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Using Mutations for Pesticide Resistance to Identify the Cause of Toxicity in Environmental Samples

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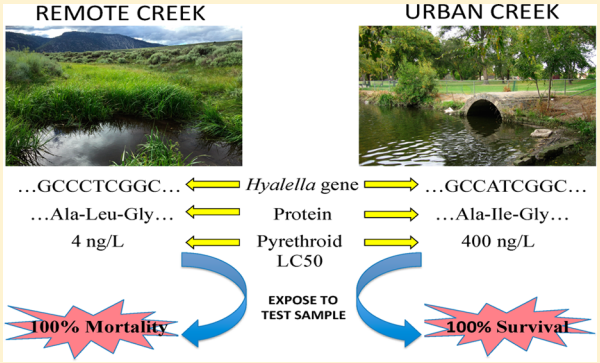
Supporting Information

ABSTRACT: Traditional Toxicity Identification Evaluations (TIE) are applied to identify causal agents in complex environmental samples showing toxicity and rely upon physical or chemical manipulation of samples. However, mutations conferring toxicant resistance provide the opportunity for a novel biologically based TIE. Populations within the *Hyalella azteca* complex from pesticide-affected waterways were 2 and 3 orders of magnitude more resistant to the pyrethroid cyfluthrin and the organophosphate chlorpyrifos, respectively, than laboratory-cultured *H. azteca* widely used for toxicity testing. Three resistant populations, as well as laboratory-cultured, nonresistant *H. azteca*, were exposed to urban and agricultural runoff. Every sample causing death or paralysis in the nonresistant individuals had no effect on pyrethroid-resistant individuals, providing strong evidence that a pyrethroid was the responsible toxicant. The lack of toxicity to chlorpyrifos-sensitive, but pyrethroid-resistant, individuals suggested chlorpyrifos was not a likely toxicant, a hypothesis supported by chemical analysis. Since these mutations that confer resistance to pesticides are highly specific, toxicity to wild-type, but not resistant animals, provides powerful evidence of causality. It may be possible to identify strains resistant to even a wider variety of toxicants, further extending the potential use of this biologically based TIE technique beyond the pyrethroid and organophosphate-resistant strains currently available.



INTRODUCTION

Environmental monitoring programs often rely on toxicity testing using laboratory-cultured organisms. When toxicity is found, mitigation typically requires identifying the chemical agent(s) responsible using a Toxicity Identification Evaluation (TIE) in which the sample is subjected to physical or chemical manipulations such as pH adjustment, aeration, C18 extraction, or ethylenediaminetetraacetic acid (EDTA) addition. Each manipulation is intended to alter the toxicity of only specific substances.[1] By noting which manipulations increase or decrease toxicity, it is sometimes possible to place the responsible toxicant within a broad class and, using additional chemical analysis and/or statistical relationships, further narrow the identification.

With no a priori reason to suspect a particular substance as the cause of toxicity, TIE manipulations necessarily target broad groups, such as metals or hydrophobic organic compounds. However, when a responsible toxicant is suspected based on historical data, it is possible to use more focused manipulations. Examples include the use of piperonyl butoxide (PBO) to identify organophosphate pesticide toxicity[2] or the use of PBO, temperature manipulation, and enzyme addition to identify pyrethroid pesticide toxicity.[3−5]

The amphipod, *Hyalella azteca* (generally recognized as a species complex) is very sensitive to pyrethroids, with 96-h LC50s to several of the compounds less than 5 ng/L.[5] Therefore, the species frequently has been used for toxicity

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testing when pyrethroids are of particular concern.[3,6,7] The recent discovery that *H. azteca* from locations chronically exposed to pyrethroids can acquire pyrethroid resistance[8,9] suggests an entirely different approach to identifying responsible toxicants—a "biological TIE." If nonresistant and pesticide-resistant *H. azteca* are concurrently exposed to a sample, and nonresistant individuals show a toxic response while pesticide-resistant individuals do not, the comparison is evidence that the cause of toxicity to the nonresistant animals is the pesticide(s) to which the other group has developed resistance.

Hyalella azteca populations from pesticide-affected waterways were characterized for their sensitivity to the pyrethroid, cyfluthrin, and the organophosphate chlorpyrifos. Pesticide-resistant populations, along with laboratory-cultured, nonresistant *H. azteca*, were exposed to stormwater runoff in the laboratory. The same populations were also used for *in situ* exposures in an area receiving pesticide runoff. Issues important to utilization of this TIE approach were also addressed, including cross-resistance to a third pesticide class and suitability of the resistant populations for laboratory culture.

MATERIALS AND METHODS

Collection of *H. azteca*. *Hyalella azteca* representing the typical laboratory strain lacking resistance to pesticides was taken from a culture maintained at the University of California Berkeley (UCB) since 2003. For one sampling event (Cache Slough, March 2016), a culture at Southern Illinois University (SIU) was used as a source of nonresistant organisms. Both groups represent the strain of *H. azteca* widely used in U.S. toxicity testing laboratories. The origins of both cultures have been traced to an initial common culture at what is now the U.S. Geological Survey laboratory in Columbia, Missouri. They have comparable sensitivity to pyrethroids, and both have been shown to fall within species C of the *H. azteca* species complex as determined by cytochrome *c* oxidase I (*COI*) sequencing (letter designations of species follow Weston et al.[8]).

