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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):

$$SA_f = 1.131^{*}\left(BW_f^{0.579}\right) \quad (3)$$

2.5. K_{oc} determination

To estimate the organic carbon normalized adsorption coefficient (K_{oc}) for each of the pesticide active ingredients tested in this study, methods follow those outlined in Adsoprtion-Desorption using a Batch-Equilibrium Method (OECD Guidelines for the Testing of Chemicals (2000)).

2.6. LC-MS instrumentation

Soil and frog sample extracts were analyzed on an Agilent 1100 Series HPLC coupled to a 6120 mass spectrometer. Chromatographic separation was achieved on an Eclipse XDB-C18 (3.5 μm particle size, 3.0 × 150 mm; Agilent Technologies). Initial mobile phase was 70% water with 0.1% formic acid (A) and 30% acetonitrile with 0.1% formic acid (B). Starting conditions were held for 2 min and ramped to 90% B over 16 min and held for 4 min, before returning to initial conditions of 30% B and being re-equilibrated for 5 min (total run time of 30 min). Samples were analyzed in positive electrospray ionization (ESI) from 0 to 19 min, then switched to negative ESI from 19 to 23 min, and back to positive ESI from 23 to 30 min; both used selected ion monitoring mode. Switching between positive and negative ESI was due to respective elution times of the pesticide active ingredients.

2.7. Data analyses

All analyses were performed in R version 2.15.2 (R Core Team, 2013). For specified statistical analyses, the seven amphibian species tested were grouped according to habitat preference (i.e., Aquatic, Terrestrial, Arboreal (adapted from Elliott et al., 2009); referred to as Habitat Factor hereafter). Leopard and cricket frogs were defined as Aquatic, Fowler's and Eastern narrowmouth toads were defined as Terrestrial, and gray, green and barking treefrogs were defined as Arboreal. To evaluate relationships between K_{ow} or K_{oc} and our response variables (amphibian whole body tissue concentrations, BCF and SPF), we developed separate linear regression models that spanned the three habitat factors and five pesticides tested. To adhere to conventional methods for reporting K_{oc} and K_{ow}, we report and used log10 transformed data in our analyses. When used as a predictor variable, $\log K_{ow}$ had no significant linear relationship with whole body tissue concentration or SPF. Due to positive correlations between $\log K_{ow}$ and $\log K_{oc}$, we could not use both as covariates in the multivariate linear regression; we used $\log K_{oc}$ since there were soil-specific measurements. We then modeled $\log K_{oc}$ as a covariate and Habitat Factor as a categorical factor in general linear models (GLM) for each dependent variable. All models were tested with an interaction term between $\log K_{oc}$ and Habitat Factor that was not found to be significant. Tissue concentration was natural-log transformed and BCF and SPF were log10 transformed to induce normality and homogeneity of the covariates and responses.

2.8. Model development & selection

Model development for BCF and SPF initially included literature reported values for seven chemical parameters in addition to our experimentally determined $\log K_{oc}$ values. The literature-based chemical properties we considered in model development were $\log K_{ow}$, molecular mass (Roberts et al., 1998; 1999), water solubility, density, half-life, vapor pressure and Henry's Law Constant (FAO, 2009; Extoxnet, 2012; NCBI, 2012; USEPA, 2013). To avoid overparameterization, we narrowed the list of eight potential chemical inputs to four by removing highly correlated variables. Final model parameters included $\log K_{oc}$, molecular mass, density and water solubility as chemical properties, along with Habitat Factor as an amphibian dermal parameter.

To assess how well BCF and SPF can be estimated, given different combinations of predictor variables of interest, (and thus prioritize efforts to measure different model inputs), linear regression models were constructed as follows:

$$Y = \sum_{1 \le i \le n} \alpha_i X_i + \beta + \varepsilon_i \quad (4)$$

where Y is one of two log10-transformed response variables (BCF or SPF), α are estimated coefficients, X are potential covariates, β is an intercept constant, and ε_i is the error term. We applied a variable screen to eliminate highly correlated variables from selected covariates. We concluded by testing all possible model sets from four quantitative covariates, represented by X_i, that include pesticide properties K_{oc} (X_1), Molecular Mass (X_2), Water Solubility (X_3) and Density (X_4) (summarized in Table 1), and one categorical variable that describes amphibian habitat preference, Habitat Factor (X_5). We implemented a parsimonious model screen followed by a Bayesian information criterion (BIC) analysis to identify the best-fit regression models.

