SETAC PRESS

Environmental Toxicology and Chemistry, Vol. 23, No. 11, pp. 2699–2708, 2004

Printed in the USA
0730-7268/04 $12.00 + .00

DEVELOPMENT OF TOXICITY IDENTIFICATION EVALUATION PROCEDURES FOR PYRETHROID DETECTION USING ESTERASE ACTIVITY

CRAIG E. WHEELOCK,† JEFF L. MILLER,‡ MIKE J. MILLER,‡ SHIRLEY J. GEE,† GUOMIN SHAN,† and BRUCE D. HAMMOCK,*†
†Department of Entomology and Cancer Research Center, University of California at Davis, Davis, California 95616, USA
‡AQUA-Science, Davis, California 95616, USA

(*Received* 7 *October* 2003; *Accepted* 25 *March* 2004)

Abstract—Recent agrochemical usage patterns suggest that the use of organophosphate (OP) pesticides will decrease, resulting in a concomitant increase in pyrethroid usage. Pyrethroids are known for their potential toxicity to aquatic invertebrates and many fish species. Current toxicity identification evaluation (TIE) techniques are able to detect OPs, but have not been optimized for pyrethroids. Organophosphate identification methods depend upon the use of piperonyl butoxide (PBO) to identify OP-induced toxicity. However, the use of PBO in TIE assays will be confounded by the co-occurrence of OPs and pyrethroids in receiving waters. It is necessary, therefore, to develop new TIE procedures for pyrethroids. This study evaluated the use of a pyrethroid-specific antibody, PBO, and carboxylesterase activity to identify pyrethroid toxicity in aquatic toxicity testing with *Ceriodaphnia dubia*. The antibody caused significant mortality to the *C. dubia*. Piperonyl butoxide synergized pyrethroid-associated toxicity, but this effect may be difficult to interpret in the presence of OPs and pyrethroids. Carboxylesterase activity removed pyrethroid-associated toxicity in a dose-dependent manner and did not compromise OP toxicity, suggesting that carboxylesterase treatment will not interfere with TIE OP detection methods. These results indicate that the addition of carboxylesterase to TIE procedures can be used to detect pyrethroids in aquatic samples.

Keywords—Esterase Toxicity identification evaluation *Ceriodaphnia dubia* Pyrethroid Organophosphate

INTRODUCTION

In recent years, increased emphasis has been placed on the use of routine toxicity tests using aquatic organisms to measure water quality [1–5]. Methods have been developed for determining the impacts of discharges of municipal [1] and industrial effluent [6], agricultural runoff [4,5,7] and storm-water discharges [8,9] on receiving water quality. Concomitantly, increased attention has focused on methods for identifying the chemical(s) that are responsible for the toxicity so that appropriate control measures can be taken. The U.S. Environmental Protection Agency (U.S. EPA) has published a series of toxicity identification evaluation (TIE) methods that can be used to identify the causes of toxicity in aqueous samples using chemical characterization, identification, and confirmation procedures [10–12].

The TIE methods have been applied broadly to identify the causes of toxicity in multiple aquatic matrices as well as sediment. Toxicity identification evaluation testing routinely has identified organophosphate (OP) insecticides, including diazinon and chlorpyrifos, as causes of toxicity in municipal effluents and surface waters in northern California, USA [3–5]. However, the use of these OP insecticides is decreasing in California, with a subsequent observed increase in pyrethroid usage [13]. Pyrethroids are difficult to identify using standard TIE methods because of their physical–chemical properties [14,15] and the lack of sensitive and selective analytical methods that detect these insecticides at biologically relevant concentrations [16,17].

New TIE techniques are needed to identify pyrethroid-caused toxicity. To achieve widespread applicability in TIEs, the procedures must be rapid, relatively inexpensive, and not require a high level of expertise or expensive equipment. We have examined a number of methods for their ability to remove, reduce, or detect pyrethroid-associated toxicity. This work investigated the use of esterase activity, a pyrethroid-selective antibody, or piperonyl butoxide (PBO) as possible treatments. Carboxylesterase is an enzyme that rapidly degrades both type I and type II pyrethroids (Fig. 1). This class of enzymes has been demonstrated to be effective in reducing pyrethroid-associated toxicity in both mammals and insects [18] and is a sensible target for removing pyrethroid toxicity in TIE samples. Previous work has identified this enzyme as a good target for identifying pyrethroid-associated toxicity in aquatic samples [19]. We determined previously that antibodies selective for diazinon and chlorpyrifos could be used to identify toxicity in surface waters caused by these OP insecticides [20]. Therefore, we evaluated these procedures using antibodies developed by our group that bind both type I and type II pyrethroids [21,22]. To our knowledge the use of antibody or enzymatic procedures to identify and confirm toxicity due to pyrethroids has not yet been reported.

Certain compounds (e.g., OPs) must be activated metabolically by the test organism before they can exert their toxic effect. Many of these activation reactions consist of oxidative metabolism by a group of enzymes collectively known as mixed-function oxidases (MFOs), of which the heme-protein cytochrome P450s are a subset [23]. Compounds such as PBO, a synthetic methylenedioxy phenyl derivative, bind to and block the catalytic activity of some MFOs, preventing the toxicity of metabolically activated OP insecticides including diazinon, chlorpyrifos, malathion, parathion, and fenthion

* To whom correspondence may be addressed (bdhammock@ucdavis.edu).

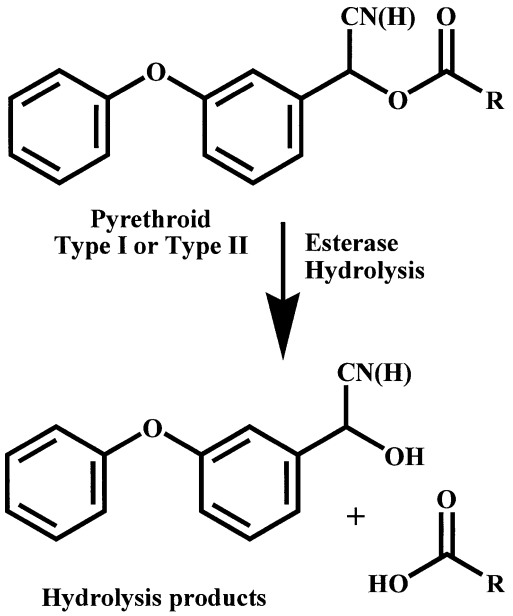


Fig. 1. Both type I and type II pyrethroids are hydrolyzed by esterases to the corresponding alcohol and acid. The benzylic carbon of the ester moiety in type II pyrethroids has a cyano group attached (forming a secondary ester), whereas the type I pyrethroids contain a hydrogen atom in this position (forming a primary ester). Cyanohydrins quickly rearrange to the corresponding aldehyde at basic pH.

[24]. Thus, when a nontoxic level of PBO is added to test samples containing one or more of these OPs, the toxicity is greatly reduced or completely blocked. The use of PBO to identify toxicity caused by metabolically activated OP insecticides has been described previously [25] and incorporated in published U.S. EPA TIE manuals [10,11]. Conversely, PBO synergizes the toxicity of pyrethroid insecticides by blocking MFO-mediated metabolism of these chemicals [26]. Thus, PBO addition to samples containing one or more pyrethroids increases and/or prolongs the toxic effect [27]. This dual action of PBO could lead to a confounding signal in TIE testing of samples that contain both OPs and pyrethroids. Alternatively, the action of PBO may be a useful tool to identify the presence/absence of pyrethroids and metabolically activated OPs in aqueous samples.

