ground-Side	Young	Adults	36	86.3 (-)	97.2 (-)	59.4 (-)	32.6 (-)
OCT	DIM	Ground-Side	Young	Nymphs	13	85.1 (-)	83.3 (-)	52.4 (+)	138.9 (+)

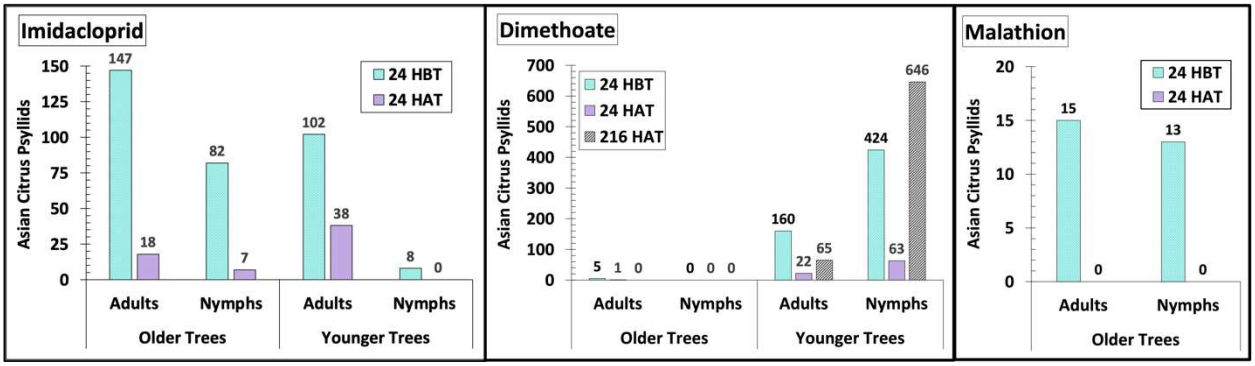


Figure 4.4. ACP population response to imidacloprid, dimethoate, and malathion treatments applied to older and younger-aged citrus trees via different foliar application methods during both field studies (FS). The data shows adult and nymph ACP population counts 24 h before treatment (HBT) and 24 h after treatment (HAT) and 216 HAT. For both imidacloprid (FS-1) and dimethoate (FS-2) treatments, older and younger trees were ground sprayed with the speed- and side-sprayers, respectively. Malathion was applied to older trees with aerial spray application (FS-2). The ACP data shown only includes ACP counts for trees that contained ACP prior to treatment (IMI: n_A=47, n_N =8, n_A =44, n_N =1; DIM: n_A =2, n_N =0, n_A =36, n_N =13; MAL: n_A =5, n_N =1) out of the n=90 trees inspected for each treatment test.

Table 4.10. Total Asian citrus psyllid counts during ground foliar application of insecticides with the aerial (younger), speed (older) and side (younger) sprayers in field study #1 and #2. IMI=Imidacloprid, MAL= Malathion, DIM=Dimethoate.

MONTH	PESTICIDE	APPLICATION METHOD	Adults			Nymphs		
			Before	After-1 day	After-9 days	Before	After-1 day	After-9 days
JULY	IMI	Ground-speed	147	29	NA	82	20	NA
JULY	IMI	Ground-side	102	53	NA	8	9	NA
JULY	MAL	Aerial	83	12	NA	0	8	NA
JULY		CONTROL	30	21	NA	0	0	NA
OCTOBER	MAL	Aerial	15	0	NA	13	0	NA
OCTOBER	DIM	Ground-speed	5	2	12	0	0	169
OCTOBER	DIM	Ground-side	160	25	92	424	85	1559

Table 4.11. Asian citrus psyllid (ACP) population changes for malathion treatment in July (FS-1) Results are reported for both adult and nymph ACP as total ACP count percent changes as well as average percent changes. Average percent is calculated by averaging the percent change of ACP populations for individual trees with ACP data. Only ACP data from trees with ACP prior to treatment were included. Only half of the sample trees were treated (n=45/90) so trees in rows 1-15 received treatment. Trees in rows 16-30 did not receive insecticide treatment and served as a control within the same tree block with similar soil conditions, tree health and canopy size. ND=no data, meaning 0 ACP so there was no population change observed. NA=not applicable, meaning we did not inspect ACP at that time.

Month	Insecticide	Application Method	Tree Age	ACP	# Trees w/ACP before insecticide	ACP Population Response (%) (-) = decrease, (+) = increase			
						1-Day After		1-Week After	
						Total	Average	Total	Average
JUL	MAL	Aerial	Young	Adults	36	80.7 (-)	89.8 (-)	NA	NA
JUL	MAL	Aerial	Young	Nymphs	0	ND	ND	NA	NA
JUL		CONTROL	Young	Adults	17	30.0 (-)	45.1 (-)	NA	NA
JUL		CONTROL	Young	Nymphs	0	ND	ND	NA	NA

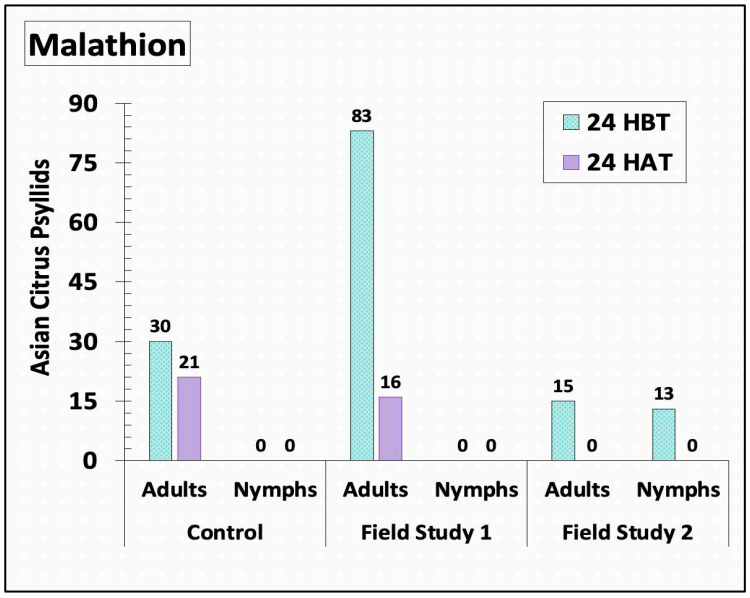


Figure 4.5. Adult and Nymph ACP population counts 24 HBT (green) and 24 HAT (purple) of malathion via aerial spray in FS-1 and FS-2. For FS-1, the control ACP counts represent trees in rows 16-30 with ACP present prior to the "non-treatment" test (n=17 out of 45 trees).The ACP counts before and after malathion treatment only include data from trees that had ACP counts prior to treatment (n=36/45 trees inspected from rows 1-15 (FS-1) and n=5/90 trees).

Nymph ACP results showed initial decreases after treatment of IMI (91.5 to 100%), DIM (85.1%), and MAL (100%) (Table 4.9). However, ACP inspections 9 days after treatment (216 HAT) revealed an increase of 52.4% in ACP nymphs after treatment of DIM during FS-2 in

October (Table 4.9, Figure 4.4). MAL showed better ACP population reductions (100%) and no ACP increases, which may be due to a lower rate constant and more persistence observed in the field, as well as no ACP remaining after application to continue reproducing (Tables 4.9, B.2-B.7). These results agree with other field studies of ACP response to insecticides that showed higher insecticide efficacy when fewer ACP were reported before and after treatment.[33,88,89] Although these observed efficacies are less than 100%, they were better than the 30% reduction observed in our control (Tables B.4-B.5, Figure 4.5) some other field studies with different crops or method parameters.[33,49,121] For instance, DIM's efficacy was 12 (adults) and 20 (nymphs) percent higher than afidopyropen, another insecticide applied under similar field and ACP population conditions.[33] This suggests DIM (a systemic organophosphate insecticide) controlled ACP better than afidopyropen (a semi-systemic pyropene insecticide). Furthermore, our ACP observations after IMI treatment showed better initial ACP decreases (mean per tree: ranging 70.5 to 100%) by 24 HAT (Table 4.9) than another study of IMI applied to Hamlin oranges, which reported varied results of insignificant increases (means ranging 105 to 112%) and decreases (mean 8%) by 96 HAT and a lower seasonal mean mortality (43 to 65%).[121] However, it's unclear how IMI was applied (foliar spray or soil drenching) and they did not observe significant ACP decreases until after a second IMI treatment, 10 days after the first treatment. According to Iqbal et al., a second treatment of the same insecticide (IMI) should be employed 2 weeks later to achieve efficacy (first: 56% versus second insecticide application: 93%).[49] However, these increased treatment frequencies may not be plausible for Florida groves due to EPA application limits and recommended rotations for reduced pest resistance.[12,28,66]

Initial ACP population reductions are important since remaining ACP may be carriers and continue to reproduce and feed, spreading HLB. Long-term ACP population control may help

population regeneration and limit continued spread of HLB. It is more important for contact insecticides to have better distribution and accurately target ACP to kill on contact whereas the flexibility of systemic insecticides allows ACP to be targeted over time as they feed on the phloem. However, both contact and systemic insecticides must be prevalent long enough in field conditions to effectively reduce ACP populations. The observed rapid regeneration of ACP by 216 HAT of DIM demonstrates how remaining ACP, either untargeted or with built-up insecticide resistance, threaten inadequately protected citrus crops. This demonstrates the adverse impact of rapid insecticide dissipation on ACP management in citrus groves.

4 IMPLICATIONS

Due to HLB destruction of citrus yields and profits, and increasing insecticide application costs,[23] growers need effective treatment methods for citrus to better fight HLB spread in groves. Rapid insecticide dissipation, with half-lives ranging from 0.6 to 4.0 h, were observed and revealed that various factors, like tree canopy size, do impact insecticide dissipation kinetics, and thus effectiveness. Therefore, growers should take insecticide class and type, application method, tree canopy size, and meteorological conditions into consideration prior to treatment to reduce these adverse impacts. Overall, we observed a wide range of ACP population reductions of 63 to 100%. Observations of ACP response to insecticides in the field vary, which is likely due to many factors: initial ACP presence, ACP counting method and inspection date after treatment, application method and parameters, field and meteorological conditions, insecticide physiochemical properties and interactions on the leaf surfaces.[93] Additionally, diverse field populations of ACP can develop different pest resistance and thus result in varied insecticide efficacies observed between different groves. Since there is no cure for HLB, for the purpose of our study, any observations less than 100% were considered inadequately effective at eradicating ACP, which

presents opportunity for ACP to continue spreading HLB. While we recognize any ACP reduction does help slow the spread of HLB, our research was to assess the effectiveness of insecticides relevant to citrus growers' applications in the field. About 20 different insecticides are used in rotation at our partnering commercial citrus grove. We selected IMI, MAL, and DIM as model insecticides, but further research should investigate dissipation of more insecticides with different chemical classes, types, and their metabolites in leaves after application in field conditions.

Furthermore, limitations in this study include variability of seasonal impacts, field conditions, limited weather data, ACP populations, ACP movement, and primarily short-term ACP population responses. Further studies should investigate larger ACP populations' responses to insecticides over longer time-periods within commercial citrus groves. There is a greater need to analyze insecticides on leaves after treatment in the field. Several researchers are striving to develop predictive models to estimate the fate of insecticides on leaves in the field. Our results will help progress these models; however, more field data from various insecticides and crops are needed to compute half-lives of insecticides on plant tissues.[26,93] These studies will also help growers understand insecticide efficacy in the field. Additional studies, along with our results presented here, will not only help growers select the best insecticides and methods to combat ACP and HLB spread, but also inform insecticide manufacturers of their products' efficacies in the field. These results are crucial for growers considering integrated pest management strategies and establish a need for further research on the fate and efficacy of insecticides used in citrus field conditions.

CHAPTER 5: SUMMARY

The purpose of the work presented in this dissertation is to better understand insecticide efficacy at controlling Asian citrus psyllids (ACP) in citrus trees infected with citrus greening disease. To do this, we investigated many factors relevant to current ACP management practices in a commercial citrus grove in Florida.

First, we studied the impacts of insecticide spatial distribution and application method on reducing total ACP populations in the field (Chapter 2). We quantified the spatial distribution of insecticides applied to individual citrus trees with three foliar spray methods: aerial, ground speed, and ground side-sprayer. After thoroughly sampling different areas of the tree canopy (cardinal sides, height, depth, and leaf-side), we learned that the undersides of leaves and lower, inner-canopy areas receive much less insecticide than top sides of leaves and upper, outer-canopy areas. Additionally, we observed differences in cardinal sides between application spray methods with better insecticide distribution to leaves closer to the spray nozzles when applied with the ground spraying methods. After quantifying total ACP population counts before and after applications, we discovered higher total ACP counts after application, suggesting inadequate insecticide treatments.

Secondly, we investigated the insecticide distribution and dissipation of a newer, semi-systemic insecticide, Afidopyropen, at the request of our industry partners (Chapter 3). Many growers are especially interested in the efficacy of newer semi-systemic insecticides at combatting sucking insects, like ACP, initially and overtime due to both their contact and semi-systemic properties. Afidopyropen showed similar distribution as the other insecticides, however better distribution was observed when application rates were increased. Afidopyropen, which is present at a lower percent in the commercial product, was detected at lower concentrations than other

common insecticides, so higher application rates may be necessary in order to achieve better distribution. However, increased application rates or number of treatments should be carefully considered while following recommendations of insecticide rotations based on various modes of action. Afidopyropen also experienced rapid dissipation with half-lives of 2.3 h and 3.4 h for the younger and older trees sprayed with the side- and speed sprayers respectively.

Lastly, we investigated the impact of different types of insecticides (contact vs. systemic) modes of action, tree-ages and canopy sizes, application methods, and field conditions on insecticide dissipation and degradation as well as effectiveness at reduction ACP populations in the field (Chapter 4). We tested three common ACP insecticides (imidacloprid, malathion, and dimethoate) as a model to assess varying insecticides' dissipation and efficacy in the field. We observed rapid dissipation initially, with half-lives ranging from 0.6 to 4.0 h, and slowed dissipation over time. When comparing different modes of action, or chemical classes, the neonicotinoid (IMI) showed lower half-lives than both organophosphates (DIM and MAL). IMI was 0.1 h-1 higher comparing all applications to older-aged trees and was 0.4 h-1 higher than DIM when comparing the two ground spray methods to younger vs. older aged trees. Overall, all insecticides applied to younger-aged trees (with the ground side-sprayer) had smaller half-lives and higher rate constants than those applied to older-aged trees with the airplane or ground speed-sprayer. We also observed faster dissipation kinetics in the summer months (July) than fall (October) sampling times. Since the insecticide applications often rotate and the method employed is based on tree size, our results elucidate that tree foliage size, temperature, and moisture, and thus application method, impact an insecticide's dissipation kinetics. Additionally, since we know application method impacts distribution and the concentration detected in leaf samples, and we've

shown the dissipation kinetics are concentration dependent, varying insecticide persistence observations in the field may be related to application method.

Overall, most of our insecticide treatment tests revealed ACP population reductions of <100%. Since there is no cure for HLB and it only takes one ACP to pass along CLas and infect a citrus tree, efficacies of 80 to 100% just aren't adequate for realistic field conditions and reducing HLB spread. We observed higher insecticide efficacies (e.g. MAL 100%) when fewer ACP were present before and after treatment, which could be due to MAL persisting longer in the field. These results may not be representative of an insecticide's performance during other times of the year or in areas of the grove with different ACP presence, but does provide understanding of how each insecticide performed in those relevant field conditions. Our results also revealed lower efficacies over time when ACP were detected in citrus trees the day after treatment. Therefore, improving distribution during application and coordinating spraying during preferred field conditions (no humidity, dew, or rainfall, hot temperatures, or sunny days) would help increase insecticide efficacy in citrus groves. However, future work should investigate more about additional insecticide metabolites and their toxicity toward ACP over longer periods of time.

Our work presented in this dissertation helps develop a better understanding of insecticide efficacy and its influencing factors in field conditions relevant to citrus growers currently battling ACP populations and citrus greening disease spread. Learning more about insecticide fate in agricultural systems helps advance the development of insecticides and predictive models, as well as promote sustainable and cost-effective crop production.