Selection criteria for the best predictive models for BCF and SPF aimed to avoid model over-fitting since the performance of regression models (when measured by R^2) always increased as additional model covariates were included. The BIC was employed to provide insight into the benefit of additional predictor variables, since it levies increasing penalties for each additional model parameter. Our first step in identifying an "optimal" model was to implement a screen to ensure identification of parsimonious regression model structures that may reasonably explain the current dataset and are more likely to be robust when applied to other active ingredients beyond the five reported in this study.

This screen utilized cross-validation results to identify robust candidate models. First, regression models were built for all possible combinations of independent variables. The full dataset was divided into a testing subset containing one pesticide (Y_j) and a training subset (Y_{-j}) containing the other four which created five pairs of testing and training datasets. For each set of Y_j and Y_{-j}, a linear regression model was fit to the training dataset and residuals were calculated ($RES_{training,-j}$). The same linear model was then applied to the testing dataset and its residuals were estimated ($RES_{testing,\ j}$). This process was repeated for all possible combinations of explanatory variables and for the remaining four pairs of datasets. Next, training residuals and testing residuals were pooled and their root mean square errors were estimated ($RMSE_{training}$, $RMSE_{testing}$), based on Equation (5) (adapted from Deakin and Kildea, 1999):

$$RMSE = \sqrt{\mathrm{var}(RES) + \mathrm{mean}(RES)^2} \quad (5)$$

The weighted RMSE was estimated based on the percentage of observations in the training (N_{-j}/N) and testing (N_j/N) datasets. Here, the training data were four times more than the testing data ($N_{-j}/N = 0.8$, $N_j/N = 0.2$):

$$RMSE_{overall} = \frac{N_{-j}}{N} \times RMSE_{training} + \frac{N_j}{N} \times RMSE_{testing} \quad (6)$$

The $RMSE_{overall}$ values were sorted in ascending order and the top 10% of the models selected. This pool should be relatively robust (i.e., training errors should fall within the same order of magnitude as testing errors). From this set of models, we identified the model with the lowest BIC and models within approximately 10 BIC units of it (Burnham and Anderson, 2004), where BIC values are estimated based on the full dataset.

3. Results

3.1. Amphibian body weights & surface areas

On the day of experimentation, individual body weights for the seven amphibian species ranged from 0.24 to 5.5 g. Mean body weight (g) (±SE) by species was 2.82 ± 0.32 for leopard frogs, 0.39 ± 0.03 for cricket frogs, 2.30 ± 0.21 for Fowler's toads, 0.45 ± 0.03 for Eastern narrowmouth toads, 2.34 ± 0.12 for gray treefrogs, 1.91 ± 0.12 for green treefrogs and 2.24 ± 0.12 for barking treefrogs. Estimated mean surface area (cm^2) (±SE) by species was 2.02 ± 0.13 for leopard frogs, 0.65 ± 0.03 for cricket frogs, 1.79 ± 0.96 for Fowler's toads, 0.70 ± 0.02 for Eastern narrowmouth toads, 1.84 ± 0.05 for gray treefrogs, 1.62 ± 0.06 for green treefrogs and 1.79 ± 0.06 for barking treefrogs.

3.2. Tissue concentrations

Control amphibians ($N = 21$ or three per species) that were not exposed to any of the five pesticide active ingredients used had no detectable pesticide load in their tissues. All amphibians exposed to pesticide active ingredients ($N = 131$) had measureable whole body pesticide tissue concentrations at the end of the 8-h exposure study. Pesticide tissue concentrations among exposed amphibians ranged from 0.019 to 14.562 μg/g across all five pesticides (Fig. 1).

On average, the highest body burdens measured were for atrazine and the lowest for imidacloprid. A univariate regression showed that $\log K_{ow}$ was not a sufficient predictor of pesticide tissue concentration in the live amphibians we tested ($P = 0.055$; Adj $R^2 = 0.02$). Our experimentally-determined $\log K_{oc}$ values had a statistically significant effect on tissue concentrations when used as a covariate with Habitat Factor, although Habitat Factor itself showed no effect ($\log K_{oc}$ $P < 0.001$; Habitat Factor $P > 0.1$; $F_{3,127} = 16.59$; Adj $R^2 = 0.26$).

