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Ca-Environmental Fate of Mosquito Adulticides and Effects on Non-target Invertebrates in Wetlands of the Sacramento Valley

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EFFECT OF PIPERONYL BUTOXIDE ON PERMETHRIN TOXICITY IN THE AMPHIPOD *HYALELLA AZTECA*

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Abstract—Piperonyl butoxide (PBO) is a synergist of pyrethroid pesticides found in many products for structural pest control, mosquito control, and home and garden uses. Because both PBO and pyrethroid residues potentially co-occur in urban creeks, this study determined if environmental levels of PBO were capable of synergizing pyrethroids in the environment. Three types of toxicity tests were conducted with the amphipod *Hyalella azteca* to determine the minimum PBO concentration required to increase toxicity of the pyrethroid permethrin: Sediment was spiked with permethrin only; permethrin and overlying water spiked with PBO; and permethrin, PBO, and overlying water spiked with PBO. In tests with PBO added to both water and sediment, PBO concentrations of 2.3 μg/L in water and 12.5 μg/kg in sediment reduced the permethrin median lethal concentration (LC50) nearly 50% to 7.3 mg/kg organic carbon (OC). Higher concentrations of PBO increased permethrin toxicity up to sevenfold. In exposures with PBO in water alone, 11.3 μg/L was required to increase permethrin toxicity. Urban creek sediments from California and Tennessee, USA, had PBO concentrations in the low μg/kg range; only one water sample was above the detection limit of 0.05 μg/L. Wetlands in northern California also were sampled after application of pyrethrins and PBO for mosquito abatement. Sediment and water PBO concentrations within 12 h of abatement spraying peaked at 3.27 μg/kg and 0.08 μg/L, respectively. These results suggest that environmental PBO concentrations rarely, if ever, reach concentrations needed to increase pyrethroid toxicity to sensitive organisms, though available data on environmental levels are very limited, and additional data are needed to assess definitively the risk.

Keywords—Pyrethroid Piperonyl butoxide *Hyalella azteca* Synergism

INTRODUCTION

Piperonyl butoxide (PBO) was first reported as a pyrethrin synergist in 1947. Today, it also is used to enhance toxicity of the newer pyrethroid pesticides, which increasingly are used for many applications since the recent withdrawal of nearly all products for residential use in the United States containing the organophosphates diazinon and chlorpyrifos. Reported commercial use of PBO (excludes retail sales) in California, USA, in 2003 totaled over 18,000 kg (www.cdpr.ca.gov). This total includes applications for structural pest control (6,900 kg), mosquito control (7,330 kg), and landscape maintenance (200 kg). For example, Drione® is used widely for structural pest control, and contains 1% pyrethrins and 10% PBO. Scourge® is used for control of adult mosquitoes, and contains 18% resmethrin and 54% PBO. Piperonyl butoxide also is used to synergize agricultural pyrethroids. However, there was 127,500 kg of pyrethroids used in California agriculture in 2003, but only 2,700 kg of PBO, so it is clear that most of the agricultural pyrethroids are applied without a PBO synergist.

Piperonyl butoxide itself is not particularly toxic to animals; typical 96-h median lethal concentrations (LC50s) are in the low ppm range: 0.53 mg/L for *Hyalella azteca*, 2.74 mg/L for *Chironomus tentans*, and 3.54 mg/L for *Lumbriculus variegatus* [1]. Erickson [2] reported a 96-h LC50 of 11.2 mg/L for rainbow trout, and 4.2 mg/L was reported for bluegill over the same test duration [3]. The toxicological significance of PBO lies in its ability to inhibit the mixed function oxidase enzymes, blocking natural detoxification pathways. Thus, PBO can enhance toxicity of compounds, such as pyrethroids, that are degraded by this pathway [4,5] and reduce toxicity of some organophosphate pesticides that require activation by mixed function oxidases [6]. Due to these distinctive effects, PBO also is used as a tool to identify certain types of pesticide toxicity in toxicity identification evaluations [7].

Technical-grade PBO is a yellow oil, soluble in water, with a log K_{OW} of 4.75. It is relatively stable in water-sediment systems: In the dark, 91% of the parent compound remained after 181 d under anaerobic conditions. In the presence of oxygen, 72% remained after 30 d in the dark. Soil degradation rates were much faster, however, with half-lives ranging from 1 to 4 d. Exposure to sunlight also rapidly increases degradation of PBO. In a neutral, aqueous solution exposed to sunlight, the half-life of PBO is just a few hours ([8]; www.ipfsaph.org). Piperonyl butoxide is detectable in surface waters, though there have only been a few attempts to measure concentrations in the environment. Piperonyl butoxide concentrations ranging from below the detection limit of 3.3 ng/L to 48.4 ng/L were measured in California rivers [9–11]. A concentration of 1,600 ng/L was reported from a single sample in a region of Spain known for rice and fruit production, though

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all other samples collected in that study were below the 50-ng/L detection limit [12]. In suspended and bed sediments, PBO was undetectable at 1.2 μg/kg in rivers from the Salton Sea watershed [13]. The only other known published PBO sediment data are from application to an experimental soil plot. Piperonyl butoxide concentrations dropped rapidly from 840 μg/kg immediately after application to 3 μg/kg in 30 d [14].

Data also are emerging on environmental concentrations of pyrethroid pesticides. In a survey throughout the Central Valley of California, an area of intensive agriculture, pyrethroids were found in 86% of the sediment samples [15]. In nearly 70% of those samples showing acute toxicity to *Hyalella azteca*, pyrethroid concentrations were high enough to account for it, even without considering potential synergism of PBO. In the Salton Sea watershed, permethrin, lambda-cyhalothrin, and bifenthrin were detected occasionally in suspended and bed sediments at concentrations ranging from above the detection limit (0.5–1.4 μg/kg) to 18.7 μg/kg [13]. Since 1991, the U.S. Geological Survey's National Water Quality Assessment has analyzed bed sediments from over 2,600 sites for permethrin; nine sites across the USA were above the detection limit (usually 5 μg/kg) with permethrin concentrations reaching 33.6 μg/kg (www.usgs.gov). Data are minimal on the distribution of pyrethroid pesticides in urban systems. Recent sampling around suburbs of Sacramento, California has found several pyrethroids, and particularly bifenthrin, in sediments of urban creeks often at concentrations sufficient to cause toxicity to sensitive invertebrates [16]. In addition, the amount of pyrethroids currently used in California for structural pest control, landscape maintenance, and public health applications is nearly twice that used in agriculture. This total does not include consumer home and garden use for pest control (it is not tracked by the State's Pesticide Use Reporting database), and therefore it seems likely that sediments in urban and suburban neighborhoods throughout California and potentially other portions of the United States may contain detectable pyrethroid concentrations.

This study was designed to determine PBO concentrations required to synergize toxicity of pyrethroids and whether or not these PBO concentrations are found in the environment. We address these issues by establishing the minimal PBO concentration required to increase permethrin toxicity to the amphipod *H. azteca*. Sediment LC50s for permethrin in spiked reference sediment were determined under three PBO exposure scenarios: No PBO, PBO exposure via water alone, and PBO spiked into both water and sediment at steady state concentrations. Additionally, sediment and water samples from urban creeks in northern California and Tennessee, USA, were collected and analyzed for PBO to determine typical urban concentrations. Wetlands in northern California sprayed with a mosquito adulticide containing PBO also were sampled shortly after application to determine PBO concentrations in water and sediment.

MATERIALS AND METHODS

Sample collection

Reference sediment for use in spiking experiments was collected in October 2003 from the South Fork of the American River (CA) about 2 km west of the confluence with Weber Creek, in Placer County near Folsom Lake. Sediment was collected by skimming the top 2 to 3 cm of sediment and sieving the material on a 1-mm screen. Sediment passing through the screen was homogenized and frozen until use. Chemical analysis showed no detectable concentrations (<1 μg/kg) of pyrethroids and the material had an organic carbon (OC) content of 1.87%.

