Ecotoxicology (2015) 24:746–759
DOI 10.1007/s10646-015-1420-1

The use of growth and behavioral endpoints to assess the effects of pesticide mixtures upon aquatic organisms

Simone Hasenbein · Sharon P. Lawler · Juergen Geist · Richard E. Connon

Accepted: 15 January 2015 / Published online: 29 January 2015

Abstract Aquatic communities are often subject to complex contaminant mixtures, usually at sublethal concentrations, that can cause long-term detrimental effects. Chemicals within mixtures can effectively interact, resulting in synergism, antagonism or additivity. We investigated the tertiary mixture effects of two pyrethroids, lambda-cyhalothrin and permethrin, and the organophosphate chlorpyrifos, evaluating sublethal endpoints; immobility and growth, on *Chironomus dilutus* in 10-day exposures. We utilized a toxic units (TU) approach, based on median lethal concentrations (LC50) for each compound. The concepts of independent action and concentration addition were used to compare predicted mixture toxicity to observed mixture toxicity. Increased immobility resulted from mixture concentrations ≥1 TU (7.45 ng/L lambda-cyhalothrin × 24.90 ng/L permethrin × 129.70 ng/L chlorpyrifos), and single pesticides concentrations ≥0.25 TU (5.50 ng/L lambda-cyhalothrin, 24.23 ng/L permethrin, 90.92 ng/L chlorpyrifos, respectively). Growth was inhibited by pesticide mixtures ≥0.125 TU (1.04 ng/L lambda-cyhalothrin × 3.15 ng/L permethrin × 15.47 ng/L chlorpyrifos), and singly by lambda-cyhalothrin ≥0.25 TU (5.50 ng/L), and permethrin ≥0.167 TU (18.21 ng/L). The no observed effect concentrations (NOEC) for immobility and growth, for both mixture and single-pyrethroid exposure, were up to 8.0 and 12.0 times respectively lower than the corresponding NOEC for survival. The median effective concentrations (EC50) for growth (mixture and single-pyrethroid exposure) were up to 7.0 times lower than the respective LC50. This study reinforces that the integration of sublethal endpoints in monitoring efforts is powerful in discerning toxic effects that would otherwise be missed by solely utilizing traditional toxicity assessments.

Keywords Aquatic toxicology · Mixture toxicity · Freshwater invertebrates · Ecotoxicological risk assessment · Sublethal endpoints

S. Hasenbein · R. E. Connon (✉)
Department of Anatomy, Physiology and Cell Biology, School of Veterinary Medicine, University of California, One Shields Avenue, Davis, CA 95616, USA
e-mail: reconnon@ucdavis.edu

S. Hasenbein · J. Geist
Aquatic Systems Biology Unit, Technische Universität München, Mühlenweg 22, 85354 Freising, Germany

S. P. Lawler
Department of Entomology and Nematology, University of California, One Shields Avenue, Davis, CA 95616, USA

Introduction

Aquatic invertebrate communities are generally exposed to multiple stressors that potentially include complex mixtures of contaminants. The impacts of these are of ecological concern, particularly during an organism's sensitive, developmental life stages (Oros and Werner 2005; Geist 2011; Brooks et al. 2012; Connon et al. 2012). Concentrations of pesticides found in waters that receive agricultural and urban runoff often do not occur at levels that result in direct mortality (Scholz et al. 2012). Although these chemicals may be present in aquatic environments at relatively high concentrations during the peak application periods of spring and early summer, water flow and adsorption of pesticides to sediments and surfaces dictate that organisms are rarely exposed to elevated concentrations that cause mortality for continuous periods of time (Phillips et al. 2012; Beketov et al. 2013; Jeon et al. 2013). However, because pesticides and their breakdown products

are retained in sediments and may gradually re-dissolve or otherwise remain available at more consistent low levels (e.g., through diet or contact), research on chronic and environmentally typical low-level exposures is needed. Exposure to low-level concentrations for extended periods of time, or to moderate concentrations for multiple brief periods, can potentially result in physiological impairments (Nyman et al. 2013; Scherer et al. 2013). Examples of reported effects in invertebrate organisms include reduction in emergence (Du et al. 2013), case-abandonment (Johnson et al. 2008) and reduced growth (Rakotondravelo et al. 2006). Many insecticides may have effects that can lead to long-term severe health impacts or reproductive impairment, which are often not detectable using traditional toxicity testing methods (Christensen et al. 2005; Connon et al. 2012). Effect-based endpoints, designed to assess sublethal impairments are often more sensitive and better predictors of deleterious effects associated with contaminated water and sediments (Maul et al. 2008; Connon et al. 2012; Deanovic et al. 2013; Rasmussen et al. 2013).

Even though aquatic organisms are generally exposed to contaminant mixtures, data used in ecotoxicological risk assessment are predominantly based on single substance evaluation (Junghans et al. 2006; Backhaus et al. 2013; Gregorio et al. 2013). This regulatory approach, though informative, may underestimate ecological relevance because the effects of contaminant mixtures is known to differ from that predicted based on the sum of individual contaminant effects (Backhaus et al. 2000; Altenburger et al. 2004; Nøargaard and Cedergreen 2010). Two fundamental concepts exist, which are devised to evaluate the general relationships between the effects of single substances and their corresponding mixtures: concentration addition (CA), in which the effect of each contaminant can be expressed as if it were a dilution of the other and is based on the assumption of a similar action, and independent action (IA), which is based on the assumption of probabilistic independence of the effects of dissimilarly acting agents (Faust et al. 2000; Backhaus et al. 2004; Belden et al. 2007; Cedergreen 2014). In brief, if chemical effects do not interact in a mixture, the influences of each compound are effectively additive, and the actual effect of the mixture is adequately described by either CA or IA as a reference model. However, interactions may occur and chemicals in a mixture may produce effects that are synergistic (more severe) or antagonistic (less severe), than predicted by either reference model (Lydy et al. 2004; de Zwart and Posthuma 2005; Jonker et al. 2005; Belden et al. 2007). Additive, synergistic, and antagonistic responses have been documented for mixtures of various classes of pesticides, and the majority of studies that have been conducted, have focused on binary mixtures. Our study expands this approach to include tertiary mixtures of contaminants that are often detected in surface waters worldwide, and makes use of sublethal endpoints that are likely to have substantial ecological relevance.

We investigated the combined effects of three commonly used insecticides: two pyrethroids; permethrin (type I pyrethroid) and lambda-cyhalothrin (type II pyrethroid), and one organophosphate; chlorpyrifos, on survival and the sublethal endpoints of immobility and growth of *Chironomus dilutus* following a 10-days exposure. *C. dilutus* is used as a standard invertebrate species used in toxicity testing, and is among the numerous non-target species that are potentially affected by pesticide runoff. It has been shown to be highly sensitive to pyrethroids and organophosphates in field and laboratory studies (Weston et al. 2004; Anderson et al. 2006; Du et al. 2013; Li et al. 2013). The life cycle of *C. dilutus* is comprised of three aquatic stages (egg, four larval instars and pupae) and a terrestrial adult stage. The larval stage is representative of organisms living in the benthic zone. During this stage organisms burrow in the upper sediments, and utilize organic matter and sediment particles to build their protective cases. Like many benthic organisms, they feed on detrital particles, making them ideal organisms for testing the sediment–water interphase.

We selected the three insecticides because they are among the most frequently detected insecticides in aquatic habitats worldwide (Amweg et al. 2006; Sprague and Nowell 2008; Hintzen et al. 2009; Trimble et al. 2009; Bereswill et al. 2013; Li et al. 2013), and are known to be highly toxic to aquatic invertebrates and fish species (Werner et al. 2010; Phillips et al. 2012). All three pesticides are used for similar pest treatments such as the cultivation of vegetables, fruits, grains, and for landscape maintenance, and were repeatedly detected in the same water or sediment samples in recent studies (Weston et al. 2008; Budd et al. 2009; Weston et al. 2013). The selected pesticides are all neurotoxins with different neurological target sites and/or modes of action. Although modulations of the voltage-gated sodium channels are similar between the two types of pyrethroids, 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 (Wouters and van den Bercken 1978; Clark and Matsumura 1982; Nasuti et al. 2003). Organophosphates (chlorpyrifos) on the other hand, inhibit acetylcholine esterase activity (Malison et al. 2010; Hua et al. 2013) directly impacting the synaptic signal. Varying modes of action could drive potential interactive exposure effect differences between single chemicals and complex mixtures.

Given that pesticides predominantly occur at concentrations below those that cause mortality, sublethal endpoints are more applicable at evaluating environmental

relevance. By assessing the effects of the three pesticides in 10-days toxicity tests, we evaluate the use and effectiveness of sublethal endpoints in mixture toxicity testing for regulatory applications and monitoring studies.

Materials and methods

Test organisms and acclimation

Chironomus dilutus were obtained from Aquatic Biosystems, Fort Collins, CO, USA. Under typical laboratory conditions, *C. dilutus* begin to pupate, and emerge as adults 21 days after hatching, thus 2nd instar larvae (8–10 days old) were used as to avoid emergence during the 10-days exposures. Upon arrival, dissolved oxygen (DO >2.5 mg/L), and temperature (23 ± 2 °C) of transport water subsamples were measured, and were within acceptable ranges stipulated by U.S. EPA standard test protocols (U.S. EPA 2000). Healthy animals were moved to aerated 7-L aquaria, fed, 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 U.S. Environmental Protection Agency (U.S. 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; U.S. EPA 2000, 2002). *C. dilutus* were fed 10 ml of 4 g/L Tetramin® slurry daily.