3.3. Bioconcentration factors

Pesticide concentrations measured in soil for all five pesticide active ingredients and for each of the seven amphibian species are given in Table 2. Across all pesticides and amphibians tested,

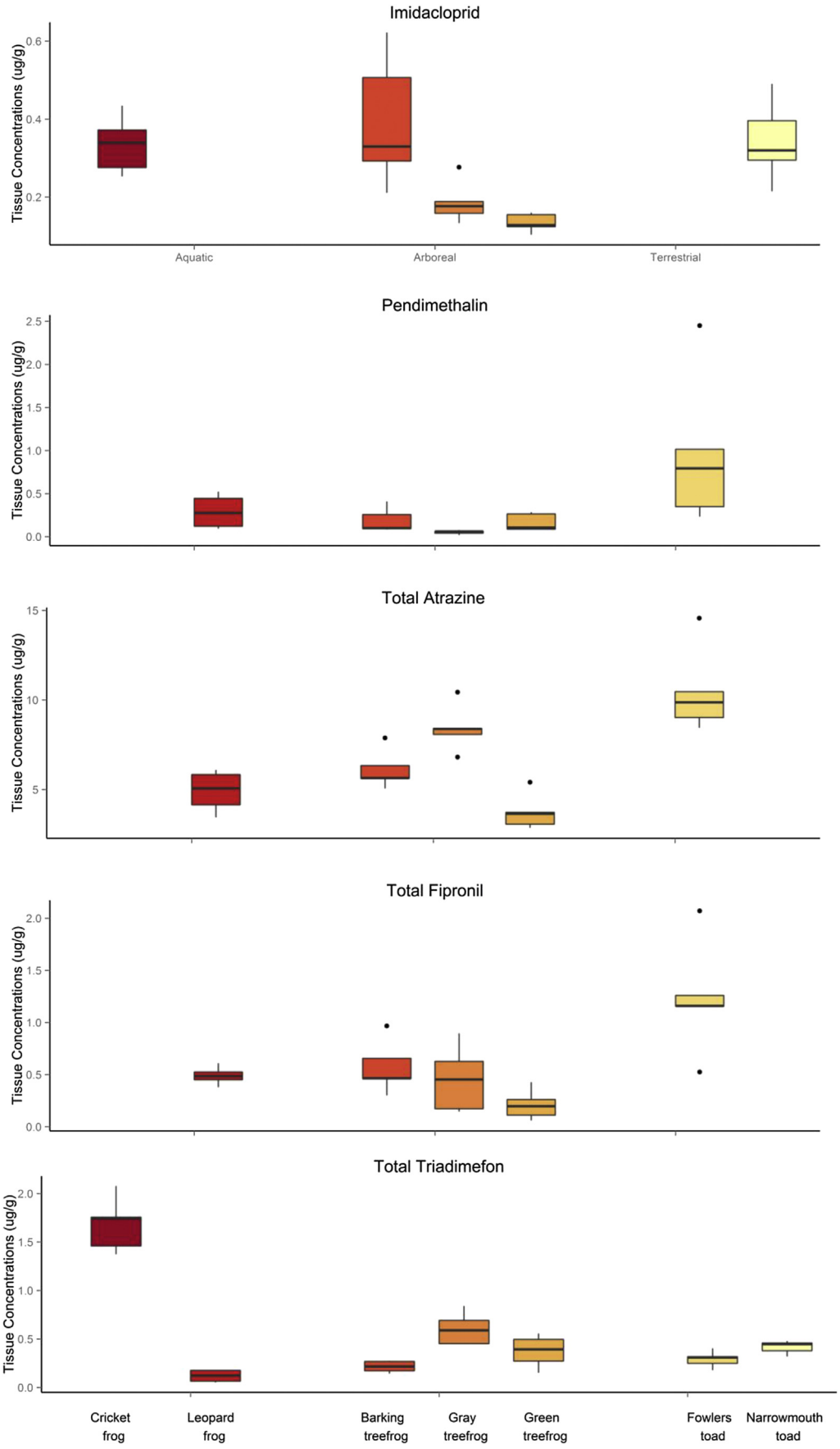

Fig. 1. Tissue concentrations (μg/g) of exposed amphibians. The seven species are grouped by habitat factor, with body burdens presented as boxplot summaries for each of the 5 samples within the pesticide treatment.