The pyrethroids permethrin, bifenthrin, cypermethrin, cyfluthrin, and esfenvalerate were identified as target compounds of interest based on quantities applied in California (http://www.cdpr.ca.gov/) and variation in structure (Table 1). In addition, λ-cyhalothrin was identified as a pyrethroid of interest in anticipation of its increased usage in controlling mosquitoes carrying West Nile virus [28]. Using these pyrethroids, we have taken three approaches to develop a method for identifying pyrethroid-caused toxicity. In the first approach, an antibody that selectively binds pyrethroids was examined for its ability to decrease toxicity. The second approach employed PBO as a known pyrethroid synergist in an effort to identify individual pyrethroids based upon the level of observed synergism. Last, a commercial preparation of carboxylesterase was used to mediate pyrethroid toxicity through hydrolysis of the parent compound to nontoxic products. These approaches may serve as cost-effective, rapid, mechanistically based methods for identifying pyrethroid-associated toxicity in aquatic samples.

MATERIALS AND METHODS

Chemicals and instrumentation

All chemicals were purchased from Aldrich Chemical (Milwaukee, WI, USA) unless otherwise noted and were used without further purification. Pyrethroid and OP standards were purchased from either Chem Services (West Chester, PA, USA) or Riedel de Haen (Seelze, Germany). Structural characterization and purity were provided by ^{1}H and ^{13}C nuclear magnetic resonance (NMR) and gas chromatography coupled to mass spectrometry (GC/MS) with electron impact (EI) ionization detection. The NMR spectra were acquired on a Mercury 300 spectrometer (Varian, Palo Alto, CA, USA). Chemical shift values were recorded in parts per million (ppm) using tetramethylsilane (TMS) as the internal reference. For GC analysis, samples were analyzed on a HP 6890 GC (Agilent Technologies, Engelwood, CO, USA) equipped with a 0.25 mm i.d. × 30 m, 0.25-μm film DB-5MS column (J&W Scientific, Folsom, CA, USA) with a constant He flow rate of 0.8 ml/min. The injector temperature was 250°C. The initial column temperature of 50°C was held for 5.00 min and then ramped at 15°C/min to 320°C and held for 2.00 min. The GC was interfaced with a HP 5973 MS run in full scan mode from 50 to 550 *m/z* with a quadrupole temperature of 186°C and a source temperature of 240°C. The GC/MS electron impact fragmentation patterns and NMR spectra supported all reported structures. Compound purity was >97% as determined by NMR and GC/MS.

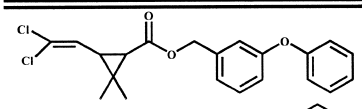
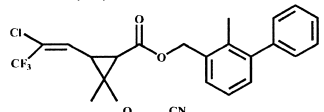
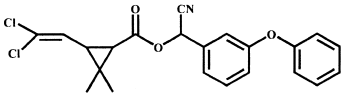
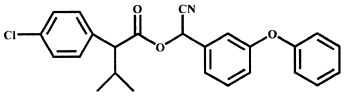
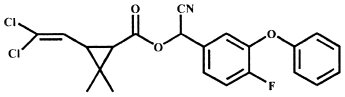
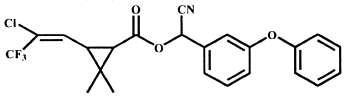
Toxicity testing

The test organism was the cladoceran, *Ceriodaphnia dubia*, a freshwater invertebrate widely used for acute toxicity tests [29], short-term chronic studies [30], and TIEs [31]. *Ceriodaphnia dubia* neonates (<24-h-old) were obtained from cultures maintained at AQUA-Science (Davis, CA, USA) in reverse osmosis–treated well water amended with dry salts to U.S. EPA moderately hard specifications (pH, 7.4–7.8; hardness, 80–100 mg/L; alkalinity, 60–70 mg/L). Cultures were maintained at 25 ± 1°C with a photoperiod of 16:8-h light:dark and were fed a mixture of the green alga, *Selenastrum capricornutum* (University of Texas Algae Type Collection, Austin, TX, USA) and blended trout chow (Silver Cup, Murray, UT, USA).

The 48-h toxicity test procedures followed those outlined by the U.S. EPA [29]. Neonates used for testing were <24-h-old, collected within 8 h from adults at least 7-d-old. All tests were conducted in 20-ml glass scintillation vials containing 18 ml of test water. A moderately hard water control, a methanol control, and five to seven concentrations of the test material with two or four replicates containing five neonates each were used for each test series. The neonates were pipetted into the vials using stratified random assortment. Test solutions were not renewed and the organisms were not fed during the exposures. Test temperature and photoperiod were identical to culture conditions. Mortalities were monitored daily. Tests were considered invalid if control survival was <90%. The concentrations required to cause an effect in 50% of the population (EC50) were calculated from the mortality data using a computer program (ToxCalc™, Tidepool Scientific, McKinnleyville, CA, USA).

Insecticide standards (99% a.i., 100 μg/L in methanol) were obtained from AccuStandard (New Haven, CT, USA). Piperonyl butoxide (90% a.i.) was obtained from Aldrich Chemical

Table 1. Structure and physical constants of pyrethroids used in this study[a]

Structure	Name[b] (Type)[c]	Log P[d] (ClogP)	Water Solubility[e]	Henry's Constant[f]
	Permethrin (I)	6.10 (6.9)	5.5	1.4×10^{-6}
	Bifenthrin (I)	6.40 (7.2)	0.014	7.2×10^{-3}
	Cypermethrin (II)	6.54 (6.1)	4.0	3.4×10^{-7}
	Esfenvalerate (II)	5.62 (6.8)	6.0	1.4×10^{-7}
	Cyfluthrin (II)	5.97 (6.4)	2.3	3.7×10^{-6}
	λ–Cyhalothrin (II)	7.00 (6.1)	5.0	1.9×10^{-7}

[a] Data are from Laskowski [39].

[b] Chemical names of each pesticide are as follows: Permethrin, ([3-phenoxyphenyl]methyl 3-[2,2-dichloroethenyl]-2,2-dimethylcyclopropane carboxylate, CAS 52645-53-1); bifenthrin, ([2-methyl(1,1′-biphenyl)-3-yl]methyl [1*R*,3*R*]-3-[(1*Z*)-2-chloro-3,3,3-trifluoro-1-propenyl]-2,2-dimethylcyclopropanecarboxylate, CAS 82657-04-3); cypermethrin, ([±] α-cyano[3-phenoxyphenyl]methyl 3-[2,2-dichloroethenyl]-2,2-dimethylcyclopropanecarboxylate, CAS 52315-07-8); esfenvalerate, ([*S*]-α-cyano[3-phenoxyphenyl]methyl [α*S*]-4-chloro-α-[1-methylethyl]benzeneacetate, CAS 66230-04-4); cyfluthrin, (α-cyano[4-fluoro-3-phenoxyphenyl]methyl 3-[2,2-dichloroethenyl]-2,2-dimethylcyclopropanecarboxylate, CAS 68359-37-5); λ-cyhalothrin, ([*S*/*R*]-α-cyano[3-phenoxyphenyl]methyl [1*S*,3*S*]-3-[(1*Z*)-2-chloro-3,3,3-trifluoro-1-propenyl]-2,2-dimethylcyclopropanecarboxylate, CAS 91465-08-6).

[c] Type II pyrethroids contain an α-cyano group on the benzylic carbon (Fig. 1).

[d] Values are the log of the partitioning constant between octanol and water (K_{OW}). *C*log*P* refers to the calculated log *P*.

[e] Units are μg/L.

[f] Units are atm m^3 mol^{-1}.

