INVESTIGATING PESTICIDE UPTAKE AND METABOLISM FOLLOWING DERMAL EXPOSURE IN TERRESTRIAL PHASE AMPHIBIANS

by

DONNA AMY GLINSKI

(Under the Direction of Marsha C. Black)

ABSTRACT

Amphibians have suffered serious global declines in recent decades. A probable cause of these declines is widespread exposure of pesticides for these non-target species. The objectives of this study were 1) identify pesticides in rainwater and surface waters (i.e. spray drift and runoff) following agricultural application in southern Georgia impacting amphibian habitats, 2) determine metabolic rate constants of select pesticides identified in these matrices in amphibian liver microsomes, 3) determine if the hydration status of an amphibian can affect the tissue concentration of pesticides, and 4) identify potential effects of exposure to pesticide mixtures on the metabolome of amphibians. To this end, surface water and rainwater samples including stemflow and throughfall were analyzed via GCxGC-ToF/MS for over 150 pesticides, overall, thirty-two different pesticides were detected in these matrices. To determine the metabolic rates for atrazine, triadimefon, and fipronil in amphibian microsomes the Michaelis-Menten equation was utilized to obtain K_M and V_{max} parameters. The V_{max} ranged from 150–834 pmol min^{-1} mg^{-1}, while K_M was measured from 5 to 147 μM which resulted in calculated intrinsic clearance rates ranging from 0.54–38.31 mL min^{-1} kg^{-1} for southern toads. The effect of hydration status on amphibians was determined by dehydrating amphibians for 0, 2, 4, 6, 8 or 10 hours prior to an 8

h exposure to pesticide-contaminated soil. Interestingly, increased time of dehydration resulted in lower tissue concentrations observed in amphibians for the five pesticides studied. Metabolomics was then utilized to identify biochemical fluxes and pathways impacted by pesticide exposure, both as singlets and in combinations, in terrestrial phase amphibians. Ultimately, this research can be utilized to create a dermal uptake model for pesticide exposure in amphibians, increasing the reliability of current regulatory efforts. Overall, this research details the consequence of pesticide exposures on amphibians and lays the foundation for identifying key biomarkers that can help identify pesticide exposure in future research efforts.

INDEX WORDS: Amphibians, pesticides, surface water, stemflow, throughfall, GCxGC-ToF/MS, microsomes, metabolism, dehydration, body burden, uptake, metabolomics, mixtures

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CHAPTER 1

INTRODUCTION

Amphibians are a unique class of vertebrates due to their varied life histories and thus can be considered valuable indicators of the health of an ecosystem. Anurans start off their life cycle as embryos and tadpoles in aquatic environments and most morph into terrestrial dwelling amphibians. Their residence time in the aquatic environment is generally short; however, many important developmental stages occur in this environment. Post-metamorphosis, most species spend many years on land (Venturino et al., 2003; Salice et al., 2011). Amphibians are the most rapidly declining and threatened taxa compared to other vertebrates such as avians and mammals (Stuart et al., 2004). Since the 1980s, over 120 species of amphibians have become extinct, and over one-third of amphibian species are currently threatened globally (Whitfield et al., 2007). In 2004, Stuart et al. noted that over 400 species of amphibians were listed as critically endangered, while presently the number has increased to 546 species (IUCN, 2017). Interestingly, in biodiverse Central America, there has been a 75% decline in amphibian density since 1970 (Whitfield et al., 2007). It has been thoroughly demonstrated that amphibian populations are in decline and since hypothesized that pesticide exposure is one of the primary causative factors in addition to habitat loss, pollution, climate change, and diseases (Sparling et al., 2001; Christin et al., 2003; Stuart et al., 2004; Lötters et al., 2014; Aldrich et al., 2016). Of note, this rapid decline in amphibian populations is not the result of a single stressor but rather the endpoint of numerous and complex stressor interactions (Buck et al., 2012).

Focusing on a single stressor of exposure to an organism has become the primary method of understanding the effects a stressor has on a certain species. However, in the environment, species are often exposed to multiple stressors at any given time. A recent national survey of U.S. streams including agricultural, urban, and mixed land use, was conducted from 1992-2001 by Gillom et al. (2006), and pesticides were detected over 90 percent of the time in these waters. The most frequently detected pesticides were herbicides, mainly atrazine and its metabolite (desethyl atrazine), metolachlor, simazine, and prometon (Gilliom et al., 2006). In the same study, pesticides were at detectable levels throughout the entire year in agricultural and urban streams. Thus, many non-targeted species will come into contact with these contaminants on a daily basis in addition to other stressors.

Pesticides and their residues are known to enter the aquatic system through precipitation, leaching, spray-drift, and discharge of wastewater, etc. (Griffini et al., 1997; Konstantinou et al., 2006; Rice et al., 2016). Interestingly, spray drift coupled with pesticide volatilization has resulted in many agriculturally-applied pesticides being transported to countries far away from application sites (Hüskes and Levsen, 1997; Ahmed et al., 1998; Coupe et al., 2000). Atmospheric transport of pesticides has also been shown to convey these chemicals to once pristine areas (McConnell et al., 1998; LeNoir et al., 1999). During a rain event, stemflow (rainwater that flows down a tree trunk) and throughfall (water that flows through a trees' canopy) have the ability to wash pesticides that have accumulated from spray drift off trees and surrounding foliage resulting in further contamination of surrounding areas. Ultimately, contamination of these once pristine areas has resulted in amphibians having median pesticide body burdens ranging from 13 to 235 µg/kg (Smalling et al., 2013).

Dehydration is another significant stressor for terrestrial amphibians. Amphibian bodies consist of 70-80 percent water by weight and water loss is known to occur daily for these species (Thorson, 1964; Dole, 1967; Hermes-Lima and Storey, 1998). Due to this vulnerability, amphibians have developed both behavioral adaptations and physiological mechanisms to combat this loss. However, regardless of these protective mechanisms, periodic droughts can create challenging terrestrial environments for amphibians. During droughts, amphibians lose their body water, this can led to an increase in their skin's hydraulic conductivity leading to a rapid rehydration when the amphibian comes in contact with water (Shoemaker and Nagy, 1977). This rapid rehydration of water may also facilitate the uptake of pesticides across the amphibians seat patch when they traverse agricultural fields or other contaminated soils (Berger et al., 2012; Fryday and Thompson, 2012).

Once pesticides enter the body, metabolism is commonly initiated by the liver. In rats, rabbits and humans, hepatic microsomes exposed to the herbicide atrazine produced two major metabolites desethyl atrazine (DEA) and deisopropyl atrazine (DIA) (Adams et al., 1990; Lang et al., 1996; Hanioka et al., 1999). In similar species, the hepatic metabolism of triadimefon is known to metabolize into triadimenol and this metabolite is considered more toxic than its parent in numerous species studied (Roberts and Huston, 1999; Kenneke et al., 2010). Due to various chemical processes, the insecticide fipronil can degrade into fipronil sulfone, fipronil sulfide, and fipronil desulfinyl (Hainzl et al., 1998; Reynaud et al., 2012). *In vitro* data can provide a high throughput method for testing pesticides in amphibians, and can fill in knowledge gaps about pesticide metabolism and estimating risk in these non-targeted species.

Exposure to pesticides and other xenobiotics in the environment usually occur as mixtures rather than a single compound, since, during agricultural application, multiple

pesticides are often applied throughout a growing season and may persist through fallow periods. Therefore, it can be hypothesized that residuals of pesticides will be present throughout most of the year, leading to non-target organisms being exposed to multiple classes of compounds. Metabolomics can be utilized in the identification of the consequence of pesticide exposure in amphibians. Following pesticide exposure, differences in metabolite fluxes in the metabolome can aid in the identification of specific pathways that are potentially affected before overt toxicity occurs.

Since pesticides can be transported significant distances away from the site of application, these contaminants pose a substantial risk to many non-target species. Due to the fact that amphibians have relatively permeable skin, dermal absorption is the major route of exposure for these contaminants (Smith et al., 2007). Thus, understanding how pesticide exposure to amphibians influences toxicity is necessary to accurately assess the ecological risks these compounds pose. The overarching objective of this research is to assess pesticide exposures and subsequent dermal uptake in amphibians by measuring the body burdens of pesticides along with utilizing metabolomics to investigate biochemical fluxes. Exposure studies incorporate both dermal absorption scenarios and biomarker elucidation. The data gained from this research will fill regulatory knowledge gaps and better allow amphibians to be more accurately assessed for the pesticide registration process. Ultimately, this research can lead to the creation of more accurate dermal exposure and uptake models in amphibians and greatly inform risk assessment efforts for these and other non-target species.

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CHAPTER 2

LITERATURE REVIEW

Pesticides in the Environment

There are numerous types of pesticides used globally to combat insects, fungi, weeds, and other nuisances. Predominantly, based on usage, they are classed as herbicides, insecticides, fungicides and other smaller classes (Gilliom et al., 2006; Potter et al., 2014). Currently, pesticides are being applied at a greater extent than in the past due in part to availability of more formulations and/or marketed varieties (Mann et al., 2009). Pesticides are largely applied in agricultural settings, however, approximately 20 percent of these compounds are used for non-agricultural purposes (Stokstad and Grullón, 2013). Once these pesticides enter the ecosystem, many environmental factors can influence their residence time. These interactions can result in processes such as translocation, environmental degradation and metabolic activation upon exposure to target and non-target species.

Pesticide translocation can occur due to spray drift, precipitation-mediated runoff, leaching into groundwater due to soil and chemical characteristics, or the pesticide can volatize and traverse the atmosphere resulting in pristine areas becoming contaminated (Van der Werf, 1996; LeNoir et al., 1999; Hapeman et al., 2003). Direct agricultural application coupled with translocation has resulted in pesticides being detected and quantified in global matrices including air, sediment, rainwater, stemflow, throughfall, fog, snow, biota and many rivers and streams (Trevisan et al., 1993; Planas et al., 1997; Rice and Chernyak, 1997; Coupe et al., 2000; Du

Preez et al., 2005; Sauret et al., 2009; Abrantes et al., 2010; Gish et al., 2011; Zhang et al., 2011; Battaglin et al., 2016).

Transformation is another main process that modifies residence time of pesticides. Environmental degradation or metabolic activation can often lead to the modification of toxicity of pesticides depending on the specific chemical processes (Van der Werf, 1996; Hapeman et al., 2003). Pesticides are susceptible to either abiotic (i.e. photolysis) or biotic processes, mainly by indigenous microbial communities (Hapeman et al., 2003). The transformation rates of pesticides are dependent on several physio-chemical factors, such as the temperature, oxygen availability and organic matter content of the soil (Van der Werf, 1996; Hapeman et al., 2003). Therefore, microbial communities can metabolize pesticides and affect rates of volatilization and leaching into groundwater, since some metabolites sorb to soil more readily than parent compounds (Hapeman et al., 2003). Within the body pesticides can become metabolically activated, often by the liver. Within the liver phase I proteins such as cytochrome P-450 are used to help with the hydrolysis, oxidation, and reduction of pesticides (Lang et al., 1996; Hanioka et al., 1999a). Many pesticides including atrazine and fipronil use cytochrome P-450 enzymes to degrade into their main metabolite(s) (Adams et al., 1990; Lang et al., 1996; Hanioka et al., 1999b). However, triadimefon, a fungicide, degrades into its main metabolite through the enzyme protein 11β-hydroxysteroid dehydrogenase type 1 (Kenneke et al., 2008).

Herbicides

Herbicides are the most frequently applied pesticides in the United States, due to their application on staple crops such as corn, soybeans, cotton and wheat (Fernandez-Cornejo et al., 2014). Overall, the type of herbicide or formulation applied depends on the crop, targeted

unwanted plant and intended land use (Gilliom et al., 2006). Several recent surveys of the U.S. have identified and quantified herbicides in streams and sediment (Kolpin et al., 2000; Gilliom et al., 2006; Smalling et al., 2012; Smalling et al., 2013b; Battaglin et al., 2016). In Gilliom et al. (2006), the main herbicides detected were atrazine, desethyl atrazine (metabolite of atrazine), simazine, prometon and metolachlor. In Smalling et al. (2012), ten different herbicides were detected in 54 water samples from 29 various sites, and atrazine, glyphosate and its metabolite aminomethylphosphonic acid (AMPA) were the most frequently detected compounds. In agreement with Smalling's research efforts, in Battaglin et al. (2016) the most frequently detected herbicides were also atrazine, AMPA and glyphosate. In Iowa, Smalling et al. (2015) also detected atrazine, metolachlor and glyphosate and these three herbicides were more frequently detected in water when compared to other pesticides. Interestingly, Kolpin et al. (2000), measured herbicide degradates in water, and the metabolites were more frequently detected than some of the parent compounds. In Kolpin et al. (2000), 55 to 99% of the time, detection of metabolites was a more effective way to measure and predict the concentration of active ingredient herbicides. Together, these surveys demonstrate that herbicides, both parents and degradates, can be quantified in most areas and should both be taken into consideration for risk assessments.

Insecticides

In 1960, more than 50% of the total pounds of pesticides applied in the U.S. were insecticides, including the high use insecticides known as organochlorines (Fernandez-Cornejo et al., 2014). Due to pesticide resistance, many farmers have increased the application rate resulting in 67 million pounds of active ingredient being applied to cotton in 1960, increasing to

105 million pounds in 1972 (Fernandez-Cornejo et al., 2014). Many of these organochlorine compounds have been banned due to persistence, bioaccumulation and toxicity, causing farmers to rely on other insecticide classes (Turusov et al., 2002; Fernandez-Cornejo et al., 2014). Even though organochlorine insecticides are banned, they are still quantifiable, if not as the parent, then as the metabolite, in many locations throughout the world in water, sediment and biota samples (Turusov et al., 2002; Zhang et al., 2011; Smalling et al., 2013a; Battaglin et al., 2016). Nowadays, the synthetic pyrethroids and neonicotinoids have replaced the organochlorines. Pyrethroids and neonicotinoids require lower field application rates and some formulations can be applied as seed treatments limiting broadcast applications and non-target exposures (Fernandez-Cornejo et al., 2014).

In Gilliom et al. (2006), diazinon, chlorpyrifos, and carbaryl were the most common insecticides detected in urban streams compared to agricultural streams. However, in Battaglin et al. (2016) the only insecticide detected in surface water samples was chlorpyrifos. Neonicotinoid insecticides were quantified in streams collected from the Midwest, and clothianidin, thiamethoxam and imidacloprid were detected (on average) more than 23% of the time (Hladik et al., 2014). Smalling et al. (2015) detected both clothianidin and thiamethoxam in agricultural wetlands with concentrations ranging from 0.002 to 0.04 µg/L. In bed sediment samples analyzed in Smalling et al. (2012), bifenthrin was the second most frequently detected pesticide. Furthermore, in Battaglin et al. (2016), bifenthrin and *p,p'*-DDE were the most and third most frequently detected insecticides, with maximum concentrations at 17.5 and 128 µg/kg, respectively. These studies verify that legacy insecticides are still being detected within the environment, indicating that pesticides can have a lasting effect on the ecosystem while new pesticides are also reaching detectable levels.

Fungicides

Fungicides are used on a wide variety of crops including many fruits, vegetables and grains, as a preventative not a curative measure (Battaglin et al., 2011). In the U.S., four different fungicides were registered in 2006 to treat soybean rust, but by 2009, this number increased to 14 (Battaglin et al., 2011). Even though fungicides are applied less than other pesticide classes, they are more frequently detected in water and sediment samples (Grube et al., 2011; Battaglin et al., 2016). This phenomenon is likely due to the relatively higher frequency of fungicide applications even though application rates are lower, increasing their detection frequencies over time compared to herbicides and insecticides (Reilly et al., 2012).

Battaglin et al. (2011) analyzed 29 streams throughout the U.S. for fungicides and detected at least one fungicide 56% of the time. In the 20 streams that had detectable levels of fungicides, the most frequently detected fungicides were azoxystrobin, metalaxyl, propiconazole, myclobutanil and tebuconazole. Concentrations of these fungicides ranged from 0.002 to 1.15 µg/L (Battaglin et al., 2011). Moreover, in water samples analyzed in Battaglin et al. (2016), azoxystrobin had the highest frequency of detection (15%), while imazalil was detected at the highest concentration (219 ng/L). In Reilly et al. (2012) water samples were analyzed for 33 fungicides and 57 other pesticides. Overall, in surface water from Maine, Idaho and Wisconsin, at least one fungicide was detected 75% of the time. Additionally, in these water samples, 12 fungicides were detected with boscalid being detected more frequently than the herbicides atrazine and metolachlor (Reilly et al., 2012). In bed sediment samples, both pyraclostrobin and tebuconazole were the most frequently detected fungicides; while tebuconazole had the highest detect concentration of 1380 µg/kg (Smalling et al., 2012; Smalling et al., 2013a). Furthermore,

in Smalling et al. (2013c) and Battaglin et al. (2016), pyraclostrobin was the most frequently detected fungicide.

Many of the studies referenced have included amphibians in their monitoring efforts, due to the fact that amphibians are great indicators of the 'state' of an environment. Amphibians have a unique life history that incorporates both aquatic and terrestrial ecosystems. Within the aquatic environment, pesticides from runoff can negatively affect a tadpole's growth and development, while spray drift and/or direct application of pesticides can impact the life cycle of an adult or juvenile terrestrial phase amphibian in agricultural fields. Numerous scientific articles have been published that co-examine pesticides and amphibians, and numerous studies have explored the effects of pesticides on tadpoles. These data include assessing pesticides of different classes as singlets or as mixtures (Boone et al., 2001; Relyea, 2003, 2004; Boone and Bridges-Britton, 2006; Hayes et al., 2006; Boone, 2008; Relyea and Diecks, 2008; Fenoglio et al., 2009; Davis et al., 2011; Stefani Margarido et al., 2013; Güngördü and Uçkun, 2015), in addition to scaling the impact of individual tadpoles up to population, community and ecosystem levels (Boone and James, 2003; Relyea, 2005a; Boone et al., 2007; Relyea, 2009; Buck et al., 2012; Hua and Relyea, 2014). However, a scarce amount of research is available that has investigated the consequence of pesticide exposure on terrestrial phase amphibians.

Amphibian Physiology

There are approximately 6000 species of amphibians in the group Lissamphibia, which are represented in the orders Gymnophiona (caecilians), Caudata (salamanders), and Anura (frogs) (Hillman et al., 2009). With more than 42 families encompassing 5300 species, anurans are classified as the most diverse and successful order (Frost et al., 2006). This success is due to

the number of species and ecological niches that have resulted in a wide distribution of anurans existing throughout the world. The amphibians' habitat ranges globally excluding many oceanic islands, low-lying arid regions, and extremely high latitudes of both hemispheres (Venturino et al., 2003; Measey et al., 2007; Hillman et al., 2009). Anuran diversity has resulted in their being placed into one of four habitat groups, aquatic, fossorial, terrestrial or arboreal (Hillman et al., 2009).

Amphibian skin is well known for being relatively permeable to both respiratory gases and water. Thus, many anurans lack a hydrophobic barrier due to the absence of epidermal scales and other protective layers (thick stratum corneum) observed in other terrestrial species (Hillman et al., 2009). This has resulted in the ability to readily exchange ions, gases and water with their surrounding environment; however, this ability comes with consequences. Due to the ease of water moving between amphibians and the environment, it is challenging for anurans to remain constantly hydrated. Therefore, multiple physiological and behavioral adaptations have evolved to compensate for this ease of water loss (Barbeau and Lillywhite, 2005; Suzuki et al., 2007; Hillman et al., 2009). Since amphibians need to stay hydrated and do not physically imbibe water, they have developed a highly vascularized seat patch. This vascularized seat patch is located in the posteroventral region of anurans and is the primary route of absorption of water (Bentley and Main, 1972). Permeability of the seat patch can be increased in the presence of arginine vasotocin, an antidiuretic hormone (Hillman et al., 2009). Arginine vasotocin has the ability to facilitate the rapid uptake of water into the body via aquaporins or small water channels that allow for the easy absorption of water from the surrounding environment (Preston and Agre, 1991).

It has been well documented that the more terrestrial an amphibian species is, the higher its skin permeability for dehydration/reabsorption of water can be (Hillman, 1982; Hillman et al., 2009). Basically, the overall thickness of anuran skin is directly correlated to habitat (Toledo and Jared, 1993). Terrestrial species also possess granulations that can increase the amount of surface area that is available for uptake of water (Lillywhite and Licht, 1974). However, aquatic amphibian species, being more tethered to their environment, have smooth skin on both the ventral and dorsal surface which is less water permeable (Lillywhite and Licht, 1974). Overall, having more permeable skin is positively correlated with the extent of dehydration tolerance the species can withstand (Shoemaker et al., 1992). Aquatic species have a lower dehydration threshold ranging from 28–33% total water loss compared to terrestrial species which can withstand up to 40–45% body mass loss without adverse effects (Shoemaker et al., 1992).

Amphibians often, on average, lose anywhere from 10–22% of their total body mass daily and can tolerate up to 25–60% of total body water lost due to dehydration (Dole, 1967; Shoemaker et al., 1992). As a behavioral adaptation, dehydrated amphibians can enter into a water conservation posture which entails hind legs and forearms being folded tightly underneath the body with the ventral surface and the chin pressed close to the substrate, resulting in minimum amount of surface area exposed to the environment (Pough et al., 1983; Zug et al., 2001).

Exposure of Terrestrial Phase Amphibians to Pesticides

Anurans come in contact with both soil and water contaminants due to their life cycle encompassing both terrestrial and aquatic environments (Bishop et al., 1999). Many amphibian species spend a significant portion of their life cycle being in contact with the aquatic

environment. Aquatic contact can occur as embryos, larvae or as adults returning to breed at a pond or water surface (Salice et al., 2011). Many of these ponds are situated around agricultural fields where pesticides are often applied (Mann et al., 2009). Typically, breeding season and embryonic and larval development occurs during spring and into early summer. Anuran breeding season frequently coincides with the planting of crops and application of pesticides to these agricultural areas (Berger et al., 2011; review in Fryday and Thompson, 2012). This timing results in herbicides, fungicides, and insecticides being applied over fields, so that amphibians, along with other non-target species, will be exposed to these compounds. In Berger et al. (2011), during pesticide application, it was estimated that upwards of 85% of the population of spadefoot toads were active during this time. During winter application of insecticides, only 20 to 40% of the spadefoot toad population was active. Many field surveys have observed a correlation between distance to agricultural fields and amphibian population decline (Bishop et al., 1999; Davidson et al., 2002; Davidson, 2004; Davidson and Knapp, 2007; Mann et al., 2009). Bishop et al. (1999) found higher population densities of seven different amphibian species upstream and downstream of an agricultural zone in Canada.

Compared to other organisms, limited journal articles (< 4%) have been published focusing on pesticide exposure in amphibians (Köhler and Triebskorn, 2013). The majority of these articles focus on tadpoles (Brühl et al., 2011;). Currently, there are few researchers investigating pesticide uptake in terrestrial phase amphibians as shown by the paucity of published literature (review in Brühl et al., 2011; Brühl et al., 2013; Smalling et al., 2013a; Van Meter et al., 2014; Smalling et al., 2015; Van Meter et al., 2015; Battaglin et al., 2016; Van Meter et al., 2016). In addition, some studies have investigated amphibian skin permeability to pesticides (Shah et al., 1983; Willens et al., 2006a, b; Quaranta et al., 2009; Storrs Méndez et al.,

2009). Grass frogs were exposed to carbaryl, DDT, dieldrin, parathion, and permethrin in Shah et al. (1983) to determine dermal penetration rates. The half time rate of dermal penetration was highest for carbaryl (6 minutes) in frogs. Dieldrin and DDT were absorbed the slowest in frogs, with dieldrin (3766 minutes) being absorbed the slowest.

The previous study demonstrated that pesticides can be readily absorbed by amphibians, while the next two assess the permeability of a pesticide in different surface locations. In Willens et al. (2006a) malathion was exposed to six different skin thicknesses that were acquired from dorsal and ventral surfaces of two different amphibian species, bullfrogs and marine toads. Exposure was done utilizing a flow-through diffusion cell with the concentration of malathion at 26 $\mu g/cm^2$ for 6 h. Overall, the authors observed that ventral skin was more permeable than dorsal skin resulting in higher absorption of malathion in the ventral skin for both species. The research in Willens et al. (2006a) was followed up with an *in vitro* model in amphibians to examine percutaneous absorption in Willens et al. (2006b). This model utilized harvested perfused anuran pelvic limb (HPAPL) from bullfrogs and again, exposure to malathion was 26 $\mu g/cm^2$ for 6 h resulting in mean absorption rates of 17.9% at 108.8 minutes. It was concluded that the HPAPL was a suitable model that can be used for percutaneous absorption kinetics and future regulatory efforts.

Pesticides can cross the amphibian skin, with ventral skin the more permeable location, the next study assessed the permeability of pesticides in amphibians versus pigs (used as mammalian skin) and further studied the relationship between several physio-chemical properties. In Quaranta et al. (2009) the authors compared permeability of green frog skin (amphibian) to pig ear skin (mammalian) by measuring the *in vitro* percutaneous passage of three herbicides atrazine, glyphosate and paraquat in a static diffusion cell. The authors calculated the

permeability coefficient (P) for each herbicide through both the pig ear and the frog skin and concluded that frogs have a greater logP value compared to mammals by an order of magnitude. Furthermore, log Kow (octanol-water partition coefficient or hydrophobicity) was positively correlated with logP of the compounds studied. Overall, the authors concluded amphibian skin was more permeable to contaminants than mammalian due to the thickness of each respective epidermis layer (Quaranta et al., 2009).

These aforementioned studies examined the permeability of the amphibian dermis to pesticides. However, Storrs Méndez et al. (2009) took it one step further by investigating the uptake, distribution and elimination of atrazine in *Bufo americanus*. This experiment demonstrated that pesticides can be absorbed across the seat patch and the greatest concentrations were observed in the gall bladder and intestines following 8 hours of exposure. Behavioral responses have also been measured *in vivo* in American toads when in contact with atrazine exposed soil (Storrs Méndez et al., 2009). Amphibians, when given the choice between atrazine contaminated soil or untreated soil, did not have a soil preference. These experiments demonstrate that adult life stages and direct contact of pesticides should also be considered in risk evaluations.

Several monitoring studies have surveyed pesticide body burdens in terrestrial phase amphibians ranging throughout the U.S. (California, Colorado, Georgia, Idaho, Iowa, Louisiana, Maine, Michigan, and Oregon) and in several countries including Argentina, Bermuda, Canada, China, and Mexico (Harris et al., 1998; Gillilland et al., 2001; Linzey et al., 2003; Loveridge et al., 2007; Jofré et al., 2008; Wu et al., 2012; Smalling et al., 2015; Valdespino et al., 2015; Battaglin et al., 2016). The influx of pesticides along with other stressors to the Sierra Nevada Mountains, an area with very little local agriculture, has been well documented in an attempt to

identify the mechanistic underpinnings of amphibian decline in this area (Bradford et al., 1994; McConnell et al., 1998; LeNoir et al., 1999; Sparling et al., 2001; Davidson et al., 2002; Davidson, 2004; Fellers et al., 2004; Davidson and Knapp, 2007; Bradford et al., 2010; Bradford et al., 2011; Smalling et al., 2013a). The body burdens for many pesticides ranged from 0.01 to 4746 ng/g for organochlorine pesticides. In Smalling et al. (2015), eight different fungicides were present in amphibian livers ranging from 5.9 to 1500 µg/kg. Herbicide and insecticide concentrations ranged from 3.8 µg/kg to 167 µg/kg and 1.8 µg/kg to 432 µg/kg, respectively. Not only can pesticides cross the dermis of amphibians, but the monitoring studies demonstrated that amphibians are accumulating pesticides within their tissues. These studies collectively demonstrate that pesticides can be transported hundreds of miles away from their application areas in the Central Valley of California and contaminate pristine areas.

However, since it is difficult to follow the exact movements of an amphibian before being captured in monitoring studies, along with estimating the direct route of exposure (dietary or dermal, and aquatic or terrestrial), focus is given to dermal absorption of pesticides via pesticide treated soil or direct overspray in terrestrial phase amphibians (adults and juveniles), similar to Brühl et al. (2011) and Fryday and Thompson (2012) (Table 2-1). Dermal absorption via pesticide treated soil was examined in the current review because this is the main pathway hypothesized that terrestrial phase amphibians uptake pesticides and water in these habitats (Brühl et al., 2011). In total, 19 studies were found to fit the criteria that examined dermal absorption of pesticides in terrestrial phase amphibians.

Anurans

Mortality Studies

This section focuses on Anuran species that were used in dermal absorption studies examining both mortality and LC values. A forest aerial overspray to examine the effects of an insecticide, aminocarb, on a forest community for two years was studied in Bracher and Bider (1982). Aminocarb formulation was applied at a rate of 175 g/ha, where short, medium and long term activity for several species was monitored. Overall, there was no significant change in activity for both *Rana* spp. and *Bufo americanus*. However, the authors concluded that an initial decrease in activity was probably due to the low availability of prey after the pesticide application.

The resistance of DDT in two species of cricket frogs was examined in Boyd et al. (1963). Amphibians were collected from both reference and cotton fields that were heavily treated with DDT. Amphibians were exposed to 10 to 50 mg/L of DDT on filter paper to simulate dermal absorption. Previous exposure to DDT had resulted in lower mortality rates compared to the amphibians obtained from reference sites. It was determined that possible resistance to DDT was observed in amphibians that had previously been exposed to the pesticide due to lower mortality being observed.

Vinson et al. (1963), similar to Boyd et al. (1963), collected amphibians from various fields with some in close proximity to cotton fields that applied insecticides. Percent mortality was recorded for both northern and southern cricket frogs exposed to aldrin and grouped by collection locality. Mortality ranged from 0 – 33.4% and 0 – 56.7% for northern cricket frogs exposed to 0.03 g/mL and 0.05 g/mL, respectively. Southern cricket frogs had an average mortality of 40% and 70% when exposed to 0.01 g/mL and 0.05 g/mL, respectively. Overall,

northern cricket frogs appeared to develop a resistance to aldrin; furthermore, one locality had no mortality including frogs collected from a bayou surrounded by 483 acres of cotton treated with other organochlorines, not aldrin, which led the authors infer that a cross-resistance had evolved.

In Ferguson and Gilbert (1967), three different amphibian species were exposed to five different organochlorines (endrin, aldrin, dieldrin, DDT and toxaphene). Amphibians were exposed to insecticides on filter paper to determine the TL50, median tolerated limit after 36 h of exposure. The authors stated that the most toxic pesticide was endrin while the least toxic were DDT and toxaphene and aldrin and dieldrin were intermediate. In this study, Fowler's toads exhibited high levels of resistance to the organochlorines ranging from 40 to 200-fold, except for endrin. However, for the northern cricket frogs, tolerance resistance to DDT was 81-fold and toxaphene was 11-fold from the different locations.

Formulations of glufosinate and glyphosate based herbicides were exposed to two species of toads to examine survival in Dinehart et al. (2009). Dermal exposure was simulated on two different substrates, paper towels or soil. Exposure to the glufosinate based formulation and Roundup WeatherMax® had no effect on survival. However, both toad species exhibited an increase in mortality when exposed, on both substrates, to Roundup Weed and Grass Killer Ready-To-Use Plus®. Only a slight increase in mortality was observed in the Great Plains toads exposed on paper towels to Roundup Weed and Grass Killer Super Concentrate®. Therefore, since high mortality rates were observed on paper towels, the authors concluded that these formulations would not cause an immediate risk to these toad species in the field.

The effect of Roundup "Weed and Grass Killer" on several amphibian species' mortality was assessed by Relyea (2005b). Roundup was applied at 1.6 mg AI/m^2 as a simulated overspray scenario so that juveniles had direct application similar to an agricultural field.

Mortality of all species exposed to Roundup for 24 h was significantly higher compared to controls, demonstrating that pesticides as a formulation can have a negative impact on amphibians causing substantial mortality to juveniles.

Brühl et al. (2013) assessed survival on juvenile European common toads after exposure to seven different pesticide formulations. Formulations were applied at 0.1, 1, and 10 times the application rate in an overspray scenario. Mortality ranged from 0 to 40% at the lowest concentration, while at the highest concentration mortality ranged from 20 to 100%. Although, at the recommended label rate mortality increased, where 100% mortality was observed in Headline® (active ingredient is pyraclostrobin) within one hour and Captan Omya® (active ingredient is captan) within 24 h. The authors concluded that the high mortality of amphibians to these formulations within seven days of application was alarming and that amphibians are not being protected by the existing risk assessment procedures.

Belden et al. (2010) studied the percent mortality of three different fungicide formulations on Great Plains toads at 0.1, 1, and 10 times the provided label rate. All three formulations were applied as an overspray and most mortality to both tadpoles and juveniles occurred within 24 h of exposure to the formulation. The active ingredients for each formulation are pyraclostrobin in Headline®, propiconazole and trifloxstrobin in Stratego®, and propiconazole and azoxystrobin in Quilt®. Headline® had the highest percent mortality for juvenile toads with over 90% within 24 h and Stratego® also killed 90% at the maximum concentration, while Quilt® was not statistically different from controls. Ultimately, in an overspray scenario in an agricultural field, many of the amphibians, including tadpoles and juveniles would not survive.

A field experiment was conducted to investigate the mortality of two different amphibian species after exposure to pyraclostrobin using Headline AMP® at 70 percent and 100 percent the application rate at three different locations (Cusaac et al., 2015). Woodhouse toads exhibited less than 12 percent mortality at all locations for both application rates. However, for Blanchard's cricket frogs, mortality was less than 8 percent after 24 h, while after 48 h the mortality was less than 25 percent for both application rates and at all three sites. Overall, at the current application rate of Headline AMP®, there was low mortality; however, presence of amphibians and application timing can still lead to exposure to these non-target species.

Eight different species of Colombian amphibians were exposed to a glyphosate formulation (Glyphos®) in conjunction with an adjuvant, Cosmo-Flux® in Bernal et al. (2009). Amphibians were exposed to a range of concentrations in an overspray scenario to determine mortality, LC1, and LC50 values for each species. LC1 values ranged from 0.32 to 7.02 kg acid equivalents/ha, while LC50 values were 4.5 to 22.8 kg acid equivalents/ha. The authors suggested that a mixture of these two chemicals does not pose a risk to amphibians in the field, due to low mortality seen at normal application rates.

Since Headline® was toxic to toads in Belden et al. (2010) at both 1 and 10 times the application rate, Cusaac et al. (2016) decided to investigate other pyraclostrobin containing formulations. The authors exposed two different pyraclostrobin formulations in an overspray scenario to Blanchard's cricket frogs to determine LC50 values for the fungicides, Headline® and Headline AMP®. The LC50 values were found to be equal to the maximum label rate which were also environmentally relevant concentrations. Therefore, these formulations applied at their label rate can pose a risk to amphibians within the environment.

