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WHOLE-SEDIMENT TOXICITY IDENTIFICATION EVALUATION TOOLS FOR PYRETHROID INSECTICIDES: I. PIPERONYL BUTOXIDE ADDITION

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Abstract—Piperonyl butoxide (PBO) is a synergist used in some pyrethroid and pyrethrin pesticide products and has been used in toxicity identification evaluations (TIEs) of water samples to indicate organophosphate or pyrethroid-related toxicity. Methods were developed and validated for use of PBO as a TIE tool in whole-sediment testing to help establish if pyrethroids are the cause of toxicity observed in field-collected sediments. Pyrethroid toxicity was increased slightly more than twofold in 10-d sediment toxicity tests with *Hyalella azteca* exposed to 25 μg/L of PBO in the overlying water. This concentration was found to be effective for sediment TIE use, but it is well below that used in previous water and pore-water TIEs with PBO. The effect of PBO on the toxicity of several nonpyrethroids also was tested. Toxicity of the organophosphate chlorpyrifos was reduced by PBO, and the compound had no effect on toxicity of cadmium, DDT, or fluoranthene. Mixtures of the pyrethroid bifenthrin and chlorpyrifos were tested to determine the ability of PBO addition to identify pyrethroid toxicity when organophosphates were present in a sample. The PBO-induced increase in pyrethroid toxicity was not seen when chlorpyrifos was present at or above equitoxic concentrations with the pyrethroid. In the vast majority of field samples, however, the presence of chlorpyrifos does not interfere with use of PBO to identify pyrethroid toxicity. Eleven field sediments or soils containing pyrethroids and/or chlorpyrifos were used to validate the method. Characterization of the causative agent as determined by PBO addition was consistent with confirmation by chemical analysis and comparison to known toxicity thresholds in 10 of the 11 sediments.

Keywords—Toxicity identification evaluation Pyrethroids Chlorpyrifos *Hyalella azteca* Sediment toxicity

INTRODUCTION

Pyrethroids are pesticides with widespread applications in agricultural and urban settings. In California (USA), for example, commercial pyrethroid use (excluding retail sales) in 2005 totaled 143,000 kg in agriculture and 325,000 kg for nonagricultural applications, which consist primarily of pest control around buildings and landscape maintenance (http://www.cdpr.ca.gov/docs/pur/purmain/htm). As a result of their ubiquitous use, pyrethroid residues have been detected in sediments from water bodies ranging from small agricultural drains to major rivers [1–3], with the vast majority of available data coming from California. These residues have reached acutely toxic thresholds for the amphipod *Hyalella azteca* in approximately 20% of the samples collected throughout California agricultural regions [2] and with even greater frequency in urban creeks [4].

Although widespread sediment toxicity to *H. azteca* has been observed in previous studies, correlation between the chemical concentrations in the sample and the toxicity thresholds measured in the laboratory has been the principal method available to identify pyrethroids as a contributing source of the observed toxicity. This approach, incorporating toxicity units (TUs) to allow comparison between compounds with different relative toxicities, has consistently provided good predictive capability and strong evidence of pyrethroid-related toxicity to *H. azteca* [2–5].

To help establish that pyrethroids are the responsible toxicant in sediment samples, it would be desirable to have multiple lines of evidence. Toxicity identification evaluation (TIE) procedures are useful in this regard. Typically, TIEs have used a tiered approach to determine causality. First, the sample is manipulated to alter its toxicity in a predictable manner unique to certain classes of compounds, and the toxicant is broadly characterized. Subsequent TIE steps may identify the specific compound and confirm its role in causing toxicity. Procedures to conduct TIEs with water samples are well established [6–8], and piperonyl butoxide (PBO) has been a useful tool in water-focused TIEs. The toxicological significance of PBO lies in its ability to inhibit mixed-function oxidase (MFO) enzymes. Thus, PBO can reduce toxicity of some organophosphate pesticides that require activation by MFO activity, and it can enhance toxicity of compounds, such as pyrethroids, that are detoxified by this pathway. The addition of PBO to the water, typically at a concentration of 100 to 200 μg/L, and a resulting decrease in toxicity frequently have been used to implicate the organophosphates, diazinon and chlorpyrifos, as the responsible agents for toxicity to cladocerans [9–13].

Piperonyl butoxide has proven to be equally useful for pore-water TIEs, but in this matrix, it usually has implicated pyrethroids by a resulting increase in toxicity. Addition of 500 μg/L of PBO to pore water (and an increased toxicity to *H. azteca*) has been an important piece of evidence in TIEs implicating pyrethroids [14–16]. Addition of an esterase enzyme is another TIE technique intended to help identify pyrethroid-related toxicity in pore water [17].

The present study examines the utility of PBO addition as a TIE tool when applied to whole sediments. A number of whole-sediment TIE procedures are available to characterize the responsible toxicant as an organic compound, such as addition to sediment of powdered coconut charcoal [18] or a nonpolar resin [19,20]. These methods likely mitigate pyre-

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throid toxicity, but they are not specific to this compound class and likely reduce the toxicity of any hydrophobic organic compound. The use of PBO, if applicable to whole sediments, could be a valuable additional tool by providing greater specificity in toxicant characterization. It has been shown that addition of PBO to overlying water can reduce pyrethroid toxicity to *H. azteca* [21,22], and in one instance, PBO has been used in a whole-sediment TIE context by addition of 500 μg/L of PBO to the overlying water to provide evidence implicating sediment-associated pyrethroids as the cause of toxicity [23].

