Environ Sci Pollut Res (2015) 22:11327–11339
DOI 10.1007/s11356-015-4374-1

RESEARCH ARTICLE

A comparison of the sublethal and lethal toxicity of four pesticides in *Hyalella azteca* and *Chironomus dilutus*

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Received: 21 January 2015 / Accepted: 12 March 2015 / Published online: 26 March 2015

Abstract Laboratory toxicity testing is the primary tool used for surface water environmental risk assessment; however, there are critical information gaps regarding the sublethal effects of pesticides. In 10-day exposures, we assessed the lethal and sublethal (motility and growth) toxicities of four commonly used pesticides, bifenthrin, permethrin, cyfluthrin, and chlorpyrifos, on two freshwater invertebrates, *Chironomus dilutus* and *Hyalella azteca*. Pyrethroids were more toxic than the organophosphate chlorpyrifos in both species. Bifenthrin was most toxic to *H. azteca* survival and growth. Cyfluthrin was most toxic to *C. dilutus*. However, cyfluthrin had the greatest effect on motility on both *H. azteca* and *C. dilutus*. The evaluated concentrations of chlorpyrifos did not affect *C. dilutus* motility or growth, but significantly impacted *H. azteca* growth. Motility served as the most sensitive endpoint in assessing sublethal effects at low concentrations for both species, while growth was a good indicator of toxicity for all four pesticides for *H. azteca*. The integration of sublethal endpoints in ambient water monitoring and pesticide regulation efforts could improve identification of low-level pesticide concentrations that may eventually cause negative effects on food webs and community structure in aquatic environments.

Keywords Species selection · Sublethal endpoint · Pyrethroid · Organophosphate · Growth · Motility · Ecological risk assessment

Responsible editor: Cinta Porte

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Introduction

Contaminants such as pesticides can pose major threats to freshwater biodiversity (Connon et al. 2012b; Dudgeon et al. 2006; Geist 2011), as aquatic ecosystems worldwide are "sinks" for contaminants discharged from areas of intense pesticide use (Scholz et al. 2012). Insecticides such as pyrethroids and organophosphates are of particular concern due to their broad-spectrum aquatic toxicities (Ankley and Collyard 1995). They are highly toxic to non-target organisms such as fish and aquatic invertebrates (Clark and Matsumura 1982; Werner and Moran 2008). Many current-use insecticides are neurotoxic compounds, which exert sublethal effects on aquatic organisms that can lead to severe health or reproductive impairment (Connon et al. 2012a; Johnson et al. 2008; Rakotondravelo et al. 2006a). Pyrethroids are known to inhibit sodium channels in the axonal membranes of nerve cells (Clark and Matsumura 1982), while organophosphates competitively inhibit the enzyme acetylcholinesterase in nerve synapses (Karnak and Collins 1974; Wheelock et al. 2005). Depending on exposure concentration, both pesticide classes result in hyperactivity and eventual failure of the nervous system (Haya 1989; Werner and Moran 2008). While acute toxicity to fish and aquatic invertebrates is rare, sublethal effects on key prey species eventually affecting food webs are of greatest concern (Brooks et al. 2012; Scholz et al. 2012).

Sublethal responses such as swimming impairment and growth are suitable endpoints for evaluating organism fitness since they integrate biochemical and physiological processes and have been shown to be highly sensitive biomarkers for low-level pesticide concentrations (Beggel et al. 2010; Christensen et al. 2005; Geist et al. 2007). However, these sublethal endpoints are not necessarily integrated in ambient water monitoring or regulatory toxicity assessments. While growth is a relatively common toxicity endpoint in fish studies, it is rarely used for invertebrates. Impaired swimming ability is generally not quantified as an endpoint in standard toxicity testing methods despite its obvious importance for the ecological fitness of a species (Christensen et al. 2005; Floyd et al. 2008; Weston and Lydy 2010). Thus, there is a pressing need for validating the effectiveness of these sublethal endpoints, if such endpoints are to be integrated in detecting water toxicity in water monitoring and regulatory toxicity assessments.

In this study we compared the lethal and sublethal toxic effects of two commonly used type-I pyrethroids (bifenthrin and permethrin), one type-II pyrethroid (cyfluthrin), and the organophosphate chlorpyrifos on *Chironomus dilutus* larvae and *Hyalella azteca*. These pesticides were selected based on their prevalence in the environment and their relative toxicities to non-target species (Bereswill et al. 2013; Hintzen et al. 2009; Li et al. 2013). In a recent study on pyrethroids encompassing 25 states across the USA, bifenthrin was the most frequently detected (58 % of samples), followed by permethrin (31 %) and cyfluthrin (14 %) (Hladik and Kuivila 2012). A study analyzing water samples from California creeks detected chlorpyrifos at concentrations between 11.8 and 1082 ng/L (Anderson et al. 2014). All four pesticides are used for similar pest treatments in agriculture and landscape maintenance and are regularly detected in the same water or sediment samples (Budd et al. 2009; Weston et al. 2008, 2013a). The selected pesticides are all neurotoxins with different neurological target sites and/or modes of action. The two types of pyrethroids cause toxicity through similar modulations of the voltage-gated sodium channels, but the degree of modification of sodium currents is different; single sodium channel currents are prolonged to a greater extent with type-II than type-I pyrethroids (Clark and Matsumura 1982; Nasuti et al. 2003; Wouters and van den Bercken 1978). Organophosphates (e.g., chlorpyrifos) inhibit acetylcholine esterase activity (Hua et al. 2013; Malison et al. 2010) directly impacting the synaptic signal. Varying modes of action could thus drive various exposure effects among different test endpoints.

C. dilutus larvae and *H. azteca* are often used in toxicity testing because of their high sensitivity to pyrethroids and organophosphates (Ankley et al. 1994b; Deanovic et al. 2013; Rakotondravelo et al. 2006b; Weston et al. 2014). Both species are highly relevant for environmental risk assessments as they are found in water bodies throughout the Americas and are important food sources for fish, amphibians, aquatic insects, and other organisms. Both species were selected for this study because they reflect differences in habitat that may result in different exposure to contaminants. The larval stage of *C. dilutus* is an endobenthic deposit feeder, where it uses the sediment and debris to build protective cases (Ankley et al. 1994a; Ding et al. 2011; Lydy and Austin 2004). *H. azteca* is an epibenthic detritivore, often found on macrophytes and other surfaces, and periodically moves into the water column. In addition to its use in sediment testing, *H. azteca* is also listed as a supplemental species for water column analyses in the US Environmental Protection Agency whole effluent toxicity testing guidance (US EPA 2002).

