Environmental Pollution 220 (2017) 375–382

Contents lists available at ScienceDirect

Environmental Pollution

journal homepage: www.elsevier.com/locate/envpol

Do pyrethroid-resistant *Hyalella azteca* have greater bioaccumulation potential compared to non-resistant populations? Implications for bioaccumulation in fish☆

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ARTICLE INFO

Article history:
Received 8 July 2016
Received in revised form
22 September 2016
Accepted 23 September 2016
Available online 15 October 2016

Keywords:
Pyrethroid insecticide
Trophic transfer
Permethrin
Biotransformation
Non-target species

ABSTRACT

The recent discovery of pyrethroid-resistant *Hyalella azteca* populations in California, USA suggests there has been significant exposure of aquatic organisms to these terrestrially-applied insecticides. Since resistant organisms are able to survive in relatively contaminated habitats they may experience greater pyrethroid bioaccumulation, subsequently increasing the risk of those compounds transferring to predators. These issues were evaluated in the current study following toxicity tests in water with permethrin which showed the 96-h LC50 of resistant *H. azteca* (1670 ng L^{-1}) was 53 times higher than that of non-resistant *H. azteca* (31.2 ng L^{-1}). Bioaccumulation was compared between resistant and non-resistant *H. azteca* by exposing both populations to permethrin in water and then measuring the tissue concentrations attained. Our results indicate that resistant and non-resistant *H. azteca* have similar potential to bioaccumulate pyrethroids at the same exposure concentration. However, significantly greater bioaccumulation occurs in resistant *H. azteca* at exposure concentrations non-resistant organisms cannot survive. To assess the risk of pyrethroid trophic transfer, permethrin-dosed resistant *H. azteca* were fed to fathead minnows (*Pimephales promelas*) for four days, after which bioaccumulation of permethrin and its biotransformation products in fish tissues were measured. There were detectable concentrations of permethrin in fish tissues after they consumed dosed resistant *H. azteca*. These results show that bioaccumulation potential is greater in organisms with pyrethroid resistance and this increases the risk of trophic transfer when consumed by a predator. The implications of this study extend to individual fitness, populations and food webs.

1. Introduction

Pyrethroids are currently the dominant insecticide in residential environments, and have major agricultural use as well (Kemble et al., 2013; Hunt et al., 2016). Pyrethroids were detected in 80% and 75% of sediment samples taken from urban and agricultural water bodies in California (USA), respectively (Amweg et al., 2006; Weston et al., 2004). Many of these sediments were found to cause mortality in test organisms during laboratory exposures and toxic unit analysis identified pyrethroid contamination as the main cause. There have been similar findings with sediments from other waterways across the United States (Ding et al., 2010; Hintzen et al., 2009; Kemble et al., 2013; Weston et al., 2005). Although pyrethroids are hydrophobic and tend to adsorb to organic matter in sediments, these compounds also have been detected in water samples at concentrations toxic to biota (Feo et al., 2010; Weston and Lydy, 2010; Weston et al., 2009). While little is known about how elevated pyrethroid concentrations will impact aquatic systems, some significant changes among populations have emerged.

Pyrethroid resistance has recently been documented in several wild populations of *Hyalella azteca*, an epibenthic amphipod commonly used in toxicity testing (Weston et al., 2013). Found in

☆ This paper has been recommended for acceptance by Charles Wong.
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http://dx.doi.org/10.1016/j.envpol.2016.09.073
0269-7491/

pyrethroid-contaminated waters in central California, these *H. azteca* were up to 550 times less sensitive to two common pyrethroids (cyfluthrin and bifenthrin) than non-resistant lab populations. Gene sequencing of the resistant animals revealed mutations in the voltage-gated sodium channels of the nervous system, the target site of pyrethroid insecticides (Weston et al., 2013). Typically, pyrethroids bind to the sodium channels, preventing or delaying their closing (Davies et al., 2007; Soderlund and Bloomquist, 1989). This results in repetitive firing of the neurons, which manifests as convulsions, tremors and loss of coordinated movements. After some time, the organism becomes paralyzed and dies (Davies et al., 2007; Soderlund and Bloomquist, 1989). However, the mutation of the sodium channels in the pyrethroid-resistant *H. azteca* prevents pyrethroid binding. Research suggests that the alternative mode of toxic action in the resistant organisms is oxidative stress, though occurring at much higher concentrations (Weston et al., 2013).