The well-documented success of PBO as a TIE tool in a water matrix, and the more limited use of PBO in whole-sediment testing, suggest that the compound may be a useful addition to whole-sediment TIE methods if its efficacy can be investigated more systematically. The present study was intended to provide more extensive evaluation of its merits through four lines of investigation. First, the acutely toxic concentration of PBO was determined so as to remain well below this level for TIE use. Second, the effect of PBO on some representative pyrethroid and nonpyrethroid toxicants was determined. Third, the effect of PBO was evaluated in the presence of pesticide mixtures in which it may enhance the toxicity of a pyrethroid while simultaneously decreasing the toxicity of an organophosphate. Finally, the PBO technique was tested on field-collected samples.

MATERIALS AND METHODS

Toxicity testing

Ten-day sediment toxicity tests with the freshwater amphipod *H. azteca* were performed using standard protocols [24]. All toxicity tests were conducted at 23°C with a 16:8-h light:dark photoperiod in 400-ml beakers containing 75 ml of sediment and 300 ml of moderately hard water reconstituted from Milli-Q® purified water (Millipore, Billerica, MA, USA). Ten amphipods (age, 7–14 d) were added to each beaker at test initiation. Most tests were done as dilution series, with three to four replicate beakers per concentration; three field sediments were tested at a single concentration with five replicates. A yeast, cerophyll, and trout chow mixture was fed daily during the 10-d tests. Water changes were performed every day, and water samples were taken before water renewal after 24 h and again on day 10 for analysis of temperature, dissolved oxygen, pH, conductivity, alkalinity, hardness, and ammonia. Temperature and dissolved oxygen were monitored regularly throughout the test. Sediment samples were taken for chemical analysis at test initiation. Tests were terminated by sieving sediments on a 425-μm screen and counting the surviving amphipods.

In previous studies, the 10-d median lethal concentrations (LC50s) for permethrin and bifenthrin were determined for *H. azteca* exposed to the pyrethroids via sediment and simultaneously to PBO in the overlying water [21,22]. Various PBO concentrations ranging from 2 to 56 μg/L were tested. Bifenthrin and permethrin LC50s responded in very similar fashion to PBO. Concentrations of approximately 4 μg/L were sufficient to significantly increase pyrethroid toxicity by a factor of 1.6, with nearly a fourfold increase in toxicity observed at 56 μg/L of PBO. Based on these data, a PBO concentration of approximately 25 μg/L was expected to produce a twofold increase in toxicity of pyrethroids, and this concentration was used for all subsequent PBO exposures conducted in the present study. Piperonyl butoxide (Sigma, St Louis, MO, USA) in methanol was added to the overlying water. Water was prepared daily by adding 10 μl/L of methanol to achieve a PBO concentration of 25 μg/L. Every day, approximately 80% of the water volume in each beaker was removed and replaced with fresh PBO solution or water, depending on the treatment. Most toxicity tests also included a solvent control (10 μl/L of methanol without PBO).

Sediments were tested in dilution series to determine LC50s with and without 25 μg/L of PBO in the overlying water. After preliminary testing to determine appropriate dilution ranges for a given sediment, the sediments were diluted with clean control sediment to at least five concentrations (e.g., 100, 50, 25, 12, and 6%), with three or four replicates per concentration. Control and test sediments were mixed thoroughly with a mixing attachment in an electric drill, and tests were initiated 24 to 48 h later. Paired tests with and without PBO were run simultaneously.

A 20:80 blend of sediment from Lake Anza Reservoir in Berkeley (CA, USA) and San Pablo Dam Reservoir (Orinda, CA, USA) was used as a control sediment in spiking experiments and dilution of field sediments in the present study. Sediments were sieved on a 1-mm screen, and material passing through the screen was homogenized. The two source sediments were blended, then frozen until use. Chemical analysis showed no detectable concentrations (<1 ng/g) of pyrethroids, and the material had an organic carbon (OC) content of 1.7 to 2.1%.

Toxicity data were analyzed using ToxCalc® 5.0 software (Tidepool Scientific Software, McKinleyville, CA, USA). The Spearman–Karber method was used to determine LC50s and associated confidence intervals (CIs). Abbott's correction was applied when necessary to account for control mortality.

Toxicity of PBO

Using control sediment, PBO was added to the overlying water in a six-step concentration series ranging from 70 to 900 μg/L. The LC50 of the compound to *H. azteca* was determined following 10-d sediment testing procedures as described above.