Table 2
Soil concentration (μg/g), bioconcentration factor (BCF) and log10 skin permeability factor (log10 SPF) by pesticide and species. Sample size (N) for Leopard Frog = 4, while all other six species $N = 5$ per pesticide tested. NT = this species and pesticide combination not tested.* indicates species with a small mass requiring 2–3 individuals as a composite sample for pesticide extraction and analysis.

Pesticide	Habitat type/Species						
	Aquatic		Terrestrial		Arboreal		
	Leopard frog	Cricket frog*	Fowler's toad	Narrowmouth toad*	Gray treefrog	Green treefrog	Barking treefrog
Imidacloprid							
Soil Conc.	NT	2.07	NT	2.02	2.87	2.03	2.57
BCF	NT	0.162 (0.016)	NT	0.170 (0.023)	0.065 (0.008)	0.066 (0.005)	0.153 (0.029)
log10 SPF	NT	4.48 (0.04)	NT	4.56 (0.06)	4.52 (0.04)	4.33 (0.04)	4.82 (0.07)
Atrazine							
Soil Conc.	21.53	NT	17.05	NT	16.64	17.61	14.44
BCF	0.228 (0.029)	NT	0.614 (0.064)	NT	0.506 (0.035)	0.213 (0.025)	0.423 (0.033)
log10 SPF	5.37 (0.07)	NT	5.65 (0.06)	NT	5.54 (0.06)	5.17 (0.07)	5.44 (0.04)
Triadimefon							
Soil Conc.	6.67	10.07	7.01	6.24	5.81	5.25	5.95
BCF	0.018 (0.005)	0.167 (0.012)	0.042 (0.005)	0.067 (0.005)	0.104 (0.013)	0.071 (0.014)	0.036 (0.004)
log10 SPF	4.62 (0.15)	5.52 (0.03)	4.95 (0.10)	4.94 (0.03)	5.33 (0.06)	5.08 (0.11)	4.88 (0.04)
Fipronil							
Soil Conc.	3.16	NT	3.37	NT	3.31	2.80	2.5
BCF	0.115 (0.015)	NT	0.366 (0.073)	NT	0.139 (0.043)	0.076 (0.023)	0.228 (0.046)
log10 SPF	5.62 (0.06)	NT	5.99 (0.11)	NT	5.58 (0.16)	5.21 (0.12)	5.67 (0.07)
Pendimethalin							
Soil Conc.	17.58	NT	12.47	NT	10.67	12.43	13.62
BCF	0.016 (0.006)	NT	0.078 (0.032)	NT	0.005 (0.000)	0.013 (0.003)	0.014 (0.005)
log10 SPF	4.07 (0.21)	NT	4.54 (0.18)	NT	3.32 (0.10)	3.90 (0.11)	3.95 (0.14)

individual BCFs ranged from 0.002 to 0.854 (Table 2). On average, the highest BCFs were associated with atrazine for all five exposed species. Pendimethalin produced the lowest BCF values among leopard frogs and all three arboreal species; triadimefon was the least bioconcentrated in both terrestrial species. In regression models, $\log K_{ow}$ was a statistically significant predictor of BCF ($P < 0.001$; Adj $R^2 = 0.21$), however, experimentally-determined $\log K_{oc}$ values were a much better predictor of BCFs when used as a covariate with Habitat Factor ($\log K_{oc}$ $P < 0.001$; Habitat Factor $P > 0.05$; $F_{3,127} = 53.12$; Adj $R^2 = 0.55$) and in simple regression models ($P < 0.001$; Adj $R^2 = 0.52$). Habitat Factor had no significant effect on BCF among the amphibians and pesticides we tested.

3.4. Skin permeability factors

Amphibian SPFs were highest for fipronil and lowest for pendimethalin among the five species exposed to them (Table 2). A general trend among SPF values emerged among all seven species: Fipronil > Atrazine > Triadimefon > Imidacloprid > Pendimethalin. $\text{Log}K_{ow}$ was not a good predictor of amphibian SPF in regression models ($P = 0.139$; Adj $R^2 = 0.009$). As with tissue concentrations and BCFs, experimentally-determined $\log K_{oc}$ values were a much better predictor of SPFs when used as a covariate in general linear models, with Habitat Factor as a categorical variable, while Habitat Factor had no effect ($\log K_{oc}$ $P < 0.001$; Habitat Factor $P > 0.05$; $F_{3,127} = 37.41$; Adj $R^2 = 0.45$).