Since the resistant *H. azteca* survive in environments with relatively high concentrations of pyrethroids, these organisms must differ from non-resistant *H. azteca* with respect to toxicokinetic processes (e.g., uptake, biotransformation and elimination) and/or bioaccumulation potential. The resistant organisms either have higher biotransformation or excretion rates, or alternatively the compounds accumulate in the organisms' tissues to higher concentrations than in non-resistant *H. azteca*, who would succumb to toxicity before attaining high body burdens. This may have implications on both environmental assessments and ecosystem functioning. For instance, significant energy allocation to biotransformation and/or high body residues of pyrethroids may reduce fitness by making individuals more sensitive to other stressors and by reducing fecundity (Chandler, 1990; Werner et al., 2002). Furthermore, a high degree of pyrethroid bioaccumulation in lower trophic level organisms increases the probability of trophic transfer of these compounds in the food web. For that reason, we investigated how resistance to the pyrethroid insecticide, permethrin affected bioaccumulation in *H. azteca* and the potential for subsequent bioaccumulation in a predator, the fathead minnow *(Pimephales promelas)*. Specifically, our objectives were to 1) determine the lethal permethrin concentration to 50% of the individuals (LC50) in non-resistant and resistant *H. azteca* through water-only exposures, 2) compare the bioaccumulation of permethrin in non-resistant and resistant populations, and 3) examine bioaccumulation potential of permethrin in fathead minnows fed resistant *H. azteca* dosed with ^{14}C-labeled permethrin.

2. Methods

2.1. Chemicals

Permethrin (40% *cis*, 60% *trans*) and two surrogates, dibromooctofluorobiphenyl (DBOFB) and decachlorobiphenyl (DCBP), were purchased from ChemService (West Chester, PA, USA) and Sigma-Aldrich Co. (St. Louis, MO, USA), respectively. Radiolabeled permethrin (^{14}C—ring-labeled, specific activity 260 mCi mmol^{-1}, purity ≥95%) was purchased from Moravek Biochemicals (Brea, CA, USA). The purity of the radiolabeled permethrin was evaluated by separating the parent compound from degradation products using an Agilent 1100 high-pressure liquid chromatograph (HPLC) equipped with a fraction collector (Agilent Technologies, Palo Alto, CA, USA) using methods similar to those in You et al. (2009) and measuring the radioactivity of resulting fractions on a Packard TriCarb 2900TR liquid scintillation counter (LSC) (Packard Instrument Company, Meriden, CT, USA). Scintillation cocktail (ScintSafe Plus 50%), all solvents (pesticide-grade acetone, hexane, methylene chloride and acetonitrile) and MS-222 were purchased from Fisher Scientific (Pittsburgh, PA, USA).

2.2. Organisms

Non-resistant and resistant *H. azteca* were cultured according to standard protocols (USEPA, 2000) at Southern Illinois University (Carbondale, IL, USA). The non-resistant culture was initiated in 2001 with organisms acquired from a culture at the U.S. Environmental Protection Agency, Duluth laboratory. Resistant *H. azteca* were collected in 2014 from Mosher Slough, Stockton, CA, USA, where resistance to cyfluthrin and bifenthrin had previously been documented (Weston et al., 2013). The Mosher Slough animals in the current study had been cultured in the laboratory for at least 16 months (approximately 16 generations) prior to testing. For all tests, juvenile *H. azteca* were collected from cultures by isolating individuals that passed through a 1 mm mesh sieve, but were retained by a 500 μm mesh sieve.

Bioaccumulation and toxicity of hydrophobic compounds can be affected by tissue lipid content; therefore, this metric was measured prior to testing to confirm lipid levels for the two different *Hyalella* populations and the fish (Klosterhaus et al., 2003). Lipid levels in all of the test organisms were determined using a phosphor-vanillin spectrometric method (Van Handel, 1985).

2.3. Water toxicity tests

Toxicity tests using both non-resistant and resistant *H. azteca* followed standard procedures for 96-h static water tests outlined by the U.S. EPA (2000). Briefly, moderately hard water (MHW) (Smith et al., 1997) was spiked using permethrin dissolved in an acetone carrier. Treatments consisted of seven permethrin concentrations determined from preliminary testing, as well as solvent and negative controls. The volume of acetone added as the carrier in permethrin treatments and added in the solvent control accounted for a minimal amount (<100 μL L^{-1}) of the total spiked water volume. Dosed water (500 ml) was distributed into five replicate 600-ml beakers for each treatment, four of which were used for toxicity testing and one was used for determination of initial permethrin concentration using methods described below in Section 2.4. The beakers were then stored at 23 °C for approximately 24 h prior to test initiation to allow for equilibration of the permethrin with the glassware.

Upon test initiation, 10 *H. azteca* were added to four replicate beakers for each permethrin concentration and control beakers after which they were stored in an incubator at 23 °C with a 16:8 h light:dark photoperiod for the duration of the tests. No feeding was provided during the tests and termination of the tests consisted of enumeration of living organisms. Final permethrin concentrations were determined by pooling 125 ml aliquots of water from each of the four replicate beakers and extracting permethrin using methods described in Section 2.4. Water quality parameters were measured at the beginning and end of each test and those details are included in Supplemental Information.