The aim of this study was to compare the effectiveness of the *C. dilutus* and *H. azteca* tests to detect toxicity caused by four current-use insecticides: three pyrethroids, bifenthrin, permethrin, and cyfluthrin, and one organophosphate, chlorpyrifos. In addition, we evaluated the use of two different sublethal endpoints, growth and motility, in detecting low-level insecticide concentrations.

Materials and methods

Test organisms

C. dilutus (second instar larvae, 10–12 days old) were obtained from Aquatic Biosystems (Fort Collins, CO, USA) and *H. azteca* (7–10 days old) from Aquatic Research Organisms (Hampton, NH, USA). Upon arrival, animals were transferred to aerated 7-L aquaria and acclimated to laboratory test conditions for 48 h. During the acclimation period, approximately 50 % of the transport water was changed twice daily and refilled with test control water, i.e., deionized water modified to attain US EPA moderately hard specifications (hardness 90–100 mg/L $CaCO_3$, alkalinity 50–70 mg/L as $CaCO_3$, SC 330–360 μS/cm and pH 7.8–8.2) (Eide and Johansson 1994; US EPA 1991). Once a day, *C. dilutus* and *H. azteca* were fed 10 ml of 4 g/L TetraMin slurry (Tetra®) and 20 ml of YCT (yeast-cerophyll-trout chow), respectively.

Exposure assessments

Ten-day toxicity tests with *C. dilutus* and *H. azteca* were conducted in a temperature-controlled room at 23±2 °C with a 12:12 h dark:light photoperiod. Bifenthrin (CAS# 82657-04-3, purity >98 %), permethrin (CAS# 52645-53-1, purity >95.7 %), cyfluthrin (CAS# 68359-37-5, purity >99 %), and chlorpyrifos (CAS# 5598-13-0, purity >99.5 %) were purchased from Chem Service (West Chester, PA, USA). Pesticide-grade methanol was used as a solvent carrier for the pesticide treatments, and in solvent controls, to a final

concentration of 0.01 % in exposure water. Corresponding stock solutions were spiked into control water according to target concentrations and mixed thoroughly. Organisms were randomly added to each replicate beaker. In total, organisms were exposed to a geometric progression of seven concentrations of each pesticide (Table 1) determined from preliminary 10-day toxicity test data (not reported), a solvent control, and a negative control. At test initiation and after each water renewal, organisms were fed 1.5 ml of 4 g/L TetraMin slurry (Tetra®) for *C. dilutus* and 1 ml of YCT for *H. azteca*.

The 10-day toxicity tests were based on US EPA protocols for static sediment toxicity testing (US EPA 2000), with the following modifications for each species. For *C. dilutus*, four replicate 1-L glass beakers, each containing a substrate of 20 g silica sand that was clean and baked (4 h at 450 °C), 750 ml of treatment water, and 10 organisms. The *H. azteca* 10-day toxicity tests were modified for water column exposures, as described in the Quality Assurance Management Plan for the State of California's Surface Water Ambient Monitoring Program (SWAMP 2002). Briefly, each concentration tested included four replicate 250-ml glass beakers, each containing 100 ml of treatment water, 10 organisms, and a 2-cm^2 piece of Nitex® screen as artificial substrate.

Mortality was recorded daily and any dead organisms were removed from the test vessels. In addition, 70 % of each test solution was renewed at 24 h (*C. dilutus*) or 48 h (*H. azteca*) time intervals, based on similar studies on *C. dilutus* (Xu et al. 2007) and *H. azteca* (Deanovic et al. 2013). At the time of water renewal, debris was removed and water quality parameters [pH, specific conductance (SC), dissolved oxygen (DO), temperature (T)] of renewal and wastewater were measured. Test vessels were randomly distributed after each water renewal.

To evaluate movement and activity of organisms at test termination, swimming behavior was measured as motility in centimeter per second. Both species are generally sedentary, but are inclined to swim when they are not provided substrate. Therefore, surviving organisms were transferred individually into corresponding filming chambers; a 5.5-cm (*C. dilutus*) or 1.3-cm (*H. azteca*) diameter well in a five-welled white PVC plate containing water from the respective beaker in which they were exposed. *C. dilutus* larvae had to be carefully teased from their cases before being transferred. To improve lighting quality and contrast of the videos, the white PVC plate was then placed on a light board. Video settings and plate position were adjusted to achieve a standardized focus point for each recording. Videos were recorded in MPEG-2 format, using a Panasonic® black and white CCTV camera (12V DC) filming all five filming chambers from the top. The camera was connected to a portable laptop-computer via a USB frame grabber (model WinTV-HVR 950, Hauppauge Computer Works, Hauppauge, NY, USA). Thirty frames per second were collected for each organism over a period of 80 s. Recorded videos were then analyzed using the Ethovision XT 6.1 Software (Noldus

Table 1 Nominal and measured concentrations (ng/L) for bifenthrin, permethrin, cyfluthrin, and chlorpyrifos used in 10-day exposures to *C. dilutus* and *H. azteca*

	Pesticide concentration (ng/L)							
	Bifenthrin		Permethrin		Cyfluthrin		Chlorpyrifos	
	Nominal	Measured	Nominal	Measured	Nominal	Measured	Nominal	Measured
C. dilutus	15.00	10.75	15.00	16.31	2.00	2.47	80.00	53.54
	29.10	18.57	29.10	24.77	4.11	3.59	131.80	91.16
	56.46	41.60	56.46	44.98	8.43	9.05	217.15	203.87
	109.54	94.41	109.54	104.60	17.32	11.93	357.77	274.19
	212.53	169.31	212.53	209.36	35.57	25.15	589.45	397.96
	412.34	378.82	412.34	310.74	73.04	63.55	971.14	632.57
	800.00	552.60	800.00	735.40	150.00	123.51	1600.00	1166.53
H. azteca	1.00	0.98	5.00	4.98	0.20	<LOD	10.00	8.33
	1.59	1.33	8.24	8.53	0.38	<LOD	17.63	12.20
	2.52	2.23	13.57	13.05	0.74	<LOD	31.07	24.48
	4.00	4.08	22.36	19.30	1.41	1.98	54.77	31.31
	6.35	5.92	36.84	34.22	2.71	2.95	96.55	65.65
	10.08	9.48	60.70	58.97	5.21	4.64	170.19	93.77
	16.00	15.08	100.00	93.66	10.00	6.62	300.00	239.46

<LOD indicates cyfluthrin concentration was below limit of detection, but concentrations were estimated by using the average factor between each available measured concentration (0.66) resulting in the following concentrations: 0.59, 0.89, and 1.33 ng/L. This data was included in the statistical analysis

Information Technology Inc., Leesburg, VA, USA) to determine motility (cm/s). The two-dimensional movement tracks were analyzed by measuring the movement of the center-point of each organism's body. While *H. azteca* move rectilinearly, *C. dilutus* display an undulating movement, resulting in a greater calculated motility than for *H. azteca*.