3.5. Model selection

Four models were identified as having high predictive capabilities for both pesticide BCF and SPF among amphibians (Table 3). The top models shared the same predictor variables: for both dependent variables, $\log K_{oc}$ and Water Solubility were always included as important parameters. Habitat Factor and Density appear in two of the top models for both BCF and SPF, while Molecular Mass was never an important parameter in any of the top regression models selected.

4. Discussion

Estimating pesticide risk to amphibians is a global concern (e.g., Kiesecker et al., 2004; Pounds et al., 2006; Alford et al., 2007), yet little emphasis has been placed on data collection during post-metamorphic terrestrial life stages. Barring normal events (e.g., predation, disease, etc.), most amphibians will spend the majority of their life cycles as terrestrial or semi-terrestrial juveniles and adults, a period when non-lethal outcomes such as reproductive success are critical to maintaining robust populations. Agricultural pesticide applications may pose the greatest risk to amphibians, given the frequency and timing of use during crop pre-emergence and growth, and as a defoliant during harvesting. Adult amphibians moving across agricultural landscapes to breeding ponds and recently metamorphosed juveniles leaving breeding ponds are likely to experience dermal exposure to pesticides. The dermal exposure and uptake data we present is a preliminary step towards

Table 3
Top regression models to predict pesticide bioconcentration factor (BCF) and skin permeability factor (SPF) among amphibians, based on weighted root mean square error (RMSE) and Bayesian Information Criterion (BIC) values. Important model parameters were Habitat Factor (HabFac), $\log K_{oc}$ or soil partition coefficient (K_{oc}), Water Solubility (Sol) and Density (Den). All numerical variables were natural log transformed for model development. "X" indicates the parameters included in each model.

Model	HabFac	K_{oc}	MolM	Sol	Den	R^2	BIC	Weighted RMSE
Bioconcentration Factor (BCF)								
1	X	X		X		0.69	98.72	0.35
2		X		X		0.65	104.94	0.36
3	X	X		X	X	0.69	102.98	0.38
4		X		X	X	0.65	109.55	0.39
Skin Permeability Factor (SPF)								
1	X	X		X		0.67	148.43	0.48
2		X		X		0.64	150.45	0.50
3	X	X		X	X	0.68	152.39	0.55
4		X		X	X	0.65	154.05	0.57

developing more comprehensive models that predict pesticide impacts to amphibians as a group of non-target organisms.

The unique physiology and morphology of amphibian skin plays a role in contaminant passage through dermal cells. Amphibian skin is very thin (~10–20 μm; Willens et al., 2006) and is the primary organ involved in both gas and water exchange (Lenfant and Johansen, 1967; Tracy, 1976). Amphibian skin thickness differs based on natural history where terrestrial species have thicker, more granulated skin to prevent water loss (Toledo and Jared, 1993). Arboreal frogs secrete a mucous layer to reduce evaporative water loss (Wygoda, 1988; Amey and Grigg, 1995). Water accumulation in amphibians is prompted by complex behavioral, environmental and physiological cues including, but not limited to, water volume stored in the bladder, plasma volume, hormones and perception of available water from the surrounding habitat (reviewed in Hillyard and Williamsun, 2011). The use of Habitat Factor in our predictive models was to distinguish between dermal characteristics of aquatic, terrestrial and arboreal species that likely play a role in contaminant uptake. Although Habitat Factor alone did not have a statistically significant effect on tissue concentration, BCF, or SPF, it was a parameter in half of the winning predictive models for BCF and SPF. Dermal characteristics and physiology are important when investigating pesticide properties and associated uptake.

LogK_{ow} is an important parameter when predicting pesticide uptake across the dermis in mammals (USEPA, 2007) and has been suggested as equally important for amphibians (Quaranta et al., 2009). Prior to data analysis, we predicted that living frog skin would be more permeable to hydrophilic pesticides and less permeable to hydrophobic pesticides, however, our data indicate that logK_{ow} is not a strong predictor of whole tissue pesticide body burdens or skin permeability factor. Based on whole body tissue concentrations, atrazine resulted in the highest body burdens while imidacloprid, even more hydrophilic than atrazine, produced the lowest. Pendimethalin, the most hydrophobic compound used, resulted in low body burdens relative to other pesticides tested. Our data differs from that collected by Quaranta et al. (2009) by showing no relationship between logK_{ow} and frog skin permeability. The primary difference between our experimental designs is that Quaranta et al. used dead frog skin on a flow-through cell and we used living amphibians placed directly on soil. Normal physiological reactions in the skin (e.g., rehydration) can take place only in live animals and this likely explains the difference in findings with respect to logK_{ow}. Given the limited number of pesticides examined in relationship to amphibian dermal uptake ($N = 5$ in this study), a broader range should be tested to verify the lack of relationship between logK_{ow} and frog skin permeability in living specimens.