2.4. Toxicity test water extractions and analyses

Non-radiolabeled permethrin was extracted from water using a liquid-liquid extraction (LLE) and concentrations were analyzed via gas chromatography (GC). Water samples (500 ml) from the beginning and end of each test were extracted with 50 ml of methylene chloride three times by hand shaking for 3 min. Surrogate compounds (DBOFB and DCBP) were added to each sample prior to extraction. Quality assurance included a matrix spike and matrix spike duplicate that were spiked with permethrin and the

two surrogates. The total 150 ml of methylene chloride from each LLE was solvent exchanged to hexane, concentrated to near dryness and reconstituted to 0.5 ml with acidified hexane (0.1% acetic acid) to stabilize permethrin isomer ratios (You and Lydy, 2007). Samples from the resistant *H. azteca* test were diluted as needed to fall within the range of the prepared calibration standards.

Sample extracts from the toxicity tests were analyzed using an Agilent 6890 series GC equipped with a microelectron capture detector (Agilent Technologies, Palo Alto, CA, USA). Dual-column confirmation was used and five or six external standards in acidified hexane were used to make calibration curves based on peak areas for quantification of the compounds (You and Lydy, 2007). Additional details on the analytical methods can be found in Supplemental Information.

2.5. Time to reach steady state

In order to determine the length of time needed to reach steady state body residues in both non-resistant and resistant *H. azteca*, a short-term toxicokinetic study was conducted following the methods detailed in Lydy et al. (2000). This test consisted of an 8 h uptake phase followed by an elimination phase that lasted up to 56 h. Additional details concerning the steady state test can be found in Supplemental Information.

The biological half-life of the parent compound ($t_{1/2}$) was determined by using the following equation fitted using an iterative least-squares procedure and a fourth-order Runga-Kutta approach in the software package Scientist® (Micromath, St. Louis, MO, USA; Lydy et al., 2000):

$$t_{(1/2)} = \frac{\ln 2}{k_{ep} + k_m} \quad (1)$$

where, k_{ep} was the parent permethrin elimination rate constant (h^{-1}), k_m was the metabolite formation constant (h^{-1}), and t was time (h). Time to reach steady state concentration in *H. azteca* was calculated by taking the $t_{1/2}$ value and multiplying by five (Landrum et al., 1992).

2.6. Bioaccumulation tests - H. azteca

These tests were conducted to examine the relative bioaccumulation potential of permethrin in the non-resistant and resistant *H. azteca* populations. Moderately hard water was spiked at the appropriate concentrations with ^{14}C-labeled permethrin solubilized in an acetone carrier (acetone accounted for <100 μL L^{-1} total volume of dosed water). Four replicates were used for each treatment as well as a negative control. The number of *H. azteca* used in each replicate ranged from 30 to 40 organisms/beaker. Non-resistant and resistant *H. azteca* were both exposed to three lower permethrin concentration treatments (24, 46 and 86 ng L^{-1}). Only the resistant *H. azteca* were exposed to higher permethrin levels (>210 ng L^{-1}) due to mortality of the non-resistant *H. azteca* at the higher concentrations. Aliquots of water (500 ml) were distributed into 600-ml beakers, which were then incubated at 23 °C overnight before adding the *H. azteca*. The toxicokinetics experiment indicated that the time to steady-state concentration for the non-resistant population was 33 h, while time to steady-state concentration for the resistant *H. azteca* was 17 h. Thus, bioaccumulation in the lower concentration treatments by both populations of *H. azteca* was measured after 72 h exposure, while an exposure time of 24 h was used for the higher permethrin treatments with resistant organisms only. The bioaccumulation tests were conducted at 23 °C with a 16:8 h light:dark photoperiod.

Water samples (3 ml) were taken upon the addition of organisms to the beakers and at the sampling time point for each replicate. Radioactivity in the water samples was determined by adding 10 ml of scintillation cocktail (ScintSafe™ Plus 50%) to each sample and allowing the samples to sit in darkness for 24 h prior to analysis via a LSC.

At each sampling time, the *H. azteca* were removed from the beaker, rinsed with MHW, patted dry, and separated into two groups for determination of total permethrin tissue concentration (15 organisms) and biotransformation of permethrin (all remaining organisms). Organisms were then weighed to the nearest 0.01 mg in 20 ml scintillation vials using a Mettler-Toledo XS105 analytical balance (Mettler-Toledo International, Inc., Columbus, OH, USA) and were stored at −20 °C until analysis. *Hyalella azteca* designated for measuring total permethrin tissue concentrations were sonicated in 10 ml scintillation cocktail for 35 s using a Tekmar model 501 sonic processor (Teledyne Tekmar, Mason, OH, USA). After allowing samples to remain in darkness for 24 h, the radioactivity of each was measured by LSC and used to calculate total permethrin tissue concentration normalized by percent lipid (wet weight).

