Tracking pyrethroid toxicity in surface water samples: Exposure dynamics and toxicity
identification tools for laboratory tests with *Hyalella azteca* (Amphipoda).

Linda A. Deanovic†, Marie Stillway‡, Bruce G. Hammock‡, Stephanie Fong§ and Inge Werner||

† Center for Aquatic Biology and Aquaculture, University of California, Davis, California
95616, USA

‡ School of Veterinary Medicine, Department of Anatomy, Physiology, and Cell Biology,
University of California, Davis, California 95616, USA

§State and Federal Contractors Water Agency, 1121 L Street, Suite 806, Sacramento, California,
95814, USA

|| Swiss Centre for Applied Ecotoxicology, Überlandstrasse 133, 8600 Dübendorf, Switzerland

Running Head: Tracking pyrethroid toxicity in surface water samples

Key Words: insecticides, environmental monitoring, toxicity identification evaluation, piperonyl
butoxide

Corresponding Author:

Linda Deanovic

Center for Aquatic Biology and Aquaculture

150 Mrak Hall

University of California,

Davis, California 95616, USA

530-752-8160

ladeanovic@ucdavis.edu

Abstract

Pyrethroid insecticides are commonly used in pest control and are present at toxic concentrations in surface waters of agricultural and urban areas worldwide. Monitoring is challenging due to their high hydrophobicity and low toxicity thresholds, which often fall below the detection limits of analytical methods. Standard daphnid bioassays used in surface water monitoring are not sensitive enough to protect more susceptible invertebrate species such as the amphipod *Hyaletta azteca*, and chemical loss during toxicity testing is of concern. In this study, we quantified toxicity loss during storage and testing, using both natural and synthetic water, and present a tool to enhance toxic signal strength for improved sensitivity of *H. azteca* toxicity tests. The average half-life during storage in LPDE cubitainers at 4°C of five pyrethroids (permethrin, bifenthrin, lambda-cyhalothrin, cyfluthrin, esfenvalerate) and one organophosphate (chlorpyrifos; used as reference) was 1.4 d, and piperonyl butoxide (PBO) proved an effective tool to potentiate toxicity. We conclude that toxicity tests on ambient water samples containing these hydrophobic insecticides are likely to underestimate toxicity present in the field, and mimic short pulse rather than continuous exposures. Where these chemicals are of concern, the addition of PBO during testing can yield valuable information on their presence or absence.

Introduction

Insecticides are among the most prevalent and toxic pollutants in surface waters of agricultural and urban areas worldwide [1,2]. In the USA, two of the most commonly used insecticide groups are the organophosphates (OPs) and pyrethroids [3-5]. For example, the OP chlorpyrifos was the dominant toxicant in water samples collected from the Sacramento-San Joaquin Delta, California, in the 1990s [6], and Weston et al. [4] demonstrated that pyrethroids were present at toxic concentrations in sediments and water of several northern California urban creeks. These neurotoxicants are effective at extremely low concentrations (e.g., *Ceriodaphnia dubia* 48-h LC₅₀s of 0.07 µg/L for bifenthrin, 0.14 µg/L for cyfluthrin, 0.55 µg/L for permethrin have been reported, as well as *Hyalella azteca* 48-h EC₅₀s of 0.0023 µg/L for lambda-cyhalothrin and 0.005 µg/L for cypermethrin [7-9]), which are often close to or below analytical detection limits. In California, pyrethroids have been used increasingly since OPs were banned for residential uses in 2001 (chlorpyrifos) and 2004 (diazinon), due to human health concerns [10,11].

In environmental samples the presence of unknown contaminant mixtures is the norm, and hydrophobic and highly toxic insecticides such as pyrethroids are notoriously difficult to detect. Besides analytical difficulties to reach detection limits below ng/L [12], target screening by chemical-analytical methods only allows limited assessment of toxic effects [13,14]. Standardized biological methods, which integrate the combined effects of pollutants, are therefore used routinely for water quality monitoring in several countries, including the USA [15]. In addition, toxicity testing is used to help identify the causative chemicals by means of toxicity identification evaluations (TIEs) [16]; or effect directed analysis [17].

1 In recent years it has been recognized that the euryhaline amphipod *Hyaletta azteca* is highly
2 sensitive to pyrethroid insecticides, making it an excellent species for use in toxicity tests [4,7].
3 For example, Deanovic et al. [7] compared the sensitivity and effectiveness of a chronic 10-d *H.*
4 *azteca* water column test to the standard 7-d *Ceriodaphnia dubia* test endpoints, and concluded
5 that the *H. azteca* test detected pyrethroid toxicity far more effectively than *C. dubia*. As an
6 additional advantage, *H. azteca* can be employed in tests for water quality monitoring in both
7 fresh and brackish water bodies [18-21]. While it is being used as a model species in standard
8 sediment and acute water column toxicity testing [15,22], there is currently no federally
9 standardized protocol for chronic *H. azteca* water column tests [23]. However, the *H. azteca*
10 water column test with acute and chronic endpoints has recently been implemented and applied
11 in California [24].

12
13 Although *H. azteca* is sensitive to pyrethroids at or below ng/L concentrations [7,8], the
14 physicochemical properties of pyrethroids still present challenges to effectively monitor water
15 quality using bioassays. Unlike most OPs, pyrethroids are highly hydrophobic (log K_{ows} 4-7),
16 and their strong affinity for the solid phase results in their quick adherence to sediments and
17 other surfaces [25-28]. However, this does not prevent off-site movement to surface waters at
18 concentrations that are problematic for non-target organisms[1] as off-site transport of
19 pyrethroids is most likely mediated by sediment movement [9,18,27]. Moreover, loss of these
20 chemicals may be high during sampling, storage and testing, as the compounds quickly bind to
21 containers and equipment, and have affinities to particular surfaces (e.g. pyrethroids bind more
22 readily to plastic than to glass) [5,26,29]. The toxic signal can therefore be lost, preventing
23 environmental resource managers from detecting or identifying the toxicants. It is important to

1 know how much of the compound(s) is lost in the testing process, to have TIE tools that provide
2 the weight of evidence regarding the identity of the toxicant(s), and to know when such TIE tools
3 can be effectively applied, especially when various types of sampling containers are used.

4 Enhancing the toxic signal of pyrethroids utilizing the synergist piperonyl butoxide (PBO) can
5 provide an important tool to detect the presence of pyrethroids in ambient water samples [20]. As
6 a pyrethroid synergist, it is used in numerous commercial pesticide formulations containing
7 pyrethroids. PBO inhibits cytochrome P450 enzymes, slowing xenobiotic metabolism and
8 inhibiting detoxification of the parent pyrethroid, thus increasing pyrethroid toxicity. In addition,
9 PBO prevents activation (oxonation) of OPs, and therefore decreases their toxicity. It has
10 successfully been used to identify OPs as primary toxicants in surface water samples [6]. Thus,
11 applying PBO as a TIE tool to enhance the toxicity signal of pyrethroids while reducing the OP
12 signal can add valuable information to the weight of evidence for toxicant identification needed
13 by regulators.