Chemicals and chemical analysis

Chlorpyrifos (purity >98 %, CAS number 2921-88-2), lambda-cyhalothrin (purity >98 %, CAS number 91465-08-6), and permethrin (purity >98 %, CAS number 52645-53-1) were purchased from Chem Service (West Chester, PA, USA). Pesticide stock solutions were prepared in methanol and spiked into laboratory control water to achieve exposure concentrations illustrated in Tables 1 and 2, with a final methanol concentration of 0.05 %. Before adding the pesticide solutions into the test beakers, three 1-L water samples for each single-chemical exposure and the tertiary mixture exposure were collected, and stored at 4 °C for subsequent chemical analyses. Within 24 h, the samples were extracted by solid phase extraction (Supelclean™ ENVI™-18 SPE Tubes, 500 mg, Sigma-Aldrich, St. Louis, MO, USA), and analyzed using an HP-6890 gas chromatograph (Agilent Technologies, Palo Alto, CA, USA) coupled to an HP-5973 N quadrupole mass spectrometer detector, operated in electron capture negative ionization mode (GC-ECNI-MS) with methane as the reagent gas, equipped with a split-splitless injector (280 °C, splitless, 1.5-min purge time) and a Supelco DB-5MS column (30 m × 0.25 mm with a 0.25 µm film thickness). Instrumental calibration was performed using nine sets of calibration standard solutions containing all three pesticides (each purchased as 100 µg/mL solution in acetonitrile, Chem Service, West Chester, PA), the surrogate trans-permethrin D6 (EQ Laboratories, Atlanta, GA), and an internal standard; dibromooctafluorobiphenyl (Chem Service, West Chester, PA) in hexane. Quantity was calculated based on peak area and comparing them to the standard curves. 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. The surrogate trans-permethrin D6 was added to each sample, including the blank, to monitor matrix effects and overall method performance. Surrogate recoveries were on average 103 % with a range between 79 and 112 % indicating 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. No pesticides were detected in the control or the method blank. Measured concentrations by GC-ECNI-MS were lower than nominal concentrations; average recoveries for lambda-cyhalothrin were 65 % (range 62–86 %), for permethrin 46 % (range 34–58 %), and for chlorpyrifos 78 % (range 71–86 %). The average proportion of the measured concentrations recovered for each pesticide was used as a factor to estimate the realized concentrations for each exposure (Tables 1, 2), and are presented as measured concentrations hereon.

Exposure tests

Mixture toxicology employs a dimensionless ratio, the toxic unit (TU), to generate a normalized scale (McCarty et al. 1992). Each toxicant concentration is considered as a fraction of its individual toxicity, which most commonly is expressed in terms of its LC50. The total TU of a mixture is the sum of the individual fractions. The TU approach assumes dose additivity (Altenburger et al. 2004). Thus, the sum of TU describes the joint chemical concentration of a mixture, given a known common effect concentration, which can be used to assess the toxicity of a tertiary mixture as follows:

$$TU_{summation} = \frac{Cw_1}{LC50_1} + \frac{Cw_2}{LC50_2} + \frac{Cw_3}{LC50_3} \quad (1)$$

where Cw_i is the concentration of chemical 1 in a mixture and $LC50_i$ is the $LC50_{96h}$ for chemical 1 (McCarty et al. 1992). For example, the sum of 1/3 of the $LC50_{96h}$ of each pesticide equals a TU value of 1. Pesticides were combined in an attempt to produce equitoxic mixtures and to evaluate

Table 1 Measured and nominal concentrations of lambda-cyhalothrin, permethrin, and chlorpyrifos at test initiation for single-chemical exposures of *C. dilutus* over 10 days

Pesticide concentration (ng/L)						
TU of single pesticide	Lambda-cyhalothrin		Permethrin		Chlorpyrifos	
	Nominal	Measured	Nominal	Measured	Nominal	Measured
0.167	6.32	4.39	31.50	18.21	78.33	55.89
0.25	9.48	5.50[a]	47.25	24.10[a]	117.50	90.48[a]
0.33	12.63	7.10	63.00	30.45	156.67	117.84
0.50	18.95	9.34	94.50	45.08	235.00	201.07
1.00	37.90	21.98[a]	189.00	96.39[a]	470.00	361.90[a]
1.50	56.85	32.97[a]	283.50	144.59[a]	705.00	542.85[a]
3.00	113.70	65.95[a]	567.00	289.17[a]	1,410.00	1,085.70[a]

[a] Indicates that concentrations were calculated based on the average proportions recovered in analytical tests for each exposure. Average factors used for each chemical: 0.58 for lambda-cyhalothrin, 0.51 for permethrin, and 0.77 for chlorpyrifos. 'TU' for each pesticide represents the toxic unit for the lethal concentration that was chosen for each treatment for each single pesticide

Table 2 Measured and nominal concentrations of lambda-cyhalothrin, permethrin, and chlorpyrifos in the mixture exposure at test initiation for mixture exposures of *C. dilutus* over 10 days

Pesticide concentration (ng/L)								
TU of mixture	TU of each pesticide	Lambda-cyhalothrin		Permethrin		Chlorpyrifos	
		Nominal	Measured	Nominal	Measured	Nominal	Measured
0.125	0.042	1.58	1.04[a]	7.88	3.15[a]	19.58	15.47[a]
0.25	0.083	3.16	2.09[a]	15.75	6.30[a]	39.17	30.94[a]
0.50	0.167	6.32	5.44	31.50	14.50	78.33	55.32
0.75	0.25	9.48	6.26[a]	47.25	18.90[a]	117.50	92.83[a]
1.00	0.33	12.63	7.45	63.00	24.90	156.67	129.70
1.50	0.50	18.95	10.25	94.50	31.94	235.00	193.40
3.00	1.00	37.90	25.01[a]	189.00	75.60[a]	470.00	371.30[a]

[a] Indicates that concentrations were calculated based on the average proportions recovered in analytical tests for each exposure. Average factors used for each chemical: 0.66 for lambda-cyhalothrin, 0.40 for permethrin, and 0.79 for chlorpyrifos. 'TU for each pesticide equals three times 'TU of mixture' and represents the percentage lethal concentration that was chosen for each treatment for each pesticide

their interactive effects (Belden and Lydy 2006; Symington et al. 2011). The treatments used in the toxicity tests were based on expected $LC50_{96h}$ values determined by other research groups using similar test methods. Targeted nominal $LC50_{96h}$ values for *C. dilutus* were 189.00 ng/L permethrin (Harwood et al. 2009), 37.90 ng/L lambda-cyhalothrin, and 470.00 ng/L chlorpyrifos (Ankley and Collyard 1995). Based on each reported $LC50_{96h}$ value, we exposed animals to single-pesticide TU values of 0.167, 0.25, 0.33, 0.5, 1, 1.5, and 3 (Table 1), and the following mixture TU values: 0.125, 0.25, 0.5, 0.75, 1, 1.5, and 3 (Table 2). These concentrations were specifically chosen to include environmentally typical concentrations as reported in previous studies, as well as high values that may occur transiently (Anderson et al. 2006; Budd et al. 2009; Werner et al. 2010; Weston and Lydy 2010). Single-pesticide responses were then used to predict the combined mixture toxicity, and compared to the observed mixture response (see Statistical analysis).

Mixture and single-chemical exposures were conducted at the same time over a 10-day period using the same batch of animals (2nd instar larvae). Tests were conducted at 23 ± 2 °C with a 16-h light: 8-h dark photoperiod, and consisted of four replicate 1 L glass beakers, each containing a layer of 10 g clean and autoclaved silica sand as a substrate, 750 mL of treatment water, and 10 organisms. The sand allowed chironomids to build their cases. Once test solutions were added to the test vessels at test initiation, organisms were randomly placed into each beaker.

Mortality was recorded daily, at which time 80 % of each test solution was renewed, and any dead organisms and debris were removed from the test vessels. Water quality parameters (pH, specific conductance, DO, and temperature) of renewal water and wastewater were

measured. At test initiation and after each water renewal, organisms were fed 1.5 ml of 4 g/L Tetramin® slurry. Test vessels were randomly redistributed within the exposure chamber at each renewal day.

Mobility of each organism was determined at test termination (day 10) using video analysis. Chironomids are generally sedentary if food and oxygen are sufficient in the immediate area, but are inclined to be mobile when they are not provided substrate. Therefore, surviving organisms were carefully teased from their cases and transferred individually into corresponding filming chambers; a 5.1 cm diameter well in a five-welled white PVC plate containing corresponding treatment water without substrate. To improve lighting quality and contrast of the videos, the white PVC plate was then placed on a light board. The positioning of the plate and video settings were standardized for each recording. Videos were recorded in MPEG-2 format, using a Panasonic® black and white CCTV Camera (12 V 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). Thirty frames per second were collected for each organism, over an 80 s period. Recorded videos were then analyzed using Ethovision XT 6.1 Software (Noldus Information Technology Inc., Leesburg, VA) to determine percentage immobility.