Four wild populations of *H. azteca* were used for the present study: Mosher Slough, American River, Medea Creek, and Calleguas Creek. Mosher Slough in Stockton, California (38.03254, −121.36511) has been shown to contain elevated concentrations of pyrethroids in sediments and pyrethroid-resistant *H. azteca*.[8−10] The American River (38.56582, −121.38406) receives urban runoff containing pyrethroids from Sacramento, California, and water samples cause toxicity to laboratory-cultured, nonresistant *H. azteca*.[11] The resident *H. azteca* in the American River demonstrate pyrethroid resistance.[9] Finally, animals were obtained from Medea Creek, Agoura Hills, California (34.14025, −118.76027), which receives urban runoff, and Calleguas Creek in Camarillo, California (34.16480, −119.06121), which receives inputs from both urban and agricultural lands. Animals from Calleguas were not always available and were used for only one study component.

Field-collected animals were obtained with a dip-net and used to start cultures in aquaria containing Milli-Q purified deionized water made moderately hard and with the addition of bromide.[12,13] Maple leaves were used as a substratum, and the animals were fed three times weekly with Tetrafin (Spectrum Brands, Blacksburg, VA). Cultures were used for all subsequent testing, with one exception. Fresh animals were collected from Mosher Slough for *in situ* exposures within Cache Slough.

***H. azteca* Genotyping.** *Hyalella azteca* were genotyped to determine species identity and the presence of pyrethroid resistance mutations. Between 10 and 20 *H. azteca* from each population were placed in ethanol for preservation, and gDNA was extracted from individuals using the Qiagen DNeasy Blood & Tissue Kit (Qiagen, Germantown, MD), measured for nucleic acid concentration and purity with a spectrophotometer (NanoDrop 2000, Thermo Scientific, Waltham, MA), and stored at −20 °C.

Species diversity was assessed using a 670-bp fragment of the *COI* gene that has previously been used in many studies to differentiate species within the *Hyalella* complex.[14,15] The target region of the *COI* gene was PCR-amplified and sequenced as described in detail in the Supporting Information.

Hyalella azteca were genotyped at the M918 and L925 loci of the voltage gated sodium channel (*vgsc*) gene that have been previously correlated with pyrethroid resistance.[8,9] A fragment of the *vgsc* (543−578 bp depending on population) was amplified and sequenced as described in the Supporting Information, following the methods of Major et al.[9] Sequences were aligned and visualized in CLC Workbench version 7.8 to manually call M918 and L925 genotypes. Homozygotes appeared as singular peaks, while heterozygotes appeared as two approximately equal peaks at the same position.

Sample Collection and Toxicity Testing. Water for *H. azteca* exposures in the laboratory was collected from six creeks draining urban or residential areas and one combined agriculture/urban drainage channel, all within 80 km of Sacramento, California (Table S1). Locations were selected based on previous findings of *H. azteca* toxicity after storm events.[8,11,16,17] Water samples were collected December 2015 and January 2016 after heavy rains when waters were turbid and flow elevated. Samples were collected from the bank, just below the water's surface, avoiding areas in close proximity to outfalls (e.g., storm drain inputs) so as to provide opportunity for mixing and ensure cross-sectional uniformity of the creek channel. Glass bottles certified clean for pesticide analysis were used. Samples were kept at 4 °C and used within 24 h for toxicity testing or 48 h for pesticide extraction.

Toxicity of creek and drain waters was determined using the UCB culture and cultures derived from the Mosher, American, and Medea wild populations. Individuals used passed through a 600-μm screen but were retained on a 500-μm screen (1.8−2.5 mm in length, corresponding to approximately 7−14 d in age).[18] Tests were performed with five replicates per site, 10 individuals per replicate, in beakers with 80 mL of test water and a 1 cm^2 piece of nylon screen for substratum. After 48 h, 1 mL of yeast-cerophyll-trout food was added, and after allowing a feeding period of 4 h, approximately 80% of the water was replaced with fresh water from samples stored at 4 °C. Tests were performed at 23 °C under a 16:8 light/dark cycle. After 96 h, tests were scored for both the total number of survivors and those survivors that were able to perform coordinated swimming. As the pesticides of interest are neurotoxins, some *H. azteca* were not killed but were paralyzed or unable to move in a controlled manner. All tests were accompanied by a laboratory control with prepared moderately hard water noted above.

Tests to derive EC50 and LC50 estimates were performed for cyfluthrin, chlorpyrifos (both ChemService, West Chester, PA), and p,p-DDT (Sigma, St. Louis, MO). Tests were performed similarly to testing of field samples, except a dilution series was done with concentration steps of 2×, with three

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Table 1. *H. azteca* Populations Used in the Study, Including Their Species Identity and Genotype at Two Loci of the Voltage Gated Sodium Channel Known to Be Associated with Mutations Conferring Pyrethroid Resistance

H. azteca population	species	M918 locus	L925 locus
UCB	C	wild-type (n = 20)	wild-type (n = 20)
SIU	C	wild-type (n = 10)	wild-type (n = 10)
American River	primarily D, B possible[a]	wild-type (n = 30)	L925I (n = 30)
Mosher Slough	primarily B, D possible[a]	wild-type (n = 20)	L925I (n = 20)
Medea Creek	D	wild-type (n = 10)	L925I (n = 10)
Calleguas Creek	D	wild-type (n = 10)	L925I (n = 10)

[a]Testing of the American River animals in culture found only species D, but species B is known from the wild population. Since species B and D co-occur at other sites in the same watershed, we believe both were initially present at the time of collection to initiate the culture, and that species D came to dominate the culture over time. However, the presence of a small number of remaining species B individuals cannot be ruled out. Similarly, the Mosher Slough animals tested were species B, but the site is known to contain species D as well, and they could have been present in small numbers.