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APPENDIX A: SUPPLEMENTARY INFORMATION FOR CHAPTER 3

Table A.1. ACP adult counts from field study #1 (April) before and after Afidopyropen application with the side-sprayer to younger citrus trees

Adult psyllid counts before-and after Afidopyropen application in younger trees						
	North		Middle		South	
Row	Pre	Post	Pre	Post	Pre	Post
1						
2		1				
3						
4		1				
5						
6	2	1				
7						
8						
9						
10	1					
11						
12						
13						
14						
15						
16						
17			1			
18		1				
19						
20						
21	2					
22						
23	2	1				
24						
25						
26	1					
27						
28		1				
29			3	2		
30						

Table A.2. ACP adult counts from field study #1 (April) before and after Afidopyropen application with the speed-sprayer to older citrus trees

Adult psyllid counts before-and after Afidopyropen application in older trees

	North		Middle		South	
Row	Pre	Post	Pre	Post	Pre	Post
1					1	
2			2			
3					1	
4						1
5					2	1
6						
7						
8					1	1
9						1
10					3	
11						
12					1	
13						
14			1			
15						
16						1
17	2		1			
18						
19						
20	2	1				
21	2				4	
22	1					
23	1	1			1	
24						
25						
26						
27						
28	1				1	
29						
30	1		1			

Table A.3. ACP adult and nymph counts from field study #2 (October) before and after Afidopyropen application with the side-sprayer to younger citrus trees

Adult psyllid counts before-and after-Afidopyropen application in young trees

	North			Middle			South		
Row	Pre	Post	Post-8	Pre	Post	Post-8	Pre	Post	Post-8
1	0	0	3	0	0	0	0	0	0
2	0	0	9	5	0	0	0	0	0
3	1	0	1	0	0	0	0	0	0
4	2	0	2	0	0	1	0	0	0
5	1	0	0	0	0	0	1	0	0
6	0	0	1	0	0	0	0	0	0
7	0	0	2	0	0	0	0	1	0
8	0	0	0	0	0	0	0	0	0
9	0	0	1	0	0	0	2	0	0
10	0	0	0	0	0	0	0	0	1
11	0	0	2	0	0	0	0	0	0
12	0	0	1	0	0	0	0	0	0
13	0	0	2	0	0	3	0	0	0
14	1	1	1	2	0	0	0	0	0
15	1	0	0	3	0	0	0	0	0
16	0	0	11	0	0	0	0	0	0
17	12	0	7	0	0	0	0	0	0
18	0	12	1	0	0	0	0	0	0
19	0	0	5	3	0	0	0	0	0
20	8	13	6	0	0	0	0	0	0
21	0	0	0	0	0	0	0	0	0
22	0	3	15	3	0	2	0	0	0
23	0	0	6	0	0	0	1	0	0
24	0	0	1	2	0	0	0	0	1
25	0	0	0	0	0	1	0	0	0
26	5	0	0	2	0	0	0	0	0
27	0	0	0	0	0	0	1	0	0
28	0	3	0	0	0	0	0	0	0
29	2	0	0	0	0	1	0	0	0
30	0	0	0	2	1	0	0	0	0

Nymph psyllid counts before-and after-Afidopyropen application in young trees

	North			Middle			South		
Row	Pre	Post	Post-8	Pre	Post	Post-8	Pre	Post	Post-8
1	0	0	0	0	0	0	0	0	31
2	0	0	0	0	0	0	0	0	0
3	0	0	44	0	0	24	0	0	0
4	0	0	0	0	0	0	0	0	0
5	0	0	0	0	0	0	0	0	25
6	0	0	112	0	0	0	0	0	0
7	0	0	0	0	0	0	0	0	29
8	0	0	0	0	0	0	0	0	0
9	0	0	0	0	0	0	0	0	33
10	0	0	0	0	0	0	0	0	0
11	0	0	47	0	0	0	0	0	0
12	0	0	11	0	0	0	0	0	0
13	0	0	51	0	0	167	0	0	0
14	0	0	37	7	0	0	0	0	0
15	0	0	0	0	0	0	0	0	0
16	0	0	4	0	0	0	0	0	0
17	21	16	81	0	4	8	0	0	0
18	0	2	3	0	0	0	0	0	0
19	0	0	49	0	0	11	0	0	0
20	0	9	189	0	0	0	0	0	0
21	0	0	0	0	0	9	0	0	0
22	0	34	78	18	0	74	0	0	0
23	0	0	28	0	0	0	0	0	0
24	0	0	15	0	0	0	0	0	7
25	0	0	0	0	0	0	0	0	0
26	0	0	4	0	0	3	0	0	0
27	0	0	0	0	0	0	0	0	0
28	0	0	94	0	0	0	0	0	0
29	0	7	11	0	0	119	0	0	0
30	0	0	0	0	2	0	0	0	0

Table A.4. ACP adult and nymph counts from field study #2 (October) before and after Afidopyropen application with the speed-sprayer to older citrus trees

Adult psyllid counts pre-and post Afidopyropen application in older trees

	North			Middle			South		
Row	Pre	Post	Post-6	Pre	Post	Post-6	Pre	Post	Post-6
1	1	0	0	0	0	0	0	0	3
2	0	0	0	0	0	0	0	0	1
3	0	0	0	0	0	0	0	0	0
4	0	0	1	0	0	0	0	0	0
5	0	0	0	1	0	0	0	0	0
6	0	0	0	0	0	0	0	0	0
7	0	0	0	0	0	0	0	0	0
8	0	0	0	0	0	0	0	0	0
9	0	0	0	0	0	0	0	0	1
10	0	0	0	0	0	0	0	0	0
11	0	0	0	0	0	0	0	0	0
12	0	0	0	0	0	0	0	0	0
13	0	1	0	0	0	0	0	0	0
14	0	0	0	0	0	0	0	0	0
15	0	0	0	0	0	0	0	0	0
16	0	0	0	0	0	0	0	0	0
17	0	0	2	0	0	1	0	0	0
18	0	0	0	0	0	0	0	0	0
19	0	0	0	0	0	0	0	0	0
20	0	0	2	0	0	0	0	0	0
21	0	0	0	0	0	0	0	0	0
22	0	0	0	0	0	0	0	0	0
23	0	0	0	0	0	0	0	0	0
24	0	0	0	0	0	0	0	0	0
25	0	0	1	0	0	0	0	0	0
26	0	0	0	0	0	0	0	0	0
27	0	0	0	0	0	0	0	0	0
28	0	0	0	0	0	0	0	0	0
29	0	0	0	0	0	0	0	0	0
30	0	0	0	0	0	0	0	0	0

Nymph psyllid counts pre-and post Afidopyropen application in older trees

	North			Middle			South		
Row	Pre	Post	Post-6	Pre	Post	Post-6	Pre	Post	Post-6
1	0	4	0	0	0	0	0	0	5
2	0	0	0	0	0	0	0	0	36
3	0	0	0	0	0	0	0	0	0
4	0	0	0	0	0	0	0	0	0
5	0	0	16	0	0	0	0	0	0
6	0	0	0	0	0	0	0	0	0
7	0	0	0	0	0	0	0	0	0
8	0	0	0	0	0	0	0	0	0
9	0	0	0	0	0	0	0	0	0
10	0	0	0	0	0	0	0	0	0
11	0	0	0	0	0	0	0	0	0
12	0	0	0	0	0	0	0	0	0
13	0	0	0	0	0	0	0	0	0
14	0	0	0	0	0	0	0	0	0
15	0	0	0	0	0	0	0	0	0
16	0	0	0	0	0	0	0	0	0
17	0	0	0	0	0	0	0	0	0
18	0	0	0	0	0	0	0	0	0
19	0	0	0	0	0	0	0	0	0
20	0	0	0	0	0	0	0	0	0
21	0	0	0	0	0	0	0	0	0
22	0	0	0	0	0	0	0	0	0
23	0	0	0	0	0	0	0	0	0
24	0	0	0	0	0	0	0	0	0
25	0	0	0	0	0	0	0	0	0
26	0	0	0	0	0	0	0	0	0
27	0	0	0	0	0	0	0	0	2
28	0	0	0	0	0	0	0	0	0
29	0	0	0	0	0	0	0	0	0
30	0	0	0	0	0	0	0	0	0

APPENDIX B: SUPPLEMENTARY INFORMATION FOR CHAPTER 4

Table B.1. Malathion, Imidacloprid, and Dimethoate insecticide half-lives from various studies (lab, greenhouse, field) and sample medium (solvent, soil, water, plant material) compiled from references relevant to our study.[25,26,110–117,122,27,29,30,93,96,105,106,109]

Insecticide	Study type-crop	Application Method	Sample Analysis	Sample Matrix	Half-life (unit)	Rate Constant	Model	Reference
Dimethoate	Greenhouse-spinach	Spiked samples	Water rinse, LC-MS	Spinach leaves	3.56 d	0.214	First-order	Hou et al. 2017
Dimethoate	Lab	Spiked onto glass slides	SERS	Water	4.13 d	0.168	First-order	Hou et al. 2017
Malathion	Lab	Std spiked in solution	Photocatalyic cell, glass slides, SPE, GC, LC	Water	5.9 min	0.117	First-order	Bavcon et al. 2007
Malathion	Lab	Product spiked in solution	Photocatalyic cell, glass slides, SPE, GC, LC	Water	8.7 min	0.079	First-order	Bavcon et al. 2007
Malathion	Lab	Std spiked in solution	Photocatalyic cell, glass slides, SPE, GC, LC	Water	8.3 min	0.083	First-order	Bavcon et al. 2007
Malathion	Lab	Product spiked in solution	Photocatalyic cell, glass-	Water	10.8 min	0.064	First-order	Bavcon et al. 2007

			slides, SPE, GC, LC					
Malathion	Lab	Std spiked in solution	Photocatalyic cell, glass slides, SPE, GC, LC	Water	2900 min (2.01 d)	0.00024	First-order	Bavcon et al. 2007
Malathion	Lab	Product spiked in solution	Photocatalyic cell, glass slides, SPE, GC, LC	Water	420 min (7 h)	0.0017	First-order	Bavcon et al. 2007
Malathion	Field-Tomato	1.5x high-dose treatment	SPE extraction, GC-FPD	Cherry tomatoes	1.73 d	0.401	First-order	Liu et al. 2020
Malathion	Greenhouse-Tomato	1.5x high-dose treatment	SPE extraction, GC-FPD	Cherry tomatoes	1.76 d	0.394	First-order	Liu et al. 2020
Malathion	Field-Broccoli	1.5x high-dose treatment	SPE extraction, GC-FPD	Broccoli	2.15 d	0.322	First-order	Liu et al. 2020
Malathion	Greenhouse-Broccoli	1.5x high-dose treatment	SPE extraction, GC-FPD	Broccoli	1.58 d	0.439	First-order	Liu et al. 2020
Malathion	Field-Mulberries	1.5x high-dose treatment	SPE extraction, GC-FPD	Mulberries	1.1 d	0.630	First-order	Liu et al. 2020

Malathion	Field-Mulberries	1.5x high-dose treatment	SPE extraction, GC-FPD	Mulberries	1.33 d	0.521	First-order	Liu et al. 2020
Malathion	Field-Cranberries	1.5x high-dose treatment	SPE extraction, GC-FPD	Cranberries	1.22 d	0.568	First-order	Liu et al. 2020
Malathion	Field-Cranberries	1.5x high-dose treatment	SPE extraction, GC-FPD	Cranberries	1.26 d	0.550	First-order	Liu et al. 2020
Malathion	Field-Figs	1.5x high-dose treatment	SPE extraction, GC-FPD	Figs	1.4 d	0.495	First-order	Liu et al. 2020
Malathion	Field-Figs	1.5x high-dose treatment	SPE extraction, GC-FPD	Figs	1.36 d	0.510	First-order	Liu et al. 2020
Malathion	Greenhouse-Amaranth	Sprayed and mixed in soil	Soil extractions, GC-MS	Soil	24 d	0.029	First-order	Al-Qurainy et al. 2009
Malathion	Greenhouse-Kidney Bean	Sprayed and mixed in soil	Soil extractions, GC-MS	Soil	23 d	0.030	First-order	Al-Qurainy et al. 2009
Malathion	Greenhouse-Lettuce	Sprayed and mixed in soil	Soil extractions, GC-MS	Soil	25 d	0.028	First-order	Al-Qurainy et al. 2009

Malathion	Greenhouse-Watercress	Sprayed and mixed in soil	Soil extractions, GC-MS	Soil	25 d	0.028	First-order	Al-Qurainy et al. 2009
Dimethoate	Greenhouse-Amaranth	Sprayed and mixed in soil	Soil extractions, GC-MS	Soil	28 d	0.025	First-order	Al-Qurainy et al. 2009
Dimethoate	Greenhouse-Kidney Bean	Sprayed and mixed in soil	Soil extractions, GC-MS	Soil	30 d	0.023	First-order	Al-Qurainy et al. 2009
Dimethoate	Greenhouse-Lettuce	Sprayed and mixed in soil	Soil extractions, GC-MS	Soil	25 d	0.028	First-order	Al-Qurainy et al. 2009
Dimethoate	Greenhouse-Watercress	Sprayed and mixed in soil	Soil extractions, GC-MS	Soil	30 d	0.023	First-order	Al-Qurainy et al. 2009
Dimethoate	Lab	Spiked onto glass slides	Direct photolysis,GC-FID,-MS	Solvent	9.35 d		Biphasic-alpha	Ishag et al. 2019
Dimethoate	Lab	Spiked samples	Direct photolysis, soil extractions, GC-FID,-MS	Soil	10.77 d		Biphasic-alpha	Ishag et al. 2019
Dimethoate	Lab	Spiked onto glass slides	Indirect photolysis, GC-FID,-MS	Solvent	2.399 d		Biphasic-alpha	Ishag et al. 2019

Dimethoate	Lab	Spiked onto glass slides	Indirect photolysis, GC-FID,-MS	Solvent	2.37 d		Biphasic-alpha	Ishag et al. 2019
Dimethoate	Lab	Spiked samples	Indirect photolysis, GC-FID,-MS	Soil	4.402 d		Biphasic-alpha	Ishag et al. 2019
Dimethoate	Lab	Spiked samples	Indirect photolysis, GC-FID,-MS	Soil	3.74 d		Biphasic-alpha	Ishag et al. 2019
Dimethoate	Lab	Spiked onto glass slides	Direct photolysis,GC-FID,-MS	Solvent	16.067 d		Biphasic-beta	Ishag et al. 2019
Dimethoate	Lab	Spiked samples	Direct photolysis, soil extractions, GC-FID,-MS	Soil	16.325 d		Biphasic-beta	Ishag et al. 2019
Dimethoate	Lab	Spiked onto glass slides	Indirect photolysis, GC-FID,-MS	Solvent	3.933 d		Biphasic-beta	Ishag et al. 2019
Dimethoate	Lab	Spiked onto glass slides	Indirect photolysis, GC-FID,-MS	Solvent	3.19 d		Biphasic-beta	Ishag et al. 2019
Dimethoate	Lab	Spiked samples	Indirect photolysis, GC-FID,-MS	Soil	5.405 d		Biphasic-beta	Ishag et al. 2019

Dimethoate	Lab	Spiked samples	Indirect photolysis, GC-FID,-MS	Soil	3.64 d		Biphasic-beta	Ishag et al. 2019
Malathion	Lab	Spiked onto glass slides	Direct photolysis,GC-FID,-MS	Solvent	2.1 d		Biphasic-alpha	Ishag et al. 2019
Malathion	Lab	Spiked samples	Direct photolysis, soil extractions, GC-FID,-MS	Soil	1.88 d		Biphasic-alpha	Ishag et al. 2019
Malathion	Lab	Spiked onto glass slides	Indirect photolysis, GC-FID,-MS	Solvent	0.96 d		Biphasic-alpha	Ishag et al. 2019
Malathion	Lab	Spiked samples	Indirect photolysis, GC-FID,-MS	Soil	0.85 d		Biphasic-alpha	Ishag et al. 2019
Malathion	Lab	Spiked samples	Indirect photolysis, GC-FID,-MS	Soil	0.87 d		Biphasic-alpha	Ishag et al. 2019
Malathion	Lab	Spiked onto glass slides	Direct photolysis,GC-FID,-MS	Solvent	3.6 d		Biphasic-beta	Ishag et al. 2019
Malathion	Lab	Spiked samples	Direct photolysis, soil extractions, GC-FID,-MS	Soil	3.056 d		Biphasic-beta	Ishag et al. 2019

Malathion	Lab	Spiked onto glass slides	Indirect photolysis, GC-FID,-MS	Solvent	2.152 d		Biphasic-beta	Ishag et al. 2019
Malathion	Lab	Spiked samples	Indirect photolysis, GC-FID,-MS	Soil	1.9 d		Biphasic-beta	Ishag et al. 2019
Malathion	Lab	Spiked samples	Indirect photolysis, GC-FID,-MS	Soil	2.156 d		Biphasic-beta	Ishag et al. 2019
Malathion	Lab-Model			Vegetation		0.0055 /h		Cahill et al. 2003
Malathion	Lab-Model			Water	36 h			Lamb et al. 2021/Wolfe et al. 1977
Imidacloprid	Field Trial-Mulberries	Knapsack sprayed	Quechers extraction, HPLC	Leaves	3.81 d	0.182	First-order	Paramasivam et al. 2014
Imidacloprid	Field Trial-Mulberries	Knapsack sprayed-2x dose	Quechers extraction, HPLC	Leaves	4.93 d	0.141	First-order	Paramasivam et al. 2014
Imidacloprid	Field-Cotton	Knapsack sprayed-Dose A	SPE extraction, LC-MS	Leaves-upper	3.89 d	0.178	First-order	Jie et al. 2021
Imidacloprid	Field-Cotton	Knapsack sprayed-Dose B	SPE extraction, LC-MS	Leaves-upper	3.94 d	0.176	First-order	Jie et al. 2021