Sediment and water samples were collected for PBO analysis from six urban creeks in the San Francisco Bay (CA, USA) area and sediments were collected from 16 sites in Sacramento, California, and Nashville, Tennessee, USA. In the San Francisco Bay region, water samples were collected in April 2004 by filling 4-L glass jars after an initial rinse with creek water. Sediment was collected at least twice: Once in late spring (April 2004) and again after the first fall storm event (October 2004). Two Sacramento, California, creeks and 14 sites from 12 creeks in Nashville also were sampled once in 2004. The surficial 2 to 3 cm of sediment was collected using a stainless steel scoop. Sediments and water samples were transported on ice to the laboratory. Water samples were extracted immediately onto C18 solid-phase extraction cartridges (Agilent Technologies, Palo Alto, CA, USA) using 500 ml of unfiltered sample. Sediments were stored at 4°C for up to one week before homogenization using a stainless steel bowl and spoon. The C18 cartridges and sediment samples were frozen at −30°C until chemical analysis. The same procedures were used for laboratory-spiked water and sediment samples.

Sediment and water samples also were collected from wetland sites in the Colusa and Delevan National Wildlife Refuges (NWRs), located 110 and 130 km north of Sacramento, respectively. The wetlands at Colusa NWR (39.13427°N, 122.04473°W and 39.13658°N, 122.04253°W) were sprayed biweekly in the late summer and early fall of 2004 to control mosquito populations. The Delevan NWR wetlands (39.19324°N, 122.05912°W and 39.19157°N, 122.05924°W) were not sprayed in 2004 or in any previous years. The Colusa Mosquito Abatement district applied Pyrenone 5–25, an adulticide mixture of 5% pyrethrin I and II and 25% PBO, at dusk using ultralow-volume foggers. The U.S. Fish and Wildlife Service collected samples the morning after mosquito adulticide application in order to evaluate possible accumulation of Pyrenone after repeated applications. Initial water and sediment samples were collected from the wetlands one week prior to the treatment period to obtain background concentrations. Additionally, water and sediment samples from the wetland inflow pipe were collected one week prior to, and also during, the initial adulticide application to obtain background PBO concentrations entering the wetlands. Sediments were collected with an Ekman hand grab (Forestry Suppliers, Jackson, MS, USA), and the upper 1 cm of sediment was retained. Occasionally, 1-L water samples were collected. At each site, three water and sediment samples were collected in individual containers and composited prior to analysis. Sampling continued throughout the 6-week abatement treatment period from September through October 2004.

Spiking of water and sediment

Permethrin (20% *cis*, 78% *trans*) was purchased from Chem Service (West Chester, PA, USA). It was chosen as a model pyrethroid because it is one of the most widely used pyrethroids in California. It also is useful for study because it has relatively low toxicity compared to other pyrethroids, allowing accurate analytical quantification of permethrin concentrations when synergized with PBO. Many other pyrethroid LC50s are near the analytical detection limit even without synergism with

PBO; exposure to PBO with more potent pyrethroids potentially could result in an LC50 below the method detection limit. Permethrin was dissolved in an acetone carrier and spiked into sediments using <200 μl acetone/kg wet sediment. This concentration of acetone previously has been shown to have no effect on pyrethroid toxicity to *H. azteca* [17]. Nominal sediment permethrin concentrations ranged from 31 to 540 ng/g.

Technical-grade PBO (90% purity) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Piperonyl butoxide was dissolved in methanol and spiked into the sediment using <75 μl methanol/kg wet sediment. Nominal sediment PBO concentrations ranged from 0.5 to 313 ng/g, and a solvent control for methanol at 75 μl methanol/kg wet sediment (and 25 μl methanol/L) was included among the treatments.

After spiking, the sediments were mixed with a steel paint mixing attachment in an electric drill and aged at 4°C for 11 to 12 d. Aliquots then were removed for chemical verification. Water was spiked with PBO in methanol using 25 μl methanol/L immediately before use, and the solution was mixed with a magnetic stirrer.

Some treatments involved spiking PBO into overlying water only. Piperonyl butoxide concentrations ranged from 0.1 to 56.3 μg/L, and the overlying water was replaced with a fresh solution containing PBO at the desired concentration every 24 h. Other treatments involved spiking PBO into sediment, and then changing the water daily with water to which PBO had been added. The treatments in which PBO was added to both the sediment and water were intended to begin the toxicity testing exposures with approximately steady state conditions so that PBO would be less likely to partition into the sediments, reducing the aqueous exposure concentration. In these cases, nominal PBO water concentrations were the same as water-only PBO exposures, and the PBO sediment spiking concentrations were determined assuming a PBO K_{OC} = 400 [18]. A 1.39% organic carbon content of the sediments was assumed based on analysis of a previous batch of sediment. When organic carbon data for the batch of sediments actually used in these experiments became available, it was determined to be 1.87%; thus, the PBO sediment concentrations may have been slightly below steady state levels. However, this error is not thought to be significant given the uncertainty in actual PBO K_{OC} values, for which estimates range from 400 to 1,800 ([8,18]; www.pesticideinfo.org).

Chemical analyses

Spiked sediment samples were analyzed for permethrin and PBO, but water samples were analyzed only for PBO. The sediment was analyzed for permethrin following the methods of You et al. [19] on an Agilent 6890 series gas chromatograph equipped with an Agilent 7683 autosampler and an electron capture detector (Agilent Technologies). Two columns from Agilent, a HP-5MS (30 m × 0.25 mm; 0.25-μm film thickness) and a DB-608 (30 m × 0.25 mm; 0.25-μm film thickness) were used for confirmation. Sediment was sonicated with acetone and methylene chloride and the extract was cleaned with deactivated Florisil. Ethyl ether and hexane were used as elution solvents, and the eluent was evaporated and redissolved in 2 ml of hexane. Half of the extract (1 ml) was used for permethrin analysis after dilution to a concentration within the calibration curve. Due to the hydrophobicity of permethrin, all pyrethroid sediment data are reported normalized to sediment organic carbon content.

The other half of sediment extract (1 ml) was used for PBO analysis that was performed on a high-performance liquid chromatography (HPLC) after further C18 solid-phase extraction cleanup. The hexane extract was solvent exchanged to 1 ml of methanol, and then diluted into 200 ml water. This solution, as well as a 6-ml water glassware rinse, was loaded on a previously conditioned solid-phase extraction cartridge, and the eluent was discarded. After drying the cartridge, PBO was eluted with 6 ml of methanol and the eluent was evaporated to 1 ml. In the case of the water samples, 500 ml of sample was loaded directly onto the solid-phase extraction cartridge.

Piperonyl butoxide concentrations then were determined on an Agilent 1100 series HPLC equipped with fluorescence detector (FLD) and ultraviolet detector (Agilent Technologies) and using a ZORBAX SB-C18 5 μm, 150 × 4.6 mm column (Agilent Technologies). A mobile phase of methanol and water was used for PBO separation. The flow rate was 1.1 ml/min and the injection volume was 25 μl. The excitation and emissions wavelengths were 295 and 335nm for fluorescence detection, and a wavelength of 287 nm was used for ultraviolet detection. With method detection limits of 0.007 μg/L and 0.3 μg/kg, the reporting limits were set at 0.05 μg/L and 5 μg/kg for water and sediment samples, respectively. The above procedure was followed for all Sacramento and San Francisco area urban creek samples. Piperonyl butoxide concentrations in a subset of these samples were verified by HPLC–mass spectroscopy (HPLC-MS) at the Marine Sciences Research Center, Stony Brook, New York, USA. Sediment and water samples collected from the Colusa and Delevan NWRs were analyzed via HPLC-MS by California Department of Fish and Game Water Pollution Laboratory, Rancho Cordova (CA, USA).