Dermal Uptake and Toxicological Studies

Webber et al. (2010) evaluated whether aquatic exposure of carbaryl and population density had an effect on the feeding, growth and survival of toads after terrestrial exposure to carbaryl (2 mg/L). This was conducted by varying larval density (low or high) and exposure of carbaryl (present or absent) followed by juveniles being exposed to carbaryl. No effect was observed on feeding or survival after amphibians were exposed to both aquatic and terrestrial treatments of carbaryl. The authors concluded that insecticides that quickly degrade may affect larval stages more than terrestrial counterparts.

Taylor et al. (1999) examined the effect of malathion (low or high dose) and malathion plus bacteria on the rate of clinical disease, hepatomegaly and mortality in Woodhouse's toads. As the dose of malathion increased so did clinical disease, hepatomegaly, and mortality rates in each group. Furthermore, when the toads were subjected to both malathion and bacteria these rates continued to increase. Additionally, the authors tested the cholinesterase (ChE) activity on malathion and control toads, and ChE was suppressed 17% in low doses and 22% in high doses. Therefore, amphibians in the field that encounter pesticides and are challenged with pathogenic bacteria will be more susceptible to diseases.

In addition to obtaining LC50 values for Headline AMP® on Blanchard's cricket frogs, Cusaac et al. (2016) also measured the tissue concentrations of both active ingredients (pyraclostrobin and metaconazole). This was done by applying 0.5 times the maximum label rate of fungicide with exposure time points at 0.25–24 h. Body burdens were similar for pyraclostrobin at all time points studied, while there were slightly higher values for metconazole at the first two time points (0.25 and 2 h). Overall, pyraclostrobin concentrations were lower than the predicted values and were acutely toxic to amphibians.

Van Meter et al. (2014) measured pesticide body burden of five pesticides after dermal exposure in seven amphibian species. These pesticides ranged in hydrophobicity (log Kow) from 0.57 to 5.18, and the amphibians represented three different habitats, aquatic, terrestrial and arboreal. All amphibians exposed to contaminated soil had quantifiable body burdens of pesticides. The authors noted that soil partition coefficient (Koc) and water solubility were the best indicators for predicting bioconcentration factor and skin permeability factor of pesticides. This study is in contrast with Quaranta et al. (2009), where they found a linear correlation between log Kow and dermal uptake.

The results of the Van Meter et al. (2014) study lead the authors to conduct a follow-up exposure involving the same pesticides at the same application rates, but examining how organic matter content influences the body burden in amphibians (Van Meter et al., 2016). American toads (*Bufo americanus*) were used to compare tissue concentrations on Plott series soil (PLE; high organic matter, 14.1%) and Orangeburg loamy-sand (OLS) soil (low organic matter, 3.1%) after an 8 h exposure. Higher body burdens were found on low organic matter soil compared to high organic matter. Therefore, the organic matter content of soil can affect the bioavailability of pesticides in many agricultural fields and impact non-targeted organisms.

Van Meter et al. (2015) compared direct application versus indirect application of pesticides in Barking treefrogs (*Hyla gratiosa*) and green treefrogs (*Hyla cinerea*) by measuring body burdens and calculating bioconcentration factors (BCF). Active ingredients examined were atrazine, fipronil, imidacloprid, pendimethalin, and triadimefon. Pesticides were applied either directly to the amphibians in an overspray scenario or indirectly via overspray to soil prior to amphibians being exposed to contaminated soil. Overall, the authors observed higher body burdens in amphibians that were exposed via direct application compared to indirect. Therefore,

direct overspray of pesticides can be a concern for amphibians that traverse or reside near agricultural fields.

Salamanders

Caudata (or salamanders) consists of over 550 species and is the second largest order after Anura under Amphibia, (Hillman et al., 2009). To date, only three dermal exposure studies examined the effects of pesticides (atrazine, carbaryl and malathion) on salamanders, where the first two are lab-based while the third study incorporated both lab and field experiments. Mitchkash et al. (2014) exposed spotted salamanders to either an atrazine or carbaryl formulation at two different concentrations to determine how locomotor performance (endurance, fatigue, speed and distance traveled), growth and mortality are affected. Atrazine exposure did not affect locomotor performance, growth or mortality. However, amphibians exposed to carbaryl had slower times and expressed greater fatigue compared to controls; while growth and mortality were not affected by carbaryl.

Tiger salamanders were used for dermal and dietary routes of exposure to malathion by measuring tissue concentration, bioconcentration, bioaccumulation and brain cholinesterase inhibition in Henson-Ramsey et al. (2008). Dermal exposure consisted of two different concentrations (50 $\mu g/cm^2$ and 100 $\mu g/cm^2$) while dietary exposure was ingestion of an earthworm that was previously exposed to malathion. Body burden of malathion and its metabolite malaoxon had an average of 0.8596 ppm with a range of 0.3472 to 1.462 ppm. Overall, brain cholinesterase was suppressed 50 to 65 percent after exposure to 50 $\mu g/cm^2$ while the higher concentration resulted in 90% suppression. Dietary exposure did not affect any of the measured endpoints, resulting in no observation of bioconcentration or bioaccumulation.

In Baker (1985) salamanders were exposed to malathion both in a laboratory setting and in the field. The laboratory experiment consisted of two applications of malathion from 2.24 kg/ha to 8.97 kg/ha to red-backed salamanders and three doses at 5.6 kg/ha to northern slimy salamanders with feeding behavior and brain cholinesterase measured as the endpoints. The author noted that feeding behavior was not affected by exposure to malathion, while brain cholinesterase was suppressed from 5.4 to 18.7% for the red-backed salamander, and 34.1% for the northern slimy salamander. The northern slimy salamander was utilized for the field exposure, which consisted of ten doses at 5.6 kg/ha applied as an overspray to the forest ground, no effect was observed on abundance or brain cholinesterase inhibition.

Conclusions

Overall, a limited number of articles have been published measuring dermal exposure to terrestrial phase amphibians, when these articles are further divided into active ingredient versus formulation, direct application or contaminated soil, the number of articles for each category dwindles. Currently, there are over several hundred active ingredient pesticides resulting in thousands of formulations, and to date, only the surface has been scratched examining pesticide effects on terrestrial phase amphibians. Considering amphibians are a taxa that is suffering a major global decline, it is imperative that more exposures and experiments are conducted to help tease out the effects both single and mixtures of pesticides on this species. Moreover, all this data can be combined to create models that are sensitive enough to protect other species that are rapidly diminishing in numbers.

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Table 2-1: Summary of studies that examined dermal exposure to terrestrial phase amphibians.*

Species	Life Stage	Chemical	Route	Endpoint	Time	App Rate	Results	Reference
American toads (*Bufo americanus)* Northern leopard frogs (*Rana pipiens)* Wood frog (*Rana sylvatica*)	Adult	Aminocarb (Matacil®)	Dermal (aerial overspray)	Short, medium, long term behavior	2 y	175 g/ha	No significant change was observed; though activity could have been reduced due to prey availability	Bracher and Bider (1982)
Northern cricket frog *(Acris crepitans)*	Juvenile & Adults	DDT	Dermal (filter paper)	Mortality	36 h	10 mg/mL 20 mg/mL 30 mg/mL 40 mg/mL 50 mg/mL	0-75% mortality 0-95% mortality 0-100% mortality 0-96% mortality 0-100% mortality	Boyd et al. (1963)
Southern cricket frog *(Acris gryllus)*	Juvenile & Adults	DDT	Dermal (filter paper)	Mortality	36 h	4 mg/mL 6 mg/mL 8 mg/mL 9 mg/mL 10 mg/mL 30 mg/mL 40 mg/mL 50 mg/mL	3.3% mortality 0-40% mortality 13.3-62.5% mortality 20-50% mortality 10-52.5% mortality 35-80% mortality 40-87.5% mortality 65-65.7% mortality	Boyd et al. (1963)
Northern cricket frog *(Acris crepitans)*	Juvenile & Adults	Aldrin	Dermal (filter paper)	Mortality	36 h	30 mg/mL 50 mg/mL	0-33.4% mortality 0-56.7% mortality	Vinson et al. (1963)
Southern cricket frog (*Acris gryllus)*	Juvenile & Adults	Aldrin	Dermal (filter paper)	Mortality	36 h	10 mg/mL 50 mg/mL	40% mortality 70% mortality	Vinson et al. (1963)
Northern cricket frog *(Acris crepitans)*	Juvenile & Adults	Endrin Dieldrin Aldrin Toxaphene DDT	Dermal (filter paper)	TL50	36 h		TL50 = 0.04-0.06 mg/mL TL50 = 0.2-0.85 mg/mL TL50 = 0.2-0.75 mg/mL TL50 = 0.5-5.4 mg/mL TL50 = 0.62-50.0 mg/mL	Ferguson and Gilbert (1967)
Southern cricket frog (*Acris gryllus)*	Juvenile & Adults	Endrin Dieldrin Aldrin	Dermal (filter paper)	TL50	36 h		TL50 = 0.02-0.045 mg/mL TL50 = 0.3-0.4 mg/mL TL50 = 0.2-0.3 mg/mL	Ferguson and Gilbert (1967)
Fowler's toad (*Bufo woodhousei fowleri*)	Juvenile & Adults	Endrin Dieldrin Aldrin Toxaphene DDT	Dermal (filter paper)	TL50	36 h		TL50 = 0.03-0.095 mg/mL TL50 = 0.1-5.4 mg/mL TL50 = 0.05-10 mg/mL TL50 = 0.57-50.0 mg/mL TL50 = 0.57-50.0 mg/mL	Ferguson and Gilbert (1967)

Table 2-1: (*continued*)

Species	Life Stage	Chemical	Route	Endpoint	Time	App Rate	Results	Reference
Great Plains toads *(Bufo cognatus)*	Juvenile	Glufosinate (Ignite®)	Dermal (overspray, paper towel)	Mortality	48 h	0.21 mL AI/m^2	25% mortality	Dinehart et al. (2009)
			Dermal (overspray, soil)				0% mortality	
		Glyphosate (Roundup WeatherMAX®)	Dermal (overspray, paper towel)			0.16 mL AI/m^2	12.5% mortality	
			Dermal (overspray, soil)				0% mortality	
		Glyphosate (Roundup Weed and Grass Killer Ready-To-Use Plus®)	Dermal (overspray, paper towel)			1.33 mL AI/m^2	100 % mortality	
			Dermal (overspray, soil)				77.5% mortality	
		Glyphosate (Roundup Weed and Grass Killer Super Concentrate®)	Dermal (overspray, paper towel)			1.33 mL AI/m^2	52.5 % mortality	
			Dermal (overspray, soil)				0% mortality	
New Mexico spadefoots *(Spea multiplicata)*	Juvenile	Glufosinate (Ignite®)	Dermal (overspray, paper towel)	Mortality	48 h	0.21 mL AI/m^2	25.9 % mortality	Dinehart et al. (2009)
			Dermal (overspray, soil)				0% mortality	
		Glyphosate (Roundup WeatherMAX®)	Dermal (overspray, paper towel)			0.16 mL AI/m^2	40.4 % mortality	
			Dermal (overspray, soil)				0% mortality	
		Glyphosate (Roundup Weed and Grass Killer Ready-To-Use Plus®)	Dermal (overspray, paper towel)			1.33 mL AI/m^2	100% mortality	
			Dermal (overspray, soil)				100% mortality	
		Glyphosate (Roundup Weed and Grass Killer Super Concentrate®)	Dermal (overspray, paper towel)			1.33 mL AI/m^2	2.8% mortality	
			Dermal (overspray, soil)				0% mortality	
Wood frog *(Rana sylvatica)* Gray treefrog *(Hyla versicolor)* Fowler's toad *(Anaxyrus fowleri)*	Juvenile	Glyphosate (Roundup Weed and Grass Killer®)	Dermal (overspray)	Mortality	24 h	1.6 mg AI/m^2	68% mortality	Relyea (2005b)
							82% mortality	
							86% mortality	
European common frog (*Rana temporaria*)	Juvenile	Pyraclostrobin (Headline®)	Dermal (overspray)	Mortality	7 d	88 mL/ha	0% mortality	Brühl et al. (2013)
						880 mL/ha	100% mortality (within 1 h)	

Table 2-1: (*continued*)

Species	Life Stage	Chemical	Route	Endpoint	Time	App Rate	Results	Reference
European common frog (*Rana temporaria*)	Juvenile	Pyraclostrobin (BAS 500 18F®)	Dermal (overspray)	Mortality	7 d	88 mL/ha 880 mL/ha 8800 mL/ha	0% mortality 20% mortality 20% mortality	Brühl l et al. (2013)
		Bromoxyniloctanoate (Curol B®)				150 mL/ha 1500 mL/ha 15000 mL/ha	0% mortality 60% mortality 100% mortality (within 24 h)	
		Captan (Captan WDG Omya®)				320 g/ha 3200 g/ha	40% mortality 100% mortality (within 24 h)	
		Fenoxaprop-P-ethyl (Dicomil ultra Royal®)				120 mL/ha 1200 mL/ha 12000 mL/ha	40% mortality 40% mortality 60% mortality	
		Spiroxamine (Prosper®)				150 mL/ha 1500 mL/ha 15000 mL/ha	0% mortality 60% mortality 100% mortality (within 24 h)	
Great Plains toads *(Bufo cognatus)*	Juvenile	Pyraclostrobin (Headline®)	Dermal (overspray)	Mortality	72 h	88 mL/ha 880 mL/ha 8800 mL/ha	10% mortality 65% mortality 100% mortality	Belden et al. (2010)
		Propiconazole & Trifloxystrobin (Stratego®)				88 mL/ha 880 mL/ha 8800 mL/ha	0% mortality 10% mortality 90% mortality	
		Propiconazole & Azoxystrobin (Quilt®)				102 mL/ha 1020 mL/ha 10200 mL/ha	7% mortality 22% mortality 18% mortality	
Woodhouse's toad (*Bufo woodhousii*)	Juvenile	Pyraclostrobin & Metconazole (Headline AMP®)	Dermal (aerial overspray)	Recovery and mortality	24 h; 48 h	731 mL/ha	Spray: 24 h - 100% recovery, 4% mortality; 48 h - 100% recovery, 0% mortality Drift: 24 h - 99% recovery, 0% mortality; 48 h - 100% recovery, 7% mortality Reference: 24 h - 100% recovery, 0% mortality; 48 h - 100% recovery, 11% mortality	Cusaac et al. (2015)

Table 2-1: (*continued*)

Species	Life Stage	Chemical	Route	Endpoint	Time	App Rate	Results	Reference
Woodhouse's toad (*Bufo woodhousii*)	Juvenile	Pyraclostrobin & Metconazole (Headline AMP®)	Dermal (aerial overspray)	Recovery and mortality	24 h; 48 h	1052 mL/ha	Spray: 24 h -100% recovery, 0% mortality; 48 h - 100% recovery, 0% mortality Drift: 24 h - 92% recovery, 3% mortality; 48 h - 100% recovery, 12% mortality Reference: 24 h - 95% recovery, 0% mortality; 48 h - 100% recovery, 2% mortality	Cusaac et al. (2015)
Blanchard's cricket frog (*Acris blanchardi*)	Juvenile	Pyraclostrobin & Metconazole (Headline AMP®)	Dermal (aerial overspray)	Recovery and mortality	24 h; 48 h	731 mL/ha	Spray: 24 h - 31% recovery, 7% mortality; 48 h - 80% recovery, 7% mortality Drift: 24 h - 60% recovery, 8% mortality; 48 h - 87% recovery, 14% mortality Reference: 24 h - 64% recovery, 4% mortality; 48 h - 92% recovery, 25% mortality	Cusaac et al. (2015)
Blanchard's cricket frog (*Acris blanchardi*)	Juvenile	Pyraclostrobin & Metconazole (Headline AMP®)	Dermal (aerial overspray)	Recovery and mortality	24 h; 48 h	1052 mL/ha	Spray: 24 h - 82% recovery, 2% mortality; 48 h - 100% recovery, 3% mortality Reference: 24 h - 73% recovery, 0% mortality; 48 h - 98% recovery, 3% mortality	Cusaac et al. (2015)
South American common toad (*Rhinella typhonius*) Common lesser toad (*Rhinella granulosa*) Cane toad (*Rhinella marina*) Tungara frog (*Engystomops pustulosus*) Red-snouted treefrog (*Scinax ruber*) Emerald glass frog (*Centrolene prosoblepon*)	Juvenile	Glyphosate (Glyphos®) & adjuvant (Cosmo-Flux®)	Dermal (overspray)	LC1, LC50, mortality	96 h	1.85 - 29.52 kg a.e./ha	LC1: 1.56 kg a.e./ha; LC50: 14.8 kg a.e./ha LC50: 6.5 kg a.e./ha LC1: 5.08 kg a.e./ha; LC50: 22.8 kg a.e./ha LC1: 7.02 kg a.e./ha; LC50: 19.6 kg a.e./ha LC1: 0.32 kg a.e./ha; LC50: 7.3 kg a.e./ha LC1: 1.97 kg a.e./ha; LC50: 4.5 kg a.e./ha	Bernal et al. (2009)

Table 2-1: (*continued*)

Species	Life Stage	Chemical	Route	Endpoint	Time	App Rate	Results	Reference
Banded robber frog (*Pristimantis taeniatus*) Yellow-striped poison frog (*Dendrobates truncatus*)	Adult	Glyphosate (Glyphos®) & adjuvant (Cosmo-Flux®)	Dermal (overspray)	LC1, LC50, mortality	96 h	1.85 - 29.52 kg a.e./ha	LC1: 1.93 kg a.e./ha; LC50: 5.6 kg a.e./ha LC1: >7.38 kg a.e./ha; LC50: >7.38 kg a.e./ha	Bernal et al. (2009)
Blanchard's cricket frog *(Acris blanchardi)*	Juvenile	Pyraclostrobin (Headline®) Pyraclostrobin & Metconazole (Headline AMP®)	Dermal (overspray)	LC50, mortality	96 h	880 mL/ha: 0.25 - 4x 1052 mL/ha: 0.25 - 4x	LC50: 2.1 µg pyraclostrobin/cm^2 LC50: 1.52 µg pyraclostrobin/cm^2	Cusaac et al. (2016)
American toad *(Bufo americanus)*	Juvenile	Dimethoate (Roxion®)	Dermal (paper towel)	Feeding behavior, weight, mortality	24 h	100 mL/ha	40% mortality	Webber et al. (2010)
Woodhouse's toad (*Bufo woodhouse*)	Adult	Malathion Malathion & bacteria	Dermal (ventral)	Disease, hepatomegaly, mortality	30 d	1000 mL/ha 10000 mL/ha 0.0011 mg/g 0.011 mg/g	40% mortality 100% mortality (within 48 h) 80% hepatomegaly, clinical disease and mortality 100% hepatomegaly, clinical disease and mortality	Taylor et al. (1999)
Blanchard's cricket frog *(Acris blanchardi)*	Juvenile	Pyraclostrobin & Metconazole (Headline AMP®)	Dermal (overspray)	Body Burden	0.25 h 2 h 6 h 12 h 24 h	526 mL/ha	Pyraclostrobin: 235 ppb; metconazole:250 ppb Pyraclostrobin: 368 ppb; metconazole: 148 ppb Pyraclostrobin: 213 ppb; metconazole: 44 ppb Pyraclostrobin: 135 ppb; metconazole: <LOQ Pyraclostrobin: 191 ppb; metconazole: 45 ppb	Cusaac et al. (2016)
Eastern narrowmouth toad (*Gastrophryne carolinensis)*	Juvenile	Imidacloprid Triadimefon	Dermal (soil)	Body Burden	8 h	5.7 µg/cm^2 2.7 µg/cm^2	[Tissue] = 0.34 ppm [Tissue] = 0.42 ppm	Van Meter et al. (2014)
Barking treefrog *(Hyla gratiosa)*	Juvenile	Atrazine Fipronil Imidacloprid Pendimethalin Triadimefon	Dermal (soil)	Body Burden	8 h	22.9 µg/cm^2 1.1 µg/cm^2 5.7 µg/cm^2 19.8 µg/cm^2 2.7 µg/cm^2	[Tissue] = 6.11 ppm [Tissue] = 0.57 ppm [Tissue] = 0.39 ppm [Tissue] = 0.19 ppm [Tissue] = 0.21 ppm	Van Meter et al. (2014)

Table 2-1: (*continued*)

Species	Life Stage	Chemical	Route	Endpoint	Time	App Rate	Results	Reference
Fowler's toad *(Anaxyrus fowleri)*	Juvenile	Atrazine	Dermal (soil)	Body Burden	8 h	22.9 µg/cm^2	[Tissue] = 10.47 ppm	Van Meter et al. (2014)
		Fipronil				1.1 µg/cm^2	[Tissue] = 1.23 ppm	
		Pendimethalin				19.8 µg/cm^2	[Tissue] = 0.97 ppm	
		Triadimefon				2.7 µg/cm^2	[Tissue] = 2.82 ppm	
Northern cricket frog *(Acris creptians)*	Juvenile	Imidacloprid	Dermal (soil)	Body Burden	8 h	5.7 µg/cm^2	[Tissue] = 0.34 ppm	Van Meter et al. (2014)
		Triadimefon				2.7 µg/cm^2	[Tissue] = 1.68 ppm	
Gray treefrog *(Hyla versicolor)*	Juvenile	Atrazine	Dermal (soil)	Body Burden	8 h	22.9 µg/cm^2	[Tissue] = 8.42 ppm	Van Meter et al. (2014)
		Fipronil				1.1 µg/cm^2	[Tissue] = 0.46 ppm	
		Imidacloprid				5.7 µg/cm^2	[Tissue] = 0.19 ppm	
		Pendimethalin				19.8 µg/cm^2	[Tissue] = 0.05 ppm	
		Triadimefon				2.7 µg/cm^2	[Tissue] = 0.60 ppm	
Green treefrog *(Hyla cinerea)*	Juvenile	Atrazine	Dermal (soil)	Body Burden	8 h	22.9 µg/cm^2	[Tissue] = 3.75 ppm	Van Meter et al. (2014)
		Fipronil				1.1 µg/cm^2	[Tissue] = 0.21 ppm	
		Imidacloprid				5.7 µg/cm^2	[Tissue] = 0.13 ppm	
		Pendimethalin				19.8 µg/cm^2	[Tissue] = 0.16 ppm	
		Triadimefon				2.7 µg/cm^2	[Tissue] = 0.37 ppm	
Southern leopard frog *(Lithobates sphenocephala)*	Juvenile	Atrazine	Dermal (soil)	Body Burden	8 h	22.9 µg/cm^2	[Tissue] = 4.91 ppm	Van Meter et al. (2014)
		Fipronil				1.1 µg/cm^2	[Tissue] = 0.36 ppm	
		Pendimethalin				19.8 µg/cm^2	[Tissue] = 0.28 ppm	
		Triadimefon				2.7 µg/cm^2	[Tissue] = 0.12 ppm	
American toad *(Bufo americanus)*	Juvenile	Atrazine	Dermal (soil; OM = 3.1%)	Body Burden	8 h	22.9 µg/cm^2	[Tissue] = 1.95 ppm; Higher concentration on lower OM soil	Van Meter et al. (2016)
			Dermal (soil; OM = 14.1%)				[Tissue] = 0.60 ppm	
		Fipronil	Dermal (soil; OM = 3.1%)			1.1 µg/cm^2	[Tissue] = 0.16 ppm; Higher concentration on lower OM soil	
			Dermal (soil; OM = 14.1%)				[Tissue] = 0.10 ppm	
		Imidacloprid	Dermal (soil; OM = 3.1%)			5.7 µg/cm^2	[Tissue] = 0.04 ppm; Higher concentration on lower OM soil	
			Dermal (soil; OM = 14.1%)				[Tissue] = 0.04 ppm	
		Pendimethalin	Dermal (soil; OM = 3.1%)			69.8 µg/cm^2	[Tissue] = 3.69 ppm; Higher concentration on lower OM soil	
			Dermal (soil; OM = 14.1%)				[Tissue] = 2.95 ppm	
		Triadimefon	Dermal (soil; OM = 3.1%)			2.7 µg/cm^2	[Tissue] = 0.33 ppm; Higher concentration on lower OM soil	
			Dermal (soil; OM = 14.1%)				[Tissue] = 0.18 ppm	
Barking treefrog *(Hyla gratiosa)*	Juvenile	Atrazine	Dermal (soil)	Body Burden	8 h	22.9 µg/cm^2	[Tissue] = 6.11 ppm	Van Meter et al. (2015)
			Dermal (overspray)				[Tissue] = 12.59 ppm	
		Fipronil	Dermal (soil)			1.1 µg/cm^2	[Tissue] = 0.57 ppm	
			Dermal (overspray)				[Tissue] = 1.94 ppm	

Table 2-1: (*continued*)

Species	Life Stage	Chemical	Route	Endpoint	Time	App Rate	Results	Reference
Barking treefrog *(Hyla gratiosa)*	Juvenile	Imidacloprid	Dermal (soil) Dermal (overspray)	Body Burden	8 h	5.7 μg/cm^2	[Tissue] = 0.39 ppm [Tissue] = 1.08 ppm	Van Meter et al. (2015)
		Pendimethalin	Dermal (soil) Dermal (overspray)			19.8 μg/cm^2	[Tissue] = 0.19 ppm [Tissue] = 2.09 ppm	
		Triadimefon	Dermal (soil) Dermal (overspray)			2.7 μg/cm^2	[Tissue] = 0.21 ppm [Tissue] = 0.63 ppm	
Green treefrog (*Hyla cinerea*)	Juvenile	Atrazine	Dermal (soil) Dermal (overspray)	Body Burden	8 h	22.9 μg/cm^2	[Tissue] = 3.74 ppm [Tissue] = 20.46 ppm	Van Meter et al. (2015)
		Fipronil	Dermal (soil) Dermal (overspray)			1.1 μg/cm^2	[Tissue] = 0.21 ppm [Tissue] = 2.10 ppm	
		Pendimethalin	Dermal (soil) Dermal (overspray)			19.8 μg/cm^2	[Tissue] = 0.17 ppm [Tissue] = 2.76 ppm	
		Triadimefon	Dermal (soil) Dermal (overspray)			2.7 μg/cm^2	[Tissue] = 0.37 ppm [Tissue] = 1.70 ppm	
Spotted salamander (*Ambystoma maculatum)*	Juvenile	Atrazine (Aatrex®)	Dermal (paper towel)	Locomotor performance, growth, mortality	24 h	50 μg/L 500 μg/L	Endurance, growth, and survival were not affected	Mitchkash et al. (2014)
		Carbaryl (Sevin®)				200 μg/L 2000 μg/L	Fatigue increased; endurance, growth and survival were not affected	
Tiger salamander (*Ambystoma tigrinum*)	Adult	Malathion	Dermal (soil)	ChE activity, body burden	24 h	50 μg/cm^2	[Tissue] = 1.462 ppm; ChE suppressed 50-65%	Henson-Ramsey et al. (2008)
			Dermal (soil)		48 h	50 μg/cm^2	[Tissue] = 1.424 ppm; ChE suppressed 50-65%	
			Dermal (soil and dietary)		48 h	50 μg/cm^2	[Tissue] = 0.347 ppm; ChE suppressed 50-65%	
			Dermal (soil and dietary)		48 h	100 μg/cm^2	[Tissue] = 0.558 ppm; ChE suppressed 90%	
Northern slimy salamander *Plethodon glutinosus*	Juvenile & Adult	Malathion	Dermal (ground forest overspray)	Abundance, ChE	59 d	6 doses 5.6 kg/ha	No effect on abundance or ChE suppression	Baker (1985)
					92 d	10 doses 5.6 kg/ha	No effect on abundance or ChE suppression	

Table 2-1: (*continued*)

Species	Life Stage	Chemical	Route	Endpoint	Time	App Rate	Results	Reference
Red-backed salamander *Plethodon cinereus*	Adult	Malathion	Dermal (filter paper)	ChE, feeding and endurance behavior	25 d	2 doses 2.24kg/ha	5.4% ChE suppression; behavior was not affected	Baker (1985)
						2 doses 5.6 kg/ha	8.9% ChE suppression; behavior was not affected	
						2 doses 8.97 kg/ha	18.7% ChE suppression; behavior was not affected	
Northern slimy salamander *Plethodon glutinosus*	Adult	Malathion	Dermal (filter paper)	ChE, feeding and endurance behavior	43 d	3 doses 5.6 kg/ha	ChE was suppressed 34.1%; behavior was not affected	Baker (1985)

*App rate = application rate; TL50 = median tolerated limit (concentration required to kill 50% of a population in 36 h); [Tissue] = body burden concentration; AI = active ingredient; a.e. = acid equivalent; LC1 = concentration that will kill 1% of the population; LC50 = concentration that will kill 50% of the population; ChE = cholinesterase

CHAPTER 3

ANALYSIS OF PESTICIDES IN SURFACE WATER, STEMFLOW, AND THROUGHFALL FROM AGRICULTURAL USE IN SOUTHERN GEORGIA, USA[1]

[1] Glinski, D.A., S.T. Purucker, R. J. Van Meter, M.C. Black, and W.M. Henderson. To be submitted to *Chemosphere*.

Abstract

To study spray drift contributions to non-targeted habitats, pesticide concentrations in stemflow (water flowing down the trunk of a tree during a rain event), throughfall (water from tree canopy only), and surface water in an agriculturally impacted wetland area near Tifton, Georgia, USA were measured (2015–2016). Agricultural fields and sampling locations were located on the University of Georgia's Gibbs research farm. Samples were analyzed for over 150 pesticides, and thirty-two different pesticides were detected in these matrices. Data indicate that herbicides and fungicides, are present in all these environmental samples, while insecticides were only detected in surface water samples. The highest concentration observed was 10.50 µg/L for metolachlor in an August 2015 surface water sample. Metolachlor, tebuconazole, and fipronil were the most frequently detected herbicide, fungicide, and insecticide, respectively, regardless of sample origin. Overall, the most frequently detected pesticide in surface water and stemflow samples was metolachlor (0.09–10.5 µg/g); however, the most commonly detected pesticide in throughfall was biphenyl (0.02–0.07 µg/g). These data help assess the importance of indirect spray drift exposures to non-targeted habitats by assessing inputs from stemflow and throughfall into surface waters.

Introduction

In 2007, approximately 5.2 billion pounds of pesticides were used worldwide and of those, 1.1 billion pounds were applied on U.S. soils (Grube et al., 2011). Along with detectable concentrations of pesticides in surface waters, pesticides have also been found globally in rainwater as well (Hüskes and Levsen, 1997; Ahmed et al., 1998; McConnell et al., 1998; Coupe et al., 2000; review in Dubus et al., 2000; Goel et al., 2005; Sauret et al., 2009; Zhang et al., 2011; Potter et al., 2014; Rice et al., 2016; Potter and Coffin, 2017). Many researchers have monitored pesticides in rainfall, where concentrations have ranged up to 22.9 µg/L near agricultural sites (Coupe et al., 2000). Frequently, in rainwater, herbicides were found to be more prevalent in samples (46–61%) and at higher concentrations compared to other insecticides and fungicides (Trevisan et al., 1993; Goel et al., 2005).

Spray drift is considered to be the amount of pesticide that does not reach the intended application zone, due to the wind transporting it elsewhere (Briand et al., 2002; Vischetti et al., 2008). Pesticide pray drift contributes to the contamination of surface waters and other non-targeted areas. Many parameters can affect spray drift like climatic conditions, land topography, as well as spray characteristics such as droplet diameter, viscosity of the formulation, and spraying technique (Briand et al., 2002; Vischetti et al., 2008). Due to variations in climatic conditions, pesticide spray drift may contaminate areas 10–100 meters away from application sites (de Snoo and de Wit, 1998). Even in the presence of a 20-meter buffer zone surrounding agricultural fields, spray drift can still pollute the surrounding area (Cunha et al., 2012).

Throughout the world, 40% of pesticides used are herbicides while 33% are insecticides. Many of these compounds can be readily volatized after application due to their physical properties (Stokstad and Grullón, 2013). Volatilization coupled with spray drift likely results in

many agriculturally-applied pesticides being transported throughout the atmosphere resulting in quantifiable amounts in rainwater far away from application sites (Hüskes and Levsen, 1997; Ahmed et al., 1998; Coupe et al., 2000). Gish et al. (2011) determined that volatilization of herbicides occurred immediately after application and consequences of this was still measurable days (>5 days) later. It has been estimated that, on average, within a few days of application, 23–65% of metolachlor can volatilize based on climatic conditions (Gish et al., 2011). While for other current-use pesticides, up to 90% of the total mass applied can become volatized following agricultural use (LeNoir et al., 1999).

Following application and subsequent volatilization, long-range atmospheric transport of pesticides can occur. These compounds can traverse many miles and have been found in remote regions, such as the Arctic (Cotham and Bidleman, 1991; Rice and Chernyak, 1997). Conveyance of pesticides to the atmosphere can be caused by spray drift, volatilization, runoff, and wind erosion of soil (Briand et al., 2002). Within environmental matrices, both runoff and volatilization have been investigated for pesticide loss, and runoff can account for less than 3% and volatilization approximately 2–25% (Gish et al., 2011). Atmospheric transport has also been shown to deposit pesticides to nearby pristine areas and it has been well documented that pesticides applied in the agriculturally intensive Central Valley of California have been transported and deposited into the Sierra Nevada mountain range (McConnell et al., 1998; LeNoir et al., 1999). Of note, in Brazil, tebuthiuron, a herbicide, had concentrations four times higher in a riparian forest potentially due to air pollution concentrating the pesticide in the tree canopy and being released into the soil during rain events (Bicalho et al., 2010).

Pesticide residues can enter the aquatic system through precipitation, leaching, spray drift, discharge of wastewater, and other routes (Griffini et al., 1997; Konstantinou et al., 2006;

Rice et al., 2016). When a rain event occurs, pesticides are washed off trees and their surrounding foliage resulting in both stemflow (precipitation that flows down along the tree trunk) and throughfall (rain that has passed through the tree canopy). These routes of environmental release have been well studied and overall, higher concentrations of pesticides are frequently determined in stemflow and throughfall samples compared to rainfall (Bernhardt and Ruck, 2004; Rice et al., 2016). This trend is likely due to the fact that stemflow will encounter more arboreal surface area and for a longer time when compared to throughfall (Rice et al., 2016). Additionally, the pesticides studied in Bernhardt and Ruck (2004) were detected in stemflow samples for a longer period of time compared to rainfall due to the trees filtering contaminants from the atmosphere. Furthermore, large pulses of pesticides are known to flow into rivers or surface waters after spray application that coincided with rain events (Thurman et al., 1991; Griffini et al., 1997; Konstantinou et al., 2006; Rice et al., 2016). Thus, understanding the concentrations of pesticides entering aquatic habitats through these routes is paramount in quantifying the cumulative exposure and risk associated with spray drift.

