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Estimating terrestrial amphibian pesticide body burden through dermal exposure

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ABSTRACT

Dermal exposure presents a potentially significant but understudied route for pesticide uptake in terrestrial amphibians. Our study measured dermal uptake of pesticides of varying hydrophobicity ($logK_{ow}$) in frogs. Amphibians were indirectly exposed to one of five pesticide active ingredients through contact with contaminated soil: imidacloprid ($logK_{ow}$ = 0.57), atrazine ($logK_{ow}$ = 2.5), triadimefon ($logK_{ow}$ = 3.0), fipronil ($logK_{ow}$ = 4.11) or pendimethalin ($logK_{ow}$ = 5.18). All amphibians had measurable body burdens at the end of the exposure in concentrations ranging from 0.019 to 14.562 μg/g across the pesticides tested. Atrazine produced the greatest body burdens and bioconcentration factors, but fipronil was more permeable to amphibian skin when application rate was considered. Soil partition coefficient and water solubility were much better predictors of pesticide body burden, bioconcentration factor, and skin permeability than $logK_{ow}$. Dermal uptake data can be used to improve risk estimates of pesticide exposure among amphibians as non-target organisms.

1. Introduction

Pesticide applications are deemed critical to maintaining high agricultural output, however, they can cause significant mortality to non-target organisms (Pimentel, 1971; Hogwarth, 2000). Amphibians are an important group of non-target organisms in agricultural landscapes (e.g., Mann et al., 2009; Bishop et al., 2010; Choung et al., 2011) and may come into contact with these chemicals directly or residually through plant material and soil. Brühl et al. (2011) recently highlighted the necessity for data collection on pesticide exposure and uptake in terrestrial phase amphibians since this life-stage has been overlooked in nearly all datasets published to date. Worldwide amphibian declines have triggered massive research efforts to improve our understanding of both biotic and abiotic factors that may be contributing to such large-scale losses (e.g., Kiesecker et al., 2004; Pounds et al., 2006; Alford et al., 2007), but there has been little emphasis on better understanding the uptake of environmental toxins and associated mortality to juvenile and adult amphibians. Terrestrial exposure to contaminants may be a significant pathway for dermal uptake in amphibians that frequent aquatic habitats only during breeding season.

Pesticides vary in their hydrophobicity, which is expressed as a specific chemical's K_{ow} or $logK_{ow}$ (octanol–water partitioning coefficient). $LogK_{ow}$ is considered an important predictor in dermal contact models for mammals (USEPA, 2007) since higher K_{ow} (hydrophobic) and lower K_{ow} (hydrophilic) chemicals have separate pathways for dermal exposure (Michaels et al., 1975). Quaranta et al. (2009) recently verified that amphibian skin allows greater permeation of pesticides compared to pig dermis. This is likely due to the lack of a hydrophobic barrier in amphibian skin and high porosity to water molecules. They also report a positive regression slope between both frog and pig skin permeability ($Log\ K_p$) and a pesticide's $LogK_{ow}$, suggesting that high K_{ow} or hydrophobic pesticides may be more readily taken up by amphibian dermis. This analysis was carried out on excised frog skin in a flow-through cell that simulates diffusive water movement, however, and may not fully represent the physiology of live, intact amphibian dermis. A comparison of dermal absorption of the moderately hydrophobic pesticide fipronil and the very hydrophobic polycyclic aromatic hydrocarbon benzo[a]pyrene (BaP) in living female green frogs

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indicated that the more hydrophilic fipronil compound was more readily taken up (Reynaud et al., 2012). This study suggests that dermal uptake in live, physiologically intact amphibians may not be predicted well by chemical properties typically linked to diffusive uptake (e.g., molecular mass, diffusivity, $\log K_{ow}$); instead, properties which may be attributed to advective processes (e.g., solubility, $\log K_{oc}$) may be better estimators. Understanding the influence of chemical properties on the permeability of pesticides across amphibian skin is necessary to make more informed decisions about potential impacts to this group of non-target organisms.