Imidacloprid	Field-Cotton	Knapsack sprayed-Dose C	SPE extraction, LC-MS	Leaves-upper	3.54 d	0.196	First-order	Jie et al. 2021
Imidacloprid	Field-Cotton	Knapsack sprayed-Dose D	SPE extraction, LC-MS	Leaves-upper	3.35 d	0.207	First-order	Jie et al. 2021
Imidacloprid	Field-Cotton	Knapsack sprayed-Dose E	SPE extraction, LC-MS	Leaves-upper	3.07 d	0.226	First-order	Jie et al. 2021
Imidacloprid	Field-Cotton	Knapsack sprayed-Dose A	SPE extraction, LC-MS	Leaves-middle	3.77 d	0.184	First-order	Jie et al. 2021
Imidacloprid	Field-Cotton	Knapsack sprayed-Dose B	SPE extraction, LC-MS	Leaves-middle	3.96 d	0.175	First-order	Jie et al. 2021
Imidacloprid	Field-Cotton	Knapsack sprayed-Dose C	SPE extraction, LC-MS	Leaves-middle	3.18 d	0.218	First-order	Jie et al. 2021
Imidacloprid	Field-Cotton	Knapsack sprayed-Dose D	SPE extraction, LC-MS	Leaves-middle	3.77 d	0.184	First-order	Jie et al. 2021
Imidacloprid	Field-Cotton	Knapsack sprayed-Dose E	SPE extraction, LC-MS	Leaves-middle	3.19 d	0.217	First-order	Jie et al. 2021

Imidacloprid	Field-Cotton	Knapsack sprayed-Dose A	SPE extraction, LC-MS	Leaves-lower	3.69 d	0.188	First-order	Jie et al. 2021
Imidacloprid	Field-Cotton	Knapsack sprayed-Dose B	SPE extraction, LC-MS	Leaves-lower	3.00 d	0.231	First-order	Jie et al. 2021
Imidacloprid	Field-Cotton	Knapsack sprayed-Dose C	SPE extraction, LC-MS	Leaves-lower	1.95 d	0.355	First-order	Jie et al. 2021
Imidacloprid	Field-Cotton	Knapsack sprayed-Dose D	SPE extraction, LC-MS	Leaves-lower	2.69 d	0.258	First-order	Jie et al. 2021
Imidacloprid	Field-Cotton	Knapsack sprayed-Dose E	SPE extraction, LC-MS	Leaves-lower	3.28 d	0.211	First-order	Jie et al. 2021
Imidacloprid	Field-Cotton	Knapsack sprayed-Dose A	Soil extraction, LC-MS	Soil	1.95 d	0.355	First-order	Jie et al. 2021
Imidacloprid	Field-Cotton	Knapsack sprayed-Dose B	Soil extraction, LC-MS	Soil	2.02 d	0.343	First-order	Jie et al. 2021
Imidacloprid	Field-Cotton	Knapsack sprayed-Dose C	Soil extraction, LC-MS	Soil	2.38 d	0.291	First-order	Jie et al. 2021

Imidacloprid	Field-Cotton	Knapsack sprayed-Dose D	Soil extraction, LC-MS	Soil	2.24 d	0.309	First-order	Jie et al. 2021
Imidacloprid	Field-Cotton	Knapsack sprayed-Dose E	Soil extraction, LC-MS	Soil	0.87 d	0.797	First-order	Jie et al. 2021
Dimethoate	Field Trial-Tea	Knapsack sprayed	SPE Extraction, GC-FPD	Leaves	1.08 d	0.642	First-order	Pan et al. 2015
Dimethoate	Field-Mango	Sprayed	SPE extraction, LC-MS	Mango	2.0 d	0.347	First-order	Bhattcherjee et al. 2016
Dimethoate	Field-Mango	Sprayed-2x dose	SPE extraction, LC-MS	Mango	2.0 d	0.347	First-order	Bhattcherjee et al. 2016
Malathion	Field & Lab Storage conditions	Sprayed, storage degradation	Quechers extraction, LC-MS	Barley	5.8 d	0.119	2, first-orders phases	Kong et al. 2016
Malathion	Field & Lab Storage conditions	Sprayed, storage degradation	Quechers extraction, LC-MS	Barley	7.0 d	0.099	2, first-orders phases	Kong et al. 2016
Malathion	Lab-Model			Plant Material	2.48 d		Model 3	Fantke et al. 2014
Imidacloprid	Lab-Model			Plant Material	3.70 d		Model 3	Fantke et al. 2014

Dimethoate	Lab-Model			Plant Material	3.61 d		Model 3	Fantke et al. 2014
Malathion	Greenhouse-Beans/Cotton	Sprayed and spiked		Leaves	1.2-3.8 d			Katagi 2011
Dimethoate	Greenhouse-Beans	Sprayed and spiked		Leaves	1.7-4 d			Katagi 2011
Imidacloprid	Greenhouse-Tomato	Sprayed and spiked		Leaves	0.7-1.4 d			Scholtz et al. 1999
Imidacloprid	Field Trial-Chick Peas	Knapsack sprayed	SPE extraction, LC-MS	Chick Pea Pods	2.07 d	0.335	First-order	Chahil et al. 2014
Imidacloprid	Field Trial-Chick Peas	Knapsack sprayed-2x dose	SPE extraction, LC-MS	Chick Pea Pods	2.31 d	0.300	First-order	Chahil et al. 2014
Imidacloprid	Field Trial-Chick Peas	Knapsack sprayed	SPE extraction, LC-MS	Leaves	1.75 d	0.396	First-order	Chahil et al. 2014
Imidacloprid	Field Trial-Chick Peas	Knapsack sprayed-2x dose	SPE extraction, LC-MS	Leaves	1.72 d	0.403	First-order	Chahil et al. 2014
Imidacloprid	Field Trial-Woody plants	Soil Injection	SPE extraction, LC-MS	Soil	107-1250 d			Mach et al. 2018

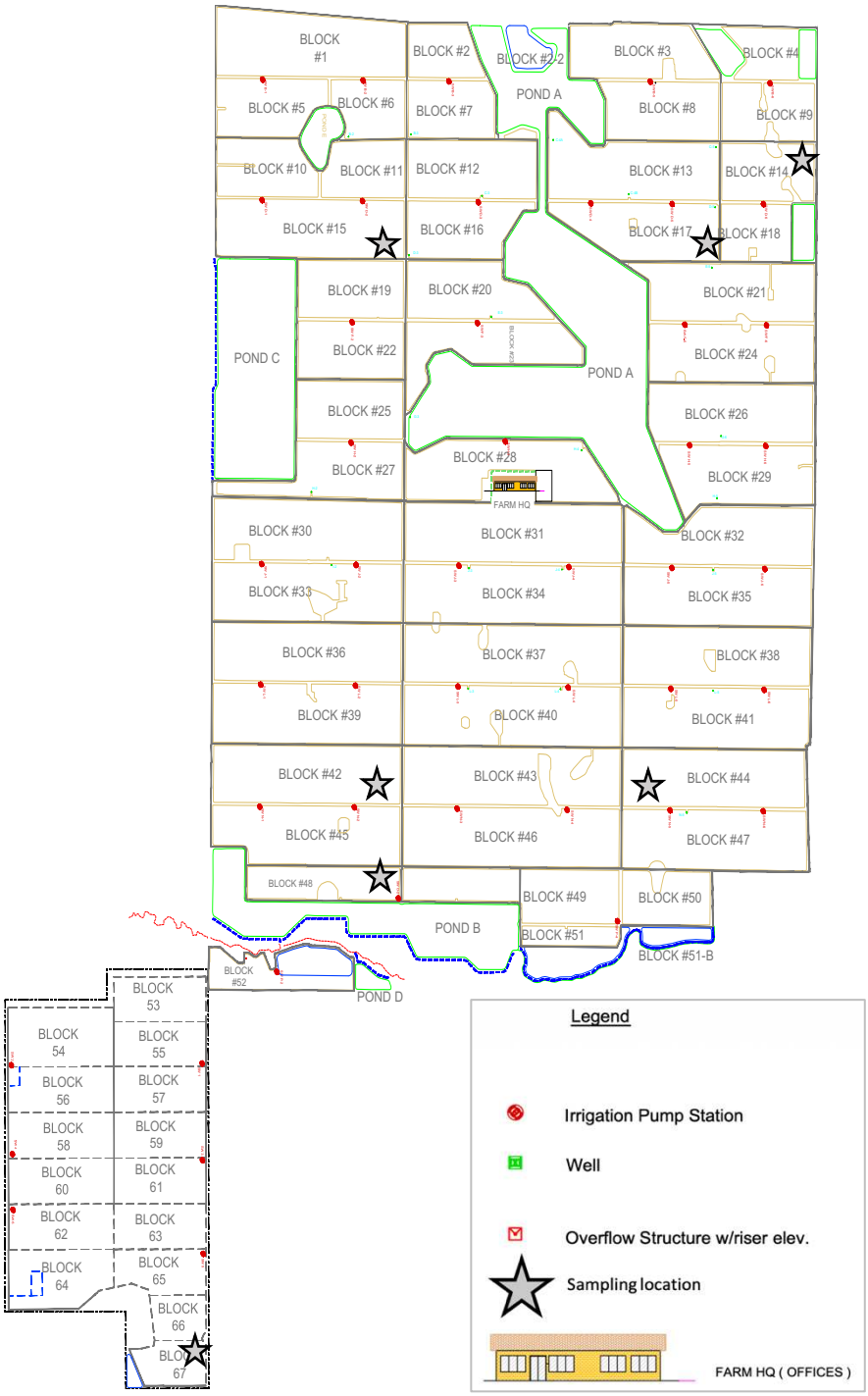

Figure B.1. Citrus grove field site and experimental locations for various block tests executed in field study #1 (FS-1) and #2 (FS-2). Stars represent block test sampling locations and correlate with Table S3 information.

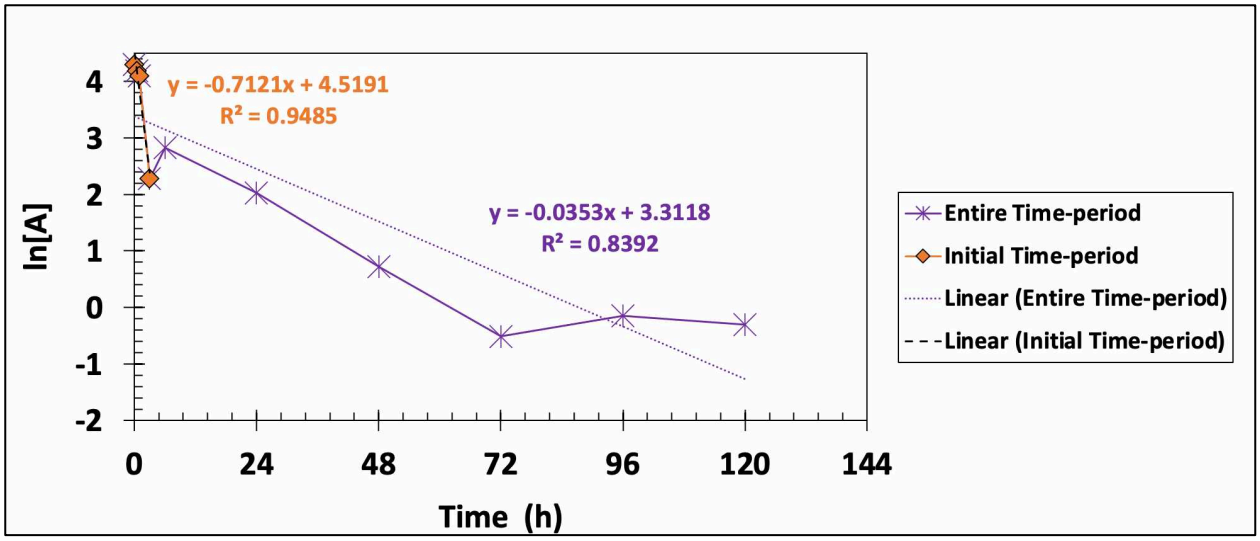

Figure B.2. Observed first-order dissipation kinetics of dimethoate over time. Residues were obtained from leaf samples after application to younger-aged trees with the ground side-sprayer. Linear regression trendlines were fitted to both the entire and initial time periods. The calculated half-lives were 20 h (entire) and 1 h (initial).

Table B.2. ACP adult and nymph counts from FS-1 (July) before and after imidacloprid application with the speed-sprayer to older citrus trees.

Adult psyllid counts pre-and post Imidacloprid application in older trees

Row	North		Middle		South	
	Pre	Post	Pre	Post	Pre	Post
1					1	
2	2		1	1	9	
3					1	
4	2					
5						
6						
7					8	
8	4				6	
9		1				
10					6	
11	5				1	
12	3				2	
13	1					1
14			1			
15	17			1	1	1
16	1		2		1	1
17	7				2	
18	1		2		2	
19						
20	6		3		2	
21			1		3	
22				1		5
23		1			2	1
24	6	2			1	
25					4	3
26	3	5	1		1	
27	2				6	
28	2		3		4	2
29	2		1		2	
30			3	2		1

Nymph psyllid counts pre-and post Imidacloprid application in older trees

Row	North		Middle		South	
	Pre	Post	Pre	Post	Pre	Post
1						
2					2	
3						
4						
5						
6		1				
7						
8	19					
9						
10						
11						
12						
13						
14						
15	6					
16	13					
17	4				18	
18						
19		12				
20	8	7				
21						
22						
23						
24						
25						
26						
27						
28	12					
29						
30						

Table B.3. ACP adult and nymph counts from FS-1 (July) before and after imidacloprid application with the side-sprayer to younger citrus trees.

Adult psyllid counts pre-and post Imidacloprid application in Younger trees

	North		Middle		South	
Row	Pre	Post	Pre	Post	Pre	Post
1					5	4
2	2	2	2		1	
3			1			
4	1		1		1	
5	2				4	2
6	5	2			1	
7						1
8	2	1				
9		1		1	5	1
10	2				2	2
11			2		2	
12	8	1			2	2
13						
14					1	
15	5	1			2	1
16			1			
17	1				1	
18					2	1
19	3	1	1	1		1
20	1	2	2		1	
21				1		
22	1		2			
23		1	1		1	
24	6	2	1	1		
25		1	1			
26	2	1		2		
27					4	
28			3	6	2	
29				2		1
30		2	6	3	1	1

Nymph psyllid counts before-and after-Imidacloprid application in younger trees

	North		Middle		South	
Row	Pre	Post	Pre	Post	Pre	Post
1						
2						
3						
4						
5						
6	8					
7						
8						
9						
10						
11						
12						
13						
14						
15						
16						
17						
18						
19						
20						
21						
22						
23						
24						
25						
26						
27						
28				9		
29						
30						

Table B.4. ACP adult and nymph counts from FS-1 (July) before and after aerial application of malathion to older citrus trees. The pilot only sprayed the trees in rows 1-15. Therefore, ACP data from rows 16-30 did not receive treatment.

Adult psyllid counts before-and after Malathion application in younger trees

	North		Middle		South	
Row	Pre	Post	Pre	Post	Pre	Post
1	5				7	
2			2		3	
3	3					
4						
5	4				3	
6	8				2	
7			1			
8			3		3	
9						
10					1	
11	13	1				
12	2				1	
13	4			1		
14					3	2
15	15	2				
16	3	6	1			
17					2	
18					1	
19			1	1	1	
20	2	1				
21		7				4
22		2	2	2		3
23	2					
24				2		
25	3	7			1	
26		1		1	2	
27	4	2	1		2	
28	1	1				
29	1	1				
30				1		

Nymph psyllid counts before-and after- Malathion application in younger trees

	North		Middle		South	
Row	Pre	Post	Pre	Post	Pre	Post
1						
2						
3						
4						
5						
6						
7						
8						
9						
10						
11						
12						
13						
14						
15						
16						
17						
18						
19						
20						
21						8
22						
23						
24						
25						
26						
27						
28						
29						
30						

Table B.5. ACP adult and nymph counts from FS-2 (October) before and after aerial Malathion application to older citrus trees. Very few ACP were counted in these trees prior to application and no ACP were found after application.

Adult psyllid counts before-and after-Malathion application in older trees

	North		Middle		South	
Row	Pre	Post	Pre	Post	Pre	Post
1	0	0	0	0	0	0
2	0	0	0	0	0	0
3	2	0	0	0	0	0
4	0	0	0	0	0	0
5	0	0	0	0	0	0
6	0	0	0	0	0	0
7	0	0	0	0	0	0
8	0	0	0	0	0	0
9	0	0	0	0	0	0
10	1	0	0	0	0	0
11	0	0	0	0	0	0
12	0	0	0	0	0	0
13	0	0	0	0	0	0
14	0	0	0	0	0	0
15	0	0	0	0	0	0
16	0	0	0	0	0	0
17	0	0	0	0	0	0
18	0	0	0	0	0	0
19	0	0	0	0	0	0
20	0	0	0	0	0	0
21	0	0	0	0	0	0
22	3	0	0	0	0	0
23	0	0	0	0	0	0
24	0	0	0	0	0	0
25	0	0	0	0	0	0
26	0	0	0	0	0	0
27	0	0	0	0	0	0
28	0	0	0	0	0	0
29	0	0	0	0	0	0
30	0	0	0	0	0	0

Nymph psyllid counts before-and after-Malathion application in older trees

	North		Middle		South	
Row	Pre	Post	Pre	Post	Pre	Post
1	0	0	0	0	0	0
2	0	0	0	0	0	0
3	0	0	0	0	0	0
4	0	0	0	0	0	0
5	0	0	0	0	0	0
6	0	0	0	0	0	0
7	0	0	0	0	0	0
8	0	0	0	0	0	0
9	0	0	0	0	0	0
10	0	0	0	0	0	0
11	0	0	0	0	0	0
12	0	0	0	0	0	0
13	0	0	0	0	0	0
14	0	0	0	0	0	0
15	0	0	0	0	0	0
16	0	0	0	0	0	0
17	0	0	0	0	0	0
18	0	0	0	0	0	0
19	0	0	0	0	0	0
20	0	0	0	0	0	0
21	0	0	0	0	0	0
22	0	0	0	0	0	0
23	0	0	0	0	0	0
24	0	0	0	0	0	0
25	0	0	0	0	0	0
26	0	0	0	0	0	0
27	0	0	0	0	13	0
28	0	0	0	0	0	0
29	0	0	0	0	0	0
30	0	0	0	0	0	0

Table B.6. ACP adult and nymph counts from FS-2 (October) before and after Dimethoate application with the speed-sprayer to older citrus trees.