Effect of PBO on known toxicants

To determine the effect of PBO on a variety of pyrethroid and nonpyrethroid toxicants, LC50s with and without PBO exposure were determined for five representative compounds: Bifenthrin (a pyrethroid), cadmium (a metal), chlorpyrifos (an organophosphate), DDT (an organochlorine), and flouranthene (a polycyclic aromatic hydrocarbon). The DDT and fluoranthene were purchased from Sigma. Cadmium (as $CdCl_2$) was purchased from Fisher Scientific (Waltham, MA, USA). Chlorpyrifos and bifenthrin were purchased from Chem Service (West Chester, PA, USA). Pesticides and fluoranthene were dissolved in an acetone carrier and spiked into sediments using less than 200 μl acetone/kg wet sediment. This concentration of acetone has been shown previously to have no effect on *H. azteca* survival [3]. Cadmium was dissolved in water for sediment spiking. After spiking, the sediments were mixed with a mixing attachment in an electric drill and aged at 4°C for 12 to 26 d (all pesticides) or for 7 to 8 d (for cadmium). Aliquots were removed for chemical verification at toxicity test initiation.

Organophosphate/pyrethroid mixtures

Because PBO inhibits MFO enzymes, exposure to PBO was expected to decrease organophosphate toxicity and increase

pyrethroid toxicity. These opposing responses may obscure a clear PBO signal from either organophosphates or pyrethroids when both are present in a sample. Therefore, it was necessary to determine what effect PBO has in a sample containing both organophosphates and pyrethroids.

To address this question, sediment 10-d LC50s were determined for chlorpyrifos and bifenthrin individually, and then tests were conducted using these compounds at five different ratios: 0:1, 1:3, 1:1, 3:1, and 1:0. The ratios were based on TUs of each compound. A TU, expressed on an OC-normalized basis because of compound hydrophobicity, is defined as

$$\text{toxic units (TU)} = (\text{actual concentration in the sediment on an OC basis}) \div (\text{10-d } \textit{H. azteca} \text{ sediment LC50 on an OC basis})$$

For example, the LC50 of chlorpyrifos was 1.77 μg/g OC, and that of bifenthrin was 0.26 μg/g OC. A sediment containing one total TU in a 1:1 ratio would contain equitoxic amounts of chlorpyrifos (0.5 TU = 0.89 μg/g OC) and bifenthrin (0.5 TU = 0.13 μg/g OC). To determine the LC50 at a 1:1 ratio, a six-step concentration series (plus a control) was prepared, extending from 0.2 total TU (0.1 TU chlorpyrifos = 0.177 μg/g OC; 0.1 TU bifenthrin = 0.026 μg/g OC) to 2.8 total TU. Four replicates beakers were used at each concentration step.

Similar concentration series were done using ratios of 1:3, 1:1, and 3:1, and each concentration series was tested in parallel tests with and without 25 μg/L of PBO in the overlying water. Thus, it was possible to determine the influence of PBO as the contribution to toxicity from an organophosphate decreased over the gradient while the contribution of a pyrethroid simultaneously increased.

Field-sediment testing

Nine field sediments and two soils were used to validate the PBO method developed in the present study. Two of the sediments were from urban water bodies: GSLI from Gilsizer Slough (Yuba City, CA, USA), and SRCP from Strong Ranch Slough (Sacramento, CA, USA). Seven of the sediments were from water bodies draining agricultural lands: SN1 from Natividad Creek (Salinas, CA, USA), CS15 from Spring Creek (Williams, CA, USA), CS12 from an unnamed agricultural drain near Orland (CA, USA), FT19 from an unnamed agricultural drain near Orange Cove (CA, USA), SED15 from an unnamed agricultural drain near Alpaugh (CA, USA), SED11 from an unnamed agricultural drain near Stockton (CA, USA), and HOSP from Hospital Creek near Vernalis (CA, USA). Two farm soils, O2 and O21, were from a pear orchard and a rice farm near Sacramento at which esfenvalerate and lambda-cyhalothrin, respectively, had been applied. All these sediments contained pyrethroids and/or chlorpyrifos at concentrations acutely toxic to *H. azteca*. Approximately 20 organochlorine pesticides were quantified and were below toxic levels, but the possibility cannot be ruled out that additional unquantified toxicants may be in some of these sediments given their diverse sources.

The sediments typically were collected over a 30-m reach of the water body by using a stainless-steel scoop to skim the upper 1 to 2 cm of the sediment column. Soil was collected over a 100-m^2 area of farmland in a similar manner. Samples were composited in the field into a 4-L glass jar and returned to the laboratory on ice. Sediments were homogenized by hand-mixing in a stainless-steel bowl, and aliquots were taken for pesticide analysis and total OC and then stored at 4°C until toxicity testing. All sediments were chosen for PBO testing based on their initially high toxicity to *H. azteca* in standard 10-d sediment toxicity tests.

Chemical analyses

Spiked sediment samples and field-collected sediments were analyzed for pyrethroids, chlorpyrifos, and DDT following the methods described by You et al. [25] on an Agilent 6890 series gas chromatograph equipped with an Agilent 7683 autosampler and an electron-capture detector (Agilent Technologies, Palo Alto, CA, USA). Two columns from Agilent, a HP-5MS (length, 30 m; inner diameter, 0.25 mm; film thickness, 0.25 μm) and a DB-608 (length, 30 m; inner diameter, 0.25 mm; film thickness, 0.25μm), were used for confirmation. Sediment was sonicated with acetone and methylene chloride, and the extract was cleaned with deactivated Florisil (Fisher Scientific). Ethyl ether and hexane were used as elution solvents, and the eluent was evaporated and redissolved in 2 ml of hexane. The extract was used for pesticide analysis after dilution to a concentration within the calibration curve.