Published pesticide exposure and toxicity data for terrestrial amphibians are limited, making it difficult to predict associated hazards through dermal contact. Our current understanding of pesticide permeability across the skin through terrestrial exposures is limited to one study: malathion uptake by fossorial tiger salamanders, *Ambystoma maculatum* (Henson-Ramsey et al., 2008). Storrs-Mendez et al. (2009) found that juvenile American toads (*Bufo americanus*) readily absorb atrazine across the pelvic seat patch, however, exposures were in an aqueous medium. In addition to these datasets, only five pesticide toxicity studies in terrestrial amphibians that included direct contact with a soil medium have been published since 2009 (Bernal et al., 2009; Dinehart et al., 2009; Belden et al., 2010; Edge et al., 2011; Brühl et al., 2013). These are critical for making progress towards understanding pesticide impacts on terrestrial amphibians, but differences in methods, species, endpoints and chemicals limit our predictive capabilities and make it difficult to draw conclusions.

The purpose of this study is to quantify dermal uptake of five pesticides of varying hydrophobicity ($\log K_{ow}$) by live juvenile frogs through indirect terrestrial exposures. The pesticide active ingredients tested are imidacloprid ($\log K_{ow} = 0.57$), atrazine ($\log K_{ow} = 2.5$), triadimefon ($\log K_{ow} = 3.0$), fipronil ($\log K_{ow} = 4.11$) and pendimethalin ($\log K_{ow} = 5.18$) (Roberts et al., 1998; 1999). We report total pesticide body burdens and bioconcentration factors for the five pesticide active ingredients across seven amphibian species. Our goal is to expand on the limited data available on uptake of pesticides through dermal exposure in terrestrial amphibians. To evaluate the effects of $\log K_{ow}$ on skin permeability among living amphibians, we also quantify skin permeability factors for the five pesticide active ingredients tested and develop regression models to explore potential relationships between $\log K_{ow}$ and skin permeability. Among live amphibians, we predict the absence of a strong positive correlation between hydrophobicity and dermal permeability as is seen in other vertebrates, and that measurable body burdens will be higher for hydrophilic relative to hydrophobic pesticides after indirect contact with contaminated soil after an 8 h exposure.

2. Materials and methods

2.1. Chemicals

All pesticide exposures were conducted with analytical grade pesticide active ingredients with purities ≥98%. Pesticides were applied at selected registered label application rates scaled to the size of a 10-gallon aquarium (Table 1) (USEPA, 2013); they represent a gradient of $\log K_{ow}$ and water solubility (Table 1).

Table 1
Pesticide application rate (μg/cm^2), $\log K_{ow}$, mean experimental $\log K_{oc}$, molecular mass (g/mol), density (g/m^3) and water solubility at 20 °C (mg/L).

Pesticide	App. Rate	$\log K_{ow}$	$\log K_{oc}$	Molecular mass	Density	Water solubility
Imidacloprid	5.7	0.57	2.56	255.7	1.543	510
Atrazine	22.9	2.5	2.3	215.7	1.187	30
Triadimefon	2.7	3.11	3.03	291.7	1.22	260
Fipronil	1.1	4	4.24	437.2	1.48	3.78
Pendimethalin	19.8	5.18	6.43	281.3	1.17	0.3

2.2. Amphibian collection & rearing

From March–July 2012, seven amphibian species were collected as ovipositing amplexed pairs, egg masses or embryos from University of Georgia's Whitehall Forest. Species used were Southern leopard frog (*Lithobates sphenocephala*), Fowler's toad (*Anaxyrus fowleri*), gray treefrog (*Hyla versicolor*), Northern cricket frog (*Acris crepitans*), Eastern narrowmouth toad (*Gastrophryne carolinensis*), barking treefrog (*Hyla gratiosa*) and green treefrog (*Hyla cinerea*). All species were reared in 375-L outdoor wading pools and fed fish food daily through metamorphosis. As metamorphs emerged, they were transferred to 600-L polyethylene tanks lined with sphagnum moss and leaf litter. All juvenile amphibians were fed cultured fruit flies and purchased crickets for 60–90 days post-metamorphosis.