Following video recording, the organisms were transferred from the filming chambers onto individual pre-weighed tin dishes (pooled per treatment replicate), desiccated at 60 °C following methods described by Nahon et al. (2010), and weighed using a Mettler® Toledo AL104 balance (0.1 mg accuracy). To examine 10-day growth (increase of weight in grams over time), the weights of five subsamples of ten organisms were measured at test initiation and compared to the weights of surviving individuals at test termination. Due to limited scale sensitivity, organisms were pooled per replicate beaker, and only treatment replicates with five or more surviving individuals are reported herein. Mean individual dry weight in milligrams was calculated for each replicate for statistical analysis. The calculated 10-day growth was compared between treatments and controls to determine pesticide effects.

Analytical chemistry

At test initiation, 1-L water samples for each treatment and the solvent control were collected and stored in amber glass bottles in the dark at 4 °C for subsequent chemical analyses (Table 1). Within 48 h, samples were spiked with trans-permethrin (dimethyl D6, EQ Laboratories, Atlanta, GA, USA) as a recovery surrogate and extracted using solid phase extraction cartridges (Supelclean ENVI™ - C18, 500 mg, Sigma-Aldrich, St. Louis, MO, USA). Cartridges were preconditioned using 12 mL 1:1 ethyl acetate:hexane, 12 mL methanol, and 12 mL Milli-Q water (Millipore). Samples were loaded on the cartridge and eluted with 10 mL 1:1 ethyl acetate:hexane and evaporated to 0.4 mL at 40 °C under a gentle stream of nitrogen. As an internal standard, 4,4′-dibromo-octafluorobiphenyl (Chem Service, West Chester, PA, USA) was added (Parry and Young 2013). Extracts were analyzed using an HP-6890 gas chromatograph (Agilent Technologies, Palo Alto, CA, USA) coupled to an HP-5973N quadrupole mass spectrometer detector operated in electron capture negative ionization mode (GC-ECNI-MS) with methane as the reagent gas (Hladik and Kuivila 2012; Weston et al. 2013a). The gas chromatograph was equipped with a Supelco DB-5MS (30 m×0.25 mm with a 0.25-μm film thickness) with helium as the carrier gas. A 1-μL of sample was injected in splitless mode (injector temperature 280 °C, purge time 1.5 min). Instrumental calibration was performed using nine sets of calibration standard solutions containing all four pesticides (each purchased as 100 μg/mL solution in acetonitrile, Chem Service, West Chester, PA, USA), the surrogate trans-permethrin (dimethyl D6) and the internal standard 4,4′-dibromo-octafluorobiphenyl in hexane. Quantification of the pesticides was based on peak areas and comparing them with a calibration curve normalized to the internal standard response. All calibration curves had an $r^2 > 0.99$. Quality-assurance/quality-control was conducted by analyzing a method blank of deionized water (Milli-Q) to ensure that no contamination occurred during sampling extraction and analysis and by analyzing two laboratory spike samples to determine whether the sample matrix contributes bias to the analytical results and to what degree the method is successful in recovering the target analytes. The surrogate trans-permethrin was added to each sample, including the blank, before extraction to monitor matrix effects and overall method performance. Surrogate recoveries were on average 111.21 % with a range between 102.01 and 116.59 % confirming high extraction efficiency. Reported values were not corrected for surrogate recovery. Dibromooctafluorobiphenyl was added to sample extracts before analysis in order to correct quantitative differences in extract volume as well as to monitor instrument conditions. Instrumental limit of detections (whole water) were as follows: 0.6 ng/L bifenthrin, 4.8 ng/L permethrin, 1.4 ng/L cyfluthrin, and 0.8 ng/L chlorpyrifos.

No pesticides were detected in the controls or the method blank. In particular, average recoveries for bifenthrin were 84.97 % (range 63.81–102.00 %), for permethrin 93.15 % (range 75.36–108.73 %), for cyfluthrin 93.79 % (range 66.20–140.43 %), and for chlorpyrifos 71.68 % (range 55.10–93.88 %). Pesticide concentrations are herein reported as measured concentrations. For cyfluthrin (exposure to *H. azteca*), three treatments were below the limit of detection. To include these treatments for statistical analysis, the concentrations were estimated by using the average factor between measured concentrations (0.66), resulting in the following concentrations: 0.59, 0.89, and 1.33 ng/L.

Statistical analysis

No observed effect concentrations (NOEC) were determined using one-way ANOVA followed by a Dunnett's multiple comparison. Where data were not normally distributed, but homogeneity of variances was met, a Kruskal–Wallis test was applied. Shapiro–Wilk test and Levene's test were used to test normality and equality of variances, respectively. All differences discussed below are significant unless otherwise noted. All analyses were carried out using Minitab 17 Statistical Software 2013 (Minitab, Inc., State College, PA, USA) with a significance level at $\alpha = 0.05$.

Concentrations that caused a 50 % reduction in survival (LC_{50}) and sublethal endpoints (EC_{50}) were determined by fitting non-linear regression curves to the measured toxicity data using the DRC package in the program R, version 2.3-96 (R Core Team 2014; Ritz and Streibig 2005). For all data, log–

logistic and Weibull functions were fitted with the optimal model fit chosen for each dataset by the distribution that had the lowest Akaike's information criterion value. The optimal model was confirmed by a goodness-of-fit test.

Results

Water quality parameters

Water quality parameters remained stable throughout all exposures. Ranges for *C. dilutus* tests were as follows: 7.5–8.6 pH, 242.7–290.7 μS/cm SC, 4.3–9.4 mg/L DO, and 20.2–22.7 °C T, and for *H. azteca*: 7.6–8.5 pH, 257.4–296.3 μS/cm SC, 4.9–9.7 mg/L DO, and 20.9–22.8 °C T. Mean control survival of *C. dilutus* and *H. azteca* was 98 % (SE=±0.03) and 100 % (SE=±0.00), respectively, meeting test acceptance criteria for these species (SWAMP 2002; US EPA 2000).

Effects on survival

Cyfluthrin was the most toxic pesticide to *C. dilutus* with an LC_{50} of 17.36 ng/L, followed by bifenthrin (101.07 ng/L), permethrin (166.80 ng/L), and chlorpyrifos (335.20 ng/L) (Fig. 1a and Table 2). The lowest $NOEC_{Survival}$ was also greatest for cyfluthrin (9.05 ng/L), followed by bifenthrin (41.60 ng/L), permethrin (44.98 ng/L), and chlorpyrifos (203.87 ng/L).