In a recent survey analyzing streams throughout the U.S., the occurrence rate was over 90% for detecting one or more pesticides in agricultural, urban or mixed use watersheds matrices (Gilliom et al., 2006). The most frequently encountered herbicides in these waters were atrazine, glyphosate, AMPA (a metabolite of glyphosate) and metolachlor (Gilliom et al., 2006; Battaglin et al., 2016). In Smalling et al. (2012), 24 pesticides were detected in water samples collected from several states throughout the U.S. The most frequently detected pesticides were again herbicides, mainly atrazine, glyphosate and AMPA. However, Battaglin et al. (2016) reported that fungicides were more frequently detected in surface water samples collected throughout the U.S. when compared to insecticides and herbicides, which is likely due to their more frequent

application schedules given their lower application rates. Regardless, concentrations of pesticides in surface waters tend to follow seasonal variation where higher values are observed during periods of heavy use, such as in spring and summer due to preparation for planting, and lower values in winter and after crop harvesting (Konstantinou et al., 2006).

To date, there are limited studies that have examined pesticides in stemflow and throughfall, along with how these concentrations impact surface water concentrations (Trevisan et al., 1993; Bernhardt and Ruck, 2004; Zhang et al., 2011; Rice et al., 2016). The overall objective of this study was to identify and quantify pesticides in surface water, stemflow and throughfall adjacent to agricultural fields over the course of a year (February 2015 through January 2016). Following quantification of these values, we compare the stemflow to throughfall at three paired sites after rain events, and estimate residence time of detected pesticides. This research will ultimately inform numerous regulatory aspects of pesticide application such as environmental residence time in rainwater, surface waters/rivers or the surrounding foliage that is impacted by spray drift and the bearing it can have on non-target organisms inhabiting these areas.

Materials and Methods

Chemicals

Pesticides (>150) were purchased in the GC multiresidue pesticide kit from Restek (Bellefonte, PA). Several additional pesticides were obtained from the U.S. EPA National Pesticides Standard Repository (Fort Meade, MD). All solvents and analytical reagents used were HPLC grade and obtained from Fisher Scientific at highest purity (Pittsburgh, PA).

Surface Water Collection

Monthly surface water samples were collected from ten different locations throughout Gibbs Research Farm in Tifton, GA from February 2015–January 2016 (Figure 3-1). Gibbs Farm is an agricultural research facility currently used by the University of Georgia (UGA) and the U.S. Department of Agriculture (USDA). In southwest Georgia, the most prominent crops are cotton and peanuts, although corn and soybeans are also farmed there (personal communication, Thomas Potter, USDA). Corn, peanuts, and cotton were visually identified on the dates that water samples were collected. One liter of water was obtained from each site, after 3 pre-rinses with surface water in glass amber jars. Duplicate 4 L samples were collected at sites 4, 5 and 8 to allow for additional detection of pesticides due to their proximity to application and cropped fields. All samples were immediately capped, labeled and placed in a cooler on ice. Once back at the U.S. EPA lab, all samples were placed in the refrigerator at 4°C until processed as described below (<2 weeks).

Stemflow and Throughfall Collection

Stemflow and throughfall samples were collected on a rain-event basis at the Gibbs Farm in Tifton, GA from March 2015 – January 2016. For stemflow collection, PVC clear vinyl tubing was wrapped ~2.5 times around a tree at three different locations throughout Gibbs Farm (adapted from Williams, 2004). These setups were adjacent to sites 3, 4, and 7, where the diameter at breast height (DBH) was 11.46, 11.46 and 13.05 inches, respectively (see Figure 3-1). Trees were selected based on distance to field, DBH, and the presence of no overlapping tree canopies. Tubing was attached to the tree using nails and silica adhesive glue with the top half removed resulting in the top portion being open for collection (adapted from Willams, 2004).

Stemflow was collected in 25 L carboys to minimize environmental/animal interactions with samples. Stemflow samples were also collected on an event basis, when rainfall was greater than 0.5 mm (>100 mL).

Throughfall was collected under the same tree canopy that was used for stemflow, with a large funnel attached to PVC pipe and tubing that was connected to a 20 L carboy (adapted from Willams, 2004). The funnel was covered with a screen mesh to remove large debris (i.e. leaves and sticks). After each rain event, stemflow and throughfall subsamples were transferred to a pre-labeled 1 L amber jar with the date and volume recorded and stored in a refrigerator at 4 °C until processed as described below.

Extraction Procedure

All water samples were filtered through a 0.45 µm GF/F filter paper using a filtration apparatus. The entire sample was passed through a pre-conditioned C18 solid phase extraction (SPE) cartridge (Oasis HLB 6cc, 500mg) at a rate of 10 mL min^{-1} that was attached to a vacuum manifold. The SPE was dried under vacuum for 45 minutes and then eluted sequentially with 6 mL each of methanol and dichloromethane into a glass disposable cell culture tube. The sample was gently evaporated under nitrogen gas and then reconstituted in 1 mL of ethyl acetate and transferred to a 2 mL vial. All samples were analyzed on a GCxGC-ToF/MS as described below.

GCxGC-ToF/MS Analysis

All water samples were analyzed on a LECO Pegasus® 4D GCxGC-ToF/MS equipped with a Rtx-CLPesticides II (30 m, 0.25 µm thickness, and 0.32 mm ID; Restek, Bellefonte, PA) primary column and a Rxi-17Sil MS (2 m, 0.15 µm thickness, and 0.15 mm ID; Restek,

Bellefonte, PA) secondary column following the method in de Koning and Gumpendobler (2007). All injections (4 µL) were made in splitless mode. The inlet, transfer line and source temperatures were 275 °C, 250 °C and 225 °C, respectively. Helium was used as the carrier gas and maintained at a constant pressure of 26 psi during the run. The primary oven's initial temperature was 95 °C and held for 5 min, ramped 10 °C min^{-1} to 200 °C followed by a ramp of 7 °C min^{-1} to 270 °C with a final ramp of 10 °C min^{-1} to 320 °C that was held for 10.5 minutes. While the secondary oven's initial temperature was 105 °C for 6 min ramped 10 °C min^{-1} to 360 °C and held for 15 min. The modulator offset was 30 °C higher than the primary oven and the modulation period for liquid nitrogen was 5 seconds with a hot pulse at 0.6 seconds. Standards containing over 150 pesticides were analyzed to develop a data processing method and a calibration method to compare all field samples against (Figure 3-2A) based on the protocols supplied by the GCxGC-ToF/MS manufacturer's guidelines. The standards were all reprocessed using the final data processing method to obtain concentrations for the corresponding pesticides against the calibration curve (Table 3-1S).

Statistical Analysis

We tested to see whether field observations of stemflow concentrations were greater than throughfall concentrations. Samples were paired across three locations and 18 different sampling dates, therefore, up to 54 observations for each of the 32 pesticides. However, many samples were non-detects in both stemflow and throughfall samples, so a non-parametric Wilcoxon Rank Sum test was implemented. Other tests requiring distribution assumptions (e.g., t-test) were considered not practical due to non-detects and data skewness. The method used to calculate the signed-rank test is by Wilcoxon (1945), which discards any tied data and then

calculates the signed ranks. We used the wilcox.test with ties (paired = TRUE, alternative = 'two.sided', exact = FALSE) from the R stats package (R Core Team, 2017) with the test being run on pooled pair-wise comparisons across all chemicals, date and sample locations.

Results and Discussion

All Matrices Sampled

A standard mixture of over 150 pesticides was analyzed at the beginning of each set of samples to process all field samples against (Table 3-1S). There were, in total, 32 different pesticides identified and quantified in all three environmental matrices. Overall, ten herbicides, eleven fungicides, five insecticides, five pesticide degradates, and one bird repellent detected across all water samples (Table 3-1). These data are similar to Battaglin et al. (2016), where more fungicides were detected than herbicides and insecticides. In this study, metolachlor was the most frequently detected herbicide with a detection frequency of 86% in surface water, 90% in stemflow, and 78.8% in throughfall (Figure 3-2B). The highest concentration of metolachlor determined was 10.50 µg/L, and observed in surface water in August. Interestingly, metolachlor is applied in southern Georgia as a pre-emergent herbicide in late April to early May on peanuts and cotton via a tractor boom pivot (personal communication, Thomas Potter, USDA). Tebuconazole was the second highest detected pesticide as well as the most frequently detected fungicide with frequencies of 62% in surface water, 30% in stemflow, and 83% in throughfall samples (Figure 3-2C). The highest concentration detected for tebuconazole was 1.8 µg/L in an August throughfall sample. Tebuconazole is one of the most commonly applied fungicides in southern Georgia with application beginning in mid-June about one month after planting, and can be applied for a total of 7–8 times using tractor boom pivots (personal communication,

Thomas Potter, USDA). However, data was not available for the exact timing of pesticide application(s).

Co-occurring pesticides were frequently observed in the water samples analyzed, with more than one pesticide being present in over 80% of surface water and stemflow samples. Over 94% of the throughfall samples contained at least two pesticides. Similar to our data, Gilliom et al. (2006) also detected two or more pesticides over 90% of the time in urban, agricultural and mixed-use streams. These results further demonstrate that mixtures of pesticides are constantly present in the environment. Furthermore, in this study, over 35% of the time more than 5 pesticides were detected in surface water, while for stemflow and throughfall this occurred 8% and 55% of the time, respectively. Dubus et al. (2000) noticed that, on average, two or more pesticides were detected in rainwater samples in Europe. However, Potter and Coffin (2017) reported, the median number of detections for pesticides in rainwater near Tifton, GA during 2007–2009 was 6, which was higher than our stemflow (2.6) and throughfall (4.4) detects. These results demonstrate that pesticide mixtures are present in several different matrices, making it difficult to determine the potential adverse effects that these compounds will have on non-target species.

Surface Water Samples (1 L)

Metolachlor was detected the most frequently in nine out of ten surface water sites (Figure 3-2B); the exception was site 7 where 2-phenylphenol and flutolanil were more frequently detected. For eight of the twelve months in the study period, metolachlor had the highest concentration relative to all other pesticides. However, metalaxyl had the highest concentration for the months of September and January, while atrazine and ethalfluralin (both

herbicides) had the highest concentration for May and December, respectively. Interestingly, metalaxyl a fungicide, is not known to be used on the crops located at our collection sites, and its detection possibly arises from agricultural use on adjacent fields.

At site one, the highest concentration detected was 0.29 µg/L for flutolanil (a fungicide) in July. Metolachlor had the highest concentrations at sites two (0.32 µg/L), three (10.50 µg/L), four (5.48 µg/L), five (5.14 µg/L), six (1.12 µg/L), eight (2.2 µg/L), nine (4.09 µg/L), and ten (8.92 µg/L) for the months of April, August, August, August, September, August, August and April, respectively. At site seven, flutolanil had the highest concentration in August (0.34 µg/L). The sites and months were ranked by total concentrations per site and month with site 10 and the month of August having pesticide total concentrations of 39.62 µg/L and 39.67 µg/L, respectively. However, January and site two had the fewest number of detections and lowest overall concentration from cumulative ranked data (5.28 µg/L) and place (1.48 µg/L).

Metolachlor and tebuconazole had the two highest frequencies of detection and were plotted against time for each site (Figures 3-3A and 3-3B) to investigate temporal trends. The initial application time for metolachlor appears to be between the sampling dates in July and August (Fig 3-3A), however, surface water samples were only collected monthly so an estimation of the exact application date is difficult. An initial spike in concentration was observed (Figs. 3-3A and 3-3B) that can be assumed to result from application. This spike was followed by a steady decline in pesticide concentration at each site, resulting in both metolachlor and tebuconazole total concentrations diminishing over time. Since all the sites were interconnected this potentially leads to some concentrations being observed in sites that earlier had no detectable levels, due to water moving from one site to next with the use of weirs and drainage pipes (Figure 3-1).

In several countries throughout Europe pesticide concentrations were observed to be higher in surface water samples after rain events (Griffini et al., 1997; Konstantinou et al., 2006). This same occurrence pattern was also observed within the U.S. throughout the Midwestern states by Thurman et al. (1991) and more recently in Maryland by Rice et al. (2016). However, in the current study, pond water samples were only collected once a month so it was not feasible to be able to observe each rain event's effect on pesticide concentration at each site. On the other hand, total precipitation between each sampling date was calculated and the highest amount of precipitation that fell was between July and August (6.05 inches, data not shown). This also corresponds to the highest total concentration of metolachlor being observed in the month of August (32.2 µg/L) compared to July (5.03 µg/L) or September (10.99 µg/L). Therefore, after the summation of all the rain events, the highest amount of metolachlor was observed in the surface waters for the month of August, which agrees with previous studies. Griffini et al. (1997) observed their highest concentration of metolachlor (3.68 µg/L) after an intense rainfall event; however, in the current study our value of metolachlor was at least three times higher. This demonstrates that metolachlor application as a pre-emergent in April to May, can lead to the occurrence of spray drift and subsequently runoff into the nearby surface waters. The highest concentrations of metolachlor in surface waters were observed after the greatest amount of precipitation.

Surface Water Samples (4 L)

In addition to collecting 1 L surface water samples, 4 L samples were collected at sites 4, 5 and 8 to further aid in identifying pesticides present at low concentrations. Compared to the 1 L surface water samples, 19 pesticides were detected in 4 L samples, with biphenyl being the

only additional pesticide detected. Cumulative concentrations of pesticides were plotted for each month and site with an increase in concentration and number of pesticides were detected from June–August (Figure 3-4). The highest number of pesticide detects was in July for site 4 with eight pesticides, while sites 5 and 8 had the highest number of pesticides in June with 12 and 9 detects, respectively. This same trend was also observed for 1 L surface water samples, with a spike in pesticide concentration observed during pesticide application and then a gradual decline over time throughout the winter months. The pesticide that contributed the most was metolachlor which was detected in 34 out of 35 samples with the highest concentration observed in August at 10.54 µg/L, however for site 8 in July no sample was collected. Tebuconazole had the second highest frequency of detects similar to the 1 L surface water samples, with concentrations that appear in April and a maximum observed in August (1.1 µg/L) followed by a gradual decline in winter months.

Stemflow Samples

Stemflow is rainwater that travels down the trunk of a tree. This can lead to higher pesticide concentrations as it washes tree surface area with significant concentrations due to spray drift. Desethyl atrazine (DEA), a metabolite of atrazine, had the highest concentration quantified at 9.54 µg/L in a stemflow sample from May. Out of the two metabolites for atrazine, DEA and DIA, DEA was detected more frequently than DIA, in accordance with several other studies that also had higher concentrations of DEA measured in surface waters, subwatersheds, surface runoff, stream, and groundwater samples (Thurman et al., 1991; Shipitalo and Owens, 2003; Gilliom et al., 2006; Hively et al., 2011). This trend is likely due to the fact that DIA is more labile than DEA (Thurman et al., 1991). The presence of bacteria, mosses, and fungi on

leaf and stem surfaces can biodegrade and metabolize pesticides (Brinkmann, 1983). This results in the appearance of the metabolite DEA from the parent compound atrazine, which had higher concentrations and frequencies of detection compared to atrazine.

Additionally, the May stemflow sample from site 4 had the highest concentration of pesticides throughout the year totaling 12.78 µg/L. While at site 3, the rain event with the highest combined concentration of pesticides was in June at 3.92 µg/L and site 7 had 3.03 µg/L in September. Trevisan et al. (1993), found pesticides in nine out of the 25 stemflow samples that were analyzed, including four different herbicides and an organophosphate. Atrazine was found in five stemflow samples with the highest concentration at 1.99 µg/L (Trevisan et al., 1993). While in the current study, out of these five compounds, only atrazine was detected nine times with the highest concentration at 3.65 µg/L. However, in Bernhardt and Ruck (2004) only four herbicides were analyzed in stemflow samples, where metolachlor had the highest concentration in May at 48 ng/L, 54 times lower than the current study which observed the highest stemflow concentration of metolachlor in September at 2.63 µg/L. Overall, stemflow concentrations were 4 to 64 times higher in the current study compared to Trevisan et al. (1993) and Bernhardt and Ruck (2004). On the other hand, both atrazine and metolachlor were observed to have similar concentrations as the year 1 data from Rice et al. (2016); however, subsequent years had concentrations 2 to 4 times higher than the current study.

Throughfall Samples

Throughfall is rainwater that comes in contact with the leaf surfaces of the tree canopy only, therefore it is the foliage capture of pesticides from spray drift. Atrazine had the highest concentration in throughfall samples (7.09 µg/L), observed in May (Figure 3-2D). Overall,

throughfall measured at site 3 had the highest concentrations observed (9.38 µg/L), while site 4 atrazine was measured at 4.25 µg/L on the same day. July had the highest summed concentration for throughfall at site 7 at 5.01 µg/L. In this study, 13 pesticides were detected in throughfall. In Trevisan et al. (1993) the authors detected ten various pesticides in twenty different samples, diazinon being the most frequently detected. In Bernhardt and Ruck (2004), throughfall was only collected for three events were herbicides were quantified. In Zhang et al. (2011) the authors measured organochlorine fluxes for hexachlorobenzenes, hexachlorocyclohexanes, and DDT (with its metabolites) and concluded that throughfall accounted for 10% of the mass found in runoff. Even though organochlorines were not detected in our current study, throughfall could result in other detectable pesticides contributing to runoff and subsequently higher concentrations in surface water. Rice et al. (2016) only analyzed throughfall samples for the herbicides atrazine and metolachlor for four years after application. They stated that higher concentrations of metolachlor were observed compared to atrazine presumably due to metolachlor's higher Koc (organic carbon adsorption coefficient) value which resulted in this pesticide being more likely to be retained in the tree canopy during days without rain compared to atrazine. This was not observed in our study, where atrazine and its metabolite DEA had on average, higher concentrations than metolachlor, however, this could be due to different application rates of the two pesticides in southern Georgia.

Stemflow vs Throughfall

Concentrations for metolachlor and tebuconazole in stemflow and throughfall were plotted against time (Figures 3-5A and 3-5B). Stemflow samples of metolachlor were higher compared to throughfall samples for the majority of the samples. This same phenomenon was

also observed by Rice et al. (2016), where they noted that the tree canopy acted as a filtering mechanism for these pesticides. Due to the higher surface area and longer period of time that rainwater is in contact with the tree to create stemflow, higher concentrations would be expected (Rice et al., 2016). This occurrence is not specific to pesticides; in addition to pesticides, heavy metals and ions, especially nitrates, exhibited higher concentrations, up to 4 times, in stemflow soil (soil that is impacted by stemflow rainwater) around trees compared to distal areas (Marchionni et al., 1999; Chang and Matzner, 2000; Morselli et al., 2004). Furthermore, in the current study, metolachlor was still detectable weeks to months post-application in stemflow samples compared to throughfall samples. However, when examining all the pesticides that were detected in throughfall and stemflow samples, throughfall samples were higher than stemflow samples 172 times (p =0.0013; Figure 3-6), while stemflow samples were higher than throughfall samples 90 times. This is contrary to Rice et al. (2016) where directly after application, higher concentrations were observed in stemflow than throughfall. In the current study, stemflow and throughfall samples were collected for an entire year, including pre-application rain events. These pre-application rain events could skew the results so that throughfall samples appeared to be higher than stemflow, while directly after application, stemflow could have higher concentrations. Furthermore, it was quite possible for the first rain event after pesticide application to result in a spike of pesticides for stemflow samples resulting in an initial flush of higher concentrations followed by a large volume of rainfall with low, diluted concentrations. This is in contrast to a corresponding throughfall sample where the rainwater would still interact with foliage and have elevated pesticides concentrations.

GCxGC-ToF/MS Analysis

Most previous research efforts that have examined pesticides in rainwater, stemflow and throughfall had been quantifying chemicals as a targeted approach. In Trevisan et al. (1993), the authors targeted 12 pesticides, analyzing for five pesticides using liquid chromatography (LC), while the other seven were analyzed on a gas chromatograph (GC). Bernhardt and Ruck (2004), utilized LC-MS in selected ion monitoring (SIM) mode to analyze for 18 pesticides in both negative and positive mode. Additionally, Zhang et al. (2011) targeted 11 organochlorine pesticides on GC, while Rice et al. (2016) was only targeting two herbicides on a LC-MS/MS, demonstrating that the targeted approach does not necessarily express all the pesticides that can be observed in a given matrix. In the current study, we utilized a non-targeted approach analyzing for over 150 pesticides using GCxGC-ToF/MS. GCxGC enables two compounds to separate out based on a second varying phase column. A non-targeted approach was useful in this study by enabling us to identify other pesticides that were not directly applied within the Gibbs Farm research fields. Therefore, in addition to the known pesticides that were applied during our sampling, other pesticides that could have entered the study site due to spray drift, volatilization, and other atmospheric transport mechanisms would be detected and quantified demonstrating that these processes occur in the environment and need to be taken into consideration for pesticide risk assessment. Furthermore, a time of flight mass spectrometer was utilized because it can capture all the ions that hit the detector at any given time, instead of scanning for a specific *m/z* value. In a pilot study, analyzing pesticides in rainwater, stemflow and throughfall, we only targeted six pesticides, however, if we had continued with these select six pesticides for this current study many compounds would have been omitted, and this would not have been an accurate assessment of spray drift within agricultural fields in southern

Georgia. Therefore, this instrumentation allowed us to maximize our efforts in identifying pesticides within these environmental matrices.

Conclusions

Overall, metolachlor was the most frequently detected pesticide in all surface water and stemflow samples, while biphenyl was more commonly found in throughfall. Tebuconazole and metolachlor were the most frequently detected fungicide and herbicide, respectively, in all the matrices analyzed. Stemflow samples had lower concentrations than throughfall samples for most of the pesticides that were analyzed in this study, possibly due to higher volumes of water collected at stemflow samplers resulting in a dilution effect. Pesticides from spray drift can accumulate on trees in buffer zones resulting in high concentrations of both parent and metabolites in stemflow and throughfall. Spray drift can result in subsequent rain events washing pesticides off trees in buffer zones leading to higher concentrations being observed in nearby streams and wetlands where non-targeted habitats become contaminated. Thus, most of the surface water samples had pesticides in them, due to the pesticides moving through forested wetlands.

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Table 3-1: Data processing parameters used for GCxGC-ToF/MS data analysis.

Parameters Used for GCxGC-TOF/MS	
Baseline Offset	1
Number of Data Points Averaged for Baseline Smoothing	Auto
1st Dimension Peak Width for Peak Finding	12
Match Required to Combine Peaks in the Second Dimension	650
2nd Dimension Peak Width for Peak Finding	0.15
Minimum SNR for Subpeak to be Retained	6
Integration Approach	Traditional
Minimum S/N Ratio for Peak Finding	50
Minimum Number of Apexing Masses for Peak Finding	2
Library Search Mode	Normal, Forward
Library Hits Returned per Peak	10
Minimum/Maximum Molecular Weight Allowed	0-800
Relative Mass Threshold for Library Searching	5
Minimum Similarity Match Before Name is Assigned	600
Libraries Used for Searching	NIST 2014
Mass Used for Area/Height Calculation	Unique

Table 3-2: Pesticide type, detection frequency and maximum concentration in surface water, stemflow and throughfall samples from Gibbs Farm in Tifton, G.

Compound	Type	Detection Frequency (%)			Maximum Concentrations		
		Surface water (n =114)	Stemflow (n=50)	Throughfall (n=52)	Surface water µg/L	Stemflow µg/L	Throughfall µg/L
2-Phenylphenol	Fungicide	28.1	30.0	44.2	0.28	0.68	0.55
Acetochlor	Herbicide	6.1	10.0	34.6	0.46	0.95	3.05
Alachlor	Herbicide	25.4	<LOD	<LOD	1.40	<LOD	<LOD
Anthraquinone	Bird Repellent	14.0	8.0	9.6	0.29	2.88	0.26
Atrazine	Herbicide	10.5	20.0	32.7	1.65	3.65	7.09
Benfluralin	Herbicide	0.9	<LOD	<LOD	0.11	<LOD	<LOD
Bifenthrin	Insecticide	1.8	2.0	<LOD	0.14	0.08	<LOD
Biphenyl	Fungicide	<LOD	26.0	84.6	<LOD	1.34	0.07
Chlorothalonil	Fungicide	4.4	<LOD	<LOD	0.33	<LOD	<LOD
Cyprodinil	Fungicide	10.5	<LOD	<LOD	0.38	<LOD	<LOD
DEA	Degradate	3.5	22.0	5.8	1.25	9.54	1.55
Diazinon	Insecticide	0.9	<LOD	<LOD	0.18	<LOD	<LOD
Diphenylamine	Fungicide	1.8	6.0	<LOD	0.16	0.21	<LOD
Endosulfan Ether	Degradate	14.9	<LOD	<LOD	0.14	<LOD	<LOD
Endosulfan Lactone	Degradate	21.1	<LOD	1.9	0.83	<LOD	0.07
Endosulfan Sulfate	Degradate	1.8	<LOD	<LOD	0.19	<LOD	<LOD
Ethalfluralin	Herbicide	1.8	<LOD	<LOD	0.74	<LOD	<LOD
Fipronil	Insecticide	4.4	<LOD	<LOD	0.19	<LOD	<LOD
Fludioxonil	Fungicide	<LOD	2.0	3.8	<LOD	0.40	0.56
Flutolanil	Fungicide	20.2	30.0	53.8	0.35	0.38	0.63
Malathion	Insecticide	0.9	<LOD	<LOD	0.11	<LOD	<LOD
Metalaxyl	Fungicide	33.3	<LOD	1.9	5.76	<LOD	0.42
Metolachlor	Herbicide	86.0	90.0	78.8	10.50	2.63	2.98
Myclobutanil	Fungicide	4.4	<LOD	<LOD	0.23	<LOD	<LOD
Oxadiazon	Herbicide	<LOD	<LOD	1.9	<LOD	<LOD	0.04
Oxyfluorfen	Herbicide	4.4	<LOD	<LOD	0.35	<LOD	<LOD

Table 3-2: (*continued*)

Compound	Type	Detection Frequency (%)			Maximum Concentrations		
		Surface water (n =114)	Stemflow (n=50)	Throughfall (n=52)	Surface water µg/L	Stemflow µg/L	Throughfall µg/L
Pendimethalin	Herbicide	1.8	<LOD	<LOD	0.20	<LOD	<LOD
Piperonyl butoxide	Insecticide, synergist	1	<LOD	<LOD	0.23	<LOD	<LOD
Propyzamide	Herbicide	1	<LOD	<LOD	0.15	<LOD	<LOD
Tebuconazole	Fungicide	62	30	83	0.48	1.64	1.80
Tetrahydrophthalimide	Degradate	1	<LOD	<LOD	0.16	<LOD	<LOD
Triadimefon	Fungicide	8	<LOD	<LOD	0.20	<LOD	<LOD

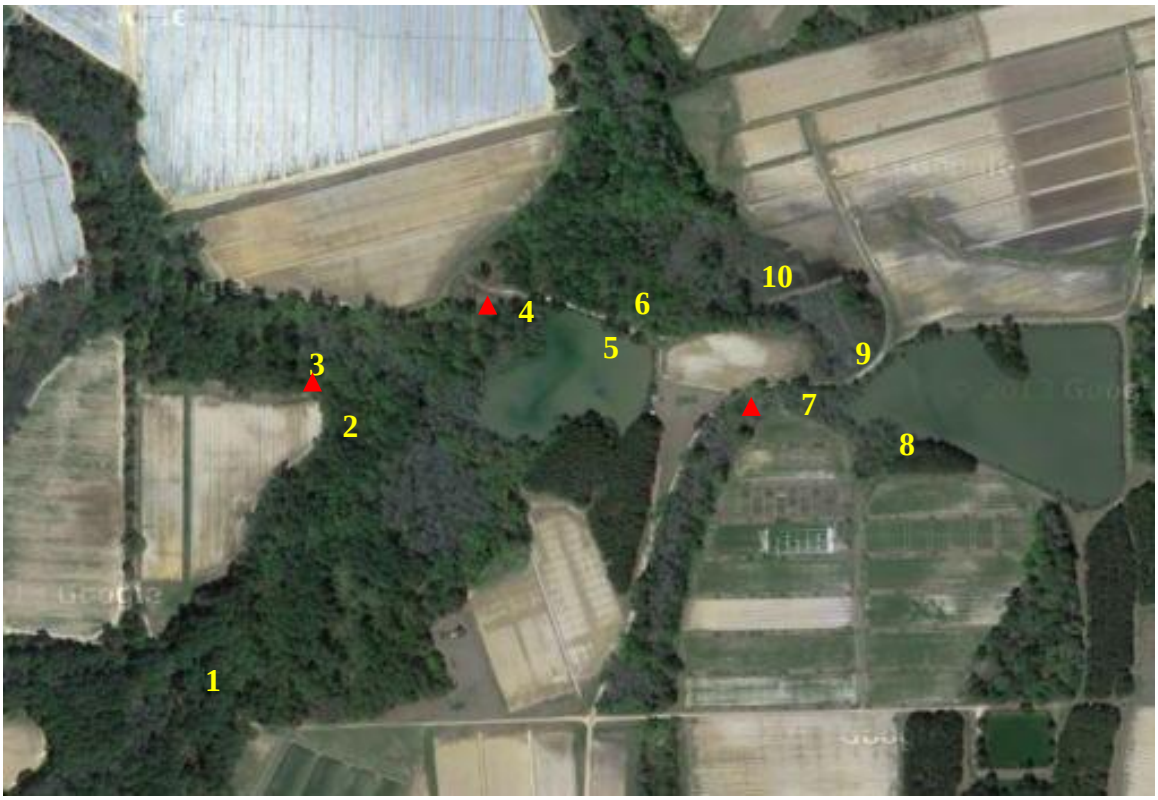

Figure 3-1: Location of study sites in Tifton, GA at the University of Georgia's Gibbs Research Farm. Triangles represent the locations of the paired stemflow and throughfall collectors located at sites 3, 4, and 7.

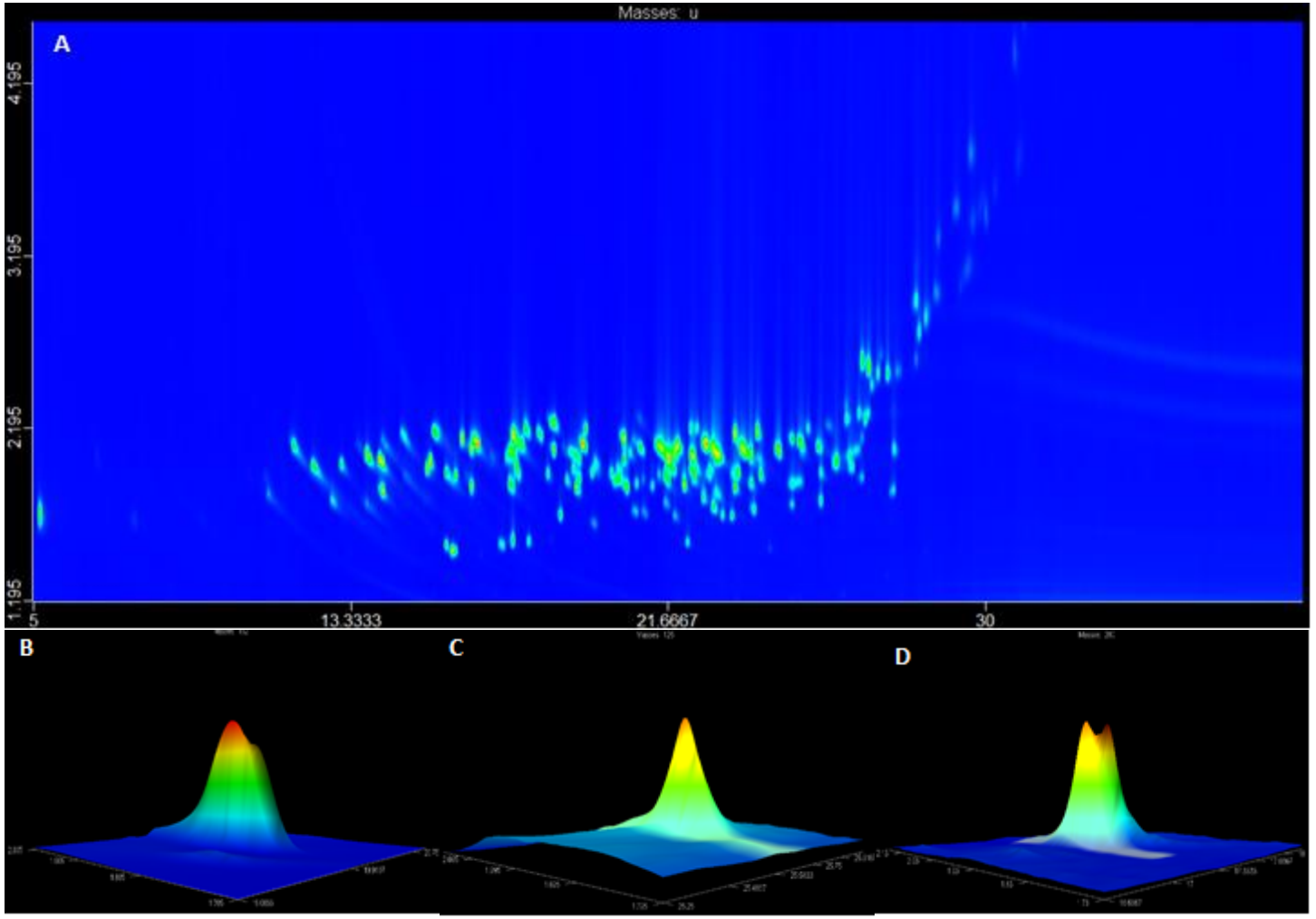

Figure 3-2: GCxGC-ToF/MS chromatograms: (A) standard with over 150 pesticides, (B) metolachlor at ion 162 *m/z* in stemflow sample, (C) tebuconazole at ion 125 *m/z* in surface water and (D) atrazine at ion 200 *m/z* in throughfall.