14
15 Toxicity Identification Evaluation protocols with *C. dubia* are well established [30,31] and have
16 been successfully applied in surface water monitoring [6]; however applying these methods
17 directly to other test species is difficult. Toxicity Identification Evaluation tests use chemicals
18 such as EDTA, sodium thiosulfate, PBO, or an adjustment of water quality parameters (e.g. pH),
19 coupled to a subsequent series of toxicity tests to identify the group of chemicals causing
20 toxicity. Sensitivity to conditions used in TIE manipulations is different for each test species and
21 must be characterized before such methods can be applied. As a general rule, TIE manipulations
22 must effectively enhance or diminish toxicity, but be non-toxic to the test species under a variety
23 of water quality conditions (e.g. water hardness) that may exist in ambient water samples. In

addition, the chemical(s) must be stable and remain bioavailable throughout the testing period and subsequent TIE manipulations in order to obtain meaningful results.

This study was conducted to improve available biological tools used to detect and identify toxicity due to pyrethroid insecticides in environmental samples and allow for better interpretation of *H. azteca* bioassay data. The Surface Water Ambient Monitoring Program (SWAMP) in California, USA, has implemented the standardization of *H. azteca* water column tests in their ambient monitoring program, which includes TIE investigations when acute toxicity is observed in the initial screening test. Therefore, we conducted three experiments. First, we quantified insecticide loss for a simulated, SWAMP and Environmental Protection Agency (EPA) compliant, 10-d *H. azteca* water column toxicity test during both sample storage and an exposure. Second, we determined the optimum concentration of the synergist PBO for use in *H. azteca* water column TIEs. The OP chlorpyrifos was used for comparison as a well-characterized, environmentally relevant, and similarly hydrophobic reference substance. Finally, we determined the potentiation factor for 25 µg/L of PBO for bifenthrin and permethrin to quantify the synergistic effects on *H. azteca*.

Materials and methods

Analytical chemistry

Analytical grade pesticides were obtained from ChemService (West Chester, PA): permethrin (CAS 52645-53-1), bifenthrin (CAS 82657-04-3), lambda-cyhalothrin (CAS 91465-08-6), cyfluthrin (CAS 68359-37-5), esfenvalerate (CAS 66230-04-4), and chlorpyrifos (CAS 2921-88-

2). Pesticide grade methanol was used as a carrier for the pesticides and did not exceed 0.05% in experimental solutions.

Pesticide concentrations in experimental solutions were quantified at the California Department of Fish and Wildlife, Water Pollution Control Laboratory (Rancho Cordova, CA). Water samples were collected in 1-L glass amber bottles and preserved with 10 mL of dichloromethane, stored in the dark at 4°C, and extracted within 7 d of sample receipt. Extraction procedures followed USEPA Method 3510C – Separatory Funnel Liquid-Liquid Extraction. One-liter samples were fortified with the surrogates triphenyl phosphate and dibromooctafluorobiphenyl to monitor extraction efficiency and extracted twice with dichloromethane using a mechanical rotating extractor. Extracts were dried using sodium sulfate, concentrated and solvent exchanged with petroleum ether using Kuderna-Danish evaporative glassware equipped with a 3-ball Snyder column followed with a micro-Snyder apparatus and adjusted to a final volume of 2 mL in iso-octane. Final extracts were analyzed for OP pesticides using USEPA method 8141AM, and for pyrethroids using USEPA method 8081BM. OP pesticides were analyzed using a dual column high resolution gas chromatography with flame photometric detectors in phosphorous mode. The five pyrethroids were measured as six analytes (bifenthrin, cyfluthrin, esfenvalerate, lambda cyhalothrin, cis-permethrin and trans-permethrin) using a dual column high resolution gas chromatograph equipped with an electron capture detector. The Method Detection Limit (MDL) and Reporting Limit for chlorpyrifos was 10 and 20 ng/L, respectively. MDLs for pyrethroids ranged from 0.2 to 0.6 ng/L and Reporting Limits ranged from 0.4 to 1 ng/L (supplemental information). The percent recovery of surrogates ranged from 71.2 to 118% for the OP pesticide scan and 76.8 to 118% for the pyrethroid scan (supplemental information).

Insecticide loss study

A simulated 10-d *H. azteca* toxicity test was performed to quantify pesticide loss (adsorption and degradation) during two interrelated components of testing: sample storage and exposure. Total loss is the sum of storage and exposure losses, and includes the adsorption to multiple surfaces including the LDPE sample storage container, the 600-mL sample preparation beaker and the 250-mL replicate beakers.

Insecticide loss during storage: Three questions related to storage loss were addressed: 1) how much loss occurs during storage over time when samples are collected in plastic containers, 2) does the matrix affect the loss rate during storage, and 3) what is the functional form of the relationship between pyrethroid concentration and time? To commence the study, test solutions were amended with insecticides, rigorously mixed in a 120-L high density polyethylene cylindrical tank and dispensed into 3.8-L LDPE cubitainers (Fisherbrand). While far from ideal when collecting ambient samples suspected of containing pyrethroids, it is a container type that is often used for projects where glass containers have a high risk of breaking due to adverse field conditions, or in instances where the required volume of water exceeds what can be reasonably collected in glass containers. Initial nominal concentrations, selected based on their environmental relevance, were 50 ng/L each for permethrin (cis and trans combined), bifenthrin, lambda-cyhalothrin, cyfluthrin and esfenvalerate, and 100 ng/L for chlorpyrifos. The insecticides were spiked into water from two sources: a synthetic moderately hard water used for laboratory control treatments and an unfiltered ambient water sample that was a composite sample of previously tested non-toxic freshwater samples collected from the Sacramento-San Joaquin Delta between January 6 and 8, 2009. Nine cubitainers with each water type were stored in the dark at

4°C to mimic typical procedures followed in ambient toxicity monitoring. Dissolved organic carbon and total suspended solids were not measured for either water type.

Aliquots (950 mL) of both water types were collected for chemical analysis from the 120-L high density polyethylene tank immediately following the addition of pesticides, and from each cubitainer after vigorous shaking for 30 s after 3 and 9 days of storage. An additional set of samples were collected from the cubitainers containing synthetic water after 4 and 10 days of storage. The analytical data from all samples were used to determine the half-life of pyrethroids during storage in LDPE cubitainers at 4°C, and to determine whether the matrix influences loss/degradation rate.

The analytical data related to storage loss were analyzed using multi-model inference [32]. Models were built in R (version 3.0.2, R Core Team, 2013) corresponding to potential relationships between storage time and pyrethroid concentration. Models included an intercept model (no predictors of concentration), a model with a linear effect of storage time, a model in which pesticide concentration declined exponentially with time [33], and the same exponential decay model but with a parameter for water type (synthetic or Delta). The exponential decay model describes loss during storage in the laboratory, rather than in a natural environment. The exponential decay model was

$$\text{Concentration} \sim ae^{b\text{Days}}$$

where a and b are estimated parameters, *Concentration* is the estimated concentration of a pyrethroid, and *Days* is the number of days since storage began. Models were fit to the concentrations of all pyrethroids because they behaved similarly through time (Fig 1). Each

model had a Gaussian distribution of error. Models were compared using Akaike information criterion corrected for small sample size (AIC_c)[32]. Better models have lower AIC_c scores, and if models have a difference in AIC_c of <2 they are considered similar [33]. Pesticide half-life (HL) in storage containers was calculated using the following equation:

$$HL = \frac{\ln(2)}{b}$$

where b is the estimated day parameter from the exponential decay model above [33].