Biotransformation was evaluated by determining the percentage of the total radioactivity attributed to parent permethrin in the *H. azteca* using methods similar to Harwood et al. (2009). Briefly, the remaining organisms of each replicate were pooled for each treatment and were homogenized in 5 ml of acetone using a glass tissue homogenizer. Extracts were solvent exchanged to acetonitrile, filtered through a Whatman™ syringeless filter (0.2 μm pore size) and concentrated to 100 μl. Samples were then analyzed by separating the parent compound from biotransformation products based on known retention times of the permethrin isomers using an Agilent Eclipse XDB-C18 column (4.6 × 150 mm) on an Agilent 1100 HPLC equipped with a fraction collector. Additional details on the biotransformation analyses can be found in Supplemental Information.

2.7. Feeding experiments - fathead minnows

The goal of this experiment was to measure the amount of permethrin and biotransformation products bioaccumulated in adult fathead minnows fed *H. azteca* that were exposed to water spiked with ^{14}C-labeled permethrin. Only resistant *H. azteca* exposed at an elevated permethrin concentration (781 ng L^{-1}) were used in this feeding experiment in order to simulate a worst-case scenario. These “feeder” *H. azteca* remained in the dosed water for 24 h prior to being fed to the fathead minnows in order to reach steady state body residues. One adult fathead minnow (41–45 mm in length, 0.43–0.58 g in mass) was placed into each of three 1-L beakers containing 800 ml of permethrin-free MHW. Each fish was fed 15 feeder *H. azteca* every morning for four days. This number of *H. azteca* is much less than what these fish can consume if more prey is available (*personal observation*). The fish consumed the *H. azteca* within five minutes after they were introduced into the beaker each day. A control beaker of MHW with no fish was also included. Dosed *H. azteca* were added to this beaker and then removed at the end of the feeding period each day to verify that transferring dosed *H. azteca* from the exposure beaker to feeding beakers did not result in significant ^{14}C-permethrin water concentrations in feeding beakers. Daily water changes (~80%) were performed to reduce fish exposure to eliminated permethrin and biotransformation products. The fish were allowed to depurate their gut contents for 24 h following the final feeding, after which they were removed from the feeding beakers and were euthanized with a lethal dose of MS-222.

Following the experiment, water, *H. azteca* and fish samples were analyzed for radioactivity to determine permethrin concentrations. The water that the fish were held in and water from the

control was tested for ^{14}C-labeled activity by removing 5 ml of water each day during the experiment, and processed following the procedure outlined in Section 2.6. After the fish were euthanized, they were placed into pre-weighed scintillation vials and then freeze dried for 72 h in a FreeZone 2.5 freeze dryer (Labconco Corporation, Kansas City, MO, USA). Each freeze-dried fish was then ground using a mortar and pestle and ~30% of the fish was used for total permethrin measurement, while the remaining ground fish was used for biotransformation product analysis. Details for the analysis of parent compound and biotransformation products in the fish tissue are provided in Section 2.6.

2.8. Data analysis

Toxicity test data were analyzed to determine LC50 values using the USEPA Trimmed Spearman-Karber (TSK) software (version 1.5) for water exposures. The average permethrin concentrations, based on the measured concentrations from samples collected at the beginning and end of each test, were used as the representative values for each exposure level to calculate the LC50 values. Data from the bioaccumulation tests were analyzed using a two-sample two-tailed *t*-test assuming equal variances to compare populations with respect to total tissue residue concentration, and homoscedasticity was confirmed using an F-test. These analyses were performed using Sigma Plot 11.0 software. The bioaccumulation data was further analyzed by comparing the total tissue residue concentrations of resistant *H. azteca* at all six exposure concentrations with an ANOVA and Tukey post-hoc test using IBM SPSS Statistics version 22.

3. Results

3.1. Toxicity tests

Average measured permethrin water concentrations (measured after the 24 h equilibration period and at the end of the test) during the toxicity tests were 60.0± 16.5% (mean ± SD) of nominal concentrations, with losses likely due to adsorption onto the glassware (Day and Kaushik, 1987; Sharom and Solomon, 1981). Ranges for surrogate and permethrin recoveries from the matrix spikes were as follows: 52–82% (DBOFB), 90–115% (DCBP) and 91–119% (permethrin). Permethrin concentrations in control samples were always below reporting limits (<10 ng L^{-1}).

Tissue lipid content was 5.9± 0.4% and 8.1± 2.6% (mean ± SD) for non-resistant and resistant populations, respectively and these values were slightly different from one another ($p = 0.05$). Water quality parameters fell within acceptable ranges for all toxicity tests (USEPA, 2000) and were as follows: temperature, 23 ± 1 °C; conductivity, 341 to 355 μS cm^{-1}; pH, 7.8 to 8.2; and dissolved oxygen > 7.5 mg L^{-1}. Less than 5% mortality was observed in negative or solvent controls during the water exposures.