Adult psyllid counts before and after Dimethoate application in older trees

	North			Middle			South		
Row	Pre	Post	Post-9	Pre	Post	Post-9	Pre	Post	Post-9
1	0	0	1	0	0	1	0	1	3
2	0	0	0	4	1	0	0		0
3	0	0	1	0	0	0	0	0	0
4	0	0	1	0	0	0	0	0	0
5	0	0	0	0	0	0	0	0	0
6	0	0	0	0	0	2	0	0	0
7	0	0	0	0	0	0	0	0	0
8	0	0	0	0	0	0	0	0	1
9	0	0	0	0	0	0	0	0	0
10	0	0	0	0	0	0	0	0	0
11	0	0	0	0	0	0	0	0	0
12	0	0	0	0	0	0	0	0	0
13	0	0	0	0	0	0	0	0	0
14	0	0	0	0	0	0	0	0	0
15	0	0	0	0	0	1	0	0	0
16	0	0	0	0	0	0	0	0	0
17	0	0	1	0	0	0	0	0	0
18	0	0	0	0	0	0	0	0	0
19	0	0	0	0	0	0	0	0	0
20	0	0	0	0	0	0	0	0	0
21	0	0	0	0	0	0	0	0	0
22	0	0	0	0	0	0	0	0	0
23	0	0	0	0	0	0	0	0	0
24	0	0	0	0	0	0	0	0	0
25	0	0	0	0	0	0	0	0	0
26	0	0	0	0	0	0	0	0	0
27	0	0	0	0	0	0	0	0	0
28	1	0	1	0	0	0	0	0	0
29	0	0	0	0	0	0	0	0	0
30	0	0	0	0	0	0	0	0	0

Nymph psyllid counts before and after Dimethoate application in older trees

	North			Middle			South		
Row	Pre	Post	Post-9	Pre	Post	Post-9	Pre	Post	Post-9
1	0	0	0	0	0	124	0	0	0
2	0	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0	0
4	0	0	0	0	0	0	0	0	0
5	0	0	0	0	0	0	0	0	0
6	0	0	0	0	0	0	0	0	0
7	0	0	0	0	0	0	0	0	0
8	0	0	0	0	0	0	0	0	0
9	0	0	0	0	0	0	0	0	0
10	0	0	0	0	0	0	0	0	0
11	0	0	0	0	0	0	0	0	0
12	0	0	0	0	0	0	0	0	0
13	0	0	0	0	0	0	0	0	0
14	0	0	0	0	0	0	0	0	0
15	0	0	0	0	0	8	0	0	0
16	0	0	0	0	0	0	0	0	0
17	0	0	23	0	0	0	0	0	0
18	0	0	0	0	0	0	0	0	0
19	0	0	0	0	0	0	0	0	0
20	0	0	0	0	0	0	0	0	0
21	0	0	0	0	0	0	0	0	0
22	0	0	0	0	0	0	0	0	0
23	0	0	0	0	0	0	0	0	0
24	0	0	0	0	0	0	0	0	0
25	0	0	0	0	0	0	0	0	0
26	0	0	0	0	0	0	0	0	0
27	0	0	0	0	0	0	0	0	0
28	0	0	14	0	0	0	0	0	0
29	0	0	0	0	0	0	0	0	0
30	0	0	0	0	0	0	0	0	0

Table B.7. ACP adult and nymph counts from FS-2 (October) before and after Dimethoate application with the side-sprayer to younger citrus trees.

Adult psyllid counts before and after Dimethoate application in younger trees

	North			Middle			South		
Row	Pre	Post	Post-9	Pre	Post	Post-9	Pre	Post	Post-9
1	16	4	1	0	0	0	0	0	0
2	0	0	1	0	0	0	1	0	0
3	0	0	0	1	0	1	0	0	0
4	19	0	2	0	0	0	0	0	0
5	1	0	1	0	0	0	0	1	1
6	3	0	0	1	0	0	0	0	0
7	5	0	5	0	0	0	0	0	1
8	2	0	3	0	0	0	0	0	0
9	3	0	1	0	0	0	0	0	0
10	24	17	6	0	0	0	3	0	0
11	1	0	6	0	0	0	0	0	0
12	1	0	0	0	0	0	0	0	0
13	3	0	12	0	0	0	0	0	2
14	7	0	2	1	0	1	2	0	3
15	15	1	5	2	0	0	1	0	0
16	1	0	2	2	0	0	3	0	0
17	0	0	0	0	0	0	2	0	0
18	0	0	0	0	0	1	0	0	0
19	2	0	3	0	0	1	0	1	1
20	7	0	4	3	0	0	0	0	1
21	0	0	2	0	0	0	0	0	1
22	0	1	0	0	0	0	0	0	3
23	5	0	0	0	0	4	0	0	0
24	4	0	2	0	0	0	0	0	0
25	6	0	3	6	0	0	0	0	0
26	0	0	1	0	0	0	1	0	0
27	0	0	0	0	0	0	3	0	1
28	2	0	1	0	0	2	0	0	2
29	0	0	0	1	0	1	0	0	2
30	0	0	1	0	0	0	0	0	0

Nymph psyllid counts before and after Dimethoate application in younger trees

	North			Middle			South		
Row	Pre	Post	Post-9	Pre	Post	Post-9	Pre	Post	Post-9
1	35	8	48	0	1	21	0	0	0
2	0	0	0	0	3	0	0	0	0
3	0	0	0	0	0	0	0	0	0
4	64	7	16	0	0	0	0	0	0
5	0	0	39	0	0	0	0	0	14
6	24	2	44	0	0	0	0	0	0
7	71	9	98	0	0	0	0	0	22
8	0	3	15	0	0	0	0	0	0
9	0	0	3	0	0	0	0	0	0
10	17	12	0	0	0	0	0	0	0
11	3	0	41	0	0	0	0	0	0
12	0	0	0	0	0	0	0	0	0
13	0	0	18	0	0	0	0	0	0
14	22	11	72	0	0	66	0	0	21
15	91	9	32	0	0	0	0	0	0
16	0	0	12	0	0	0	0	0	0
17	0	0	0	0	0	0	0	0	0
18	0	0	0	0	0	32	0	0	14
19	16	5	61	0	0	43	0	0	20
20	29	0	85	0	0	0	0	0	8
21	0	0	51	0	0	9	0	0	0
22	4	0	34	0	0	0	0	0	0
23	34	0	78	0	2	69	0	0	0
24	0	0	4	0	13	0	0	0	0
25	14	0	37	0	0	16	0	0	0
26	0	0	64	0	0	19	0	0	0
27	0	0	0	0	0	7	0	0	87
28	0	0	6	0	0	0	0	0	0
29	0	0	19	0	0	76	0	0	53
30	0	0	59	0	0	0	0	0	26

APPENDIX C: CO-AUTHOR CONTRIBUTIONS TO PESTICIDE DISTRIBUTION STUDY

Reprinted from Ruth F. Menger, Rachelle A. Rehberg, Pankaj Trivedi, Charles S. Henry, and Thomas Borch, 2022, High spatial resolution fluorescence imagery for optimized pest management in a Huanglongbing-infected citrus grove, Phytopathology, 112:173-179.

According to CRediT criteria, my co-author contributions to this work included conceptualization, methodology, investigation, data curation, writing-review and editing, visualization, and supervision.

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Disease Control and Integrated Management

e-Xtra*

High Spatial Resolution Fluorescence Imagery for Optimized Pest Management in a Huanglongbing-Infected Citrus Grove

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ABSTRACT

Huanglongbing (HLB), or citrus greening disease, has significantly decreased citrus production all over the world. The disease management currently depends on the efficient application and adequate distribution of insecticides to reduce the density of the disease vector, the Asian citrus psyllid. Here, we use a novel fluorescent-based method to evaluate insecticide distribution in an HLB-infected citrus grove in Florida. Specifically, we evaluated six different locations within citrus trees, the top and bottom sides of leaves, the effect of application approach (tractor versus airplane), and different application rates. We found that despite the insecticide distribution being highly variable among the different locations within a tree, the top of the leaves received an average increase of 21 times more than the bottom of the leaves. Application by tractor also resulted in a 4- to 87-fold increase in insecticide coverage compared with aerial application, depending on the location in the tree and side of the leaf. When taken to context with the type of insecticide that is applied (systemic vs. contact), these results can be used to optimize a pest management strategy to effectively target psyllids and other pests while minimizing the time and money spent on insecticide application and reducing risk to the environment.

Keywords: canopy penetration, citrus greening disease, contact, crop protection, huanglongbing, pesticide distribution, pest management, systemic

Crop protection depends on the efficient application of insecticide to control the insects or vectors that spread crop diseases. For example, the Asian citrus psyllid, *Diaphorina citri*, is the vector for the bacterial pathogen *Candidatus* Liberibacter spp., which causes huanglongbing (HLB). When the psyllids feed on the phloem sap, they infect the tree with *C.* Liberibacter, resulting in citrus trees with blotchy, mottled leaves, discolored fruit, and a weakened root system (Wang and Trivedi 2013). HLB has significantly affected productivity and fruit quality, with severe economic effects (Halbert and Manjunath 2004). In the United States, the production of oranges for processing decreased 72% between 2007 and 2018 due to HLB (Dala-Paula et al. 2019; USDA-NASS 2018). At present, there is not a "silver bullet cure" for HLB (Huang et al. 2021; Wang and Trivedi 2013; Yuan et al. 2021). Management and reduction of this disease requires a systemic approach, including destroying infected trees, using disease-free rootstock and scion grafts, and optimizing insecticide application (Gottwald et al. 2007; Martini et al. 2015; Quarles 2013).

The use of insecticides is one of the main management strategies to reduce the Asian citrus psyllid population and therefore the transmission of HLB (Gottwald 2010; Lopes et al. 2009). Because female psyllids prefer to lay their eggs on the underside of new flush, protecting the bottom of those young leaves on the outside of the tree is vital for preventing the spread of HLB (Halbert and Manjunath 2004). However, insecticide application is expensive, with yearly costs of >$1,000 per acre for just insecticides, compared with pre-HLB costs of $800 per acre for both insecticides and fertilizers (Farnsworth et al. 2014). Ensuring adequate and efficient distribution of insecticides within the citrus tree can improve psyllid management and reduce the cost and environmental impact of insecticide application (Uk and Courshee 1982; Wolf et al. 2000).

There are two types of insecticides, contact and systemic. Contact insecticides kill the insect by coming into contact with it and absorbing through the exoskeleton. It is important for the leaves to receive a good distribution of insecticide to ensure the highest probability of actually hitting the pest. Systemic insecticides are absorbed by the roots or leaves (depending on drench or foliar application) and translocate through the plant via the xylem and the phloem (Cloyd et al. 2011). It kills the pest when the pest ingests the phloem sap. Systemic insecticides can reach other areas of the tree and still be effective against pests if the area with the pest did not initially get covered with insecticide. Full coverage of the leaves is not as important for the systemic insecticide to be effective (Boina and Bloomquist 2015).

The spatial distribution of insecticides within crops has not been fully studied. Water-sensitive papers or Kromekote cards have been used to evaluate the effect of application rate, spray volume, droplet size, sprayer type, ground speed, and meteorological conditions on spray deposition on individual leaves (Bretthauer et al. 2008; Brusselman et al. 2012; Ferguson et al. 2020; Pergher and Gubiani 1995; Salyani and Hoffman 1996; Whitney et al. 1989). However, the spatial distribution within the entire crop canopy has not been evaluated in full. Onions, tomato, pepper, oat, wheat, and pineapple plants have been analyzed for insecticide canopy penetration, but these are all small plants with sparse plant material to be covered with insecticide (Ferguson et al. 2016; Foqué et al. 2012; Llop et al. 2015; MacIntyre-Allen et al. 2007; Olivet et al. 2011; Wang et al. 2020; Wolf et al. 2000). The results from these studies cannot be translated to larger orchard crops such as fruit trees because they have a larger and denser canopy that is more difficult to penetrate with insecticide. The canopy penetration in citrus trees specifically has been evaluated by a few studies, with the finding that the outer

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canopy has a higher spray deposition than the inner canopy (Farooq and Salyani 2002, 2004; Juste et al. 1990; Rehberg et al. 2021; Whitney et al. 1989). These studies applied metal or fluorescent tracers that were collected on the leaves or a cotton ribbon and then rinsed off for analysis (Juste et al. 1990; Whitney et al. 1989). However, this approach does not account for differences between the top and bottom of the leaf. For the management of HLB, differentiation between the side of the leaf is essential because psyllids are located primarily on the underside of the leaf (Farooq and Salyani 2002; Halbert and Manjunath 2004). Knowing exactly where the insecticide is distributed within the tree can inform better management practices for optimizing insecticide application and development of more efficient spraying technology. In addition, applications of nutrients, peptides, and other antimicrobials are becoming more common as promising methods to treat, slow down, or prevent HLB (Atta et al. 2021; Huang et al. 2021; Li et al. 2021). Because these treatments are applied to the tree by a spray, ensuring the best distribution method is important to make it most effective.

Here we use a novel method to evaluate the spatial distribution of pesticides in citrus trees in a citrus grove in Venus, Florida (Menger et al. 2020). In a large study with >1,000 samples collected, we show the heterogeneity of pesticide application within the trees based on canopy height, canopy depth, and side of leaf. We also show how changing the application rate resulted in altered pesticide distribution within the trees and on either side of the leaf. Finally, the results obtained by these studies provide conclusions that are used to inform best practices for applying systemic and contact pesticides in citrus trees. These best practices are recommended to properly target psyllids for the management of citrus greening disease but can also be applied to other insects and pests.

MATERIALS AND METHODS

Overview. A novel fluorescent-based method was used to evaluate the spatial distribution of insecticides (Menger et al. 2020). The method was previously developed and validated and is briefly described here (Supplementary Fig. S1C). Circles (diameter 47 mm) were cut out of Whatman filter paper no. 1 with an Epilog Zing CO_2 laser cutter. These filter circles (samplers) were clipped to the leaves of the citrus tree with mini binder clips (9/16") (Supplementary Fig. S1B). To measure the pesticide distribution, a red fluorescent dye (Risk Reactor IFWBC7) was added to the insecticide mixture, which was then sprayed onto the crop and filters via conventional sprayers. Samplers were collected within an hour of spraying to minimize photodegradation and stored in foil packets until analysis. Pictures of each sampler were taken with a lightbox and simplified computer with a camera (Raspberry Pi) (Supplementary Fig. S1A). The pictures were analyzed for percentage coverage with a custom Python script (Menger et al. 2020). Percentage coverage is defined as the percentage of a sampler that is covered by the dye–insecticide mixture.

Field study description. Samplers were collected during two field studies at a commercial citrus grove (Valencia oranges) in Venus, Florida, in October 2018 and April 2019. Meteorological data for each trip were provided by the grove. Pesticide application details are summarized in Supplementary Table S1. For all studies, the filter paper samplers were hung in citrus trees in various locations to test variables such as canopy height (upper, middle, lower), canopy depth (inner, outer), and side of leaf (top, bottom) (Fig. 1). For each of the six locations within the canopy (e.g., upper inner top), 20 filters were collected across five trees, for a total of 240 samplers for each sampling group. Field blanks (three per tree) were also collected to ensure that there were no interfering substances on the leaves before pesticide application. Four groups of trees were sampled to evaluate the effect of application approach (aerial, ground), and age of tree (young, old) on spatial distribution: aerial young, aerial old, ground young, ground old. All trees were sprayed aerially with an airplane equipped with 86 flat fan #15 nozzles (Fig. 1A). For ground application, there were two different sprayers. The young trees (1 year old, 1.1 × 1.4 m) were sprayed with a side sprayer (Newton Crounch). The horizontal nozzle boom has 2 TXR80049VK nozzles (Teejet) facing down, and each vertical boom has four TXR80017VK nozzles (Teejet) facing inward (Fig. 1C). The older trees (3 years old, 1.4 × 1.7 m) were sprayed with an airblast sprayer (FM Copling) with 18 D3-C25 nozzles (Albuz/Teejet) on each side (Fig. 1B).

Statistical analysis. Statistical analysis was performed in the JMP Pro 13 software package (SAS Institute Inc., Cary, NC). A level of $P < 0.05$ was used for statistical significance. The assumption of normality, homogeneity of variance, and linearity were checked with normal probability plots and residual plots, respectively. Due to violation of the normality assumption, a base-10 logarithm transformation was performed on percentage coverage. A mixed model was used to assess the significance (Student's t test, $P < 0.05$) of canopy depth, canopy height, side of leaf, and all two-way interactions (fixed effects) on percentage coverage within each application type, application rate, and tree age. A random effect was applied for the individual leaf within the tree to account for the samplers being attached to the top and bottom of the same leaf. Factorial ANOVAs and Tukey post hoc tests were done for each parameter combination within each sampling group to check for statistical significance. To compare the results from the October and April field studies at a certain location within the tree, Student's t test was used with significance at $P < 0.05$. Throughout all analyses, a random effect for leaf was included to account for measurements on both the top and bottom of each leaf.