(Milwaukee, WI, USA). Working standards were prepared by diluting the chemicals in high-performance liquid chromatography (HPLC)–grade methanol (Fisher Scientific, Fairlawn, NJ, USA). Aliquots of the working solutions were then used to prepare the test solutions in moderately hard dilution water. Methanol concentration in all test solutions was <0.1%. All stock and working solutions were stored in the dark at 4°C.

Dissolved oxygen, pH, conductivity, alkalinity, hardness, and temperature were measured at the initiation of exposure. Dissolved oxygen, temperature, and conductivity were determined with Yellow Springs Instrument probes and meters (Yellow Springs, OH, USA), and pH was measured with a Hach pH meter (Loveland, CO, USA). Alkalinity and hardness were determined with Hach titrant kits. Temperature was measured continuously in the temperature-controlled room throughout the exposure period. Dissolved oxygen, temperature, and pH also were measured at the conclusion of each test. All instrumentation were cleaned thoroughly between analyses using hot water and soap followed by rinsing with methanol.

Pyrethroid removal methods

All three of the methods described below were performed using the toxicity testing procedures described in the previous section. Any method deviations are listed; otherwise, assays were all performed identically.

The antibody used in this study was a polyclonal permethrin–selective antibody developed by our laboratory, which had a high level of cross-reactivity for cypermethrin (65%) [21]. The stock solution was prepared in sodium phosphate buffer (0.1 M, pH 7.4) by diluting crude serum 50-fold. Working antibody solutions of 100- and 10,000-fold dilutions were prepared in the same buffer. Different amounts of these working solutions (10–100 μL) were added into the test containers containing the pyrethroids 1 to 3 h prior to addition of *C. dubia*.

Technical grade (90% a.i.) PBO was diluted in HPLC-grade methanol (0.1 mg/ml) and added to test containers containing the pyrethroids for a final concentration of 100 μg/L. Samples were incubated for 1 to 3 h prior to the addition of *C. dubia*.

Porcine esterase was obtained from Sigma Chemical (catalog E-2884, lot 107H7016, 250 U/ml, 15 mg/ml; St. Louis, MO, USA). Stock enzyme solution was prepared in sodium phosphate buffer (0.1 M, pH 7.4) and diluted appropriately for each assay. Enzyme was added to test containers containing the pyrethroid for a final activity of 2.5×10^{-3} units/ml (U/ml) unless otherwise noted (one unit of activity will hydrolyze

1.0 μmole of ethyl butyrate to butyric acid/min at 25°C as defined by the supplier). Test solutions were incubated with the enzyme for 1 h prior to addition of *C. dubia*.

Esterase activity assays

Esterase assays with *p*-nitrophenyl acetate (PNPA) were performed using sodium phosphate buffer (0.1 M, pH 7.4) according to methods of Wheelock et al. [32] as adapted from Ljungquist and Augustinsson [33]. Assays were preformed in 96-well microtiter styrene flat bottom plates (Dynex Technologies, Chantilly, VA, USA) and analyzed on a Spectramax 340PC plate reader (Molecular Devices, Sunnyvale, CA, USA). Porcine esterase was used at a final concentration of 0.1 μg/ml (~1.8 mU/ml; catalog no. E-2884, lot no. 102K7062, 184 U/ml, 10 mg/ml). All assays were designed such that no more than 10% of the substrate was hydrolyzed over the length of the assay and solvent content never exceeded 1% of the total assay volume. Reported results are all corrected for background hydrolysis of the substrate. Activity was monitored using a 2.0-min kinetic read at 405 nm.

Pyrethroid hydrolysis assays were performed as described in Wheelock et al. using porcine esterase [34]. Briefly, assays were conducted in 2 ml of sodium phosphate buffer (0.1 M, pH 7.4 or 8.0) at either 25 or 37°C. Protein concentration varied depending upon pyrethroid and assay conditions, but ranged from a low of 2.6 μg/ml (~0.5 U/ml; lot no. 102K7062) for permethrin at 37°C and pH 8.0 to a high of 20.8 μg/ml (~3.8 U/ml; lot no. 102K7062) for bifenthrin at 25°C and pH 7.4. Assays were linear over the life of the assay and were configured such that no more than 10% of the substrate was consumed. Pyrethroid substrate was added and the mixture incubated for 5 min at the indicated temperature, followed by addition of 1 ml of brine and 1 ml of ethyl acetate (EtOAc). Samples were vortexed for 30 s, centrifuged for 5 min, and then a 250-μL aliquot of the EtOAc was analyzed by GC/MS. Upon hydrolysis, pyrethroids form the corresponding cyanohydrin as shown in Figure 1, which spontaneously rearranges to the aldehyde at basic pH [34]. The rate of pyrethroid hydrolysis was measured by quantifying the resultant aldehydes. Quantification standards were: 3-phenoxy-benzaldehyde for cypermethrin, esfenvalerate, and λ-cyhalothrin; 3-phenoxybenzylalcohol for permethrin; 2-methyl-3-biphenyl methanol for bifenthrin; and 4-fluoro-3-phenoxy-benzaldehyde for cyfluthrin. All standards were purchased from Aldrich Chemical except for 4-fluoro-3-phenoxy-benzaldehyde, which was synthesized as described below.

All assays to determine the concentration of inhibitor that reduced enzyme velocity by 50% (IC50) were designed such that there were at least two datum points above and below the IC50 value in the linear range. Inhibitor solutions were prepared in ethanol and diluted as required for each assay. The IC50 determinations with permethrin or cypermethrin as the substrate were conducted as described above using porcine esterase at 5.2 μg/ml (~1.0 U/ml; lot no. 102K7062). Assays with PNPA were performed as described above using 0.2 μg/ml (~3.7 U/ml; lot no. 102K7062) porcine esterase. Solvent never exceeded 1% of the assay volume and no solvent effects were observed.

Synthesis of 4-fluoro-3-phenoxy-benzaldehyde

Cyfluthrin (106.9 mg) was dissolved in 2-ml tetrahydrofuran and 300 μL of 1M NaOH was added. The solution was stirred slowly at room temperature for 96 h, after which the mixture was washed with 3 × 20 ml of water and 10 ml of EtOAc was added. The organic fraction was washed with 1 × 25 ml of brine and dried over $MgSO_4$, filtered, and stripped to give a viscous yellow oil. The oil was extracted with hexane and stripped to give a viscous pale yellow oil. The oil was then dissolved in minimal EtOAc and loaded onto a preparative thin-layer chromatography plate (PK6F silica gel 60 Å, 20 × 20 cm, 1,000-μM thick; Whatman, Clifton, NJ, USA), which was developed in 70:30 hexanes:EtOAc. The product was extracted from the silica gel using EtOAc, which was stripped to give a dark orange oil (4.5 mg, 8.5% yield). The ^{1}H NMR ($CDCl_3$) showed δ ppm 7.03 (2, J = 7.8 Hz, 2H), 7.18 (t, J = 7.2 Hz, 1H), 7.38 (m, 2H), 7.52 (dd, J = 7.5, 1.8 Hz, 1H), 7.64 (m, 1H), 9.87 (s, 1H). GC/electron impact-MS (*m/z*) 216 (M^+, 100%), 187 (38%), 159 (35%), 139 (20%), 133 (24%), 77 (56%).

Table 2. Effect of pyrethroid antibody (Ab) on the toxicity of cypermethrin to *Ceriodaphnia dubia*

Sample treatment	Antibody pmole IgG/ml[a]	48-h % mortality[b]
Lab control	0	0
Ab control	0.1	10
Ab control	10	20
Cypermethrin[c]	0	90
	0.01	90
	0.1	60
	1.0	30
	10	30
Inactive Ab[d]	10	40

[a] Antibody concentration in immunoglobin G (IgG) per ml of test solution.
[b] Mean mortality in two replicates of 5 neonate *C. dubia.*
[c] Cypermethrin was spiked at 800 ng/L nominal water concentrations (~1 toxic unit).
[d] Antibody was heated at 75°C for 1 h and sample contained 800 ng/L cypermethrin.