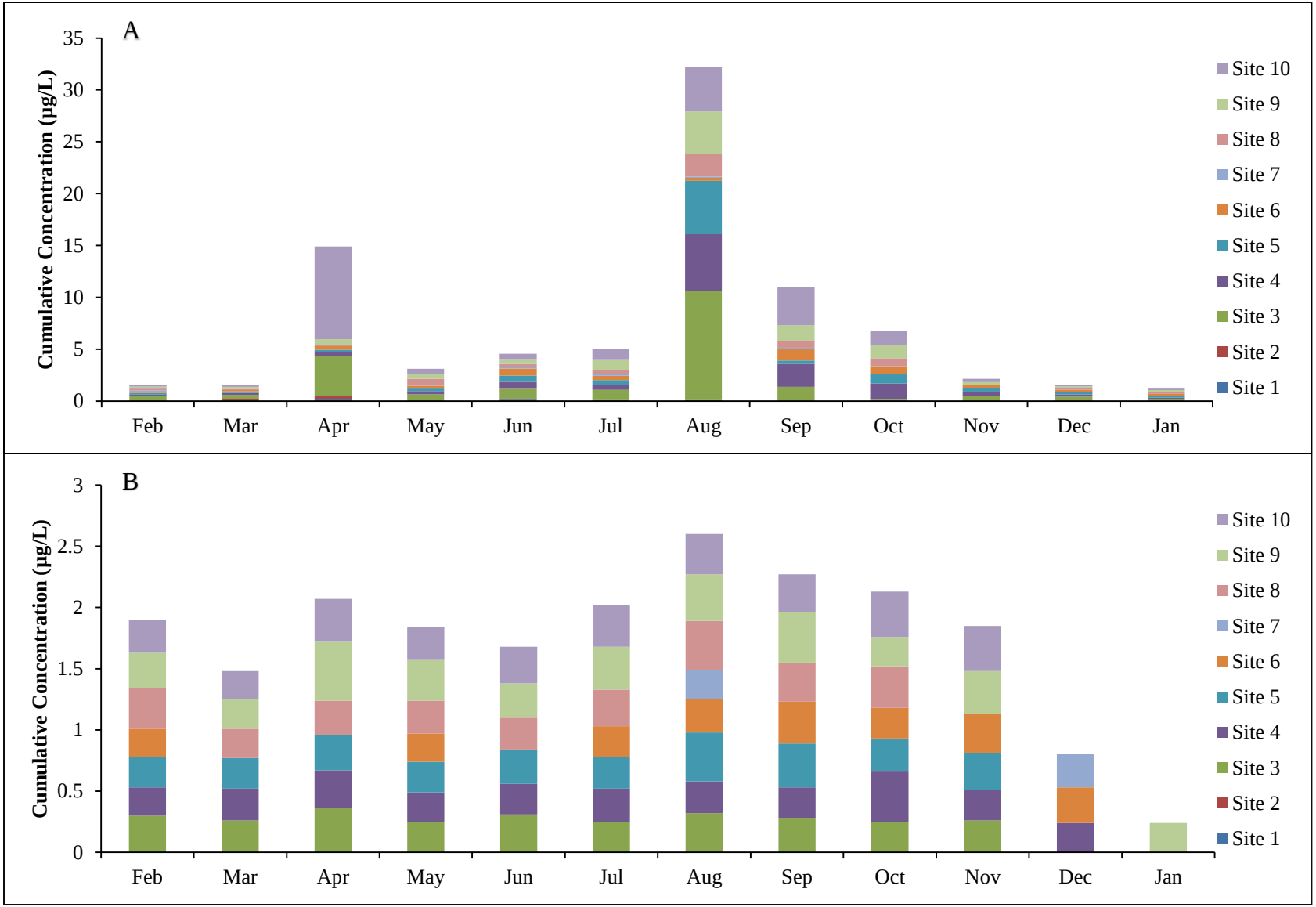

Figure 3-3: Surface water concentration (µg/L) of metolachlor (A) and tebuconazole (B) at each site (1-10) during the sample month.

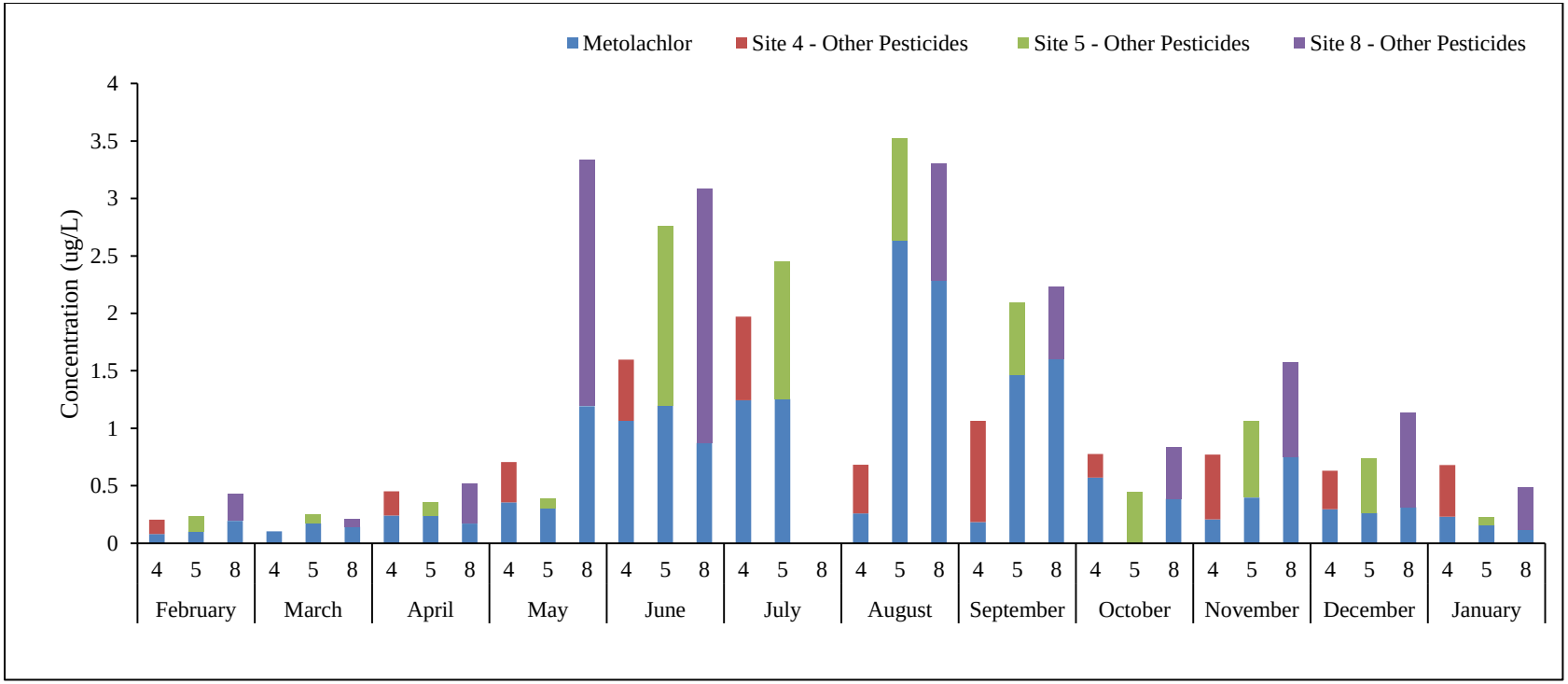

Figure 3-4: Temporal metolachlor concentrations as well as cumulative pesticide concentrations (µg/L) for all pesticides detected in 4 L surface water samples for sites 4, 5, and 8.

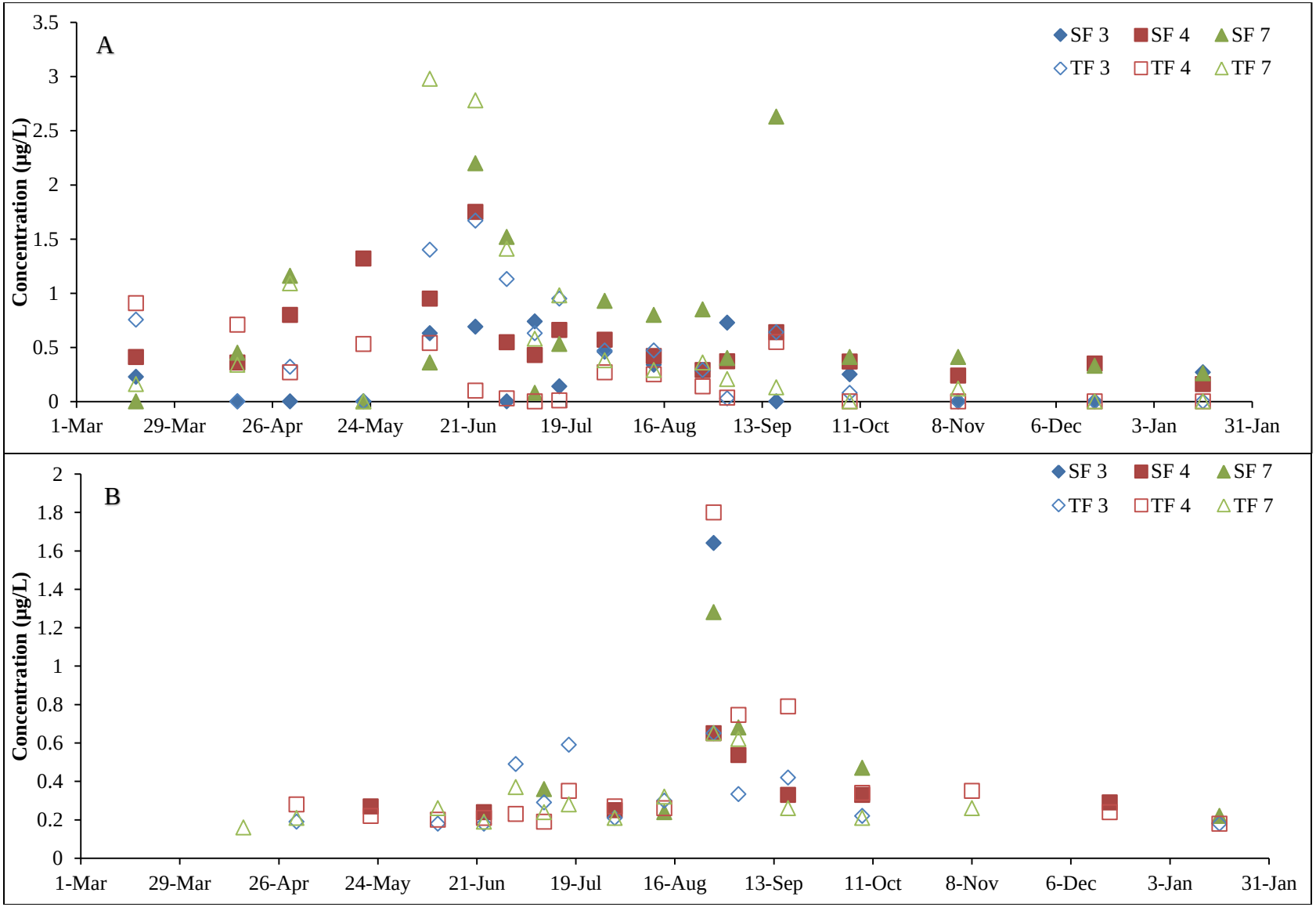

Figure 3-5: Plot of concentration (µg/L) versus time for stemflow and throughfall at sites 3, 4, and 7 for metolachlor (A) and tebuconazole (B).

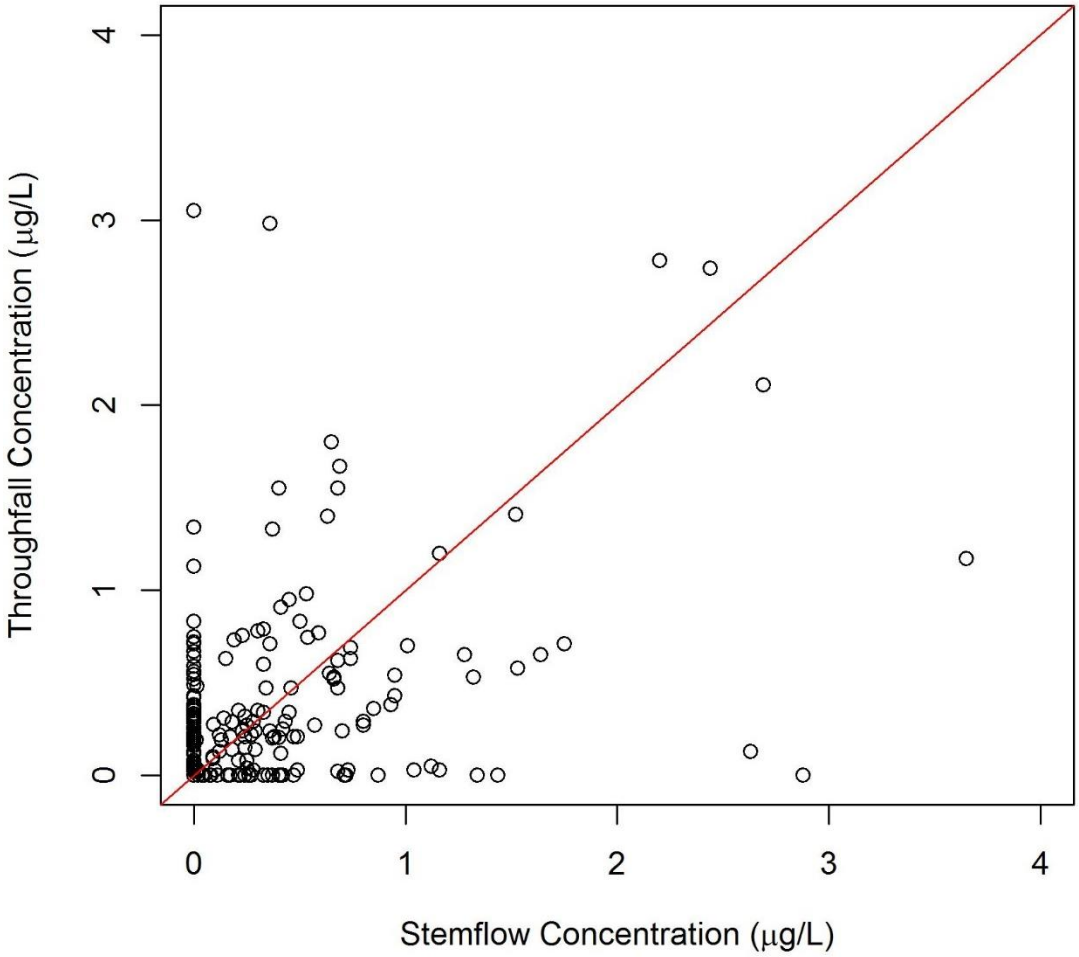

Figure 3-6: Comparison of throughfall concentration (µg/L) versus stemflow concentration (µg/L) for all pesticides that were detected in these matrices.

Table 3-1S: List of pesticides that were analyzed in all sample matrices including absolute retention time from the GCxGC-ToF/MS and concentrations (µg/L) for standards ranging from 0.0781–1.25 µg/L. N/A = the data processing method could not find a hit.

Compound	Absolute R.T. (min , sec)	0.0781	0.156	0.3125	0.625	1.25
2,3,5,6-Tetrachloroaniline	15.5 , 2.185	0.12	0.04	0.33	0.63	1.36
2,4'-DDD	23.8333 , 2.080	N/A	N/A	N/A	0.47	1.34
3,4-Dichloroaniline	13.5833 , 2.085	N/A	0.22	0.24	0.22	1.26
4,4'-Dichlorobenzophenone	20.8333 , 2.010	-0.01	0.21	0.38	0.59	0.39
4,4'-Methoxychlor olefin	24.5 , 2.085	0.25	0.24	0.25	0.49	1.09
9,10-Anthracenedione	20.9167 , 2.255	0.27	0.3	0.3	0.46	0.87
Acetochlor	19 , 1.905	0.1	0.14	0.28	0.66	1.24
Alachlor	19.3333 , 1.915	0.07	0.12	0.31	0.71	1.21
Aldrin	19.4167 , 2.120	0.08	0.13	0.26	0.67	1.32
Allidochlor	11.1667 , 1.820	0.2	0.14	0.38	0.51	1.08
alpha-BHC	16.6667 , 2.110	0.06	0.15	0.24	0.75	1.22
Atrazine	17.4167 , 2.035	N/A	0.15	0.26	0.54	1.3
Benfluralin	16.0833 , 1.490	0.11	0.11	0.37	0.78	1.14
beta-BHC	17.9167 , 2.205	N/A	N/A	N/A	N/A	1.19
Bifenthrin	25.6667 , 1.780	0.18	0.16	0.31	0.59	1.09
Bioallethrin	21 , 1.725	0.15	0.25	0.12	0.12	1.28
Biphenyl	11.8333 , 2.100	0.23	0.1	0.48	0.37	1.16
Botran	17.75 , 2.090	0.19	N/A	0.23	0.56	1.19
Bromfenvinphos	22.75 , 1.920	0.13	0.18	0.19	0.68	3.52
Bromfenvinphos-methyl	21.9167 , 2.025	0.07	0.11	0.19	0.45	1.83
Bromophos	20.6667 , 2.060	0.21	0.25	N/A	0.49	0.98
Bromophos-ethyl	21.5833 , 1.940	0.17	0.23	0.28	0.5	1.17
Bromopropylate	26.0833 , 2.020	0.14	0.15	0.26	0.45	1.49
Bupirimate	23.4167 , 1.855	0.21	0.22	0.27	0.47	1.18
Captan	22.25 , 2.200	0.16	0.17	0.26	0.57	2.27
Carbophenothion	24.5833 , 2.045	N/A	N/A	N/A	N/A	1.25
Carfentrazone ethyl	24.9167 , 1.760	0.22	0.2	0.25	0.56	1.1
Chlorfenapyr	23.9167 , 1.730	0.23	0.24	0.27	0.53	1.01
Chloroneb	14.0833 , 2.020	0.02	0.06	0.23	0.53	1.84
Chlorothalonil	19.25 , 2.050	N/A	N/A	0.34	0.58	1.26
Chlorpropham	16 , 1.895	0.11	0.19	0.32	0.58	1.19
Chlorpyrifos	20.1667 , 1.945	0.17	0.22	0.35	0.48	1.1
Chlorpyrifos methyl	19.0833 , 2.070	0.15	0.13	0.28	0.53	1.36
Chlozolinate	21.5833 , 1.775	0.16	0.14	0.27	0.46	1.44
cis-Chlordane	21.8333 , 2.060	0.06	0.11	0.24	0.54	1.67
cis-Nonachlor	23.6667 , 2.065	0.14	0.14	N/A	N/A	1.21

Table 3-1S: (*continued*)

Compound	Absolute R.T. (min , sec)	0.0781	0.156	0.3125	0.625	1.25
Coumaphos	29.5833 , 3.405	N/A	N/A	0.25	0.69	4.38
Cycloate	15.3333 , 1.970	0.1	0.1	0.34	0.58	1.34
Cyfluthrin	29.3333 , 3.030	0.2	0.24	0.33	0.45	1.08
Cyfluthrin:2	29.4167 , 3.075	0.15	0.18	N/A	0.64	0.76
Cyfluthrin:3	29.5833 , 3.150	0.18	0.19	0.47	0.46	1.2
Cypermethrin	29.6667 , 3.395	0.06	0.14	0.17	0.67	1.51
Cypermethrin:2	29.9167 , 3.520	0.21	0.21	0.51	0.41	1.14
Cyprodinil	20.8333 , 2.115	0.24	0.29	0.28	0.4	1.06
DCPA	20.4167 , 1.900	-0.12	-0.01	0.27	0.68	1.93
Deethylatrazine (DEA)	16.9167 , 2.015	0.08	0.19	1.89	0.93	1.71
Deisopropylatrazine (DIA)	16.75 , 2.155	N/A	N/A	N/A	0.25	0.85
delta-BHC	18.6667 , 2.235	0.14	0.16	0.31	0.62	1.14
Diallate (cis and trans)	16.0833 , 1.930	N/A	N/A	N/A	0.68	1.26
Diallate (cis and trans)	16.4167 , 1.905	0.6	0.82	0.43	0.61	1.06
Diazinone	17.5833 , 1.855	0.19	0.27	0.35	0.62	1.16
Dichlofluanid	20.5 , 2.025	0.15	0.22	0.35	0.5	1.1
Dieldrin	22.6667 , 2.110	0.07	0.12	0.21	N/A	1.73
Dimethachlor	19.1667 , 1.965	0.11	0.16	0.33	0.69	1.05
Dimethazone	17.5833 , 2.060	0.06	0.16	0.27	0.57	1.33
Diphenamid	21.3333 , 2.120	N/A	N/A	N/A	0.48	1.02
Diphenylamine	15.5833 , 2.200	0.19	0.23	0.3	0.59	0.96
Edifenphos	25.3333 , 2.200	0.25	0.25	0.31	0.46	0.97
Endosulfan	21.9167 , 2.105	N/A	0.23	N/A	0.43	1.26
Endosulfan:2	24 , 2.180	0.23	N/A	N/A	0.55	1.14
Endosulfan ether	18.3333 , 2.155	0.09	0.2	0.29	0.62	1.2
Endosulfan sulfate	25.5833 , 2.095	0.16	0.15	0.29	0.52	1.3
Endrin	23.4167 , 2.170	N/A	N/A	N/A	0.51	1.24
Endrin aldehyde	24.9167 , 2.140	0.18	0.2	0.25	0.59	1.59
Endrin ketone	26.9167 , 2.555	0.16	0.19	0.32	0.52	1.2
EPN	26.8333 , 2.255	0.22	0.23	0.31	0.47	1.06
Ethalfluralin	15.8333 , 1.525	N/A	N/A	N/A	N/A	0.95
Ethion	24.0833 , 1.930	0.28	0.28	0.28	0.5	0.87
Ethylan (Perthane)	22.9167 , 2.025	0.21	0.25	0.32	0.33	2.46
Etofenprox	29.5833 , 3.795	0.13	0.16	0.3	0.55	1.29
Etridiazole	13.0833 , 1.995	0.2	0.17	0.32	0.51	1.15
Fenarimol	28.1667 , 2.945	0.23	0.25	0.32	0.48	0.98
Fenitrothion	20.4167 , 1.960	0.24	0.27	0.34	0.45	0.92

Table 3-1S: (*continued*)

Compound	Absolute R.T. (min , sec)	0.0781	0.156	0.3125	0.625	1.25
Fenpropathrin	26.5 , 1.975	0.15	0.15	0.33	0.56	1.17
Fenson	21.3333 , 2.100	N/A	N/A	N/A	0.66	0.71
Fipronil	22.1667 , 1.540	0.18	0.19	0.17	0.39	1.59
Fluazifop-p-butyl	23.0833 , 1.700	0.22	0.23	0.26	0.36	1.32
Fluchloralin	18 , 1.550	0.16	0.24	0.31	0.55	1.04
Flucythrinate	30 , 3.430	0.23	0.25	0.28	0.49	1.05
Flucythrinate:2	30.25 , 3.590	0.19	0.22	0.31	0.51	1.1
Fludioxonil	24 , 2.035	0.25	0.25	0.3	0.48	0.98
Fluquinconazole	29.25 , 3.480	0.22	0.19	0.33	0.51	1.06
Flusilazole	23.4167 , 1.870	0.18	0.19	0.23	0.49	1.33
Flutolanil	22.9167 , 1.835	0.18	0.19	0.23	0.37	1.51
Flutriafol	23.0833 , 2.015	0.21	0.15	0.27	0.65	0.59
Folpet	22.4167 , 2.200	N/A	0.28	0.31	0.48	2.38
Fonofos	17.6667 , 2.110	N/A	0.35	0.4	0.46	0.98
Fonofos oxon	17.0833 , 2.025	-0.03	0.18	0.38	0.68	1.21
Heptachlor	18.6667 , 2.075	0.17	0.22	0.09	0.71	1.22
Heptachlor epoxide	20.9167 , 2.090	0.13	0.23	0.32	0.58	1.05
Hexachlorobenzene	16.25 , 2.140	0.02	0.1	0.3	0.74	1.3
Hexazinone	26.6667 , 2.290	0.19	0.19	0.2	0.52	1.32
Iodofenphos	22.5833 , 2.110	0.19	0.19	0.29	0.45	1.72
Isazophos	18.5 , 1.940	0.12	0.16	0.32	0.68	1.06
Isodrin	20.5 , 2.145	N/A	N/A	N/A	0.63	1.2
Isopropalin	20.8333 , 1.715	0.18	0.24	0.33	0.52	1.04
lambda-Cyhalothrin	27.5833 , 1.845	0.15	0.18	0.31	0.55	1.19
lambda-Cyhalothrin:2	27.5833 , 2.080	0.22	0.25	0.21	0.54	1.12
Lenacil	25.8333 , 2.220	0.14	0.14	0.29	0.58	1.28
Leptophos	27.1667 , 2.520	0.22	0.21	0.31	0.49	1.08
Malathion	20.3333 , 1.900	0.08	0.16	0.32	0.49	1.45
Metalaxyl	19.75 , 1.975	0.18	0.22	0.35	0.56	0.96
Metazachlor	21.5833 , 2.060	0.23	N/A	N/A	0.49	1.28
Methacrifos	14.1667 , 1.840	0.15	0.16	0.28	0.48	1.37
Methoxychlor	26.3333 , 2.255	0.22	0.23	0.2	0.56	1.12
Methyl parathion	19.75 , 2.020	0.25	0.26	0.32	0.49	0.89
Metolachlor	20.25 , 1.895	0.13	0.21	0.35	0.54	1.1
Mevinphos	13.9167 , 1.830	0.21	0.47	0.2	0.34	1.01
MGK-264	21 , 1.905	0.2	0.25	0.33	0.49	1.01
Mirex	26.75 , 2.590	0.08	0.14	0.31	0.63	1.27
Mitotane	23.8333 , 2.060	N/A	N/A	0.27	0.51	1.11

Table 3-1S: (*continued*)

Compound	Absolute R.T. (min , sec)	0.0781	0.156	0.3125	0.625	1.25
Myclobutanil	23.8333 , 1.885	0.21	0.2	0.24	0.52	1.22
N-(2,4-dimethylphenyl)formamide	14.75 , 1.925	0.23	0.24	0.24	0.53	1.08
Nitrofen	23.8333 , 2.025	0.28	N/A	0.31	0.51	1.03
Norflurazon	26.1667 , 1.980	0.09	0.12	0.2	0.84	1.18
o,p'-DDE	21.5 , 2.085	N/A	N/A	0.26	0.65	1.35
o,p'-DDE:2	22.3333 , 2.035	0.14	0.23	0.26	0.51	2.92
o,p'-DDT	23.5833 , 2.085	N/A	N/A	N/A	N/A	1.53
o,p'-Methoxychlor	25.0833 , 2.130	0.2	0.18	0.29	0.65	1.15
o-Hydroxybiphenyl	14.25 , 2.100	0.16	0.23	0.19	0.53	1.32
Ovex	23 , 2.050	N/A	0.22	0.24	0.35	1.43
Oxadiazon	22.8333 , 1.745	0.17	0.19	0.24	0.37	1.51
Oxyfluorfen	23.3333 , 1.695	0.2	0.22	0.21	0.45	1.35
p,p'-DDT	23.5 , 2.110	N/A	N/A	N/A	0.48	0.78
p,p'-DDT:2	24.5833 , 2.070	0.31	0.33	0.36	0.52	0.85
Paclobutrazol	22.3333 , 1.905	0.14	0.16	0.14	0.41	1.74
Parathion	20.8333 , 1.880	0.21	0.25	0.32	0.5	1
Pebulate	12.8333 , 1.785	0.17	0.21	0.33	0.45	1.2
Penconazole	21.4167 , 1.935	0.14	0.19	0.27	0.47	1.39
Pendimethalin	21.25 , 1.870	0.17	0.22	0.3	0.55	1.07
Pentachloroaniline	18.5833 , 2.240	0.16	0.18	0.33	0.63	0.99
Pentachloroanisole	16.5 , 2.080	-0.1	0.45	0.41	1.2	1.25
Pentachlorobenzene	13.75 , 2.050	0.03	0.1	0.29	0.67	1.41
Pentachlorobenzonitrile	17.8333 , 2.105	0.07	0.18	0.29	0.63	1.25
Pentachloronitrobenzene	17.5 , 2.070	0.15	0.19	0.24	N/A	1.25
Pentachlorothioanisole	19.5 , 2.200	0.14	0.18	0.32	0.61	1.11
Phenothrin	26.4167 , 2.040	0.2	0.21	0.35	0.5	1.02
Phosalone	27.6667 , 2.535	0.26	0.27	0.29	0.46	0.98
Phosmet	27.25 , 2.630	N/A	0.36	0.38	0.47	0.97
Piperonyl butoxide	24.9167 , 1.870	0.2	0.19	0.31	0.56	1.07
Pirimiphos ethyl	20.5833 , 1.850	0.23	0.27	N/A	0.47	0.98
Pirimiphos methyl	19.6667 , 1.945	0.17	0.24	0.35	0.57	0.93
Pretilachlor	22.5833 , 1.805	0.12	0.17	0.14	0.39	1.78
Procymidone	22.0833 , 1.870	0.12	0.17	0.27	0.61	2.57
Prodiamine	19.75 , 1.645	0.18	0.22	0.35	0.59	0.94
Profluralin	17.3333 , 1.520	0.18	0.58	0.37	1.63	1.3
Propachlor	15.8333 , 1.945	0.13	0.17	0.33	0.68	1
Propanil	19.6667 , 1.990	N/A	N/A	N/A	0.83	0.94
Propanil:2	19.9167 , 1.920	N/A	N/A	N/A	0.75	1.06

Table 3-1S: (*continued*)

Compound	Absolute R.T. (min , sec)	0.0781	0.156	0.3125	0.625	1.25
Propargite	25.0833 , 1.905	0.23	0.22	0.32	0.54	0.92
Propyzamide	17.5 , 1.885	0.15	0.22	0.3	N/A	1.25
Prothiofos	22.25 , 1.960	0.13	0.18	0.29	0.56	2.92
Pyrazophos	28.1667 , 2.600	0.22	0.24	0.31	0.48	1.04
Pyridaben	28.6667 , 2.985	0.14	0.17	0.31	0.49	1.31
Pyridaphenthion	26.9167 , 2.290	N/A	N/A	0.4	0.57	1.18
Pyrimethanil	17.75 , 2.135	0.14	0.21	N/A	0.45	1.33
Pyriproxyfen	27 , 2.450	0.21	0.18	0.18	0.54	1.32
Resmethrin	25 , 1.885	0.21	0.18	0.29	0.56	1.08
Ronnel	19.3333 , 2.015	0.16	0.18	0.31	0.61	1.07
Sulfotep	16.5 , 1.865	0.08	0.03	0.39	0.77	1.12
Tebuconazole	25.6667 , 1.945	0.24	0.24	0.22	0.53	1.09
Tebufenpyrad	26 , 1.995	0.11	0.12	0.29	0.53	1.42
Tefluthrine	17.5833 , 1.550	0.11	0.21	0.29	0.6	1.18
Terbacil	19 , 2.065	0.19	0.2	0.36	0.55	0.98
Terbuthylazine	17.6667 , 1.990	0.17	0.17	0.27	0.58	1.27
Tetrachloronitrobenzene	15.4167 , 2.015	0.08	0.13	0.29	0.64	1.32
Tetrachlorvinphos	22.4167 , 1.965	0.17	0.2	0.26	0.51	2.72
Tetradifon	27.4167 , 2.520	0.23	0.22	0.21	0.57	1.08
Tetrahydrophthalimide	14.75 , 2.150	0.21	0.19	0.28	0.5	1.18
Tetramethrin	26.5833 , 2.070	0.25	0.31	0.12	0.43	4.59
Tolylfluanid	21.75 , 1.970	0.1	0.31	0.38	0.63	1.14
Transfluthrin	18.8333 , 1.695	0.11	0.15	0.33	0.7	1.05
trans-Nonachlor	21.75 , 2.000	0.17	0.29	0.29	0.52	0.23
trans-Nonachlor:2	23.6667 , 2.100	0.14	0.21	0.25	0.66	1.21
Triadimefon	20.5833 , 1.860	N/A	0.24	0.31	0.58	1.14
Triadimenol	21.6667 , 1.890	0.12	0.16	0.24	0.46	1.52
Triallate	17.75 , 1.925	0.13	0.22	0.3	0.53	1.19
Triazophos	25.1667 , 2.105	0.24	0.23	0.3	0.47	1.07
Tricyclazole	25.5833 , 2.110	N/A	0.13	0.25	0.79	15.58
Triflumizole	21.8333 , 1.695	0.08	0.12	0.2	0.46	1.75
Trifluralin	16.75 , 1.470	0.09	0.14	0.32	0.03	1.12
Vinclozoline	19.25 , 1.860	-0.05	0.05	0.31	0.82	1.41

CHAPTER 4

USING *IN VITRO* DERIVED METABOLIC RATE CONSTANTS TO INFORM PESTICIDE BODY BURDEN IN AMPHIBIANS[2]

[2] Glinski, D.A., W.M. Henderson, R.J. Van Meter, and S.T. Purucker. To be submitted to *Toxicology Letters*.

Abstract

Understanding how pesticide exposure to non-target species influences toxicity is necessary to accurately assess the ecological risks these compounds pose. To assess the potential metabolic activation of broad use pesticides in amphibians, *in vitro* and *in vivo* metabolic rate constants were derived from toad (*Anaxyrus terrestris*) livers in experiments measuring the depletion of atrazine (ATZ), triadimefon (TDN), and fipronil (FIP) as well as formation of their metabolites. To determine the predictability of these *in vitro* derived rate constants, Fowler's toads (*Anaxyrus fowleri*) were exposed to soil contaminated with each of the pesticides at maximum application rate. Desethyl atrazine (DEA) and deisopropyl atrazine (DIA), both metabolites of ATZ, exhibited similar velocities (V_{max}) while the K_M constant for DIA was two times higher than DEA. TDN was metabolized into two diastereomers of triadimenol (TDL A and TDL B), where TDL B had a V_{max} around two times higher than TDL A. The metabolite fipronil sulfone's V_{max} and K_M were 150 pmol min^{-1} mg^{-1} and 29 µM, respectively. While intrinsic clearance rates for the pesticides ranged from 0.54–38.31 mL min^{-1} kg^{-1}. Thus, gaining knowledge on differences in metabolism of pesticides within amphibians is important in estimating risk to these non-target species since the inherent toxicity of metabolites can differ from the parent compound.