The toxicity test data confirmed that the resistant *H. azteca* were much less sensitive to permethrin than the non-resistant population when exposed in water. The LC50 of the resistant population was 53 times higher than that of the non-resistant *H. azteca* in water (1670 ng L^{-1} [95% confidence interval = 1380–2010] and 31.2 ng L^{-1} [26.4–36.9], respectively). In fact, the non-resistant organisms rarely survived at permethrin water concentration exceeding 86 ng L^{-1} (the highest concentration used for non-resistant organisms in the bioaccumulation test).

3.2. Bioaccumulation tests

Overall, steady state tissue concentrations of parent permethrin and biotransformation products attained by resistant *H. azteca* increased as exposure concentrations increased (Fig. 1; $p < 0.001$ for the resistant population). There were no statistically significant increases in bioaccumulation of permethrin by resistant *Hyalella* between the lower exposures of 24, 46 and 86 ng L^{-1}, but bioaccumulation significantly increased as exposure concentrations were raised to 210 ng L^{-1} and with every higher concentration (Fig. 1). At the concentrations in which the non-resistant animals were able to survive (24–86 ng L^{-1}), the non-resistant and resistant population's bioaccumulated comparable amounts of permethrin. Additionally the ratio of parent to biotransformation products was similar among populations at each of the exposure concentrations (Fig. 1). It should be noted that there was ~30% mortality of the non-resistant population in the 86 ng L^{-1} (at 72 h) exposure, since this concentration was nearly three times its 96-h LC50, and the mobility of those individuals still living was impaired; this may have been the cause for the slightly greater variance in those data.

Only the resistant population could tolerate the exposure concentrations of 210 ng L^{-1} and above, and body burdens attained at these higher exposures continued to increase (Fig. 1). The maximum body burden attained in resistant animals was 89.2 μg g^{-1} lipid at the 843 ng L^{-1} exposure level. Whereas the maximum body burden attained by the non-resistant animals was 9.1 μg g^{-1} lipid in those individuals surviving the 86 ng L^{-1} exposure level.

Biotransformation in *H. azteca* was evaluated by measuring the percent of the total permethrin tissue residues that was attributed to the parent permethrin. Both populations of *H. azteca* were capable of biotransforming permethrin (Fig. 1; Table S1). Nearly all permethrin was biotransformed in 72 h at the lowest exposure concentration (24 ng L^{-1}), and very little difference was observed

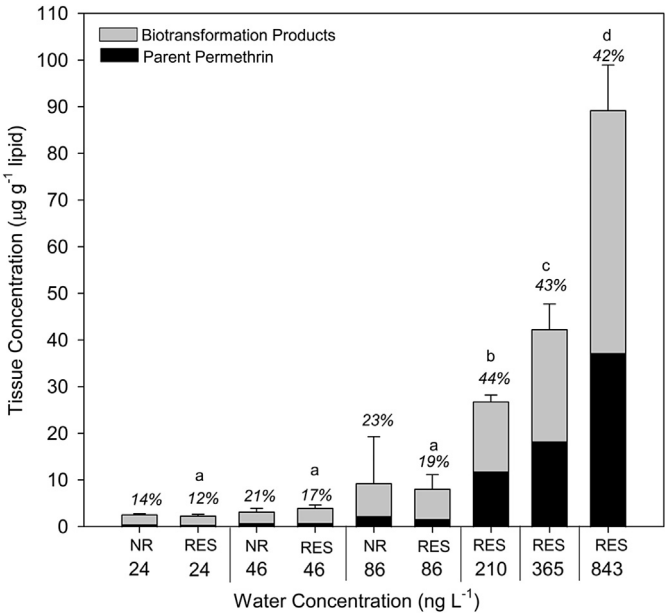

Fig. 1. Steady state concentrations of permethrin residues in tissues of non-resistant (NR) and resistant (RES) *Hyalella azteca* exposed to several permethrin concentrations in water. Exposures at low concentrations (24 ng L^{-1} to 86 ng L^{-1}) took place for 72 h and exposures at high concentrations (210 ng L^{-1} to 843 ng L^{-1}) took place for 24 h. Error bars represent the standard deviation and n = 4. Tissue concentrations were normalized for lipid fraction in *H. azteca*. Control organisms had no detectable tissue concentrations of ^{14}C-labeled permethrin. Contributions of parent compound (permethrin) and biotransformation products to total tissue residue concentrations are shown by the black and grey bar, respectively. The percent permethrin relative to the total is shown above the bars. The letters (a, b, c, d) above the resistant bars indicate statistically significant groups from a pair-wise comparison after ANOVA using Tukey's HSD.

between non-resistant and resistant populations (14% and 12% parent permethrin remaining, respectively). The extent of biotransformation declined as the exposure concentration increased. At the three highest exposure concentrations ($\geq$210 ng L^{-1}) more than 40% of the total permethrin in the resistant *H. azteca* tissue was still parent compound. Due to the need to pool replicate samples to measure permethrin biotransformation products, statistics could not be used to assess similarity between populations or concentrations.