RESULTS

Effect of treatment upon pyrethroid toxicity

A series of 48-h toxicity tests were conducted with a pyrethroid-selective antibody developed in our laboratory [21]. Antibody concentrations of 0.1 to 10 pmole IgG/ml were effective in reducing cypermethrin toxicity to *C. dubia* (Table 2). However, both antibody controls produced detectable toxicity to *C. dubia*. To examine nonspecific binding effects, the antibody was deactivated by incubation for 1 h at 75°C. The denatured antibody reduced pyrethroid-associated mortality by approximately 50%, being nearly as effective in reducing cypermethrin toxicity as the highest active antibody concentration tested.

The effect of PBO on the toxicity of a suite of type I and type II pyrethroids to *C. dubia* was investigated. Solutions containing PBO-synergized individual pyrethroid toxicity to *C. dubia* by 9- to 137-fold compared to solutions without PBO (Table 3). The largest PBO toxicity ratio (137-fold) was observed with cypermethrin; an intermediate toxicity ratio (40-fold) was observed with λ-cyhalothrin; and lower toxicity ratios (9- to 17-fold) were observed with bifenthrin, cyfluthrin, esfenvalerate, and permethrin. No distinct patterns between pyrethroid type and level of synergism were observed among the different pyrethroids. Permethrin, bifenthrin, and cyfluthrin all had approximately the same ratio, yet are a mixture of type

Table 3. Effect of piperonyl butoxide (PBO) on pyrethroid toxicity to *Ceriodaphnia dubia*

Pyrethroid (type)[b]	EC50 (ng/L)[a] PBO[c] (−)	PBO (+)	Ratio[d]
Permethrin (I)	250 ± 119	18 ± 7	14
Bifenthrin (I)	142 ± 122	16 ± 22	9
Cypermethrin (II)	683 ± 72	5 ± 2	137
Esfenvalerate (II)	250 ± 71	21 ± 19	12
Cyfluthrin (II)	344 ± 41	20 ± 4	17
λ-Cyhalothrin (II)	200 ± 90	5 ± 3	40

[a] Mean EC50 (concentration at which 50% of the population exhibits an effect) of 3 to 4 paired toxicity tests ± the standard deviation. All values are based on nominal water concentrations.
[b] Indicates type I or type II pyrethroid as determined by the presence of the α-cyano group.
[c] PBO was spiked at 100 μg/L in all PBO (+) samples.
[d] Ratio of PBO(−)/PBO(+).

I and type II pyrethroids. The differences between cypermethrin (type II) and permethrin (type I) were extremely large and these two compounds differ only by the presence of the α-cyano group, suggesting effects upon ester hydrolysis. No structure-activity relationships were observed among the different pyrethroids examined.

A 48-h toxicity study was conducted to determine if the porcine carboxylesterase could degrade high concentrations of the pyrethroid and OP insecticides to nontoxic levels to *C. dubia*. Exposure concentrations were approximately 2 to 3 times the estimated EC50 for each compound. In all cases, the carboxylesterase hydrolyzed the six pyrethroids to nontoxic levels, whereas samples that contained no esterase had 100% mortality (Table 4). The enzyme had no detectable effect on the toxicity of the two OP insecticides (diazinon and chlorpyrifos) to *C. dubia*, with all samples exhibiting 100% mortality in the presence or absence of esterase. No selectivity towards type I versus type II pyrethroids was observed, with the enzyme removing 2 to 3 toxic units (TUs) of all pyrethroids examined. Studies with bifenthrin used four TUs, which were remediated successfully by the enzyme. A TU is defined as 100/EC50 (the concentration at which 50% of the population demonstrates the desired effect, in this case mortality). The enzyme did not have any observable toxic effects upon *C. dubia*, with enzyme controls showing 0% mortality.

Table 4. Effect of esterase addition on pyrethroid-associated toxicity to *Ceriodaphnia dubia*

Treatment	Concn.[a] (ng/L) (TUs)	48-h % mortality[b] Esterase (−)	Esterase (+)
Control[c]	0	0	0
Permethrin	600 (2)	100	0
Bifenthrin	660 (4)	100	0
Cypermethrin	1,450 (2)	100	0
Esfenvalerate	700 (3)	100	0
Cyfluthrin	560 (2)	100	0
λ-Cyhalothrin	600 (3)	100	0
Diazinon	760 (2)	100	100
Chlorpyrifos	160 (2)	100	100

[a] Concentration at which samples were spiked. All values are nominal water concentrations. Values in parentheses are toxic units (TUs) defined as 100/EC50 (the concentration at which 50% of the population exhibits an effect).
[b] Mean mortality in two replicates of five neonate *C. dubia.* Standard deviations for all samples are zero. In all samples, either 0 or 100% mortality was observed. Esterase (+) samples contain enzyme spiked at 2.5×10^{-3} U/ml. Esterase (−) samples contain no added enzyme.
[c] Controls were performed with and without the addition of the esterase.

Table 5. Hydrolysis of selected pyrethroids by porcine liver esterase

Substrate	Temp. (°C)	pH	Activity[a]
Permethrin	25	7.4	160 ± 3
		8.0	250 ± 13
	37	7.4	549 ± 16
		8.0	786 ± 76
Bifenthrin	25	7.4	2.7 ± 0.1
		8.0	3.6 ± 0.1
	37	7.4	11.5 ± 2.4
		8.0	15.7 ± 0.3
Cypermethrin	25	7.4	64.2 ± 6.2
		8.0	91.6 ± 1.1
	37	7.4	194 ± 4
		8.0	221 ± 16
Esfenvalerate	25	7.4	6.0 ± 0.1
		8.0	6.3 ± 0.5
	37	7.4	21.7 ± 2.1
		8.0	21.3 ± 1.6
Cyfluthrin	25	7.4	23.3 ± 6.3
		8.0	32.5 ± 2.2
	37	7.4	62.3 ± 1.8
		8.0	88.7 ± 1.6
λ-Cyhalothrin	25	7.4	ND[b]
		8.0	ND
	37	7.4	ND
		8.0	ND
PNPA[c]	25	7.4	25.1 ± 2.8
		8.0	45.9 ± 4.9
	37	7.4	49.7 ± 3.3
		8.0	72.9 ± 4.6

[a] Activity is in units of nmol/min/mg protein. All assays were performed in triplicate and data are the average ± the standard deviation. Background hydrolysis at 25°C and pH 7.4 was 0.07 nmol/min for bifenthrin, 0.11 nmol/min for esfenvalerate, 0.38 nmol/min for cypermethrin, and 0.39 nmol/min for permethrin.
[b] Not detected. The method detection limit was 0.1 μM, which corresponds to 0.2 nmol/min/mg protein.
[c] *p*-Nitrophenyl acetate. Activity is in units of μmol/min/mg protein.

Esterase characterization

Initial studies suggested that the porcine liver esterase was the most promising technique for removing pyrethroid-associated toxicity from TIE samples. The esterase preparation, therefore, was characterized further to understand its performance under potential TIE conditions. The esterase hydrolysis of all six pyrethroids examined in this study as well as the standard esterase substrate PNPA was measured at a range of assay conditions (Table 5). The porcine esterase hydrolyzed all of the pyrethroids to some extent, except for λ-cyhalothrin, with hydrolysis increasing with temperature and pH. Of particular interest was the lack of observable hydrolysis of λ-cyhalothrin even though toxicity assays with λ-cyhalothrin showed that the esterase was capable of removing all pyrethroid-associated toxicity (Table 4). The rate of pyrethroid hydrolysis observed was approximately 1,000-fold slower than that of PNPA under all conditions examined.