Survival of *H. azteca* was most sensitive to bifenthrin (LC_{50}=2.01 ng/L), followed by cyfluthrin (2.89 ng/L), permethrin (40.90 ng/L), and chlorpyrifos (58.41 ng/L) (Fig. 1a and Table 2). The $NOEC_{Survival}$ of cyfluthrin and bifenthrin were 1.33 ng/L, for permethrin 19.30 ng/L, and for chlorpyrifos 31.31 ng/L.

Effects on motility

Average control motility of *C. dilutus* was 1.88 cm/s (SE±0.25). Exposure to all three pyrethroids caused a decrease in motility of *C. dilutus*, while chlorpyrifos did not affect this endpoint (Fig. 2a). At the lowest concentrations causing a significant effect, bifenthrin was most potent in reducing the motility by 62 % to 0.72 cm/s (SE±0.24) at 94.41 ng/L followed by permethrin and cyfluthrin which reduced motility by 56 % to 0.82 cm/s (SE±0.09) at 44.98 ng/L and by 53 % to 0.88 cm/s (SE±0.17) at 9.05 ng/L, respectively. Cyfluthrin was the most toxic pyrethroid affecting *C. dilutus* motility at an EC_{50} of 4.81 ng/L, followed by permethrin (44.59 ng/L) and bifenthrin (52.67 ng/L) (Table 2). The lowest $NOEC_{Motility}$ was determined for cyfluthrin (3.59 ng/L), followed by permethrin (24.77 ng/L) and bifenthrin (41.60 ng/L).

Average control motility of *H. azteca* was 0.56 cm/s (SE±0.05). Exposure to all three pyrethroids caused a decrease in motility of *H. azteca*; however, no effect of chlorpyrifos on motility was observed (Fig. 2b). At the lowest concentrations causing a significant effect, cyfluthrin was most potent in reducing the motility by 32 % to 0.38 cm/s (SE±0.08) at 0.89 ng/L followed by bifenthrin and permethrin which reduced motility by 23 % to 0.43 cm/s (SE±0.06) at 0.98 ng/L and 0.53 cm/s (SE±0.03) at 8.53 ng/L, respectively. Cyfluthrin was the most toxic pyrethroid on *H. azteca* motility (EC_{50}=0.53 ng/L), followed by bifenthrin (1.40 ng/L) and permethrin (38.63 ng/L) (Table 2). The lowest $NOEC_{Motility}$ was determined for cyfluthrin (0.59 ng/L), followed by bifenthrin (<0.98 ng/L), permethrin (4.98 ng/L), and chlorpyrifos (<8.33 ng/L). The $NOEC_{Motility}$ of *H. azteca* for cyfluthrin (0.59 ng/L) was higher than the EC_{50} value (0.53 ng/L) due to the use of an estimated concentration rather than the measured concentration which was below the limit of detection.

Effects on growth

Average initial dry weight of *C. dilutus* at test initiation was 0.17 mg (SE=±0.01) per individual compared to an average final 10-day dry weight of 1.55 mg (SE=±0.05) per individual in the controls. These results indicate an average growth that was 9.12 times the initial weight over the 10-day test period. All pyrethroids significantly affected growth of *C. dilutus*, while exposure to the organophosphate did not cause any effect (Fig. 3a). At the lowest concentration causing a significant effect, bifenthrin was most potent in growth inhibition reducing weight by 36 % to 0.99 mg (SE±0.13) per individual at 10.75 ng/L, followed by permethrin and cyfluthrin which reduced weight by 29 % to 1.11 mg (SE±0.23) per individual at 24.77 ng/L and by 21 % to 1.23 mg (SE±0.14) per individual at 9.05 ng/L, respectively. Cyfluthrin was the most toxic pyrethroid affecting *C. dilutus* weight (EC_{50}=14.48 ng/L), followed by bifenthrin (15.08 ng/L) and permethrin (26.81 ng/L) (Table 2). The $NOEC_{Weight}$ for cyfluthrin was 3.59 ng/L, followed by bifenthrin (<10.75 ng/L) and permethrin (16.31 ng/L).

All four pesticides significantly affected growth of *H. azteca* (Fig. 3b). Average initial dry weight of a subsample of *H. azteca* at test initiation was 0.040 mg (SE=±0.004) per individual compared to an average final 10-day dry weight of 0.100 mg (SE=±0.000) per individual in the controls. These results indicate an average growth of 2.50 times the initial weight over the 10-day test period. At the lowest concentration causing a significant effect, chlorpyrifos was most potent in growth inhibition reducing weight by 49 % to 0.051 mg (SE±0.014) per individual at 24.48 ng/L followed by permethrin, bifenthrin, and cyfluthrin which reduced weight by 42 % to 0.058 mg (SE±0.006) per individual at 4.98 ng/L, by