Weight was determined using the same organisms used for mobility assessments. Following video recording, the organisms were transferred from the filming chambers onto 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® AE 100 balance (0.1 mg accuracy). To examine 10-day growth, the weights of a separate set of four replicates of ten organisms were measured at test initiation and compared to the weights of surviving individuals at test termination. Weight is presented as mg/surviving individual. Data for both sublethal endpoints is presented up to 1 TU as mortality in higher treatments was greater than 50 %.

Statistical analysis

Median lethal and effective concentrations (LC50 and EC50) and no observed effect concentrations (NOEC) were determined for mortality and the sublethal endpoints of immobility and growth, based on measured exposure concentrations (Tables 1 and 2). We tested for significant differences of the treatments compared to the controls using Analysis of Variance, or where parametric assumptions were not met, a Kruskal–Wallis test, including a Dunnett's post hoc comparison, using Minitab 16 Statistical Software 2010 (Minitab, Inc., State College, PA, USA). The significance level or α used in all these tests was $p \leq 0.05$. All differences discussed below are significant unless otherwise noted. Concentrations that caused a 50 % reduction in survival (LC50) and growth (EC50) were determined by fitting non-linear regression curves to the toxicity data used in the DRC package version 2.3-96 (Ritz and Streibig 2005) R (R Core Team 2014). EC50 concentrations for the effects on immobility and for chlorpyrifos on weight were not calculated because the levels of responses did not amount to 50 % relative to the control. For all data, log-normal, 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 fit of the optimal model was confirmed by a goodness of fit test. Three-parameter regression models were fitted, assuming a lower limit of 0.

The dose–response data were described with a log-logistic dose–response model with an upper limit of

$$1 : y = \frac{1}{1 + (x/e)^b} \tag{2}$$

Where e is the effect concentration (LC50), and the parameter b denotes the relative slope around e. Once the dose–response curves were fitted, joint effect predictions in relation to concentration addition (CA) and independent action (IA) were made. For the prediction of mixture toxicities the complete concentration–response range between 1 and 99 % effect was predicted according to both the CA as well as the IA concept. CA assumes that the chemicals act as dilutions of each other (Bliss 1939; Hewlett and Plackett 1952). For the concept of CA the calculation was conducted as outlined in Backhaus et al. (2000). Briefly, the total concentrations of each mixture were calculated in steps of 1 % as:

$$LCx_{mix} = \left(\sum_{i=1}^{n} \frac{p_i}{ECx_i} \right)^{-1} \tag{3}$$

Where p_i is the fraction of compound i present in the mixture. The resulting 99 pairs were connected with straight lines, visualizing the predicted concentration response-curve.

To calculate the mixture effects according to IA, the individual compounds were expressed as fractions pi of the total concentration c_{mix}. The overall effect of any given total mixture concentration can be calculated as

$$L(c_{mix}) = 1 - \prod_{i=1}^{n} [1 - F_i(c_i)] \tag{4}$$

where $L(c_{\text{mix}})$ denotes the predicted effect (scaled from 0 to 1) of an n-compound mixture, c_i is the concentration of the ith compound, and F_i is the effect of that concentration if the compound is applied singly. To calculate the mixture effects predicted according to IA, the 99 predicted mixture concentrations obtained from the concentration–response range for the CA model described in Eq. (3) were used.

The observed data were considered to be significantly different from the predicted model if the 95 % confidence intervals of the observed toxicity values did not overlap the value predicted by the model.

The toxicity of each of the chemicals in a mixture can differ substantially, therefore each chemical's relative contribution to toxicity was expressed by using toxicity units rather than their actual concentrations (LC50) to predict the joint toxicity (Lydy and Austin 2004; Jonker et al. 2005). An LC50 resulting in a TU >1 represents a synergistic type of joint action, whereas a TU <1 indicates an antagonistic type of action (Pape-Lindstrom and Lydy 1997; Sørensen et al. 2007).

Results

Water quality parameters remained stable throughout all exposures and mean control survival of *C. dilutus* was 75 % (SE = ± 0.13) meeting the U.S. Environmental Protection Agency minimum acceptance criteria for this species (U.S. EPA 2000). Control mortality was only observed within 24 h after test initiation, and was likely caused by random handling stress when transferring the chironomids into treatment beakers.

The tertiary mixture exposure resulted in a less severe response than the pesticides applied singly. In single-chemical tests, lambda-cyhalothrin was the most toxic pesticide to *C. dilutus* resulting in an LC50 value of 32.99 ng/L (±2.56 SE), followed by permethrin (159.41 ng/L, ±16.36 SE), and chlorpyrifos (571.49 ng/L, ±88.68 SE) (Table 3). These values were 1.1–1.5 times higher than the corresponding single-pesticide NOEC values for lambda-cyhalothrin, permethrin, and chlorpyrifos (21.98, 144.59, and 361.90 ng/L, respectively). The LC50 value for the observed mixture exposure was 1.90 TU (±0.28 SE), indicating an antagonistic response (>1 TU). Survival of *C. dilutus* was reduced in concentrations ≥1.5 TU of lambda-cyhalothrin (32.97 ng/L) and chlorpyrifos (542.85 ng/L), whereas the mixture exposure caused no significant reduction in survival ≤1.5 TU (10.25 ng/L lambda-cyhalothrin, 31.94 ng/L permethrin, 193.40 ng/L chlorpyrifos) indicating an IA response (Fig. 1). The CA and IA concept were applied to predict mixture toxicity based on the single-pesticide exposures, and compared to the observed mixture response (Fig. 2). The observed mixture response was most consistent with the IA concept, whereas the CA concept overestimated the combined effect.

Average control immobility was 77.89 % (±6.69 SE) over the recording time of 80 s. Single-pesticide exposure caused a decrease in mobility across all concentrations tested with an average immobility of 93.68 % (±0.44 SE) at 0.25 TU for all three pesticides (Fig. 3). The exposure to the tertiary mixtures decreased mobility at 1 TU with an average immobility of 94.23 %. Exposure to chlorpyrifos individually caused the greatest inhibition of mobility at 1 TU (361.90 ng/L), with an average immobility of 93.15 % (±0.71 SE), followed by permethrin (90.42 %, ±0.59 SE), mixture (88.58 %, ±0.99 SE), and lambda-cyhalothrin (87.48 %, ±1.79 SE). Comparing NOEC values for immobility and survival, permethrin exposures caused the greatest difference (NOEC for immobility 8 times smaller than for survival), followed by chlorpyrifos (6.5 times), lambda-cyhalothrin (5 times), and the mixtures (3 times).

Initial weight (T_0) of four subsamples of *C. dilutus* was 0.345 mg/individual (±0.04 SE; Fig. 4). Final weight of control organisms was 1.932 mg/individual (±0.07 SE) equaling a 5.6-fold growth over the 10-days test period. Both pyrethroids and the mixture led to an inhibition in *C. dilutus* growth, while chlorpyrifos had no detectable effect on this endpoint. Compared to the final control weight, reductions were recorded at concentrations ≥0.25 TU for lambda-cyhalothrin (from 32.6 % up to 65.8 % in the highest concentration) and the mixture (25.2–63.1 %), and ≥0.167 TU for permethrin (33.0–77.8 %). In detail, exposure to lambda-cyhalothrin resulted in a growth inhibition up to a final weight of 0.661 mg/surviving individual (±0.08 SE) at 1 TU (21.98 ng/L lambda-cyhalothrin). Mixture exposures resulted in a final weight of 0.712 mg/surviving individual (±0.03 SE) at 1 TU (7.45 ng/L lambda-cyhalothrin, 24.90 ng/L permethrin, 129.70 ng/L chlorpyrifos). Exposure to permethrin resulted in a final weight

Table 3 Effect concentrations calculated for 10-days exposures of *C. dilutus* to lambda-cyhalothrin (LC), permethrin (Perm), and chlorpyrifos (CLF) applied singly and in mixture (Mixture) of *C. dilutus*. *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. '—' indicates value was not calculable

Chemical	Effect concentration (ng/L)						
	$NOEC_{Survival}$	LC50	SE	$NOEC_{Weight}$	$EC50_{weight}$	SE	$NOEC_{Immobility}$
LC	21.98	32.99	2.56	4.39	18.13	5.20	<4.39
Perm	144.59	159.41	16.36	<18.21	22.51	7.75	<18.21
CLF	361.90	571.49	88.68	–	–	–	<55.89
Mixture	1.50 TU	1.90 TU	0.28	0.125 TU	0.49 TU	0.19	0.50 TU