3.3. Feedings experiments

The feeding experiment indicated that permethrin bioaccumulated by resistant *H. azteca* is capable of being transferred to their fish predators by ingestion (Fig. 2). The average total permethrin concentration in fish tissue was 0.22 μg g^{-1} lipid. Although this value was less than the average tissue concentration in the *H. azteca* used to feed the fish (96.5 μg g^{-1} lipid), feeding was only done for an arbitrary 4-d period and may not reflect steady state levels in the fish. The percentage of total tissue permethrin that was parent compound was also less in the fish than in the *H. azteca* used to feed them (32% and 47%, respectively), suggesting further biotransformation of permethrin within the fish. Measurements of radioactivity in the water verified that the permethrin concentration to which fish were exposed through the water (as a result of amphipod transfer from dosed water or elimination of permethrin and biotransformation products by fish) never exceeded two times background levels during the experiment.

4. Discussion

Pyrethroid contamination of aquatic habitats has been well documented, and this widespread exposure has ultimately led to the development of resistant populations of *H. azteca*. The permethrin sensitivity differences noted in the current study between non-resistant and resistant *H. azteca* populations were striking, with the latter having substantially higher LC50 values. The resistant *H. azteca* were 53 times less sensitive than their non-resistant counterparts. These results are similar to those previously obtained, as Weston et al. (2013) showed that *H. azteca* collected from Mosher Slough, where the resistant population in the current study originated, were approximately 100 times less sensitive to the pyrethroid cyfluthrin than non-resistant *H. azteca*.

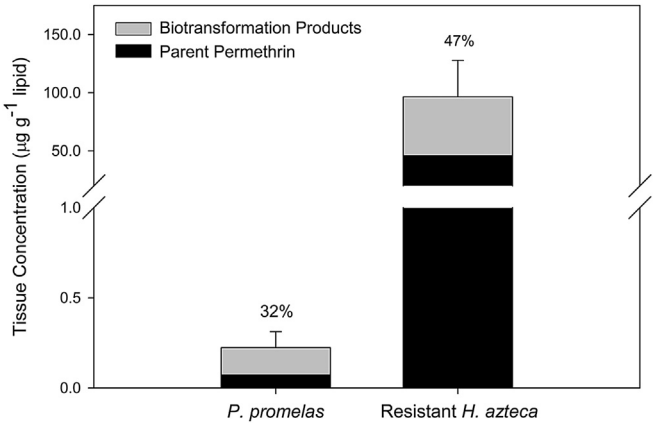

Fig. 2. Tissue concentrations for *Pimephales promelas* after consuming resistant *Hyalella azteca* exposed to 781 ng L^{-1} permethrin in water. Error bars indicate standard deviation of the average lipid normalized tissue concentrations of the *P. promelas* (A) and *H. azteca* averaged over four days (B). Contributions of parent compound (permethrin) and biotransformation products to total tissue residue concentrations are shown by the black and grey bars, respectively. The percent parent permethrin relative to the total is shown above the bars. Control organisms had no detectable tissue concentrations of ^{14}C-labeled permethrin.

It has previously been shown that the Mosher Slough population has a L925I mutation in the voltage gated sodium channel gene, a mutation known to confer pyrethroid resistance in several insect species (Rinkevich et al., 2013; Weston et al., 2013). The resistant *H. azteca* used in the current study were cultured in uncontaminated conditions for at least 16 months prior to testing. Additionally, since bioaccumulation was similar at lower exposure concentrations it is unlikely that toxicokinetic differences resulted in the decreased sensitivity. Thus, our results show that the mutation responsible for conferring resistance in these organisms appears to be retained within the population for many generations after exposure to pyrethroids has ceased.

It should be noted, however that we refer to our species as *Hyalella azteca sensu lato*, though recognize the name often has been applied to what is known to be a *Hyalella* complex. While phylogenetic differences between species may affect sensitivity to pyrethroids, recent research suggests environmental pressures could play a greater role. Work by Weston et al. (2013) identified four separate clades when comparing pyrethroid sensitivity among organisms of the *H. azteca* species complex. Genetic analyses coupled with toxicity testing revealed that resistance to pyrethroids was more closely associated with environmental pyrethroid concentrations than clade (Weston et al., 2013). For instance, individuals in clade B did not exhibit resistance when collected from sites with low pyrethroid contamination, but showed significant resistance when collected from sites with higher pyrethroid concentrations. Likewise, some populations of clade D displayed pyrethroid resistance, while others did not (Weston et al., 2013). The organisms used in the current study were originally collected from a site that Weston et al. (2013) found to have individuals belonging to clade D. They also showed that pyrethroid resistance in *H. azteca* emerged independently in multiple populations. All of this taken together suggests the pyrethroid resistance and increased potential for bioaccumulation will likely be highly influenced by environmental concentrations in the wild for the *H. azteca* species complex and potentially other aquatic organisms.