Test sediments were wet sieved to determine grain-size distribution and analyzed for organic carbon content on a CE-440 elemental analyzer from Exeter Analytical (Chelmsford, MA, USA), following acid vapor treatment to remove inorganic carbon.

Toxicity testing

Ten-day toxicity tests with the freshwater amphipod *H. azteca* were performed on sediments using standard U.S. Environmental Protection Agency protocols [20]. All toxicity tests were conducted at 23°C with a 16:8-h light:dark cycle in 400-ml beakers (four replicates per concentration) containing 50 to 75 ml of spiked sediment and 300 ml of moderately hard water reconstituted from Milli-Q® purified water (Millipore, Billerica, MA, USA). Ten amphipods, 7 to 10 d in age, were added to each beaker at test initiation. A yeast, cerophyll, and trout chow mixture was fed daily during the 10-d tests. Water changes (80%) using freshly prepared PBO-spiked water were performed every day, and water samples were taken prior to water renewal after 24 h and again on day 10 for analysis of temperature, dissolved oxygen, pH, conductivity, alkalinity, hardness, and ammonia. Sediment samples were taken for permethrin and PBO analysis at test initiation. Water samples were taken for PBO analysis at test initiation, again after 24 h before water exchange, and finally on day 10 at the conclusion of the test. All beakers were aerated gently and continuously. Tests were terminated by sieving contents of the beakers over a 425-μm screen and counting the surviving amphipods.

Table 1. Piperonyl butoxide (PBO) and permethrin concentrations and recoveries measured by high-performance liquid chromatography with fluorescence detection in spiked water and sediments. Data shown are from experiments in which PBO was spiked into both the sediment and overlying water. NA = Not available

Compound	Nominal concn.	Measured concn. (initial)	Measured concn. (24 h)	Measured concn. (10 d)	% Recovery initial	% Recovery 24 h	% Recovery 10 d
PBO in water (μg/L)	56.3	59.4	39.1	38.7	105.6	65.8	65.2
	11.3	11.6	7.2	7.8	103.1	62.1	67.2
	11.3	12.9	8.2	5.9	114.4	63.5	45.8
	2.3	2.7	2.3	1.7	120.0	85.2	63.0
	0.5	0.5	0.4	NA	117.8	81.1	NA
PBO in sediment (μg/kg)	312.7	135.0	NA	NA	43.2	NA	NA
	62.6	26.6	NA	NA	42.5	NA	NA
	12.5	10.8	NA	NA	86.3	NA	NA
	2.5	<5	NA	NA	NA	NA	NA
	0.5	<5	NA	NA	NA	NA	NA
Permethrin in sediment (μg/kg)	352.0	217.7	NA	NA	61.8	NA	NA
	235.0	144.1	NA	NA	61.3	NA	NA
	157.0	110.7	NA	NA	70.5	NA	NA
	157.0	117.1	NA	NA	74.6	NA	NA
	104.0	80.2	NA	NA	77.1	NA	NA

Statistics

Toxicity data were analyzed using ToxCalc 5.0 software (Tidepool Scientific Software, McKinleyville, CA, USA). Survival data were arc–sin transformed prior to analysis. The Spearman-Karber method was used to determine LC50s and associated confidence intervals for each permethrin/PBO mixture. Abbott's correction was applied in a few cases to account for control mortality.

RESULTS

Spiking recovery

Mean recovery of PBO spiked into water in the toxicity tests was 112 ± 11% of the initial nominal concentrations (Table 1). Over the 24-h period between water renewal in the beakers, PBO declined to 70 ± 10% of the initial concentrations. Because PBO is susceptible to photodegradation, this loss most likely is due to breakdown in the test water, although additional losses to adsorption, evaporation, or other loss mechanisms have not been evaluated.

Sediments spiked with permethrin and PBO had recoveries lower than nominal concentrations. In five samples, mean permethrin recovery at test initiation was 69 ± 7%. Low recoveries may have been due to degradation during the 12-d aging period, adsorption on container walls (glass), or incomplete chemical extraction from the sediments. Recoveries in this range have been typical of past uses of the same spiking and analytical techniques [17]. Piperonyl butoxide recovery in sediments averaged 58 ± 25%, with loss possibly due to degradation during sediment aging. All data reported are based on nominal concentrations.

Toxicity testing

Control survival in all tests averaged 96 ± 4%, and all water-quality parameters were acceptable. Addition of PBO in the absence of permethrin had no toxic effect at any concentration used (up to 56 μg/L); mean survival in all tests with PBO in sediment and/or water, but with no permethrin was 96 ± 3%. Permethrin LC50s for *H. azteca* in this study were 14.2 mg/kg OC (confidence interval = 11.8–17.1) and 21.3 mg/kg OC (14.7–30.5) as determined in two independent tests. No statistically significant differences were found between these values and permethrin LC50 determined in the acetone solvent control (13.2 mg/kg OC [11.5–15.2]) or the permethrin LC50 reported by Amweg et al. [17] in similar American River test sediment (17.9 mg/kg OC [14.8–19.9]). Because no difference existed between solvent control and control LC50s determined in this study, the median test LC50 (14.2 mg/kg OC) was used for statistical evaluation of PBO effects.

Hyalella azteca exposed to PBO and permethrin in laboratory toxicity tests exhibited higher mortality than those exposed to permethrin alone. In the tests conducted with PBO-spiked sediment and water, permethrin was approximately twice as toxic if the organisms were concurrently exposed to PBO at 12.5 μg/kg and 2.3 μg/L (permethrin LC50 reduced from 14.2 to 7.3 mg/kg OC; Table 2, Figure 1A). Higher concentrations of PBO reduced the LC50 by even greater amounts. A 3.5-fold increase in toxicity was noted for permethrin when

Table 2. Nominal water and sediment piperonyl butoxide (PBO) concentrations in all laboratory exposures and the resulting permethrin 10-d median lethal concentration (LC50) to *Hyalella azteca*. The PBO treatments with asterisks indicate LC50s significantly lower ($p < 0.05$) than the permethrin-only LC50. OC = Organic carbon; CI = 95% confidence interval

Aqueous PBO (μg/L)	Sediment PBO (μg/kg)	Permethrin LC50 (CI) (mg/kg OC)
Permethrin with no PBO		
Permethrin (1)–0.0	0	14.2 (11.8–17.1)
Permethrin (2)–0.0	0	21.3 (14.7–30.5)
Permethrin, solvent control–0.0	0	13.2 (11.5–15.2)
Permethrin with PBO in water and sediment		
0.1	0.5	13.3 (11.7–15.2)
0.5	2.5	14.3 (13.0–15.8)
2.3	12.5	*7.3 (6.5–8.0)
11.3	62.6	*5.5 (5.0–6.1)
56.3	312.7	*2.0 (1.9–2.1)
Permethrin with PBO in water only		
2.3	0	10.5 (9.3–11.8)
11.3	0	*8.6 (7.7–9.6)
56.3	0	*4.1 (2.6–5.0)