TU Toxicity Units

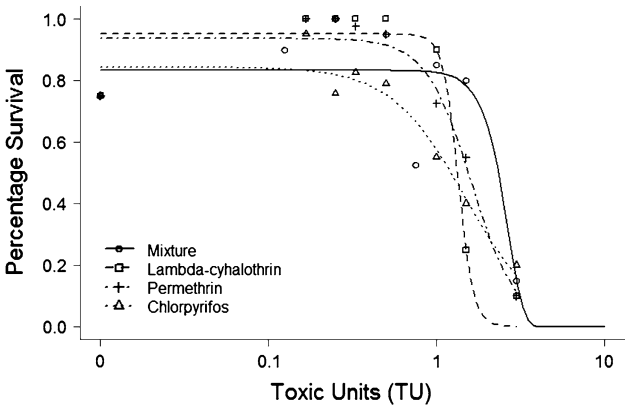

Fig. 1 Percent survival of *C. dilutus* following 10-days exposures to lambda-cyhalothrin, permethrin and chlorpyrifos, and corresponding mixtures up to 3 TU. Non-linear regression curves were fitted to the toxicity data using the DRC package version 2.3-96 (Ritz and Streibig 2005) in R (R Core Team 2014). Average control survival = 75 %

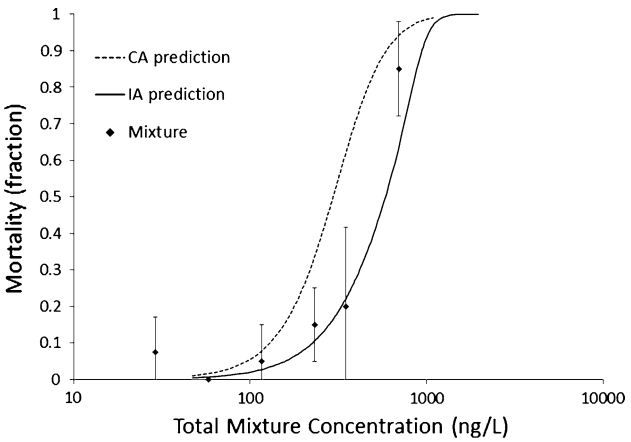

Fig. 2 Observed and predicted toxicities of tertiary mixtures of lambda-cyhalothrin, permethrin and chlorpyrifos on *C. dilutus*. Mixture concentrations derived from LC50 values of the individual components. *Mixture* = observed toxicity of the tertiary mixture; *CA prediction* = Prediction according to concentration addition model; IA prediction = Prediction according to independent action model

of 0.429 mg/surviving individual (±0.03 SE) at 1 TU (96.39 ng/L permethrin). NOEC values for the growth endpoint, for both mixture and single-pyrethroid concentrations were 5.0–12.0 times lower than the respective NOEC for survival (Table 3), with the mixture exposure (NOEC = 0.125 TU) causing the greatest difference (12 times lower than for survival), and permethrin (NOEC < 18.21 ng/L, 8 times lower) and lambda-cyhalothrin (NOEC = 4.39 ng/L, 5 times lower) the smallest differences. EC50 values for growth were on average 1.8–7.0 times lower than the respective LC50 for survival, with permethrin (EC50 = 22.51 ng/L, ±7.75 SE) representing the largest difference and lambda-cyhalothrin (EC50 = 18.13 ng/L, ±5.20 SE) the smallest. The EC50 value for the mixture (0.49 TU) was 3.9 times lower than the respective LC50 (1.90 TU).

Discussion

The assessment of three insecticides, lambda-cyhalothrin, permethrin, and chlorpyrifos, demonstrates that mixtures can affect survival in an antagonistic manner (1.90 TU) and result in effects on sublethal endpoints that are up to 12.0 times lower than the corresponding NOEC for survival. The sublethal effect concentrations for growth inhibition caused by lambda-cyhalothrin (EC50 = 18.13 ng/L), permethrin (EC50 = 22.51 ng/L) and the tertiary mixture (0.25 TU = 2.09 ng/L lambda-cyhalothrin, 6.30 ng/L permethrin, and 30.94 ng/L chlorpyrifos) in this study are within the range of environmentally relevant concentrations reported in previous monitoring studies in different states of the USA (Anderson et al. 2006; Smith and Lizotte 2007; Werner et al. 2010). For example, studies in Californian streams by Budd et al.(2009) and Weston et al.(2014) detected lambda-cyhalothrin at concentrations of 1.4–27.0 ng/L, and permethrin between 4 and 470 ng/L. Chlorpyrifos was detected at concentrations between 1.2 and 226.0 ng/L (Weston and Lydy 2010). Even though concentrations for lambda-cyhalothrin in these monitoring studies were on average lower than for the other two pesticides, the lower effective concentrations of 18.13 ng/L (EC50 Growth) and <3.69 ng/L (NOEC Immobility) determined in this study suggest that lambda-cyhalothrin is the most toxic, and of the three, the pesticide of greatest concern in terms of potential ecotoxicological effects on invertebrate populations and aquatic communities.

The EC50 of the mixture of 0.49 TU indicates that 0.163 TU (LC8) of each chemical in a tertiary mixture results in a 50 % growth inhibition relative to controls. The levels of 0.167 TU as measured in our single-chemical exposures were 3.67 ng/L lambda-cyhalothrin, 16.07 ng/L permethrin, and 60.31 ng/L chlorpyrifos; these too are lower than the reported environmentally relevant concentrations. These results highlight the pressing need to adequately assess the sublethal effects of contaminant mixtures as they co-occur in the environment.

Treatment concentrations for each pesticide were initially chosen from literature values to achieve equitoxic concentrations in each mixture, the measured concentrations were, as anticipated, below nominal concentrations, and toxicity levels are known to vary among studies (Wheelock et al. 2005). Differences between nominal and measured concentrations are not unusual, especially when target concentrations are near the limit of detection (Farmer et al. 1995; Amweg et al. 2005). Wheelock et al. (2005) showed that up to 50 % of the pyrethroid can adsorb to the

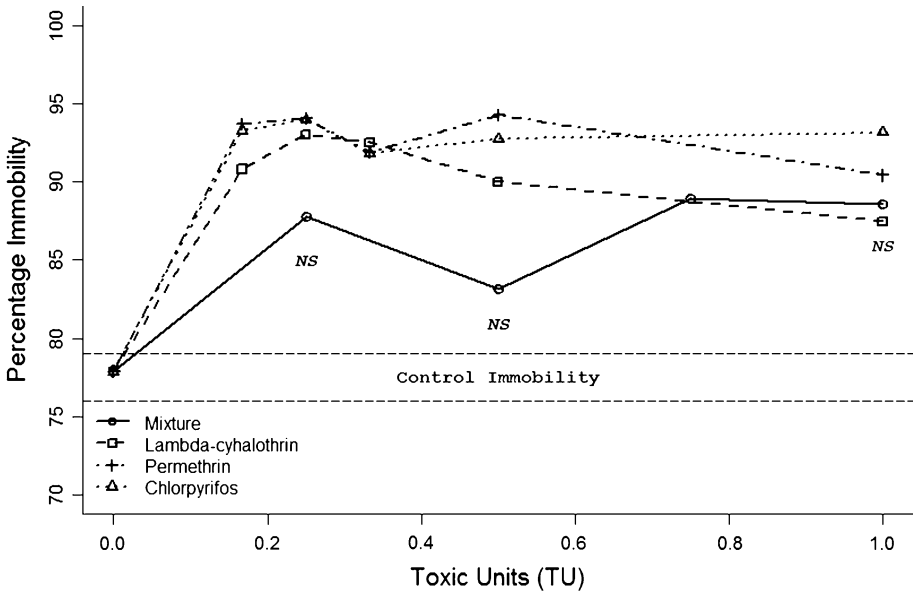

Fig. 3 Percentage immobility over 80 s of recording time of *C. dilutus* following 10-days exposures to lambda-cyhalothrin, permethrin, chlorpyrifos, and corresponding mixtures up to 1 TU (survival ≥50 %). *NS* non-significant to controls, all others $p < 0.05$. Data points are connected to aid visualization. Average control Immobility = 77.88 %

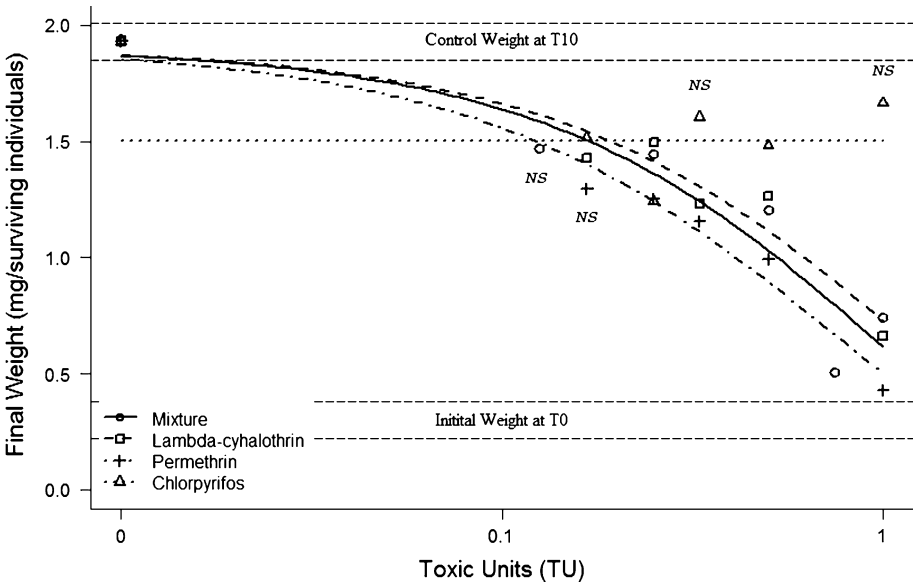

Fig. 4 Final weight in mg/surviving individual of *C. dilutus* following 10-days exposures to lambda-cyhalothrin, permethrin, chlorpyrifos, and corresponding mixtures up to 1 TU (survival ≥50 %). *NS* non-significant to controls, all others $p < 0.05$. Non-linear regression curves were fitted to the toxicity data using the DRC package version 2.3-96 (Ritz and Streibig 2005) R (R Core Team 2014). *Horizontal bars* represent average initial weight at test initiation (T0) (0.345 mg/individual) and average weight of the control at test termination (T10) (1.932 mg/surviving individual), and are presented to facilitate the comparison with initial and final weights along increasing toxic units

sampling container in 24 h which may be one explanation for the lower measured concentrations in our study.