RESULTS AND DISCUSSION

Overview. In this study, we evaluated the spatial distribution in different locations in citrus trees as well as on each side of the leaf. During the first field study in October 2018, four groups of trees were selected to evaluate the effect of application approach (aerial vs. ground) and tree age (young vs. old). Samplers were distributed throughout the tree as shown in Figure 1. At an initial glance, the application pattern between ground and aerial application is quite different (Fig. 1). The aerial application has individual and distinct droplets, whereas the ground application samplers are more evenly covered for both types of sprayers.

Percentage coverage is defined as the percentage of a sampler that is covered by the dye–insecticide mixture. In 78% of the samplers, the tops of the leaves received an average of 22 times more dye than the bottom of the leaves (Supplementary Table S2). In addition, trees with insecticide applied via tractors (ground application) had a significantly higher average percentage coverage compared with the aerial application (Supplementary Fig. S2), indicating that ground application results in more complete coverage of the leaves. The coverage on top of the leaves when sprayed by a tractor was 10 times higher than when sprayed by airplane, and the bottom of the leaves had 35 times higher coverage with tractor application (Supplementary Table S3). However, the implication that ground application is better than aerial application because it has a higher percentage coverage must be considered in context with the type of insecticide being applied (i.e., contact vs. systemic mode of action). Because contact insecticides must come into direct contact with the insect to kill it by absorbing through its skin, the small, distinct spots in Figure 2A are not ideal because the probability of hitting the insect with a small droplet is low. This method also leaves unprotected areas on the leaves for the insect to avoid the insecticide (Nansen et al. 2011). It could still be effective if the insect walks through the spot to encounter the insecticide; however, volatilization and degradation decrease the active concentration of insecticide over time (Bedos et al. 2002; Boina and Bloomquist 2015). Good coverage on the bottom of the leaf is important for contact insecticides to kill the Asian citrus psyllid because they reside and feed primarily on this side of the leaves (Halbert and Manjunath 2004). However, the presence of insecticide and uniform coverage on the bottom of the leaf are less

important for systemic insecticides because the insecticide will be absorbed into the leaf from both sides.

It is important to note that the variability of the field study data is quite high, and the error bars often overlap (Supplementary Figs. S3 and S5). This is not a result of the method used for determining the spatial distribution of pesticides. However, it does indicate the nonuniformity of insecticide application, even within each location in the tree, with percentage relative standard deviation ranging from 24 to 346% among the sampling groups (Supplementary Tables S5 and S6).

Aerial application. It is important to consider the distribution of insecticide within the citrus tree and how it is affected by the application approach and age of the tree. Figure 3 shows the percentage coverage for the top (orange) and bottom (blue) of the leaf at the six locations evaluated in each tree with pesticide application by airplane. The samplers on the tops of leaves did not yield a significant difference between any of the locations except for the inner lower location of the young trees. Equal coverage between all locations within the tree shows efficient application and is ideal for pest management to protect all parts of the tree. Systemic insecticides, such as imidacloprid, can take days to weeks for full uptake in mature citrus trees, so insecticide coverage on the inside of the tree as well as the outside is recommended for full protection (Grafton-Cardwell et al. 2008).

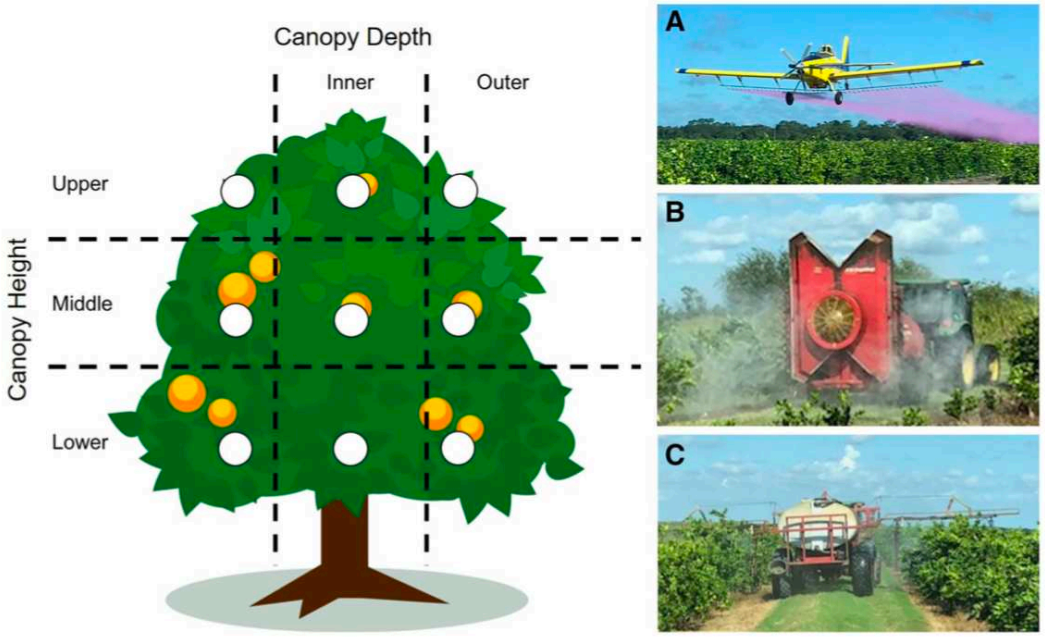

Fig. 1. (Left) Representative citrus tree to show sampler locations. Trees were divided to investigate canopy depth (inner and outer) and height (upper, middle, and lower). Each sampler was attached on the top and bottom of a leaf in each canopy height and depth section, along with each coordinate side of the tree (north, east, south, and west) for additional replicates. This scheme generated 48 samplers per tree. **A,** An airplane was used to apply pesticide to both young and old trees. **B,** An airblast sprayer was used to spray pesticide on older trees (>2 years old), and **C,** the side sprayer was used to spray young trees (<2 years old).

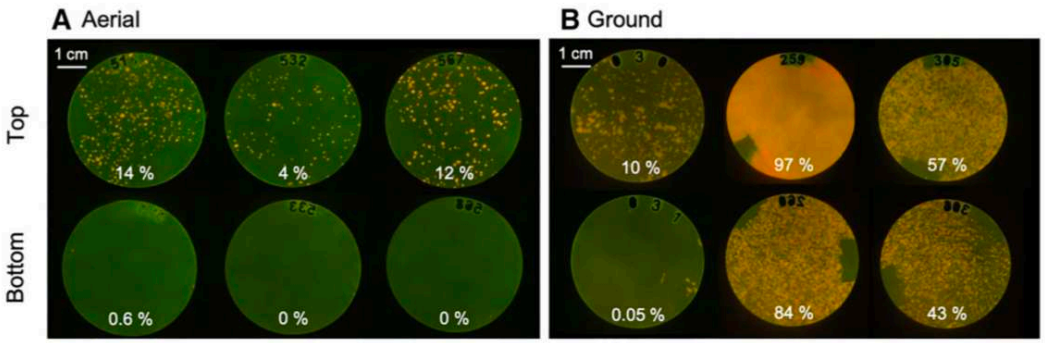

Fig. 2. Representative images of Whatman filter paper circles (samplers) collected from the field demonstrating the varying spray pattern for insecticide sprayed via **A,** aerial and **B,** ground application, as well as the difference between the top and bottom of the leaf. The percentage coverage (%) is labeled for each sampler.

As would be expected with application from above, the top of the leaves of the young trees received significantly more insecticide than the bottom in most cases, by a factor of 47 (Supplementary Figs. S3 and S4, Supplementary Table S2). For a systemic insecticide that absorbs into the leaf, higher coverage on the top of the leaf would suffice as long as the insecticide concentration inside the plant is high enough to kill the insect being targeted. The old trees were also sampled, but because of a miscommunication with the pilot, the trees were not sprayed properly. Therefore, those data are not presented.

Ground application. For ground application, two sprayer types were evaluated: a side sprayer for the young trees (1 year old) (Fig. 1C) and an airblast sprayer for the older (3 years old) trees (Fig. 1B). The two sprayers have different nozzle arrangements. The side sprayer has three panels of nozzles: a vertical panel on either side of the tree and a horizontal panel that passes over the tops of the trees. One would expect the outer and upper leaves of the young trees to get higher coverage than the inner, middle, and lower sections of the tree because of their closer proximity to the nozzles (Whitney et al. 1989). However, our findings show no statistical difference between the top of the leaves for the different locations, but the general trend does match what is expected based on the arrangement of the nozzles. The outer and upper regions of the tree received more pesticide (Fig. 4). Because of the horizontal panel of nozzles traveling above the trees, the tops of the leaves receive nine times more pesticide compared with the bottom, with significant differences at all locations except for the inner and outer upper locations (Supplementary Table S4, Fig. 4). Visually, the upper canopy is less dense than the rest of the tree, which means the samplers and leaves are not blocked by other leaves.

The airblast sprayer for the old trees has one vertical panel of nozzles that spray the insecticide with a high pressure and application rate (200 psi, 35 gallons per acre [GPA]), which is meant to increase the spray deposition to the inside of the tree (Farooq and Salyani 2002). Ideally, there would be no difference in canopy height (upper vs. middle vs. lower) because of the vertical panel or canopy depth (inner vs. outer); however, this is not the case. There is a significant difference between the outer upper and lower locations, the outer middle and lower, and the inner upper and lower locations (Fig. 4). This may be due to improper adjustment of the nozzle boom in relation to the canopy, where the end of the boom does not reach high or low enough. There is also a significant difference between the outer and inner canopy at the middle and lower locations, with the outer canopy receiving on average 8.8 times more dye–insecticide mixture (Supplementary Table S4). It has always been a challenge to reach the inside canopy of an orchard tree (Farooq and Salyani 2002, 2004; Salyani et al. 2007). Our results show that an application rate of 35 GPA does not provide insecticide coverage on the inside of the tree. The force of spray is also meant to agitate the leaves enough so that more pesticide gets on the bottom of the leaves, but there is a significant difference between the top and the bottom of the leaves at each location (Supplementary Fig. S5). The tops of the leaves receive 8.9 times as much dye–insecticide mixture, indicating that pesticide application is not as uniform as it was thought to be (Supplementary Table S2).

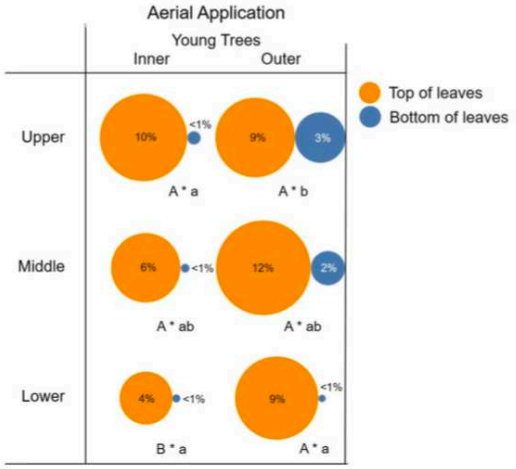

Fig. 3. Bubble plot showing the percent area coverage of samplers with insecticide applied aerially to young trees (1 year old). Samplers are grouped by canopy depth (outer, inner) and canopy height (upper, middle, lower). The left bubble (orange) of the pair is the top of the leaf, and the right bubble (blue) is the bottom of the leaf. The size of the bubbles corresponds to percentage coverage, with the text in each bubble showing the average of 20 filter samplers. Letters indicate significant difference: Bubbles connected by different letters are significantly different from one another, and bubbles with the same letter are not significantly different (Tukey's honestly significant difference test, $P < 0.05$). Capital letters are for the top of the leaf, and lowercase letters are for the bottom. Stars indicate significant differences between the top and bottom sides of the leaf at that location ($P < 0.05$).

With both sprayers, the outer canopy receives more insecticide than the inner canopy, by a factor of 8.8 for the old trees (airblast sprayer) and 3.0 for the young trees (side sprayer) (Supplementary Table S4). Depending on where the insect prefers to reside, higher coverage on the outer canopy may suffice instead of having equal coverage throughout the tree. To target a specific insect, their movement within the crop should be used to inform the best application. For example, the psyllids that carry *Candidatus* Liberibacter asiaticus prefer the underside of the new flush, which is on the outer part of the tree, but they can still move to the inside of the tree (Sétamou and Bartels 2015). An application approach that effectively covers the bottom of the leaves is necessary for a contact insecticide, whereas adequate coverage on the top of the leaves would suffice for a systemic insecticide. However, although "adequate" coverage has been recommended as 15% coverage (Deveau 2016), the concentration of the pesticide still needs to be high enough to kill the pest being targeted. Based on preliminary results, there is a significant correlation ($P = 2.2e\text{-}16$, Supplementary Fig. S6) between pesticide concentration and percentage coverage (Rehberg et al. 2021). Spraying the tree with a low pesticide concentration but high spray coverage is ineffective because the pesticide concentration is probably not high enough to be toxic to the insect, leading to pesticide resistance (Rehberg et al. 2021). The trees probably need to be sprayed again, increasing the time and money associated with pest management (Nansen et al. 2015).

Impact of changing spraying settings. To evaluate the impact of changing the application rate on pesticide distribution, a second field study was performed in April 2019. The nozzle pressure was increased or decreased (thus changing the application rate), and all other sampling parameters remained the same. For the young trees (1 year old), the application rate was decreased from 35 to 20 GPA, resulting in a 2.3-fold decrease in coverage for the top of the leaves and a 5.8-fold decrease for the bottoms (Fig. 5A, Supplementary Table S5). There was a significant decrease in coverage on both the tops and bottoms of the leaves at most locations as a result of the decreased application rate (Supplementary Fig. S7A).

For the old trees (3 years old), the application rate was increased from 50 to 90 GPA. This increased application rate resulted in higher coverage on both the top and bottom of the leaves (Fig. 5B). Most notable is the statistically significant increase on the bottom of the leaves at each location (Supplementary Fig. S7B). The coverage on the bottom of the leaves increased by a factor of 12, most likely due to increased agitation of the leaves, whereas the tops of the leaves increased only by a factor of 1.6 (Supplementary Table S6). The tops of the leaves at the inner lower and middle locations also had a significant increase in coverage (Supplementary Fig. S6B).

Overall, the percentage coverage of pesticide on the leaves improved with this higher application rate. Although increasing the

application rate may seem like an obvious choice to improve insecticide application, this higher application rate with the same insecticide concentration cannot be used for every application because the insecticide concentration would reach the limits set by the Environmental Protection Agency. These regulations are in place to reduce health risks to humans and the environment (Federal Insecticide, Fungicide, and Rodenticide Act 1996). In addition, intensive insecticide application can lead to insecticide resistance and have negative impacts on other insects that naturally help reduce the psyllid population (Tiwari et al. 2011). A higher application rate could be used with a lower insecticide concentration (in the tank mixture) to ensure that the bottom of the leaves and the inside of the leaves are targeted, but the insecticide concentration on the leaf might not be high enough to kill the psyllids. In addition, a higher application rate results in smaller droplets that are more susceptible to drift, resulting in more insecticide loss to the environment (Nansen et al. 2015).

Conclusions and implications. In this study, we have demonstrated how application rate and type affect the pesticide distribution within a citrus tree. Application by airplane resulted in significantly higher coverage on the tops of the leaves. The young trees were sprayed with a side sprayer, which resulted in higher pesticide coverage on the upper and outer parts of the tree and higher coverage on the top of the leaves. When the application rate was decreased from 35 to 20 GPA, overall coverage decreased. The older trees were sprayed with an airblast sprayer and a higher application rate (50 GPA). Although there was still more coverage on the top of the leaves, the difference was not as great compared with application by the airplane or side sprayer. When the application rate was increased from 50 to 90 GPA, coverage on the bottom of the leaves significantly increased.

The results of a systematic study of pesticide application should be used to evaluate the effectiveness of current spraying techniques to make improvements given the pest to be targeted and the pesticide being applied (Fig. 6). For long-term protection of a large grove, a systemic insecticide applied by airplane is recommended. Although it may take a few weeks for the insecticide to be fully taken up by a tree, it offers weeks to months of protection (Grafton-Cardwell et al. 2008). With a systemic insecticide, coverage on the bottom of the leaves and inner part of the tree is less critical, so the time saved by aerial application is more beneficial. When psyllids are present and a quick knockdown of the psyllid population is needed, the areas of high psyllid population, such as the borders of the grove and on the new flush (Halbert and Manjunath 2004; Sétamou and Bartels 2015), can be sprayed with a high application rate and high insecticide concentration (Rehberg et al. 2021). For routine maintenance, contact insecticides should be applied on a rotating basis via tractor-based approaches to ensure high coverage within the entire tree and both sides of the leaf. High coverage will increase the probability of directly hitting the insect with pesticide. Care should be taken to rotate between different classes of insecticides to reduce the progression of resistance.

With regard to the management of HLB, insecticides are currently the primary management technique to reduce the psyllid population. Other treatment methods such as the application of nutrients, peptides, and other antimicrobials are also becoming common as part of a diversified management plan. As with the insecticide application, the distribution of foliar spray is an important consideration for maximum efficacy, increasing the implications for this study. It should also be noted that this study focused on newer and small citrus trees (<3 years old). Additional studies should be performed to confirm the results on larger, denser, and more mature citrus trees (≥6 years old). Although this study used the management of HLB in citrus trees as a case study, the fluorescent-based filter paper method can be used in any crop to evaluate the efficiency of any pest management approach.