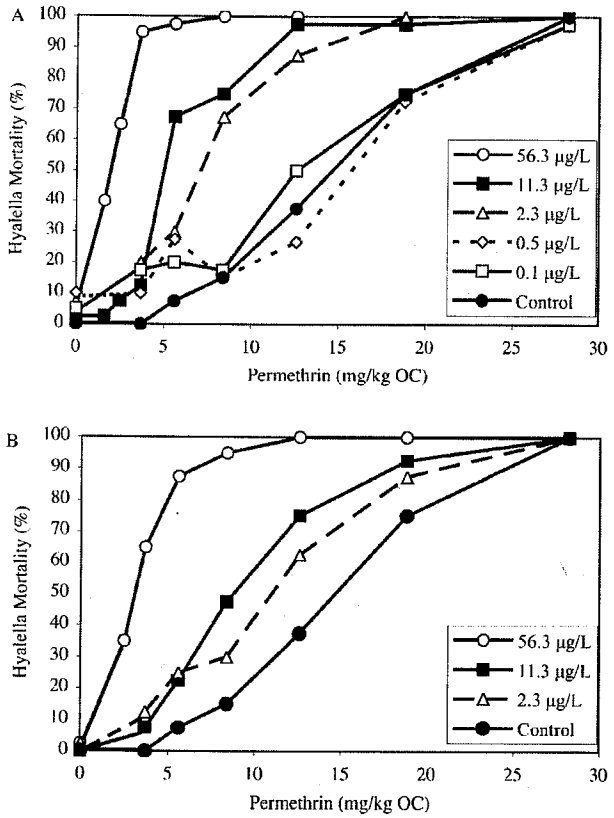

Fig. 1. Permethrin median lethal concentration (LC50) curves for *Hyalella azteca*. Exposures with piperonyl butoxide (PBO) in sediment and water are shown in (**A**), and exposures with PBO in water only are shown in (**B**). The legend indicates water concentrations of PBO; concurrent sediment concentrations are shown in Table 2. OC = organic carbon.

also exposed to PBO at 11.3 µg/L and 62.6 µg/kg. Piperonyl butoxide concentrations of 56.3 µg/L and 313 µg/kg resulted in a permethrin LC50 of just 2.0 mg/kg OC, a sevenfold increase in toxicity. The synergistic effect of PBO on permethrin toxicity is seen clearly by a dose-dependent shift in the dose-response curve to the left and an increase in the slope of the curve at higher PBO concentrations.

In toxicity tests conducted with only PBO-spiked water, permethrin toxicity also was increased (Table 2, Figure 1B). A PBO concentration of 2.3 µg/L, which significantly changed permethrin toxicity in the spiked sediment and water treatment, resulted in a slight, but not statistically significant, increase in permethrin toxicity in the spiked water-only tests. A PBO concentration of 11.3 µg/L was necessary to increase significantly permethrin toxicity, reducing the LC50 from 14.2 to 8.6 mg/kg OC. Permethrin toxicity was increased by a greater amount in tests where PBO was spiked into both sediment and water, as compared to tests with PBO exposure via water alone. Because both types of tests were conducted using the same nominal PBO water concentrations, this effect probably was due to two factors. First, PBO in the water-only tests would partition into sediment, reducing the bioavailable fraction in the overlying water and pore water. Sediments were spiked with steady state concentrations of PBO to eliminate PBO flux to sediments in tests with PBO in sediment and water. Additionally, *H. azteca* probably is exposed to a mixture of overlying and pore water [21–23]. When only the overlying water is spiked, dilution of PBO with pore water in the surficial sediments occupied by *H. azteca* would reduce the exposure concentration below that measured in the overlying water.

Urban creek sampling

Sediment collected from urban creeks in the San Francisco Bay area contained detectable PBO at least once at every site (Table 3). In April 2004, the reporting limit for PBO was 1 µg/kg; concentrations measured by HPLC-FLD ranged from 2.6 to 8.9 µg/kg. In October 2004, only two of eight sediments contained PBO above the reporting limit of 5 µg/kg. Piperonyl butoxide concentrations in Kirker and San Leandro sediments were verified by HPLC-MS, a more accurate method for determining compound identity. Both were found to contain 1.8 µg/kg PBO, compared to the 3.6 and 8.9 µg/kg reported by HPLC-FLD. Because HPLC-MS offers compound identification, the lower values of 1.8 µg/kg PBO are considered more accurate and suggest that other contaminants may be misidentified as PBO by the HPLC-FLD at times. With the only two verified samples containing 20 and 50% PBO of that reported by HPLC-FLD, it is not possible to generalize whether these ratios are typical of the other environmental sediment samples.

Water samples typically were below the detection limit for both methods, although Lauterwasser Creek did contain 0.13 µg/L PBO as measured by HPLC-MS (0.068 µg/L by HPLC-FLD). It is unclear why HPLC-FLD detected a lower concentration than HPLC-MS in this sample. Theoretically, peaks detected by HPLC-FLD could contain both PBO and other co-eluting contaminants, whereas HPLC-MS offers more accurate compound identification, resulting in PBO concentrations typically lower than determined by HPLC-FLD. However, in the absence of co-eluting contaminants, HPLC-FLD performs quite well: Recoveries were 112% of nominal in laboratory water spiked with PBO (Table 1). Taken together, these data suggest that HPLC-FLD would be an appropriate screening tool for detecting PBO in environmental samples, with PBO detection verified by HPLC-MS.

Out of the 14 urban creek sediments sampled in Nashville, only three sediment samples contained PBO in sediment above the reporting limit of 5 µg/kg. These concentrations were 5.5, 7.7, and 26.2 µg/kg PBO. This range overlaps with effective synergistic concentrations in laboratory tests with PBO-spiked sediment and water. Neither of the two creek sediments collected from Sacramento contained detectable concentrations of PBO.

Mosquito abatement sampling

None of 20 sediment samples or 12 water samples collected at the control site, Delevan NWR, contained detectable levels of PBO (data not shown). In Colusa NWR wetlands, PBO was detected in 10 of 18 sediment samples taken during mosquito abatement activities, although only two samples contained PBO above the 2.0 µg/kg reporting limit (Table 4). Sediment PBO concentrations 12 h following ultralow-volume fogger application of Pyrenone 5–25 peaked at 3.3 µg/kg. Prior to the initial Pyrenone 5–25 application and one week after applications had ceased, sediment PBO concentrations were below the reporting limit. In water samples, PBO was detectable in seven of eight water samples collected within 12 h of abate-

Table 3. Piperonyl butoxide (PBO) concentrations in sediment and water collected from urban creeks in California and Tennessee, USA. The reporting limit in sediments for high-performance liquid chromatography with fluorescence detection (HPLC-FLD) is 1 μg/kg for April 2004, 5 μg/kg for October 2004, and 0.050 μg/L for water samples. For samples verified by HPLC–mass spectroscopy, the results of these confirmatory analyses are shown in parentheses after the HPLC-FLD values. OC = organic carbon