Table 2. Cyfluthrin and Chlorpyrifos 96-h LC50 Estimates and 95% Confidence Intervals for Each *H. azteca* Population Tested[a]

	cyfluthrin		chlorpyrifos	
H. azteca population	LC50 (ng/L)	date (source)	LC50 (ng/L)	date (source)
UCB	2.3 (0.9−3.8)[b]	Sept. 2008 (L)	135 (115−158)[e]	June 2009 (L)
	1.7 (1.1−2.3)[b]	Sept. 2008 (L)	172 (128−231)	Oct. 2014 (L)
	4.8 (3.9−6.2)[c]	Sept. 2010 (L)		
	2.9 (2.3−3.9)[c]	Sept. 2010 (L)		
	4.3 (3.4−5.5)[d]	Apr. 2015 (L)		
	4.9 (4.0−5.9)[d]	Apr. 2015 (L)		
	4.9 (3.9−6.0)[d]	Feb. 2016 (L)		
	4.4 (3.5−5.5)[d]	Feb. 2016 (L)		
SIU	1.3 (1.0−1.5)[c]	Oct. 2010 (L)	no data	
	2.6 (2.3−3.0)[c]	Oct. 2010 (L)		
American River	52(42−65)[d]	Nov. 2014 (W)	132 (103−169)	Oct. 2014 (W)
	379 (329−437)	Sept. 2015 (L, 7−9)	190 (161−225)	Oct. 2014 (W)
	101(81−126)	Apr. 2016 (L, 14−16)	132 (111−156)	Sept. 2015 (L, 7−9)
Mosher Slough	193 (158−226)[c]	July 2010 (W)	542 (442−665)	Oct. 2014 (W)
	211 (176−244)[c]	July 2010 (W)	551 (413−735)	Sept. 2015 (L, 2)
	99 (80−123)[d]	July 2015 (W)		
	158 (128−194)	Sept. 2015 (L, 2)		
	268(224−319)	Apr. 2016 (L, 9)		
Medea Creek	629 (504−785)[d]	June 2015 (W)	676 (403−1130)	July 2015 (W)
	474 (423−532)[d]	July 2015 (W)		
	310(243−395)	Apr. 2016 (L, 9)		
Calleguas Creek	456 (364−569)[d]	June 2015 (W)	156 000 (101 000−242 000)[f]	July 2015 (W)

[a]Multiple tests are shown when available, including the date tested and whether the test animals were from a wild collection (W) or laboratory culture (L). If the laboratory culture was originally derived from a field-collected population, the number of months in culture is shown, with a range given if multiple field collections had been made (e.g., L, 7−9). [b]Data from ref 5. [c]Data from ref 8. [d]Data from ref 9. [e]Data from ref 4. [f]Though a definitive LC50 value was provided by only a single test, a preliminary range-finding test up to 13 000 ng/L showed no effect.

replicates per step. The pesticide in an acetone carrier was spiked into the moderately hard water. The acetone concentration was <40 μL/L, a concentration found to have no effect in solvent controls.

In situ toxicity testing was done in Cache Slough, in the Sacramento−San Joaquin Delta of California. Cache Slough is a large water body compared to the creek sites, with a 100−300 m width and a length of 18 km. The Upper Cache site was at the western end where Ulatis Creek, a major source for runoff containing pyrethroids and occasionally chlorpyrifos, discharges to it.[16] The Middle and Lower Cache sites were approximately 4 and 8 km farther seaward, respectively. A control site was located 2 km outside of Cache Slough where pesticide exposure has been low in past monitoring (Figure S1).[16]

Nonresistant laboratory cultures (UCB January event, SIU March event) and Mosher and American resistant populations were deployed *in situ* in Cache Slough. Resistant American River animals were taken from the same laboratory cultures used for the creek testing, but fresh collections of wild individuals were made from Mosher Slough. Animals were temperature acclimated prior to deployment (10 °C in January, 14 °C in March). The amphipods were held in 150 mL polyethylene containers with 5 cm openings on the top and bottom, screened with 500-μm nylon mesh. To prevent escape, larger animals (individuals passing through a 1000 μm screen, but retained on a 600 μm screen) were chosen for field deployment. The cages were placed at three Cache Slough sites (five replicates per site) the day before rain began, suspended approximately 1−2 m below the surface and 2 m above the bottom. They were left for 4−5 days throughout the rain and subsequent runoff, and then retrieved and scored for death and paralysis. Water samples for chemical analysis were collected

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daily at each site. Rainfall accumulation during each deployment was 6−9 cm.