The realized toxicity levels were therefore not strictly equitoxic. An equitoxic concentration approach was originally chosen to evaluate the joint action of the tertiary mixture by using CA and IA models, and to determine the degree of interaction, similarly to other studies (Denton et al. 2003; Belden and Lydy 2006; Symington et al. 2011; Larras et al. 2013; Norwood et al. 2013; Villa et al. 2014). The CA and IA concepts are usually evaluated using a "fixed ratio design", where the constituents ratio is kept constant throughout the studies (Barata et al. 2006), however, strictly equitoxic ratios are not essential for mixture toxicity assessments, and non-equitoxic ratios are more reflective of pesticide levels in the environment (Altenburger et al. 2004; Brodeur et al. 2014).

Ecotoxicological effects are known to differ significantly depending on routes of uptake, e.g., aqueous versus

dietary exposures, as demonstrated by Werner et al. (2002a). *C. dilutus* are detritivores, therefore dietary exposure to contaminants is likely (Laskowski 2002; Liu et al. 2004; Yang et al. 2006). Pyrethroids and organophosphates are known to adsorb to particulate matter. *C. dilutus* spends most of its larval stage in the substrate, therefore aqueous exposure may be reduced for this species, but cuticular exposure from the substrate may be greater. Pyrethroid exposure resulted in reduced growth of *C. dilutus,* which could have been caused by food avoidance due to insecticide-bound organic material. 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. Sublethal, behavioral effects such as case abandonment in the caddis fly *Brachycentrus americanus*, has been reported on exposures to esfenvalerate (type II pyrethroid), and associated with energetically costly activities such as case-rebuilding (Johnson et al. 2008). Induced case-rebuilding has further been associated with reduced growth in the caddis fly *Odontocerumalbicorne* (Stevens et al. 1999). Growth- and/or behaviorally-related effect concentrations will likely impact population dynamics.

This could occur through several mechanisms, e.g., chironomids are short-lived as adults, and since fecundity is largely determined by size at emergence, contaminant exposure may lead to reduced number of offspring in subsequent generations (Xue and Ali 1994). Smaller individuals may also be more susceptible to predators, may have reduced resistance to other environmental stressors as homeostatic energy demands are increased to contend with contaminant stress (Liber et al. 1996; Sibley et al. 1997; McKenney et al. 1998).

The observed mixture effects on survival (Fig. 2) suggest that the IA model is the most suitable concept to predict the combined toxicity effects of the three chemicals tested in this study, likely due to the differences in modes of action of the assessed pesticides. Other studies have also found that the IA concept provides a reasonable prediction of toxic effect for mixtures containing compounds with different modes of action (including pyrethroids and organophosphates, Backhaus et al. 2000; Faust et al. 2003; de Zwart and Posthuma 2005; Barata et al. 2012). However, the CA model has been highlighted by others (Könemann 1981; Hermens et al. 1984; Cedergreen 2014; Chen et al. 2014) to better predict mixture toxicity (e.g., chlorpyrifos and esfenvalerate; Belden and Lydy (2006)), indicating that the IA under-predicts toxicity. Variable results between studies, which evaluated different types of mixtures containing organophosphates and pyrethroids, may have been due to the toxicity of the most toxic constituent, rather than their modes of action. In this study, lambda-cyhalothrin was 5–12 times more potent than permethrin and chlorpyrifos, respectively, and thus is likely the driver of toxicity in the mixtures. This supports previous postulations that CA and IA models can be driven by one chemical within mixtures if its potency is substantially higher than the other members of the mixture (Heindel et al. 1995; Olmstead and LeBlanc 2005).

Effects on sublethal endpoints of *C. dilutus* were observed at environmentally relevant concentrations. This was also found in a study by Maul et al. (2008) where the individual exposure to lambda-cyhalothrin or permethrin significantly affected immobilization and growth rate of *C. dilutus*. Due to the complex life cycle of *C. dilutus* (involving pupation and emergence events), larval growth is frequently demonstrated to be a more sensitive endpoint than survival during the larval period, because growth may predict survival to adulthood and fecundity (Ankley et al. 1993, 1994; Burton et al. 1996; Maul et al. 2008). Growth inhibition could lead to failure to mature and reproduce. In previous studies, reduced larval growth in *C. dilutus* negatively affected pupation, emergence (86–100 % reduction), adult female size, number of eggs per female, and fecundity (Liber et al. 1996; Sibley et al. 1997; Ristola et al. 1999).

Agra and Soares (2009) also report that environmentally relevant concentrations of chlorpyrifos did not affect growth in *C. dilutus* supporting the lack of detectable effect on growth in this study. While daily food provision was equal throughout the tests, increased mortality resulting from exposure may have resulted in increased food availability *per capita*, thus concealing potential growth effects; as reported in other studies (Sibley et al. 1996; Martinez-Jeronimo et al. 2000; Hooper et al. 2003; Rakotondravelo et al. 2006). Furthermore, density can have negative effects on growth, development, and reproduction, as reported by Hooper et al. (2003). But regardless of food *per capita* and density, growth was also affected by the pyrethroid pesticides, thus the effects observed following pyrethroid exposure, but not following exposure to chlorpyrifos, are potentially due to differences in mode of action of the two insecticide groups. Pyrethroids work by preventing closure of the sodium channels in neuronal membranes affecting both the peripheral and central nervous system which may directly lead to decrease in feeding activity (Landrum et al. 2002), assimilation efficiency (Jager et al. 2006), protein synthesis, and rates of biotransformation and damage repair (Kooijman and Metz 1984). Chlorpyrifos can block the function of acetylcholinesterases (AChE), an important enzyme involved in neurotransmission. A number of studies found that despite a significant inhibition of AChE chironomid larvae are able to survive (Rakotondravelo et al. 2006; Rebechi et al. 2014), while behavioral effects such as limited mobility are present in organisms (Azevedo-Pereira et al. 2011) potentially having a lesser effect on feeding ability.

Mobility was the most sensitive endpoint in this study. A variety of neurotoxic contaminants in aquatic systems may affect mobility at sublethal exposure levels (Christensen et al. 2005; Werner and Moran 2008; Jin et al. 2009), thus suggesting to be a highly environmentally-relevant endpoint. It is especially useful for estimating effects on individual level in fish (Little and Finger 1990; Heath et al. 1993; Geist et al. 2007; Floyd et al. 2008; Beggel et al. 2010), and has also been applied in experiments involving *C. dilutus* (Hatch and Burton 1999). The assessment of swimming performance incorporates biochemical and physiological effects and directly evaluates the impacts of neurotoxic contaminants on nerve cell transmissions and resulting muscle activity (Heath et al. 1993; Jin et al. 2009). Inability to swim normally after an exposure to insecticides will therefore negatively affect individual fitness and survival, with potential consequences at the population level (Little and Finger 1990; Floyd et al. 2008). In the current study, significant effects on mobility were detected at exposures that are within the range of reported environmental concentrations (Anderson et al. 2006; Budd et al. 2009; Werner et al. 2010; Weston and Lydy 2010).

In ecological risk assessment, safety factors are applied to account for the uncertainty of extrapolating from laboratory toxicity tests to the real environment (EC 2003; U.S. EPA 2004; Jager et al. 2006; Hanson and Stark 2012). Despite these regulation efforts, pesticides exceed sublethal effective concentrations (NOEC and EC50) determined for growth and immobility in this study (Amweg et al. 2006; Weston and Lydy 2010; Bereswill et al. 2013; Li et al. 2013), and have been shown to impact aquatic organisms worldwide at concentrations that current legislation considers environmentally protective (Werner et al. 2002b; Schulz 2004; Weston and Lydy 2012; Beketov et al. 2013). Thus, it is evident that the toxicity of contaminants, and complex mixtures, may be significantly underestimated, by solely utilizing mortality as an endpoint for monitoring ambient water quality, as well as in ecological risk assessments, indicating the need for more sensitive endpoints to adequately assess ecological risk.