Site	Location	Date	Latitude	Longitude	% OC	Sediment (μg/kg)	Sediment (mg/kg OC)	Water (μg/L)
San Francisco Bay Area, CA								
Glen Echo Creek	Oakland	04/20/2004	37.97500	−122.50833	1.55	7.7	0.50	<0.050
		10/26/2004			0.52	5.9	1.13	
Kirker Creek	Pittsburg	04/20/2004	38.01655	−121.83914	2.12	3.6 (1.8)	0.17 (0.08)	<0.050 (<0.005)
		10/18/2004			1.35	<5.0	<0.37	
		10/25/2004			2.91	<5.0	<0.17	
Lion Creek	Oakland	04/21/2004	37.76037	−122.19512	4.54	6.8	0.15	<0.050
		10/28/2004			7.81	12.7	0.16	
Lauterwasser Creek	Orinda	04/21/2004	37.89754	−122.19238	0.36	2.6	0.73	0.068 (0.130)
		10/18/2004			1.13	<5.0	<0.44	
		10/25/2004			1.60	<5.0	<0.31	
San Pablo Creek	Richmond	04/20/2004	37.88611	−122.25500	0.92	2.9	0.31	<0.050
		10/26/2004			0.94	<5.0	<0.53	
San Leandro Creek	Oakland	04/20/2004	37.72547	−122.18278	1.95	8.9 (1.8)	0.46 (0.09)	<0.050
		10/29/2004			2.16	<5.0	<0.23	
Sacramento area, CA								
Arcade Creek	Sacramento	10/15/2004	38.64217	−121.36695	1.40	<5.0	<0.36	
Curry Creek	Roseville	10/15/2004	38.75813	−121.35860	3.64	<5.0	<0.14	
Nashville area, TN								
Cedar Creek	Nashville	06/28/2004	36.23131	−86.50636	1.34	<5.0	<0.37	
Mill Creek	Nashville	06/29/2004	36.09185	−86.68623	1.58	<5.0	<0.32	
Mill Creek	Nashville	06/29/2004	36.11757	−86.71921	3.16	26.2	0.83	
West Fork Hamilton Creek	Nashville	06/29/2004	36.08896	−86.62771	1.88	5.5	0.29	
Little Harpeth River	Nashville	06/29/2004	36.01928	−86.82078	5.17	<5.0	<0.10	
Harpeth River	Nashville	06/29/2004	36.02869	−86.92424	1.80	<5.0	<0.28	
Harpeth River	Nashville	06/29/2004	36.07711	−86.95721	1.72	<5.0	<0.29	
Dry Creek	Nashville	06/28/2004	36.28455	−86.70625	3.08	7.7	0.25	
Madison Creek	Nashville	06/28/2004	36.31434	−86.66566	1.46	<5.0	<0.34	
Drake Creek	Nashville	06/28/2004	36.31261	−86.60865	1.74	<5.0	<0.29	
Station Camp Creek	Nashville	06/28/2004	36.34674	−86.52545	3.07	<5.0	<0.16	
East Fork Station Camp Creek	Nashville	06/28/2004	36.38684	−86.48183	2.94	<5.0	<0.17	
Gills Creek	Nashville	06/28/2004	36.34583	−86.44000	4.43	<5.0	<0.11	
Hays Branch	Nashville	06/28/2004	36.25513	−86.55940	1.27	<5.0	<0.39	

ment spraying. Three of these samples were above the 0.01 μg/L reporting limit with concentrations ranging from 0.04 to 0.08 μg/L. Water PBO concentrations were below the reporting limit one week after abatement activities.

DISCUSSION

The results of this study suggest that PBO synergism in the environment is possible, although not likely under most conditions. In laboratory tests, 2.3 μg/L PBO in water and 12.5 μg/kg in sediment significantly increased 10-d permethrin toxicity. The synergistic sediment concentration of PBO was exceeded by concentrations in two of the 30 urban creek samples: 26.2 μg/kg in a Nashville creek and 12.7 μg/kg in Lion Creek, but considering the possibility of overestimation by HPLC-FLD, urban sediment concentrations actually may be lower. Peak sediment concentrations of PBO following mosquito abatement activities were fourfold below effective synergistic concentrations. No tests were conducted with PBO-spiked sediment only; however, assuming *H. azteca* is exposed to a mixture of pore and overlying water [21], in the absence of overlying water PBO exposure, sediment PBO concentrations necessary to cause synergy are probably far higher than any seen in these environmental samples.

Only one of the six urban creek water samples contained PBO concentrations above the 0.050 μg/L reporting limit. This sample, from Lauterwasser Creek, contained 0.068 μg/L (by HPLC-FLD) or 0.13 μg/L (by HPLC-MS); the concentration was at least 18 times too low to affect permethrin toxicity based on these laboratory experiments. The concentration of PBO necessary to synergize other pyrethroids was not determined, but presumably would be comparable to that for permethrin, because a PBO-induced inhibition of p450 activity would affect detoxification of all pyrethroids.

Given the results from this study, higher environmental PBO concentrations would be necessary in order to exert a synergistic effect with pyrethroids, perhaps in the case of pulses of PBO carried in to surface waters by storm events, or more likely when surface waters are contaminated directly during mosquito spraying. However, even 12 h following mosquito abatement applications of PBO using ultralow-volume foggers, peak water concentrations were just 0.08 μg/L, about 3% of synergistic levels. Although PBO frequently was de-

Table 4. Piperonyl butoxide (PBO) concentrations in sediment and water collected from the Colusa National Wildlife Refuge (CA, USA) following application of Pyrenone 5-25 for mosquito abatement purposes. The reporting limit for sediment and water was 2.0 μg/kg and 0.01 μg/L, respectively; the detection limit was 1.0 μg/kg and 0.005 ng/L, respectively. <RL = detected but less than reporting limit; ND = not detected; NA = not available because no sample was collected

	Location	Date	Sediment PBO concn. (μg/kg)	Water PBO concn. (μg/L)
1 Week prior to Pyrenone 5-25 application	Inflow	9/2/04	ND	ND
	Wetland	9/2/04	ND	ND
	Wetland	9/2/04	ND	ND
Mosquito abatement treatments with Pyrenone 5-25 (samples collected 12 h following each abatement treatment)	Inflow	9/10/04	<RL	NA
	Wetland	9/10/04	<RL	NA
	Wetland	9/10/04	<RL	NA
	Wetland	9/15/04	ND	0.08
	Wetland	9/15/04	ND	<RL
	Wetland	9/17/04	ND	<RL
	Wetland	9/17/04	ND	0.04
	Wetland	9/22/04	ND	NA
	Wetland	9/22/04	ND	NA
	Wetland	9/24/04	<RL	<RL
	Wetland	9/24/04	ND	<RL
	Wetland	9/29/04	<RL	NA
	Wetland	9/29/04	3.00	NA
	Wetland	10/1/04	<RL	NA
	Wetland	10/1/04	3.27	NA
	Wetland	10/13/04	ND	ND
	Wetland	10/13/04	<RL	0.06
1 Week after abatement applications concluded	Wetland	10/20/04	<RL	<RL
	Wetland	10/20/04	ND	<RL

tected in water samples following mosquito abatement application of Pyrenone 5–25, concentrations were not elevated above those found in urban creek samples. Sediment pyrethroid residues that co-occurred with PBO contamination also would be required in order for increased toxicity to invertebrates with similar sensitivities to *H. azteca*. Piperonyl butoxide synergism could occur only under a limited set of environmental circumstances, and it seems likely environmental PBO concentrations typically would not alter pyrethroid toxicity. However, this conclusion must be somewhat qualified by the relatively small data set available for this study on environmental PBO concentrations.

It is important to bear in mind that only the amphipod *H. azteca* was tested in this study. It is one of the more sensitive species to pyrethroids tested to date [15], and therefore the effect of PBO and pyrethroids on other aquatic organisms is not immediately clear. Insect populations are known to develop insecticide resistance by increasing detoxification enzymes, including increased p450 metabolism. In a pyrethroid and organophosphate pesticide resistant tobacco budworm population, PBO was two to 11 times more effective as a synergist than in a nonresistant population [24]. In beet army-worms, PBO was 14 times more effective as a synergist in pyrethroid-resistant populations than nonresistant populations [25]. Thus, if pesticide resistance exists in wild populations, exposure to PBO may enhance pyrethroid toxicity at concentrations lower than found in the present study.

Pyrethroid resistance in insects also has been shown to confer crossresistance among pyrethroids and DDT, with partial reversal to susceptibility with exposure to PBO [26–28]. Although several molecular resistance mechanisms are known, the efficacy of PBO in these cases suggests the mechanism involves enhanced p450-mediated detoxification [29,30]. Pesticide-resistant populations, including aquatic invertebrates, with increased p450 metabolism therefore also could face PBO synergism of DDT if these two compounds co-occurred at biologically relevant concentrations. This effect has been shown in laboratory exposures with DDT-resistant *Drosophila* populations capable of metabolizing DDT via a p450-mediated pathway [31,32].