Loss to exposure containers: Questions related to loss associated with the exposure itself were addressed with samples taken during a simulated exposure. The simulated exposure started after 24 h of sample storage to mimic the sample holding time typically applied to freshwater toxicity tests (Table 1). The exposure followed the toxicity test methods described below but did not include organisms or food, thus loss related to their presence was not evaluated. The questions addressed during this portion of the study were: 1) does the matrix affect the loss rate during the exposure, and 2) does a more frequent renewal period increase the concentration of insecticides available to the test organism?

The exposure loss portion of the study had three treatments: 1) synthetic water with daily renewals, 2) synthetic water with every-other-day renewals, and 3) Delta water with every-other-day renewals. In order to generate enough analytical sample water for each treatment and mimic typical 10-d *H. azteca* water column methods, the typical number of beakers used for sample preparation and exposing organisms were tripled, from 4 to 12 replicates. Each LDPE storage

1 cubitainer was shaken vigorously for 30 s just prior to pouring 500 mL of treatment water into
2 three 600-mL sample preparation beakers where water was warmed to the test temperature of 23
3 $\pm 2^{\circ}\text{C}$ for two hours and aerated at about 100 bubbles/minute during the last half hour of sample
4 warming. Then, four 100 mL aliquots were poured from each 600-mL beaker into 250-mL glass
5 exposure beakers, for a total of 12 replicates of each water type and renewal regime.

6
7 To mimic organism exposure during a toxicity test, samples for chemical analyses were collected
8 from twelve replicate exposure beakers on days 4 and 10, following the full duration of either a
9 24-h (daily) or 48-h (every-other-day) renewal regimen. These two time points represent the
10 acute (4-d) and chronic (10-d) exposure periods in *H. azteca* water column tests and also the
11 lowest possible concentrations expected for these two test durations. Each analytical sample was
12 generated by pooling 75 mL from each of the 12 replicate beakers at the end of the renewal
13 period. A single analytical sample was collected for each time point and water type, thus
14 statistical analyses could not be conducted to compare losses between water types or renewal
15 periods. Although the MDLs for the analytes measured in this study are considered to be very
16 sensitive, analyte concentrations sometimes fell below the MDL, especially towards the end of
17 the simulated toxicity test. In these instances, the MDL values were used in the loss calculations
18 and therefore underestimate loss.

20 **Toxicity tests**

21 All tests were conducted at the University of California Aquatic Health Program Laboratory
22 (Davis, California, USA). *H. azteca* were purchased from Aquatic Research Organisms
23 (Hampton, NH, USA). Upon receipt, amphipods were moved to 10-L aquaria, fed, and

acclimated to laboratory test conditions for 48 h. The 10-d testing procedure used in this study was based on protocols described in the Quality Assurance Management Plan for the State of California's Surface Water Ambient Monitoring Program [34] and by USEPA [22]. Water was warmed to test temperature ($23 \pm 2^{\circ}\text{C}$) in 600-mL beakers and briefly aerated at approximately 100 bubbles/min until the dissolved oxygen (DO) concentration was 4.9 - 8.9 mg/L. De-ionized water amended to USEPA moderately hard specifications [22] was used as a synthetic control water. Tests were initiated with 9 to 14 d old *H. azteca*. Each of four replicate 250-mL glass beakers contained 100 mL of water, a small piece of nitex screen (approx. 4 cm^2) for use as substrate for the *H. azteca*, and 10 organisms. Animals were fed a mixture of yeast, organic alfalfa and trout chow (blended dispersion; 1 mL per replicate) at test initiation and on Days 2, 4, 6, and 8. Tests were conducted at $23 \pm 2^{\circ}\text{C}$ with a 16h: 8h L: D photoperiod. Mortality was recorded daily and 75% of the test water was renewed every other day or daily, as prescribed. On day 10, the surviving *H. azteca* were counted, dried to constant weight at 103 to 105°C , and weighed using a Mettler AE 163 balance. When evaluated, lethal and sub-lethal effect concentrations were calculated using CETIS v.1.1.2 (Tidepool Scientific Software, 2006). Values for no-effect concentrations and low-effect concentrations were calculated using USEPA standard statistical protocols [15]; LC_{50} and EC_{25} concentrations were calculated using linear regression, non-linear regression or linear interpolation methods.

Effectiveness of PBO as a TIE tool

A 10-d *H. azteca* test was conducted to determine the optimal concentration of PBO in TIEs - a concentration that does not produce significant mortality in the method control, but elicits strong TIE signals in the ambient samples. The 10-d LC_{50} of PBO for *H. azteca* is $444\text{ }\mu\text{g/L}$ (10-d LC_{25}

260 µg/L, 10-d LC₁₀ 160 µg/L; UCD AHP, unpublished data). The PBO concentrations used in this test were approximately 1/16, 1/8 and 1/4 of a TU of PBO or 25, 50 or 100 µg/L respectively. The upper concentration of 100 µg/L has rarely caused elevated mortality in method blanks in our laboratory tests. Previous tests also revealed that 25 µg/L PBO neither affected mortality nor growth in the 10-d *H. azteca* water column test [24]. PBO at 25, 50 or 100 µg/L was added to solutions containing insecticide concentrations at the 96-h LC₅₀ value (approximately 1 TU) for permethrin, bifenthrin and chlorpyrifos. The TU concentrations for three common use pesticides were derived from nominal LC₅₀ values determined by Deanovic et al. [7] and spiked at 94 ng/L permethrin, 10 ng/L bifenthrin and 143 ng/L chlorpyrifos. The test included a solvent control (0.004% MeOH) with and without 25 µg/L PBO. In treatments with PBO, we expected toxicity of the two pyrethroids to be enhanced and toxicity of the OP pesticide to be reduced. For data analysis, each PBO treatment was statistically compared to its non-PBO counterpart using the Wilcoxon statistical test.

Potential of pyrethroid toxicity by PBO

To determine the extent to which PBO synergizes the toxicity of permethrin and bifenthrin, a range of pyrethroid concentrations (1/32 to 1 TU) and a solvent control (0.002% MeOH) were tested with and without 25 µg/L PBO in a 10-day *H. azteca* test. The methods were identical to those used in the initial PBO test. Effect concentrations were calculated using CETIS software as described above and compared.