Aqueous PBO concentrations were not confirmed in the present study, but previous work using the same procedures has shown initial spiking accuracy of 112% ± 11% of nominal concentrations (mean ± standard deviation). Recovery of PBO after 24 h in the test chambers was 70% ± 10%, presumably because of photolysis [21]. Test sediments were analyzed for OC content on a CE-440 elemental analyzer from Exeter Analytical (Chelmsford, MA, USA) following acid-vapor treatment to remove inorganic carbon.

RESULTS

Chemical recovery

In tests involving addition of a known toxicant to the sediment, a subsample at one concentration step was set aside and used for analytical confirmation of the nominal spike concentration. Recovery of chlorpyrifos and bifenthrin was 67 to 71 and 83%, respectively. Cadmium recovery was 86%, and DDT recovery was 70%. Fluoranthene concentrations were not confirmed.

Control survival

Survival of *H. azteca* in control sediments ranged from 90 to 100% (average, 96%). No appreciable difference in survival was observed in solvent controls for the acetone carrier added to the sediment in the pesticide and fluoranthene additions (mean, 94%), the solvent control for the methanol carrier for the PBO in the overlying water (mean, 98%), or PBO at 25 μg/L in the overlying water in the absence of sediment toxicants (mean, 96%).

Toxicity of PBO

No effect of PBO on *H. azteca* survival was observed at concentrations up to 320 μg/L. At the next concentration step of 540 μg/L, survival over a 10-d exposure was reduced to 60%, with a calculated LC50 of 555 μg/L (95% CI, 493–625 μg/L). On the basis of these results, the 25 μg/L concentration of PBO initially chosen for TIEs was considered to be suitable for continued use.

Table 1. Ten-day sediment median lethal concentration (LC50) and 95% confidence interval for the contaminants studied, with and without the presence of piperonyl butoxide (PBO) in the overlying water[a]

Contaminant	10-d *Hyalella azteca* LC50		LC50 ratio (without PBO:with PBO)
	Without PBO	With PBO (25 μg/L)	
Bifenthrin (μg/g OC)	0.26 (0.24–0.28)	0.12 (0.11–0.14)	2.2
Cadmium (mg/kg)	134 (119–150)	159 (140–181)	0.84 (NS)
Chlorpyrifos (μg/g OC)	1.77 (1.47–1.96)	2.65 (2.34–3.01)	0.67
DDT (μg/g OC)	128 (108–154)	142 (121–156)	0.90 (NS)
Fluoranthene (μg/g OC)			
First trial	8,136 (4,973–13,307)	9,152 (5,849–14,323)	0.89 (NS)
Second trial	8,510 (7,508–9,645)	9,506 (8,661–10,433)	0.90 (NS)

[a] OC = organic carbon; NS = no statistically significant difference between the LC50s.

Effect of PBO on known toxicants

By inhibiting the biotransformation and detoxification of pyrethroids, PBO increases the toxicity of the parent compound. The bifenthrin LC50 in spiked sediment without addition of PBO was 0.26 μg/g OC (95% CI, 0.24–0.28 μg/g OC), comparable to previously reported values of 0.37 to 0.63 μg/g OC [5] and 0.62 μg/g OC [22]. In the presence of 25 μg/L of PBO in the overlying water, the LC50 decreased to 0.12 μg/g OC (95% CI, 0.11–0.14 μg/g OC), reflecting an increased toxicity by a factor of 2.2 (Table 1).

Because many organophosphates are metabolized to the toxic form by MFO-mediated biotransformation, inhibition of this process by addition of PBO would be expected to decrease their toxicity. As predicted, chlorpyrifos was 1.5-fold less toxic with exposure to PBO. The LC50 increased from 1.77 μg/g OC (95% CI, 1.47–1.96 μg/g OC) without PBO to 2.65 μg/g OC (95% CI, 2.34–3.01 μg/g OC) with PBO.

Cadmium and DDT toxicities were not significantly affected by PBO exposure, as would be expected given that neither is biotransformed by MFO enzymes. Cadmium LC50s determined in the present study were 134 to 159 mg/kg. The measured LC50s for DDT (128–142 μg/g OC) were similar to literature values of 100 to 470 μg/g OC for DDT [26,27].

Fluoranthene toxicity was not affected by the presence of PBO, with LC50s of 8,136 to 9,506 μg/g OC. Because MFO enzymes act on polycyclic aromatic hydrocarbons, an effect was anticipated, but a second trial yielded the same negative results as the first trial.

Organophosphate/pyrethroid mixtures

Median lethal concentrations for chlorpyrifos (1.77 μg/g OC) and bifenthrin (0.26 μg/g OC) were first determined independently in the absence of PBO (Table 1). These LC50s were then used to prepare mixtures of the two compounds in which they were in an equitoxic ratio (1:1 ratio of TUs) as well as mixtures in which chlorpyrifos made up three-fourths of the toxicity (3:1) and in which bifenthrin made up three-fourths of the toxicity (1:3). At each ratio, a concentration series was done extending from 0.2 to 2.8 total TU. Survival at each step along the concentration series was used to determine the LC50, expressed as total TUs. Parallel tests were performed with and without PBO.