In addition to resistance, species differences in p450 metabolism also appear to have a dramatic influence on PBO efficacy. Exposure to PBO did not alter organophosphate toxicity to *L. variegatus*, whereas PBO exposure in *C. tentans* and *H. azteca* caused the expected mitigation of toxicity [1]. The authors postulate that *L. variegatus* has a relatively low p450 metabolic activity and pesticides and xenobiotics are detoxified via other pathways, such as carboxylesterases or glutathione-*S*-transferases. Oligochaetes and other organisms with low p450 activity will be much less sensitive to the synergistic effects of PBO under environmental conditions as well.

Regardless, populations of *H. azteca* do exist in creeks throughout the United States. In order to protect this sensitive species and others, effective monitoring programs should consider the cumulative effect of exposure to contaminant mixtures, and additional information on environmental PBO levels should be acquired before dismissing potential synergism of pyrethroids under realistic environmental conditions.

CONCLUSION

Laboratory toxicity tests conducted with the amphipod, *H. azteca*, show that PBO effectively synergizes toxicity of the pyrethroid pesticide permethrin. Permethrin sediment 10-d LC50s conducted with exposure to PBO via spiked water and sediment caused increased toxicity at concentrations as low as 2.3 μg/L and 12.5 μg/kg. In permethrin sediment toxicity tests

with PBO exposure via water only, 11.3 μg/L PBO was required before permethrin toxicity to *H. azteca* was enhanced.

In field samples from urban creeks in California and Tennessee, and samples from a California wetland following mosquito abatement treatments applied with ultralow-volume foggers, PBO water concentrations were far below those necessary to synergize permethrin. In a small proportion of the samples, sediment PBO concentrations did approach such levels, although PBO concentrations in overlying water were not measured or were below synergistic levels at these times. Given that synergistic levels of PBO appear to occur infrequently, and they would have to co-occur with toxicologically relevant levels of pyrethroids in the environment to be of concern, it appears environmental synergism of pyrethroids by PBO would be unlikely, except perhaps under exceptional circumstances.

Pyrethroid use has been increasing in recent years, especially in urban areas. Given the recent concern over West Nile virus and the withdrawal of some alternative pesticides from the consumer market, this trend likely will continue. Although, when based on the limited data the risk of environmental synergy appears low, more information on pyrethroid distribution in urban creeks, as well as additional information on PBO concentrations in surface waters and sediments, is necessary to assess properly risk from pesticide residues.

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Does synergized pyrethrin applied over wetlands for mosquito control affect *Daphnia magna* zooplankton or *Callibaetis californicus* mayflies?

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Abstract

BACKGROUND: Public health agencies may apply aerosolized synergized pyrethrin over wetlands repeatedly to control mosquitoes. This concerns wildlife managers because studies have shown the accumulation of pyrethroids, which are chemically similar to pyrethrin, in sediments in amounts that can be toxic to invertebrates. The authors tested whether repeated applications of synergized pyrethrin over wetlands caused mortality of two aquatic invertebrates: the zooplankton *Daphnia magna* Straus and a mayfly, *Callibaetis californicus* Banks. Fifteen wetland mesocosms were either exposed to repeated pyrethrin sprays or were protected by lids. Invertebrates in screened cages were placed in mesocosms before the fifth and eleventh spray, and directly into wetlands before spray 11. Six mesocosms were exposed to spray deposition. Caged adult mosquitoes were used to verify that sprays drifted over mesocosms. Sediments were analyzed for insecticide residues.

RESULTS: There were no detectable effects of synergized pyrethrin on 36 h survival of *Daphnia* or mayflies, but most exposed adult mosquitoes died. Some exposed sediments yielded pyrethrin ($\leq$34.5 ng g^{-1}); most showed piperonyl butoxide (PBO) ($\leq$14.9 ng g^{-1}).

CONCLUSIONS: Deposition of aerosolized 25% pyrethrin + 5% PBO may contaminate wetlands, but its application at rates used for mosquito control did not produce detectable effects on indicator species.
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Keywords: pyrethrin; piperonyl butoxide; ULV; adulticide; Culicidae; Baetidae; Cladocera; ecological impact

1 INTRODUCTION

Integrated pest management programs employed by mosquito abatement districts commonly include ultralow-volume (ULV) aerosolized applications of pyrethrin, pyrethroids or organophosphates as 'adulticides' (chemicals used to control adult mosquitoes over wetlands and other areas). ULV pesticides are being applied over wider areas of the USA following the invasion of West Nile virus. Typically, adulticides are used to control mosquitoes that could not be controlled effectively in breeding sources at the larval stage. Sometimes ULV insecticides are applied over the same areas multiple times within a season, but the effects of repeated applications on non-target invertebrates have rarely been quantified. This study focuses on pyrethrin, which is in rotational use with organophosphates such as malathion in northern California, USA. The authors quantified the effects on non-target aquatic invertebrates of repeated applications of ULV synergized pyrethrin for mosquito control under operational conditions. The formulation used was Pyrenone® 25–5 Public Health (Bayer Environmental Science, Montvale, NJ). This contains 50 g L^{-1} pyrethrin synergized with 250 g L^{-1} piperonyl butoxide. The typical composition of pyrethrin is pyrethrin I (38.0%), cinerin I (7.3%), jasmolin I (4.0%), pyrethrin II (35.0%), cinerin II (11.7%) and jasmolin II (4.0%); the composition of piperonyl butoxide is 80% 5-[2-(2-butyloxyethoxy)ethoxymethyl]-6-propyl-1,3-benzodioxole and 20% related compounds (Brill J, Bayer Environmental Science, private communication).

Although generally regarded as safe for birds and mammals, pyrethrin and pyrethroids are more toxic to fish and invertebrates.[1–3] Pyrethrin is a

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distillate of the chrysanthemum plant. Pyrethroids are chemically similar but have been modified for enhanced stability and/or insecticidal activity. These are considered together in this brief review owing to their similarities. Natural resource managers have expressed concern about the environmental safety of pyrethrin and pyrethroids because laboratory studies have shown that these insecticides are toxic to zooplankton and aquatic insects at concentrations of less than $1\,\mu g\,L^{-1}$.[3–8] Such levels or higher may occur after ULV applications for mosquito control.[9] In general, pyrethrin and pyrethroids break down rapidly in sunlight and adsorb to sediments, which may reduce exposure of some invertebrates.[2,5,10] However, these chemicals can persist in sediments,[9,11,12] so that benthic organisms may experience more exposure.[10]

The few published field studies of how ULV pyrethrin or pyrethroids affect aquatic invertebrates have had variable outcomes, and study designs have not reflected repeated applications to realistic wetlands. Jensen *et al.*[13] found no detectable effects on field populations of aquatic stages of insects after one-time applications of pyrethrin or permethrin. A field study in which zooplankton were placed outside in 50 mL beakers with clean water and no sediments showed some mortality in zooplankton after exposure to ULV permethrin.[6] A regression study showed declines in several zooplankton taxa at $\geq 0.13\,\mu g\,L^{-1}$ cypermethrin in 200 L enclosures without sediment, where insecticide was added to the water.[7]

The authors used realistic field mesocosms with wetland vegetation and sediments. Research sites were the Colusa and Sacramento National Wildlife Refuges in Colusa Co., CA (hereafter, Colusa NWR and Sacramento NWR). Colusa NWR is typically treated once or twice weekly with ULV synergized pyrethrin during September and October, while the southern part of Sacramento NWR is untreated. The survival of field-exposed sentinel species to acute and cumulative sprays was assessed. Sentinels were 'water fleas', *Daphnia magna* Straus, and larvae of the mayfly *Callibaetis californicus* Banks. *Daphnia magna* is a standard organism for toxicity assays. Its sensitivity to pyrethroids is comparable with other zooplankton.[4] Mayflies were included in this study because they live in contact with surfaces and use gill respiration; both their microhabitat and permeable cuticle should result in exposure to any pyrethrin present.