To test the efficacy of the esterase over a range of pyrethroid concentrations, the enzyme was challenged with 1 to 5 TUs of bifenthrin (350–1,750 ng/L) or cypermethrin (750–3,750

Table 6. Effect of varying esterase concentration upon pyrethroid toxicity to *Ceriodaphnia dubia*

Sample	Pyrethroid concn.[b]	48-h % mortality[a]			
		Enzyme activity[c]			
		0	1.25	2.5	5.0
Control[d]	0	0	—[e]	—	0
Inactive enzyme[f]	0	—	—	—	0
Cypermethrin[g]	750	80	0	0	0
(type II)	1,500	100	0	0	0
	2,250	100	0	0	0
	3,000	100	10	10	0
	3,750	100	10	0	0
Bifenthrin[h]	350	100	100	40	0
(type I)	700	100	100	50	0
	1,050	100	100	100	30
	1,400	100	100	60	20
	1,750	100	100	100	10

[a] Mean mortality in two replicates of five neonate *C. dubia.*
[b] Concentrations, which are all nominal water concentrations in ng/L.
[c] Enzyme activity is in units of 10^{-3} U/ml (~0, 75, 150, and 300 ng/ml protein, respectively). These numbers give the final concentration of enzyme in each 20-ml scintillation vial. One unit of esterase will hydrolyze 1.0 μmole of ethyl butyrate to butyric acid and ethanol per min at pH 8.0 at 25°C.
[d] Controls were performed both with and without the addition of enzyme.
[e] — indicates that the test was not performed.
[f] Enzyme was heated at 80°C for 1 h.
[g] Concentrations of cypermethrin range from 1 toxic unit (TU) at 750 ng/L to 5 TU at 3,750 ng/L. A toxic unit is defined as 100/EC50 (the concentration at which 50% of the population exhibits an effect).
[h] Concentrations of bifenthrin range from 1 TU at 350 ng/L to 5 TU at 1,750 ng/L.

ng/L). Additionally, three different concentrations of enzyme were used to examine the dose-response relationship. Data provided a dose-response relationship with increasing levels of enzyme capable of mediating more pyrethroid TUs (Table 6). All samples with no enzyme caused 100% mortality (except for the lowest concentration of cypermethrin, which caused 80% mortality). At the highest concentration of enzyme examined in this study (5.0×10^{-3} U/ml), all cypermethrin toxicity was removed. Bifenthrin samples exhibited some toxicity at the highest level of enzyme used; however a significant amount (70–90%) of the toxicity was removed. No toxicity to *C. dubia* was observed with either the enzyme control or the inactivated enzyme.

The amount of compound required to reduce esterase velocity by 50% (IC50) was measured for both the parent and oxon-forms of chlorpyrifos and diazinon. Results showed that the oxon form of both diazinon and chlorpyrifos are extremely potent inhibitors of the esterase-mediated hydrolysis of permethrin (type I) and cypermethrin (type II) pyrethroids, as well as PNPA (Table 7). However, the parent OPs are poor inhibitors, with IC50 values >100 μM for all substrates examined.

DISCUSSION

In the phase I TIE process, the toxic environmental sample is subjected to a number of treatments that identify cationic metals, oxidants, ammonia, and polar and nonpolar organic toxicants [10–12,29]. The TIE procedures include pH adjustment, addition of chelating and oxidizing reagents, separation on solid-phase extraction (SPE) columns, and fractionation of the SPE column eluates using HPLC, followed by analysis of the toxic HPLC fractions by advanced instrumentation (GC/MS and/or HPLC/MS) as shown in Figure 2. Once the toxicant(s) is identified, toxicity tests are conducted to determine if the concentrations of suspected toxicant(s) can account for the amount of toxicity measured in the sample.

Two TIE techniques are useful in identification of OP- and pyrethroid-caused toxicity. Use of SPE columns removes toxicity due to nonpolar organic toxicants, including OP and pyrethroid insecticides. Alternatively, treatment with PBO blocks activation of OP insecticides to their toxic form [25] and increases the toxicity of pyrethroid insecticides [26]. Thus, if PBO decreases the toxicity, metabolically activated OP insecticides (e.g., diazinon and/or chlorpyrifos) are suspected. If PBO increases the sample toxicity, pyrethroid insecticides are suspected. However, if there is a mixture of compounds, the agents causing the toxicity are more difficult to pinpoint and the signal is convoluted. Therefore, we propose intervening in the TIE process at this junction with an additional step to either mediate or identify pyrethroid-associated toxicity as shown in Figure 2.

Table 7. Inhibition of esterase-mediated pyrethroid hydrolysis by organophosphates

Substrate	Inhibitor IC50[a]	
	Diazinon-oxon[b]	Chlorpyrifos-oxon[b]
Permethrin	35.1 ± 2.7	34.8 ± 3.3
Cypermethrin	24.8 ± 2.3	29.2 ± 0.8
PNPA[c]	1.67 ± 0.02	0.87 ± 0.05
	Diazinon[d]	Chlorpyrifos[d]
Permethrin	>100	>100
Cypermethrin	>100	>100
PNPA[c]	>100	>100

[a] IC50 = Concentration of inhibitor required to reduce enzyme velocity by 50%. All assays were performed in triplicate and each IC50 value is the average of three different experiments ± the standard deviation.
[b] IC50 values are in nM.
[c] *p*-Nitrophenyl acetate.
[d] IC50 values are in μM.

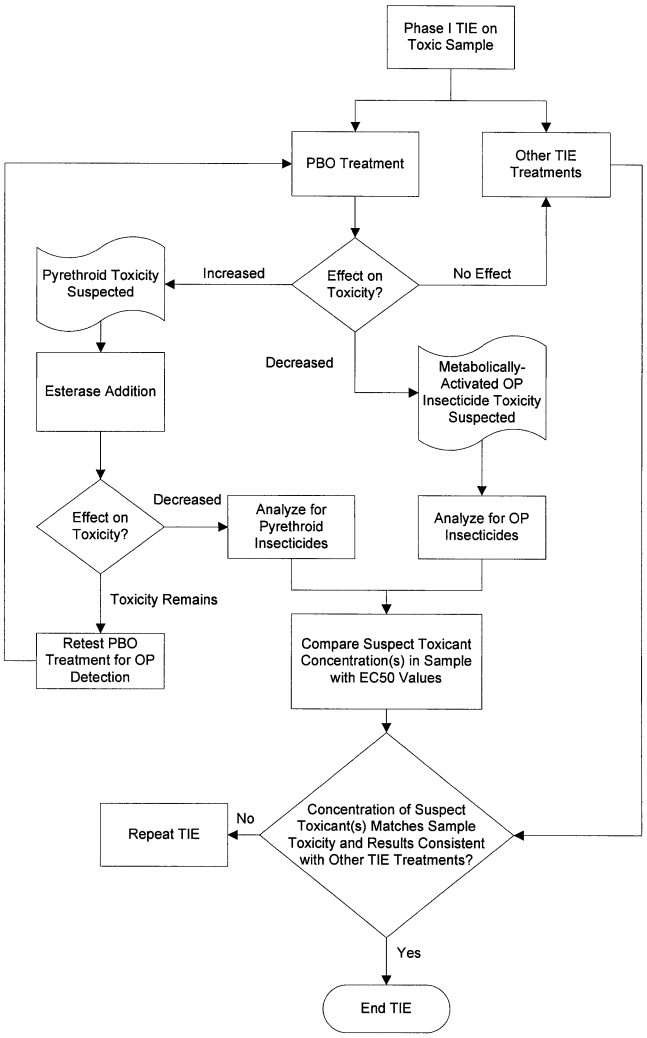


Fig. 2. Application of esterase procedures to the phase I toxicity identification evaluation (TIE) process. Abbreviations used in figure are piperonyl butoxide (PBO), organophosphate (OP), and concentration to cause 50% effect (EC50).