The degree of resistance in the *H. azteca* of the current study is similar to the observed pyrethroid resistance in some wild populations of target species. Chandre et al. (1998) found that resistant mosquitoes were 9.5–82 times less sensitive to permethrin than susceptible strains. Similarly, pyrethroid-resistant cotton bollworm larvae and resistant houseflies were up to 81 and 70 times (respectively) less sensitive than non-resistant populations (Khan et al., 2013; Mironidis et al., 2013). Pyrethroids are repeatedly applied to terrestrial habitats in order to control the populations of certain insect pests. As such, it is not surprising that resistance is eventually selected for in these target species. Our findings of similar resistance levels in *H. azteca* are concerning, however as they indicate that non-target, aquatic species are being exposed to terrestrially-applied pesticides at concentrations sufficient to apply a selective pressure to the population.

The presence of insecticide resistance in wild populations can have serious impacts on their use as an indicator species in environmental evaluations. Environmental quality assessments often utilize the presence of *H. azteca* to imply low levels of local contamination. If pyrethroid resistance allows *H. azteca* to live in relatively contaminated habitats, an evaluation of environmental quality based on their presence could be misleading and inaccurately depict the degree of contamination of the local habitat. Other issues may arise if resistant organisms are unknowingly introduced into cultures used in laboratory toxicity testing as some cultures have historically been supplemented with wild-caught organisms.

The results of subsequent testing then could be skewed, not only for those involving pyrethroids, but perhaps in other testing as well since resistance to one stressor may alter the sensitivity to other stressors. For instance, cross-resistance to pesticides with similar modes of action may occur as it has in other non-target and target species (Bendis and Relyea, 2016; Brengues et al., 2003). DDT-like organochlorines act on the voltage-gated sodium channels of insects in a manner similar to that of pyrethroids (Coats, 1990). This is likely why the development of resistance to DDT has been found to coincide with resistance to pyrethroids for some mosquito populations (Brengues et al., 2003). Such cross-resistance in a species used for environmental assessments or toxicity testing would obviously be detrimental to habitat evaluations and studies. On the other hand, resistance to a contaminant may increase the sensitivity to other stressors including food deprivation, temperature change or other types of contaminants (Stone et al., 2001; Xie and Klerks, 2003), again potentially skewing test results.

Although resistance may be a necessary adaptation for survival in contaminated habitats, it may also have fitness costs to *H. azteca* that this study did not directly address. The costs of retaining resistance may limit the amount of energy that an organism can allocate to development and reproduction. Insecticide resistance in various insect taxa has been associated with a lower proportion of egg-laying females, fewer eggs produced, a lower proportion of viable eggs and longer larval development time in uncontaminated laboratory settings (Brito et al., 2013; Chan and Zairi, 2013; Martins et al., 2012). Furthermore, maintaining a resistance mechanism may deplete resources needed to cope with other environmental stressors. Survival time upon food deprivation, ability to tolerate additional contaminants and tolerance to temperature change were significantly reduced for heavy-metal resistant organisms (beetles and fish, respectively) relative to non-resistant strains in several studies (Stone et al., 2001; Xie and Klerks, 2003). Significant energy allocation to detoxification (biotransformation or elimination) in resistant organisms in contaminated habitats could have similar effects. Such impacts on reproduction and stress tolerance could potentially translate into effects on the population as a whole.

Additionally, the resistant *H. azteca* were shown to bioaccumulate increasing amounts of permethrin as exposure concentrations increased. This bioaccumulation occurs at elevated concentrations, when it appears that the amount of biotransformation occurring decreases due to the increased presence of parent compound. Since resistant organisms can survive in habitats that have higher pyrethroid concentrations, our results indicate they may be able to accumulate more of these compounds in their tissues than their non-resistant counterparts. In the current study, resistant *H. azteca* were able to accumulate up to 96.5 $\mu g\ g^{-1}$ lipid. It is important to note that the highest exposure concentration used was only half of the determined LC50. This may indicate an even greater bioaccumulation potential as these organisms would be able to survive much higher pyrethroid concentrations than those tested. A LC50 in sediment of approximately 540 $\mu g\ g^{-1}$ OC can be estimated if it is assumed that the resistant *H. azteca* population would also be 50 times less sensitive to permethrin in sediment exposures. This suggests that they would survive even at some of the highest concentrations ever detected in the field (Weston et al., 2004, 2005). Therefore, it is not unreasonable to assume that wild resistant animals could exhibit even greater bioaccumulation than those exposed in the current study. This bioaccumulation could have health implications for both the *H. azteca* and their predators.