Results

Insecticide loss during storage

Loss of insecticide during storage was severe and constituted the majority of loss in the simulated toxicity test. The greatest loss occurred during the first 72 h of storage, as the two exponential decay models received an AIC_c weight proportion of 1. Water matrix (synthetic or Delta water) was not an important predictor of insecticide concentration, as the exponential decay model with a parameter for water type received little AIC_c support (AIC_c weight = 0.23; Table 2). Instead, the simple exponential decay model, which did not distinguish between water types, received the highest proportion of AIC_c weight (0.77, Table 2; Figure 1). The concentration in ng/L of pyrethroids stored in LDPE cubitainers at 4°C is given by the following:

$$Concentration = ae^{bDay}$$

The ‘a’ parameter estimate is 32.23 (95% CI: 29.18-35.28) and the ‘b’ parameter estimate is -.056 (95% CI: -0.63, -0.37). Thus, neither 95% confidence interval for the exponential decay parameter estimates overlapped zero. The half-life calculated from this model is 1.39 days (95% CI -1.1, -1.87). Thus, roughly half of the pyrethroids are lost during storage at 4°C in LDPE ~1.4 days after collection, despite vigorous shaking. There was no apparent difference between the less hydrophobic OP chlorpyrifos and the highly hydrophobic pyrethroids (Figure 1). Consistent with exponential decay, percent loss after 72 h of storage for the seven analytes (chlorpyrifos and six pyrethroids) averaged $74.4 \pm SE 2.8$ in Delta water and $83.6 \pm SE 1.2$ in synthetic water. After 9 d of storage, average percent loss was $88.1 \pm SE 3.5$ in Delta water and $93.1 \pm SE 1.5$ in synthetic water.

Insecticide loss during exposure procedures

Exposure loss was minor relative to storage loss in relation to initial concentrations of pesticides (Figure 2). Chlorpyrifos was not included in this figure due to the high frequency at which concentrations fell below the MDL of 10 ng/L; chlorpyrifos was detected in only 2 of the 12 relevant samples. The total loss (storage plus exposure) for pyrethroids in replicate beakers for synthetic (for both renewal regimes) and Delta waters ranged from 90.2 to 93.8% at the 4-d time point and from 94.5 to 98.1% at the 10-d time point. These small differences ($\leq 3.6\%$) suggest that neither this specific water matrix nor the renewal frequency in synthetic water had a noteworthy effect on losses relative to the initial concentration.

Loss in the replicate beakers at the end of renewal period was also calculated relative to the starting concentration in the sample preparation beakers to better understand how much pesticide was lost during the toxicity test itself. These losses averaged across pyrethroids ($n=6$) for the 96-h time point were 21.9, 54.4 and 76.3% for the synthetic water with daily renewals, the synthetic water with every-other-day renewals and Delta water with the every-other-day renewals, respectively. For the 10-d time point, the losses were 36.7, 13.9 and 71.1%. These losses were greatest in the Delta water. Several pyrethroid insecticides have a narrow range of concentrations that would be toxic under ideal conditions, but instead degrade and adsorb to such an extent that the concentrations in an ambient sample become non-toxic. Under these circumstances, the addition of PBO can still produce a TIE signal (increased mortality), even though the ambient sample itself is no longer toxic. Thus, the relationship between fold loss in pyrethroids and the potentiation potential from the addition of PBO are important. Fold losses relative to the initial

concentration of pyrethroids are presented in Figure 3. At best, the highest average concentration of pyrethroids was one quarter of their original concentration or a fourfold loss (Delta water on Day 2 of the test). Regardless of the water type or renewal regimen, fold losses generally continued to increase over time and with increasing exposure to container surfaces with the greatest fold loss (79.2) occurring in the Delta water in the replicate beakers on Day 10 of the exposure. Overall, this loss data suggests that PBO must potentiate the toxicity of pyrethroids a minimum of four fold simply to recover the toxicity that may be lost during storage and testing procedures.

The fold loss for chlorpyrifos ranged from 5.1 to 7.2 for both water renewal periods and water types. However, the upper value in this range would have been considerably larger had the MDL for chlorpyrifos been lower.

Effectiveness of PBO as a TIE tool

All concentrations of PBO accelerated mortality of the two pyrethroids and reduced mortality of the OP pesticide (Figures 4A, B and C). Although the nominal concentrations for bifenthrin, permethrin and chlorpyrifos were not confirmed with chemical analyses, the time to death in this test confirms that approximately one TU of each insecticide was present in test solutions.

Survival in the control and methanol control treatments was 100%. No statistical differences in mortality occurred in the PBO method blanks and the methanol control, suggesting that all PBO concentrations were non-lethal; however, significantly reduced growth was measured at 50 and 100 µg/L PBO (supplemental information). All three concentrations of PBO with permethrin were statistically different from the permethrin alone on Day 2 of the test only, suggesting that there is a limited window of opportunity to observe a TIE signal in the presence of

approximately 1 TU. For bifenthrin, the higher two concentrations of PBO, 50 and 100 µg/L, were statistically different from bifenthrin alone for three consecutive days during the experiment, while the 25 µg/L PBO treatment showed a similar signal for two consecutive days. PBO at 25 µg/L produced a less distinct TIE signal, both in duration and by in percent survival, than the 50 and 100 µg/L PBO concentrations for permethrin and bifenthrin. The weight endpoint with PBO could not be evaluated for both pyrethroids, as all organisms in treatments containing pyrethroids exhibited 100% mortality by the end of the test. For chlorpyrifos, the highest concentration of PBO reduced mortality for the final five days of the test, while the lower two concentrations of PBO reduced mortality for the last four days. There were no significant differences in growth in chlorpyrifos treatments, both with and without PBO, suggesting that PBO may not be an effective TIE tool for identifying OP pesticides at any concentration for the growth endpoint.

Potential of pyrethroid toxicity by PBO

Table 4 summarizes results of *H. azteca* exposures to permethrin and bifenthrin alone and in the presence of 25 µg/L PBO. PBO potentiated mortality of pyrethroids by ≥ 2.48 -fold. The permethrin LC₅₀ was potentiated 2.48 and 8.81-fold for the 96-h and 10-d LC₅₀s, respectively. Synergism for bifenthrin was 3.95 and 3.22-fold for the 96-h and 10-d LC₅₀s, respectively. Similar increases were observed for each chemical for the 96-h and 10-d no-effect concentration and low-effect concentration values. Potentiation related to low-effect concentration values and EC₅₀s could not be evaluated for the 10-d growth endpoint due to high mortality rates associated with the pesticide exposures, or in the case of permethrin alone, an irregular dose response curve. The 10-d growth no-effect concentration was synergized 8 and 2-fold for permethrin and

bifenthrin, respectively, which closely followed the potentiation related to the 10-d survival endpoints.

Discussion

Managers involved in designing environmental monitoring programs that emphasize the identification of contaminants, such as SWAMP and other programs like it, benefit from understanding the limitations of the tools applied. Laboratory toxicity tests can detect environmental pollutants where analytical methods may fail; however, highly hydrophobic chemicals may be lost during transport, storage and testing of ambient samples, especially when less-than ideal sample containers are used. Laboratory tests are therefore likely to underestimate the toxicity and risk of such chemicals to aquatic organisms in the field. Our study demonstrated that this is the case for several pyrethroids, but also for the less hydrophobic OP chlorpyrifos. The addition of PBO can address these problems by enhancing the toxic signal of pyrethroids.