Effect of treatment upon pyrethroid toxicity

The first treatment examined was a pyrethroid-selective antibody that was hypothesized to remove pyrethroids from solution, thereby reducing toxicity. At higher levels of IgG, this phenomenon was in fact observed. However, significant mortality was associated with the antibody controls. In addition, antibody inactivated by heating reduced toxicity almost as effectively as the highest concentration of native antibody used in this study. Heated antibody should be denatured and therefore unable to actively bind cypermethrin, suggesting that the observed effects upon toxicity were nonspecific. Due to their extreme hydrophobicity, pyrethroids will adsorb to lipophilic surfaces [14]. Protein addition, therefore, could be sufficient to remove some of the pyrethroid from solution. It is unclear what percentage of this bound pyrethroid would remain bioavailable. This technique might be a useful method for pyrethroid removal through the addition of a sufficiently hydrophobic material to the test solution. However, it would most likely remove OPs as well, thus eliminating any selectivity for detecting OPs versus pyrethroids.

Additional studies utilizing a wider range of antibody concentrations will be needed to establish if direct antibody addition can be used to prevent cypermethrin or other pyrethroid toxicity. To determine the extent of the nonspecific protein adsorption, subsequent studies should utilize an antibody that does not bind pyrethroids rather than denatured antibody. Further studies could examine a range of pyrethroid antibodies, including class-specific (e.g., type I or type II) antibodies. In addition, purifying the antibody and attaching it to a solid matrix (such as glass beads) may improve the binding capacity while eliminating the toxicity of the antibody itself [20]. The increased mortality observed likely is due to factors in the serum other than the antibody. Future work thus will need to be performed with enriched antibody fractions or monoclonal antibodies. The resulting lower protein concentration should decrease the reduction in toxicity seen with inactive antibody (Table 2). Further research will be necessary to develop these antibody techniques for use in TIE procedures.

The use of PBO in aquatic toxicity testing has been reported by several researchers [2,4,25,35] and recommended by the U.S. EPA [29]. However, with the co-occurrence of OPs and pyrethroids in TIE samples, the use of PBO to identify specifically the compounds responsible for the toxicity could result in convoluted test results. It also is possible that the synergistic effects of PBO upon pyrethroid toxicity could be used as a pyrethroid signature in TIE protocols. We therefore examined the ability of PBO toxicity ratios to identify individual pyrethroids (vs the entire class as a whole) in the absence of OPs. It was hypothesized that each pyrethroid could be identified by its individual PBO toxicity signature in the presence and absence of PBO. However, the lack of structure-activity relationships and differential toxicity between many of the pyrethroids makes it unlikely that this technique will be useful (Table 3), with the possible exception of cypermethrin identification. The PBO treatment did synergize pyrethroid toxicity in all cases and, therefore, may be a useful general identifier for the presence of pyrethroids. None of these studies examined the effects of PBO treatment on samples containing both OPs and pyrethroids. Additional research will be needed to determine if PBO toxicity ratios can be used to identify toxicity due to OPs and pyrethroids when both classes of toxicants co-occur in samples.

The esterase treatment provided the most promising results as the enzyme eliminated all pyrethroid-associated toxicity. An advantage of working with the esterase is that the mechanism of toxicity reduction is well understood [18] as shown in Figure 1. The hydrolysis of pyrethroids by esterases has been characterized by several researchers [15,18,36]. The esterase preparation used for these studies is from porcine liver and contains a mixture of esterase isozymes [37] with varying affinity for pyrethroids (data not shown). The amount of esterase used was sufficiently high to hydrolyze quickly all pyrethroids and no pyrethroid-specific effects were observed. It might be possible to detect differences between type I and type II pyrethroids at lower concentrations of esterase.

The esterase was incubated with the pyrethroids for 1 h before the addition of *C. dubia* to allow for pyrethroid hydrolysis. We did not attempt to optimize the length of the incubation and it is possible that a shorter incubation time would be acceptable. If the enzyme is assumed to be operating under V_{max} conditions (an assumption that most likely is invalid), the theoretical amount of pyrethroid hydrolyzed over 1 h can be calculated using the activity numbers in Table 5 and the amount of enzyme added per assay ($\sim$2.7 μg). Porcine esterase theoretically can hydrolyze approximately 2,000 TUs

of permethrin (1.5 mg/L) and 30 TUs of bifenthrin (10.5 μg/L) in 1 h. However, as substrate concentrations decrease, the enzyme will no longer function at V_{max} (i.e., non–Michaelis-Menton conditions) due to decreased substrate concentration and catalytic activity. Thus the actual amount of pyrethroid hydrolyzed most likely will be substantially less than the theoretical max calculated here. However, given that most samples will contain significantly less pyrethroid, the esterase should be capable of hydrolyzing a significant amount of the pyrethroid as shown in Table 6. These numbers represent ideal conditions and it is likely that complicated matrices, such as effluent or pore water, will reduce the catalytic efficiency and/or lifetime of the enzyme.

The presence of esterase had no observable affect upon the toxicity of the OPs diazinon and chlorpyrifos, indicating specificity in the esterase mechanism of action (Table 4). Results from these studies suggest that the enzyme could be used to selectively degrade pyrethroid toxicity in OP-containing samples. It should then be possible to perform the PBO treatment afterwards to test for an OP signature. It also is possible that esterases of varying specificity could be used to distinguish between individual pyrethroids. Even if it is not possible to identify specific pyrethroids, it should be achievable to find an esterase that can distinguish between type I and type II pyrethroids. Numerous esterases are available commercially as well as in different research groups. Further work should examine several esterases to determine which one has superior performance in TIE formats. One could envision a TIE procedure where a battery of esterases was used to identify quickly the pyrethroids responsible for the observed toxicity.

Esterase characterization

Initial studies showed that, out of the three methods examined in this study, esterase was the most promising treatment for detecting or removing pyrethroid toxicity and therefore was chosen for further characterization for use in TIE assays. Esterase assays with mammalian enzymes generally are performed at pH 8.0 at 37°C [38]. However, most TIE assays are performed at 25°C or cooler at pH 7.4 [29]. The efficiency of the enzyme at hydrolyzing pyrethroids therefore was examined under multiple conditions. The observed responses were predictable in that higher temperature and higher pH resulted in increased rates of pyrethroid hydrolysis. The hydrolysis rates of the pyrethroids examined varied by approximately three orders of magnitude, demonstrating that there are strong structural effects upon pyrethroid hydrolysis. Sterically unhindered type I pyrethroids are hydrolyzed quicker than type II pyrethroids. This effect can be seen most dramatically in the difference in hydrolysis rates for permethrin versus cypermethrin shown in Table 5 (106 vs 64 nmol/min/mg protein, respectively at 25°C at pH 7.4). However, the type I pyrethroid bifenthrin exhibited a slower hydrolysis rate relative to cypermethrin or cyfluthrin (type II pyrethroids). This observation is most likely due to the ortho methyl substituent that sterically hinders the ester, affecting the hydrolysis rate analogously to the α-cyano moiety of the type II pyrethroids. Hydrolysis activity for λ-cyhalothrin could not be measured with these methods, yet all λ-cyhalothrin–associated toxicity could be removed by the addition of esterase as shown in Table 4. This observation confirms that the enzyme still can serve its function even on pyrethroids that are recalcitrant to esterase-mediated hydrolysis.