Results are presented as the ratio of LC50 without PBO to the LC50 with PBO (Fig. 1). When only chlorpyrifos was present (1:0 ratio in Fig. 1), the presence of PBO significantly reduced toxicity. The ratio of chlopyrifos LC50 without PBO to that with PBO was 0.67. With addition of a relatively small amount of bifenthrin, such that the pyrethroid comprised only one-fourth of the toxicity in the sample (3:1 ratio in Fig. 1), the PBO-induced reduction of chlorpyrifos toxicity was no longer evident, and in fact, the LC50 of the mixture was essentially unaffected by addition of PBO.

Conversely, when only bifenthrin was present (0:1 ratio of Fig. 1), the presence of PBO substantially increased toxicity. The ratio of the bifenthrin LC50 without PBO to that with PBO was 2.2. Comparison of this ratio to the chlorpyrifos-only ratio of 0.67 noted above indicates that PBO was more effective at increasing pyrethroid toxicity than it was at mitigating organophosphate toxicity. Addition of a small amount of chlorpyrifos (1:3 ratio of Fig. 1) slightly diminished the PBO enhancement of pyrethroid toxicity, but it remained quite evident. When chlorpyrifos was present at an equitoxic ratio (1:1 ratio of Fig. 1), the increased toxicity as a result of PBO was no longer statistically significant. The 95% CIs of the LC50s with and without PBO barely overlapped, indicating

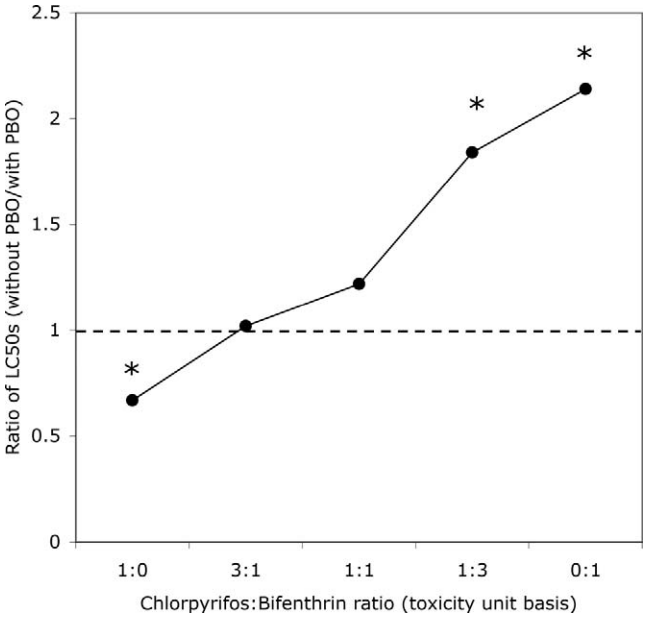

Fig. 1. The effect of piperonyl butoxide (PBO) on sediment toxicity as the responsible toxicant gradually shifts from chlorpyrifos (left) to bifenthrin (right). The horizontal line indicates the point of no PBO effect on toxicity; values above the line indicate an increase in toxicity because of PBO and values below the line a decrease. The asterisks indicate points for which the toxicity was significantly changed by the presence of PBO (nonoverlapping 95% confidence intervals of the median lethal concentrations [LC50s].

Table 2. Toxicity of laboratory-spiked and field-collected sediments as measured by the 10-d sediment median lethal concentration (LC50) to *Hyalella azteca* when tested with and without 25 μg/L of piperonyl butoxide (PBO)[a]

Sample	Mortality in original sample (%)	Toxicant > 0.2 TU	TU	Concn. (ng/g)	LC50 without PBO (95% confidence interval)	LC50 with PBO (95% confidence interval)	Ratio (without PBO:with PBO)
Spiked compounds (μg/g OC)							
Bifenthrin[b]					0.26 (0.24–0.28)	0.12 (0.11–0.14)	2.2
Bifenthrin[c]					0.61 (0.54–0.68)	0.27 (0.25–0.29)	2.3
Permethrin[c]					14.2 (11.8–17.1)	~7.2	2.0
Chlorpyrifos[b]					1.77 (1.47–1.96)	2.65 (2.34–3.01)	0.7
Field-collected sediments (%)							
GSLI (11/21/05)	91	Bifenthrin	1.8	33	27.32 (23.74–31.44)	11.01 (9.38–12.92)	2.5
		Lambda-cyhalothrin	1.0	17			
		Cypermethrin	0.6	8.3			
		Cyfluthrin	0.3	13			
		Permethrin	0.2	80			
O21 (6/27/05)	100	Lambda-cyhalothrin	6.7	48	5.61 (4.77–6.60)	2.05 (1.56–2.70)	2.7
SRCP (8/12/05)	100	Bifenthrin	2.3	48	Slightly >6	1.62 (1.42–1.86)	~3.7
		Cyfluthrin	0.6	26			
		Lambda-cyhalothrin	0.4	6.6			
		Cypermethrin	0.3	4.3			
SN1 (9/23/05)	90	Lambda-cyhalothrin	1.7	7.0	29.8 (23.3–38.2)	19.8 (16.2–24.3)	1.5 (NS)
SED15 (8/18/05)	100	Bifenthrin	26.1	110	0.61 (0.50–0.74)	0.30 (0.22–0.40)	2.0
		Chlorpyrifos	24.4	588			
		Lambda-cyhalothrin	9.9	36			
CS15 (8/9/05)	96	Chlorpyrifos	1.6	23	33.98 (29.43–39.23)	19.09 (16.11–22.62)	1.8
		Bifenthrin	0.6	1.6			
		Esfenvalerate	0.2	1.8			
CS12 (8/9/05)	99	Chlorpyrifos	0.8	14	22.70 (19.79–26.05)	8.62 (7.71–9.62)	2.6
		Fenpropathrin	0.6[d]	52			
FT19 (8/19/05)	100	Chlorpyrifos	5.4	150	7.29 (6.22–8.55)	10.86 (9.55–11.35)	0.7