Conclusion

Determining the effect of water pollution remains a great challenge for environmental policy and management. This study reinforces the pressing need of integrating sublethal endpoints into regulatory toxicity assessments and monitoring studies to adequately assess the effects of contaminants. Mortality alone, as typically used for ecological assessment and management of industrial chemicals and pesticides, provides limited, to no information on organism fitness caused by pesticide exposure, especially since environmentally relevant concentrations do not generally occur at concentrations that result in direct mortality to aquatic communities. The use of sublethal endpoints, such as growth or mobility, in toxicity tests can indicate the presence of low levels of contaminants in water or sediment samples, sometimes at concentrations below the limit of detection of current-use analytical methods. Therefore it is essential to incorporate such tests into ambient water monitoring efforts and ecological risk assessment. In order to be able to monitor the effects of contaminant mixtures and to safeguard human health and the environment, a more holistic approach is required that includes assessing the combined effects of cumulative exposures to multiple stressors utilizing sublethal endpoints on toxicity exposures.

Acknowledgments The authors thank Linda Deanovic, Matthias Hasenbein, Keith Miles, Emily Parry, Marie Stillway, Inge Werner and Thomas Young for their support. This study was funded by the California State Water Resources Control Board (contract No. 06-447-300to REC), California Department of Pesticide Regulation (contract No. 10-C0096 to SPL), the State and Federal Contractors Water Agency (contract No. 201301957 to REC), and a postgraduate scholarship (contract No. DOK-121-10 to JG) by Bayerische Forschungsstiftung, Germany. The authors acknowledge the support by the Faculty Graduate Center Weihenstephan of TUM Graduate School at Technische Universität München, Germany.

Conflict of interest The authors declare that they have no conflict of interest.

References

Agra AR, Soares AM (2009) Effects of two insecticides on survival, growth and emergence of Chironomus riparius Meigen. Bull Environ Contam Toxicol 82(4):501–504

Altenburger R, Walter H, Grote M (2004) What contributes to the combined effect of a complex mixture? Environ Sci Technol 38(23):6353–6362

Amweg EL, Weston DP, Ureda NM (2005) Use and toxicity of pyrethroid pesticides in the Central Valley, California, USA. Environ Toxicol Chem 24(4):966–972

Amweg EL, Weston DP, You J, Lydy MJ (2006) Pyrethroid insecticides and sediment toxicity in urban creeks from California and Tennessee. Environ Sci Technol 40(5):1700–1706

Anderson BS, Phillips BM, Hunt JW, Worcester K, Adams M, Kapellas N, Tjeerdema RS (2006) Evidence of pesticide impacts in the Santa Maria River watershed, California, USA. Environ Toxicol Chem 25(4):1160–1170

Ankley GT, Collyard SA (1995) Influence of piperonyl butoxide on the toxicity of organophosphate insecticides to three species of freshwater benthic invertebrates. Comp Biochem Physiol C: Pharmacol Toxicol Endocrinol 110(2):149–155

Ankley GT, Benoit DA, Hoke RA, Leonard EN, West CW, Phipps GL, Mattson VR, Anderson LA (1993) Development and evaluation of test methods for benthic invertebrates and sediments: effects of flow rate and feeding on water quality and exposure conditions. Arch Environ Contam Toxicol 25(1):12–19

Ankley GT, Call DJ, Cox JS, Kahl MD, Hoke RA, Kosian PA (1994) Organic carbon partitioning as a basis for predicting the toxicity of chlorpyrifos in sediments. Environ Toxicol Chem 13(4):621–626

Azevedo-Pereira HMVS, Lemos MFL, Soares AMVM (2011) Effects of imidacloprid exposure on Chironomus riparius Meigen larvae: linking acetylcholinesterase activity to behaviour. Ecotoxicol Environ Saf 74(5):1210–1215

Backhaus T, Scholze M, Grimme LH (2000) The single substance and mixture toxicity of quinolones to the bioluminescent bacterium Vibrio fischeri. Aquat Toxicol 49(1–2):49–61

Backhaus T, Arrhenius Å, Blanck H (2004) Toxicity of a mixture of dissimilarly acting substances to natural algal communities: predictive power and limitations of independent action and concentration addition. Environ Sci Technol 38(23):6363–6370

Backhaus T, Faust M, Kortenkamp A (2013) Cumulative risk assessment: a European perspective on the state of the art and the necessary next steps forward. Integr Environ Assess Manag 9(4):547–548

Barata C, Baird DJ, Nogueira AJ, Soares AM, Riva MC (2006) Toxicity of binary mixtures of metals and pyrethroid insecticides to Daphnia magna Straus. Implications for multi-substance risks assessment. Aquat Toxicol 78(1):1–14

Barata C, Fernandez-San Juan M, Luisa Feo M, Eljarrrat E, Soares AMVM, Barcelo D, Baird DJ (2012) Population growth rate responses of Ceriodaphnia dubia to ternary mixtures of specific acting chemicals: pharmacological versus ecotoxicological modes of action. Environ Sci Technol 46:9663–9672

Beggel S, Werner I, Connon RE, Geist JP (2010) Sublethal toxicity of commercial insecticide formulations and their active ingredients to larval fathead minnow (*Pimephales promelas*). Sci Total Environ 408(16):3169–3175

Beketov MA, Kefford BJ, Schäfer RB, Liess M (2013) Pesticides reduce regional biodiversity of stream invertebrates. Proc Natl Acad Sci 110(27):11039–11043

Belden JB, Lydy MJ (2006) Joint toxicity of chlorpyrifos and esfenvalerate to fathead minnows and midge larvae. Environ Toxicol Chem 25(2):623–629

Belden JB, Gilliom RJ, Lydy MJ (2007) How well can we predict the toxicity of pesticide mixtures to aquatic life? Integ Environ Assess Manag 3(3):364–372

Bereswill R, Streloke M, Schulz R (2013) Current-use pesticides in stream water and suspended particles following runoff: exposure, effects, and mitigation requirements. Environ Toxicol Chem 32(6):1254–1263

Bliss CI (1939) The toxicity of poisons applied jointly1. Ann Appl Biol 26(3):585–615

Brodeur JC, Poliserpi MB, D'Andrea MF, Sanchez M (2014) Synergy between glyphosate- and cypermethrin-based pesticides during acute exposures in tadpoles of the common South American Toad Rhinella arenarum. Chemosphere 112:70–76

Brooks ML, Fleishman E, Brown LR, Lehman PW, Werner I, Scholz N, Mitchelmore C, Lovvorn JR, Johnson ML, Schlenk D, van Drunick S, Drever JI, Stoms DM, Parker AE, Dugdale R (2012) Life histories, salinity zones, and sublethal contributions of contaminants to pelagic fish declines illustrated with a case study of San Francisco Estuary, California, USA. Estuar Coasts 35(2):603–621

Budd R, ÓGeen A, Goh KS, Bondarenko S, Gan J (2009) Efficacy of constructed wetlands in pesticide removal from tailwaters in the Central Valley, California. Environ Sci Technol 43(8):2925–2930

Burton GA, Norberg-King TJ, Benoit DA, Ankley GT, Winger PV, Kubitz J, Lazorchak JM, Smith ME, Call DJ, Ingersoll CG, Greer E, Dwyer FJ, Day KE, Kennedy P, Stinson M (1996) Interlaboratory study of precision: *Hyalella azteca* and *Chironomus tentans* freshwater sediment toxicity assays. Environ Toxicol Chem 15(8):1335–1343

Cedergreen N (2014) Quantifying synergy: a systematic review of mixture toxicity studies within environmental toxicology. PLoS ONE 9(5):e96580

Chen C, Wang Y, Zhao X, Wang Q, Qian Y (2014) The combined toxicity assessment of carp (Cyprinus carpio) acetylcholinesterase activity by binary mixtures of chlorpyrifos and four other insecticides. Ecotoxicology 23(2):221–228

Christensen BT, Lauridsen TL, Ravn HW, Bayley M (2005) A comparison of feeding efficiency and swimming ability of Daphnia magna exposed to cypermethrin. Aquat Toxicol 73(2):210–220

Clark JM, Matsumura F (1982) Two different types of inhibitory effects of pyrethroids on nerve Ca- and Ca + Mg-ATPase activity in the Squid, *Loligo pealei*. Pestic Biochem Physiol 18(2):180–190

Connon RE, Geist J, Werner I (2012) Effect-based tools for monitoring and predicting the ecotoxicological effects of chemicals in the aquatic environment. Sensors 12(9):12741–12771

de Zwart D, Posthuma L (2005) Complex mixture toxicity for single and multiple species: proposed methodologies. Environ Toxicol Chem 24(10):2665–2676

Deanovic LA, Markiewicz D, Stillway M, Fong S, Werner I (2013) Comparing the effectiveness of chronic water column tests with the crustaceans Hyalella azteca (order: Amphipoda) and Ceriodaphnia dubia (order: Cladocera) in detecting toxicity of current-use insecticides. Environ Toxicol Chem 32(3):707–712

Denton DL, Wheelock CE, Murray SA, Deanovic LA, Hammock BD, Hinton DE (2003) Joint acute toxicity of esfenvalerate and diazonin to larval fathead minnows (*Pimephales promelas*). Environ Toxicol Chem 22(2):336–341

Du J, Pang J, You J (2013) Bioavailability-based chronic toxicity measurements of permethrin to Chironomus dilutus. Environ Toxicol Chem 32(6):1403–1411

EC EC (2003) Technical guidance document in support of Commission Directive 93/67 EEC on risk assessment for new notified substances, Commission Regulation (EC) no. 1488/94 on risk assessment for existing substances and Directive 98/8/EC of the European Parliament and of the Council concerning the placing of biocidal products on the market. European Chemical Bureau, Ispra, Italy