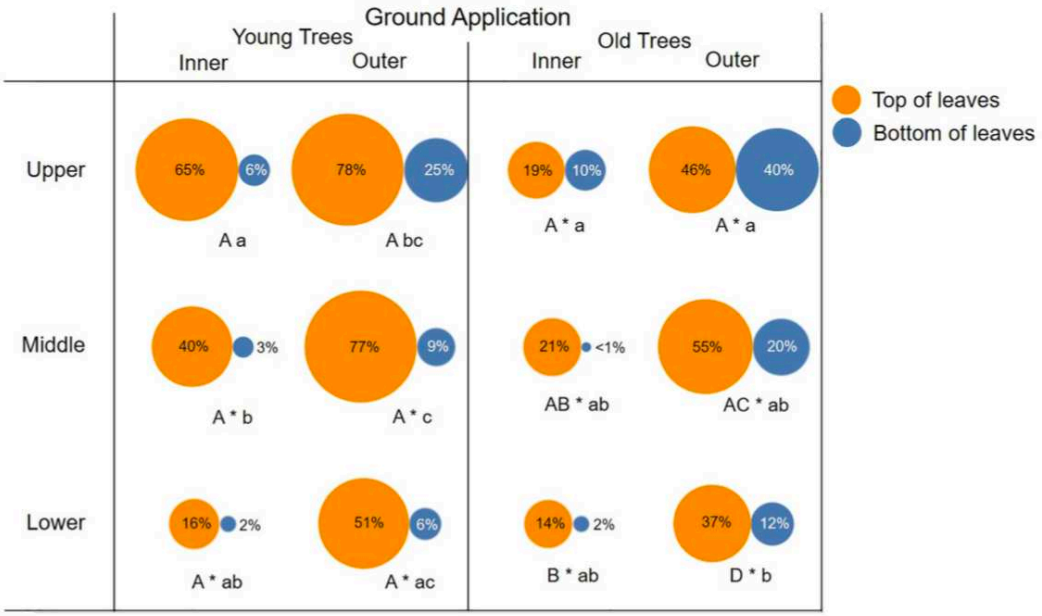

Fig. 4. Bubble plot showing the percent area coverage of samplers with insecticide applied via tractors (ground application). Samplers are grouped by tree age (young, <1 year old; old, 3 years old), then by canopy depth (outer, inner) and canopy height (upper, middle, lower). The left bubble (orange) of the pair is the top of the leaf, and the right bubble (blue) is the bottom of the leaf. The size of the bubbles corresponds to percentage coverage, with the text in each bubble showing the average of 20 samplers. Letters indicate significant differences: Bubbles with different letters are significantly different from one another, and bubbles with the same letter are not significantly different (Tukey's honestly significant difference test, $P < 0.05$). Capital letters are for the top of the leaf, and lowercase letters are for the bottom. Stars indicate a significant difference between the top and bottom sides of the leaf at that location ($P < 0.05$).

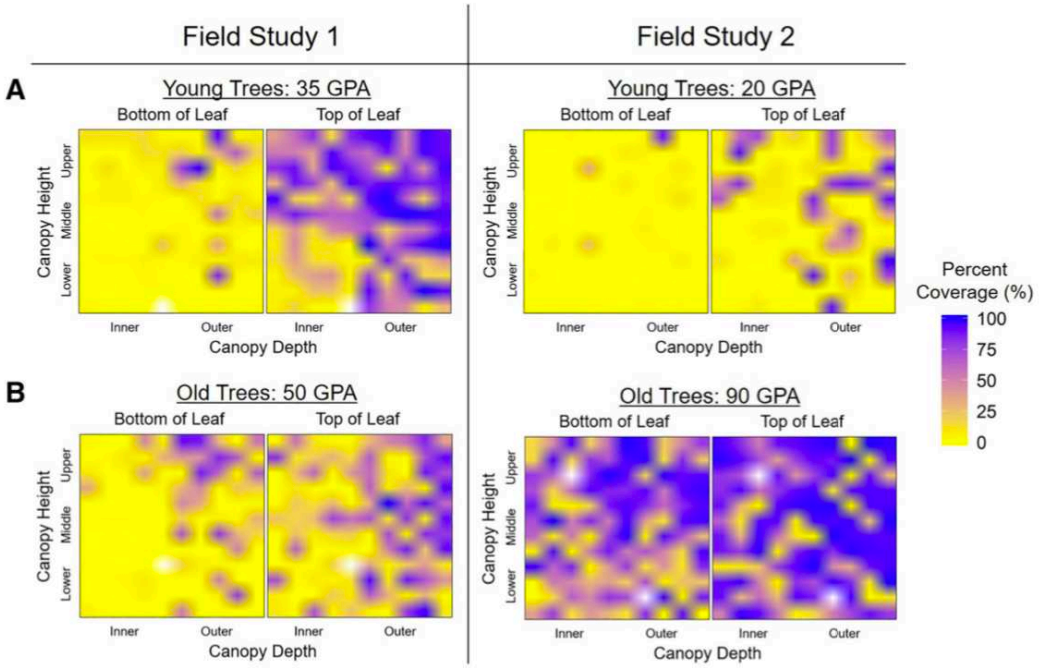

Fig. 5. Comparison of field studies 1 (October 2018) and 2 (April 2019) in **A,** young and **B,** old trees. For the young trees (1 year old), the application rate was decreased from 35 to 20 gallons per acre (GPA) during field study 2. For the old trees (3 years old), the application rate was increased from 50 to 90 GPA during field study 2. Each two-dimensional density plot is representative of half a tree, divided into the six sampling locations. Each cell corresponds to one sampler, with a total of 20 samplers per location. The percentage coverage is represented by a color scale, from 0% (yellow) to 100% coverage (blue). The white cells are missing samplers.

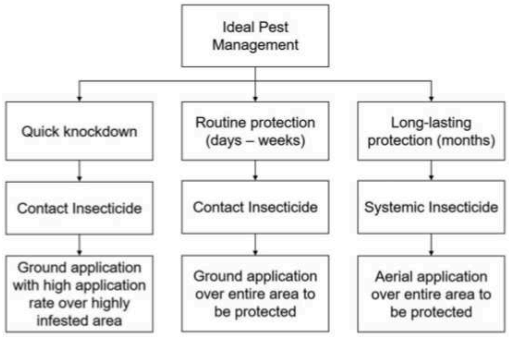

Fig. 6. Decision tree for ideal pest management based on desired pesticide protection.

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APPENDIX D: CO-AUTHOR CONTRIBUTIONS TO WHEAT PLANT UPTAKE STUDY

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According to CRediT criteria, my co-author contributions to this work included methodology, validation, formal analysis, investigation, and writing-review and editing.

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Irrigation of wheat with select hydraulic fracturing chemicals: Evaluating plant uptake and growth impacts☆

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ABSTRACT

Oilfield flowback and produced water (FPW) is a waste stream that may offer an alternative source of water for multiple beneficial uses. One practice gaining interest in several semi-arid states is the reuse of FPW for agricultural irrigation. However, it is unknown if the reuse of FPW on edible crops could increase health risks from ingestion of exposed food, or impact crop growth. A greenhouse experiment was conducted using wheat (*Triticum aestivum*) to investigate the uptake potential of select hydraulic fracturing additives known to be associated with health risks. The selected chemicals included acrylamide, didecyldimethylammonium chloride (DDAC), diethanolamine, and tetramethylammonium chloride (TMAC). Mature wheat grain was extracted and analyzed by liquid chromatography-triple quadrupole mass spectrometry (LC-QQQ) to quantify chemical uptake. Plant development observations were also documented to evaluate impacts of the chemicals on crop yield. Analytical results indicated that TMAC and diethanolamine had significantly higher uptake into both wheat grain and stems than control plants which were not exposed to the four chemicals under investigation. Acrylamide was measured in statistically higher concentrations in the stems only, while DDAC was not detected in grain or stems. Growth impacts included lodging in treated wheat plants due to increased stem height and grain weight, potentially resulting from increased nitrogen application. While analytical results show that uptake of select hydraulic fracturing chemicals in wheat grain and stems is measurable, reuse of FPW for irrigation in real world scenarios would likely result in less uptake because water would be subject to natural degradation, and often treatment and dilution practices. Nonetheless, based on the outstanding data gaps associated with this research topic, chemical specific treatment and regulatory safeguards are still recommended.

1. Introduction

Water volumes used in hydraulic fracturing vary widely across the United States. Directional drilling operations in the West and Permian Basin use less than one million gallons per well while horizontal drilling operations in the East can use over six million gallons per well (Kondash and Vengosh, 2015). After well stimulation, 10–70% of the original injected volume flows back to the surface (API, 2010). In addition to flowback water, native formation produced water also surfaces over the production life of the well. The combination of flowback and produced water (FPW) surfacing during the first 5–10 years of production can reach over 3.7 million gallons per well (Kondash et al., 2017).

All surfacing FPW requires either disposal or treatment and reuse, posing a significant water management challenge. Water management options include injecting FPW into the subsurface through Underground Injection Control (UIC) wells, reuse for oil and gas operations, and beneficial reuse for activities such as dust suppression, industrial power generation, and agricultural irrigation (Veil et al., 2004). If the FPW stays in the surface water cycle instead of undergoing subsurface injection, it could bolster

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freshwater resources (Gleick, 1993). Due to droughts in Western states, one surface water reuse option gaining attention in recent years is the application of FPW for agricultural irrigation (Dolan et al., 2018; Echchelh et al., 2018; GWPC, 2019; Miller et al., 2019; Miller et al., 2020; Scanlon et al., 2020; Sedlacko et al., 2019).

FPW, however, can contain high salinity, measured by total dissolved solids (TDS), total organic carbon (TOC), natural constituents such as metals and radionuclides, and additive chemicals from oilfield processes which may need to be treated and/or diluted before the water is viable for beneficial reuses such as agricultural irrigation (Elsner and Hoelzer, 2016; GWPC, 2019; Kahrilas et al., 2015; Kahrilas et al., 2016; McDevitt et al., 2019; McLaughlin et al., 2020a; McLaughlin et al., 2020b). In general, FPW reused for agricultural irrigation at minimum undergoes natural dilution and degradation, and likely some form of treatment, before application. Natural dilution occurs as injected hydraulic fracturing fluid is mixed with formation water throughout the course of the operation. Initially, when fluid returns to the surface as flowback water, additive chemicals and their degradants are present in measurable amounts. As the operation progresses, native formation produced water returns to the surface in larger proportions than flowback water (Oetjen et al., 2018), diluting the additive chemical concentrations.

Peer reviewed literature suggests that concentrations of TDS and TOC in irrigation water should remain under 3500 mg/L and 5 mg/L respectively to maintain biomass yield and plant health (Pica et al., 2017; Sedlacko et al., 2019). Due to the variety of natural formation properties across the United States, TDS can range from less than 1000 to over 400,000 mg/L. Lower TDS concentrations of less than 50,000 mg/L are present west of Kansas (Otton and Mercier, 1995), the same area of the United States using irrigated agriculture due to reduced rainfall (USGS, 2015). While irrigating crops with water above current salinity guidelines can cause reduced yield and germination issues (Kondash et al., 2020), and high treatment costs can make reuse uneconomical, several locations in California and Wyoming have naturally low TDS produced water. As a result, select locations in California have successfully produced crops using FPW with minimal TDS treatment (CVRWQCB, 2019). Since some projects have targeted low-TDS FPW to grow agricultural crops, the research presented here specifically focuses on the remaining data gaps surrounding the uptake of and growth impacts from additive chemicals.

At the time of the experiment, limited data on FPW chemical concentrations were published and minimal public data on additive chemicals in oil recovery processes, other than hydraulic fracturing, were available. For this reason, the experiment was designed using the median hydraulic fracturing fluid concentrations reported in FracFocus (U.S.EPA, 2015a) to show a worst-case scenario situation where irrigation was a result of a long-term spill. Stock chemical solutions were also stored at colder temperatures to prevent degradation to reflect a potential ongoing spill. The high concentration/low degradation design was employed as a proof of concept to verify whether the plants were capable of taking up the chemicals in measurable concentrations. Since chemicals used in hydraulic fracturing are also used in enhanced oil recovery (EOR) processes such as waterflooding and steamflooding (Taylor et al., 2014), we believe that this research can be applicable to other FPW reuse operations depending on the operator selected chemical constituents.

The experiment tested the hypotheses that 1) uptake concentrations in wheat plants would not be statistically different between the control and treated plants, 2) uptake concentrations would not be present at elevated health risk levels after irrigation at worst-case scenario concentrations, and 3) applied chemicals would not impact crop growth. If experiment results failed to reject these null hypotheses, then actual irrigation practices which use more diluted and degraded FPW chemical concentrations would not pose a risk to human health based on available health hazard assessment procedures or impact the growth of wheat cultivated in this experiment.

Experimental results were additionally used to identify other hydraulic fracturing chemicals with uptake potential and propose treatments to reduce the concentrations of the selected hydraulic fracturing chemicals in reused water. Since the presented research focused on a small subset of the additive chemicals used in oil and gas operations in low salinity waters and does not represent the true complexity of fluids that would be reused, the additional evaluations were performed to help guide existing reuse of FPW for agriculture irrigation and suggest topics and chemicals of interest for future research inquiries.

2. Materials and methods

2.1. Hydraulic fracturing chemical selection

First, we identified hydraulic fracturing chemicals of interest with a four-round elimination process. The first round of elimination focused on data availability; we removed chemicals lacking a CAS number, toxicity data, or physiochemical data (Long et al., 2015). Solid proppants were also removed. The second round only retained chemicals with significant oral toxicity values. Thresholds included LD_{50} values of $\leq$500 mg/kg, National Fire Protection Association (NFPA) or Hazardous Materials Identification System (HMIS) health hazard ratings of 3 or 4 which represent serious or severe hazards, chronic HMIS value of 1 representing chronic health risk, an acute toxicity Globally Harmonized System (GHS) value of <4 representing an oral LD_{50} value of <300 mg/kg, or cell mutagenicity or reproductive GHS values of 1 or 2 indicating confirmed or suspected adverse effects (Long et al., 2015; TOXNET, 2014). Additionally, suspected or confirmed carcinogens and endocrine disruptors were retained in this round even if other acute toxicity thresholds were not met.

The third round of elimination focused on physiochemical properties. Literature review indicated that constituents with log octanol-water partition coefficient (K_{OW}) values below 5 were more able to cross the Casparian strip, a waxy barrier around endodermis root cells that forces water traveling through a root to enter the cell through the lipid bilayer membrane before passing into the xylem. (Dettenmaier et al., 2009; Miller et al., 2016; Tao et al., 2009). While higher K_{OW} values were documented to have significantly reduced uptake, the threshold was set at 5 to be initially inclusive of chemical properties associated with at least minimal uptake. Due to the execution of the experiment in a greenhouse, we excluded volatile chemicals by only retaining chemicals with Henry's Constant (K_H) values less than 10^{-7} atm-m^3/mol at 25 °C (Watts, 1998). Inorganic acids and bases such as potassium hydroxide and hydrochloric acid were assumed to be neutralized during the hydraulic fracturing process and were also eliminated in this phase (U.S. EPA, 2004). The last elimination round focused on prevalence of use. At least one third of 21 states reporting to the FracFocus database mined for this experiment had to report using the chemical for it to be retained in this round.

The final chemicals selected were acrylamide (residual in friction reducers and flocculants; polyacrylamide degradation product), didecyldimethylammonium chloride (DDAC; biocide), diethanolamine (surfactant and corrosion inhibitor), tetramethylammonium chloride (TMAC; biocide), and tetrasodium ethylenediaminetetraacetic acid (EDTA; corrosion inhibitor and metal chelator). EDTA was included in the treatment water to evaluate its role in metal uptake for a concurrent experiment (Shariq, 2019).

The addition of EDTA was not expected to have a significant impact on the uptake of organic constituents.

In 2015, the U.S. EPA compiled maximum concentrations in fracturing fluid (% by mass) for all states reporting to the FracFocus database (U.S. EPA, 2015a). The chemical concentrations used for this experiment were calculated using the median value reported by the U.S. EPA for all oil and gas operations. For acrylamide, the concentration also accounted for the residual monomer in polyacrylamide by adding 0.05% of the polyacrylamide concentration to acrylamide median as reported by the U.S. EPA (Lentz et al., 2008). Final concentrations used for irrigation were: acrylamide (1.2 mg/L), DDAC (30 mg/L), diethanolamine (37 mg/L), TMAC (694 mg/L), and EDTA (37 mg/L). Both FracFocus and the U.S. EPA report the maximum concentration in fracturing fluid (% by mass) as well as the maximum concentration in the additive (% by mass). An independent investigation conducted comparing original chemical usage logs with those published on FracFocus revealed that the value reported on FracFocus as the maximum concentration in fracturing fluid already accounted for the maximum concentration in the additive (Chesapeake Energy, 2011). Thus, the maximum concentration in fracturing fluid was used for the calculation.

2.2. Crop determination

When evaluating crops to use for the experiment, staple crops were targeted because they are not easily removed from the diet and have high consumption rates. Thus, even low uptake concentrations could still impact human health. Wheat was selected because it was shown in peer reviewed articles to uptake selected organic chemicals (Collins and Willey, 2009), it was the most consumed crop by humans and livestock in 2008 (Pimentel and Pimentel, 2008), it grows well in greenhouses, and it has been used in a series of recent studies (Miller et al., 2019, 2020; Sedlacko et al., 2019; Shariq, 2019).