Statistical analysis of toxicity tests was done using CETIS (Tidepool Scientific Software, McKinleyville, CA). A comparison of effects between sites was done by *t* test if parametric assumptions were met, or by Wilcoxon Rank Sum if not. EC50 and LC50 estimates were derived by the Spearman-Karber method. When doing a dilution series, water from one concentration in the midpoint of the range, which was generally close to the LC50, was set aside at the beginning of the test and composited with a sample of the same nominal concentration at the 48-h water replacement. After pesticide analysis of this sample, the deviation between nominal and actual values was used to adjust the reported EC50 and LC50 for that test. Actual concentrations were a median 80% of nominal for cyfluthrin (range 42−110%), 76% for chlorpyrifos (range 69−119%), and 79% for DDT (range 77−81%).

Analytical Chemistry. Full details of the analytical methods are provided in the Supporting Information. Briefly, for all water samples other than Cache Slough, the sample was liquid/liquid extracted with dichloromethane, cleaned on a solid phase extraction cartridge, and analyzed by gas chromatography−mass spectroscopy (GC-MS).[19] Analytes included chlorpyrifos and eight pyrethroids, and all had reporting limits of 3 ng/L.

In Cache Slough samples, the dissolved and particulate phases were separately quantified and then summed. The particulate phase was recovered on a 0.45 μm GF/F filter, and the dissolved phase was recovered from the filtrate on an Oasis HLB cartridge (Waters, Milford, MA). Analysis was done by GC-MS using a high-resolution time of-flight (QTOF) mass spectrometer.[20] Twelve pyrethroids as well as chlorpyrifos were quantified, with reporting limits of 0.1−1 ng/L.

RESULTS AND DISCUSSION

Characterization of the Various *H. azteca* Populations. Both the UCB and SIU laboratory cultured *H. azteca* populations were species C and were wild-type at the 918 and 925 loci of the *vgsc*, previously reported to be associated with pyrethroid resistance mutations in *H. azteca*[8,9] (Table 1). The four populations derived from field collections (American, Mosher, Medea, Calleguas) all had a mutation at the 925 locus of the *vgsc* that resulted in an isoleucine substitution for the wild-type leucine. Though mutation at the 918 locus has been reported in other pyrethroid-resistant populations, it was not present in any of the populations used for the present study. These four populations all were either species B or D.

Sensitivity to pyrethroids was evaluated for all populations, since such information is critical for the TIE tests as envisioned and the degree of resistance cannot be predicted by species identity.[9] Standard laboratory-reared *H. azteca* with no prior pesticide exposure, as represented by both UCB and SIU cultures, were extremely pyrethroid sensitive, with cyfluthrin 96-h LC50 values of 1−5 ng/L (Table 2). All four of the wild populations from pyrethroid-affected waterways were cyfluthrin resistant to varying degrees, with LC50s ranging from 52 to 629 ng/L.

Multiple cyfluthrin LC50 estimates for any given population were generally consistent, and approximately within a factor of 2, even over a period of five years or more (UCB and Mosher Slough; Table 2). However, the American River population was a notable exception. While animals from the site were always pyrethroid resistant to some extent, LC50 estimates were erratic. An LC50 of 52 ng/L was first measured by collection of wild animals in November 2014. Collections 1−3 months later were used to start a laboratory culture. When tested in September 2015, after 7−9 months in culture, juveniles had an LC50 of 379 ng/L. After an additional 7 months, LC50 had decreased to 101 ng/L. These differences were likely attributable to the wild population consisting of largely homozygous L925I mutants, but with lesser numbers of wild-type and heterozygous individuals.[9] LC50 results may have varied depending on the relative abundance of these groups in the test at any given time.

The LC50s for chlorpyrifos were in the range of 100−200 ng/L for nonresistant *H. azteca*, typified by the UCB culture. Though American River animals showed pyrethroid resistance, they had no apparent resistance to chlorpyrifos. Mosher Slough and Medea Creek animals had moderate chlorpyrifos resistance, with LC50s at approximately 600 ng/L. Calleguas Creek animals were extraordinarily resistant. Their LC50 was 156 000 ng/L, approximately 1000-fold greater than nonresistant *H. azteca*. Though we have documented the mutations that confer pyrethroid resistance,[8,9] the mechanism for chlorpyrifos resistance at Calleguas Creek remains under investigation.

Toxicity of Field Samples. Our biological TIE approach utilizes the resistance profiles of laboratory versus multiple wild resistant populations to identify the substance causing toxicity. As shown in Figure 1, if these populations are simultaneously exposed to an environmental sample, and pyrethroids or organophosphates are responsible, the toxicant can be identified (i.e., sample 2 or 3). If all populations succumb to toxicity, it would suggest that a different substance is the cause (i.e., sample 4). This result could also arise if pyrethroid concentrations exceeded even the tolerance of the resistant populations, but this would be unusual given the concentrations observed in past monitoring in the region.[11,16,17]