[a] All compounds ending with *-thrin* (as well as esfenvalerate) are pyrethroids, and chlorpyrifos is an organophosphate insecticide. The LC50s are given as μg/g organic carbon (OC) for spiked compounds and as the percentage original sediment for the field-collected material. The ratio of the LC50s without and with PBO is shown. The locations from which the various sediments (e.g., GSLI and O21) were collected are given in the text. NS = no significant difference as indicated by overlapping confidence intervals; TU = toxic units.

[b] Present study (see Table 1).

[c] Weston et al. [22].

[d] Fenpropathrin TUs based on an estimated LC50. The 5th percentile of water LC50s of fenpropathrin in tests with a variety of aquatic life is 114 ng/L, compared to 68 ng/L for permethrin [35]. This ratio was used to adjust the 10.83 μg/g OC *H. azteca* sediment LC50 for permethrin [5] to an estimated 18.2 μg/g OC sediment LC50 for fenpropathrin.

that the 1:1 equitoxic ratio is a threshold beyond which additional chlorpyrifos obscures the effect of PBO on pyrethroid toxicity. Given that at least an equitoxic chlorpyrifos–bifenthrin mixture is necessary to obscure the PBO enhancement of pyrethroid toxicity, and given that bifenthrin is approximately sixfold more toxic than chlorpyrifos to *H. azteca* (comparison of LC50s of Table 1), the chlorpyrifos concentration in a sediment sample would need to be at least approximately sixfold the bifenthrin concentration for PBO to fail to elicit a typical pyrethroid response (an increase in toxicity).

Field-sediment testing

Eight field sediments were tested in dilution series, using control sediment as the diluent, to determine 10-d LC50s to *H. azteca* with and without exposure to 25 μg/L of PBO (Table 2). All sediments had been tested previously in undiluted form and found to be highly toxic to *H. azteca*. They also had been analyzed chemically and found to contain pyrethroids and/or chlorpyrifos in high enough concentrations to expect acute toxicity.

Four of the sediments (GSLI, O21, SRCP, and SN1) contained only pyrethroids among the analytes for which the actual concentrations exceeded those concentrations known to be toxic to *H. azteca* [5,28]. Pyrethroid TUs in these samples ranged from approximately two to seven, with bifenthrin and lambda-cyhalothrin often making the greatest contribution to toxicity. In all four cases, addition of PBO to the overlying water increased the toxicity of the sample, but in one of these instances (sample SN1), a slight overlap was found between the CIs of the LC50s with and without PBO, making the comparison not statistically significant. With this exception, the other pyrethroid-containing sediments showed the expected response of increased toxicity in the presence of PBO, approximately doubling toxicity in two cases, as did the pyrethroids in the spiked trials, and showing an even greater effect in one instance (sample SRCP).

Three of the eight field sediments (SED15, CS15, and CS12) contained substantial concentrations of both chlorpyrifos and pyrethroids, with the chlorpyrifos to pyrethroid TU ratio of the two groups ranging from approximately 0.7:1 to 2:1. The laboratory-spiking study with chlorpyrifos and bifenthrin had indicated that a PBO effect would begin to become obscured when the chlorpyrifos contribution to toxicity equaled that of the pyrethroids, but in all three of the field sediments, a strong PBO response of increased toxicity remained, indicative of the presence of the pyrethroids. Thus, the potential complications in identifying pyrethroid-induced toxicity in the presence of co-occurring organophosphates may be even more minimal than initially suggested.

Finally, one of the eight sediments (FT19) contained only

chlorpyrifos among the toxicants known to be in high enough concentration to cause acute toxicity. In the presence of PBO, this sample showed a reduction in toxicity, with a ratio of LC50s without PBO to those with PBO of 0.7—precisely the ratio seen in the spiked trials with chlorpyrifos.

Three additional sediments were tested at a single concentration rather than a dilution series as in Table 2. These sediments all contained several TUs of pyrethroids (sediments SED11 and O2 contained esfenvalerate, and sediment HOSP contained bifenthrin and lambda-cyhalothrin). Because all these sediments caused complete *H. azteca* mortality when undiluted, preliminary tests were used to determine a dilution with control sediment that yielded partial survival (52, 66, and 88% survival in SED11, O2, and HOSP, respectively). In parallel treatments with PBO in the overlying water, complete mortality was observed in all three sediments, providing evidence in support of the putative pyrethroid toxicants. These single-concentration trials were done early in our development of the PBO procedures, when 50 μg/L of PBO was being used, but otherwise followed the same procedures as in all other tests described herein using 25 μg/L.