2.3. Experimental design

Sample size selection was based on Pearson and Hartley power function charts (Pearson and Hartley, 1951), and the calculation of a critical value from previous experimental data (Tao et al., 2009). The final layout of the experimental units was in a completely randomized design containing three treatments with eight replications each.

The experiment was conducted at the University of California, Davis (UC Davis) Orchard Park greenhouse facility. The planting method was designed to allow for uniform seed distribution across experimental units. Initially, 0.016 m^3 of UC Davis "Ron's Mix" soil was used to fill each experimental unit. Ron's Mix soil is composed of 1 part coarse sand, 1 part compost (redwood shavings and turkey manure), 1 part peat moss, and 3 pounds/yard of Dolomite. Additional soil quality data are presented in Supplemental Information Fig. S1. Seeds were then planted using a template 1.5 inches apart and 2 inches deep. A total of 59 seeds were planted per experimental unit with the objective of aggregating grain from the mature wheat plants for analytical analysis at the end of the experiment on day 76.

Irrigation began immediately after planting. Each pot was irrigated with 1.2 L of control or chemically amended water 2 times a week for the first 3 weeks then 3 times for the remaining 7.5 weeks for a total of 29 irrigation applications. Irrigation water was applied via treatment specific watering cans directly to the soil of each experimental unit. A total of 34.8 L of treatment water, containing a cumulative 41.76 mg of acrylamide, 1.044 g of DDAC, 1.288 g of diethanolamine, and 24.15 g of TMAC, was applied to each pot over the course of the experiment.

The experiment was divided into three irrigation sources, the control, Treatment 1, and Treatment 2. The control water consisted of reverse osmosis (RO) fertilized water. The selection of RO water was made in order to isolate the growth impacts and uptake potential of the additive chemicals from miscellaneous constituents in the background water. The water was produced from an Applied Membranes RO system and the fertilizer consisted of Growmore 4-18-38, CALCINIT (a calcium nitrate amendment), and magnesium sulfate. Fertilizer was introduced with Dosatron injectors to yield an N-P-K concentration of 100-200-100 mg/L. Treatment 1 irrigation water consisted of RO fertilized water (control water) amended in the laboratory with all the selected chemicals of interest. Treatment 2 water consisted of RO fertilized water (control water) amended in the laboratory with all the chemicals of concern except EDTA.

Stock solutions were created for each experimental chemical, stored at 4 °C for the duration of the experiment, and diluted in RO fertilized water before each irrigation event to achieve the median concentrations identified in hydraulic fracturing fluid. Acrylamide (Product Number (PN) A4058), diethanolamine (PN 31589), TMAC (PN T3411), and EDTA (PN 03699) were purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA). DDAC (PN 98484) was purchased from Matrix Scientific (Columbia, SC, USA). The acrylamide stock solution was created at 1 g/L, DDAC and diethanolamine at 30 g/L, TMAC at 548 g/L, and EDTA at 1 g/L.

Wheat plants were sampled at maturity, 76 days after planting. Stems were cut at their base from each pot separately. Grains from each experimental unit were extracted by gloved hand from the stems. Stems and grain were then separately packaged in two layers of aluminum foil and two Ziploc bag before storing at −20 °C until analysis.

2.4. Analytical methods

2.4.1. Extraction procedure

Prior to extraction, wheat kernels and stems were freeze-dried and ground in a wheat mill. The mill was vacuumed, wiped and rinsed with methanol between each sample to prevent contamination. For each plant, three kernel extracts and three stem extracts were made. Prior to analysis, equal volumes of the three kernel extracts were combined to create a composite kernel sample for each plant. Similarly, equal volumes of the stem extracts were combined to create a composite stem sample. Homogenized samples were weighed (100 mg) into centrifuge tubes and 750 μL of cold (4 °C) 95% methanol/5% water (HPLC grade) was added to each tube. Samples were vortexed for 1 h, sonicated for 20 min and vortexed a second time for 20 min, all while maintained at 4 °C. Next, samples were centrifuged at 4 °C for 15 min at 3000 rpm. A 500 μL portion of each liquid extract was then removed and added to a new centrifuge tube. Samples were dried under a gentle stream of nitrogen and then reconstituted with 100 μL of 95% methanol. Reconstituted samples were shaken by hand and replicate samples were composited as described above. Samples were transferred to autosampler vials prior to analysis, stored at 4 °C and analyzed within 48 h of extraction. In addition to the samples collected from the experiment, a pre-experiment kernel (PEK) was run through the extraction procedure. This was a kernel from the seed packet that the planted kernels were from, however, this kernel was never planted. Finally, extraction blanks that contained 750 μL solvent and no wheat were run through this extraction procedure.

2.4.2. Liquid chromatography analysis

Extracts were analyzed using an Agilent 1290 Infinity Series liquid chromatograph coupled with an Agilent Jet Stream electrospray ionization source (ESI) and an Agilent 6460 triple quadrupole

mass spectrometer (LC-QQQ). Analysis was performed using a Zorbax Eclipse C18 column (150 mm × 4.6 mm, 3.5 μm particle size). To analyze extracts for TMAC, diethanolamine and acrylamide, mobile phases were A (1% formic acid) and B (methanol). A 4-min isocratic method was developed with 95% A and 5% B at a flow rate of 0.6 mL/min. Injection volume was 2 μL. A separate LC-QQQ method was developed for DDAC with mobile phases A (0.1% formic acid) and C (acetonitrile + 0.1% formic acid). A 5-min isocratic method was developed with 80% A and 20% C. Flow rate was 0.6 mL/min and injection volume was 20 μL. The following operational parameters were used for both methods: gas temperature, 330 °C; gas flow, 10 L/min; nebulizer pressure, 45 psi; sheath gas temperature, 350 °C; sheath gas flow, 11 L/min; capillary voltage, 3500 V; and cell acceleration voltage, 7 V. MassHunter Optimizer was used to optimize parameters for detection of each compound, which are provided in Supplemental Information Table S1. Quantification was conducted using TMAC, diethanolamine, acrylamide and DDAC standards. A solvent blank (95% methanol/5% water) was also run between each sample. In general, only one blank was needed, however, additional blanks were run in some cases (e.g. after higher concentration samples) until the concentration of all chemicals was below limit of quantification (LOQ; See Table S2).

3. Results

3.1. Plant uptake of hydraulic fracturing chemicals

The concentrations of TMAC, diethanolamine, acrylamide, and DDAC in treated and control wheat stems and grain were quantified by LC-QQQ. Analytical results indicated that all chemicals except DDAC were taken up into treated plants at concentrations above limit of quantification (Table S2). One-way Analysis of Variance (ANOVA) evaluations and Tukey mean separation tests were performed at a significance level of 0.05 for each set of analytical results to distinguish treatment effects. In the figures presented, Tukey test results are indicated by letters "a" and "b". Treatment means with the same letter are not statistically different from each other while means with different letters are significantly different. Additional details on the statistical analysis process and assumption tests are available in the Supplemental Information. Analytical and statistical results are displayed in Table 1 and Fig. 1 with significant p-values indicated by an asterisk.

Both TMAC and diethanolamine were detected in the treated grains and stems but absent from the controls. As a result, the differences in TMAC and diethanolamine concentrations between the treated and control samples were significant ($p < 0.05$). Concentrations of TMAC in both grain and stems were higher than concentrations of both diethanolamine and acrylamide, by at least three orders of magnitude. Tukey test results for TMAC in stems and grain, diethanolamine in stems and grain, and acrylamide in stems indicate that mean concentrations in Treatment 1 and Treatment 2 were not significantly different from each other, but both mean treatment concentrations were significantly different from the mean control concentration.

In contrast to TMAC and diethanolamine, acrylamide was detected in both the treated and control grains and stems. Acrylamide concentrations in treated stems were significantly higher than control stems ($p < 0.05$), but acrylamide concentrations in treated grain were not significantly higher than control grain ($p > 0.05$). Acrylamide was also detected in the PEK but was not detected in the solvent blanks or extraction blanks.

3.2. Impacts on plant growth

Plants irrigated with hydraulic fracturing chemical-spiked water showed accelerated emergence of the grain bearing portion (head), increased stalk height, heavier grain yield, and increased lodging (i.e., the bending of stems near the soil making the plant unable to stand upright). On day 39 of the experiment, the treated plants began the head emergence growth stage approximately 5 days earlier than the control plants (Fig. 2a). Emerged heads were counted in each experimental unit and ANOVA results indicate that treated plants had significantly more emerged heads than control plants ($p < 0.05$). On day 45, plant stalk heights were measured. The ANOVA performed on stalk heights indicate that treated plants were significantly taller than control plants ($p < 0.05$) (Fig. 2b and Fig. S2). Grain yields were weighed after harvest by determining the average grams per stalk for each treatment. ANOVA results indicate that grain yield per stalk from plants irrigated with treated water was significantly greater than control plants ($p < 0.05$) (Fig. 2c).

Tukey test results for emerged heads, stalk height, and grain weight indicate that mean concentrations in Treatment 1 and Treatment 2 were not significantly different from each other, but both mean treatment concentrations were significantly different from the mean control concentration. The combination of increased stem height, heavier grain weight, and accelerated growth, leaving stems thinner and weaker, likely contributed to the noticeable lodging in treated plants (Fig. S2).

3.3. Physiochemical property comparison

A list of hydraulic fracturing chemicals with similar physiochemical properties to the two chemicals taken up into wheat grain was created to identify compounds that may share similar plant uptake potential. Physiochemical properties of TMAC and diethanolamine were characterized with respect to molecular

Table 1
Summary table displaying the analytical results and statistical evaluation of acrylamide, diethanolamine, and TMAC uptake in wheat grain and stems.

Chemical	Treatment	Count	Grain (mg/kg)			Stems (mg/kg)		
			Average	Std Dev	ANOVA p-value	Average	Std Dev	ANOVA p-value
TMAC	Control	8	0	0		0	0	
	Treatment 1	8	120	20.0	2.44E-14[b]	745	169	1.28E-11[b]
	Treatment 2	8	126	14.7		675	102	
Diethanolamine	Control	7[a]	0	0		0	0	
	Treatment 1	8	0.039	0.008	2.38E-09[b]	0.052	0.011	1.69E-09[b]
	Treatment 2	8	0.04	0.012		0.043	0.011	
Acrylamide	Control	7[a]	0.286	0.051		0.617	0.149	
	Treatment 1	8	0.351	0.103	0.122	2.24	0.464	1.71E-08[b]
	Treatment 2	8	0.356	0.062		1.90	0.273	

[a] One stem analytical result was out of calibration range and therefore not included in statistical analysis.
[b] Significant p-values.

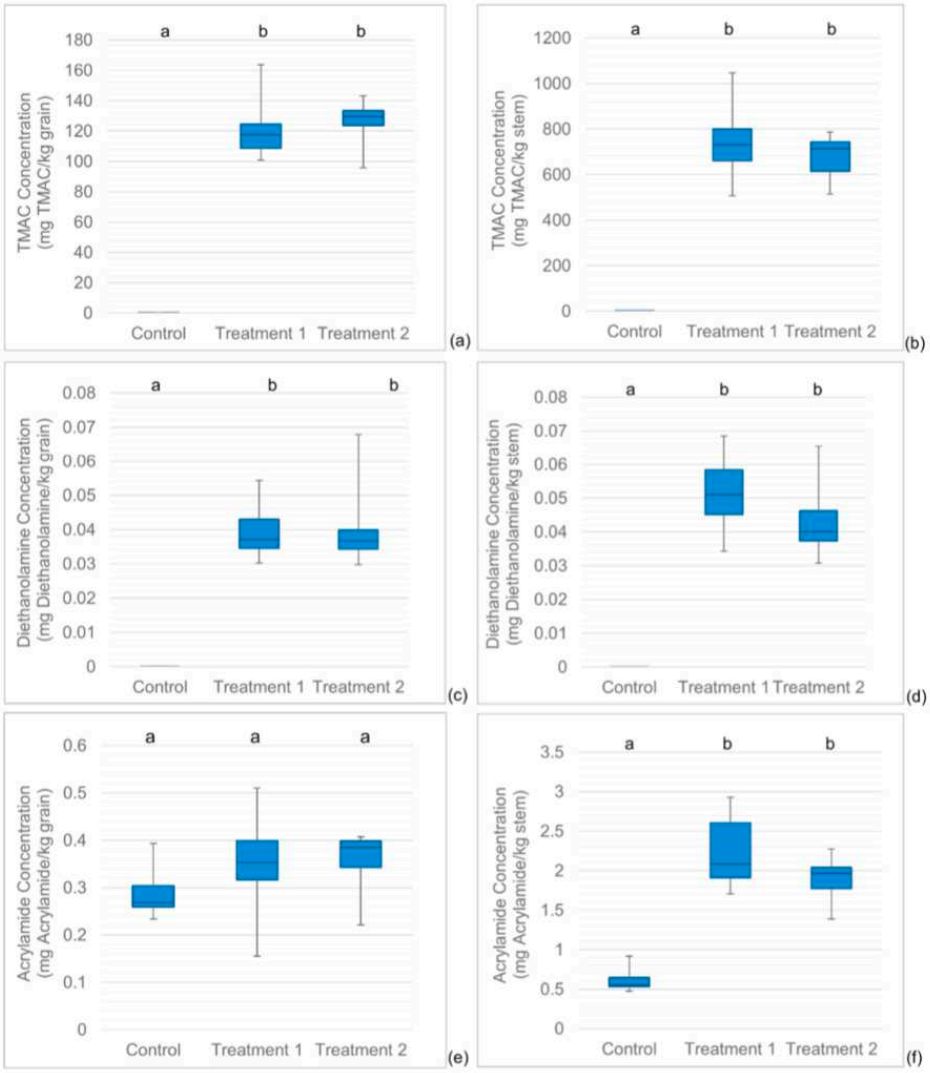

Fig. 1. Distribution of uptake concentrations in control and treated wheat plants for (a) TMAC in grain, (b) TMAC in stems, (c) diethanolamine in grain, (d) diethanolamine in stems, (e) acrylamide in grain, and (f) acrylamide in stems. Letters indicate the results of the Tukey pairwise comparison test run at a 0.05 significance level.

weight, water solubility, K_{OW}, organic carbon-water partition coefficient (K_{OC}), and K_H (U.S. EPA, 2013), then compared to the 517 hydraulic fracturing chemicals documented by the U.S. EPA (U.S. EPA, 2015b). Both TMAC and diethanolamine fall into the same physiochemical categories described in the U.S. EPA's Interpretive Assistance Document of Assessment of Discrete Organic Chemicals (2013). The parameter limits of these categories were applied as filters to the U.S. EPA list of hydraulic fracturing chemicals and 31 chemicals with similar weight, solubility, hydrophilicity, sorption, and volatility properties were identified (Table 2).

4. Discussion

4.1. Evaluation of hypotheses

The first hypothesis proposed that uptake concentrations of the selected hydraulic fracturing additives would not be statistically different between the control and treated plants. Based on the statistically significant increase of TMAC and diethanolamine in wheat stems and grain, and acrylamide in wheat stems, this hypothesis can be rejected for acrylamide, diethanolamine, and TMAC, but cannot be rejected for DDAC.

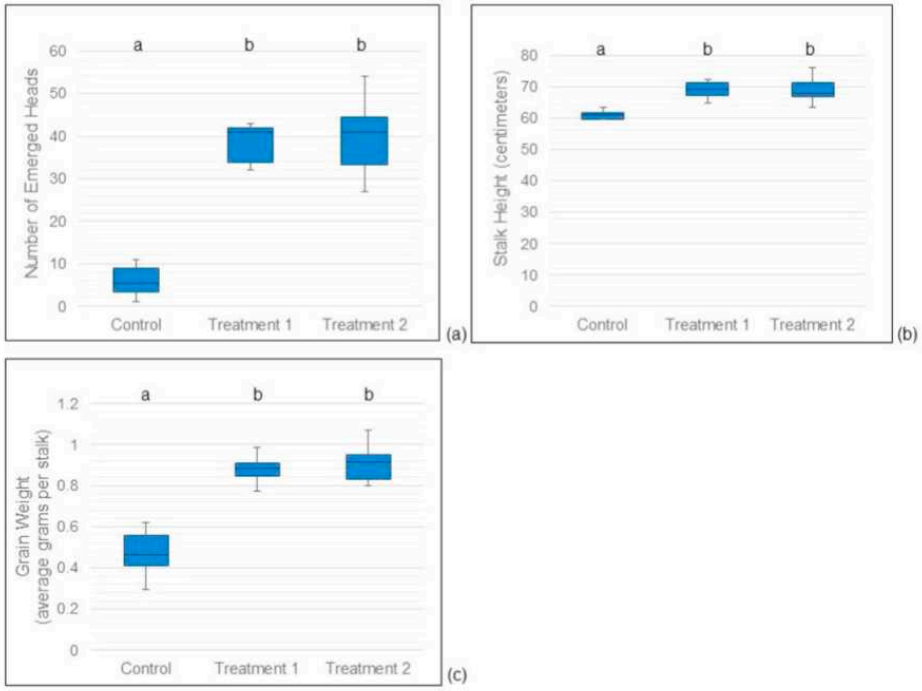

Fig. 2. Distribution of (a) average emerged heads counted on day 39, (b) average stalk height measured on day 45, and (c) average grain yield measured on day 76. All treatment groups were statistically different from the control.