Introduction

Over 2.4 billion kilograms of pesticides have been used worldwide in preventing diseases, dealing with nuisance animals, and aiding in crop management (Stokstad and Grullón, 2013). Worldwide, herbicides have the most prevalent usage and compromise approximately 40% of all pesticides applied for both commercial and industrial use. The percentages of the total global use of pesticides are 33 percent for insecticides and 10 percent for fungicides (Stokstad and Grullón, 2013). In the United States, pesticide use surpassed 1 billion pounds in 2007 and their use continues to increase (Grube et al., 2011). Of note, 80% of pesticides being applied in the U.S. are for agricultural purposes (Stokstad and Grullón, 2013). Atrazine (ATZ), triadimefon (TDN), and fipronil (FIP) are broad use pesticides in the U.S. where ATZ is one of the most commonly used herbicides to control weeds in agricultural crops (Fernandez-Cornejo et al., 2014), TDN is a broad-spectrum fungicide used in both fruit and evergreen farming (Kenneke et al., 2008), and FIP is an insecticide with both industrial and consumer applications (Hainzl et al., 1998).

Although pesticides are used to control insects, diseases and general nuisance organisms, exposure to non-target species frequently occurs. Amphibians are important sentinel environmental species to pesticide exposure because they have the ability to integrate stressors from both aquatic and terrestrial ecosystems. Numerous species begin early life stages in aquatic environments and then migrate to land. It has been demonstrated that amphibian populations are in decline and pesticide exposure, in these non-target species, has been identified as one of the primary causative factors (Wake, 1991; Davidson et al., 2002; Mann et al., 2009a). Amphibians are often exposed to pesticides during direct and indirect agricultural application and also during

rain events when water sources become contaminated with pesticides where amphibians breed and deposit broods (Hayes et al., 2002; Houlahan and Findlay, 2003).

Through investigating the consequence of pesticide exposure in amphibians, Brühl et al. (2013) observed in an agricultural overspray scenario, that mortality of the frogs exposed to pesticides ranged from 100% after one hour to 40% after seven days. Reynaud et al. (2012) observed that FIP was found in various organs throughout the female green frog (*Pleophylax kl. esculentus*) after one day of exposure to contaminated water, while the gall bladder had the highest bioconcentration factor after eight days of exposure. Additionally, fipronil sulfone (F. sulfone), a biomarker of FIP exposure, was the most prominent metabolite found in the female green frogs exposed to FIP contaminated water (Reynaud et al., 2012). Hayes et al. (2003) reported ATZ concentrations in both water and amphibians that were collected from the same sites, along with its main metabolites. Previous work in our laboratory has shown that seven species of amphibians exposed to five different pesticides bioaccumulated atrazine in the greatest concentrations; however, when application rates were taken into account, fipronil was the most permeable to amphibian skin (Van Meter et al., 2014).

After entering the body, pesticide metabolism is commonly initiated by the liver, where phase I proteins such as cytochrome P-450 enzymes are used to help with the oxidation, reduction, and hydrolysis of xenobiotics (Lang et al., 1996; Hanioka et al., 1999a). In *in vitro* exposures, liver degraded ATZ into two major metabolites: desethyl atrazine (DEA) and deisopropyl atrazine (DIA) in rats, guinea pigs, goats, rabbits and humans (Adams et al., 1990; Lang et al., 1996; Hanioka et al., 1999b). Hanioka et al. (1998) observed that rats exposed to ATZ formed DIA ten times faster than DEA. Hepatic metabolism of TDN can only result in triadimenol (TDL) formation through 11β-hydroxysteroid dehydrogenase type 1 (Kenneke et al.,

2008). Triadimenol is considered more toxic than the parent in an array of species examined and interestingly, is also used as a pesticide (Roberts and Huston, 1999; Kenneke et al., 2010). Fipronil can degrade into fipronil sulfone (F. sulfone), fipronil sulfide, and fipronil desulfinyl depending on different chemical processes such as metabolic oxidation or photolysis (Hainzl et al., 1998; Reynaud et al., 2012). In human liver microsomes, F. sulfone was the major metabolite observed through oxidation (Tang et al., 2004). F. sulfone is also known to be more toxic than its parent FIP in many marine invertebrates, fish, and avian species (U.S. EPA, 1996; Baird et al., 2013).

Although data on pesticide metabolism exists for many species, limited data are available for amphibians. To better understand the role of amphibian hepatic metabolism following pesticide exposure, the objective of this study was two-fold: 1) to establish metabolic rate constants for both parent and metabolites *in vitro* in toad microsomes and 2) to compare *in vitro* data and their predictive abilities to estimate body burdens determined through *in vivo* exposure studies using amphibians.

Materials and Methods

Pesticide active ingredients and their metabolites were obtained from the U. S. Environmental Protection Agency's National Pesticides Standard Repository (Fort Meade, MD, USA). Active ingredients (AI) and metabolites analyzed in the study were ≥96.5% purity for atrazine (ATZ; CAS 1912-24-9), deisopropyl atrazine (DIA; CAS 1007-28-9), desethyl atrazine (DEA; CAS 6190-65-4), triadimefon (TDN; CAS 43121-43-3), triadimenol (TDL; CAS 55219-65-3), fipronil (FIP; CAS 120068-37-3), fipronil sulfone (F. sulfone; CAS 120068-36-2) and tetraconazole (CAS 112281-77-3). RapidStart NADPH (nicotinamide adenine dinucleotide

phosphate) regenerating system along with all solvents used for pesticide extraction and analysis were of highest grade and purchased from Fisher Scientific (Pittsburgh, PA, USA). Southern toads (n=46) were purchased from Backwater Reptiles (FL, USA) with half female and half male and shipped live to Celsis *In Vitro* Technologies where they were immediately euthanized for liver microsome preparation.

In Vitro Studies

Pooled southern toad (*Anaxyrus terrestris*) liver microsomes were purchased from Celsis *In Vitro* Technologies (Baltimore, MD, USA) and stored in a -80 °C freezer until used for metabolic assays. The total P450 concentration for the toad liver microsomes was 1.249 nmol/mg provided by the vendor. Metabolism assays were performed based on the method of Mazur et al. (2007) with slight modifications and done in triplicate. Briefly, microsomes were pre-incubated at 30 °C for 10 min in potassium phosphate buffer (100 mM, pH 7.42) in microcentrifuge tubes prior to the addition of substrate (total volume 500 µL). Concentrations of AI used in the study ranged from 0.7-200 µM (organic solvent in reaction medium was less than 1%) with a final microsomal protein concentration of 0.2 mg/mL. The reaction was initiated by the addition of RapidStart NADPH regenerating system for a final yield of 500 µM. After concurrent addition of both AI and NADPH, the assays were vortexed and incubated for a specified time (0–90 min). Reactions were quenched using 0.5 mL of 60% MeOH:H_2O (v:v) with internal standard (tetraconazole), vortexed and then immediately placed on ice. The samples were centrifuged at 4 °C for 10 min at 13500 rpm. Following centrifugation and protein precipitation, aliquots of the assay were placed in 2 mL vials and analyzed by liquid chromatography coupled to mass spectrometry (LC-MS).

V_{max} and K_M Values

Utilizing the data analysis described in Crowell et al. (2010), the maximum velocity (V_{max}, pmol min^{-1} mg^{-1}) and Michaelis constant (K_M, µM) were calculated using the Michaelis-Menten equation:

$$V_S = \frac{V_{max}[S]}{K_M + [S]}$$

Briefly, substrate concentration for each pesticide was plotted against time to calculate the initial reaction rates (pmol min^{-1}). These values were then normalized to microsomal protein (MSP) to obtain an initial reaction velocity (V, pmol min^{-1} mg^{-1}). V_{max} and K_M parameters along with standard error for each parent and corresponding metabolite(s) were calculated by plotting initial reaction rates versus substrate concentration in RStudio (version 3.2.3, 2015).

Intrinsic Clearance, CLint

Intrinsic clearance was similarly calculated based on the formula in Crowell et al. (2010):

$$CL_{int} = \left(\frac{V_{max}}{K_M}\right)\left(\frac{mg\ of\ MSP}{1\ g\ of\ liver\ weight}\right)\left(\frac{g\ of\ liver\ weight}{1\ kg\ of\ body\ weight}\right)$$

For toads, 5.4 mg of MSP per gram of liver was used due to taxonomic relations (Noshiro and Omura, 1984) and the liver weight used for toads was 52 g/kg body (based on liver weight being 5.2% of body weight).

In Vivo Studies

Exposure studies were carried out based on the methods in Van Meter et al. (2014). Fowler's toads (*Anaxyrus fowleri*) were reared at U.S. EPA in Athens, GA from egg masses collected at University of Georgia's Whitehall Forest. Toads were reared in 375 L outdoor

wading pools and fed Tetra Fin fish food *ad libitum* until metamorphosis. As toad metamorphs emerged they were transferred to 600 L polyethylene tanks padded with sphagnum moss and leaf litter to simulate a terrestrial environment. All juvenile toads were fed purchased crickets and cultured fruit flies until 60-90 days post-metamorphosis. Rearing, housing and animal studies were all done in accordance with approved animal use and care protocols. Soil collected from a reference study site in Newton, GA in June 2013 was processed through a 2 mm sieve and stored at < 4 °C until used. Soil was applied to the bottom of an aquarium (10 gallon) and each pesticide was applied to the surface of the soil using a compressed air propellant Spray Gun® canister attached to a graduated glass jar.

Maximum labeled application rates were scaled down to the size of the aquarium and final rates were 22.9, 2.7, and 1.1 μg/cm^2 for ATZ, TDN, and FIP, respectively. Fowler's toads (n=5) were then placed into aquaria (n=4) where replicate individuals were separated by PVC pipes (10 cm internal diameter) for a total of 20 toads per pesticide. One replicate from each tank was randomly removed and placed in a pre-weighed 50 mL centrifuge tube at each time step: 2 h, 4 h, 12 h, 24 h, and 48 h and euthanized in a -80 °C freezer. Additionally, three soil samples were collected at each time point from each tank into pre-weighed 15 mL centrifuge tubes and stored at -20 °C freezer until analyzed. Prior to pesticide extraction, amphibians were brought to room temperature to obtain final weights and 5 mL of MQ water was added to facilitate homogenization with a tissue grinder. All samples were spiked with 10 μL of 1000 ppm tetraconazole and placed on a freeze drier overnight. Next, 5 mL of methanol (MeOH) was added to each 50 mL centrifuge tube and placed into a sonicator (Model Branson B-22-4) for 30 minutes. Samples were centrifuged for 20 minutes at 3250 rpm and the supernatant was transferred to a 20 mL scintillation vial. The above procedure was repeated to ensure that all the

pesticide was extracted. The pooled supernatants were placed under a steady stream of nitrogen gas and blown down until approximately 1 mL of MeOH remained. Next, 10 mL of MQ water and 3 mL of methyl *tert*-butyl ether (MTBE) were added to each vial along with < 1 g of sodium sulfate to remove any emulsion. After complete separation of the two phases, the top organic layer was transferred to a 2 mL centrifuge tube and centrifuged for 15 minutes at 13500 rpm. From this sample, a 1 mL aliquot was taken and placed into a 2 mL vial to be blown dry under nitrogen and finally reconstituted in 1 mL 30% MeOH (v:v) and analyzed via liquid chromatography mass spectrometry (LC-MS).

LC-MS Instrumentation

All analytical conditions have been previously reported in Van Meter et al. (2014). Active ingredients and metabolites were quantified on an Agilent 1100 Series HPLC coupled to a 6120 mass spectrometer equipped with an Eclipse XDB-C18 (3.5 µm particle size, 3.0 x 150 mm; Agilent Technologies, CA, USA). Initial conditions were held for 2 minutes at 70% water with 0.1% formic acid (A) and 30% acetonitrile with 0.1% formic acid (B). Ramped to 90% B over 16 minutes and held there for 4 minutes before returning to starting conditions. FIP and F. sulfone were analyzed in negative electrospray ionization (ESI) while all other active ingredients and metabolites were analyzed in positive ESI in selected ion monitoring mode (SIM; Table 4-1).

Statistical Analysis

V_{max} and K_M constants were calculated using RStudio (version 3.2.3, 2015) all values presented in Table 2 are average ± standard error. For TDL B the 200 µM data point was an outlier and excluded from all calculations.

Results and Discussion

V_{max} and K_M Values

To determine pesticide metabolic rate constants in amphibians, a series of toad microsomal experiments were conducted to determine both the depletion rates of ATZ, TDN, FIP, and formation rates for their corresponding metabolites. In all the microsomal experiments the formation of the metabolites plateaued in the concentration range of 0.7–250 µM.

In the toad microsomes, ATZ depletion rate was 834 pmol min^{-1} mg^{-1} while the K_M was 77 µM (Figure 4-1; Table 4-2). Compared to other species, the V_{max} for Sprague-Dawley and Fischer rats were 720 and 1160 pmol min^{-1} mg^{-1} (respectively), resulting in toads' metabolic parameters falling between the two species (Adams et al., 1990). However, the K_M constant for Sprague-Dawley and Fischer rats were 27.5 and 27.8 µM, which was significantly lower than the toads.

DEA and DIA were metabolized at equal rates with a V_{max} of 112 and 124 pmol min^{-1} mg^{-1}, respectively (Figure 4-1; Table 4-2). The velocity for DEA in toads was similar to the one observed by Hanioka et al. (1998) in male rats at 133 pmol min^{-1} mg^{-1}. Furthermore, Hanioka et al. (1999b) exposed microsomes from mice and guinea pigs to atrazine, where the DEA V_{max} for guinea pigs at 109 pmol min^{-1} mg^{-1} was slightly lower than toads, while the mouse was slightly higher at 277 pmol min^{-1} mg^{-1}. On the other hand, DIA was produced ten times faster in rats at a

rate of 1511 pmol min^{-1} mg^{-1} compared to toads (Hanioka et al., 1998). The toad had the lowest V_{max} by 3 to 6.5-fold for DIA when compared to mice and guinea pigs, 805 and 413 pmol min^{-1} mg^{-1}, respectively (Hanioka et al., 1999b). Lang et al. (1996) investigated pig and human liver microsomes for atrazine metabolism and found the pigs produced DIA around an order of magnitude higher than toads, while humans were 1–8 times faster than toads. Conversely, in both pig and human liver microsomes, DEA was produced much faster in toads, ranging from 3–23 times higher (Lang et al., 1996). However, Joo et al. (2010) found that human liver microsomes produced DEA and DIA at 224 and 220 pmol min^{-1} mg^{-1}, respectively, which appears two times faster than toads. A major difference observed in the *in vitro* study was that the metabolite diaminochlorotriazine (DACT) was not found in the toad microsomes even though it was a major metabolite found *in vivo* (rats) and a minor one *in vitro* (rat microsomes) (Brzezicki et al., 2003; Ross and Filipov, 2006).

The depletion rate for TDN was 639 pmol min^{-1} mg^{-1}, which was several fold slower compared to both rats and mice as studied in Crowell et al. (2010). Furthermore, the K_M was 5 µM for toads and this was found to be at least 7 times lower compared to rats and mice (Crowell et al., 2010). In Barton et al. (2006) the V_{max} was only 4.5 times higher for rats compared to toads, however, when the rats were pretreated with a high dose of TDN the $V_{max\ app}$ increased, resulting in a 5-fold difference.

Approximately 30% of the pesticides in commercial use are chiral enantiomers of one another, leading to racemic mixtures often being used in pesticide formulations (Ulrich et al., 2009). However, with chiral pesticides, one enantiomer is often more toxic than the other, and information on the concentrations of each enantiomer are essential to protect non-target species (Garrison, 2006). For instance, TDL consists of two diastereomers A and B (A: 1S,2R; 1R,2S;

B: 1S,2S; 1R,2R). The 1S,2R TDL enantiomer was the most toxic to rats; furthermore, in rainbow trout it was produced at the slowest rate out of all the TDL enantiomers (Kenneke et al., 2010). Additionally, in Kenneke et al. (2008) the diastereomer TDL B was formed at a greater rate than the TDL A diastereomer in rats. This was also observed in toad microsomes, where the enantiomer TDL B was metabolized faster than TDL A (Figure 4-2). The TDL B formation velocity was around two times higher at 537 pmol min^{-1} mg^{-1} than TDL A which was 281 pmol min^{-1} mg^{-1} in toads (Figure 4-2; Table 4-2). The K_M concentrations for TDL A was three times higher than TDL B in toads with values at 147 and 40 µM, respectively. However, TDL A formation was similar to female rats with a velocity of 344 pmol min^{-1} mg^{-1}, while being at least an order of magnitude slower than the male rat (Crowell et al., 2010). While for TDL B, the toad microsomes were slightly higher than the female rats, but half the velocity of the male and female mouse (Crowell et al., 2010).

There was only formation of fipronil sulfone in the microsomal systems of amphibians, which was consistent with the *in vivo* study (Figure 4-3). Additionally, this same observation was noted in Tang et al. (2004) where only F. sulfone was quantified in both human and rat liver microsomes. Fipronil sulfone formation has been determined *in vivo* in green frogs (*Pelophylax kl. esculentus*), barking treefrogs (*Hyla gratiosa*), and green treefrogs (*H. cinerea*) (Reynaud et al., 2012; Van Meter et al., 2015). In the current study, toad microsomes metabolized FIP into F. sulfone with a V_{max} of 150 pmol min^{-1} mg^{-1} and a K_M of 29 µM (Table 4-2). Rat microsomes were shown to metabolize FIP 3.8 times faster than human liver microsomes with a V_{max} of 0.39 and 0.11 nmol min^{-1} mg^{-1}, respectively (Tang et al., 2004). Toad microsomes again fall intermittent to these two values, however, the toads had a K_M that was three times higher than either rats or humans (27.2 and 19.9 µM, respectively; Tang et al., 2004).

Intrinsic Clearance, CL_{int}

The intrinsic clearance rates were calculated for the depletion of the parent compounds and the formation of the metabolites (Table 4-2). In this study, intrinsic clearances ranged from 0.54 to 38.31 mL min^{-1} kg^{-1} with those calculated for TDL A and TDN being the lowest and highest rates, respectively. The metabolites of atrazine, DIA and DEA, had the lowest clearance rates at 0.97 and 1.86 mL min^{-1} kg^{-1}, compared to ATZ which was 3.04 mL min^{-1} kg^{-1}. Of all the pesticides tested, TDN had the highest intrinsic clearance at 38.31 mL min^{-1} kg-^{1}, while TDN's metabolites were much lower at 0.54 and 3.77 mL min^{-1} kg^{-1} for TDL A and TDL B, respectively. The intrinsic clearance for F. sulfone was 1.47 mL min^{-1} kg^{-1}. Intrinsic clearance can be a flow-limited system if the metabolism and clearance exceeds the hepatic blood flow rate, meaning the rate-limiting step becomes the transportation of the substrate and its product to that tissue (Crowell et al., 2010). In female rats exposed to TDN, the CL_{int} values were calculated to be near the hepatic blood flow rate of rodents suggesting that metabolism may not be blood-flow limited (Crowell et al., 2010). The hepatic blood flow for toads (*Bufo arenarum*) was calculated to be 25 mL min^{-1} kg^{-1} by Uranga (1969), (where the hepatic blood flow was divided by the average weight of the toads in kg) thus most of the intrinsic clearances for the pesticides, both parents and metabolites, were below this value, except TDN. This suggests that the metabolism and clearance for these pesticides are not flow-limited and can be problematic for amphibians. In this study, the microsomes were of a mixed sex, while examining the differences between sexes could be further investigated. Additionally, examining liver microsomes from toads at various developmental stages could result in different biotransformation abilities.

Rats appear to be acceptable surrogates for predicting the V_{max} for DEA. However, all other analytes examined in this study were several-fold to an order of magnitude different than

other published kinetic rate values for various species. Within the select few species (rats, mice and humans) that are mainly examined for *in vitro* studies, the kinetic rates are different and this could be due to differences in species, sex or function/abundance of the cytochrome P450 enzymes (Hanioka et al., 1999a; Crowell et al., 2010). However, there is very little research that examines these kinetic rates in amphibians. These kinetic parameters can be used for physiologically based pharmacokinetic models for pesticide exposure in amphibians, which can aid in creating amphibian models for risk assessment purposes.

In Vivo Studies

For ATZ the maximum concentration was reached at 4 h in this study, and decreased at 48 h while for DIA and DEA both were observed at the earliest time tested (Figure 4-4). This is comparable to Storrs Méndez et al. (2009), who observed that atrazine rapidly crossed the seat patch in toads and equilibrated at 5 h. However, DEA concentrations were the same throughout the entire exposure, while DIA concentrations increased up to 24 h. This resulted in the combined DEA and DIA concentration being 10–130% of the total atrazine body burden. TDN was observed to increase in concentration until 24 h and then it decreased at 48 h (Figure 4-4). It appears that TDN could have a slower absorption rate compared to the other two pesticides examined in this study because it took longer to reach its maximum threshold. The metabolites of TDN reached maximum concentration at 4 h then decreased, which could be due to further metabolism of these metabolites, however, the degradates of TDL were not analyzed in this experiment. Throughout the entire *in vivo* exposure, TDL B had higher concentrations than TDL A, which corresponds to the *in vitro* experiments where TDL B was formed at a faster rate than TDL A. Fipronil decreased slowly over the entire study, while F. sulfone increased, even

surpassing fipronil's concentration at the 24 and 48 h time points (Figure 4-4). While these same pesticides were examined in Van Meter et al. (2014) and Van Meter et al. (2016), the exposure length was only 8 h and the body burden for pesticides was a summation of parent and metabolites, making it difficult to draw inferences about metabolism across data sets. Reynaud et al. (2012) observed F. sulfone to be the primary metabolite in female green frogs exposed to FIP, which was similar to the current *in vivo* study. Furthermore, Reynaud et al. (2012) observed an increase in bioconcentration factor for fipronil over six days in green frogs, which was the opposite of the current study.

In Vitro to In Vivo Extrapolations

Extrapolation from V_{max} *in vitro* liver microsomes to V_{max} *in vivo* for risk assessments was similar to the method in Lipscomb et al. (1998) (Table 4-2). DIA and DEA had the lowest values at 0.47 and 0.46 mg h^{-1} kg^{-1}, respectively, while the highest value was for TDN at 2.38 mg h^{-1} kg^{-1}. Atrazine had a V_{max} *in vivo* value of 2.30 mg h^{-1} kg^{-1}, which was slightly higher than TDL B (2.10 mg h^{-1} kg^{-1}). TDL A (1.29 mg h^{-1} kg^{-1}) was lower than TDL B, but higher than DEA, DIA and F. sulfone (1.11 mg h^{-1} kg^{-1}). One difference between the *in vitro* and *in vivo* studies was the *in vivo* study had used only one concentration, which was the maximum application rate for each pesticide. Even though the *in vitro* and *in vivo* study were similar for TDL A and B, where higher concentrations were predicted for TDL B. This was not true for DIA and DEA, where DIA was much higher than DEA for *in vivo* studies. However, we did not examine the depletion rates for any of the metabolites, but the clearance rate for DIA was two times higher than DEA, which could result in different tissue concentrations. It is also possible that enzyme induction could cause differences in tissue concentration considering the *in vivo*

exposures lasted 48 h and other studies have noted an increase in P450 content after exposure to ATZ (Dong et al., 2009; Fu et al., 2013). Overall, these V_{max} *in vivo* values can also be used for physiologically based pharmacokinetic models to determine pesticide tissue concentrations in amphibians for risk assessment.

Conclusions

Estimating kinetic rate constants for chemicals is a well-established method for estimating the metabolism rates for compounds. Identifying the main metabolites of pesticides utilizing *in vitro* microsomes can aid in estimating the kinetic rate constants for broad-spectrum use pesticides that are ubiquitous in the environment. Obtaining a better understanding for these kinetic rate parameters, V_{max} and K_M, can help elucidate the fate and toxicity of these chemicals *in vivo*. Even though *in vitro* exposures minimize time and expense that are required for testing and estimating potential toxicity, little research has been done on amphibians and other non-targeted species in the environment. This study shows that species extrapolation cannot always be viable considering that most of the pesticides examined in this study were different when compared to common laboratory species.

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Table 4-1: LC-MS analytical parameters for active ingredients and metabolites in *in vitro* and *in vivo* studies.

Compound	Retention Time	Mass 1 (*m/z*)	Mass 2 (*m/z*)	Ionization Mode
DIA	4.3	174	176	(+)
DEA	6.2	188	146	(+)
ATZ	13.8	216	174	(+)
TDL A	15.8	296	227	(+)
TDL B	16.3	296	227	(+)
TDN	17.6	294	225	(+)
Tetraconazole	17.7	372		(+)
FIP	19.7	435	330	(-)
F. Sulfone	20.1	451	415	(-)

Table 4-2: Michaelis-Menten parameters for depletion and formation of pesticides in mixed gender southern toad (*Anaxyrus terrestris*) liver microsomes, intrinsic clearance rates, and extrapolation of V_{max} *in vitro* to V_{max} *in vivo* (values are average ± standard error, ND = not determined).

Compound	Depletion/ Formation	V_{max} (pmol min^{-1} mg^{-1})	K_M (µM)	CL_{int} (mL min^{-1} kg^{-1})	V_{max} (*in vivo*) (mg h^{-1} kg^{-1})$^{0.75}$
ATZ	Depletion	834±210	77±52	3.04	2.30
DIA	Formation	124±32	36±37	1.86	0.46
DEA	Formation	112±31	17±26	0.97	0.47
TDN	Depletion	639±71	5±4	38.31	2.38
TDL A	Formation	281±192	147±201	0.54	1.29
TDL B	Formation	1712±2441	239±566	2.02	5.00
FIP	Depletion	ND	ND	ND	ND
F. Sulfone	Formation	150±27	29±23	1.47	1.11

Table 4-3: Tissue concentrations (µg/g) of parent pesticide and corresponding metabolites in Fowler's Toads (*Anaxyrus fowleri*) following exposure on contaminated soil.

Compound	2 h	4 h	12 h	24 h	48 h
ATZ	3.34 ± 0.59	5.36 ± 1.38	3.12 ± 0.63	1.55 ± 0.30	1.00 ± 0.18
DIA	0.33 ± 0.06	0.94 ± 0.38	1.08 ± 0.25	1.99 ± 0.67	0.66 ± 0.27
DEA	0.013 ± 0.002	0.044 ± 0.014	0.054 ± 0.006	0.037 ± 0.007	0.020 ± 0.010
TDN	0.39 ± 0.03	0.55 ± 0.15	0.75 ± 0.16	0.87 ±0.43	0.25 ± 0.04
TDL A	0.06 ± 0.01	0.08 ± 0.02	0.04 ± 0.008	0.02 ± 0.004	0.02 ± 0.002
TDL B	0.08 ± 0.008	0.13 ± 0.03	0.08 ± 0.02	0.04 ± 0.006	0.03 ± 0.002
FIP	1.87 ± 0.67	1.30 ± 0.26	0.57 ± 0.12	0.27 ±0.026	0.21 ± 0.07
F. sulfone	0.35 ± 0.14	0.40 ± 0.24	0.61 ± 0.29	1.25 ± 0.26	0.91 ± 0.28

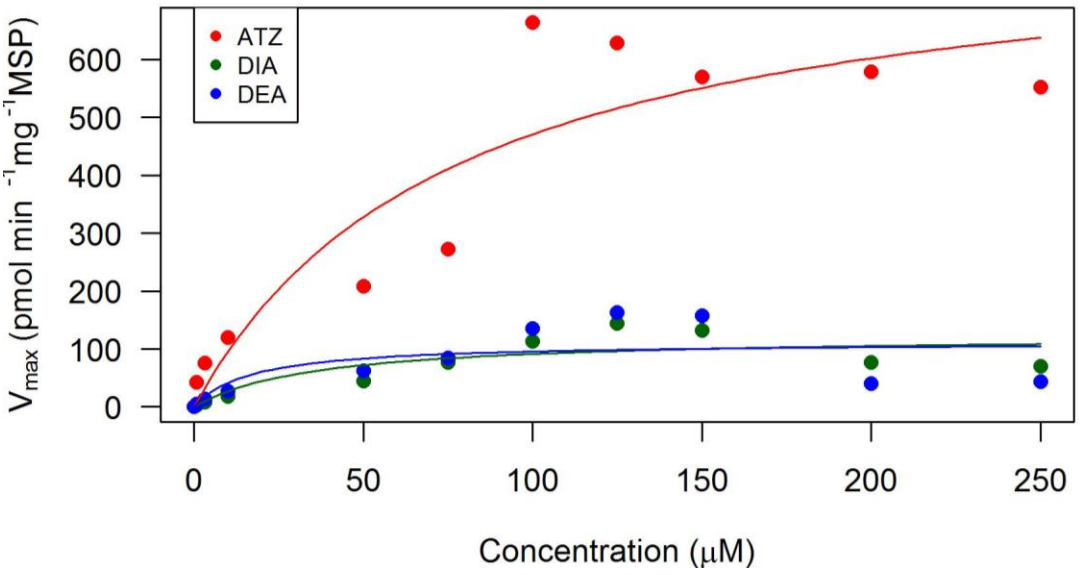

Figure 4-1: Michaelis-Menten kinetic analyses of atrazine (ATZ), deisopropyl atrazine (DIA), and desethyl atrazine (DEA) in southern toad (*Anaxyrus terrestris*) liver microsomes. Atrazine is the parent compound in red, DIA and DEA are metabolites in green and blue, respectively.

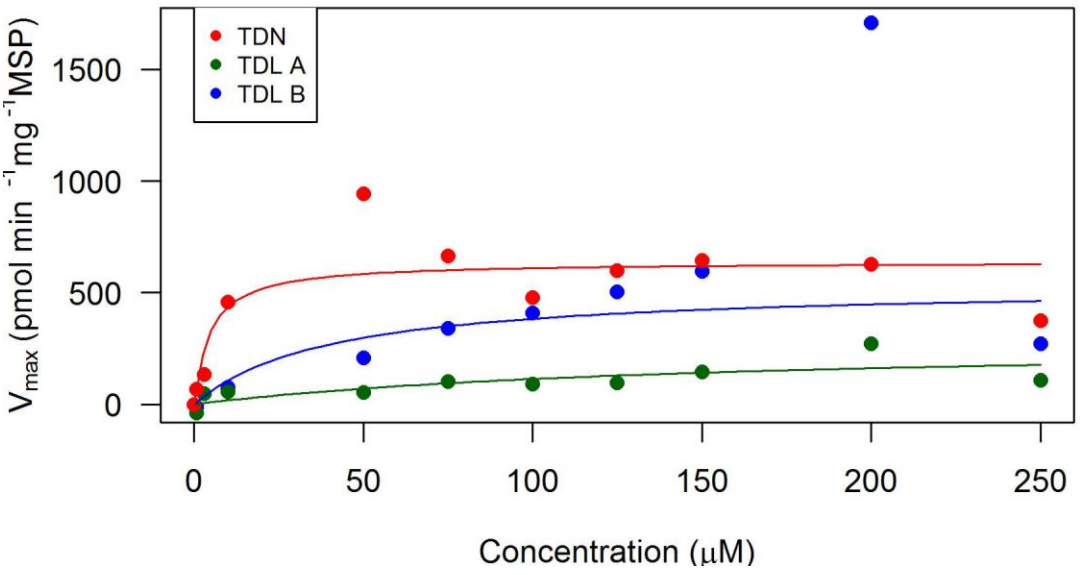

Figure 4-2: Michaelis-Menten kinetic analyses of triadimefon (TDN), triadimenol A (TDL A), and triadimenol B (TDL B) in southern toad (*Anaxyrus terrestris*) liver microsomes. Triadimefon is the parent compound in red, TDL A and TDL B are metabolites in green and blue, respectively.

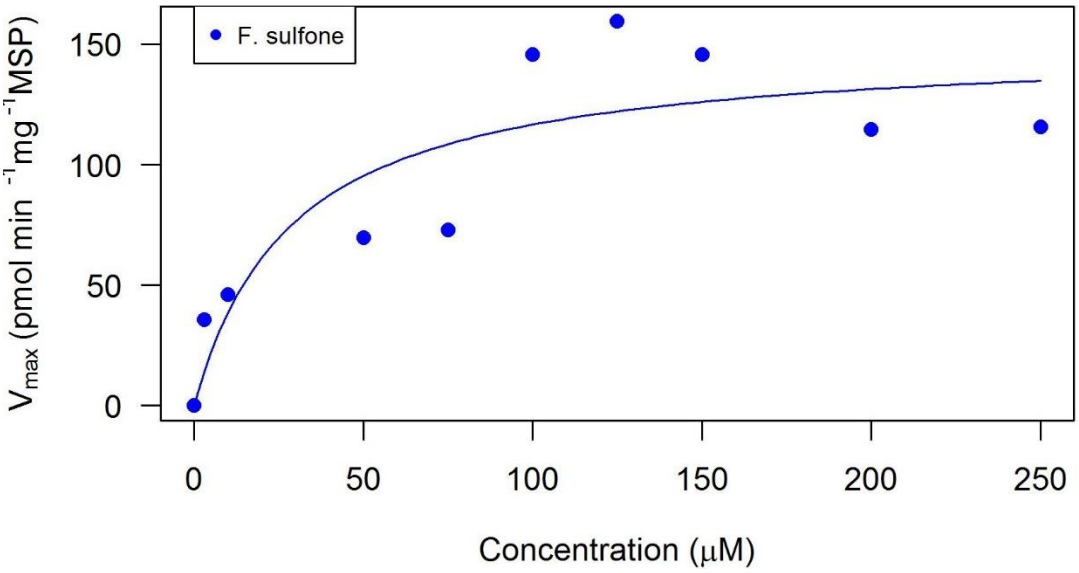

Figure 4-3: Michaelis-Menten kinetic analyses of fipronil sulfone (F. sulfone) in southern toad (*Anaxyrus terrestris*) liver microsomes. Fipronil (parent) appeared to be immediately depleted in these exposures.