Dose-response studies with the enzyme demonstrated that it is capable of reducing multiple TUs of both bifenthrin and cypermethrin. Table 5 shows that of all the pyrethroids for which hydrolysis could be detected, bifenthrin had the slowest hydrolysis rate. However, at the highest concentration of esterase used in Table 6, 90% of the mortality of five TUs of bifenthrin was eliminated. It will be necessary to quantify this effect for multiple pyrethroids in a variety of matrices. Matrix effects (such as organics, other toxicants, pH, etc.) could be very large for this system, possibly resulting in a decrease of esterase activity. At the highest concentration of enzyme used in Table 6, no enzyme-associated toxicity was observed. However, it is possible that the enzyme could exert toxic effects at increased levels. Further studies should examine a range of enzyme concentrations to determine the maximum concentration of enzyme that does not cause significant toxicity. In order to achieve optimal efficiency for removal of pyrethroids, it is desirable to use the highest concentration of esterase possible. The lack of toxicity of heat-inactivated enzyme was very promising and suggests that it is an appropriate control for nonspecific binding effects, unlike the heat-inactivated antibody.

As it is likely that OPs and pyrethroids will co-occur in TIE samples [9], the inhibition of the esterase by OPs was examined using diazinon and chlorpyrifos as model compounds. The effects of OPs upon esterase activity were examined by measuring the concentration of OP (inhibitor) required to reduce enzyme velocity by 50% (IC50). The active form of OP inhibitor is the oxon metabolite following cytochrome P450 oxidation [23]. Therefore, both parent and oxon OP were examined for their ability to inhibit porcine liver esterase. The parent OPs as thiones (P = S) did not inhibit the enzyme as would be expected. However, the oxon forms (P = O) were potent inhibitors of both permethrin and cypermethrin hydrolysis as shown in Table 7. This observed inhibition most likely is not a concern for use of the esterase in TIE assays for a number of reasons. First, formation of the oxon form of the OPs usually requires metabolic activation by MFOs, which can occur only inside of the test organism. For the oxon metabolite to inhibit the esterase, it would have to diffuse out of the organism into the test water, which is unlikely to occur in significantly high enough concentrations to cause inhibition of the esterase. Secondly, the amount of oxon formed relative to the total amount of esterase added to the test solutions is very small. Additionally, the esterase was added to the test solutions 1 h before the addition of the *C. dubia*, allowing the enzyme ample opportunity to hydrolyze pyrethroids. Therefore, even if the esterase was inhibited by oxon formed in vivo in the test organism, it should not influence the ability of esterases to detoxify the test samples prior to organism addition.

The ability of the esterase preparation to function with additional organisms should be examined. These studies were limited to *C. dubia*, but it is likely that the technique will be applicable to a wide range of aquatic testing organisms. One potential limitation will be enzyme use in sediment-based assays. It is possible that high levels of sediment or organic material will reduce significantly enzyme activity. However, in assays that are based upon a partitioning of pyrethroid from sediment to solution phase, it is likely that the esterase can be used successfully to remove pyrethroid toxicity.

An interesting observation in this study was the apparent reduction in pyrethroid levels in the aqueous phase over the life of the toxicity test (data not shown). A time-dependent effect of pyrethroid adsorption to the container was observed

and is a cautionary point for the formatting of aquatic toxicity testing. These observations have been noted by other researchers as well and are a known difficulty in working with pyrethroids [14,17,39]. Further work should attempt to quantify pyrethroid adsorption to sampling and test containers as well as determine its effects upon the outcomes of toxicity testing. Given the extreme hydrophobicity of many pyrethroids, it is possible that sorption to test containers significantly affects test outcome.

CONCLUSION

Three different techniques were compared for their ability to identify or remediate pyrethroid toxicity in aquatic toxicity tests, with esterase activity proving to be the most promising. An advantage to the use of esterase activity to remediate pyrethroid toxicity is its commercial availability and relatively low cost. It will be necessary to characterize the enzyme preparation fully and examine the variability in activity and stability of different commercial lots. Additional studies will be needed to confirm that the enzyme procedures are effective in reducing or eliminating pyrethroid-caused toxicity. Specifically, work should focus on matrix effects upon enzyme activity. If such studies demonstrate the effectiveness of the procedures, Figure 2 shows how they can be used either singly or in combination in the TIE process. If the sample toxicity was decreased by the enzyme treatment, pyrethroid analyses would be conducted on the original test sample and/or the SPE column eluates and toxic HPLC fractions. Use of the esterase preparation is an inexpensive, simple, mechanistically based method for removing pyrethroid toxicity from aquatic samples. Addition of esterase to the TIE procedure allows for the selective detection of the presence of pyrethroids, and does not interfere with subsequent detection of OP toxicity with standard TIE procedures. This esterase preparation should serve as a new tool in the development of TIE procedures for characterizing the contribution of pyrethroid insecticides to the toxicity of water samples.

Acknowledgement—C.E. Wheelock was supported by National Institute of Health (NIH) post doctoral training grant T32 DK07355-22 and a University of California Toxic Substances Research and Teaching Program Graduate Fellowship. This work was supported in part by State Water Resources Control Board Contract 0-079-250-0, National Institute of Environmental Health Sciences (NIEHS) grant R37 ES02710, NIEHS Superfund grant P42 ES04699, NIEHS Center for Environmental Health Sciences grant P30 ES05707, and NIH/National Institute of Allergy and Infectious Diseases grant U01 AI058267. The authors thank Jim Sanborn and Åsa Karlsson for critical reading of the manuscript.