Insecticide loss during storage

According to the exponential decay model, the majority of insecticide was lost from solution after 1.4 d of storage, leaving little insecticide in the aqueous mixture thereafter. Immediate pyrethroid adsorption to storage containers is becoming a widely recognized problem and several researchers have reported similar losses within a comparable timeframe during storage. Sharom and Solomon [35] reported greater than 60% adsorption of permethrin to borosilicate glass scintillation vials within the first 48 h of storage. Zhou et al. [36] had similar losses of permethrin, telfluthrin, cypermethrin and lambda-cyhalothrin to glass centrifuge tubes within the first 24 h of storage, and Oudou and Hansen [37] also observed a 20 to 60% loss of cypermethrin, fenvalerate, lambda-cyhalothrin and deltamethrin to centrifuge tubes within 24 h.

1 Wheelock et al. [29] reported up to 50% loss of lambda-cyhalothrin to 20 mL glass vials in the
2 first 24 h of storage with more than 75% lost by 96 h. Lee et al. [5] measured respective
3 reductions of recovery of 37, 28, 21 and 32% of bifenthrin, *cis*-permethrin, *trans*-permethrin and
4 deltamethrin in de-ionized water within the first 24 h of sample storage, with starting
5 concentrations of 2000 ng/L for each pyrethroid. These same researchers also illustrated that
6 pyrethroids adsorbed to container walls can be recovered, but with varying results. Wheelock et
7 al. [29] found that vortexing glass vials eliminated pyrethroid loss for up to 96 h of storage and
8 Hladik et al. [26] were successful in recovering pyrethroids from container walls with one
9 minute of shaking. In contrast, Oudou and Hansen [37] and Zhou et al. [36] found that water
10 samples still exhibited significant loss after 24 h of shaking. Sharom and Solomon [35] were able
11 to recover pyrethroids from glass after centrifugation but not from polyethylene.

12
13 In the current study, standard sample preparation techniques [15] were consistent throughout the
14 10-d exposure and included 30 s of vigorous shaking prior to pouring sample waters out of
15 storage containers. While this method is adequate to re-suspend solids, it was inadequate to
16 recover hydrophobic compounds from the LDPE storage containers. Sharom and Solomon [35]
17 and Wheelock et al. [29] demonstrated that pyrethroids are especially difficult to recover from
18 plastics. The results of their studies and ours suggest that glass should be used whenever possible
19 and constructing protective cases to prevent breakage in adverse field conditions are worth the
20 effort. LDPE cubitainers should be reserved for only the most challenging field conditions.

1 It is important to recognize that the severe loss observed in our study will cause studies using
2 similar methodology to grossly underestimate the toxicity experienced by the test organism at
3 any time point during an exposure for both pyrethroid insecticides and chlorpyrifos.

4 **Insecticide loss during the exposure**

5 Relative to the initial concentration, we observed minimal differences in total loss ($\leq 3.6\%$)
6 between the two renewal methods and water matrices at both the 96-h and 10-d time points, with
7 slightly more loss occurring in the Delta water matrix. The exposure component of a typical
8 toxicity test has several conditions that vary from storage conditions that would be expected to
9 increase loss, including the presence of organisms and food, which were absent in the current
10 study. The use of smaller sample preparation and replicate containers (increased surface to
11 volume ratio) increases the potential for additional loss due to adsorption. Additionally, methods
12 to desorb pyrethroids such as shaking or vortexing cannot be applied when organisms are present
13 in replicate beakers. The exposures are also conducted under fluorescent lighting and at higher
14 temperatures, both of which can increase degradation [5,38]. Wheelock et al. [29] estimated that
15 the three step process from storage container to sample preparation container to replicate
16 containers could result in a 90% loss of pyrethroids. Our results confirm this estimation.

17
18 In our study, the loss associated with the exposure alone was high for pyrethroids (13.9 to
19 76.3%). Exposure loss is not only relevant to ambient monitoring with one-time grab samples,
20 but also to effect concentration testing where pesticide solutions are typically made daily. As in
21 our study, Wheelock et al. [29] examined loss during an exposure. Although concentrations were
22 not measured, these authors compared loss due to adsorption in acute *H. azteca* and *C. dubia*
23 tests and found that allowing permethrin solutions to sit in replicate scintillation vials for 4 h

prior to loading *C. dubia* resulted in a 50% loss of toxicity. While the authors reported the loss of toxicity to *H. azteca* was not as severe, the pesticide loss rate to replicate container walls was high nonetheless.

Effectiveness of PBO as a TIE tool

In the interest of expediting chemical analyses, the decision to send a sample in for a particular analytical scan is not based on statistical analysis of the daily mortality observations in a TIE, but rather on the signal strength of treatment manipulations. For our purposes, we define a strong signal as an obvious difference in mortality between a non-manipulated sample and its manipulated counterpart which is sustained for multiple days. Strong TIE signals guide researchers to prioritize the most appropriate analytical scans in the shortest amount of time.

In the current study, PBO was an effective tool to synergize pyrethroid and antagonize OP toxicity for the survival endpoint. All three PBO concentrations evaluated either increased or decreased survival as anticipated. Both 50 and 100 µg/L PBO accelerated the toxicity of pyrethroids and delayed the toxicity of OP pesticides without negatively affecting the 10-d survival endpoint in the method controls. These concentrations equated to 1/8 and 1/4 TU of PBO for our 10-d *H. azteca* test and were the most effective concentrations examined. Two studies have also shown that higher concentrations of PBO will produce stronger signals for both OPs and pyrethroids. Ankley et al. [39] demonstrated that higher concentrations of PBO were more effective at reducing toxicity of OP pesticides for three cladoceran species. Similarly, their most effective concentrations were 1/8 and 1/4 of a TU for a 48-h test duration, which was 125 and 250 µg/L. Likewise, Amweg et al. [20] reported that higher concentrations of PBO synergized the toxicity of permethrin better than lower concentrations of PBO in 10-d *H. azteca*

1 sediment tests. The highest concentration tested in this 10-d *H. azteca* sediment test was 1/10 TU
2 (56.3 µg/L) of PBO.

3
4 Higher concentrations of PBO produced stronger TIE signals in our water column study,
5 although relative to *C. dubia*, the signal for *H. azteca* is subtle. For OPs administered at 1 TU,
6 addition of 100 µg/L PBO delayed toxicity to *C. dubia* and other cladoceran by at least 24 h [39].
7 Moreover, Bailey [40] showed that 200 µg/L PBO completely blocked the toxicity of diazinon
8 and chlorpyrifos at concentrations greater than 1 TU for more than 24 h. A ≥24-h delay in
9 toxicity by PBO treatment is therefore considered a reliable signal that OPs are contributing to
10 toxicity. In the current study with *H. azteca*, chlorpyrifos was present at slightly less than 1 TU
11 and the three PBO concentrations tested produced statistically different signals ranging from a 28
12 to 77% difference in mortality between the chlorpyrifos-plus PBO and chlorpyrifos-only
13 treatments.