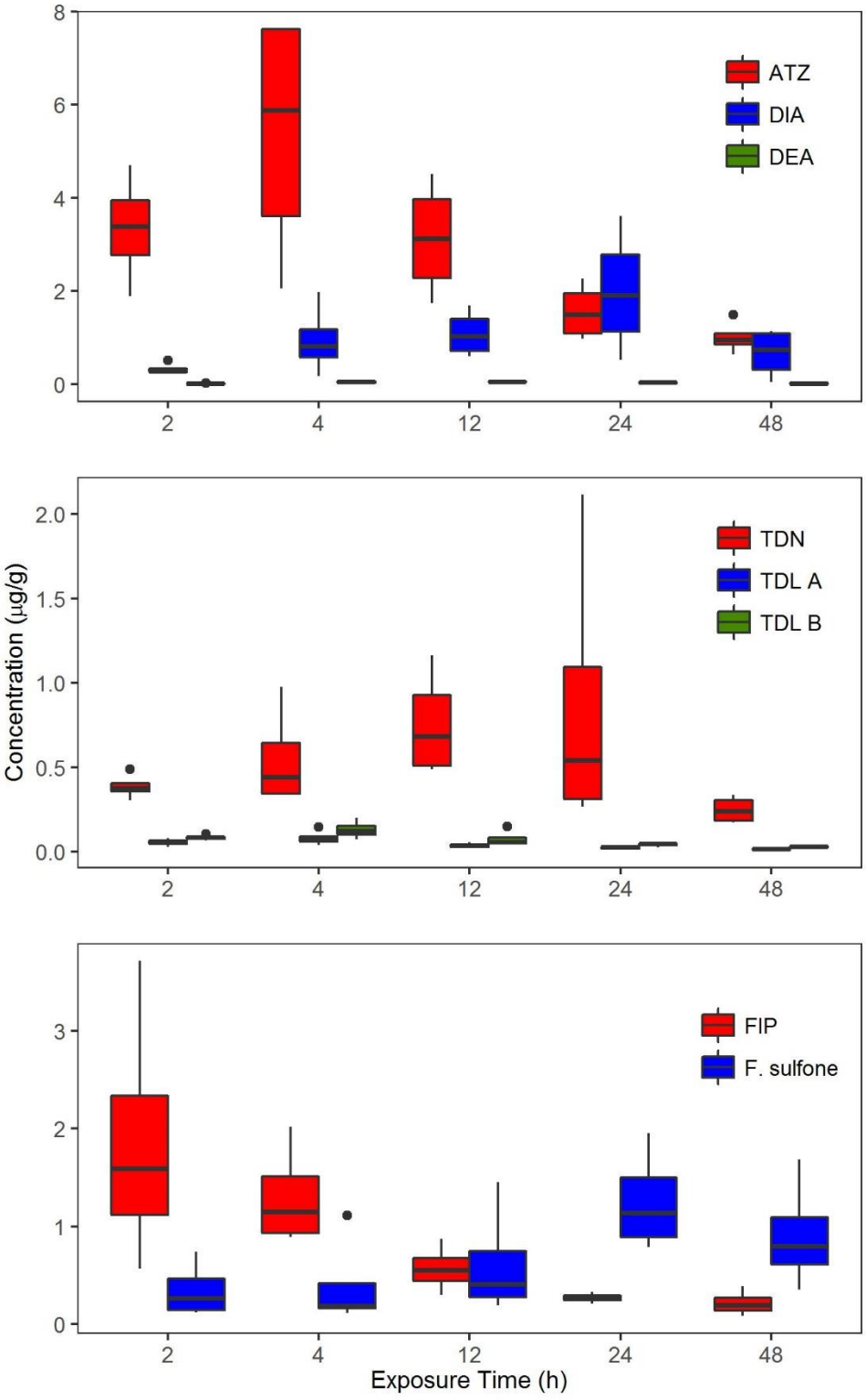

Figure 4-4: Pesticide tissue concentrations (µg/g) in Fowler's toads (*Anaxyrus fowleri*) exposed for 8 h on pesticide contaminated soil.

CHAPTER 5

EFFECT OF HYDRATION STATUS ON DERMAL PESTICIDE UPTAKE IN AMPHIBIANS[3]

[3] Glinski, D.A., W.M. Henderson, R.J. Van Meter, and S.T. Purucker. To be submitted to *Journal of Exposure Science and Environmental Epidemiology*.

Abstract

In this study, the impact of hydration status on dermal uptake of pesticides in two species of amphibians is examined. Absorption of pesticides in anurans occurs primarily through a highly vascularized dermal seat patch, however, pesticides can also enter through the superficial dermis following exposure. Despite the growing body of literature on dermal exposure in amphibians, little is known on how hydration status influences uptake. Thus, the objective of this study was to investigate the influence of hydration status on absorption of pesticides (atrazine, triadimefon, metolachlor, chlorothalonil, and imidacloprid) in southern leopard frogs (*Lithobates sphenocephala*) and Fowler's toads (*Anaxyrus fowleri*). Amphibian treatments included dehydration periods of 0, 2, 4, 6, 8 or 10 hours prior to exposure to pesticide-contaminated soils for 8 hours. Following exposure, soil and whole body homogenates were extracted and analyzed by LC-MS/MS. Dehydration time was then regressed against post-exposure concentrations to infer the impact of dehydration on dermal pesticide uptake. Increased dehydration time resulted in significantly lowered pesticide concentrations in both species ($F_{6,293} = 67.66$, $p < 0.01$) for the five pesticides studied. This phenomenon could be due to an energy and/or dilution effect.

Introduction

Environmental factors, including temporal weather patterns, are known to influence the absorption and evaporation of water in amphibians (Shoemaker and Nagy, 1977; Shoemaker et al., 1992). Amphibians lack a hydrophobic barrier, allowing their skin to be extremely permeable to the surrounding environment, facilitating gas and water exchange (Smith et al., 2007; Hillman et al., 2009; Quaranta et al., 2009). Once an amphibian has morphed into its terrestrial form, however, it must remain hydrated to avoid desiccation (Smith et al., 2007). Thus, numerous adaptations for regulating water content and varying fluids osmolality in the amphibian body have been described (McClanahan, 1967; Heatwole et al., 1969; Churchill and Storey, 1993; Toledo and Jared, 1993; Barbeau and Lillywhite, 2005; Lillywhite, 2006). One main route for terrestrial amphibians to uptake water and rehydrate themselves is to place the ventral pelvic region (seat patch) in contact with moist substrate (Taylor et al., 1999). In addition to water uptake, amphibians have been known to absorb metals and pesticides through their skin (James et al., 2004; Willens et al., 2006; Van Meter et al., 2014; Van Meter et al., 2015; Van Meter et al., 2016). Such passive absorption leads to transport across the skin as the most significant route for exposure to contaminants, compared to dietary and pulmonary routes (Smith et al., 2007).

Amphibians are extremely sensitive to environmental conditions that result in water loss, such as extreme arid conditions, diurnal and seasonal variations in climate. Amphibian bodies consist of 70–80 percent water by weight, and their daily water loss can range from 10–22 percent of total mass (Thorson, 1964; Dole, 1967; Hermes-Lima and Storey, 1998). Due to this inherent susceptibility to dehydration, amphibians have developed both physiological and behavioral mechanisms to combat water loss. One physiological characteristic is the ability to

reabsorb water from dilute urine in times of need, along with having a high storage capacity for urine (Suzuki et al., 2007; Ogushi et al., 2010a; Ogushi et al., 2010b). Another mechanism is the use of a posteroventral seat patch that is highly vascularized and extremely permeable and responsive to water balance hormones that result in rapid rehydration of water (Bentley and Main, 1972; Hillman et al., 2009; Ogushi et al., 2010b); this leads to the seat patch becoming the primary means for water absorption across the skin (Hillman et al., 2009).

Variation in skin permeability is a critical adaptation for amphibians. Bentley and Main (1972) observed that species from different habitats have distinct differences in seat patch permeability, with terrestrial species having more permeable skin than aquatic species. Aquatic amphibians have smooth ventral and dorsal skin, which is not very permeable, compared to the more granular skin of terrestrial amphibians (Toledo and Jared, 1993; Hillman et al., 2009). The granulation or "epidermal sculpturing" of the ventral skin found in terrestrial species such as toads facilitates water absorption due to increased surface area, where this sculpturing can come in contact with a moist substrate that allows the skin to reabsorb water (Lillywhite and Licht, 1974; Hillman et al., 2009).

Overall, terrestrial species have highly vascularized seat patches compared to aquatic and semi-terrestrial species (Hillman et al., 2009). The responsive hormones such as arginine vasotocin (AVT) and hydrins, can also facilitate the permeability of the seat patch in amphibians (Bentley and Main, 1972; Hillman et al., 2009). Arginine vasotocin is known to increase amphibian skin water permeability as well as slowing or halting the formation of urine, thereby conserving water (Shoemaker and Nagy, 1977; Hillman et al., 2009). Furthermore, AVT and hydrin hormones enable absorption of water into the amphibian's body by utilizing aquaporins (Tracy and Rubink, 1978; Hillman et al., 2009). Aquaporins are water-conducting channels

classified as integral membrane proteins (Preston and Agre, 1991; Ogushi et al., 2010a). Arginine vasotocin induces the insertion of aquaporin containing vesicles into the apical membrane, allowing water to be more rapidly absorbed (Suzuki et al., 2007; Hillman et al., 2009). When water is absorbed, it moves into cutaneous capillaries via basolateral membranes, utilizing more aquaporins which can increase rehydration rates (Suzuki et al., 2007; Hillman et al., 2009).

Behavioral characteristics have also been observed that allow amphibians to prevent water loss such as higher activity levels at night than during the day; residing near or in aquatic habitats; burrowing; changing activity level or posture; aggregating together; or smearing lipids over their bodies (McClanahan, 1967; Heatwole et al., 1969; Toledo and Jared, 1993; Barbeau and Lillywhite, 2005; Lillywhite, 2006). Many amphibians will stop moving to conserve water, but some species locomote again when dehydration becomes too severe, which can lead to an increase in the amount of water lost (Heatwole et al., 1969; Putnam and Hillman, 1977; Pough et al., 1983). Amphibians also prevent dehydration from air exposure with a water-conserving posture that places the ventral surfaces and head against a substrate while their legs are folded so the feet are under the body (Heatwole et al., 1969; Pough et al., 1983). Amphibians can also aggregate so that very little surface area is exposed to the environment as a means to conserve water. Last, to aid in slowing water loss, several species display a body-wiping behavior that puts an extra-epidermal water barrier comprised of lipids between the dermis and the environment to reduce evaporative water loss (Barbeau and Lillywhite, 2005).

Many amphibian breeding grounds can be found within agricultural landscapes, leading to significant exposure potential to applied chemicals (Lenhardt et al., 2015). Terrestrial species are often mobile and must respond to changing water and habitat availability. Such changes can

result in migration to other habitats, and irrigated agricultural fields may be preferable in water-scarce areas and seasons. Many species of amphibians have a terrestrial life stage which allows them to disperse far from their breeding grounds. These migration periods can lead to dehydration events and associated increases in conductivity that allows rapid rehydration when the amphibian does come in contact with water (Shoemaker and Nagy, 1977). Rapid rehydration events may also allow exposure and absorption of fertilizers and pesticides when they occur on agricultural landscapes (Berger et al., 2012; Fryday and Thompson, 2012). Climate change can also trigger habitat changes that may make agricultural landscapes a relatively desirable habitat for amphibians (U.S. EPA, 2014).

This study investigated how hydration status can affect whole body concentrations of pesticides in terrestrial phase amphibians. We investigated the influence of hydration status on dermal absorption of pesticides (atrazine, triadimefon, metolachlor, chlorothalonil, and imidacloprid) in two frog species, southern leopard frogs *(Lithobates sphenocephala*) and Fowler's toads (*Anaxyrus fowleri*), to measure uptake from predetermined pesticide exposures and after variable periods of dehydration. Using these data, we infer whether duration of the dehydration period has a significant effect on pesticide uptake across five pesticides studied. Our null hypothesis was that the dehydration period would have no effect on pesticide uptake and our expectation was that longer dehydration periods could increase the uptake of pesticides during the rehydration period.

Materials and Methods

All solvents used for pesticide extraction and analysis were of HPLC-grade purity and purchased from Fisher Scientific (Pittsburgh, PA, USA). Pesticide active ingredients and their

metabolites were obtained from the U.S. Environmental Protection Agency's National Pesticides Standard Repository (Fort Meade, MD, USA). Active ingredients (AI) and metabolites analyzed in the study were ≥ 98% purity for atrazine (ATZ), deisopropyl atrazine (DIA), desethyl atrazine (DEA), triadimefon (TDN), triadimenol (TDL), metolachlor, metolachlor ethane sulfonic acid (MESA), metolachlor oxanilic acid (MOXA), chlorothalonil, chlorothalonil metabolite, and imidacloprid.

Dehydration Experiments

Animal collection and rearing were described in detail in Van Meter et al. (2014) while soil collection and pesticide application to soils contained in Pyrex® bowls were detailed in Van Meter et al. (2016) Briefly, soil was collected from a control study site in Newton, GA during July 2014, and sifted through a 2-mm sieve before being stored at 4 °C. A thin layer of OLS soil (~150 g) from Van Meter et al. (2016) was added to the bottom of each Pyrex® bowl and individual pesticides were applied to the soil in 100% methanol (MeOH) using a compressed air propellant Spray Gun® canister attached to a graduated glass jar. To minimize spraying time and have as little error as possible between replicates, maximum application rates were scaled down to the area of six bowls at 1350 cm^2 (each bowl 15 cm x 15 cm): ATZ 23.95 $\mu g/cm^2$, MET 31.01 $\mu g/cm^2$, chlorothalonil 44.30 $\mu g/cm^2$, imidacloprid 5.39 $\mu g/cm^2$ and TDN 2.91 $\mu g/cm^2$. After application, bowls were placed under a fume hood overnight to evaporate all MeOH, the following morning 50 mL of water were used to rehydrate the soil of each individual bowl using a standard spray bottle. Control bowls were sprayed with 100% MeOH, and followed the same procedure as pesticide applied bowls. Leopard frogs and Fowler's toads used in the study were at least 60 days post-metamorphosis and had an average weight of ~1 g. Both amphibian species

were last fed 48 h prior to exposure. Amphibians were dehydrated for 0, 2, 4, 6, 8, and 10 hours before an exposure commenced with laboratory conditions: temperature ranged between 20–22 °C with 12 h light/12 h dark cycle. Dehydration data entailed taking an initial composite weight before culled frogs were moved to an empty glass aquarium with a screen lid to allow adequate air flow for dehydration; individual replicates were n=8 for leopard frogs and n=6 for Fowler's toads for each of the five dehydration time periods and pesticides tested. After the allotted dehydration time, composite amphibians from the same dehydration time point were reweighed to obtain an approximate percent of body weight lost during the dehydration period. Subsequently, each amphibian was placed into a Pyrex® bowl and covered with a mesh screen that was secured with a rubber band for an 8 h exposure (n=6 for leopard frogs and n=5 for Fowler's toads) and 1 control for each time point per pesticide exposure (total n=7 for leopard frogs and n=6 for Fowler's toads). Exposures commenced at 08:00 and took place under laboratory conditions. Rehydrated weights during exposure studies were used to determine analytical body concentrations and burdens.

Amphibian and Soil Extraction

All pesticide extraction procedures have been previously described in Van Meter et al. (2014). Briefly, whole body homogenates were spiked with 10 µL of 1000 ppm internal standard (tetraconazole) and placed on a freeze-dryer overnight. Both amphibian and soil samples were extracted twice with MeOH, using both sonication and centrifugation. Methanol was then combined and evaporated down to ~1 mL using a gentle stream of nitrogen; next, a liquid-liquid extraction was done using methyl *tert*-butyl ether (MTBE). For chlorothalonil, a 1 mL aliquot of the MTBE sample was taken and placed into a GC vial and analyzed on a GC-MS;

for all other analytes, the 1 mL aliquot was blown dry under nitrogen, reconstituted with 30% MeOH (v:v), and analyzed on a LC-MS/MS. To obtain the whole body concentration for each amphibian, the parent active ingredient was summed with its corresponding metabolite(s) and divided by the amphibians rehydrated weight.

LC-MS/MS Instrumentation

Active ingredients and metabolites were quantified on a Varian Prostar HPLC linked to a Varian 1200L triple quadrupole mass spectrometer with an Eclipse XDB-C18 column (3.5 µm particle size, 3.0 x 150 mm; Agilent Technologies, CA, USA). Initial conditions were held for 2 minutes at 70% water with 0.1% formic acid (A) and 30% acetonitrile with 0.1% formic acid (B) then ramped to 90% B over 16 minutes and held there for 4 minutes before returning to starting conditions. The drying gas was set at 225 °C and the capillary voltage was at 50 V for all compounds analyzed. MOXA, MESA, and chlorothalonil metabolite were the only analytes analyzed in negative mode; all other pesticides were analyzed in positive mode (Table 5-1).

Data Analysis

The effects of dehydration time on the slope of post-exposure pesticide concentrations were tested with an ANCOVA that controlled for species and chemical categorical variables. The sample design was completed for both species (n=7 for leopard frogs; n=6 for Fowler's toads); treatments were conducted for each combination of chemical (atrazine, chlorothalonil metabolite, imidacloprid, metolachlor, triadimefon) and time (0, 2, 4, 6, 8, and 10 hours). Statistical test results are presented for the experiment as a whole, then examined for each individual chemical. All analyses were performed in R version 3.3 (R Core Team, 2017).

Results

To determine the influence of hydration status on exposure to pesticides in amphibians, anurans were dehydrated for 0–10 h and exposed to a pesticide contaminated soil for 8 h to observe differences in whole body concentrations. On average, dehydrated leopard frogs lost 12.5, 18.9, 19.8, 21.0 and 24.5 percent body weight when dehydrated for 2, 4, 6, 8 and 10 h, respectively. Fowler's toads had slightly higher body loss with 11.4, 19.5, 25.7, 34.6 and 34.7 percent body weight for 2, 4, 6, 8 and 10 h, of dehydration respectively. Rehydration weights for composite time points demonstrated an increase in weight with dehydration time, however, it was not significant ($p > 0.05$). Hydration status significantly affected the absorption of pesticides in both leopard frogs and Fowler's toads. The two amphibian species were considered to be statistically different from one another with Fowler's toads having higher concentrations ($p < 0.01$). Furthermore, when taking into account the five different pesticides and two amphibian species for each dehydration time point, there was a statistically significant decline in concentration with increase in dehydration time ($F_{6,293} = 67.66$, $p < 0.01$). Although this relationship between concentration and dehydration time was significant across the entire experiment, all pesticides did not demonstrate uniform declines in whole body amphibian concentrations; therefore, each chemical is addressed individually below.

Leopard frogs exposed to ATZ-contaminated soils showed a decrease in pesticide concentrations as dehydration status increased ($p < 0.01$); the total body burden decreased from 8.84 ± 2.34 ppm (0 h) to 7.65 ± 2.12 ppm (10 h). The Fowler's toads exposed to ATZ exhibited an initial increase in absorption from 0 h (20.59 ±3.96 ppm) to 2 h (27.44 ± 2.62 ppm), followed by a steady decline as dehydration time increased (10 h, 9.62 ± 3.53 ppm). Overall, Fowler's toads demonstrated a decrease in ATZ tissue concentration with an increase in dehydration time

($p < 0.01$). Atrazine and its metabolites were found at higher concentrations in Fowler's toads than leopard frogs at each dehydration time point ($p < 0.01$; Figure 5-1).

For leopard frogs exposed to TDN, there was a negative slope indicating that body concentrations decreased with increased dehydration time; however, it was not statistically significant ($p > 0.05$). At 0 h the average was 0.99 ± 0.12 ppm and at 10 h the average was 0.60 ± 0.04 ppm. An upward, increasing trend was observed for the first few time points (≤ 4 h) in Fowler's toads exposed to TDN; afterward, a decline was exhibited (4 h was 1.68 ± 0.50 ppm and 10 h was 0.82 ± 0.13 ppm), but was not statistically significant ($p > 0.05$; Figure 5-1).

Both leopard frogs and Fowler's toads exhibited minimal decreases in metolachlor pesticide concentrations with an increase in dehydration time, however, neither amphibian species had concentrations that differed significantly with time ($p > 0.05$; Figure 5-1). Overall, leopard frogs decreased from 4.48 ± 1.43 ppm at 0 h to 1.75 ± 0.24 ppm at 10 h. For Fowler's toads, metolachlor had an initial increase from 0 h (1.26 ± 0.45 ppm) to 2 h (5.05 ± 2.82 ppm), followed by a decrease in absorption as dehydration time increased to 10 h (2.16 ± 0.70 ppm).

Exposure to chlorothalonil only resulted in the observation of its metabolite. For the chlorothalonil metabolite in leopard frogs, dehydration time exhibited a decline in concentration from 0.074 ± 0.010 at 0 h to 0.038 ± 0.007 ppm at 10 h (Figure 5-1). In Fowler's toads, there was also an inverse relationship between whole body concentrations and dehydration time. Time was significant ($p < 0.05$), while species was close but does not meet the significant threshold ($p = 0.104$).

For the insecticide imidacloprid, the parent pesticide was the only compound analyzed; additionally, imidacloprid was only exposed to leopard frogs due to the limited number of

Fowler's toads. For leopard frogs, imidacloprid had a negative correlation with dehydration time; however, it was not statistically significant ($p > 0.05$; Figure 5-1).

Rehydration rates (g/h) were determined for each pesticide and species combination at every time point (Figure 5-2). For leopard frogs, the rehydration rate appears to have reached steady state around 4 h. However, for Fowler's toads there was a slight increase over time from 0 h – 10 h, but this was minimal.

Discussion

Amphibian species have declined in the last several decades and pesticides are one potential causative factor. A recent U.S. survey conducted by Gilliom et al. (2006) analyzed pesticides in water samples from agricultural, urban, and mixed land use areas, observing at least one pesticide 90 percent of the time. Similarly, Smalling et al. (2012) analyzed water and bed-sediments for pesticides in eleven areas inhabited by amphibians throughout the U.S., where 24 pesticides (in water) and 22 pesticides (in bed-sediments) were identified and quantified. Moreover, several studies have found quantifiable amounts of current-use pesticides in frog tissues from various sites throughout the U.S., presumably due to agricultural application (Smalling et al., 2013; Smalling et al., 2015; Battaglin et al., 2016). Bradford et al. (2010) noted that pesticide body burden in amphibians from the Sierra Nevada mountains was linked to atmospheric transport of pesticides from the agricultural San Joaquin Valley. Pesticides therefore are present in most areas where amphibians live and breed, so coming into contact with pesticides can be a daily occurrence.

Dehydration tolerance has been well documented within amphibians and the more terrestrial species can withstand a higher loss in body water (Hillman, 1982). This increased

tolerance is due to overall skin thickness which correlates to what habitats they occupy (Toledo and Jared, 1993). Aquatic frogs usually have smooth dorsal and ventral skin, making water less permeable; terrestrial species have granulations on their ventral skin for a greater surface area enabling them to uptake water quickly (Lillywhite and Licht, 1975; Toledo and Jared, 1993; Hillman et al., 2009). Overall, amphibian skin has a low resistance to water; however, studies indicate that higher skin resistance to evaporative water loss is more indicative of arboreal species than non-arboreal species, which is due to exposure to higher temperatures and radiation for the arboreal species (Lillywhite, 2006). This skin permeability feature places anurans in one of four habitat groups: aquatic, semi-aquatic, terrestrial or arboreal (Hillman et al., 2009).

Amphibians can tolerate up to 50-60% loss of their total body water due to dehydration (Hermes-Lima and Storey, 1998). Since Fowler's toads are able to reabsorb water more quickly than leopard frogs, the hypothesis for this study was that concentrations and body burdens would be higher in Fowler's toads. Moreover, we thought these concentrations would be higher at longer dehydration time points. Within the present study, Fowler's toads had statistically higher pesticide concentrations at each dehydration time compared to leopard frogs. Increased hydration correlated with the overall uptake of pesticides in dehydrated frogs. In the current study, whole body concentrations for each pesticide were shown to decrease with increasing dehydration time (Figure 5-1); this was likely due to the decrease of aerobic metabolism when up to 30% of initial body weight was lost. Hillman (1978a) illustrated that anurans lost the ability to conduct aerobic metabolism when they were dehydrated, which resulted in increased glycolysis. Gatten Jr (1987) also observed that a major effect of dehydration was a decrease in ATP being synthesized via aerobic means. In amphibian livers, ATP was observed to drop by 44 percent in anurans dehydrated to 50 percent total body water (Churchill and Storey, 1995). It is therefore

possible that anurans in this study did not have enough energy to facilitate uptake of pesticides from the soil, or energy was utilized for more vital processes.

Dehydration in *Xenopus laevis* resulted in a decline in circulatory oxygen and cardiovascular capabilities that led to anoxia within the tissues (Hillman, 1978a; Hillman, 1980). This decrease in oxygen, measured as maximal oxygen consumption rates from dehydration, was due to osmotic effects in the tissues (Hillman, 1978a). Loss of body water resulted in increased blood viscosity and hematocrit (cellular fraction of the blood); thus, it became more difficult to deliver oxygen which resulted in anoxic tissues (Hillman, 1978a; Hillman, 1978b; Shoemaker et al., 1992; Hermes-Lima and Storey, 1998; Hillman et al., 2009). The decline in oxygen pressure at rest due to dehydration decreases cardiac output which can lead to cells experiencing hypoxic conditions and ultimately death (Hillman, 1978a; Hillman et al., 2009). It is stressful for the amphibian to rehydrate following dehydration, however, due to wide ranges in oxygen forming free radicals which can cause oxidative stress and compromise ATP production (Hermes-Lima and Storey, 1998; Hillman et al., 2009). This stress occurs because dehydration lowers systemic O_2 transport; once the amphibian rehydrates, reoxygenation of the tissues allows reactive oxygen species to form (Hermes-Lima and Storey, 1998; Hillman et al., 2009).

Due to dehydration, tissues become hypoxic or anoxic from impairment of blood circulation throughout the body. It is therefore possible that pesticides were not accumulated in dehydrated amphibians at initiation of the experiment. Once the amphibian is rehydrated to a threshold value or targeted percent hydration, its aerobic metabolism and metabolic demand can facilitate uptake of pesticides into the body. This will result in lower concentrations of pesticides being observed in more dehydrated amphibians. Since hydrated anurans did not need to

overcome a lack of metabolic demand on physiological processes, they continued to uptake pesticides at a regular rate.

Another mechanism that could have occurred in amphibians, in addition to the loss of energy, is a dilution scenario. More water would be uptaken in the severely dehydrated amphibians, resulting in a higher amphibian mass leading to a lower pesticide tissue concentration for amphibians dehydrated the longest. Since aquaporins are permeable only to water, by affecting the rate at which water moves across the membrane via osmosis, these channels would be too small for pesticides (Campbell and Reece, 2005). Therefore, the dilution scenario occurred at 0, 2, and 4 h dehydration time points based on Figure 5-2, where there was a slight increase in rehydration rate. Additionally, in Viborg and Rosenkilde (2004), dehydrated toads demonstrated a decrease in water uptake over a 2 h time span. Moreover, these rehydration values for dehydrated toads were significantly above hydrated toad values for the same length of time (Viborg and Rosenkilde, 2004). This demonstrates that amphibians rapidly uptake water when they are dehydrated, and once they are hydrated again, their rehydration rate decreases. However, in the current study, during the 6–10 h time points, 20 percent body mass loss was observed, resulting in a decline in liver ATP which demonstrates that a loss of energy occurred within amphibians (Churchill and Storey, 1995). Hence, both of these mechanisms are possible and appear to operate simultaneously.

Several articles recently examined effects of pesticide uptake in amphibians. Van Meter et al. (2015) noted that dermal contact from pesticides to amphibians was the main route for exposure in anurans, and simulated overspray scenarios resulted in higher concentrations compared to indirect contaminated soil. Van Meter et al. (2014) observed that water solubility and soil partition coefficients were the best indicators for uptake of pesticides in amphibians.

Furthermore, Van Meter et al. (2016) showed that organic matter content of agricultural soil can significantly affect pesticide body burdens. This study built on past experiments by testing hydration status on terrestrial phase amphibians, and utilized a dehydration time of 10 h as a worst-case scenario. The current study demonstrates that more dehydrated amphibians have lower pesticide body burdens resulting after 8 hours of exposure. Future experiments do not need to dehydrate amphibians for an extended period of time if the goal is to evaluate maximum uptake rates. These tissue concentrations calculated post dehydration can also be incorporated into ecological risk assessments.

Climate change can result in weather patterns that make irrigated agricultural landscapes relatively desirable amphibian habitat in otherwise xeric landscapes or time periods (U.S. EPA, 2014). In these situations, anurans may dehydrate faster, requiring them to search for water sources in order to remain hydrated, leading to a higher likelihood of being exposed to pesticides in agricultural areas. These interactions can add to the list of potentially interacting stressors that amphibian populations are confronting (Davidson et al., 2002; Davidson, 2004). Results of this study help to address the interaction of hydration status and pesticide uptake in two different species of terrestrial phase amphibians. In conclusion, our study demonstrates that hydration status plays a surprisingly important role in pesticide uptake within anurans: more severely dehydrated amphibians exhibited lower concentrations of pesticides, contrary to our initial expectation. Understanding the mechanisms behind this process and how it governs exposure of pesticides in amphibians is critical in estimating how hydration status interacts with other natural and anthropogenic stressors in specific landscapes (Purucker et al., 2007). Interactions of hydration status and pesticide exposure in amphibians also needs to be studied at levels beyond

individual body burdens (biomarkers to population) for a broader understanding of the stressors being experienced by amphibians (Grant et al., 2016; Snyder et al., 2017).

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Table 5-1: LC-MS/MS analytical parameters for both parent and metabolite pesticides in dehydration exposures.

Compound	Type	Mode	Rt (min)	Parent (*m/z*)	Daughter (*m/z*)	CE (V)
DIA	Atrazine degradate	+	4.165	174	68 104	-23 -19
Imidacloprid	Insecticide	+	5.937	256	175 209	-19 -15
DEA	Atrazine degradate	+	5.984	188	104 146	-22 -14
Atrazine	Herbicide	+	15.869	216	104 174	-27 -16
Triadimenol	Fungicide	+	18.158 18.583	296	70 90	-4 -10
Triadimefon	Fungicide	+	20.032	294	197 225	-15 -13
Tetraconazole	Fungicide	+	20.078	372	70 159	-9 -25
Metolachlor	Herbicide	+	21.616	284	176 252	-25 -14
MESA	Metolachlor degradate	-	7.716	328	80 121	25 17
MOXA	Metolachlor degradate	-	14.771	278	158 206	20 9
Chlorothalonil metabolite	Chlorothalonil degradate	-	17.181	245	175 182	22 26

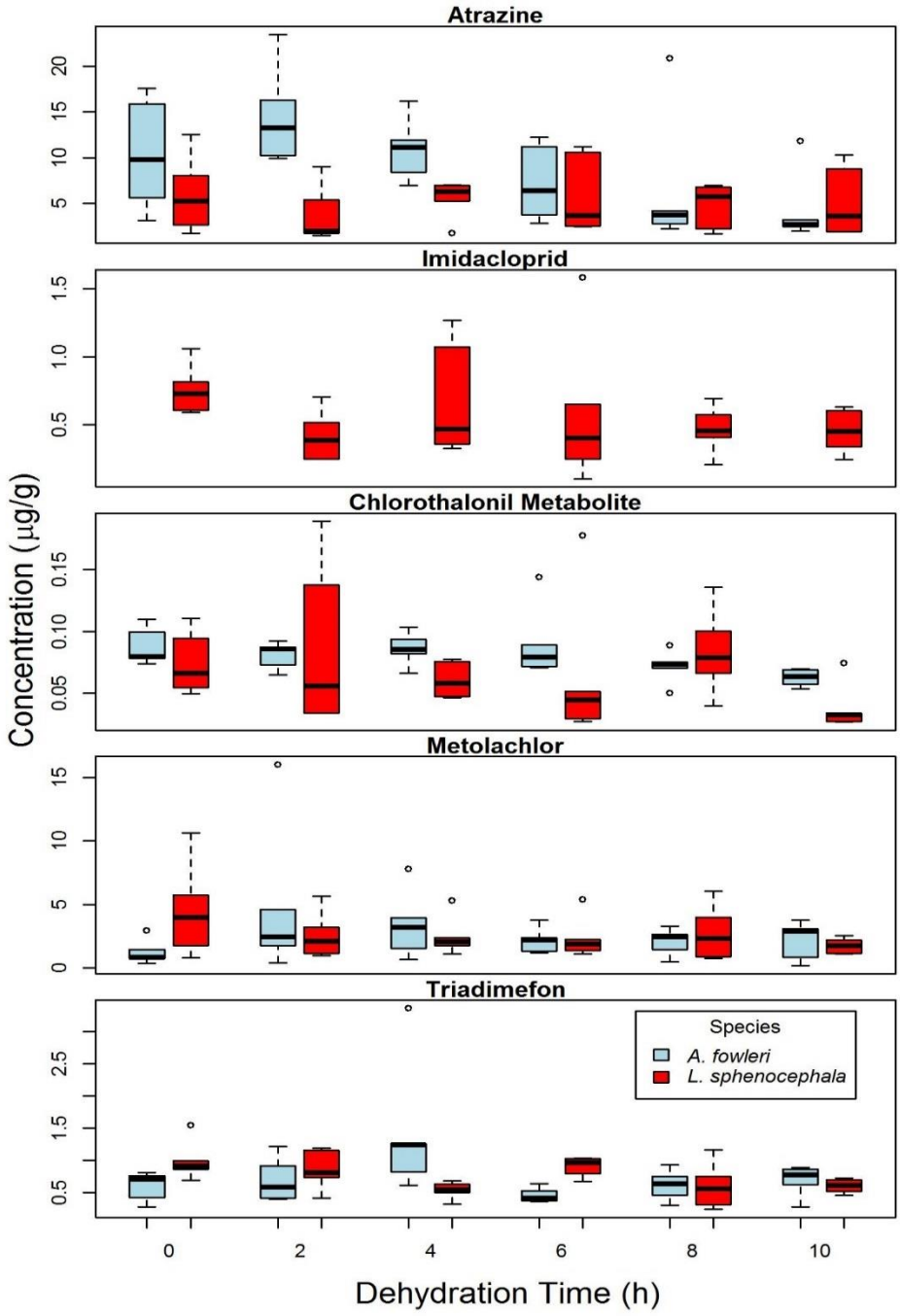

Figure 5-1: Tissue concentrations (µg/g) of pesticides in Fowler's toads (*Anaxyrus fowleri*) and leopard frogs (*Lithobates sphenocephala*). The two species are shown next to each other with body burdens presented as boxplot summaries for each of the dehydration time points following 8 h exposure within the pesticide treatment. Fowler's toads (*Anaxyrus fowleri*) (blue) and leopard frogs (*Lithobates sphenocephala*) (red). Individually atrazine and chlorothalonil metabolite are significant ($p < 0.05$). Overall body burden was significant across time, species, and pesticide ($p < 0.01$).