Eide I, Johansson E (1994) Statistical experimental design and projections to latent structures analysis in the evaluation of fuel blends with respect to particulate emissions. Chemometr Intell Lab Syst 22(1):77–85

Farmer D, Hill IR, Maund SJ (1995) A comparison of the fate and effects of two pyrethroid insecticides (lambda-cyhalothrin and cypermethrin) in pond mesocosms. Ecotoxicology 4(4):219–244

Faust M, Altenburger R, Backhaus T, Blanck H, Boedeker W, Gramatica P, Hamer V, Scholze M, Vighi M, Grimme LH (2003) Joint algal toxicity of 16 dissimilarly acting chemicals is predictable by the concept of independent action. Aquat Toxicol 63:43–63

Faust M, Altenburger R, Backhaus T, Bödeker W, Scholze M, Grimme LH (2000) Predictive assessment of the aquatic toxicity of multiple chemical mixtures. J Environ Qual 29(4):1063–1068

Floyd EY, Geist JP, Werner I (2008) Acute, sublethal exposure to a pyrethroid insecticide alters behavior, growth, and predation risk in larvae of the fathead minnow (*Pimephales promelas*). Environ Toxicol Chem 27(8):1780–1787

Geist J (2011) Integrative freshwater ecology and biodiversity conservation. Ecol Ind 11(6):1507–1516

Geist J, Werner I, Eder KJ, Leutenegger CM (2007) Comparisons of tissue-specific transcription of stress response genes with whole animal endpoints of adverse effect in striped bass (*Morone saxatilis*) following treatment with copper and esfenvalerate. Aquat Toxicol 85(1):28–39

Gregorio V, Chevre N, Junghans M (2013) Critical issues in using the common mixture toxicity models concentration addition or response addition on species sensitivity distributions: a theoretical approach. Environ Toxicol Chem 32(10):2387–2395

Hanson N, Stark JD (2012) Utility of population models to reduce uncertainty and increase value relevance in ecological risk assessments of pesticides: an example based on acute mortality data for daphnids. Integr Environ Assess Manag 8(2):262–270

Harwood AD, You J, Lydy MJ (2009) Temperature as a toxicity identification evaluation tool for pyrethroid insecticides: toxicokinetic confirmation. Environ Toxicol Chem 28(5):1051–1058

Hatch AC, Burton GA (1999) Sediment toxicity and stormwater runoff in a contaminated receiving system: consideration of different bioassays in the laboratory and field. Chemosphere 39(6):1001–1017

Heath AG, Cech JJ, Zinkl JG, Steele MD (1993) Sublethal effects of three pesticides on Japanese medaka. Arch Environ Contam Toxicol 25(4):485–491

Heindel JJ, Chapin RE, George J, Gulati DK, Fail PA, Barnes LH, Yang RSH (1995) Assessment of the reproductive toxicity of a complex mixture of 25 groundwater contaminants in mice and rats. Fundam Appl Toxicol 25:9–19

Hermens J, Canton H, Steyger N, Wegman R (1984) Joint effects of a mixture of 14 chemicals on mortality and inhibition of reproduction of Daphnia magna. Aquat Toxicol 5(4):315–322

Hewlett PS, Plackett RL (1952) Similar Joint Action of Insecticides. Nature 169(4292):198–199

Hintzen EP, Lydy MJ, Belden JB (2009) Occurrence and potential toxicity of pyrethroids and other insecticides in bed sediments of urban streams in central Texas. Environ Pollut 157(1):110–116

Hooper HL, Sibly RM, Maund SJ, Hutchinson TH (2003) The joint effects of larval density and 14C-cypermethrin on the life history and population growth rate of the midge Chironomus riparius. J Appl Ecol 40(6):1049–1059

Hua J, Cothran R, Stoler A, Relyea R (2013) Cross-tolerance in amphibians: wood frog mortality when exposed to three insecticides with a common mode of action. Environ Toxicol Chem 32(4):932–936

Jager T, Heugens E, Kooijman S (2006) Making sense of ecotoxicological test results: towards application of process-based models. Ecotoxicology 15(3):305–314

Jeon J, Kurth D, Ashauer R, Hollender J (2013) Comparative toxicokinetics of organic micropollutants in freshwater crustaceans. Environ Sci Technol 47(15):8809–8817

Jin M, Zhang X, Wang L, Huang C, Zhang Y, Zhao M (2009) Developmental toxicity of bifenthrin in embryo-larval stages of zebrafish. Aquat Toxicol 95(4):347–354

Johnson KR, Jepson PC, Jenkins JJ (2008) Esfenvalerate-induced case-abandonment in the larvae of the caddisfly (*Brachycentrus americanus*). Environ Toxicol Chem 27(2):397–403

Jonker MJ, Svendsen C, Bedaux JJM, Bongers M, Kammenga JE (2005) Significance testing of synergistic/antagonistic, dose level-dependent, or dose ratio-dependent effects in mixture dose-response analysis. Environ Toxicol Chem 24(10):2701–2713

Junghans M, Backhaus T, Faust M, Scholze M, Grimme LH (2006) Application and validation of approaches for the predictive hazard assessment of realistic pesticide mixtures. Aquat Toxicol 76(2):93–110

Könemann H (1981) Fish toxicity tests with mixtures of more than two chemicals: a proposal for a quantitative approach and experimental results. Toxicology 19(3):229–238

Kooijman SALM, Metz JAJ (1984) On the dynamics of chemically stressed populations: the deduction of population consequences from effects on individuals. Ecotoxicol Environ Saf 8(3):254–274

Landrum PF, Gedeon ML, Burton GA, Greenberg MS, Rowland CD (2002) Biological Responses of Lumbriculus variegatus Exposed to Fluoranthene-Spiked Sediment. Arch Environ Contam Toxicol 42(3):292–302

Larras F, Lambert A-S, Pesce S, Rimet F, Bouchez A, Montuelle B (2013) The effect of temperature and a herbicide periphytic algae mixture on freshwater. Ecotoxicol Environ Saf 98:162–170

Laskowski DA (2002) Physical and chemical properties of pyrethroids. Rev Environ Contam Toxicol 174:49–170

Li H, Sun B, Lydy MJ, You J (2013) Sediment-associated pesticides in an urban stream in Guangzhou, China: implication of a shift in pesticide use patterns. Environ Toxicol Chem 32(5):1040–1047

Liber K, Call DJ, Dawson TD, Whiteman FW, Dillon TM (1996) Effects of Chironomus tentans larval growth retardation on adult emergence and ovipositing success: implications for interpreting freshwater sediment bioassays. Hydrobiologia 323(3):155–167

Little EE, Finger SE (1990) Swimming behavior as an indicator of sublethal toxicity in fish. Environ Toxicol Chem 9(1):13–19

Liu W, Gan JJ, Lee S, Kabashima JN (2004) Phase distribution of synthetic pyrethroids in runoff and stream water. Environ Toxicol Chem 23(1):7–11

Lydy MJ, Austin KR (2004) Toxicity assessment of pesticide mixtures typical of the Sacramento-San Joaquin Delta Using Chironomus tentans. Arch Environ Contam Toxicol 48(1):49–55

Lydy M, Belden J, Wheelock C, Hammock B, Denton D (2004) Challenges in regulating pesticide mixtures. Ecol Soc 9(6):1

Malison RL, Benjamin JR, Baxter CV (2010) Measuring adult insect emergence from streams: the influence of trap placement and a comparison with benthic sampling. J N Am Benthol Soc 29(2):647–656

Martinez-Jeronimo F, Espinosa-Chavez F, Villasenor R (2000) Effect of culture volume and adult density on the neonate production of Daphnia magna, as a test organism for aquatic toxicity tests. Environ Toxicol 15(3):155–159

Maul JD, Brennan AA, Harwood AD, Lydy MJ (2008) Effect of sediment-associated pyrethroids, fipronil, and metabolites on *Chironomus tentans* growth rate, body mass, condition index, immobilization, and survival. Environ Toxicol Chem 27(12):2582–2590

McCarty LS, Dixon DG, Ozburn GW, Smith AD (1992) Toxicokinetic modeling of mixtures of organic chemicals. Environ Toxicol Chem 11(7):1037–1047

McKenney JCL, Weber DE, Celestial DM, MacGregor MA (1998) Altered growth and metabolism of an estuarine shrimp (*Palaemonetes pugio*) during and after metamorphosis onto fenvalerate-laden sediment. Arch Environ Contam Toxicol 35(3):464–471

Nahon S, Charles F, Lantoine F, Vétion G, Escoubeyrou K, Desmalades M, Pruski AM (2010) Ultraviolet radiation negatively affects growth and food quality of the pelagic diatom skeletonema costatum J Exp Mar Bio Ecol 383:164-170

Nasuti C, Cantalamesa F, Falcioni G, Gabbianelli R (2003) Different effects of Type I and Type II pyrethroids on erythrocyte plasma membrane properties and enzymatic activity in rats. Toxicology 191(2–3):233–244

Nørgaard K, Cedergreen N (2010) Pesticide cocktails can interact synergistically on aquatic crustaceans. Environ Sci Pollut Res 17(4):957–967