2 MATERIALS AND METHODS

2.1 Mesocosms and spray application

The authors prepared 12 wetland mesocosms on Colusa NWR in polyethylene cattle watering tanks (1150 L; High Country Plastics, Caldwell, ID). Half were open to sprays and half were covered with tarps on spray nights. Treatments were randomized with the constraint that not all of one treatment could be on the same side or end of the array; the second randomization met this criterion. Three additional, uncovered mesocosms were located ~22.5 km northwest, in the unsprayed area of Sacramento NWR, as extra controls in case there were lid effects. Tank bottoms were half-covered with dirt (5–10 cm deep), with the other half containing grass sod. Both substrates were from unsprayed seasonal wetlands at Sacramento NWR. The 12 tanks were placed in a double row 18 m from a spray route along the south levee. Pools were filled to ~30 cm on 30 August with certified clean water and maintained at that level for trials. Fields were then flooded. The wetland substrates provided resting stages of many invertebrates and insects oviposited in the mesocosms.

The Colusa Mosquito Abatement District (CMAD) applied synergized pyrethrin along a levee south of the mesocosms once weekly from 5 to 21 September, then twice weekly through October ($0.458\,g\,AI\,ha^{-1}$). Southerly prevailing winds carried all or part of the spray across the array. The control tanks at Colusa NWR were covered with tarp lids at dusk on each spray night, and lids were removed early the next morning. USFWS collected sediment samples from the mesocosms within 12 h (5:00–8:00 a.m.) after each application. Invertebrate mortality trials took place on 28–30 September (spray 5), 17–19 October (spray 10) and 19–21 October (spray 11).

2.2 Chemical analysis

Core samples of sediment and associated water were collected from mesocosms on mornings following sprays. The top 2 cm of the core was placed in a chemical clean I-Chem amber jar, placed on ice and transported to the California Department of Fish and Game Water Pollution Control Laboratory, Rancho Cordova, CA, for analysis. Samples were kept at 4 °C and extracted within 7 days of receipt. Pyrethrins and piperonyl butoxide were extracted from water samples with solid-phase extraction (SPE) columns packed with octadecyl (C18) bonded-phase silica. Pressurized fluid extraction using a Dionex Accelerated Solvent Extractor (ASE-200) was used for sediment samples. Pyrethrin I and II and its isomers, as well as the synergist piperonyl butoxide (PBO), were analyzed with an Agilent 1100 LC-MSD coupled to a diode array UV-Vis detector (DAD) using atmospheric pressure ionization-electrospray ionization (API-ES) in positive mode. The system used a reversed-phase C18 column and an acidified methanol:water gradient. Data were collected in both SIM and scan modes. A surrogate (fluridone) was added to monitor extraction efficiency. The method detection limit for water was $0.010\,\mu g\,L^{-1}$ and sediment was $2.00\,\mu g\,kg^{-1}$ dry weight. Mean fortified sample recoveries ranged from 74.7 to 107% for water samples with a relative standard deviation (RSD) of 1.41–18.9%. Recoveries from fortified sediment samples ranged from 84.8 to 103% with 6.97–16.4% RSD.

2.3 Bioassays

On trial nights, the efficacy of sprays on mosquitoes was measured using caged adult female *Culex*

tarsalis (Coquilliet) mosquitoes from an insecticide-susceptible laboratory strain. Standard screened adult cages contained 20 recently emerged females plus wicks soaked with sugar solution.[14] Four cages were staked 2 m apart and 0.6 m above the wetland surface within the tank array shortly before spray application. Four similar cages were staked near the three mesocosms at Sacramento NWR. Cages were collected 20–40 min after sprays, wicks were refreshed with sugar solution and the treated and control cages were held in separate coolers until mosquito mortality was recorded ~20 h later.

The survival of commercially raised adult female *D. magna* and *C. californicus* mayflies collected at the Sacramento NWR control site was assessed. *Daphnia magna* arrived from the supplier (Aquatic Biosystems, Fort Collins, CO) 12–24 h before experiments. These were fed algae and yeast cultures prior to use. Mayflies were collected from unsprayed wetlands at Sacramento NWR on the day of experiments, using gentle sweeps of a 'd-ring' net. Mayfly body lengths ranged from 4 to 10 mm.

For the first two trials, two cages containing 25 *D. magna* each and two cages containing ten mayflies each were placed in every mesocosm. Cages for *D. magna* were floating buckets (3.79 L) with fine-screened bottoms, lids and side windows. Cages for mayflies were plastic buckets (18.93 L) with fine-screened bottoms and sides. Both cage types had screening for at least 50% of total surface area and 80% of cage bottoms. Mayfly cages were pushed into the substrate so that there was a layer of sediment on the cage bottoms. The cage volumes were large enough to minimize the relative amount of pyrethroid adsorption to bucket surfaces.[10] Mortality was recorded after ~19 and 36 h.

The third trial included the following modifications. *Daphnia* per cage were reduced to 10 because the supplier had fewer available. The lids were left off half of the sentinel cages to check whether direct deposition might lead to stronger effects than the indirect deposition monitored in the first trials. Because the wind had been shifting to northerly, CMAD added a spray along the north levee. Additional cages of sentinel organisms were placed directly into the north end of the wetland at Colusa NWR and in another wetland at Sacramento NWR. Four stakes were placed approximately 5 m apart in each of these wetlands, approximately 9 m from levees. Four additional containers of mayflies and three of *Daphnia* were attached to the stakes, so that the mayfly cages rested on the bottom and the *Daphnia* were at the surface. Three adult mosquito cages were placed at the array on Colusa and two at the north end of the wetland next to the new aquatic sentinels, and five adult cages were arranged similarly at Sacramento NWR.

2.4 Statistical analysis

The survival of control and exposed adult mosquitoes was compared via non-parametric analysis of variance (Mann–Whitney test) owing to unequal variances between treatments. For zooplankton and mayflies, survival values were the means of the two buckets per species, per tank, since buckets within a tank are not independent. Data were analyzed with repeated-measures analysis of variance (ANOVA) on the mean proportion surviving at 12 and 36 h, using both arcsin square root transformed data and non-parametric ANOVA on untransformed data with dates. Two containers of *Daphnia* and one container of mayflies were eliminated from the analysis owing to problems with the cages. One control bucket of *Daphnia* had unexplained 100% mortality (possibly owing to excess chilling in the cooler), and one treatment bucket had missing animals plus a small rip in the netting that admitted a predatory insect (Belostomatidae). A mayfly cage was eliminated from the 21 October survival data because seven of the ten surviving mayflies were spilled. In each case, the survival of animals in the other cage was used for that mesocosm.

3 RESULTS

Two applications of synergized pyrethrin resulted in considerable mortality of *C. tarsalis* adult females (Fig. 1). Spray 5 on 28 September killed 71% of the mosquitoes, whereas only one control female died (Mann–Whitney $U = 16, P = 0.018$). Spray 11 on 19 October killed 82% of the mosquitoes and only one control female died ($U = 25$, $P = 0.007$). Mortality was 60–95% next to the mesocosms and 95–100% at the north end of the wetland. However, for spray 10 on 17 October, winds were weak and changeable, and the exposed adult mosquitoes showed only 20–30% mortality. Virtually all aquatic sentinel organisms were alive the next morning. These data were discarded as being uninformative.