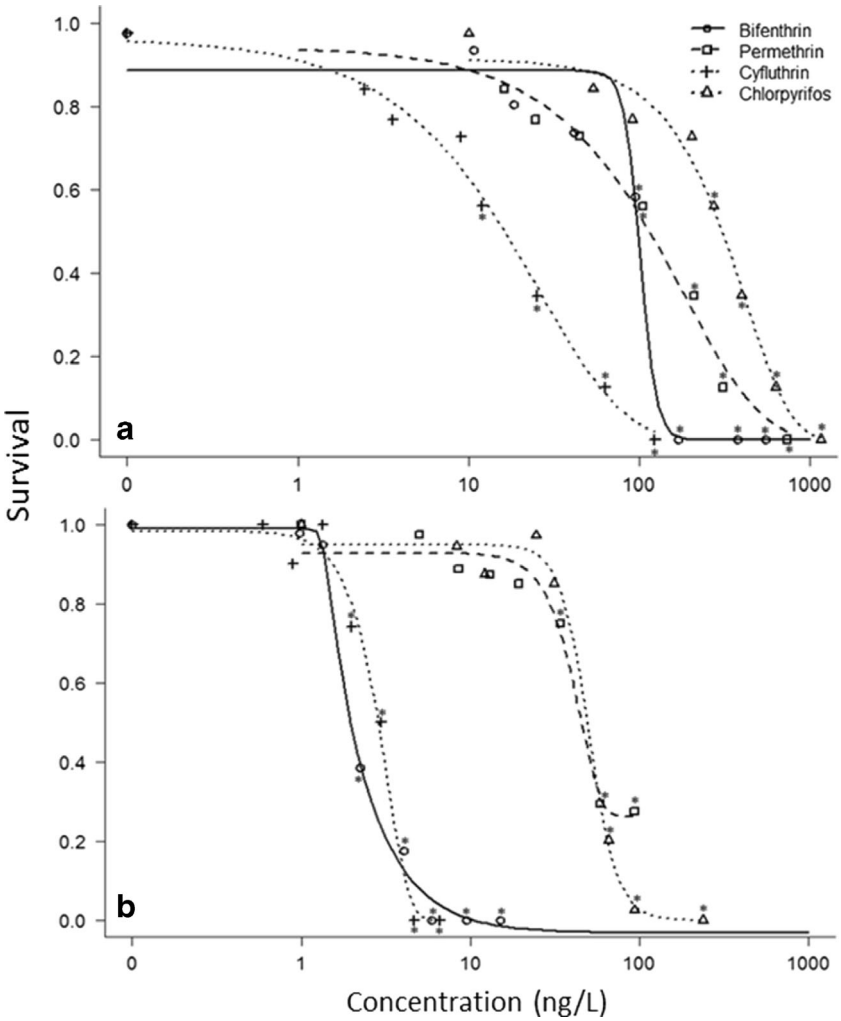

Fig. 1 Lethal effects of bifenthrin, permethrin, cyfluthrin, and chlorpyrifos to **a** *C. dilutus* and **b** *H. azteca*. Specific dose–response models (log–logistic or Weibull) were fitted to survival data for both species using the "mselect" function in the "drc" package. Y-axis = survival. X-axis = concentration (ng/L) for each pesticide. *Asterisks* indicate significant differences compared to the control ($p<0.05$)

41 % to 0.059 mg (SE ±0.012) per individual at 1.33 ng/L, and by 11 % to 0.089 mg (SE±0.006) per individual at 0.89 ng/L, respectively. Cyfluthrin was the most toxic pesticide on *H. azteca* weight (EC_{50}=1.19 ng/L), followed by bifenthrin (1.65 ng/L), permethrin (4.03 ng/L), and chlorpyrifos (25.08 ng/L) (Table 3b). The lowest $NOEC_{Weight}$ was determined for cyfluthrin (0.59 ng/L), followed by bifenthrin (0.98 ng/L), permethrin (<4.98 ng/L), and chlorpyrifos (12.20 ng/L).

Comparison of endpoints for each species

Comparing effective concentrations for each species, motility was the most sensitive endpoint across both species. The motility EC_{50} for *C. dilutus* were 1.9 (bifenthrin) to 3.7 (permethrin) and for *H. azteca* 1.1 (permethrin) to 5.5 (cyfluthrin) times lower than corresponding LC_{50}. $NOEC_{Motility}$ differed between 1.8 (permethrin) and 2.5 (cyfluthrin) times for *C. dilutus* and 1.4 (bifenthrin) and 3.9 (permethrin) times for *H. azteca* compared to $NOEC_{Survival}$.

Weight EC_{50} values for *C. dilutus* were 1.2 (cyfluthrin) to 6.7 (bifenthrin) times lower than corresponding LC_{50} values, while the $NOEC_{Weight}$ differed between 2.5 (cyfluthrin) and 3.9 (bifenthrin) times compared to the corresponding $NOEC_{Survival}$. For *H. azteca* weight, the EC_{50} was 1.2 (bifenthrin) to 10.2 (permethrin) times lower than corresponding LC_{50} values, while $NOEC_{Weight}$ differed between 1.4 (bifenthrin) and 3.9 (permethrin) times compared to $NOEC_{Survival}$.

Comparing chemical classes, the type-II pyrethroid cyfluthrin represented the most toxic pesticide class, resulting in effective concentrations that were up to 73 times lower than type-I pyrethroids [$EC_{50\text{-Velocity}}$ (*H. azteca*) for cyfluthrin compared to permethrin] and 21 times lower than the organophosphate [$EC_{50\text{-Weight}}$ (*H. azteca*) of cyfluthrin compared to chlorpyrifos]. Exposure to cyfluthrin elicited the greatest effect on motility and growth of both species, and on survival of *C. dilutus*, while bifenthrin was most toxic to *H. azteca* survival.

Compared to *C. dilutus*, *H. azteca* was more sensitive across all pesticides tested. The LC_{50} was up to 50, the EC_{50}

Table 2 Effect concentrations calculated for 10-day exposures of *C. dilutus* and *H. azteca* to bifenthrin (BIF), permethrin (PERM), cyfluthrin (CYF), and chlorpyrifos (CLF)

		Effect concentration (ng/L)										
	Chemical	$NOEC_{Survival}$	LC_{50}	SE	95 % C.I.	$NOEC_{Motility}$	$EC_{50Motility}$	SE	95 % C.I.	$NOEC_{Weight}$	$EC_{50Weight}$	SE	95 % C.I.
C. dilutus	BIF	41.60	101.07	32.33	34.95–167.20	41.60	52.67	29.39	1.01–114.96	<10.75	15.08	3.50	7.70–22.44
	PERM	44.98	126.16	23.43	78.23–174.91	24.77	33.96	17.57	11.05–56.85	16.31	26.81	4.71	16.97–36.63
	CYF	9.05	17.36	3.04	11.15–23.57	3.59	4.81	2.46	0.05–57.64	3.59	14.48	5.84	2.15–26.80
	CLF	203.87	335.20	34.52	264.59–405.80	–	–	–	–	–	–	–	–
H. azteca	BIF	1.33	2.01	0.10	1.71–2.30	<0.98	1.40	0.27	0.81–1.99	0.98	1.65	0.22	1.18–2.12
	PERM	19.30	40.90	3.26	34.22–47.56	4.98	38.63	11.61	14.88–62.37	<4.98	4.03	0.85	2.25–5.80
	CYF[a]	1.33	2.89	0.13	2.61–3.16	0.59	0.53	0.13	0.26–0.79	0.59	1.19	0.10	0.96–1.40
	CLF	31.31	50.41	4.93	40.32–60.50	<8.33	–	–	–	12.20	25.08	10.23	3.48–46.67

NOEC no observed effective concentration, *LC50* lethal concentration resulting in 50 % mortality of the population, *EC50* effect concentration resulting in 50 % reduction in growth, *SE* standard error, *C.I.* confidence interval

"–" indicates that value was not calculable because the levels of response did not amount to 50 % relative to the control (EC_{50} values) or did not cause significant effects (NOEC)

[a] Determined effect concentrations are based on a calculation that includes estimated concentrations (see Table 1)

for motility up to 43, and EC_{50} for weight up to 12 times lower for *H. azteca* than for *C. dilutus*. The only exception was permethrin for the motility endpoint, where the EC_{50} for *C. dilutus* was 1.2 times lower than for *H. azteca*. The $NOEC_{Motility}$ and $NOEC_{Weight}$ of *H. azteca* were 5–43 times and 3–11 times lower than for *C. dilutus*, respectively, with bifenthrin displaying the largest and permethrin the smallest difference. While the weight of *H. azteca* was a more sensitive endpoint across all chemicals tested, it displayed the smallest differences in sensitivity between the two species.