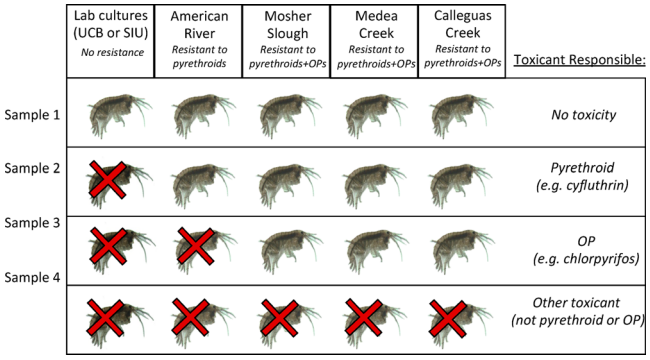

Figure 1. Conceptual diagram of the biological TIE approach. Mosher, Medea, and Calleguas animals were resistant to both pyrethroids and organophosphates (OPs; specifically chlorpyrifos). American animals were resistant to pyrethroids but not OPs; UCB and SIU animals were resistant to neither. In sample 1, there is survival in all populations including the wild-type lab populations, indicating no toxicity in the sample. In sample 2, toxicity only occurs in the UCB and SIU populations (shown by the red crosses) with no effect on any of the pyrethroid-resistant populations, suggesting that pyrethroids were responsible for toxicity. In sample 3, toxicity is seen to UCB or SIU, and the American River population, but none of the others, suggesting OPs as the cause. In sample 4, toxicity to the UCB or SIU animals as well as any of the Mosher, Medea, or Calleguas populations would suggest a toxicant that is neither a pyrethroid nor an OP. *H. azteca* image credit: Helen Poynton, CC-BY.

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Environ. Sci. Technol. 2018, 52, 859−867

For field samples tested in the laboratory, controls consisted of exposure to prepared moderately hard water. For *in situ* deployments in Cache Slough, control deployments were made just outside of the Slough at a site minimally affected by runoff where chlorpyrifos never exceeded 1 ng/L and no pyrethroid exceeded 0.2 ng/L during the present study. Control performance in the laboratory exposures, quantified as the percent of individuals alive and capable of swimming, ranged from 94 to 98% (UCB culture), 84−86% (American), 68−78% (Medea), and 92% (Mosher). Control performance for the *in situ* Cache Slough exposures were 96% (UCB culture), 78% (SIU culture), 78−80% (American), and 94−96% (Mosher; Table S2).

Widespread toxicity was seen among the laboratory-cultured, nonresistant UCB or SIU organisms (Figure 2, Table S2). Six

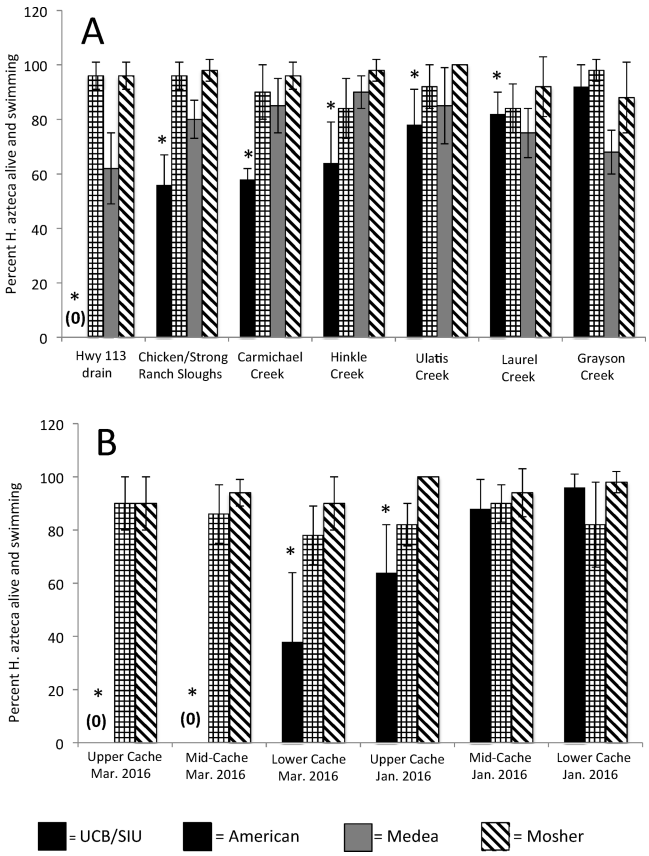

Figure 2. Results of toxicity tests when 3−4 *H. azteca* populations (shown in legend) were simultaneously exposed to water from (A) creeks in laboratory testing or (B) three locations in Cache Slough during two rain events by *in situ* deployments. Asterisks indicate statistically significant toxicity (*t* test) relative to that same population's response in controls. Numerical data in Table S2.

of the seven creek or drain samples tested in the laboratory (all except Grayson) were toxic based on their inability to swim normally, and three were also toxic based on mortality alone. Among the six *in situ* Cache Slough exposures, four caused paralysis or death to UCB or SIU animals. In January, the inability to swim was seen only at the upstream site, near where Ulatis Creek brings urban and agricultural runoff to the slough. The March storm caused toxicity at all three Cache Slough sites.

Yet in every case when toxicity to nonresistant UCB or SIU animals occurred, the pyrethroid-resistant organisms from American River, Medea Creek, and Mosher Slough were all unaffected (comparable to Figure 1, sample 2 example). None of these populations showed death or impairment of swimming in any sample that was significantly greater than in their respective control samples. American and Mosher populations showed 84−100% of the test individuals unaffected at all sites. The Medea population tended to do the poorest, with 64−93% unaffected, but as the control performance for this population was the lowest, none of the test sites had significantly greater effects.