Norwood WP, Borgmann U, Dixon DG (2013) An effects addition model based on bioaccumulation of metals from exposure to mixtures of metals can predict chronic mortality in the aquatic invertebrate hyalella azteca. Environ Toxicol Chem 32(7):1672–1681

Nyman A-M, Hintermeister A, Schirmer K, Ashauer R (2013) The insecticide imidacloprid causes mortality of the freshwater amphipod gammarus pulex by interfering with feeding behavior. PLoS ONE 8(5):e62472

Olmstead AW, LeBlanc GA (2005) Toxicity assessment of environmentally relevant pollutant mixtures using a heuristic model. Integr Environ Assess Manag 1:114–122

Oros DR, Werner I (2005) Pyrethroid insecticides an analysis of use patterns, distributions, potential toxicity and fate in the Sacramento-San Joaquin Delta and Central Valley. SFEI contribution 415. Oakland, California, San Francisco Estuary Institute, 112

Pape-Lindstrom PA, Lydy MJ (1997) Synergistic toxicity of atrazine and organophosphate insecticides contravenes the response addition mixture model. Environ Toxicol Chem 16(11):2415–2420

Phillips BM, Anderson BS, Hunt JW, Siegler K, Voorhees JP, Tjeerdema RS, McNeill K (2012) Pyrethroid and organophosphate pesticide-associated toxicity in two coastal watersheds (California, USA). Environ Toxicol Chem, n/a–n/a

Rakotondravelo M, Anderson T, Charlton R, Zhu K (2006) Sublethal effects of three pesticides on larval survivorship, growth, and macromolecule production in the aquatic midge, *Chironomus tentans* (Diptera: Chironomidae). Arch Environ Contam Toxicol 51(3):352–359

Rasmussen JJ, Wiberg-Larsen P, Kristensen EA, Cedergreen N, Friberg N (2013) Pyrethroid effects on freshwater invertebrates: a meta-analysis of pulse exposures. Environ Pollut 182:479–485

Rebechi D, Richardi VS, Vicentini M, Guiloski IC, Assis HCSd, Navarro-Silva MA (2014) Low malathion concentrations influence metabolism in Chironomus sancticaroli (Diptera, Chironomidae) in acute and chronic toxicity tests. Rev Bras de Entomol 58:296–301

Ristola T, Pellinen J, Ruokolainen M, Kostamo A, Kukkonen JVK (1999) Effect of sediment type, feeding level, and larval density on growth and development of a midge (*Chironomus riparius*). Environ Toxicol Chem 18(4):756–764

Ritz C, Streibig J (2005) Bioassay analysis using R. J Stat Softw 12(5):1–22

Scherer C, Seeland A, Oehlmann J, Mueller R (2013) Interactive effects of xenobiotic, abiotic and biotic stressors on Daphnia pulex-results from a multiple stressor experiment with a fractional multifactorial design. Aqua Toxicol (Amsterdam) 138:105–115

Scholz NL, Fleishman E, Brown L, Werner I, Johnson ML, Brooks ML, Mitchelmore CL, Schlenk D (2012) A perspective on modern pesticides, pelagic fish declines, and unknown ecological resilience in highly managed ecosystems. BioScience 62(4):428–434

Schulz R (2004) Field studies on exposure, effects, and risk mitigation of aquatic nonpoint-source insecticide pollution. J Environ Qual 33(2):419–448

Sibley PK, Ankley GT, Cotter AM, Leonard EN (1996) Predicting chronic toxicity of sediments spiked with zinc: an evaluation of the acid-volatile sulfide model using a life-cycle test with the midge Chironomus tentans. Environ Toxicol Chem 15(12):2102–2112

Sibley PK, Benoit DA, Ankley GT (1997) The significance of growth in Chironomus tentans sediment toxicity tests: relationship to reproduction and demographic endpoints. Environ Toxicol Chem 16(2):336–345

Smith S Jr, Lizotte RE Jr (2007) Influence of selected water quality characteristics on the toxicity of lambda-cyhalothrin and gamma-cyhalothrin to Hyalella azteca. Bull Environ Contam Toxicol 79(5):548–551

Sørensen H, Cedergreen N, Skovgaard I, Streibig J (2007) An isobole-based statistical model and test for synergism/antagonism in binary mixture toxicity experiments. Environ Ecol Stat 14(4):383–397

Sprague LA, Nowell LH (2008) Comparison of pesticide concentrations in streams at low flow in six metropolitan areas of the United States. Environ Toxicol Chem 27(2):288–298

Stevens DJ, Hansell MH, Monaghan P (1999) Developmental trade-offs in caddisflies: increased investment in larval defence alters adult resource allocation. Proc R Soc B: Biol Sci 266:1049–1054

Symington SB, Hodgdon HE, Frisbie RK, Clark JM (2011) Binary mixtures of pyrethroids produce differential effects on Ca^{2+} influx and glutamate release at isolated presynaptic nerve terminals from rat brain. Pestic Biochem Physiol 99(2):131–139

Trimble AJ, Weston DP, Belden BJ, Lydy MJ (2009) Identification and evaluation of pyrethroid insecticide mixtures in urban sediments. Environ Toxicol Chem 28(8):1687–1695

R Core Team (2014) R: a language and environment for statistical computing, R Foundation for Statistical Computing, Vienna, Austria, http://www.R-project.org/

U. S. Environmental Protection Agency (2000) Methods for measuring the toxicity and bioaccumulation of sediment-associated contaminants with freshwater invertebrates. EPA 600/R-99/064. Duluth, MN

U. S. Environmental Protection Agency (2002) Methods for measuring the acute toxicity of effluents and receiving waters to freshwater and marine organisms. EPA-821-R-02-012

U. S. Environmental Protection Agency (2004) Overview of the ecological risk assessment process in the office of pesticide programs. Washington DC: Office of Pesticide Programs. http://www.epa.gov/espp/consultation/ecorisk-overview.pdf

Villa S, Vighi M, Finizio A (2014) Experimental and predicted acute toxicity of antibacterial compounds and their mixtures using the luminescent bacterium Vibrio fischeri. Chemosphere 108:239–244

Werner I, Moran K (2008) Effects of pyrethroid insecticides on aquatic organisms. Synthetic Pyrethroids, Am Chem Soc 991:310–334

Werner I, Deanovic LA, Hinton DE, Henderson JD, De Oliviera GH, Wilson BW, Krueger W, Wallender WW, Oliver MN, Zalom FG (2002a) Toxicity of stormwater runoff after dormant spray application of diazinon and esfenvalerate (Asana) in a French prune orchard, Glenn County, California, USA. Bull Environ Contam Toxicol 68(1):29–36

Werner I, Geist J, Okihiro M, Rosenkranz P, Hinton DE (2002b) Effects of dietary exposure to the pyrethroid pesticide esfenvalerate on medaka (Oryzias latipes). Mar Environ Res 54(3–5):609–614

Werner I, Deanovic LA, Markiewicz D, Khamphanh M, Reece CK, Stillway M, Reece C (2010) Monitoring acute and chronic water column toxicity in the northern Sacramento-San Joaquin estuary, California, USA, using the euryhaline amphipod, *Hyalella azteca*: 2006 to 2007. Environ Toxicol Chem 29(10):2190–2199

Weston DP, Lydy MJ (2010) Urban and agricultural sources of pyrethroid insecticides to the Sacramento-San Joaquin Delta of California. Environ Sci Technol 44(5):1833–1840

Weston DP, Lydy MJ (2012) Stormwater input of pyrethroid insecticides to an urban river. Environ Toxicol Chem, n/a–n/a

Weston DP, You J, Lydy MJ (2004) Distribution and toxicity of sediment-associated pesticides in agriculture-dominated water bodies of California's Central Valley. Environ Sci Technol 38(10):2752–2759

Weston DP, Zhang M, Lydy MJ (2008) Identifying the cause and source of sediment toxicity in an agriculture-influenced creek. Environ Toxicol Chem 27(4):953–962

Weston DP, Ding Y, Zhang M, Lydy MJ (2013) Identifying the cause of sediment toxicity in agricultural sediments: the role of pyrethroids and nine seldom-measured hydrophobic pesticides. Chemosphere 90(3):958–964

Weston DP, Asbell AM, Lesmeister SA, Teh SJ, Lydy MJ (2014) Urban and agricultural pesticide inputs to a critical habitat for the threatened delta smelt (Hypomesus transpacificus). Environ Toxicol Chem 33(4):920–929

Wheelock CE, Miller JL, Miller MJ, Phillips BM, Gee SJ, Tjeerdema RS, Hammock BD (2005) Influence of container adsorption upon observed pyrethroid toxicity to Ceriodaphnia dubia and Hyalella azteca. Aquat Toxicol 74(1):47–52

Wouters W, van den Bercken J (1978) Action of pyrethroids. Gen Pharmacol 9:387–398

Xue R-D, Ali A (1994) Oviposition, fecundity, and body size of a pestiferous Midge, Chironomus crassicaudatus (Diptera: Chironomidae). Environ Entomol 23(6):1480–1484

Yang W, Gan J, Hunter W, Spurlock F (2006) Effect of suspended solids on bioavailability of pyrethroid insecticides. Environ Toxicol Chem 25(6):1585–1591