14
15 Similarly, the statistically different signals produced by the three concentrations of PBO ranged
16 from 20 to 58% in the presence of approximately one TU of bifenthrin or permethrin. Even in the
17 highest concentration of PBO, the stronger signals were only 57 and 58% difference in survival
18 on Day 2 alone for bifenthrin and permethrin, respectively, and the difference in survival
19 diminished in the following days. More studies should be conducted to identify a signal strength
20 (difference in survival and duration) for *H. azteca* that will reliably lead to the detection of
21 pyrethroids in analytical chemistry measurements, especially if a sample is not submitted to the
22 analytical laboratory until the Phase I TIE is completed.

PBO potentiation

PBO's synergistic effect becomes important when one considers the significant loss of insecticide concentrations that takes place during storage and testing. In essence, PBO can act as a recovery tool to help determine if pyrethroids are present at potentially biologically relevant concentrations. Using bifenthrin as an example, only 38.1% of the original concentration remained in the ambient Delta water used in this study after 72 hours of storage. If an ambient sample starts with 1 TU of bifenthrin, storage loss alone may reduce the concentration down to 1/3 TU in a very short time period, which may no longer elicit a response in the toxicity test, especially in a delayed TIE. In our study, 25 µg/L PBO (the lowest concentration of PBO tested), synergized the 96-h bifenthrin toxicity approximately four-fold, and would allow the detection of potentially toxic concentrations of pyrethroids in an environmental sample via organism response. The application of this tool in environmental monitoring may help avoid false negatives, thus assisting environmental managers in drawing the correct conclusions regarding the toxicity of a water body. Results of the current study generally support the use of 100 µg/L PBO for 96-h tests and 50 µg/L for 10-d tests when conducted at 23°C. A concentration of 25 µg/L PBO should only be used if the sub-lethal endpoint growth is being examined.

Conclusions and recommendations

When toxicity is detected in ambient surface water samples, environmental managers have to identify the toxic contaminant(s) before remedial action can be taken. The challenges related to toxicant identification are, however, complex and influenced by multiple factors including the toxicant's initial concentration, loss during storage and testing, the availability of reliable TIE

1 methods, and adequate analytical detection limits. For pyrethroids and chlorpyrifos, the high loss
2 rate during standard storage conditions and toxicity testing leads to an underestimation of
3 toxicity to the extent that it may frequently go undetected. Even if toxicity is detected, our study
4 shows that valuable resources may be wasted if TIE procedures are not initiated within an
5 appropriate time frame and with the appropriate tools. The following recommendations are based
6 on the findings of this study and are intended to inform managers about more cost effective
7 approaches in detecting and identifying toxicity due to pyrethroids in ambient water samples.

8
9 1) TIEs using PBO treatments should only be conducted with samples that cause at least 50%
10 mortality in the first 72 h of the initial screening test, and initiated immediately when this occurs,
11 in order to obtain the strongest TIE signal, which will aid managers in data interpretation and
12 decision making, e.g. to send in samples for chemical analysis.

13
14 2) Where available data suggests that pyrethroids are likely to be present in the sample,
15 simultaneous testing of a treatment with PBO added increases the likelihood of successful
16 pyrethroid identification.

17
18 3) In order to maximize the signal in *H. azteca* TIEs, we recommend the use of 100 µg/L (1/4
19 TU PBO) PBO. This PBO concentration did not affect survival of *H. azteca* in the 10 d toxicity
20 test.

Acknowledgments

We would like to thank the staff of the UC Davis Aquatic Health Program Laboratory for conducting the toxicity tests. Funding was provided by the Interagency Ecological Program, Sacramento, California (Contract No. 4600008070 to I. Werner) and the Central Valley Regional Water Quality Control Board (Contract No. 06-2662-150-0 to I. Werner). We would also like to thank the California Department of Fish and Wildlife Water Pollution Control Laboratory for conducting our chemical analyses. All authors declare that there are no conflicts of interest.

References

1. Stehle S and Schulz R. 2015. Agricultural insecticides threaten surface waters at the global scale. *PNAS* 2015 112 (18) 5750-5755; published ahead of print April 13, 2015, doi:10.1073/pnas.1500232112. www.pnas.org/cgi/doi/10.1073/pnas.1500232112
2. Alvanja MCR. 2009. Pesticides Use and Exposure Extensive Worldwide. *Rev. Environ. Health* 24(4): 303-309.
3. You J, Brennan A, Lydy MJ. 2009. Bioavailability and biotransformation of sediment-associated pyrethroid insecticides in *Lumbriculus variegatus*. *Chemosphere* 75:1477-1482.
4. Weston DP, Amweg EL, Mekebri A, Ogle RS, Lydy MJ. 2006. Aquatic effects of aerial spraying for mosquito control over an urban area. *Environ. Sci. Tech.* 40:5817-5822.
5. 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.
6. 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.
7. Deanovic LA, Markiewicz D, Stillway M, Fong S, Werner I. 2013. Comparing the effectiveness of chronic water column tests with *Hyaella azteca* (Order: Amphipoda) and

1 *Ceriodaphnia dubia* (Order: Cladocera) in detecting toxicity of current-use insecticides. *Environ.*
2 *Toxicol. Chem.* 32(3):707-712.

3

4 8. Werner I and Moran K. (2008) Effects of pyrethroid insecticides on aquatic organisms. In:
5 Weston DP. 2006. Temperature Dependence of Pyrethroid Toxicity: TIE Applications and
6 Environmental Consequences. *Proceedings*, SETAC, National Meeting, Montreal, QC, Canada.
7 P26.

8

9 9. Oros DR and Werner I. 2005. Pyrethroid Insecticides: An Analysis of Use Patterns,
10 Distributions, Potential Toxicity and Fate in the Sacramento-San Joaquin Delta and Central
11 Valley. White Paper for the Interagency Ecological Program. SFEI Contribution 415. San
12 Francisco Estuary Institute, Oakland, CA.

13

14 10. Rauh VA, Perera FP, Horton MK, Whyatt RM, Bansal R, Hao X, Liu J, Barr DB, Slotkin
15 TA, Peterson BS. 2012. Brain anomalies in children exposed prenatally to a common
16 organophosphate pesticide. *PNAS*. 109(20):7871-7876.

17

18 11. Dahlgren JG, Takhar HS, Ruffalo CA, Zwass M. 2004. Health Effects of Diazinon on a
19 Family. *Journal of Toxicology: Clinical Toxicology*. 42(5):579-591.

20

21 12. Moschet C, Wittmer I, Simovic J, Junghans M, Piazzoli A, Singer H, Stamm C, Leu C,
22 Hollender J. 2014. How a Complete Pesticide Screening Changes the Assessment of Surface
23 Water Quality. *Environ. Sci. Technol.* 48:5423 – 5432.