The difference in toxic effects among the populations was most stark at the Highway 113 drain and at the Upper and Mid-Cache sites in March. At all three sites there was total or near-total mortality of the UCB or SIU animals. When exposed to Highway 113 drain water, all UCB animals quickly became immobile within minutes. Yet all of the resistant populations were unaffected at these three sites even after a four-day exposure.

The absence of toxicity in multiple populations known to be pyrethroid resistant, but paralysis or death in those lacking such resistance, provides strong evidence that pyrethroids were the cause of toxicity to the nonresistant animals. These results provide convincing support for the "biological TIE" approach even in the absence of analytical chemistry, and when coupled with analytical data, the case becomes even stronger. Of the 10 samples with toxicity, eight contained one or more pyrethroids exceeding the reported 96-h EC50 or LC50 for standard laboratory-cultured *H. azteca* (Table 3). In the remaining toxic samples, both had pyrethroids nearing their published EC50 (cyhalothrin at 73% of its EC50 at Mid-Cache in March; cypermethrin at 88% of its EC50 at Lower Cache in March). Bifenthrin was the pyrethroid most frequently found at toxic concentrations in the urban creeks, but high cyhalothrin and/or cyfluthrin concentrations were responsible for toxicity at the Highway 113 drain and Cache Slough that are both agriculture-affected. Deltamethrin was in relatively high concentrations in Carmichael Creek, but its role in toxicity cannot be assessed, since no *H. azteca* LC50 data are available for this compound.

Several of these sites have been tested by more traditional chemical TIE methods in the past, specifically by the addition of PBO, a pyrethroid synergist. *Hyalella azteca* toxicity due to pyrethroids was suggested by PBO addition at Laurel Creek, Ulatis Creek, and Upper and Mid-Cache Slough sites.[4,16,17] Our present results using the biological TIE are highly consistent with those findings.

Of the three sites that showed no toxicity even to UCB/SIU animals, no pyrethroid-related toxicity would have been expected at two of them given the concentrations (i.e., below reported EC50s or LC50s). Only the Grayson Creek sample contained a pyrethroid in sufficient concentration to expect toxicity, but no effects were observed, possibly due to high suspended solids and organic matter likely associated with the runoff, though quantitative data on these parameters is lacking for this sample.

Had chlorpyrifos been responsible for toxicity in any of the samples, toxicity to UCB or SIU animals and the chlorpyrifos-sensitive American population would have occurred, but chlorpyrifos-resistant populations (Medea and Mosher) would have been unaffected (Figure 1, sample 3). That pattern was never observed, and the analytical data showed the maximum chlorpyrifos concentration at only 5% of its reported 96-h EC50 of 96 ng/L.[4] Most chlorpyrifos-containing urban use products in the U.S. were withdrawn approximately a decade

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Table 3. Pyrethroid, Chlorpyrifos, and Suspended Solids Concentrations Measured in the Water at All Sites, with Bold Values Indicating Concentrations Exceeding Reported 96-h EC50 or LC50 Values Provided in Footnote

sampling location	toxicity results[a]	pyrethroid conc. (ng/L)[b]	chlorpyrifos conc. (ng/L)[b]	suspended solids (mg/L)
creek samples tested in the laboratory				
highway 113 agricultural drain	only UCB affected	**14.9(ch)**	4.9	427
Chicken/Strong Ranch Sloughs	only UCB affected	**18.5(b)**, 4.3(d)	4.9	186
Carmichael Creek	only UCB affected	**26.5(b)**, 27.3(d)	<3	423
Hinkle Creek	only UCB affected	**4.1(b)**	<3	32
Ulatis Creek	only UCB affected	**5.2(b)**	<3	102
Laurel Creek	only UCB affected	**3.9(b)**	<3	21
Grayson Creek	no toxicity	**9.9(b)**, 3.3(d)	<3	no data[c]
in situ Cache Slough testing[d]				
Upper Cache Slough: Mar. 2016	only SIU affected	0.9(b), 1.2(cf), **4.4(ch)**	0.7	427
Mid-Cache Slough: Mar. 2016	only SIU affected	1.1(b), 1.7(ch)	0.7	155
Lower Cache Slough: Mar. 2016	only SIU affected	1.5(cp)	1.0	25
Upper Cache Slough: Jan. 2016	only UCB affected	3.2(b), 0.4(ch), **9.1(cf)**	2.0	72
Mid-Cache Slough: Jan. 2016	no toxicity	0.7(b), 0.1(ch), 1.1(cp)	1.0	10
Lower Cache Slough: Jan. 2016	no toxicity	0.1(ch), 3.0(p)	0.7	11

[a]UCB amphipods used in all cases except SIU during Cache Slough March event. Quantitative toxicity results were presented in Figure 1 and Table S2 but are summarized here to facilitate comparison with chemistry data. [b]For comparison with the reported concentrations, published 96-h point estimates are as follows: Bifenthrin (b) EC50 = 3.3 ng/L, cyfluthrin (cf) EC50 = 1.9 ng/L, and cypermethrin (cp) EC50 = 1.7 ng/L (median values from Weston and Jackson[5]); lambda-cyhalothrin (ch) EC50 = 2.3 ng/L;[37] permethrin (p) LC50 = 21.1 ng/L;[38] chlorpyrifos EC50 = 96 ng/L.[4] No data available for EC50 or LC50 of deltamethrin (d). [c]Water was highly turbid, but no quantitative suspended solids data available. [d]Cache Slough analyses were done daily throughout 4−5 days of amphipod deployment, and the highest value found is shown.