2.3. Dermal pesticide exposures

Soil was collected from the Coweeta Long-term Ecological Research (LTER) site in Otto, NC in July and August 2012. All amphibians were dehydrated overnight for 12 h in dry glass aquariums prior to pesticide exposure. This facilitated uptake across the dermis through rehydration when the exposure was initiated. Experimental chambers were 10-gallon glass aquariums, lined with 750 g of soil. The day before experimentation, single pesticide active ingredients dissolved in 75 mL methanol (MeOH) were sprayed evenly over the surface of the soil using compressed air propellant Spray Gun® canisters attached to glass jars. Following pesticide application, aquariums were placed in a fume hood overnight to allow the methanol to evaporate. The next morning aquariums were removed from the fume hood and rehydrated with 300 mL distilled water. Five conspecifics were added to each aquarium immediately after soil rehydration for an 8-h pesticide exposure. Gray, green and barking treefrogs were exposed to all 5 pesticide active ingredients. Due to limitations in quantity, size and timing of metamorphosis, leopard frogs and Fowler's toads were exposed to 4 of the tested active ingredients (atrazine, triadimefon, fipronil and pendimethalin) while cricket frogs and narrowmouth toads were exposed to 2 of the tested active ingredients (imidacloprid and triadimefon). At the termination of each study, individual amphibians were placed in pre-labeled scintillation vials and put into a −80 °C freezer for euthanasia, as permitted by our approved IACUC Animal Use Permit #A2012 05–018-R1.

2.4. Amphibian and soil pesticide extraction

At the end of the eight-hour exposure, two composite soil samples were collected from each aquarium. Roughly 1.0 g of the composite soil was weighed out into a 15 mL centrifuge tube for pesticide extraction and quantification. All samples were spiked with 10 μL of 1000 ppm tetraconazole, internal standard. To initiate extraction, 5 mL of MeOH was added to each 1.0 g sample, followed by vortexing and sonication for 30 min. Afterward, the samples were centrifuged (3200 rpm) for 20 min and the resulting supernatant was transferred to scintillation vials. The solid pellet remaining at the bottom of the centrifuge tubes was extracted a second time by repeating the above steps. The resulting supernatant was pipetted into the vial containing the first sample for continued evaporation and concentration.

After evaporation, 10 mL of deionized water, 3 mL of methyl *tert*-butyl ether (MTBE) and a small scoop of sodium sulfate (for emulsion) was added to the vial, with vortexing between each addition. The top organic MTBE layer containing the pesticides was pipetted off the top of the mixture and centrifuged (14,000 rpm) for 15 min. Samples were placed under UHP-grade nitrogen gas again to evaporate off the MTBE and reconstituted with 30% MeOH for LC-MS analysis.

To initiate extraction from whole body tissue homogenates, frogs were thawed at room temperature and sprayed with compressed air to remove any remaining soil particles before obtaining individual weights. Whole frogs were homogenized using dissection scissors and a tissue homogenizer, therefore we report whole body burden. After homogenization, 1.0 g aliquots of the frog tissues were placed into centrifuge tubes and spiked with 10 μL of 1000 ppm tetraconazole as an internal standard. The samples were returned to the −80 °C freezer for four hours and then placed on a bench top freeze dryer overnight. After freeze drying, the tissue samples were broken up with a spatula and then analyzed by LC-MS following the extraction procedure for soil outlined above.

After analysis, bioconcentration factors (BCFs) were determined for each species and pesticide:

$$\mathrm{BCF} = C_f / C_s \quad (1)$$

where C_f is the frog whole body tissue concentration (μg/g) and C_s is the average composite soil concentration within an experimental chamber (μg/g), both at the end of the 8-h exposure. Frog skin permeability factors (SPFs), defined here as the μg of pesticide taken up per cm^2 of frog dermis, divided by the μg of pesticide applied per cm^2, were also determined for each species and pesticide:

$$\mathrm{SPF} = \left(C_f * \mathrm{BW}_f / \mathrm{SA}_f\right) / \mathrm{AR}_{ec} \quad (2)$$

where C_f is the frog whole body tissue concentration (μg/g), BW_f is the body weight (g) of the frog, SA_f is the surface area of the frog (cm^2), and AR_{ec} is the application rate of the pesticide applied to each unique experimental chamber (μg/cm^2). Frog surface area (SA_f) was calculated as follows (Hutchison et al., 1968):