Discussion

C. dilutus and *H. azteca* differed greatly in their sensitivities to the four pesticides investigated and showed different sublethal responses at fractions of LC_{50} concentrations.

Cyfluthrin was the most toxic pesticide in all endpoints tested for *C. dilutus* and both sublethal endpoints tested for *H. azteca*. Like other type-II pyrethroids, cyfluthrin is chemically modified via the addition of functional groups (cyano and halogen group) and therefore hydrolyzes more slowly than type-I pyrethroids, resulting in a toxic potency up to 73 times greater than that of the type-I pyrethroids investigated in this study. However, *H. azteca* survival was most sensitive to bifenthrin, rather than cyfluthrin, which also caused the biggest difference in species sensitivity in terms of survival (LC_{50} value for *H. azteca* was 50 times lower than for *C. dilutus*) and motility (EC_{50} value for *H. azteca* was 38 times lower than for *C. dilutus*). This difference in sensitivity between the two species was also reported in Weston et al. (2013a), where the contribution of pyrethroids to sediment toxicity was investigated. This study found that bifenthrin was approximately twelvefold more toxic to *H. azteca* than to *C. dilutus* whereas differences among cyfluthrin, permethrin, and chlorpyrifos were only twofold. Similar results were found in other studies (Amweg et al. 2005; Maul et al. 2008; Maund et al. 1998). Weight was the most sensitive endpoint to detect pyrethroid toxicity using *C. dilutus* in this study. Significant effects on *C. dilutus* weight were observed at concentrations of 9.05 ng/L cyfluthrin, 10.75 ng/L bifenthrin, and 24.77 ng/L permethrin. For *H. azteca*, both sublethal endpoints were effective to detect low-level pesticide concentrations. The concentrations causing significant effects on all three endpoints in both species are within the range of environmentally relevant concentrations as reported in previous monitoring studies in different states of the USA (Anderson et al. 2006; Phillips et al. 2012; Smith and Lizotte 2007; Werner et al. 2010). For example, studies in Californian creeks by Budd et al. (2009) and Weston and Lydy (2012) detected bifenthrin at concentrations up to 37.3 ng/L, permethrin up to 470.0 ng/L, and cyfluthrin up to 8.7 ng/L. Chlorpyrifos was detected at concentrations up to 226.0 ng/L (Weston and Lydy 2010).

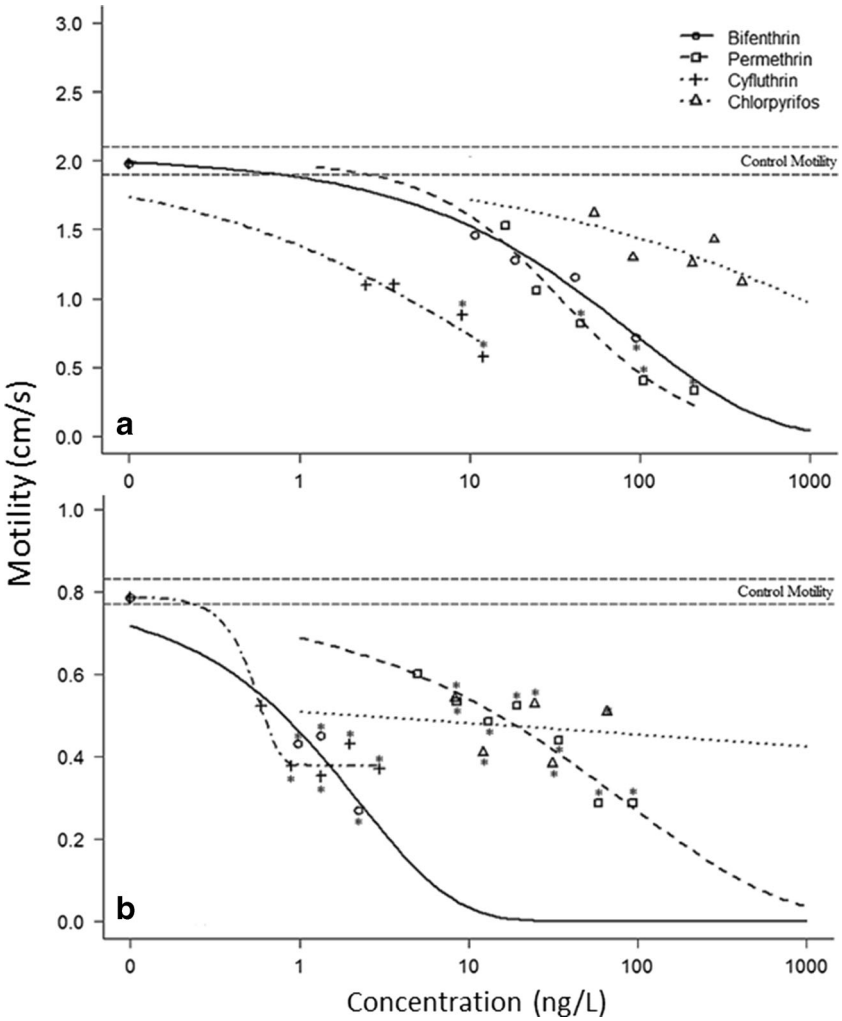

Fig. 2 Sublethal effects of bifenthrin, permethrin, cyfluthrin, and chlorpyrifos on motility of **a** *C. dilutus* and **b** *H. azteca*. Specific dose–response models (log–logistic or Weibull) were fitted to motility data for both species using the "mselect" function in the "drc" package. Y-axis = motility (cm/s). X-axis = concentration (ng/L) for each pesticide. *Asterisks* indicate significant differences compared to the control ($p<0.05$)

Mortality as an endpoint

The determined LC_{50} for the pesticides used in this study match results reported in other studies using *H. azteca*. Brander et al. (2009) reported a 10-day LC_{50} for permethrin of 48.90 ng/L (40.90 ng/L in this study), and Deanovic et al. (2013) a 10-day LC_{50} for bifenthrin of 2.3 ng/L (2.0 ng/L in this study) and for cyfluthrin 1.9 ng/L (2.89 ng/L in this study), while Phipps et al. (1995) reported a higher 10-day LC_{50} for chlorpyrifos of 86.0 ng/L (50.41 ng/L in this study). The difference in chlorpyrifos toxicity is likely caused by a different experimental setup, as Phipps et al. (1995) used a flow-through system, while Deanovic et al. (2013) and Brander et al. (2009) used a static system as was used in this study. No 10-day LC_{50} was reported for *C. dilutus* in the literature for cyfluthrin, and values reported for the other three chemicals differed from the ones determined in this study. Ding et al. (2012) determined different LC_{50} of bifenthrin (23.0 ng/L), permethrin (99.0 ng/L), and chlorpyrifos (140.0 ng/L) for *C. dilutus* using a static system, without solution renewal and a decreased feeding interval, which possibly caused the differing values compared to this study.