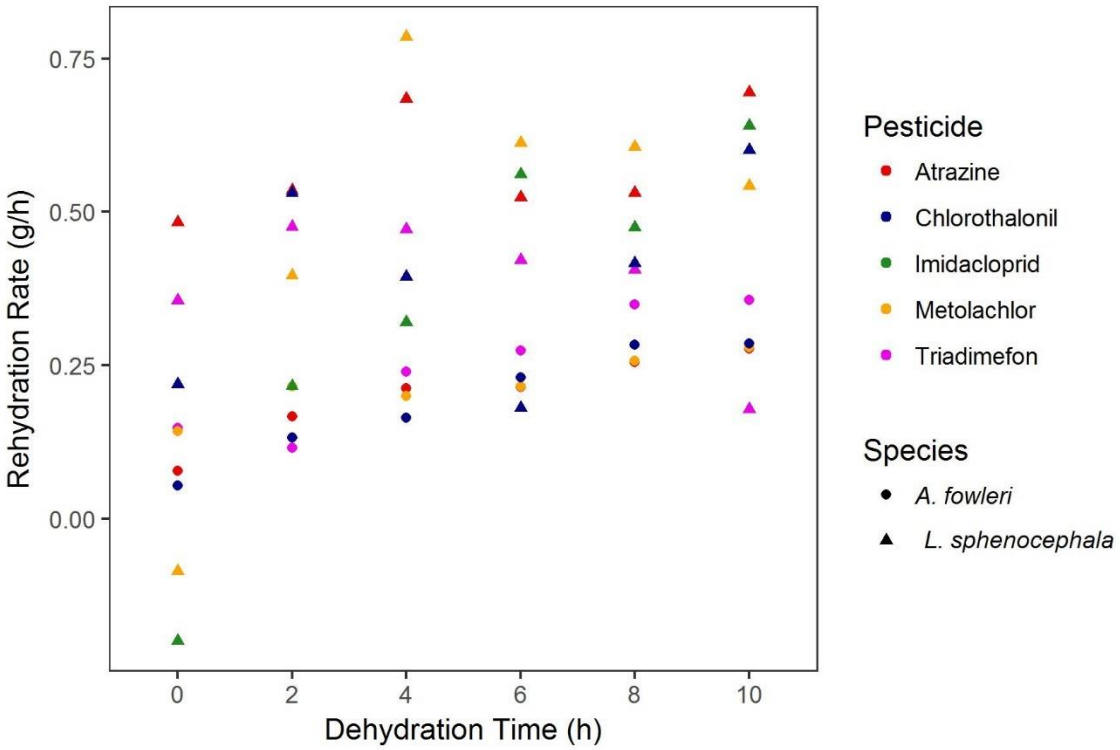

Figure 5-2: Rehydration rates (g/h) for each pesticide and species examined in dehydration exposures.

CHAPTER 6

ENDOGENOUS AND EXOGENOUS BIOMARKER ANALYSIS IN TERRESTRIAL PHASE AMPHIBIANS FOLLOWING DERMAL EXPOSURE OF PESTICIDE MIXTURES [4]

[4] Glinski, D.A., S.T. Purucker, R.J. Van Meter, M.C. Black, and W.M. Henderson. To be submitted to *Environmental Toxicology and Chemistry*.

Abstract

Agricultural application of pesticides frequently contains pesticide mixtures that are co-applied throughout a growing season to maximize crop yield. Therefore, it is likely that non-target species will be exposed to several pesticides at any given time. The objectives of this study were to quantify body burdens along with elucidating the metabolites and corresponding biochemical pathways affected by exposure to pesticides as both singlets and in combination in terrestrial phase amphibians. Southern leopard frogs (*Lithobates sphenocephala*) were exposed either at maximum or $1/10^{th}$ maximum application rate to single, double or triple pesticide mixtures of bifenthrin (insecticide), metolachlor (herbicide), and triadimefon (fungicide). Tissue concentrations demonstrate both facilitated and competitive uptake of pesticides when in mixtures. Metabolomic profiling of amphibian livers identified metabolites of interest for both application rates, however; magnitudes of changes varied for the two exposure rates. Exposure to lower concentrations demonstrated down regulation in amino acids due to their being utilized for glutathione metabolism or energy demands. Amphibians exposed to the maximum application rate resulted in upregulation of amino acid metabolites because energy resources were depleted. Endogenous and exogenous biomarkers of pesticide exposure can be utilized to form vital links in an ecological risk assessment for non-target species.

Introduction

Varying types and amounts of pesticides are applied to agricultural fields depending on the season, crop, or nuisance pest targeted (Laetz et al., 2009). Within the broad classification of pesticides applied, the most common classes are herbicides, insecticides, and fungicides (Laetz et al., 2009). However, due to the continuous application of pesticides, some pests have developed resistance, resulting in higher application rates and greater application frequencies. Therefore, due to post-application persistence and the use of mixtures, non-target species may be exposed to multiple pesticides concurrently. Interestingly, pesticides and fertilizers are now being applied together at a higher extent and in more commercially available combinations (Mann et al., 2009). These combinations of pesticides and/or fertilizers help minimize application costs and ensure that multiple pests can be combated (Cloyd, 2012). As a result, multiple pesticides are constantly being quantified in agricultural settings; moreover, low level residues are still observed well after they have been applied resulting in mixtures exposures in non-targeted organisms. Mixtures of pesticides have been detected in streams, sediment, surface water, and rainwater (Gilliom et al., 2006; Smalling et al., 2012; Potter and Coffin, 2017). Not only does assessing the effects of pesticide mixtures have challenges but incorporating them into ecotoxicological assessments or regulatory context also has its own set of hurdles (Backhaus and Faust, 2012).

Amphibians are a unique class of vertebrates due to their biphasic life histories that incorporate both aquatic and terrestrial habitats, thus, they are often considered indicators of the health of an ecosystem. As tadpoles, amphibians are continuously exposed to contaminants in the aquatic environment. However, terrestrially, they must remain near aquatic environments to stay hydrated, thus they can become co-contaminated by the terrestrial and aquatic ecosystem.

Moreover, within the past several decades' amphibian populations have been declining globally due to exposure of multiple stressors such as pesticides, deforestation, pollution and diseases (Blaustein et al., 1994; Sparling et al., 2001; Christin et al., 2003). Since the 1980s, more than 120 species have become extinct globally, while over one-third of amphibian species are threatened globally (Whitfield et al., 2007).

Multiple studies have investigated the effects of multiple pesticides on aquatic stage amphibians. When investigating the time to metamorphosis in tadpoles, Boone (2008) found that exposure to three different insecticides had an effect on their mass at metamorphosis. This was supported by Hayes et al. (2006) where pesticide mixtures resulted in greater effects being observed on the inhibition of growth and development in *Rana pipiens*. Additionally, Relyea (2004) noted that combinations of four pesticide formulations reduced survival and growth within the amphibian larvae that were exposed. This same phenomenon was also observed in Relyea (2009) and Hua and Relyea (2014), where mixtures reduced survival more than their corresponding individual pesticides. A synergistic relationship occurred between atrazine and organophosphates in midges and in *Xenopus laevis* (Pape-Lindstrom and Lydy, 1997; Wacksman et al., 2006). Furthermore, in amphibian larvae that were exposed to a mixture of atrazine and alachlor, toxicity was observed to be greater than additive (Howe et al., 1998). This was contradicted by Boone and Bridges-Britton (2006) where the authors observed no effects of multiple chemical stressors on tadpoles; however, this could be due to the fact that the compounds they used had different modes of action. Similar observations of no effects have been observed in other studies (Boone and James, 2003; Relyea, 2009). Of note, many of these articles that focus on pesticide mixtures in amphibians, examine exposures from the aquatic

environment and in tadpoles; therefore, there is a dearth of information on terrestrial phase amphibians being exposed to pesticide mixtures.

Biomarker analysis and metabolite or pathway identification can be utilized in the assessment of pesticide exposures. These sublethal biochemical consequences of pesticide exposure can contribute to the assessment of the ecosystem (Venturino et al., 2003). Metabolomics is an experimental technique that examines the endogenous levels of metabolites such as amino acids, fatty acids, and sugars that are likely to be either up or down regulated following exposure to pesticides or other xenobiotics prior to the onset of overt toxicity. Following exposures, differences in fluxes in the metabolome make it possible to identify specific pathways (i.e. citric acid cycle or fatty acid biosynthesis) that are potentially impacted. Therefore, metabolomics can be utilized to identify effects of exposure that are occurring within the metabolome before changes occur in measured apical endpoints (growth, reproduction, and mortality). The identification of biomarkers can also aid in bridging the gaps for an adverse outcome pathway where biochemical pathways generated from metabolomics can help inform the knowledge found from effects endpoints.

The objectives of this study were to observe the body burdens of individual and mixture effects, among three pesticides – the herbicide metolachlor, the insecticide bifenthrin, and the fungicide triadimefon – in terrestrial phase amphibians (southern leopard frogs, *Lithobates sphenocephala*) at both maximum and $1/10^{th}$ maximum application rates. Obtaining a metabolomic profile of the amphibian liver to observe perturbations that occurred at both application rates can help detect differences in pesticide classes and in mixtures. Maximum application would be considered a worst-case scenario, while $1/10^{th}$ maximum application would mimic the concentration of residual pesticide in agricultural fields. Understanding how

exposures of single and multiple pesticides to non-target species influences toxicity is necessary to accurately assess the ecological risks these compounds pose.

Materials and Methods

Chemicals

Active ingredients (AI) and corresponding metabolites for metolachlor (Met), metolachlor ethane sulfonic acid (MESA), metolachlor oxanilic acid (MOXA), triadimefon (Tdn), triadimenol (Tdl), bifenthrin (Bif), permethrin, and tetraconazole were obtained from the U.S. Environmental Protection Agency's National Pesticides Standard Repository (Fort Meade, MD, USA). All pesticides used in the study were ≥ 96.5% purity. All solvents used for pesticide extraction and analysis were of highest grade and purchased from Fisher Scientific (Pittsburgh, PA, USA).

Amphibian Exposures

Amphibian collection and rearing was previously described in Van Meter et al. (2014) and exposure studies were carried out based on the methods in Van Meter et al. (2016). Briefly, soil was processed through a 2 mm sieve and stored in a cold room < 4 °C until used; all soil was collected from Newton, GA in July 2015. Each Pyrex® bowl contained 150 g of sieved soil and a full factorial exposure design with three pesticides (Tdn, Met, and Bif) at two concentrations (maximum or 1/10th maximum application rate) was applied to the soil with 8 individual replicates per treatment. Application rates were scaled down to the size of 8 Pyrex® bowls (1800 cm^2; each bowl 15 cm x 15 cm) to minimize spraying time and have as little error as possible between replicates. Application rates of bifenthrin, metolachlor and triadimefon were 3.45,

30.62, and 2.87 μg/cm^2, respectively, for maximum application rate, while for 1/10th maximum application rate bifenthrin, metolachlor and triadimefon were 0.345, 3.062, and 0.287 μg/cm^2, respectively. Pesticides were dissolved in 75 mL of methanol (MeOH), while 100% MeOH was used as a control, and applied to the 8 Pyrex® bowls using a Spray Gun® canister attached to a graduated glass jar. After application, all bowls were placed in a fume hood overnight to evaporate the MeOH. The following morning each bowl was rehydrated with 50 mL of deionized water. Amphibians were dehydrated for 10 h prior to 8 h exposure on pesticide contaminated soil. Leopard frogs used in the study were at least 180 days post-metamorphosis. After the experiment amphibians were euthanized and stored at -80 °C while soil sample aliquots (5 g) were collected and stored in -20 °C freezer until processing and analysis.

Metabolomics and Pesticide Analysis

Leopard frogs were thawed and an initial weight was obtained before livers were excised and placed into pre-labeled 2 mL centrifuge tube and immediately placed on ice. Endogenous hepatic metabolites were extracted and analyzed following the bi-phasic procedure described in Viant (2007). Briefly, livers were extracted with a mixture of methanol and chloroform using 2 mm beads in a tissue homogenizer to separate metabolites into a polar and non-polar fraction. Individual fractions were placed into 2 mL vials and evaporated to dryness overnight on a speedvac. Samples were derivatized with 100 μL of methoxyamine hydrochloride in pyridine (20 mg/mL; 2.5 h, 60 °C) and then with 100 μL of *N*,*O*-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) containing 10% TCMS (1.5 h, 60 °C) for a total of 2.5 h. After derivatization, all polar samples were analyzed via gas chromatography coupled with mass spectrometry as described below.

Methods for pesticide and soil extractions for body burden analysis were described in Van Meter et al. (2014). Briefly, remaining whole body homogenates were spiked with 10 µL of 1000 ppm tetraconazole (internal standard) and placed on a freeze drier overnight. Soil (also spiked with tetraconazole) and amphibian samples were extracted two times with MeOH via sequential sonication and centrifugation. Supernatants from both extractions were combined and blown down to 1 mL under a gentle stream of nitrogen gas. Then, liquid-liquid extraction was utilized with methyl *tert*-butyl ether (3 mL MTBE). A 1 mL aliquot was transferred to a 2 mL vial and blown dry under nitrogen and then reconstituted with 1 mL 30% MeOH (v:v) and analyzed on a liquid chromatograph coupled to a mass spectrometer (LC-MS/MS) as described below.

GC-ToF/MS

Metabolomic samples were analyzed on a LECO Pegasus® 4D GC-ToF/MS (St. Joseph, MI) with an Agilent 7890B gas chromatograph and all data were processed with ChromaTOF® software. The separation column was a DB-5ms (30m, 0.25 µm thickness, and 0.25 mm ID, Agilent Technologies, CA). All injections were made in splitless mode (2 µL). The injector temperature was 275 °C, the transfer line temperature was held constant at 280 °C and the ion source temperature was 225 °C. The carrier gas was UHP grade helium and maintained at a constant flow of 0.8 mL/min. The initial oven temperature was held for 2 min at 60 °C and ramped at 8 °C/min to 300 °C with a hold time of 5 min. Mass spectra were acquired from 50-650 *m/z* at an acquisition rate of 20 spectra/second. All chromatograms were exported as netcdf files and imported into MetAlign 041012 for data alignment and processing using recommended parameters for time of flight mass spectrometry (Lommen, 2009). After alignment, spectra were

filtered and duplicate retention times were removed using Excel as described in Niu et al. (2014). To generate t-test filtered chromatograms Excel was used, while for ANOVA and metabolite pathway analysis MetaboAnalyst 3.0 was used (Xia and Wishart, 2016). Metabolites were identified using NIST 2014 spectral database.

LC-MS/MS

Active ingredients as well as their metabolites were analyzed on a Varian Prostar HPLC interfaced to a Varian 1200L triple quadrupole mass spectrometer. Compounds were separated with an Eclipse XDB-C18 column (3.5 µm particle size, 3.0 x 150 mm; Agilent Technologies, CA, USA). Initial conditions were held for 2 minutes at 70% water with 0.05% formic acid and 2 mM ammonium acetate (A) and 30% methanol with 0.05% formic acid and 2 mM ammonium acetate (B) then ramped to 95% B over 22 minutes and held for 6 minutes before returning to starting conditions (total runtime 40 minutes). The flow rate was 400 µL/min and injection volume was 20 µL. The drying gas was set at 225°C and the capillary voltage was at 60 V for all compounds analyzed. MOXA and MESA were the only analytes analyzed in negative mode from 0–17 minutes; all other pesticides were analyzed in positive mode from 17–40 minutes (Table 6-1S).

Results

Tissue Concentration for Maximum Application Rate

The objectives of this study were to identify individual and combined effects on pesticide uptake using three different classes of pesticides in terrestrial phase amphibians after an 8 h dermal exposure. We hypothesized that mixtures would lead to higher tissue concentrations of

each pesticide. Amphibians exposed to metolachlor had a body burden of 7.53 ± 2.20 µg/g, however, when exposed as a component of a mixture the concentration decreased, where the lowest tissue concentration was 1.99 ± 0.42 µg/g for the BifMetTdn mixture (Figure 6-1). Tissue concentration for Bif (0.367 ± 0.046 µg/g) increased in the presence of Met (0.461 ± 0.089 µg/g) and Tdn (0.670 ± 0.084 µg/g). Together Met and Tdn had no apparent effect on the uptake of Bif (0.381 ± 0.045 µg/g). However, for Tdn, the tissue concentration increased when Bif was added from 0.326 ± 0.051 µg/g to 0.413 ±0.060 µg/g. When Met was applied with Tdn, the concentration of Tdn was similar 0.326 ± 0.051 µg/g (Tdn only) and 0.313 ± 0.065 µg/g (MetTdn). Overall, both of these compounds did not affect Tdn absorption in the three-way mixture only resulting in an insignificant decrease in body burden (0.311 ± 0.078 µg/g). Both Bif and Tdn appeared to facilitate the uptake of each other, resulting in higher body burdens for both compounds. However, a decrease in Met when any other pesticide was co-exposed demonstrated either a competitive or an inhibitory uptake.

Tissue Concentration for 1/10th Maximum Application Rate

Metolachlor had the highest tissue concentration at 0.163 ± 0.032 µg/g compared to the other pesticides as singlets (Figure 6-2). There appeared to be a facilitated uptake for Met, where exposure to the BifMetTdn mixture resulted in the highest amphibian body burden concentration of Met at 0.359 ± 0.055 µg/g. While Bif body burden was lower in the presence of Met (0.039 ± 0.008 µg/g), Tdn only slightly lessened the dermal uptake from 0.088 ± 0.016 µg/g (Bif, singlet) to 0.061 ± 0.009 µg/g (BifTdn, doublet). Independently, Met and Bif did not statistically affect Tdn total body burden; however, when all three were exposed together, Tdn levels increased from 0.038 ± 0.006 µg/g (Tdn, singlet) to 0.063 ± 0.009 µg/g (BifMetTdn, triplet).

Metabolomics

To assess the consequence of exposure to pesticide and pesticide mixtures on the hepatic biochemical profile in amphibians, polar metabolites were analyzed on a GC-ToF/MS to identify potential biochemical pathways affected. To aid in visualizing the 'biological impact' on the metabolome the statistically significant peaks from t-test filtering were summed (Figure 6-3). Interestingly, the biological impact of the maximum application rate was smaller in spectral features compared to $1/10^{th}$ maximum application rate (Figure 6-3). For the $1/10^{th}$ maximum application exposure, Met had the largest abundance of spectral features, however, for maximum application rate, BifMet exhibited the greatest number of spectral features modified (Figure 6-3).

The chromatograms were further processed to determine the retention times that were statistically different between each pesticide or pesticide mixture and control to determine similar metabolites affected by exposure (Table 6-1). Overall, 75 metabolites were identified as statistically up or down regulated for any exposure compared to controls. The main metabolites that were up or down regulated were amino acids (i.e., alanine, serine, leucine and proline), carbohydrates (i.e, galactose, maltose and mannose) and nucleic acid content (adenine, thymine and hypoxanthine). The $1/10^{th}$ maximum exposure to bifenthrin had the fewest number of metabolites (n=8) that were up or down regulated, while BifTdn at maximum application had the highest number of metabolites (n= 44). On average the maximum application rate had 38 metabolites that were perturbated compared to 22 metabolites in the $1/10^{th}$ maximum application rate treatments. Furthermore, pesticides applied as singlets had on average fewer metabolites up or down regulated compared to the doublet and triplet mixtures.

These metabolites were then used to identify biological pathways that were affected by pesticide exposure in amphibian livers (Table 6-2S). Overall, only three pathways were similar

amongst all pesticide treatments and both application rates: 1) aminoacyl-tRNA biosynthesis; 2) alanine, aspartate and glutamate metabolism; and 3) arginine and proline metabolism.

Discussion

The majority of articles published focusing on amphibians and pesticide exposure primarily focus on tadpoles (Brühl et al., 2011). To date, limited studies have assessed the effects of pesticide exposure on terrestrial phase amphibians (Dinehart et al., 2009; review in Brühl et al., 2011; Van Meter et al., 2014; Cusaac et al., 2015; Van Meter et al., 2015; Cusaac et al., 2016; Van Meter et al., 2016). Therefore, there is a paucity of data on mixtures of pesticides impacting terrestrial phase amphibians. To date and to our knowledge, only one article has been published that assessed tissue concentrations of multiple active ingredients on terrestrial post-metamorphic amphibians (Van Meter et al., 2017). As in the present study, Van Meter et al. (2017) utilized a full factorial exposure design by comparing all combinations/pairings with three pesticides. Therefore, the objectives of the current study were to identify effects from pesticides applied as singlets and mixtures in terrestrial phase amphibians by measuring body burdens and examining the metabolic profiles of the liver.

Few studies have investigated the modification of biochemical profiles in tadpoles exposed to pesticides (Zaya et al., 2011; Dornelles and Oliveira, 2014; Güngördü et al., 2016). Furthermore, to date, only two studies have used metabolomics to examine the effects of pesticide exposure on amphibians (Snyder et al., 2017; Van Meter et al., 2017). From these studies, little information can be concluded about the metabolic impacts of pesticides on amphibians. In the current study, up and down regulations were observed for various metabolite classes such as amino acids, purine nucleosides, carbohydrates, and others. This was similar to

Snyder et al. (2017) where the authors pointed out that these metabolites can affect purine metabolism, amino acid metabolism, and aminoacyl-tRNA biosynthesis after exposure to atrazine and disrupt amino acid and energy metabolism. In our study, three different biological pathways were considered to be affected by all classes of pesticide exposure. In contrast to Van Meter et al. (2017), the main metabolites of significance resulted in eight different pathways identified as being affected by triple herbicides, while for the mixed pesticides twelve biological pathways were affected. However, aminoacyl-tRNA biosynthesis and alanine, aspartate and glutamate metabolism were the only two pathways that were the same between the current study and Van Meter et al. (2017).

In this study, we hypothesized that exposure to multiple pesticides would influence the dermal uptake of other constituents and that these tissue concentrations would be indicative of the magnitude of fluxes observed in the hepatic profiles. Furthermore, we hypothesized that this phenomenon would also be more pronounced in amphibians exposed to the maximum application rate compared to $1/10^{th}$ maximum application rate. Our hypothesis for the $1/10^{th}$ maximum application rate was correctly observed for Met; higher concentrations were observed when Met was combined with another pesticide, leading to the triple mixture having the highest tissue concentration for Met. However, this same phenomenon was not observed in the maximum application rate experiments; instead, there appeared to be competition or inhibition for Met uptake in the amphibians. In Van Meter et al. (2017) the authors observed that atrazine as the singlet pesticide had the highest concentration, resulting in competitive uptake for the mixtures that were studied. However, when atrazine was combined with malathion the authors noted an increased uptake of atrazine, while a facilitated effect was observed for malathion (Van Meter et al., 2017).

In the current study, 1/10th maximum exposures were observed to have a higher abundance of statistically different spectral features compared to maximum application exposures. This could be due to the organism overextending itself to eliminate low levels of xenobiotics or that at high concentrations toxicity could result in a shutdown of pathways. The triple pesticide at maximum exposure had the second highest abundance for spectral features, which could indicate that the pesticides, all having different modes of action, overwhelmed the system. However, for the 1/10th maximum treatments the triple pesticide mixture was fifth highest in abundance. In Van Meter et al. (2017) the authors observed that the triple pesticide exposure had the lowest overall biological impact on the metabolome, which they stated was potentially due to the different modes of action, also observed in the current study.

Metabolomics

T-tested 'filtered' spectra were used to determine statistical differences between pesticide treated and control samples for all exposures tested. Both up and down regulated metabolites were found in groups of endogenous metabolite classes such as amino acids, carbohydrates, organic acids, and sugar/phosphoric acid derivatives. These endogenous metabolite classes were similar to what was observed in Snyder et al. (2017), which examined the effects of atrazine exposure in tadpoles and in Van Meter et al. (2017), which explored the effects of mixtures both one of each class and three different herbicides on terrestrial phase amphibians. The identification of these metabolites was not unexpected as GC-MS is amendable to derivatized lipids, sugars, aldehydes, ketones esters, amino acids and fatty acids.

Overall, in the current study, we observed a decrease in the amino acids alanine, glycine, methionine, proline, serine, threonine, and tyrosine in the 1/10th maximum application rate

exposure group, while at the same time there was an upregulation of lactose, maltose, galactose. This same phenomenon was detected by Nagato et al. (2016), in diazinon exposed *Daphnia magna*, where a decrease in amino acids happened concurrently with an increase in sugar, which suggested that energy resources were being utilized to combat stress. Moreover, at maximum application rates, aspartic acid, alanine, glutamic acid, lysine, methionine, proline, serine, threonine, and valine were upregulated, while galactose, mannose, maltose and lactose were down regulated. When *D. magna* were exposed to high concentrations of diazinon and malathion, the authors concluded that the organism was slowing down due to the depletion of energy which resulted in lowered protein synthesis (Nagato et al., 2016). This demonstrates that at low pesticide levels, more energy resources are being used to help combat stress, such as aerobic respiration and catabolism of amino acids; however, at higher levels of exposure all the organism's energy resources are depleted, and cannot fully compensate (Sokolova et al., 2012; Nagato et al., 2016).

In the current study, 15 amino acids were affected by pesticide exposure, either as a singlet or in combination, at both application rate exposures. The amino acid arginine is known for being incredibly versatile, since it is a precursor for the synthesis of proteins, other amino acids (glutamate and proline), ornithine, and urea (Wu and Morris, 1998). In the groups, receiving the maximum application rate both proline and glutamic acid were upregulated compared to controls due to the production of these compounds, conversely at lower exposures ($1/10^{th}$ maximum application rate) proline and glutamic acid were down regulated compared to controls. The three branched-chain amino acids (BCAAs) leucine, isoleucine and valine are vital for energy production and protein synthesis (Brosnan and Brosnan, 2006; Kimball and Jefferson, 2006; Ch et al., 2015; Xu et al., 2015). Leucine is the most important amino acid in skeletal

muscle protein synthesis, while isoleucine is best for being incorporated into lymphocytes (Calder, 2006; Kimball and Jefferson, 2006). In the 1/10th maximum application treatments for bifenthrin exposures valine, leucine and isoleucine were all down regulated compared to controls, though for metolachlor and triadimefon valine, leucine and isoleucine were both up and down regulated. Metolachlor, at maximum application rate, expressed an upregulation for valine and leucine. Similar to amphibians Ekman et al. (2006), observed an upregulation in valine when rats were exposed to a high dose of triadimefon. Xu et al. (2015), measured a significant increase in these three amino acids when goldfish were exposed to butachlor. Conversely, in Ch et al. (2015), cypermethrin treated earthworms expressed a decrease in both valine and isoleucine, due to neurological defects and stress within the muscles. Therefore, in this study, the down regulation of BCAAs observed in the 1/10th maximum exposures would potentially be due to the increased synthesis of proteins.

Two of the aromatic amino acids phenylalanine and tyrosine are major constituents in producing the biogenic amines dopamine and octopamine, which are utilized by the nervous system (Miller et al., 1970; McCoole et al., 2012). In the 1/10th maximum application treatments, all pesticides exhibited a down regulation in tyrosine. However, in butachlor treated goldfish an increase in abundance for these aromatic amino acids was expressed (Xu et al., 2015). In the current study a down regulation of phenylalanine and tyrosine in the 1/10th maximum application treatments suggest that biogenic amines were being produced. Therefore, the catabolism of both BCAAs and aromatic amino acids can produce ketones within the liver which is utilized for metabolic energy within amphibians (Nelson and Cox, 2008).

The endogenous amino acid, aspartic acid, aids in the development of the nervous system by acting as a neurotransmitter and a neuromodulator, in addition to partaking in the

neuroendocrine system (D'Aniello, 2007). Aspartic acid is also essential for amino acid metabolism (Wu and Rittenberg, 1949). Triadimefon at 1/10th maximum application was the only treatment to exhibit a down regulation in aspartic acid when compared to controls, both bifenthrin and metolachlor at this rate were associated with upregulation of aspartic acid. Although, all pesticides at maximum application expressed an upregulation in aspartic acid. Ralston-Hooper et al. (2008) noted that aspartic acid was the only amino acid to be down regulated when freshwater amphipods were exposed to atrazine. Additionally, atrazine treated marine mussels *Mytilus edulis* expressed a decrease in aspartic acid (Tuffnail et al., 2009). Therefore, a decrease in aspartic acid would result in an increase in amino acid metabolism within amphibians.

As noted by Liu et al. (2015) glutamate and aspartate are excitatory neurotransmitters, where glutamate can produce GABA through a decarboxylation reaction. GABA is the main inhibitory neurotransmitter within the nervous system, where it regulates psychological and physiological processes along with mediating fast inhibitory synaptic transmissions (Kumar and Goyal, 2008). Within the current study, both glutamic acid and GABA were down regulated in the 1/10th maximum application group; however, they were upregulated in the maximum application groups. Madl and Royer (2000), noted that GABA levels can increase due to hypoxia inhibiting GABA metabolism and the depletion of ATP inducing glutamate decarboxylase. Both compounds, glutamate and GABA were significantly increased in goldfish that were exposed to the organophosphate dichlorvos (Liu et al., 2015). Moreover, goldfish exposed to butachlor also expressed an increase in glutamate, which showed that the tissues were hypoxic (Xu et al., 2015). Therefore, this upregulation in glutamic acid and GABA at maximum application would also indicate that amphibian tissues were hypoxic.

Amphibians that are dehydrated can express a decrease in ATP synthesis via aerobic metabolism, therefore other routes must be used to synthesize energy (Gatten Jr, 1987). One route is through anaerobic metabolism, or glycolysis, which results in the accumulation of lactate (Gatten Jr, 1987). Since the amphibians in this study were dehydrated for 10 h prior to exposure and exposed for 8 h this could have resulted in a buildup of lactic acid. Therefore, due to the upregulation of lactic acid in all of the amphibians, anaerobic metabolism was used to produce energy, even at a less efficient ratio compared to aerobic metabolism due to the hypoxic conditions. This was also observed in Chang et al. (2006), where the authors noted that lactic acid formed under hypoxic conditions within freshwater prawns due to the oxidation of pyruvate.

Myo-inositol is known for generating second messengers by being a precursor of the inositol phospholipids (Downes and Macphee, 1990). However, in this study fluxes in myo-inositol were only detected in the treatments at $1/10^{th}$ maximum application rate where it was both and up and down regulated. Whereas inositol was only detected in maximum application for bifenthrin, where it was down regulated compared to controls. This could mean the production of second messengers by myo-inositol was upregulated due to the down regulation of this compound when the organism was under low stress. In Ch et al. (2015), cypermethrin treated earthworms expressed an increase in myo-inositol, demonstrating that cypermethrin disrupted the phosphatidyl inositol phosphate metabolism.

Nucleic acids are the main building blocks of life and are known for regulating the synthesis of proteins; however, any change in nucleic acid content can lead to alterations in protein synthesis (Tilak et al., 2009). An upregulation was observed for hypoxanthine and the bases of nucleic acids purine and thymine in amphibians exposed to bifenthrin, metolachlor and triadimefon at maximum application. Additionally, uridine and the nucleoside 5-methyluridine

were down regulated in 1/10th maximum application exposure, while uracil was down regulated for singlets. The down regulation of the nucleic acids, would lead to a disruption in nucleic acid and protein synthesis along with other biological processes being hindered. In Tilak et al. (2009) freshwater fish that were exposed to alachlor had expressed a decrease in nucleic acid content. Moreover, when the freshwater fish *Punctius arenatus* was exposed to the pesticides fenvalerate and monocrotophos there was a significant decrease in nucleic acid content, which could affect amino acids and proteins (Rathod and Kshirsagar, 2010).

In the current study, galactose, lactose, and maltose were upregulated for the 1/10th maximum treatment groups, also resulting in energy metabolism being impacted. Another route to obtain energy, when it is in high demand, is through the metabolism of amino acids (Zaya et al., 2011). Additionally, a decrease in amino acids compared to controls can be attributed to DNA repair mechanisms and defense proteins (Taylor et al., 2009; Spann et al., 2011). Ultimately, the changes observed in amphibians suggest biochemical fluxes in energy which can aid in detoxifying pesticides from organisms (Dornelles and Oliveira, 2014).

Pathways

Using the metabolites identified, the main biochemical pathways that were perturbed in this study were: aminoacyl-tRNA biosynthesis; alanine, aspartate and glutamate metabolism; and arginine and proline metabolism.

Aminoacyl-tRNA biosynthesis creates an aminoacyl-tRNA for each amino acid which is used for protein building, which is vital for DNA replication (Ibba and Söll, 2000; O'Donoghue and Luthey-Schulten, 2003). The function of an aminoacyl-tRNA is to deliver its appropriate amino acid to the ribosomal A site, so the corresponding anticodon and codon can match up in

mRNA (Ibba and Söll, 2000). The main metabolites that were identified to affect this pathway were the amino acids identified, resulting in the aminoacyl-tRNA biosynthesis pathway being perturbed for each pesticide at both application rates. This same pathway was impacted in studies by Ch et al. (2015), Snyder et al. (2017) and Van Meter et al. (2017). Therefore, pesticide exposure to amphibians can affect the aminoacyl-tRNA biosynthesis pathway and in turn impact DNA replication and protein synthesis.

The alanine, aspartate and glutamate metabolism pathway had alanine, aspartic acid, glutamic acid, GABA, and succinic acid as the major metabolites that were affected. Both the amino acids, aspartic acid and glutamic acid hold a vital position within amino acid metabolism (Wu and Rittenberg, 1949). These compounds undergo a transamination reaction, which results in the removal of the amino group within the liver for urea formation (Wu and Rittenberg, 1949). Additionally, within *Clostridium welchii* aspartic acid can be converted into alanine via an enzymatic decarboxylation reaction (Meister et al., 1951). This pathway was affected by all exposure treatments. Through the amino acid glutamate, many other metabolism pathways are connected to this pathway, they are nitrogen metabolism, glutathione metabolism, butanoate metabolism, glyoxylate and dicarboxylate metabolism and arginine and proline metabolism. The impact on this pathway would affect the synthesis of other vital amino acids within amphibians. A down regulation in this pathway would result in a smaller supply of amino groups for synthesizing amino acids.

The main metabolites that were found in the arginine and proline metabolism pathway were aspartic acid, glutamic acid, proline, urea, ornithine, and putrescine. Additionally, one of the main metabolites of this pathway is nitric oxide, due to the breakdown of arginine, where nitric oxide is synthesized in neurons and is an important neurotransmitter and neuromodulator

within the central nervous system (Wu and Morris, 1998; Ichu et al., 2014). This pathway was impacted in each exposure scenario. Furthermore, alanine, aspartate and glutamate metabolism and butanoate metabolism pathways are connected to the arginine and proline metabolism pathway through putrescine. After putrescine undergoes two reactions to form first 4-aminobutyraldehyde and subsequently GABA, it can then partake in both butanoate metabolism and alanine, aspartate and glutamate metabolism (Houen, 1998; Schneider and Reitzer, 2012). Down regulation in this pathway would negatively impact the biosynthesis of proteins and amino acids in amphibians.