REFERENCES

1. Bailey HC, Miller JL, Miller MJ, Dhaliwal BS. 1995. Application of toxicity identification procedures to the echinoderm fertilization assay to identify toxicity in a municipal effluent. *Environ Toxicol Chem* 14:2181–2186.
2. Bailey HC, DiGiorgio C, Kroll K, Miller JL, Hinton DE, Starrett G. 1996. Development of procedures for identifying pesticide toxicity in ambient waters: Carbofuran, diazinon, chlorpyrifos. *Environ Toxicol Chem* 15:837–845.
3. Bailey HC, Deanovic LA, Reyes E, Kimball T, Larson K, Cortright K, Connor V, Hinton DE. 2000. Diazinon and chlorpyrifos in urban waterways in Northern California, USA. *Environ Toxicol Chem* 19:82–87.
4. Werner I, Deanovic LA, Connor V, de Vlaming V, Bailey HC, Hinton DE. 2000. Insecticide-caused toxicity to *Ceriodaphnia dubia* (Cladocera) in the Sacramento–San Joaquin River Delta, California, USA. *Environ Toxicol Chem* 19:215–227.
5. de Vlaming V, Connor V, DeGiorgio C, Bailey HC, Deanovic LA, Hinton DE. 2000. Application of whole effluent toxicity test procedures to ambient water quality assessment. *Environ Toxicol Chem* 19:42–62.
6. Maltby L, Clayton SA, Yu H, McLoughlin N, Wood RM, Yin D. 2000. Using single-species toxicity tests, community-level responses, and toxicity identification evaluations to investigate effluent impacts. *Environ Toxicol Chem* 19:151–157.
7. Anderson BS, de Vlaming V, Larson K, Deanovic LA, Birosik S, Smith DJ, Hunt JW, Phillips BM, Tjeerdema RS. 2002. Causes of ambient toxicity in the Calleguas Creek watershed of southern California. *Environ Monit Assess* 78:131–151.
8. Bailey HC, Miller JL, Miller MJ, Wiborg LC, Deanovic LA, Shed T. 1997. Joint acute toxicity of diazinon and chlorpyrifos to *Ceriodaphnia dubia*. *Environ Toxicol Chem* 16:2304–2308.
9. Werner I, Deanovic LA, Hinton DE, Henderson JD, de Oliveira GH, Wilson BW, Krueger W, Wallender WW, Oliver MN, Zalom FG. 2002. Toxicity of stormwater runoff after dormant spray application of diazinon and esfenvalerate (Asana) in a French prune orchard, Glenn County, California, USA. *Bull Environ Contam Toxicol* 68:29–36.
10. U.S. Environmental Protection Agency. 1991. Methods for aquatic toxicity identification evaluations. Phase I toxicity characterization procedures. EPA-600/6-91/0303. Office of Research and Development, Duluth, MN.
11. U.S. Environmental Protection Agency. 1993. Methods for aquatic toxicity identification evaluations: Phase II toxicity identification procedures for samples exhibiting acute and chronic toxicity. EPA-600/R-92/080. Office of Research and Development, Duluth, MN.
12. U.S. Environmental Protection Agency. 1993. Methods for aquatic toxicity identification evaluations: Phase III toxicity confirmation procedures for samples exhibiting acute and chronic toxicity. EPA-600/R-92/081. Office of Research and Development, Duluth, MN.
13. Epstein L, Bassein S, Zalom FG. 2000. Almond and stone fruit growers reduce OP, increase pyrethroid use in dormant sprays. *Calif Agric* 54:14–19.
14. Sharom MS, Solomon KR. 1981. Adsorption and desorption of permethrin and other pesticides on glass and plastic materials used in bioassay procedures. *Can J Fish Aquat Sci* 38:199–204.
15. Casida JE, Gammon DW, Glickman AH, Lawrence LJ. 1983. Mechanisms of selective action of pyrethroid insecticides. *Annu Rev Pharmacol Toxicol* 23:413–438.
16. Leng G, Lewalter J, Rohrig B, Idel H. 1999. The influence of individual susceptibility in pyrethroid exposure. *Toxicol Lett* 107: 123–130.
17. Lee S, Gan J, Kabashima J. 2002. Recovery of synthetic pyrethroids in water samples during storage and extraction. *J Agric Food Chem* 50:7194–7198.
18. Abernathy CO, Casida JE. 1973. Pyrethroid insecticides: Esterase cleavage in relation to selective toxicity. *Science* 179:1235–1236.
19. Denton DL, Wheelock CE, Murray S, Deanovic LA, Hammock BD, Hinton DE. 2003. Joint acute toxicity of esfenvalerate and diazinon to fathead minnow (*Pimephales promelas*) larvae. *Environ Toxicol Chem* 22:336–341.
20. Miller JL, Miller MJ, de Vlaming V. 2004. Use of antibody-mediated procedures to identify and confirm the role of diazinon and chlorpyrifos in surface water toxicity. In Norberg-King TJ, Ausley L, Burton D, Waller T, Miller JL, eds, *Toxicity Identification Evaluations (TIE) for Effluents, Ambient Waters, and Other Aqueous Media*, Vol 1. SETAC Special Publication Series. SETAC, Pensacola, FL, USA (in press).
21. Shan G, Leeman WR, Stoutamire DW, Gee SJ, Chang DPY, Hammock BD. 2000. Enzyme-linked immunosorbent assay for the pyrethroid permethrin. *J Agric Food Chem* 48:4032–4040.
22. Watanabe T, Shan G, Stoutamire DW, Gee SJ, Hammock BD. 2001. Development of a class-specific immunoassay for the type I pyrethroid insecticides. *Anal Chim Acta* 444:119–129.
23. Hodgson E. 1982. Production of pesticide metabolites by oxidative reactions. *J Toxicol Clin Toxicol* 19:609–621.
24. Hamm JT, Wilson BW, Hinton DE. 2001. Increasing uptake and bioactivation with development positively modulate diazinon toxicity in early life-stage medaka (*Oryzias latipes*). *Toxicol Sci* 61: 304–313.
25. Ankley GT, Dierkes JR, Jensen DA, Peterson GS. 1991. Piperonyl butoxide as a tool in aquatic toxicological research with organophosphate insecticides. *Ecotoxicol Environ Saf* 21:266–274.

26. Casida JE. 1970. Mixed-function oxidase involvement in the biochemistry of insecticide synergists. *J Agric Food Chem* 18:753–772.
27. Casida JE, Quistad GB. 1995. Metabolism and Synergism of Pyrethrins. In Casida JE, Quistad GB, eds, *Pyrethrum Flowers: Production, Chemistry, Toxicology, and Uses*, Vol 1. Oxford University, New York, NY, USA, pp 258–276.
28. McAbee RD, Kang K, Stanich MA, Christiansen J, Wheelock CE, Inman A, Hammock BD, Cornel AJ. 2004. Pyrethroid tolerance in *Culex pipiens pipiens* var *molestus* from Marin County, California. *Pest Manag Sci* 60:359–368.
29. U.S. Environmental Protection Agency. 1993. Methods for measuring the acute toxicity of effluent and receiving waters to freshwater and marine organisms. EPA-600/4-90/027F. Office of Research and Development, Duluth, MN.
30. U.S. Environmental Protection Agency. 1994. Methods for estimating the chronic toxicity of effluents and receiving waters to freshwater organisms. EPA-600/4-91/002. Office of Research and Development, Duluth, MN.
31. U.S. Environmental Protection Agency. 1992. Toxicity identification evaluation: Characterization of chronically toxic effluents, phase I. EPA-600/6-91/005F. Office of Research and Development, Duluth, MN.
32. Wheelock CE, Severson TF, Hammock BD. 2001. Synthesis of new carboxylesterase inhibitors and evaluation of potency and water solubility. *Chem Res Toxicol* 14:1563–1572.
33. Ljungquist A, Augustinsson KB. 1971. Purification and properties of two carboxylesterases from rat-liver microsomes. *Eur J Biochem* 23:303–313.
34. Wheelock CE, Wheelock ÅM, Zhang R, Stok JE, Le Valley SE, Green CE, Hammock BD. 2003. Evaluation of α-cyanoesters as fluorescent substrates for examining interindividual variation in general and pyrethroid-selective esterases in human liver microsomes. *Anal Biochem* 315:208–222.
35. Hunt JW, Anderson BS, Phillips BM, Nicely PN, Tjeerdema RS, Puckett HM, Stephenson M, Worcester K, De Vlaming V. 2003. Ambient toxicity due to chlorpyrifos and diazinon in a central California coastal watershed. *Environ Monit Assess* 82:83–112.
36. Glickman AH, Lech JJ. 1981. Hydrolysis of permethrin, a pyrethroid insecticide, by rainbow trout and mouse tissues in vitro: A comparative study. *Toxicol Appl Pharmacol* 60:186–192.
37. Ashour MBA, Hammock BD. 1987. Substituted trifluoroketones as potent, selective inhibitors of mammalian carboxylesterases. *Biochem Pharmacol* 36:1869–1879.
38. Satoh T, Hosokawa M. 1998. The mammalian carboxylesterases: From molecules to functions. *Annu Rev Pharmacol Toxicol* 38: 257–288.
39. Laskowski DA. 2002. Physical and chemical properties of pyrethroids. *Rev Environ Contam Toxicol* 174:49–170.