13. Hladik ML and Kuivila K. 2009. Assessing the Occurrence and Distribution of Pyrethroids in Water and Suspended Sediments. *J. Agric. Food Chem.* 57:9079-9085.
14. Hladik, ML. 2007. Methods Development for the Analysis of Pyrethroid Pesticides in Environmental Samples. *Final Report for CALFED*. U.S. Geological Survey. Sacramento, CA.
15. USEPA. 2002. Short-term Methods for Measuring the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms. 4th ed. *EPA/821/R-02/013*. Washington, DC.
16. USEPA. 1991. Technical Support Document for Water Quality-based Toxins Control. *EPA/505/2-90/001*. Washington, DC.
17. Brack W. 2003. Effect-directed analysis: a promising tool for the identification of organic toxicants in complex mixtures. *Anal. Bioanal. Chem.* 377(3):397-407.
18. Weston DP and Lydy MJ. 2010. Urban and Agricultural Sources of Pyrethroid Insecticides to the Sacramento-San Joaquin Delta of California. *Environ. Sci. Technol.* 44(5):1833–1840.
19. Werner I, Deanovic LA, Reece C, Markiewicz D, Stillway M, Khamphanh J. 2008. Pelagic Organism Decline (POD): Acute and Chronic Invertebrate and Fish Toxicity Testing in the Sacramento-San Joaquin Delta 2008-2010. Progress Report II. Aquatic Toxicology Laboratory, University of California, Davis. Davis, CA.

- 1
- 2 20. Amweg EL, Weston DP, Jonson CS, You J, Lydy MJ. 2006. Effect of Piperonyl Butoxide on
- 3 Permethrin Toxicity in the Amphipod *Hyaella azteca*. *Environ. Toxicol. Chem.* 25(7):1817-
- 4 1825.
- 5
- 6 21. Burkepile DE, Moore MT, Holland MM. 2000. Susceptibility of Five Non-Target Organisms
- 7 to Aqueous Diazinon Exposure. *Bull. Environ. Contam. Toxicol.* 64:114-121.
- 8
- 9 22. USEPA. 2000. Methods for Measuring the Toxicity and Bioaccumulation of Sediment-
- 10 associated Contaminants with Freshwater Invertebrates. 2nd ed. *EPA/600/R-99/064*. Washington,
- 11 DC.
- 12
- 13 23. Ankley GT and Collyard SA. 1995. Influence of piperonyl butoxide on the toxicity of
- 14 organophosphate insecticides to three species of freshwater benthic invertebrates. *Comp.*
- 15 *Biochem. Physiol.* 110C(2):149-155.
- 16
- 17 24. Werner I, Deanovic LA, Markiewicz D, Kamphanh J, Reece CK, Stillway M, Reece C. 2010.
- 18 Monitoring acute and chronic water column toxicity in the Northern Sacramento-San Joaquin
- 19 Estuary, California, USA, using the euryhaline amphipod, *Hyaella Azteca*: 2006 to 2007.
- 20 *Environ. Toxicol. Chem.* 29:2190-2199.
- 21
- 22 25. Lao W, Tiefenthaler L, Greenstein DJ, Maruya KA, Bay S M, Ritter K, Schiff K. 2012.
- 23 Pyrethroids in Southern California Coastal Sediments. *Environ. Toxicol. Chem.* 31(7):1649-1656.

- 1
- 2 26. Hladik ML, Orlando JL, and Kuivila KM. 2009. Collection of pyrethroids in water and
3 sediment matrices: development and validation of a standard operating procedure. *U.S.*
4 *Geological Survey Scientific Investigations Report*. 2009–5012, 22. Sacramento, CA.
- 5
- 6 27. Gan J, Lee SJ, Liu WP, Haver DL, Kabashima JK. 2005. Distribution and Persistence of
7 Pyrethroids in Runoff Sediments. *J. Environ. Qual.* 34:836-841.
- 8
- 9 28. Laskowski DA. 2002. Physical and chemical properties of pyrethroids. *Rev. Environ.*
10 *Contam. Toxicol.* 179:49-170.
- 11
- 12 29. Wheelock CE, Miller JL, Miller MJ, Phillips BM, Gee SJ, Tjeerdema RS, Hammock BD.
13 2005. Influence of container adsorption upon observed pyrethroid toxicity to *Ceriodaphnia dubia*
14 and *Hyaella azteca*. *Aquat. Toxicol.* 74(1):47-52.
- 15
- 16 30. USEPA. 1992. Toxicity Identification Evaluation: Characterization of Chronically Toxic
17 Effluents, Phase I. *EPA/600/6-91/005F*. Washington, DC.
- 18
- 19 31. USEPA. 1993. Methods for Aquatic Toxicity Identification Evaluations: Phase II Toxicity
20 Identification Procedures for Samples Exhibiting Acute and Chronic Toxicity. *EPA/600/R-*
21 *92/080*. Washington, DC.
- 22

32. Burnham KP and Anderson DR. 2002. *Model selection and multimodel inference: a practical information-theoretic approach*. Springer Verlag, New York, New York.
33. Bolker BM. 2008. *Ecological models and data in R*. Princeton University Press. Princeton, New Jersey.
34. Surface Water Ambient Monitoring Program. 2008. Quality Assurance Program Plan. Developed for the State Water Resources Control Board. Sacramento, CA. 2008.
35. Sharom MS and 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.
36. Zhou JL, Rowland S, Mantoura RFC. 1995. Partition of synthetic pyrethroid insecticides between dissolved and particulate phases. *Wat. Res.* 29(4):1023-1031.
37. Oudou HC and Bruun Hansen HC. 2002. Sorption of lambda-cyhalothrin, cypermethrin, deltamethrin and fenvalerate to quartz, corundum, kaolinite and montmorillonite. *Chemosphere* (49):1285-1294.
38. Albaser SS, Nagesawara R, Swamy YV, Mukkanti, K. 2011. Analytical artifacts, sample handling and preservation methods of the environmental samples of synthetic pyrethroids.

1 *Trends*. 30(11).

2

3 39. Ankley G, Dierkes JR, Jensen DA, Peterson GS. 1991. Piperonyl Butoxide as a Tool in
4 Aquatic Toxicological Research with Organophosphate Insecticides. *Ecotoxocol. Environ.*
5 *Safety*. 21:266-274.

6

7 40. Bailey HC, DiGiorgio C, Kroll K, Miller JL, Hinton DE, Starrett G. 1996. Development of
8 procedure for identifying pesticide toxicity in ambient waters: Carbofuran, Diazinon,
9 Chlorpyrifos. *Environ. Toxicol. Chem.* 15(6):837-845.

10

11

Legends

Figure 1. Evaluation of insecticide loss (measured concentrations) during storage of spiked water samples in 3.8L low-density polyethylene cubitainers at 4°C in the dark. ‘Day’ reflects selected time points during daily (days 4 and 10) and every other day (days 3 and 9) water renewal regimes in a simulated 10-d water column toxicity test. Nominal initial concentrations were 50 ng/L for pyrethroids and 100 ng/L for chlorpyrifos. The circles represent six different pyrethroids and the squares represent the organophosphate chlorpyrifos. The solid line shows the exponential decay model, and the dashed lines are the 95% confidence interval of the model. Note: the chlorpyrifos data was not used to fit the model. Chlorpyrifos concentrations are plotted at the minimum detection limit on Days 4, 9 and 10 of storage, and cis-permethrin on Day 9.