Valine, leucine and isoleucine biosynthesis pathway regulates brain amino acid uptake, muscle protein synthesis, and insulin secretion (Brosnan and Brosnan, 2006). These BCAAs are considered dietary essential amino acids; however, when metabolizing BCAAs, the first step is a transamination to a ketoacid, which is reversible (Platell et al., 2000; Brosnan and Brosnan, 2006). Dietary restrictions to BCAAs in mice, resulted in the immune system being impaired along with an increase in susceptibility to other pathogens (Calder, 2006). Additionally, the amino acid leucine partakes in the insulin signaling pathway which can regulate protein synthesis (Layman and Walker, 2006). Furthermore, these three amino acids when they are upregulated within the brain can prevent the uptake of the aromatic amino acids (phenylalanine, tyrosine and tryptophan), which would lead to a decline in the synthesis and release of neurotransmitters (Fernstrom, 2005).

Since the BCAAs were both up and down regulated within several treatments, this infers they could also be degrading. Valine, leucine and isoleucine are all catabolized as a group extrahepatically within the skeletal muscle (Platell et al., 2000; Brosnan and Brosnan, 2006). The three BCAAs go through a deamination step to produce branched-chain ketoacids that are

incorporated into protein or aid as an energy substrate in several areas of the body (brain, heart, kidney and mainly the skeletal muscle) (Abumrad et al., 1982; Platell et al., 2000). Additionally, these amino acids are nitrogen donors, within the brain and peripheral tissues; where the amino group is transported from the muscle to the liver in the form of alanine and glutamine, while in the brain for the synthesis of glutamate and GABA (Hutson et al., 2005).

Glutathione is involved in Phase II metabolism of xenobiotics and can help control reactive oxygen species that are harmful to the organism (Hansen et al., 2006). Glutathione is made up of three amino acids, glycine, cysteine and glutamate, while the main metabolites that were observed in this pathway were glutamate, glycine and pyroglutamic acid. The glutathione pathway was observed in two of the treatments exposed at $1/10^{th}$ maximum application rate, bifenthrin and metolachlor. In Zaya et al. (2011) this pathway was over-represented or upregulated, after *Xenopus laevis* tadpoles were exposed to atrazine, which could have assisted in the removal of atrazine and its metabolites. Therefore, the role of the glutathione metabolism pathway in the current study may have been to facilitate the excretion of pesticides from the body.

For nitrogen metabolism, only two metabolites, glutamate and glycine were putative hits for this pathway. Nitrogen metabolism was impacted in both of the metolachlor treatments and at $1/10^{th}$ maximum application for triadimefon. Stitt et al. (2002) noted that glutamate, malic acid and low sugars can hinder the nitrogen metabolism pathway. Snyder et al. (2017) observed that nitrogen metabolism was altered when *Hyla versicolor* tadpoles were exposed to atrazine.

The metabolites that partake in the butanoate metabolism pathway that were observed in the exposures were succinic acid, malic acid, glutamate and GABA. This pathway was unique in this study, because it was only impacted in bifenthrin exposures at both application rates.

Although, in Ch et al. (2015), only one metabolite was identified that affected butanoate metabolism when earthworms were exposed to cypermethrin, Snyder et al. (2017), found this pathway was only impacted in *H. versicolor* tadpoles with three metabolites of interest.

Conclusions

The effects of pesticides as mixtures on body burden and the metabolome of terrestrial phase amphibians was assessed. Metolachlor had the most impact on the amphibians by having the highest body burden for the maximum application rate, and displaying the highest number of spectral features. The $1/10^{th}$ maximum application rate's metabolomic profile was more impacted than the maximum application rate. Pesticide mixtures at both application rates appeared to equally affect the metabolome of amphibians, possibly due to the same metabolites being required to bring the organism back to a homeostatic state. Exposure at low concentrations, exhibited a down regulation in metabolites that are necessary to combat stress of xenobiotics. Prominent amino acids were down regulated because they are being used for glutathione metabolism, or energy demands such as protein formation. However, at higher exposure concentrations necessary metabolites for energy and synthesis of new compounds would demonstrate the opposite trend. Therefore, elucidating the effects of pesticides both as singlets and mixtures is vital in assessing the risk these compounds pose to non-target species.

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Table 6.1: Metabolites that were statistically ($p \leq 0.05$) up or down regulated following exposure to bifenthrin, metolachlor or triadimefon compared to control at maximum application rate and $1/10^{th}$ maximum application rate. Highlighted rows are metabolites observed in the three main pathways: aminoacyl-tRNA biosynthesis; alanine, aspartate and glutamate metabolism; and arginine and proline metabolism.

	Maximum Application Rate							***1/10th Maximum Application Rate***						
Compound	**Bif**	**Met**	**Tdn**	**BifMet**	**BifTdn**	**MetTdn**	**BifMetTdn**	**Bif**	**Met**	**Tdn**	**BifMet**	**BifTdn**	**MetTdn**	**BifMetTdn**
Acetic acid		↑				↑								
Adenine	↓			↓			↓	↓	↑	↑		↓		↓
Alanine		↑		↑		↑	↑	↓	↓	↓	↓	↓		↓
Allose											↓			↓
2-alpha-mannobiose		↓				↓	↓				↓	↑	↓	
Aminomalonic acid								↓	↓					
Arabinonic acid			↑		↑	↑	↑							
Arabinose								↓	↓	↓				
Arabitol											↑	↑		↑
Aspartic acid			↑	↑	↑	↑	↑	↑	↑	↓	↓	↓	↓	↓
Azelaic acid											↑		↑	
Butanoic acid											↓	↓		
Cellobiose		↓		↓		↓		↓	↓	↓		↑	↑	↑
2-Deoxy-D-ribose												↓	↓	
Erythose								↓		↓				
Erythronic acid				↑		↑	↑	↑	↑		↑	↓	↑	↑
Fructose											↓		↓	↓
GABA	↑	↑	↑	↑		↑	↑	↓	↓	↓		↓		↓
Galactopyranoside												↑		↑
Galactose	↓	↓	↓	↓	↓	↓	↑	↑	↓	↓	↑	↑	↓	↑
Gluconic acid								↓	↓	↓	↓	↓		↓

Table 6-1: (*continued*)

	Maximum Application Rate							***1/10th Maximum Application Rate***						
Compound	**Bif**	**Met**	**Tdn**	**BifMet**	**BifTdn**	**MetTdn**	**BifMetTdn**	**Bif**	**Met**	**Tdn**	**BifMet**	**BifTdn**	**MetTdn**	**BifMetTdn**
Glucuronic acid			↑			↑	↑					↑	↑	
Glutamic acid				↑		↑	↑		↓	↓				
Glutaric acid											↑		↑	↑
Glyceric acid									↓	↓		↑	↑	↑
Glycerol												↓	↓	
Glycine				↑		↑	↑	↓	↓	↓				
Glycolic acid													↑	↑
Glycoside								↑	↑		↓			↓
Hypoxanthine	↑	↑		↑	↑	↑	↑	↓		↓				
Inosine											↑	↑		
Inositol	↓			↓	↓									
Isoleucine									↓	↓		↓	↓	↓
Lactic acid		↑		↑	↑	↑	↑	↑	↑	↓		↑	↑	↑
Lactose					↓	↓	↓	↓	↓	↓		↑		↑
Lactulose				↑	↑		↑	↓	↓		↓	↓	↓	↓
Leucine		↓		↓		↑	↑	↓	↓	↓		↓	↓	↑
Lysine		↑		↑		↑	↑	↑	↑		↑	↓	↓	↑
Malic acid		↑		↑		↑	↑						↓	↓
Maltose		↑	↓	↑	↓	↓	↑	↓	↓	↓	↓	↓	↑	↑
Mannobiose												↓		↓
Mannose		↓		↓		↓					↓		↓	
Methionine				↑		↑	↑	↓	↓					
Methylmalonic acid			↑			↑	↑							
5-Methyluridine								↓	↓	↓				

Table 6-1: (*continued*)

	Maximum Application Rate							***1/10th Maximum Application Rate***						
Compound	**Bif**	**Met**	**Tdn**	**BifMet**	**BifTdn**	**MetTdn**	**BifMetTdn**	**Bif**	**Met**	**Tdn**	**BifMet**	**BifTdn**	**MetTdn**	**BifMetTdn**
Myo-inositol								↑	↑	↓	↓	↓	↓	↓
N-acetyl-D-glucosamine					↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
N-alpha-acetyllysine												↓		↓
Ornithine	↑		↑	↑		↑	↑				↓	↓		↓
Oxalic acid				↑		↑		↓	↓	↓				
Phenylalanine			↑	↑		↑	↑				↑			↑
Proline	↑	↑	↑	↑		↑	↑	↓	↓	↓	↓	↓		↓
Purine		↑	↑			↑	↑				↓		↓	↓
Putreanine											↓	↓	↓	↓
Putrescine				↑	↑		↑	↓	↓		↑	↓	↓	↓
Pyroglutamic acid		↓	↓			↓	↓	↓	↓	↓	↓	↓		↓
Ribitol			↑			↑	↑	↓	↓	↓	↓	↑	↓	↓
Ribofuranose								↓	↓	↓	↑	↑	↑	
Ribose								↓	↓	↓	↓	↓		
Serine			↑			↑	↑					↓	↓	
Sorbofuranose													↑	↑
Succinic acid	↓		↑	↑	↑	↑	↑				↑	↑		
Talopyranose												↓		↓
Threonic acid			↑	↑		↑	↑							
Threonine				↑	↑	↑	↑					↓		↓
Thymine			↑			↑	↑							
Turanose											↓	↓	↓	↓
Tyrosine								↓	↓	↓	↓		↓	
Uracil								↓	↓	↓	↑	↑	↑	

Table 6-1: (*continued*)

	Maximum Application Rate							***1/10th Maximum Application Rate***						
Compound	**Bif**	**Met**	**Tdn**	**BifMet**	**BifTdn**	**MetTdn**	**BifMetTdn**	**Bif**	**Met**	**Tdn**	**BifMet**	**BifTdn**	**MetTdn**	**BifMetTdn**
Urea								↓	↓	↓			↓	↓
Uridine								↓	↓	↓		↓		↓
Valine		↑		↑		↑	↑				↓	↓	↑	↑
Xylopyranose								↓		↓				
Xylose								↓	↓	↓				

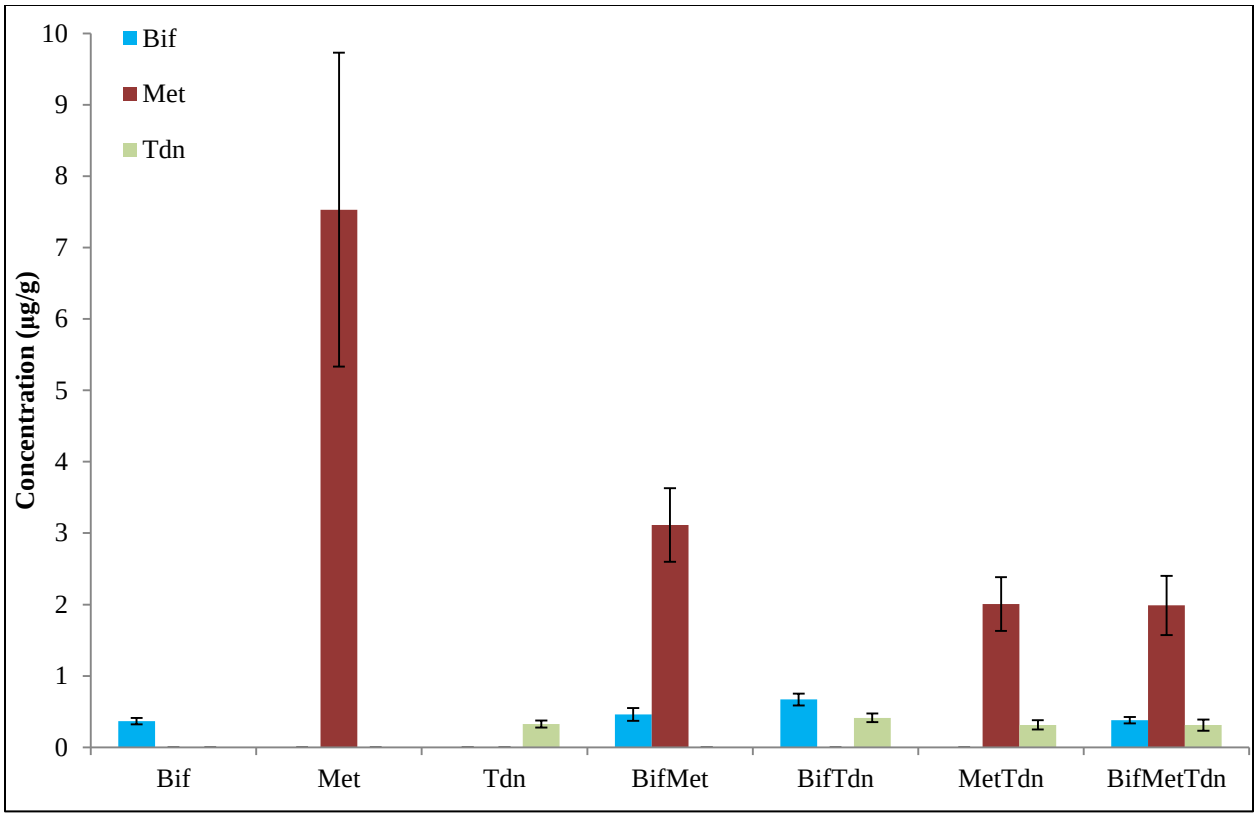

Figure 6-1: Tissue concentrations (µg/g) of pesticide(s) in amphibians exposed at maximum application. Mean ± S.E. of each pesticide that was exposed.

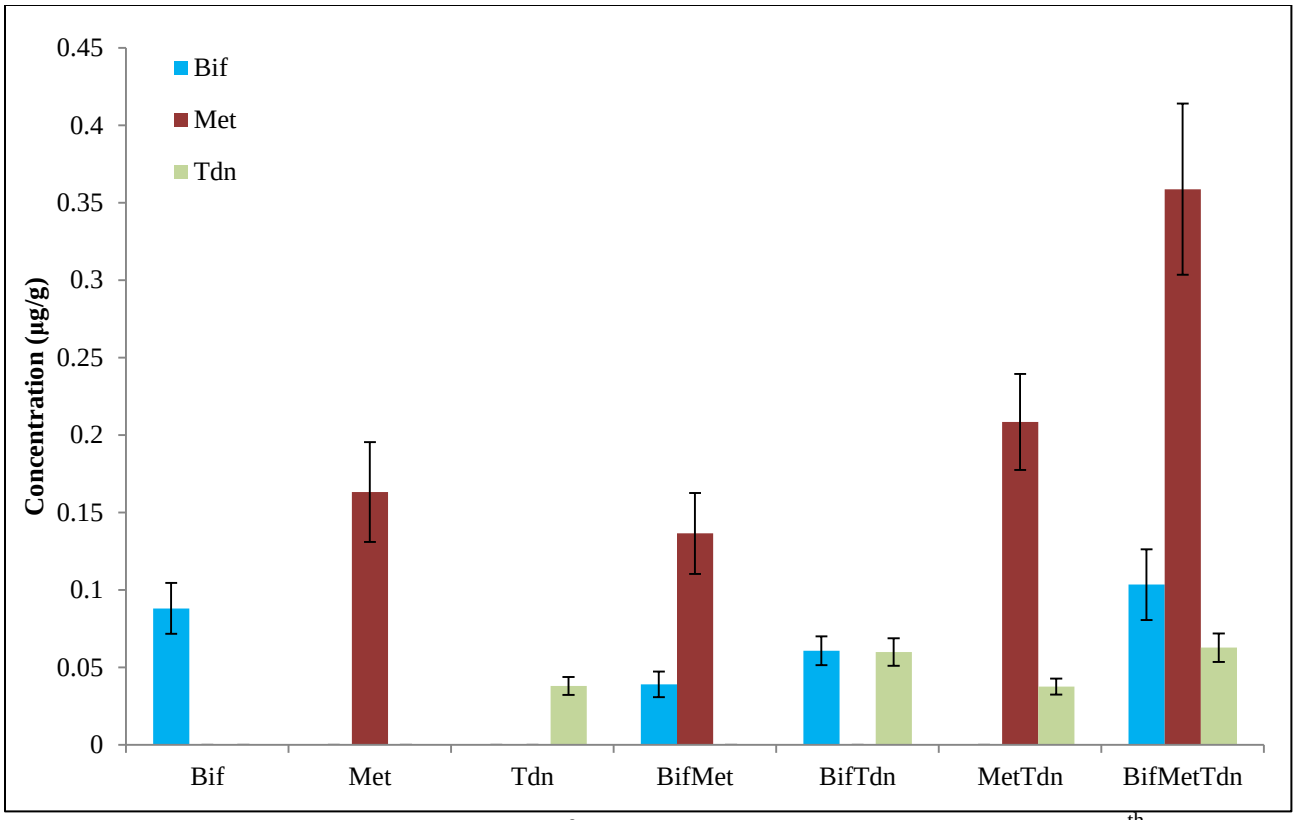

Figure 6-2: Tissue concentrations (µg/g) of pesticide(s) in amphibians exposed at 1/10th maximum application. Mean ± S.E. of each pesticide that was exposed.

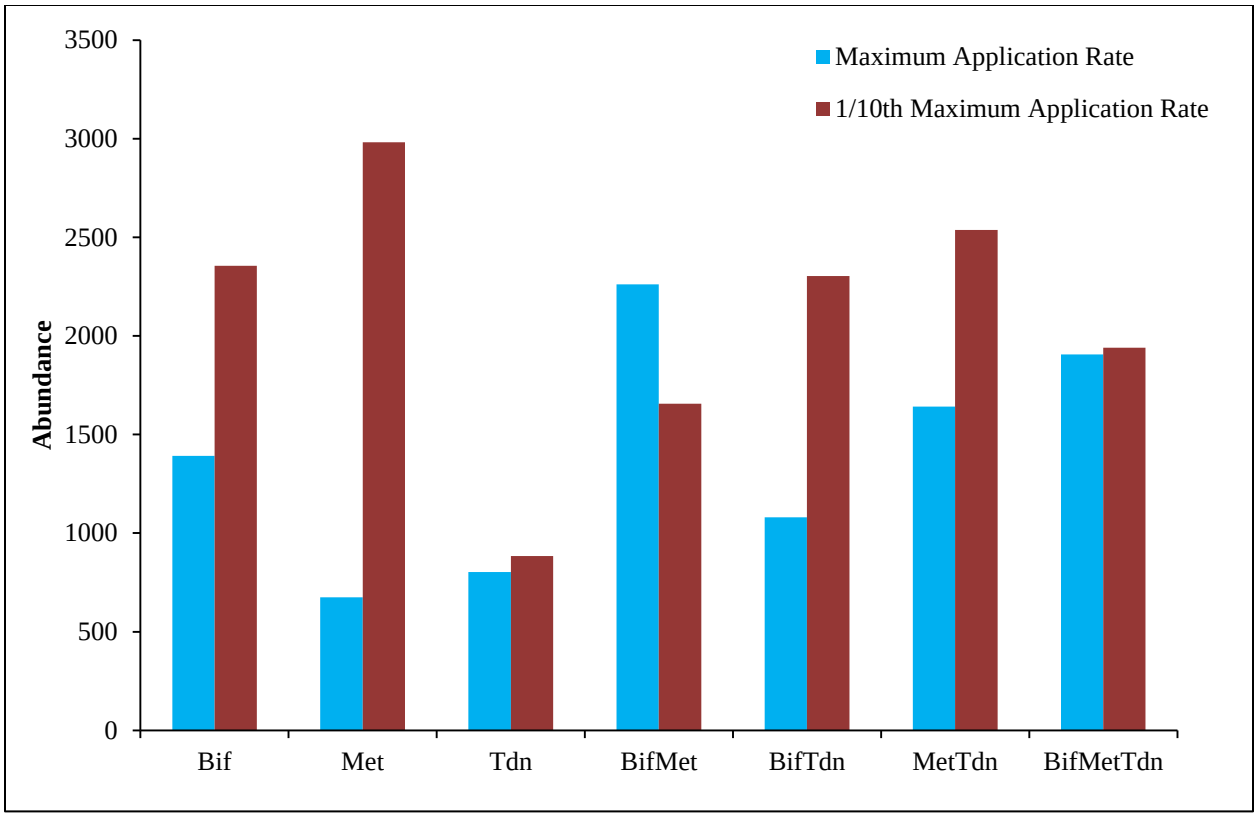

Figure 6-3: Number of spectral features that were effected by either pesticide treatment either at maximum or 1/10th maximum application rate.

Table 6-1S: LC-MS/MS analytical parameters for active ingredients and metabolites in pesticide exposures as singlets or as mixtures.

Compound	Type	Mode	Retention Time (min)	Parent (*m/z*)	Daughter (*m/z*)	CE (V)
Triadimenol	Triadimefon degradate	+	18.16 18.58	296	70 90	-4 -10
Triadimefon	Fungicide	+	20.03	294	197 225	-15 -13
Tetraconazole (IS)	Fungicide	+	20.08	372	70 159	-9 -25
Bifenthrin	Insecticide	+	24.2	440	166 181	-40 -21
Permethrin (IS)	Insecticide	+	24.5 26.3	408	149 183	-20 -22
Metolachlor	Herbicide	+	21.62	284	176 252	-25 -14
MESA	Metolachlor degradate	-	7.72	328	80 121	25 17
MOXA	Metolachlor degradate	-	14.77	278	158 206	20 9

Table 6-2S. Biological pathways that were affected by pesticide treatment in amphibians. Bold indicates pathways shared between the same application rate; * pathways between the same pesticide.

Pathway	Hits	Total	p value
Maximum_Bifenthrin			
Arginine and proline metabolism*	4	43	0.0005
Alanine, aspartate and glutamate metabolism*	3	24	0.0013
Butanoate metabolism*	2	22	0.0180
Aminoacyl-tRNA biosynthesis*	3	67	0.0239
Maximum_Metolachlor			
Aminoacyl-tRNA biosynthesis*	11	67	0.0000
Alanine, aspartate and glutamate metabolism*	4	24	0.0007
Valine, leucine and isoleucine biosynthesis*	3	13	0.0013
Arginine and proline metabolism*	4	43	0.0061
Pyruvate metabolism	3	22	0.0062
Nitrogen metabolism*	2	9	0.0103
Histidine metabolism	2	14	0.0246
Selenoamino acid metabolism	2	17	0.0355
Maximum_Triadimefon			
Arginine and proline metabolism*	4	43	0.0052
Alanine, aspartate and glutamate metabolism*	3	24	0.0071
Aminoacyl-tRNA biosynthesis*	4	67	0.0247
1/10th maximum _Bifenthrin			
Aminoacyl-tRNA biosynthesis*	12	67	0.0000
Valine, leucine and isoleucine biosynthesis	4	13	0.0006
Arginine and proline metabolism*	6	43	0.0022
Phenylalanine, tyrosine and tryptophan biosynthesis	2	4	0.0061
Alanine, aspartate and glutamate metabolism*	4	24	0.0067
Glutathione metabolism	4	26	0.0089
Butanoate metabolism*	3	22	0.0330
Phenylalanine metabolism	2	11	0.0481
1/10th maximum _Metolachlor			
Aminoacyl-tRNA biosynthesis*	11	67	0.0000
Arginine and proline metabolism*	6	43	0.0022
Alanine, aspartate and glutamate metabolism*	4	24	0.0067
Valine, leucine and isoleucine biosynthesis*	3	13	0.0075
Glutathione metabolism	4	26	0.0089
Ascorbate and aldarate metabolism	2	6	0.0146

Table: 6-2S: (*continued*)

Pathway	Hits	Total	p value
1/10th maximum _Metolachlor			
Glyoxylate and dicarboxylate metabolism	3	18	0.0191
Amino sugar and nucleotide sugar metabolism	4	37	0.0304
Nitrogen metabolism*	2	9	0.0328
Starch and sucrose metabolism	3	22	0.0330
1/10th maximum _Triadimefon			
Aminoacyl-tRNA biosynthesis*	11	67	0.0000
Arginine and proline metabolism*	6	43	0.0027
Alanine, aspartate and glutamate metabolism*	4	24	0.0078
Valine, leucine and isoleucine biosynthesis	3	13	0.0085
Glutathione metabolism	4	26	0.0105
Galactose metabolism	4	26	0.0105
Cyanoamino acid metabolism	2	6	0.0159
Glyoxylate and dicarboxylate metabolism	3	18	0.0216
Nitrogen metabolism	2	9	0.0357
Methane metabolism	2	9	0.0357

CHAPTER 7

ECOLOGICAL SCREENING

Amphibians are often likely to be exposed to pesticides as a consequence of direct or indirect agricultural application (Houlahan and Findlay, 2003). Amphibians are known to breed and deposit eggs in agricultural ponds and streams where runoff and rainwater can contaminate the water sources (Houlahan and Findlay, 2003). Due to the permeability of the adult amphibian dermis, pesticides can be readily taken up into their body (Van Meter et al., 2014; Van Meter et al., 2015; Van Meter et al., 2016). During rain events, atmospheric water is known to wash pesticides from leaves and stems and further contaminate the surrounding soil or aquatic systems. Pesticides are also known to migrate through the atmosphere, post-application, and contaminate pristine areas. However, this influx of pesticides can be harmful to amphibians either due to the additional pulses of pesticides being released or environmental transformation of the pesticides that form metabolites that are often more toxic than parent pesticides (Boone et al., 2001; Relyea and Diecks, 2008). Exposure to environmental contaminants, especially pesticides, can affect the mass, size, and time of metamorphosis of amphibians that reside in these aquatic ponds (Boone et al., 2001). Additionally, pesticide application and the drying up of these small streams and ponds during the summer can lead to higher concentrations of pesticides concentrating in these aquatic systems, which will result in more adverse effects being expressed in non-target organisms (Boone and James, 2003; Relyea, 2005; Boone, 2008; Smalling et al., 2012; Battaglin et al., 2016; Potter and Coffin, 2017).

Even though there are measurable concentrations of pesticides in different environmental matrices, this does not necessarily mean that these levels will be toxic to aquatic life (Gilliom et al., 2006; Potter and Coffin, 2017). Therefore, in the current study, stemflow, throughfall and surface water concentrations were compared against aquatic life benchmarks and environmental water screening values to determine if adverse effects would occur for non-targeted organisms (Table 7-1). Over 80 percent of the time mixtures are present in the different matrices and thus it is difficult to determine the potential adverse effects that these individual mixture components will have on aquatic life, due to the fact that the pesticides could cause additive, synergistic or antagonist effects.

To assess the possible risk that agricultural pesticides have on non-target organisms, particularly amphibians, samples from surface water, stemflow, and throughfall were collected and analyzed. Using the U.S. EPA's aquatic life benchmark data for freshwater, a table was compiled that included the most sensitive values to be evaluated against the pesticides detected in these matrices similar to Potter and Coffin (2017) (U.S. EPA, 2016a). Of the 32 pesticides detected, only eight had concentrations that exceeded the aquatic life benchmark value. These pesticides were acetochlor, atrazine, bifenthrin, diazinon, ethalfluralin, fipronil, malathion, and metolachlor. Acetochlor and atrazine exceeded benchmark values for throughfall samples, these pesticide levels may pose a risk to many organisms that live under tree canopies. Two pesticides, atrazine and bifenthrin had stemflow values that exceeded the benchmark level. These values place many amphibians, especially treefrogs, at risk for hazardous adverse effects because they reside on trees. Since stemflow samples comprise of rainwater that washes down the tree trunk, it is a composite of all the pesticides, both parents and metabolites, impacted by spray drift. Furthermore, seven pesticides were detected in surface water samples that could

directly cause harm to amphibians, either when adults return to ponds to breed or tadpoles while they are growing and developing. Several of these pesticides were detected in colder months, which would be harmful to tadpoles that overwinter is these areas.

Atrazine exceeded the most sensitive aquatic benchmark value for eleven samples (5% detection frequency). Atrazine is an herbicide that has been shown to elicit neuroendocrine effects in mammals, in addition to, affecting developmental and reproductive systems by altering hormone levels in rats (U.S. EPA, 2016b). Six of these eleven samples were collected on the same date in May 2015, one surface and six stemflow and throughfall sites. Therefore, atrazine application most likely occurred in April between the two collection dates. This corresponds with time of application for atrazine because it is applied as a pre-emergent. Additionally, pesticide concentrations in stemflow and to a lesser extent, in throughfall, were still detectable even at lower concentrations, however, they were still above the benchmark value.

In contrast, fipronil was detected in five surface water samples in the beginning of the year. This could have resulted from application in the previous year and residues remained over the winter, or early application of the insecticide in spring which resided for several weeks (Greenberg et al., 2010; Greenberg et al., 2014). Fipronil is known to affect the GABA receptors in the central nervous system, by blocking the GABA-gated chloride channels (Das et al., 2006). Therefore, detecting concentrations that are above benchmark values may be hazardous for tadpoles that overwinter in ponds. Furthermore, the metabolites of fipronil (especially fipronil sulfone) are known for being more toxic than the parent, which can also pose a risk to these non-target organisms (U.S. EPA, 1996; Baird et al., 2013).

Metolachlor was the most frequently detected pesticide in both surface water and stemflow samples. In particular, the concentration in two samples (8.92 and 10.5 µg/L)

exceeded the aquatic benchmark value. This pre-emergent herbicide is mainly used on corn, and interestingly, site 3 is adjacent a corn field at Gibbs Farm.

Bifenthrin is an insecticide that affects the central nervous system and exceeded the aquatic benchmark value in at least 3 samples: two surface water and one stemflow samples. The herbicide ethalfluralin exceeded the limit twice in surface water samples collected in December. Malathion and diazinon, both organophosphate insecticides, were above the limit once each in surface water samples. Even though these compounds were detected above the aquatic benchmark value once, they can still pose a potential risk to aquatic life considering other pesticides were detected in the water samples at the same time. For the malathion sample that exceeded the aquatic benchmark value atrazine was also detected in the same sample. This is problematic considering several studies have demonstrated that atrazine can increase the toxicity of organophosphates when present in mixtures (Pape-Lindstrom and Lydy, 1997; Wacksman et al., 2006).

However, if the chronic fish endpoints are used, because of more similarities between fish and amphibians than invertebrates and plants, five samples would still exceed the aquatic benchmark value. These include ethalfluralin and bifenthrin, for four surface water samples and one stemflow. The pesticides mixture studied above, (triadimefon, metolachlor, and bifenthrin) had more of an impact on the metabolome at the $1/10^{th}$ maximum application rate, which could imply that pesticides detected in surface water, stemflow and throughfall samples at lower than benchmark values, can still have adverse effects on non-targeted organisms. Moreover, the average number of pesticides detected in a stemflow samples was 2.6, throughfall was 4.4, while surface waters were 3.6 pesticides per collected date and site. While concentrations of these pesticides were below the aquatic benchmark values for each individual compound, it is possible

that the summation of pesticide type (i.e. herbicides, fungicides, and insecticides) or specific mode of action could have an impact on these non-target organisms.

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Table 7-1: Sensitive aquatic freshwater benchmark values (µg/L) and endpoint type for pesticides that were detected in surface water (SW), stemflow (SF) and throughfall (TF) from Tifton, GA, USA. (NR = not reported).[a]

Pesticide	Value (µg/L)	Endpoint Type	Matrix Found In	Number of Samples that Exceeded Value
2-Phenylphenol	NR	NR	SW, SF, TF	0
Acetochlor	1.43	Nonvascular Plants – Acute	SW, SF, TF	1 (1 – TF)
Alachlor	1.64	Nonvascular Plants – Acute	SW	0
Anthraquinone	NR	NR	SW, SF, TF	0
Atrazine	<1	Nonvascular Plants – Acute	SW, SF, TF	11 (1 – SW, 5 – SF, 5 -TF)
Benfluralin	1.9	Fish – Chronic	SW	0
Bifenthrin	0.04	Fish – Chronic	SW, SF	3 (2 – SW, 1 – SF)
Biphenyl	NR	NR	SF, TF	0
Chlorothalonil	0.6	Aquatic Invertebrates – Chronic	SW	0
Cyprodinil	8	Aquatic Invertebrates – Chronic	SW	0
DEA	NR	NR		0
Diazinon	0.105	Aquatic Invertebrates – Acute	SW	1 (1 – SW)
Diphenylamine	NR	NR	SW, SF	0
Endosulfan Ether	NR	NR	SW	0
Endosulfan Lactone	NR	NR	SW, TF	0
Endosulfan sulfate	1.9	Fish – Acute	SW	0
Ethalfluralin	0.4	Fish – Chronic	SW	2 (2 – SW)
Fipronil	0.011	Aquatic Invertebrates – Chronic	SW	5 (5 – SW)
Fludioxonil	19	Fish – Chronic	SF, TF	0
Flutolanil	220	Fish – Chronic	SW, SF, TF	0
Malathion	0.035	Aquatic Invertebrates – Chronic	SW	1 (1 – SW)
Metalaxyl	100	Aquatic Invertebrates – Chronic	SW, TF	0
Metolachlor	8	Nonvascular Plants – Acute	SW, SF, TF	2 (2 – SW)
Myclobutanil	830	Nonvascular Plants – Acute	SW	0
Oxadiazon	5.2	Nonvascular Plants – Acute	TF	0
Oxyfluorfen	1.1	Nonvascular Plants – Acute	SW	0
Pendimethalin	5.2	Nonvascular Plants – Acute	SW	0

Table 7-1: (*continued*)

Pesticide	Value (µg/L)	Endpoint Type	Matrix Found In	Number of Samples that Exceeded Value
Piperonyl Butoxide	30	Aquatic Invertebrates – Chronic	SW	0
Propyzamide	NR	NR	SW	0
Tebuconazole	12	Fish – Chronic	SW, SF, TF	0
Tetrahydrophthalimide	>56500	Aquatic Invertebrates – Acute	SW	0
Triadimefon	41	Fish – Chronic	SW	0

[a] = OPP Aquatic Life Benchmark values for freshwater (U.S. EPA, 2016a).

CHAPTER 8

CONCLUSIONS

We conclude pesticides from agricultural spray drift can accumulate on foliage and trunks in buffer zones leading to concentrations being detected in stemflow, throughfall, and nearby surface waters following rain events. Overall, screening the prominent pesticides detected in these matrices, *in vitro* metabolic experiments were conducted, and no current animal model accurately predicted amphibian derived metabolic rate constants. Furthermore, dehydration status can reduce the body burdens of pesticides in amphibians by both energy depletion and/or dilution scenarios. Finally, mixtures of pesticides present as residues can potentially facilitate the uptake of each other in terrestrial phase amphibians, which was not observed in maximum application rate exposures. While, in the metabolome of amphibians, low pesticide exposures can down regulate metabolites to combat stress levels; however, high pesticide exposures cause the opposite scenario, due to energy resources being depleted leading to high stress levels. Together these data can be used for furthering amphibian risk assessment to pesticides.