Table 4. Comparison of DDT 96-h EC50s and LC50s and Their 95% Confidence Intervals Determined for Four *H. azteca* Populations, with Mosher Slough Tested on Two Occasions

source of *H. azteca*	control (% alive and swimming)	96-h EC50	96-h LC50
UCB	98	898(744−1084)	1833(480−6994)
SIU	94	912(793−1050)	1609(1298−1995)
Mosher Slough	75	208(174−249)	228(188−276)
	85	700(596−823)	914(760−1100)
Calleguas Creek	82	673(535−847)	984(773−1252)

ago, and the compound is rarely found at toxicologically meaningful concentrations in urban runoff. However, the agriculture-affected Highway 113 drain has contained up to 453 ng/L chlorpyrifos in the past,[16] but it contained <5 ng/L in the present study.

Laboratory Rearing of Resistant Populations. Routine collection of resistant individuals from field sites is unlikely to be feasible or desirable for most laboratories. Therefore, use of resistant animals for TIE application will require long-term maintenance of cultures. We have maintained wild-collected American River, Mosher Slough, and Medea Creek *H. azteca* in culture for approximately 2−3 years (21−34 months). Culturing procedures for the resistant populations have been identical to those for the laboratory-cultured *H. azteca* widely used for toxicity testing.[18]

Consistent with a genetic mechanism of resistance, our data indicate no loss of pyrethroid resistance during laboratory culture in pesticide-free conditions extending over multiple generations (given a *H. azteca* generation time of approximately 34 days[21]). After 9 months in the laboratory, juveniles taken from a Mosher Slough culture had a cyfluthrin LC50 comparable to the original wild population (268 ng/L compared to 99−211 ng/L for wild population; Table 2). Similarly, juveniles from a 9-month old Medea Creek culture had a cyfluthrin LC50 only 35% less than the original wild population used to start the culture (Table 2). American River animals in culture for 14−16 months had an LC50 twice that of the wild population, with possible reasons for the apparent increase discussed above. Maintenance of resistance over time is consistent with an earlier study that reported approximately 50% decline in pyrethroid resistance between the parent and F1 generation while in culture, but at least 40-fold greater resistance than the wild type still persisting even after nearly two years.[10]

Chlorpyrifos resistance also showed no decline in culture, though testing did not continue for as long as that for cyfluthrin. Juveniles harvested from a Mosher Slough culture initiated with animals collected 2 months earlier had a chlorpyrifos LC50 virtually identical to that of the wild population (551 ng/L versus 542 ng/L; Table 2).

Specificity of Resistance Mutations. Traditional physical/chemical TIE manipulations have a specific chemical or chemical group they are designed to target but can have unintended influence on the toxicity of nontarget chemicals. For example, a reduction in toxicity upon zeolite addition is generally taken as indicative of ammonia-related toxicity, but zeolite may also unintentionally remove trace metal toxicity.[22] The proposed biological TIE approach could also lack specificity if the mutation induced by exposure to a particular pesticide confers resistance to another. Such cross-resistance is most commonly found among pesticides having the same mode of action.

Because DDT and pyrethroids share the same target site, mutations in the *vgsc* that confer pyrethroid resistance often affect sensitivity to DDT. This possibility was explored by testing the sensitivity to DDT of the UCB and SIU amphipods

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Environ. Sci. Technol. 2018, 52, 859−867

and pyrethroid-resistant populations from Mosher Slough and Calleguas Creek (Table 4). The resistant populations consistently had lower EC50s and LC50s (i.e., greater sensitivity) to DDT than the UCB and SIU populations, though generally the differences were not significant as defined by overlapping 95% confidence intervals. In only one Mosher Slough test were the EC50 and LC50 values statistically lower than the UCB and SIU cultures, and in that test the control performance was atypically low. If pyrethroid resistance is associated with greater sensitivity to DDT, the effect is slight, but the data certainly provide no indication that pyrethroid resistance also lessens DDT sensitivity.

DDT cross-resistance does not appear to be a significant TIE issue, but this finding has another important implication. DDT use began in the 1940s and ceased in the U.S. in the early 1970s. Pyrethroids were first developed in the 1960s, but many of the more potent current-use compounds came into broad use in the 1980s. Since pyrethroid resistance in *H. azteca* is associated with mutations in the *vgsc*, and DDT also targets the sodium channel, the question arises whether the pyrethroid resistance mutation seen in *H. azteca* arose due to pyrethroid exposure, or if it arose much earlier as a result of DDT exposure and only fortuitously provided resistance to pyrethroids. Since pyrethroid-resistant *H. azteca* of both Mosher Slough and Calleguas Creek lack DDT resistance, our finding suggests adaptation has specifically been in response to pyrethroid exposure.

Future Applications. In the many urban and agricultural water bodies sampled, toxicity to nonresistant *H. azteca* coupled with unaffected pyrethroid-resistant animals provided strong evidence that pyrethroids were responsible for the toxicity. This result is consistent with past work in the region that implicated pyrethroids based on either a toxic unit approach or conventional TIE methods.[4,16,17] The biological TIE approach is intuitively an attractive and powerful technique to establish causality, and when coupled with the other supporting approaches, the evidence implicating pyrethroids becomes overwhelming.