Figure 2. Average total percent loss (storage loss and exposure loss) for pyrethroids relative to the initial concentrations. Error bars represent the SE between the six single measurements of pyrethroid analytes (bifenthrin, cyfluthrin, esfenvalerate, lambda-cyhalothrin, cis-permethrin and trans-permethrin). Upward error bars apply to total percent loss. Downward error bars apply loss associated with storage and exposure loss. Asterisks denote an average percent loss with one or more individual analytes below MDLs.

Figure 3. Average fold loss for pyrethroids relative to the initial concentrations.

Asterisks denote an average percent loss with one or more individual analytes
below MDLs.

Figure 4A. Concentration dependent effects of PBO on survival of *H. azteca* in the presence of
permethrin near the nominal 96-h LC50 concentration.

*: $P < 0.05$ with corresponding non-PBO permethrin spike

+: $P < 0.05$ with Synthetic Water + MeOH (100% survival, not shown)

Figure 4B. Concentration dependent effects of PBO on survival of *H. azteca* in the presence of
bifenthrin near the nominal 96-h LC50 concentration.

*: $P < 0.05$ with corresponding non-PBO bifenthrin spike

+: $P < 0.05$ with Synthetic Water + MeOH (100% survival, not shown)

Figure 4C. Concentration dependent effects of PBO on survival of *H. azteca* in the presence of
chlorpyrifos near the nominal 96-h LC50 concentration.

*: $P < 0.05$ with corresponding non-PBO chlorpyrifos spike

+: $P < 0.05$ with Synthetic Water + MeOH (100% survival, not shown)

Table 1. The temporal relationship between the days that the samples were stored and the simulated

H. azteca exposure was conducted. Day 0 during storage represents the time point at which initial concentrations were measured.

Shaded cells indicate time points and conditions that analytical samples were collected.

		Days											
Synthetic Water: Daily renewal	During storage	0^a	1	2	3	4	5	6	7	8	9	10	11
	At end of renewal period during exposure	-1	0	1	2	3	4	5	6	7	8	9	10
Synthetic Water: Every-other-day renewal	During storage	0^a	1	2	3	4	5	6	7	8	9	10	11
	At end of renewal period during exposure	-1	0	1	2	3	4	5	6	7	8	9	10
Delta Water: Every-other-day renewal	During storage	0	1	2	3	4	5	6	7	8	9	10	11
	At end of renewal period during exposure	-1	0	1	2	3	4	5	6	7	8	9	10

a. One synthetic water sample shared between two renewal regimes collected prior to pouring water into storage containers.

Table 2. Model comparison. a and b are estimated parameters, the response variable is chemical concentration. ΔAIC_c is the change in Akaike information criterion corrected for small sample size, df is degrees of freedom, AIC_c wt is AIC_c weight expressed as a proportion.

Model	ΔAIC_c	df	AIC_c wt
$\sim ae^{bDay}$	0.0	3	0.77
$\sim ae^{bDay} + cWater$	2.4	4	0.23
$\sim a + bDay$	45.9	3	<0.001
$\sim a$	76.3	2	<0.001

Table 3. Parameter estimates and 95% confidence intervals for the (top-ranked) exponential decay model.

Parameter	Estimate	95% CI
a	32.23	29.18, 35.28
b	-0.56	-0.63, -0.37

Table 4. Synergistic effect of 25 µg/L PBO on 96-h and 10-d *H. azteca* toxicity endpoints for permethrin and bifenthrin (nominal concentrations).

Toxicity Endpoint	Chemical	NOEC	Fold Increase	LOEC	Fold Increase	LC50/EC25	Fold Increase
		(ng/L)	Due to PBO	(ng/L)	Due to PBO	(ng/L)	Due to PBO
96-h Survival	Permethrin	47		94.0		76.9	
	Permethrin + 25 µg/L PBO	23.5	2x	47	2x	31.0	2.5x
10-d Survival	Permethrin	23.5		47.0		52.9	
	Permethrin + 25 µg/L PBO	2.94	8x	5.88	8x	6.0	8.8x
10-d Growth	Permethrin	47		>47		14.9 ^a	
	Permethrin + 25 µg/L PBO	5.88	8x	>5.88	n/a	-	n/a
96-h Survival	Bifenthrin	5		10		15.8	
	Bifenthrin + 25 µg/L PBO	1.25	4x	2.5	4x	4.0	4.0x
10-d Survival	Bifenthrin	1.25		2.5		2.9	
	Bifenthrin + 25 µg/L PBO	0.625	2x	1.25	2x	0.9	3.2x
10-d Growth	Bifenthrin	2.5		>2.5		-	
	Bifenthrin + 25 µg/L PBO	1.25	2x	>1.25	n/a	-	n/a

^a Unreliable estimate because dose-response was not consistent with a single trend line and growth endpoint of some treatments was anomalous.

Supplemental Information

Method detection, reporting limits and surrogate recovery ranges for organophosphate and pyrethroid insecticides.

Compound	Chemical Class	Method	Method Detection Limit (ng/L)	Reporting Limit (ng/L)	Percent Recovery Range (%)
Chlorpyrifos	Organophosphate	EPA 8141AM	10	20	71.2 to 118
Bifenthrin	Pyrethroid	EPA 8081BM	0.2	0.4	76.8 to 118
Cyfluthrin, total	Pyrethroid		0.4	0.8	
Esfenvalerate/ Fenvalerate, total	Pyrethroid		0.2	0.4	
Cyhalothrin, lambda, total	Pyrethroid		0.2	0.4	
Permethrin, cis-	Pyrethroid		0.6	1	
Permethrin, trans-	Pyrethroid		0.6	1	

Summary of 10-day *H. azteca* water column toxicity test initiated on 2/11/09 examining the effects on growth of PBO alone and in combination with permethrin, bifenthrin and chlorpyrifos. Missing growth data is due to high mortality in the treatment.

Treatment	Weight (mg/surviving individual) ¹	
	Mean	SE
Control	0.076	0.002
Control + MeOH @ 0.02%	0.083	0.005
Control + MeOH + 50 µg/L PBO	0.062	0.007
Control + MeOH + 100 µg/L PBO	0.062	0.005
94 ng/L Permethrin	0.045	0.015
94 ng/L Permethrin + 25 µg/L PBO	-	-
94 ng/L Permethrin + 50 µg/L PBO	-	-
94 ng/L Permethrin + 100 µg/L PBO	-	-
10 ng/L Bifenthrin	-	-
10 ng/L Bifenthrin + 25 µg/L PBO	-	-
10 ng/L Bifenthrin + 50 µg/L PBO	-	-
10 ng/L Bifenthrin + 100 µg/L PBO	-	-
143 ng/L Chlorpyrifos	0.055	0.035
143 ng/L Chlorpyrifos + 25 µg/L PBO	0.068	0.004
143 ng/L Chlorpyrifos + 50 µg/L PBO	0.071	0.011
143 ng/L Chlorpyrifos + 100 µg/L PBO	0.041	0.003

1. Highlighted areas indicate a significant reduction in weight compared to the Control + MeOH @0.02%. Comparisons were performed by pairwise t-tests and by Tukey's tests. No statistical differences were detected between a pesticide-spiked treatment and any of its related PBO amended treatments.