DISCUSSION

Selection of PBO concentration

For TIE purposes, a concentration of PBO that reliably and significantly increases the toxicity of pyrethroids is desired. The 25 μg/L concentration used in the present study achieved slightly more than a doubling of pyrethroid toxicity, reliably provided a measurable increase in toxicity of field-collected samples, and is far below the PBO concentrations capable of causing acute toxicity to *H. azteca* (10-d LC50 of 555 μg/L in the present study, and 96-h LC50 of 530 μg/L in the study by Ankley and Collyard [29]). Past uses of PBO as a TIE tool have used higher concentrations, such as the 100 to 200 μg/L used to test for organophosphate toxicity to *Ceriodaphnia dubia* [9–11,13]. Past uses of PBO with *H. azteca* often have used 500 μg/L, either when testing pore water or by addition of PBO to the water overlying bulk sediment [14,15,23]. Not only do these higher concentrations raise concerns regarding toxicity of the PBO itself when used with *H. azteca*, they also carry a substantial risk of false-positive responses. The increase in pyrethroid toxicity achieved is a function of the log of the PBO concentration [30]. The greater the PBO concentration, the less pyrethroid needed to see a PBO-mediated enhancement in toxicity. Therefore, the higher the PBO concentration used, the greater the chance that a toxicologically insignificant amount of pyrethroid could be made to become toxic, leading to the incorrect conclusion that the pyrethroid was responsible for toxicity in the original sample. This concern applies to any TIE procedure that, like PBO addition, amplifies rather than eliminates toxicity. In such instances, the smallest toxicity increase necessary to achieve a measurable response is desired. The doubling of toxicity observed with 25 μg/L of PBO was sufficient to see this response but also insures that if the response is seen, the concentration of pyrethroid in the original sample was at least very near if not above acutely toxic levels in the unaltered sample.

Potential of PBO addition as a TIE technique

Three sediments evaluated in the present study were tested at a single concentration, but most sediments were tested in a dilution series to derive LC50s with and without PBO. The dilution series approach was used to better quantify the effect of PBO (e.g., a factor-of-two reduction in LC50, indicating a doubling of toxicity) and because it allowed working with sediments that, when undiluted, caused complete mortality, making it impossible to show greater mortality on addition of PBO. For routine use of the PBO procedure, however, most testing is likely to be done at only a single sediment concentration. For sediments showing at least some survival, the sediment could be used unaltered, and the addition of PBO is straightforward. For those sediments causing complete mortality, it would be necessary to conduct preliminary tests to determine a dilution at which partial survival occurred for PBO-enhanced mortality to be demonstrated. The window of acceptable dilution probably is quite broad, and a survival rate of between 20 and 80% should be sufficient to statistically demonstrate greater toxicity in a paired comparison with and without PBO.

One advantage of PBO addition as a TIE tool is its ability to identify some toxicants (e.g., pyrethroids) by an increase in toxicity if they are detoxified by MFO activity and to identify others (e.g., many organophosphates) by a decrease in toxicity if they are activated by these enzymes. This bidirectional response is advantageous in terms of maximizing the information gained by a single TIE procedure, but it raises concerns that the expected toxicity response to PBO addition could be masked if members of both compound classes are simultaneously present, one increasing in toxicity in response to PBO and the other decreasing. With regards to pyrethroids and organophosphates, this concern is somewhat mitigated by their relative hydrophobicities. Organophosphates tend to be less sediment-associated and more water soluble than the pyrethroids; therefore, the more hydrophilic members of the class may not be in sediments.

Chlorpyrifos is one of the more hydrophobic of the widely used, metabolically activated organophosphates, and it has been shown to be present in agriculture-affected sediments [23]. The data from the current study indicate that the presence of pyrethroids could readily obscure the decrease in toxicity expected of chlorpyrifos with the addition of PBO. Because PBO is more effective at increasing pyrethroid toxicity than it is in decreasing chlorpyrifos toxicity, very little pyrethroid in the sample is necessary to mask the chlorpyrifos response. Thus, whereas a decrease in toxicity seen with the addition of PBO can be used as evidence to implicate an organophosphate as the toxicant, the absence of this response does not eliminate this class as a contributor to toxicity unless the co-occurrence of a pyrethroid can be ruled out.