H. azteca is a cryptic species complex, comprised of dozens of distinct provisional species distinguishable by *COI* sequencing.[23−25] In the present study, the nonresistant UCB and SIU animals represented species C, whereas resistant wild populations represented species B and/or D. Various *H. azteca* species have been found to have approximately 2-fold differences in sensitivity to copper, nickel, chloride, and nitrate[26,27] and approximately 6-fold differences in selenium sensitivity.[28] These differences are small in comparison to the 2 order-of-magnitude differences in pyrethroid resistance or the 3 order-of-magnitude difference in chlorpyrifos resistance among populations in the present study. Nevertheless, even these small differences in sensitivity to other contaminants could confound interpretation of results from a biological TIE in some circumstances.

The proposed approach could be improved by comparing resistant and nonresistant populations within the same species group to ensure that differences in toxicity are a consequence of the mutation conferring resistance. We recently located both nonresistant and resistant populations of all species groups we used (B, C, D). Pyrethroid-resistant individuals of species B are found in Mosher Slough and have the L925I mutation. Wild-type, nonresistant animals of species B are found in California localities with minimal pyrethroid exposure including Blodgett Reservoir and Russian River.[8,9] Species C is the widely used laboratory strain of *H. azteca* and was not resistant in the present study, but wild populations of species C found near the California−Mexico border contain the L925I mutation and are resistant to cyfluthrin.[9] Pyrethroid-resistant individuals of species D were collected from several sites in the present study. There is a nonresistant species D population in the Mojave Desert.[9] Species D from the Mojave is also not resistant to chlorpyrifos (unpub. data), providing a possible comparison to the extreme chlorpyrifos resistance shown by species D from Calleguas Creek.

In the case of *H. azteca*, the genetic difference between a nonresistant population that experiences toxicity and a pyrethroid-resistant population that does not can be as small as a single DNA base substitution.[8] This feature makes comparing toxicity in different populations a powerful tool for identifying the causative agent. Pyrethroid resistance mutations may carry a "cost" to resistant individuals that increase their susceptibility to other stressors, so even the use of intraspecies comparisons of resistant and nonresistant organisms does not ensure absolute toxicant specificity, but differences in susceptibility to other toxicants are likely to be less than those for interspecies comparisons.

Chemical data can often be helpful in identifying likely toxicants, and the present study showed good agreement between those samples in which toxicity would have been expected based on pyrethroid concentrations alone and those in which toxicity was attributed to pyrethroids based on the responses of the differentially sensitive strains. However, bioavailability issues can confound such inferences at times, as was seen in the Grayson Creek sample, making TIE data important in establishing causality. TIE data are particularly necessary for pyrethroids because the analytical limit of quantitation (approximately 1 ng/L in many laboratories) is nearly the same as the concentration at which paralysis or death occur in *H. azteca*. This issue with analytical limits means that pyrethroid-induced toxicity may be observed in field samples that contain no detectable pyrethroids. In such samples, pyrethroids can only be implicated as the source of toxicity by TIE methods.[16,29] The biological TIE technique provides such an approach, and one that is quite distinct from the physical/chemical TIE methods traditionally employed.

Once resistant populations are established in culture in the laboratory, they can routinely be used as needed for TIE purposes simply by testing them concurrently with the nonresistant *H. azteca*. While the present study found no evidence for loss of resistance over time, occasional confirmation of resistance of the animals in culture would be prudent, either by periodic determination of LC50 and/or gene sequencing to confirm the underlying mutation. The greatest obstacle to broader adoption of the approach among testing laboratories may be obtaining the resistant animals to culture, but this may not be as difficult as it may seem. In our work to date, we have identified 11 water bodies in California from which pyrethroid-resistant *H. azteca* can be obtained.[8,9] There are undoubtedly countless more, since we have found resistance throughout most of California, wherever there is intensive agriculture or major population centers. Although testing for pyrethroid resistance has not been done outside of California, it may be equally common elsewhere, as pyrethroids and associated toxicity to aquatic life have been found in many other states and countries.[30−33] Pyrethroid contamination of aquatic systems is a global concern, and the presence of resistant populations likely is as well.

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The present study shows the biological TIE approach with resistant *H. azteca* to be effective in identifying pyrethroid toxicity. Toxicity due to chlorpyrifos, and presumably other organophosphates, is likely to be equally identifiable using the resistance profiles of the groups in the present study. The samples we collected lacked toxicologically meaningful concentrations of chlorpyrifos, and thus the TIE approach was only able to show the compound was not responsible for observed effects. We have shown chlorpyrifos resistant *H. azteca* to be available, but further validation is necessary to verify the same success for chlorpyrifos as shown for pyrethroids. Pyrethroids and chlorpyrifos are responsible for much of the *H. azteca* toxicity that has been observed in California,[34,35] and pyrethroids appear to be responsible for much of the toxicity to the species across the U.S.[32,36] Thus, the biological TIE approach is quite useful even with the limited suite of contaminants to which it is currently applicable. If populations resistant to additional toxicants are found, even greater potential in identifying the causal factors underlying toxicity appears possible.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.7b05071.

Hyalella azteca genotyping methods, analytical chemistry methods, sampling site information, toxicity testing results, and a map of Cache Slough (PDF)

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Notes

The authors declare no competing financial interest.

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