Motility as an endpoint

Motility was a highly sensitive endpoint to detect toxicity of cyfluthrin and bifenthrin on *H. azteca* below 1 ng/L. Swimming behavior is ecologically important since a reduction could make invertebrates more vulnerable to predation, drift, or food competition (Holomuzki et al. 2010). It is an especially relevant endpoint when investigating neurotoxic substances, such as organophosphates and pyrethroids, because paralysis is the first visible symptom of acute exposure (Rubach et al. 2011). Several studies have demonstrated the suitability of swimming performance for assessing effects of insecticides on fish, as it integrates biochemical and physiological processes and is an important indicator of fitness in aquatic species (Beggel et al. 2010; Geist et al. 2007; Heath et al. 1993). Motility is not an established endpoint in toxicity testing using invertebrates, but Rubach et al. (2011) who investigated the

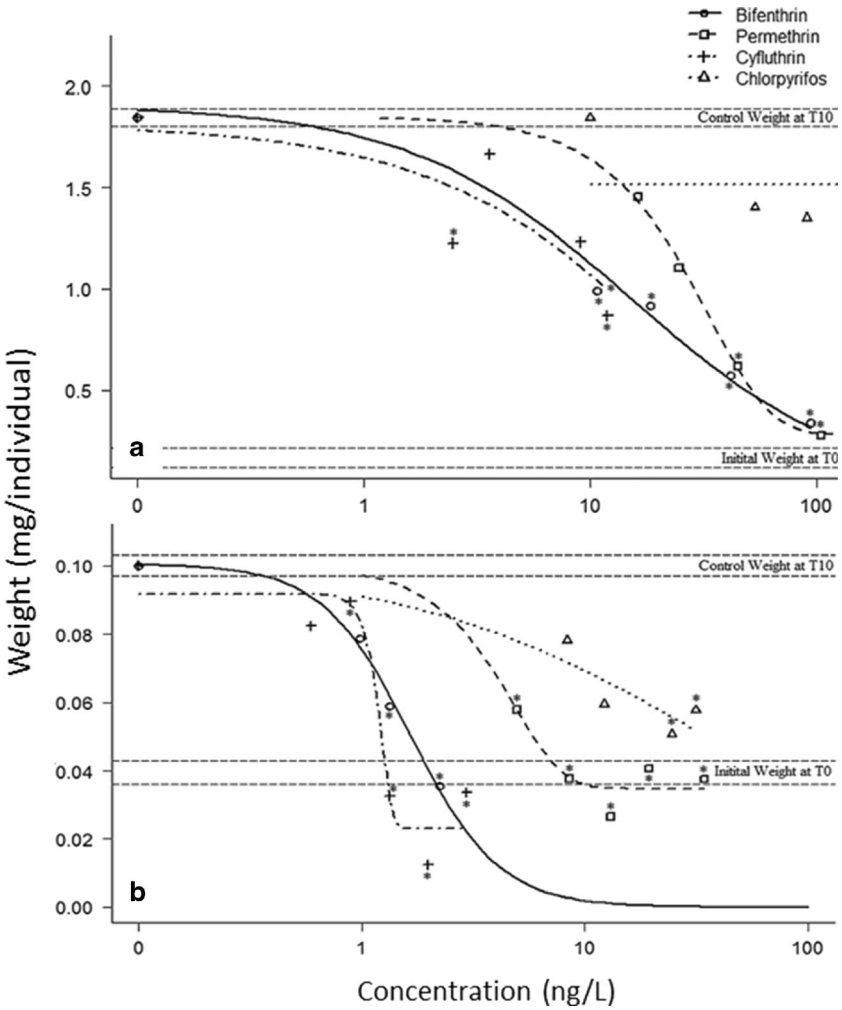

Fig. 3 Sublethal effects of bifenthrin, permethrin, cyfluthrin, and chlorpyrifos on weight of **a** *C. dilutus* and **b** *H. azteca*. Specific dose–response models (log–logistic or Weibull) were fitted to weight data for both species using the "mselect" function in the "drc" package. Y-axis = final weight (mg/surviving individuals). X-axis = concentration (ng/L) for each pesticide. *Asterisks* indicate significant differences compared to the control ($p<0.05$)

species sensitivity of 15 arthropod species, including the amphipod *Gammarus pulex*, on exposure to chlorpyrifos, found swimming behavior, rather than mortality, to be the most sensitive endpoint to use for risk assessment of neurotoxic compounds. This was also found in an exposure of the rotifer species *Brachionus calyciflorus* to the organophosphate dimethoate that resulted in adverse effects on the swimming behavior (Chen et al. 2014). These results as well as those from our study clearly demonstrate that motility is an important indicator to detect low-level pesticide concentrations which should be considered in ambient water monitoring and regulatory toxicity assessments.

Weight as an endpoint

Pyrethroid exposure resulted in reduced growth of both species. This could have been caused by food avoidance due to pyrethroids bound to organic material or decreased ability to ingest food (Maul et al. 2008). Alternatively, feeding rates may have been maintained, in which case reduced growth could be a direct effect of these insecticides; e.g., energetic reserves are allocated toward detoxification (Campero et al. 2007). Growth was the most sensitive endpoint for *C. dilutus* in this study, reflecting previously reported results. Maul et al. (2008) investigated the toxicity of bifenthrin, permethrin, and lambda-cyhalothrin on *C. dilutus* and found dramatic growth inhibition within the 10-day exposure. Growth is an established endpoint in fish toxicity studies as it represents an important ecological endpoint affecting predator avoidance and reproduction (Connon et al. 2009; Haya 1989). For smaller organisms such as invertebrates, growth is likely to be of similar ecological relevance as for fish. For example, reduced larval growth in *C. dilutus* negatively affected pupation, emergence (86 to 100 % reduction), adult female size, number of eggs per female, and fecundity (Liber et al. 1996; Ristola et al. 1999; Sibley et al. 1997). Sufficient growth during the larval stages of chironomids that successfully leads to pupation and emergence may therefore be even more crucial than growth of purely aquatic species such as amphipods (Agra and Soares 2009), as chironomid reproduction occurs during the adult terrestrial stage. Additionally, smaller individuals may also be more susceptible to predators and may have reduced

resistance to other environmental stressors as homeostatic energy demands are increased to contend with contaminant stress (Liber et al. 1996; McKenney et al. 1998; Sibley et al. 1997). Therefore, impairment of this endpoint could have profound population-level effects and is thus a highly important endpoint to consider in toxicity testing and ambient water monitoring.