In the current study, fathead minnows were shown to bioaccumulate permethrin after consumption of contaminated resistant *H. azteca*. The total permethrin body residues were much less in the fish than in the *H. azteca*; however, fish were only fed 15 contaminated *H. azteca* per day. This number is just a fraction of what the fish may consume if this prey is plentiful (*personal observation*). In addition, this feeding experiment took place over only four days. Regular, continued consumption of larger quantities of a contaminated food source could result in even greater bioaccumulation. A recent study by Corcellas et al. (2015) also found that pyrethroids bioaccumulated in fish. In their study, they analyzed for eight different pyrethroids and found the total pyrethroid concentrations ranged from 0.012 to 4.94 $\mu g\ g^{-1}$ lipid in wild caught fish from the Iberian river basin. This study suggests there is potential for much greater bioaccumulation to occur in fish than what was demonstrated in the present study if they are regularly exposed to pyrethroids. Furthermore, the risk of pyrethroid bioaccumulation is compounded for fish that live in contaminated habitats as exposure likely occurs through water as well as contaminated prey.

The consumption of contaminated prey and subsequent bioaccumulation of pyrethroids has the potential to impact predators in a number of ways. A downward trend in fecundity, fertilization success, and larval viability with increasing pyrethroid concentration in the diet has been observed in fish (*Oryzias latipes*) (Werner et al., 2002). Exposure to pyrethroids has the potential to also alter swimming behavior, respiration and biochemical mechanisms in aquatic invertebrates and fish (Başer et al., 2003; Christensen et al., 2005; Haya, 1989; Velisek et al., 2009). These sublethal contaminant effects on vital functions may subsequently affect survival. For instance, altered swimming behavior could reduce prey capture efficiency or make affected individuals more susceptible to predation themselves (Floyd et al., 2008; Rasmussen et al., 2013; Schulz and Dabrowski, 2001). Additionally, it has been reported that some pyrethroids and their biotransformation products act as endocrine disruptors in fish, with biotransformation products having higher endocrine activity than the parent compound (Brander et al., 2012; Nillos et al., 2010). Our results show these fish were able to biotransform permethrin as the percent parent compound was less in the fish tissue than in the tissues of the *H. azteca* they consumed. Furthermore, the *H. azteca* prey contained nearly 50% biotransformation products which could have also been assimilated into the fish. Thus, the potential toxicity of biotransformation products is of particular concern.

While at this time only a few California populations of *H. azteca* have been shown to be pyrethroid-resistant, there is the potential for this type of insecticide resistance in aquatic species to be more widespread. A number of aquatic species have developed resistance to terrestrial pesticides that were commonly used prior to the introduction of pyrethroids. For example, wild populations of daphnia, amphipods, frogs and shrimp have demonstrated resistance to organophosphate and/or organochlorine insecticides (Anguiano et al., 2008; Bendis and Relyea, 2014; Brausch and Smith, 2009; Cothran et al., 2013; Naqvi and Ferguson, 1970). This suggests there is potential for a variety of aquatic organisms to become resistant to pyrethroids as well, if chronically exposed. Furthermore, pyrethroid concentrations comparable to those at the sites where resistant *H. azteca* inhabit will likely become more common if trends in the usage of these pesticides in the United States (Hintzen et al., 2009; Ding et al., 2010; Weston et al., 2011) and in other areas of the world continue (Feo et al., 2010; Li et al., 2011; Peluso et al., 2013; Hunt et al., 2016).

5. Conclusions

In the current study, we demonstrated that resistance to pyrethroid pesticides increases the bioaccumulation potential of the resistant organisms and that this can lead to subsequent transfer of pyrethroids to predators. Implications of the observed pyrethroid resistance in *H. azteca* range from impacts on their use in

environmental assessments and laboratory studies, to affecting populations and ecosystem functioning. To further evaluate potential impacts, future studies could explore how pyrethroid resistance affects fitness in terms of fecundity or the ability to cope with additional stressors, as well as if these things cause effects at the population level. The present research may also be expanded by examining how bioaccumulation of pyrethroids via consumption of contaminated resistant organisms affects a predator's behavior, endocrine system functioning, fecundity and ultimately survival. Although resistance to environmental stressors may be necessary for survival of a population and prevention of detrimental trophic cascades (Bendis and Relyea, 2016), our study highlights the risk to higher trophic level organisms that is associated with development of resistance in a prey species.

Acknowledgements

The authors would like to thank Southern Illinois University for providing partial funding for this project through a Morris Doctoral Fellowship for S. Nutile and a fellowship for A. Derby through the Center for Undergraduate Research and Creative Activities.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.envpol.2016.09.073.

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