Conversely, the potential for chlorpyrifos to mask the PBO-mediated increase in toxicity expected of a pyrethroid will depend on the specific pyrethroid present. The chlorpyrifos–bifenthrin mixture experiments indicated that a PBO-induced increase in pyrethroid toxicity was not seen when the chlorpyrifos TUs in a sample at least equaled the pyrethroid TUs. Assuming PBO affects all pyrethroids similarly [22], previously published data on the relative toxicities of the pyrethroids [5,29] can be used to estimate the concentration of chlorpyrifos needed to mask the expected pyrethroid response to PBO (Table 3). For the pyrethroids such as bifenthrin, cypermethrin, and lambda-cyhalothrin, all of which are considerably more toxic to *H. azteca* than is chlorpyrifos, a chlorpyrifos concentration six- to eightfold greater than that of the pyrethroid would have to be present in the sample to obscure the pyrethroid response to PBO. On the other hand, the pyrethroid

Table 3. The amount of chlorpyrifos necessary to achieve an equitoxic mixture with selected pyrethroids and, thus, mask the increase in toxicity expected by piperonyl butoxide (PBO) addition[a]

	Hyalella azteca 10-d LC50 (μg/g OC)	Chlorpyrifos concentration required to prevent pyrethroid detection[b]
Bifenthrin[c]	0.52	5.7×
Cypermethrin	0.38	7.8×
Cyfluthrin	1.08	2.8×
Deltamethrin	0.79	3.8×
Esfenvalerate	1.54	1.9×
Lambda-cyhalothrin	0.45	6.6×
Permethrin	10.83	0.3×
Chlorpyrifos[d]	2.97	

[a] Data derived from published median lethal concentrations (LC50s) [5,28].
[b] X = pyrethroid concentration.
[c] The bifenthrin LC50 to *H. azteca* of 0.52 μg/g organic carbon (OC) derived from multiple sediments [5] has been used rather than the single-sediment value of 0.26 μg/g OC from the present study.
[d] The chlorpyrifos LC50 to *H. azteca* of 1.77 μg/g OC determined in the present study has been averaged with 2.96 μg/g OC [32] and 4.17 μg/g OC (unpublished data).

permethrin is among the least toxic of the class to *H. azteca*; thus, chlorpyrifos concentrations one-third those of permethrin would prevent an increase in toxicity by PBO addition. Because of its relatively low toxicity, however, permethrin rarely is present in either agricultural or urban sediments at concentrations high enough to cause *H. azteca* toxicity [2–4].

Field data suggest very few instances in which the presence of chlorpyrifos would prevent identification of pyrethroid toxicity by a PBO TIE procedure. In 326 sediment samples taken from agricultural and urban-affected water bodies in California [2–4,22] (unpublished data), 155 samples contained concentrations of pyrethroids approaching acutely toxic levels for *H. azteca* (total pyrethroids, >0.5 TU), yet only five of these samples contained equal or greater TUs of chlorpyrifos and would potentially have given misleading results on addition of PBO. Thus, in the vast majority of cases (150 of 155, or 97%), PBO is expected to reliably detect the presence of pyrethroids in field sediments when they are contributing to observed toxicity without interference from co-occurring chlorpyrifos.

An increase in toxicity seen on addition of PBO is suggestive of pyrethroid-related toxicity but is not conclusive evidence, because PBO could increase toxicity of any substance detoxified by MFO activity. For example, pyrethrins (plant-derived insecticides on which the synthetic pyrethroids are based) are synergized by PBO but, because of rapid degradation, are not likely to be in environmental samples except under unusual circumstances [22]. Carbamates also show increased toxicity in the presence of PBO [31]. Carbamates are far more water soluble than the pyrethroids, however, and are unlikely to occur in sediment samples. Our tests with a variety of other representative toxicants, including an organochlorine (DDT), an organophosphate (chlorpyrifos), a polycyclic aromatic hydrocarbon (fluoranthene), and a metal (cadmium), showed either a response to PBO opposite to that of pyrethroids (i.e., chlorpyrifos) or no response to PBO at all. Nevertheless, the potential exists that other compounds not included in these trials may show an increase in toxicity because of PBO, as do the pyrethroids, so the results of a PBO-addition TIE should be used in concert with other procedures to infer causality. These other procedures might include temperature manipulation [15,16], esterase addition [17,32], any of several water-based TIE methods applied to the interstitial water [6–8], or comparison of chemical concentrations with known toxicity thresholds [5].

Another consideration of the PBO-addition method is its suitability for use in TIEs with other test organisms. First, because PBO is added to the overlying water, the organism should have close contact with this water either by virtue of being epibenthic (*H. azteca*) or actively irrigating its tube or burrow. Second, for PBO to enhance toxicity effectively, test animals must have active enzymatic detoxification of pyrethroids by the MFO system. Although various cytochrome P450 enzymes are present in nearly all taxa tested, notable differences in activity are evident, with invertebrates typically having a much lower metabolic capacity than vertebrates [33]. Therefore, some species may be inappropriate for use with the PBO addition method. Use of PBO with the oligochaete *Lumbriculus variegatus* in the presence of organophosphates did not cause the expected decrease in toxicity, presumably because of limited MFO biotransformation in this species [30]. Mollusks in general have relatively limited xenobiotic metabolism as well [34], making them useful for bioaccumulation studies but, potentially, not in TIEs with PBO addition. Both of the species typically used for sediment toxicity testing, *H. azteca* and *Chironomus dilutus* (formerly *C. tentans*), however, have shown a mitigation of organophosphate toxicity by PBO [29], suggesting active MFO biotransformation and, thus, suitability of the PBO technique to detect pyrethroid toxicity as well. Moreover, the method is relatively simple and can be readily adapted to any standard sediment toxicity testing protocol.

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