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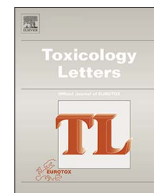

Using *in vitro* derived enzymatic reaction rates of metabolism to inform pesticide body burdens in amphibians

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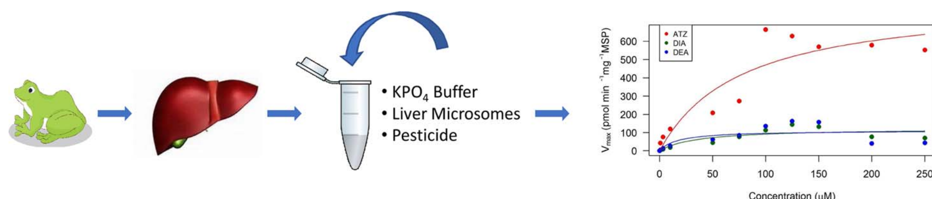
GRAPHICAL ABSTRACT



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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 to 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.

1. 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% for insecticides and 10% 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, triadimefon, and fipronil are broad use pesticides in the U.S. where atrazine is one of the most commonly used herbicides to control weeds in agricultural crops (Lang et al., 1996), triadimefon is a broad-spectrum fungicide used in both fruit and

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evergreen farming (Kenneke et al., 2008), and fipronil 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., 2009). 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 fipronil 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, a biomarker of fipronil exposure, was the most prominent metabolite found in the female green frogs exposed to fipronil contaminated water (Reynaud et al., 2012). Hayes et al. (2003) reported atrazine 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).

Amphibian skin is well known for being extremely 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). Since amphibians need to stay hydrated and they 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 where the primary route of absorption of water occurs (Bentley and Main, 1972). Pesticides and metals are known to be absorbed through the skin of amphibians (James et al., 2004; Willens et al., 2006; Van Meter et al., 2014, 2015, 2016, 2018). Therefore, the skin is presumed to be the major route of exposure for contaminants in amphibians compared to pulmonary and oral routes (Smith et al., 2007).

After entering the body, pesticide metabolism is commonly initiated by the liver, where phase I proteins such as cytochrome P450 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 atrazine into two major metabolites: desethyl atrazine and deisopropyl atrazine 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 atrazine formed deisopropyl atrazine ten times faster than desethyl atrazine. Hepatic metabolism of triadimefon can only result in triadimenol 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, 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, fipronil sulfone was the major metabolite observed through oxidation (Tang et al., 2004). Fipronil sulfone is also known to be more toxic than its parent fipronil 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 obtain values of the maximum velocity (V_{max}) and Michaelis constant (K_M) 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.

2. 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.

2.1. *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).

2.2. *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:

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

Briefly, substrate concentration ([S], µM) 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

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