Maximum allowable application rates for pesticide active ingredients vary among formulated products and by crop type (USEPA, 2013). We chose the maximum application rate among all food crops for each active ingredient tested to reflect actual pesticide application rates in agricultural landscapes. Our SPF measure allowed us to remove the effect of application rate variability and evaluate skin permeability. In doing so, we found fipronil was the most permeable across amphibian dermis despite a very low application rate. This indicates that chemical properties beyond hydrophobicity or lowK_{ow} are important considerations for dermal pesticide exposure in amphibians.

Our experimentally determined pesticide soil partition coefficients, logK_{oc}, were much better predictors of BCF and SPF compared to logK_{ow}. Soils high in organic carbon tend to bind more readily with organic chemicals than soils low in organic carbon (e.g., Grathwohl, 1990). Given this, it was important to determine K_{oc} values for the specific soil we used. We found that K_{oc} values obtained from the literature were not good predictors of BCF or SPF in our models. While our measured logK_{oc} was a better predictor, we have only been able to test five pesticides of the thousands legally registered for use in the US. Therefore, while including logK_{oc} was a better fit for our data, we encourage other researchers to test laboratory-estimated K_{oc} values. This will aid in developing a more comprehensive data set for inclusion in future modeling and meta-analysis efforts. While laboratory determinations of K_{oc} are time-consuming, they may offer better predictive capabilities for estimating pesticide exposure and accumulation in terrestrial phase amphibians.

Parameterizing models that accurately predict BCF and SPF related to dermal pesticide exposure in amphibians is complex, but necessary for ecological risk assessment of non-target species. For both BCF and SPF, the top model includes Habitat Factor, logK_{oc} and water solubility. Of the four top models, water solubility and logK_{oc} were always included, indicating these chemical properties may be the most significant in determining dermal accumulation of pesticides in amphibians. Water solubility is likely to be a very good predictor of BCF and SPF, given that amphibians rely solely on skin physiology for water absorption. Given that molecular mass was never included as a parameter in our top models, it may have little significance for pesticide uptake in frogs. Including a larger suite of pesticides to encompass a broader spectrum of chemical properties is necessary for more complete understanding of relationships between unique pesticide properties such as water solubility and dermal uptake, among amphibians.

Our study included seven different amphibian species placed in groups according to preferred habitat or Habitat Factor (detailed in Methods; aquatic, terrestrial or arboreal). Although grouping them by habitat type is reasonable for distinguishing unique dermal properties associated with degree of terrestriality, we acknowledge that, for some species, preferred habitat does not fall strictly within a category. Dermal properties are also likely to vary by species within a habitat type. Creating species-specific models would facilitate development of precise conservation strategies, but would also create enormous data requirements for pesticide registration processes. It is worth noting that two of the four winning models did not include Habitat Factor, indicating that modeling efforts aimed at estimating pesticide accumulation in amphibians may be informed solely by chemical properties.

Determining if current and proposed regulatory models, which are typically based on mammal and/or avian data, are robust enough to estimate dermal exposure and subsequent ecological risk for amphibians is a general concern (Smith et al., 2007). Screening models are typically implemented for a single proposed chemical and are therefore based on a combination of chemical properties, proposed label rates, and species data. Although we present statistical regression models with the greatest support from our observations, we recognize they must be challenged by observations from other studies before there is sufficient basis for their use in screening and higher-tier assessments. In addition, exposure processes are best estimated with algorithms that account for specific transfer processes and rates such as diffusion and advection across the dermal barrier (James et al., 2004; Willens et al., 2006). Since body burden data were collected under controlled laboratory conditions with approved labeled application rates for several amphibian species, the data collected in this study can serve as a basis for testing and verifying proposed models. In addition, they are useful for model selection among proposed algorithms designed to estimate amphibian body burdens and associated rate parameters. For dermal exposure assessment in amphibians significant data gaps remain in documenting short-term dermal transfer of pesticides, estimating bioaccumulation for pesticides that may have multiple applications, and quantifying metabolism products and rates for commonly used pesticides.

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