There were no differences in survival of control *Daphnia* or mayflies between the lidded control mesocosms at Colusa NWR and the open controls located at Sacramento NWR, so subsequent analyses present results from the Colusa mesocosms alone

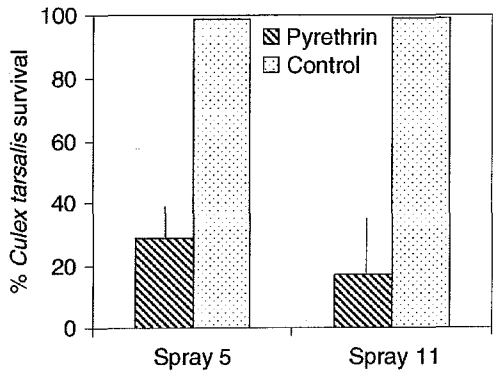

Figure 1. Mean survival of four cages of 20 adult *Culex tarsalis* per treatment either exposed to or shielded from sprays of ultralow-volume synergized pyrethrin. Spray 5 was 28 September 2006, and spray 11 was 19 October 2006. Bars are standard errors.

Table 1. Levels of sediment contamination after repeated applications of ULV pyrethrin and piperonyl butoxide over wetland mesocosms, and mean percentage survival of two screened cages of *Daphnia magna* or *Callibaetis californicus* mayflies held in exposed or control mesocosms during sprays and 36 h after sprays. Control mesocosms were protected by tarps (PBO = piperonyl butoxide; ND = not detected; spray 5 was 28 September 2006; spray 11 was 19 October 2006)

		Contaminants ($\mu g\,kg^{-1}$ dry weight)			Mean survival (%)	
Treatment	Mesocosm	Pyrethrin I	Pyrethrin II	PBO	*Daphnia*	Mayflies
			Spray 5			
Spray	1	<2.00	23.1	8.37	96	40
Spray	4	<2.00	<2.00	14.4	84	55
Spray	7	<2.00	<2.00	14.9	80	80
Spray	8	<2.00	<2.00	<2.00	96	75
Spray	11	<2.00	33.1	8.51	86	55
Spray	12	<2.00	<2.00	10.9	86	95
Control	2	<2.00	<2.00	<2.00	96	50
Control	3	<2.00	<2.00	<2.00	90	60
Control	5	<2.00	3.09	<2.00	86	100
Control	6	<2.00	<2.00	<2.00	90	85
Control	9	<2.00	<2.00	<2.00	96	90
Control	10	<2.00	<2.00	<2.00	88	75
			Spray 11			
Spray	1	<2.00	<2.00	<2.00	75	100
Spray	4	<2.00	<2.00	<2.00	90	95
Spray	7	<2.00	4.03	2.55	75	100
Spray	8	<2.00	<2.00	<2.00	80	95
Spray	11	<2.00	34.5	1.93	90	100
Spray	12	<2.00	<2.00	<2.00	95	100
Control	2	<2.00	<2.00	<2.00	80	100
Control	3	<2.00	<2.00	<2.00	90	100
Control	5	<2.00	<2.00	<2.00	70	100
Control	6	<2.00	<2.00	<2.00	75	100
Control	9	<2.00	<2.00	<2.00	80	100
Control	10	<2.00	<2.00	<2.00	75	100

for a balanced design (control *Daphnia* survival in Sacramento wetland versus Colusa mesocosms for September: $F_{1,\,7} = 0.957$, $P = 0.36$; for October: $F_{1,\,7} = 2.489$, $P = 0.159$; mayflies for September: $F_{1,\,7} = 0.603$, $P = 0.463$; all controls survived in October).

There was no detectable effect of ULV synergized pyrethrin on *Daphnia* in the mesocosms for either trial (Table 1) (spray 5, treatment $F_{1,\,10} = 2.34$, $P = 0.16$; spray 11, treatment $F_{1,\,10} \sim 0$, $P = 0.997$). There did not appear to be a relationship between *Daphnia* survival and levels of pyrethrins or piperonyl butoxide found in sediments (Table 1). In spray 11, a date × treatment interaction was detected, but survival of *Daphnia* was, if anything, slightly higher in the treated mesocosms than in control mesocosms (Table 1) (date × treatment effect $F_{1,\,10} = 5.313$, $P = 0.04$). Results were nearly identical for the sentinel buckets placed directly into wetlands, and combining these datasets for greater power had no effect on the outcome other than to remove the date × treatment interaction (treatment $F_{1,\,10} = 0.282$, $P = 0.603$; date × treatment $F_{1,\,16} = 1.51$, $P = 0.237$). There was no difference in 36 h survival between treated cages left without screened lids during the spray 11 trial and control cages ($F_{1,\,10} = 0.626$, $P = 0.447$).

Mayflies did not show mortality attributable to ULV synergized pyrethrin. For spray 5, mean survival was ~66–80% regardless of treatment (treatment $F_{1,\,10} = 1.034$, $P = 0.333$, date × treatment interaction $F_{1,\,10} = 0.107$, $P = 0.75$). For spray 11, only two of 380 mayflies died during the entire experiment and there was insufficient variance to justify statistical analysis.

Maximum–minimum thermometers recorded water temperatures ranging from 28 to 16 °C during the spray 5 trial and from 24 to 11 °C in the spray 11 trial. pH was approximately neutral.

4 DISCUSSION AND CONCLUSIONS

In spite of ~70–80% mortality of caged adult mosquitoes exposed to ULV synergized pyrethrin, aquatic invertebrate sentinel species showed no significant differences in survival between exposed and control animals. The 36 h *D. magna* survival was more than 85% for both trials. The 36 h mayfly survival ranged from 67–77% after spray 5 to nearly 100% after spray 11. The reason for better mayfly survival in the second trial is unknown, but may have been due to the cooler, more oxygenated water conditions. These results were robust to slight changes in methodology during the second trial: survival of sentinel taxa did

not differ between cages held in mesocosms and those placed directly into wetlands, nor between cages with screen lids and those left completely open during sprays.

These trials not only tested acute exposures but also show that a series of 5–11 weekly or semi-weekly sprays of synergized pyrethrin did not accumulate in the mesocosms or wetland substrates to levels that were harmful to test species during either of the 12–36 h periods assessed. Sentinel organisms were only exposed to potentially contaminated sediments for 36 h. However, additional observational data support the idea that invertebrates survive longer exposures as well. There were natural populations of mayflies in exposed mesocosms (plus many other invertebrate taxa), and these mayflies were observed emerging as adults. Also, in prior years, late-instar mayflies were common in samples from the repeatedly sprayed wetland (unpublished data).

Overall, the present study supports the results of other studies indicating that aquatic stages of invertebrates in wetlands are not measurably affected by the amounts of synergized pyrethrin used in ULV applications for mosquito control.[9,13] However, wetland managers should be aware that piperonyl butoxide could synergize pre-existing contaminants from non-agricultural and agricultural use.[9]

Results have several implications for resource management. Natural resource managers are concerned by pesticide applications around wildlife, and wish to preserve invertebrates both as forage for wildlife and for their intrinsic value. Aquatic invertebrates in wetlands are particularly important because they are food for a great variety of aquatic species (fish, aquatic and riparian birds, reptiles, amphibians). They contribute to biodiversity and ecosystem function, and some have aesthetic value to the public (e.g. dragonflies). Mosquito control agencies use a variety of methods and materials in IPM to minimize public health risk from mosquito-transmitted diseases such as West Nile virus. The IPM process is intended to minimize impacts to non-target species while achieving pest reduction objectives. The results of this study suggest that, if ULV adulticide applications are necessary for mosquito control, synergized pyrethrin has minimal impacts on two aquatic invertebrate taxa often used as indicators in ecotoxicology and ecosystem health studies. However, the wider ecological significance of finding residual chemicals in sediments at up to $34\,\mu g\,kg^{-1}$ is unknown.

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