The second hypothesis proposed that uptake concentrations of the selected hydraulic fracturing additives in wheat grain would not be present at toxic levels after irrigation at worst-case scenario concentrations. Results from non-carcinogenic health risk assessments (U.S. EPA, 1989) indicate that the second hypothesis cannot be rejected for all chemicals except for TMAC. At application concentrations of 694 mg/L per single irrigation event, elevated risk levels are associated with the consumption of TMAC in treated grain in adults and children. Based on these results, TMAC is a good candidate to target for quantification in FPW so that a concentration more representative of irrigation water can be evaluated, as well as potential synergistic effects.

The third hypothesis proposed that applied chemicals would not impact crop growth. Observational results of experimental plant growth indicated that the third hypothesis can be rejected. The number of emerged heads, stalk height, and grain weight were all significantly higher in treated plants compared to control plants. Additionally, lodging only occurred in treated plants. Application of the selected chemicals at worst case scenario concentrations therefore did impact crop growth.

4.2. Chemical uptake

Of all the chemicals applied throughout this study, TMAC was taken up into the stems and grain at the highest concentrations. This might be expected due to the high application concentration. However, TMAC is ionizable, which makes predicting its behavior in the environment more complex. TMAC was originally selected for the experiment in part because it has a low log K_{OW} of −4.18, suggesting it would be hydrophilic and transport with the irrigation water into the plant bypassing soil sorption (Dettenmaier et al., 2009). Yet since TMAC is usually ionized at neutral pH values between 5 and 9 (TOXNET, 2014), it was likely present as a TMA cation and chloride anion in the approximately pH 7.5 irrigation water. Cation sorption to soil occurs due to the negative charges of soil organic matter and clay, decreasing its availability for plant uptake (Sparks, 2003); however in this experiment, the statistically significant uptake of TMA into the wheat plant indicates that sorption of TMA to the soil did not entirely prevent its uptake into plants.

Acrylamide results were also of interest due to the occurrence of quantifiable acrylamide in the control grain and stems. While it is clear that acrylamide contamination occurred at some step in this experiment, we do not believe it affected TMAC or diethanolamine results because acrylamide was not detected in the solvent blanks or extraction blanks, and therefore the contamination was not from the lab. Acrylamide was detected in the PEK on the same order of magnitude as that detected in the treated kernels and control stems (Table S2). The PEK detection indicates that acrylamide contamination may have occurred prior to the start of the experiment, potentially from the paper packaging in which the seeds were shipped (Yang et al., 2014).

4.3. Growth impact observations

Growth impacts from the application of acrylamide, DDAC, diethanolamine, and TMAC included accelerated plant maturity, increased height and grain weight, and significant lodging compared to control plants. While the control plants were irrigated

Table 2
Hydraulic fracturing chemical additives with similar physiochemical properties to TMAC and diethanolamine.

Chemical Name	CASRN	Molecular Weight (g/mol)	Water Solubility (mg/L at 25 °C)	Log K_{OW}	Log K_{OC} (log L/kg)	Henry's Law Constant (atm-m^3/mole at 25 °C)
Diethanolamine	111-42-2	105.1356	1.00E+06	−1.71	0	3.92E-11
Tetramethylammonium chloride	75-57-0	109.5978	1.00E+06	−4.18	0.791	4.17E-12
1-Amino-2-propanol	78-96-6	75.1097	1.00E+06	−1.19	0.253	4.88E-10
1-Methoxy-2-propanol	107-98-2	90.121	1.00E+06	−0.49	0	5.56E-08
2-(Hydroxymethylamino)ethanol	34375-28-5	91.1091	1.00E+06	−1.53	0.3	1.62E-12
2-Amino-2-methylpropan-1-ol	124-68-5	89.1362	1.00E+06	−0.74	0.404	6.48E-10
2-Aminoethanol hydrochloride	2002-24-6	97.544	1.00E+06	−1.61	0.067	3.68E-10
2-Ethoxyethanol	110-80-5	90.121	7.55E+05	−0.42	0	5.56E-08
2-Methoxyethanol	109-86-4	76.0944	1.00E+06	−0.91	0	4.19E-08
3-(Dimethylamino)propylamine	109-55-7	102.1781	1.00E+06	−0.45	1.454	6.62E-09
3-Hydroxybutanal	107-89-1	88.1051	1.00E+06	−0.72	0	4.37E-09
Acetic acid, mercapto-, monoammonium salt	5421-46-5	109.1475	2.56E+05	0.03	0.158	1.94E-08
Acrylamide	79-06-1	71.0779	5.04E+05	−0.81	0.755	5.90E-09
Ammonium hydrogen carbonate	1066-33-7	79.0553	8.42E+05	−0.46	0	6.05E-09
Diethylene glycol	111-46-6	106.1204	1.00E+06	−1.47	0	2.03E-09
Dimethylaminoethanol	108-01-0	89.1362	1.00E+06	−0.94	0.088	1.77E-09
Ethanolamine	141-43-5	61.0831	1.00E+06	−1.61	0.067	3.68E-10
Ethylenediamine	107-15-3	60.0983	1.00E+06	−1.62	1.172	1.03E-09
Formamide	75-12-7	45.0406	1.00E+06	−1.61	0	1.53E-08
Glycerol	56-81-5	92.0938	1.00E+06	−1.65	0	6.35E-09
Glycolic acid	79-14-1	76.0514	1.00E+06	−1.07	0	8.54E-08
Glycolic acid sodium salt	2836-32-0	98.0332	1.00E+06	−1.07	0	8.54E-08
Glyoxylic acid	298-12-4	74.0355	1.00E+06	−1.4	0	2.98E-09
Methoxyacetic acid	625-45-6	90.0779	1.00E+06	−0.68	0	4.54E-08
N,N-Dimethylformamide	68-12-2	73.0938	9.78E+05	−0.93	0	7.38E-08
N,N-Dimethyl-methanamine-N-oxide	1184-78-7	75.1097	1.00E+06	−3.02	1.004	3.81E-15
N-Methyl-2-pyrrolidone	872-50-4	99.1311	2.48E+05	−0.11	0.869	3.16E-08
N-Methylethanolamine	109-83-1	75.1097	1.00E+06	−1.15	0.115	8.07E-10
Sodium bicarbonate	144-55-8	84.0066	8.42E+05	−0.46	0	6.05E-09
Sodium carbonate	497-19-8	105.9884	8.42E+05	−0.46	0	6.05E-09
Thioglycolic acid	68-11-1	92.117	2.56E+05	0.03	0.158	1.94E-08
Trimethanolamine	14002-32-5	107.1085	1.00E+06	−3.95	0	1.42E-08
Urea	57-13-6	60.0553	4.26E+05	−1.56	0.499	3.65E-10

with fertilizer that had an estimated total nitrogen content of 3.48 g per pot over the course of the 29 irrigations, the nitrogen content of the additive organic chemicals resulted in an additional 3.40 g per pot over the duration of the experiment. Most of this nitrogen was added by the TMAC which accounted for 3.09 g. Excessive nitrogen fertilization has been documented to cause such impacts as those observed in the experimental wheat plants (Crook and Ennos, 1995).

While increased grain weight may appeal to growers, lodging impacts can decrease yield by up to 40% by impacting grain formation, lengthening and reducing the efficiency of harvesting efforts, and inducing molding (Brook, 2001). Because of these potential repercussions, excess nitrogen application may present an unanticipated challenge that farmers and regulators should consider when evaluating the suitability of FPW water for reuse. Potential mitigation measures may include testing FPW irrigation water pre-application for nitrogen and adjusting applied fertilizer to balance the nutrient ratio or decreasing routine nitrogen fertilization to account for the additional amount in the irrigation water.

4.4. Wastewater treatment options

Literature review indicated that RO and nanofiltration (NF) treatment options are particularly well suited for the chemicals of concern. DOW Chemical FilmTec™ RO membranes can remove sub-100 amu particles, making the treatment feasible for the 105.14 and 109.60 amu weights of diethanolamine and TMAC respectively (Dow Chemical, 2019). A diethanolamine-specific research study reiterated that greater than 96.5% rejection can be achieved through RO (Seyoum et al., 2012). While initial evaluations of NF indicated both TMAC and diethanolamine are too small for complete removal by its 300−400 amu target size range (Roth et al., 2014), the Long Beach Water Department in California demonstrated up to 90% removal of aqueous salts for particles as small as 60 amu with NF (Roth et al., 2014). This treatment option could therefore be applied for partial removal of the 74.14 amu TMA cation.

The RO or NF treatment steps are predominantly used towards the end of a treatment train necessary to remove suspended solids, larger organic constituents, and some dissolved organics which can increase membrane fouling (Chang et al., 2019). Treatment train designs vary based on the influent water characteristics and effluent water quality goals, but may include steps such as dissolved air flotation or coagulation/flocculation to remove suspended solids, and biologically activated carbon filtration, granular activated carbon adsorption, or microfiltration to reduce additional suspended solids, dissolved organics, and large organic constituents before RO or NF targets dissolved organic and inorganic compounds (GWPC, 2019). Osmotic membrane bioreactors (OMBR), which combine the processes of forward osmosis with

activated sludge, have also been shown to provide high level pretreatment with lower capital and energy costs than traditional trains (Achilli et al., 2009; Coday et al., 2014).

Singular treatment options such as dissolved air floatation or adsorption are unlikely to remove diethanolamine and the intact TMAC compound due to their physiochemical properties (Camarillo et al., 2016). Dissolved air floatation works best on chemicals with $K_H > 1 \times 10^{-3}$, however TMAC and diethanolamine have K_H values less than 1×10^{-10} and are therefore unlikely volatilize. Adsorption removal by activated carbon and media such as clay works best for chemicals that havGo to page 10 MAC and diethanolamine have log K_{OC} values less than 1, they are not good candidates for this type of treatment. When ionized, however, the TMA cation is more likely to sorb to negative media such as clay, organic matter, and ion exchange resins. Graphene oxide adsorbent, for example, was demonstrated to removed up to 94% TMA in a study setting (Chang et al., 2014), and strong cation exchange resins were demonstrated to remove 89.4% of TMA from aqueous solution (Shibata et al., 2006). Based on these studies, adsorption treatments may be viable for TMAC when present as the TMA ion.

The cost of RO treatment varies depending on the treatment plant capacity, level of pretreatment required, and energy prices. Operational costs for small (50 m^3/day capacity) RO treatment plants with NF pretreatment run approximately \$3.7/m^3 (Muraleedaaran et al., 2009), or approximately \$1.3/m^3 for a 100 m^3/day capacity plant without NF (Meng et al., 2016). Operation costs can reduce down to \$0.31/m^3 for larger treatment plants with capacities greater than 20,000 m^3/day (Sauvet-Goichon, 2007). Small scale RO treatment costs are higher than other membrane technologies such as a microbial capacitive desalination/deionization cell which runs \$0.63/m^3, or thermally-driven membrane distillation with heat integration which costs \$0.74/m^3, but lower than microfiltration, membrane distillation without heat integration, and ion exchange which cost \$4.8/m^3, \$5.7/m^3, and \$13.6/m^3 respectively (Chang et al., 2019). Since calculating costs for users such as water districts, industry, or farmers can be complicated, the National Alliance for Water Innovation (NAWI) is developing Water-TAP3, a tool that can be used to estimate treatment costs for future projects (NAWI, 2020).

While RO and NF treatment trains can be more costly than some alternative treatments, reuse of FPW for agriculture is unique in that it is indirectly associated with human ingestion. Therefore, FPW that is specifically destined for agricultural reuse should be run through pretreatment options such as RO or NF treatment trains, or new technologies that demonstrate sufficient chemical of interest removal, to improve water quality and protect human health.

4.5. Application of experimental results

This experiment used median concentrations calculated in hydraulic fracturing fluid for irrigation to test the uptake potential of selected chemicals. In practice, however, these organic chemicals are subject to degradation and sorption (King, 2012), and reused water may also be subject to water treatment (CVRWQCB, 2019; GWPC, 2019). All of these processes would likely reduce the concentrations of additive chemicals in FPW.

One example of a reuse operation currently treating FPW for agricultural irrigation is located in Bakersfield, California (Kondash et al., 2020). In the Cawelo Water District Chevron reuse operation, FPW is treated with mechanical separation, sedimentation, WEMCO™ air flotation units, and walnut hull filtration (CVRWQCB, 2012) before blending with surface and groundwater at variable ratios based on seasonal volumetric needs. In the first half of 2019, Wood Environment & Infrastructure Solutions, Inc. (Wood) reported blending ratios of FPW to surface and groundwater ranged from undiluted in January to 1:4.25 in June (Wood, 2019b; 2019c). 2018 ratios indicate a decrease in freshwater contribution after June (Wood, 2018; 2019a). Based on these ratios, treated FPW accounts for 23.5–100% of the water applied to agricultural fields depending on the time of year.

As research in this field progresses, the list of 31 chemicals with similar physiochemical properties to diethanolamine and TMAC can be used to guide future crop uptake experiments, and a catalog of maximum recommended concentrations can be developed. Reuse operations can then apply these recommendations to refine their testing, treatment, and dilution practices. This practice could help reassure consumers that select oilfield additive chemicals of concern are being evaluated and treated to consider safety concerns.

4.6. Policy recommendations

While this study concludes that acrylamide, DDAC, and diethanolamine would not pose a risk to human health through consumption of exposed crops at worst case scenario concentrations, and TMAC would need to be diluted or treated from median hydraulic fracturing fluid concentrations before it is safe for reuse, additional data gaps still exist preventing comprehensive health risk evaluations of FPW reuse for agricultural irrigation. Data gaps include the 76% of hydraulic fracturing associated chemicals still lacking toxicological information (Elliott et al., 2017), the lack of analytical methods for several additive chemicals (Stringfellow et al., 2015), an incomplete understanding of degradation byproducts, the fate and transport of additive chemicals in the environment and within the crops themselves, the high variability of natural constituents, and the unknown cumulative impacts of chemicals and degradants present in reused water. The additive impacts of several diluted chemicals could pose a cumulative risk greater than any of the individual chemicals alone. Given these significant data gaps, it is difficult to develop beneficial and enforceable regulations able to holistically ensure the safe reuse of FPW for agricultural irrigation (Danforth et al., 2019).

The traditional experimental approach to addressing existing data gaps can be a costly and lengthy undertaking. Alternatively, stakeholders could work towards implementing effect-based testing such as the whole effluent toxicity (WET) approach (U.S. EPA, 2000), or developing a list of chemical additives that pose little or no risk (PLONOR) to human health when reused for agriculture. With the WET approach, water is not tested for specific chemicals, but rather evaluated with respect to its toxicity on organisms in the receiving environment. This approach has been applied specifically to aquatic organisms but would need to be adapted to terrestrial environments if it were to apply to FWP reuse in an agricultural setting (Danforth et al., 2019; GWPC, 2019). With the PLONOR approach, operators planning to reuse FPW for crop irrigation specifically could then select their chemical additives from this list for a streamlined approval process. A similar approach was developed by OSPAR and is being used in the North-East Atlantic Ocean to ensure safe discharge of produced water to the marine environment (OSPAR, 2018).

Given the outstanding uncertainties in the field, application of the effect-based approach would allow a more holistic toxicological evaluation of the water quality while the PLONOR approach would restrict additives to those known to be safe. Both approaches would be able to provide an additional layer of safety when targeting FPW for reuse in irrigation. A first cut at a PLONOR list was conducted for this report and revealed 31 chemicals with similar physiochemical properties to TMAC and diethanolamine. Further investigation into the toxicity and prevalence of these chemicals would contribute to

the PLONOR exploration process. The development of a PLONOR list would greatly benefit from ongoing stakeholder engagement in order to develop an approach that oil and gas companies, regulators, and the public can agree upon.

5. Conclusion

The evaluation of FPW reuse for agriculture is a growing topic of inquiry with a wide range of research possibilities. Future inquiries could include the evaluation of more complex chemical mixtures, various TOC and TDS levels, and additional plant species. The research presented here can inform these forthcoming investigations by providing uptake quantification methodologies and a statistical approach for experimental designs. Additionally, the quantitative results, growth observations, and data gaps highlighted in this experiment can help inform farmers and regulators of potential FPW reuse impacts and challenges. For example, farmers may view the potential economic benefit from increased grain yield could be appealing, however, this benefit could be counteracted by the lodging impacts and uptake of the toxic substances such as TMAC, diethanolamine, and acrylamide. For regulators and operators, the expense and resources required to conduct the numerous uptake experiments necessary to address existing data gaps may challenge aspirations of increasing produced water reuse for beneficial purposes. Ultimately, alternative methods of ensuring consumer safety, such as the development of a PLONOR list or adapting the WET approach to terrestrial environments, may be the path to ease consumer concerns while still moving forward with reuse objectives.

Author statement

We have decided not to provide a statement regarding **Author contributions** since its not mandatory (only encouraged according to the instructions to authors).

Declaration of Competing interest

The authors report no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2020.116402.

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List of Abbreviations

Ai: Active ingredient

ACP: Asian citrus psyllid(s)

CLas: Candidactus liberbacter asiaticus

DIM: Dimethoate

EPA: Environmental Protection Agency

FS: Field study

IMI: Imidacloprid

LC-MS: Liquid chromatography mass spectrometry

LC-MS/MS: Liquid chromatography tandem mass spectrometry

MAL: Malathion

MALX: Malaoxon

OME: Omethoate

US: United States

WF: Whatman Filter