Differences in sensitivity of species

Chlorpyrifos affected growth of *H. azteca*, but not of *C. dilutus* in this study. Generally, differences in the sensitivity of species to pesticides can be explained by their differences in behavior and habitat, as well as differences in toxicokinetics (uptake, distribution, biotransformation, elimination) and toxicodynamics (interaction with biological target sites) with differences in the mode of action being the most likely explanation in this specific case (McCarty and Mackay 1993; Rubach et al. 2012; Vaal et al. 2000). The metabolism of pesticides, their target sites, and the binding affinity at target sites, is known to differ even with only slightly different chemical structures (Nasuti et al. 2003; Soderlund et al. 2002; Vais et al. 2003). Variations in toxicokinetics among species can result from differences in lipid content, body size, and respiratory strategy (Baird and Van den Brink 2007; Nyman et al. 2014). In addition, the biotransformation capacity of a species to inactivate or activate specifically acting compounds has been considered an important factor causing differences in sensitivity (Chambers and Carr 1995; Escher and Hermens 2002). While both *C. dilutus* and *H. azteca* possess cytochrome P450-mediated mono-oxogenases capable of metabolizing organophosphate insecticides (Ankley and Collyard 1995), metabolic enzyme profiles can vary greatly across species (Clark 1989; Godin et al. 2006). As an organophosphate, chlorpyrifos is metabolically activated to a more toxic intermediate, chlorpyrifos-oxon that mainly acts on the nervous system by inhibiting acetylcholinesterase (ACh), leading to continuous neurotransmission, acute cholinergic syndrome, and eventually paralysis and death (Hsieh et al. 2001). The difference in response to chlorpyrifos exposure between the two species could result from the capability of *C. dilutus* larvae to withstand an increased inhibition of ACh as shown in previous studies (Rakotondravelo et al. 2006a; Rebechi et al. 2014).

Habitat differences are other major contributing factors to sensitivity differences between chironomids and amphipods. *H. azteca* are epibenthic grazers primarily occurring at the interface of the water column and sediment or detritus (Wang et al. 2004), while *C. dilutus* burrow into the sediment and feed on organic particles in the walls of their tube (Proulx and Hare 2014). This could lead to differences in exposure of *C. dilutus* to pyrethroids. Pyrethroids are highly non-polar chemicals of low water solubility and high K_{ow} values resulting in a high affinity to any type of surface. Laskowski (2002) summarized physical and chemical environmental properties of pyrethroids confirming that log K_{ow} values for bifenthrin, permethrin, and cyfluthrin are similar, ranging between 6.0 and 6.4. Chlorpyrifos is slightly less hydrophobic than pyrethroids with a log K_{ow} of 4.7 (Kravvariti et al. 2010). The binding properties of pyrethroids have been shown to inhibit their degradation (Lee et al. 2004), suggesting an accumulation of these compounds in the benthos causing an increased exposure to benthic organisms such as *C. dilutus*. Maund et al. (2001), on the other hand, reported that epibenthic and benthic organisms bioaccumulated a similar amount of sediment-bound pyrethroids. This indicates that bioaccumulation may be driven by cuticular uptake of the dissolved fraction, rather than ingestion of or direct contact with pyrethroid-contaminated sediments.

This study supports the use of *C. dilutus* and *H. azteca* as reliable indicators of pyrethroid presence in water samples; however, ecological implications cannot be directly assessed from toxicity demonstrated in laboratory species. Different species of chironomids are hard to identify, and there are additionally important genetic and physiological differences between laboratory and field populations of both *H. azteca* (Major et al. 2013; Weston et al. 2013b) and chironomids (Hoffman and Fisher 1994; Nowak et al. 2008; Woodworth et al. 2002). Consequently, the exposure concentrations at which effects were observed in *C. dilutus* and *H. azteca* cannot necessarily be seen as universally valid. In any case, the observed pronounced differences in the sensitivity of both species is not surprising since considerable interspecies variation in response to chemical stress exists for a wide range of animals and plants (Baird et al. 1991; Bridges and Semlitsch 2000; Jensen and Forbes 2001; Naylor et al. 1990).

Conclusion

Our data highlights the importance and usefulness of integrating sublethal endpoints on invertebrates into water-monitoring efforts and ecological risk assessment, especially to evaluate low-level contaminant concentrations. Sublethal endpoints revealed significant effects even below the limit of detection of current-use analytical methods. Our results show that pesticide sensitivities are not easily extrapolated from one species to another or between chemicals. Environmental risk may therefore be underestimated if surface water bodies are monitored assuming broad representation from a single invertebrate species, from a single-test endpoint, or by assuming that similar pesticides have similar effects. Our results demonstrate that the choice of the toxicity test, especially with respect to test species and endpoint, can be crucially important for the detection of insecticide toxicity at low concentrations. It is important to characterize not only the toxicity of common

aquatic contaminants, but also the variability in effects across species. Doing so will improve ambient water monitoring efforts and ecological risk assessment by determining the most sensitive species and endpoints that should be used to detect contaminants in water bodies. Understanding the variability in response across species will also help conservation efforts to understand the extent to which species will be affected by contaminant stress.

Acknowledgments The authors thank Linda Deanovic, Krista Hoffmann, A. Keith Miles, Jade Peralta, and Marie Stillway for their support. We are particularly grateful to Inge Werner who was involved in a prestudy for this project and who served as a mentor for Simone Hasenbein during her entire Ph.D. project. This study was funded by the State and Federal Contractors Water Agency (contract no. 15-33 to REC), California Department of Pesticide Regulation (contract no. 10-C0096 and 13-C0022 to SPL), and a postgraduate scholarship by Bayerische Forschungsstiftung, Germany (contract no. DOK-121-10 to JG). The authors acknowledge the support by the Faculty Graduate Center Weihenstephan of TUM Graduate School at Technische Universität München, Germany.

Compliance with ethical standards Accepted principles of ethical and professional conduct have been followed in the study. The authors declare no potential conflicts of interest (financial or non-financial), and the welfare of animals was considered according to the relevant laws (only invertebrates were used here).

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