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Joint toxicity of sediment-associated permethrin and cadmium to *Chironomus dilutus*: The role of bioavailability and enzymatic activities

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ABSTRACT

Pyrethroid insecticides and metals commonly co-occurred in sediment and caused toxicity to benthic organisms jointly. To improve accuracy in assessing risk of the sediments contaminated by insecticides and metals, it is of great importance to understand interaction between the contaminants and reasons for the interaction. In the current study, permethrin and cadmium were chosen as representative contaminants to study joint toxicity of pyrethroids and metals to a benthic invertebrate *Chironomus dilutus*. A median effect/combination index-isobologram was applied to evaluate the interaction between sediment-bound permethrin and cadmium at three dose ratios. Antagonistic interaction was observed in the midges for all treatments. Comparatively, cadmium-dominated group (the ratio of toxicity contribution from permethrin and cadmium was 1:3) showed stronger antagonism than equitoxicity (1:1) and permethrin-dominated groups (3:1). The reasons for the observed antagonism were elucidated from two aspects, including bioavailability and enzymatic activity. The bioavailability of permethrin, expressed as the freely dissolved concentrations in sediment porewater and measured by solid phase microextraction, was not altered by the addition of cadmium, suggesting the change in permethrin bioavailability was not the reason for the antagonism. On the other hand, the activities of metabolic enzymes, glutathione S-transferase and carboxylesterase in the midges which were exposed to mixtures of permethrin and cadmium were significantly higher than those in the midges exposed to permethrin solely. Cadmium considerably enhanced the detoxifying processes of permethrin in the midges, which largely explained the observed antagonistic interaction between permethrin and cadmium.

1. Introduction

The occurrence and toxicological effects of pyrethroid insecticides in the environment have been well documented following their increasing use as alternatives of now-restricted organochlorine and organophosphate insecticides (Li et al., 2014; Siegler et al., 2015). Due to their high hydrophobicity, pyrethroids readily associated with sediment particles after entering aquatic systems and became one of the major threats to benthic invertebrates in urban waterways (Ding et al., 2010; Gan et al., 2005; Mehler et al., 2011a).

The occurrence of other contaminants in urban environment and their contribution to the toxicity should neither be overlooked. Heavy metals were frequently detected in sediment along with pyrethroids and results of whole-sediment toxicity identification evaluation testing suggested that the toxicity of sediment samples from urban waterways in China to the midge, *Chironomus dilutus*, was caused by insecticides and metals jointly (Yi et al., 2015). Interaction between pyrethroids and metals may change their individual toxicity (Liu et al., 2009; Mehler et al., 2011b), introducing uncertainty in evaluating ecological risk when only focusing on the toxicity of individual constituents. Rather, it was imperative to take mixture effects into consideration when assessing the risk, particularly the contaminants commonly co-occurred in the environment.

Concentration addition (CA) and independent action (IA) models have been proposed to assess joint toxicity of contaminants with similar and dissimilar modes of toxic action, respectively (Altenburger et al., 2003; Barata et al., 2006; Jonker et al., 2005; Mehler et al., 2011b). In some cases, however, modes of action of the toxicants were unclear, making model selection a challenge. To circumvent the requirement of determining the modes of action for

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individual chemicals, a median effect/combination index (ME/CI)-isobologram equation was introduced in pharmacology to evaluate the interactions among chemicals (Chen et al., 2015; Chou, 2006). This method allowed for modeling the degree of synergistic, additive and antagonistic effects at different concentrations and effect levels (Chou, 2006).

The observed joint toxicity may result from toxicodynamic processes on target sites, as well as alterations of bioavailability and toxicokinetic processes of the chemicals in a mixture. Svendsen et al. (2010) explained synergistic interactions between organophosphate and neonicotinamide insecticides in the nematode *Caenorhabditis elegans* from toxicodynamic and toxicokinetic aspects. Because organophosphate-oxon is an inhibitor of acetylcholinesterase and neonicotinamide is an agonist of postsynaptic acetylcholine receptors, these insecticides synergistically enhanced neuroexcitation at the target site. From toxicokinetic aspect, neonicotinoides induced the activities of P450 enzymes, which speeded up the biotransformation of organophosphates to their more toxic oxon forms, and subsequently increased their toxicity to *C. elegans*. So far, most studies have been conducted for mixture effects among pesticides (Svendsen et al., 2010). Pesticides and metals commonly co-occurred in urban waterways, but little information is known about the reasons for their joint toxicity.

The objectives of the current study were (1) to evaluate the joint toxicity between sediment-bound pyrethroids and metals to *C. dilutus*, (2) to determine the type of interaction between the contaminants at different concentrations using the ME/CI-isobologram, and (3) to elucidate the reasons for the joint toxicity by assessing chemical bioavailability and enzymatic activity. Permethrin was one of the dominant pyrethroids in sediment, for example, permethrin was detected in 96% and 45% of the sediment samples collected from the Pearl River Delta (PRD), China and Illinois, USA, respectively (Ding et al., 2010; Mehler et al., 2011a). In addition, cadmium was frequently detected in sediments in the PRD and posed high ecological risk in this area (Li et al., 2007). To more accurately perform sediment risk assessment in areas with high co-occurrence of pyrethroids and metals, like the PRD, it is of great importance to study the joint toxicity of these two risky constituents. Therefore, the frequently detected permethrin and cadmium in field sediments were selected as the representative pyrethroid and metal, respectively.

2. Materials and methods

2.1. Sediment and chemicals

Control sediment was collected from a drinking water reservoir in Conghua, China and this sediment was also used to prepare the spiked sediments for toxicity testing. More information on chemicals and reagents used in the current study and the procedures for sediment collection and spiking and the measurements of organic carbon (OC) content in sediment are presented in the Supplementary Material.

2.2. Joint toxicity test

The midge, *C. dilutus* was chosen as the test organism because it was recommended as model organism for sediment toxicity testing by the U.S. Environmental Protection Agency (USEPA) (2000) and has been previously applied to evaluate toxicity of a variety of sediment-bound contaminants, including pyrethroids and metals (Maul et al., 2008; Mehler et al., 2011a; Yi et al., 2015). The midges were cultured at Guangzhou Institute of Geochemistry, Chinese Academy of Sciences (GIGCAS) according to the standard protocols by the USEPA (2000).

Joint toxicity tests of permethrin and cadmium were conducted at fixed ratios of toxicity contribution (1:1, 3:1 and 1:3) and nominal sediment concentrations were selected according to toxicity data of individual constitutes. The 10-d median lethal concentration (LC50) of cadmium to *C. dilutus* was 170 μg/g dry weight (dw) and was obtained in a pilot experiment, and a value of 24.5 μg/g OC from the literature (Maul et al., 2008) was used for permethrin. Toxicity testing with permethrin only was also conducted along with joint toxicity tests. As shown in Table 1 and Fig. S1 ("S" represents figures and tables in the Supplementary Material thereafter), the tests included four groups, namely permethrin-only (G0), equitoxicity (G1, the ratio of permethrin and cadmium was 1:1), permethrin-dominated (G2, 3:1), and cadmium-dominated (G3, 1:3) groups. Toxicity tests in each group were conducted using five concentrations. For groups G0 and G1, the highest concentration was set at 4 times of LC50 for each component and then was divided by a dilution factor of 1.83 to generate a series of concentrations at 0.35, 0.65, 1.19, 2.18 and 4 times of LC50, respectively. Similarly, the highest concentration was set at 6 times of LC50 for permethrin and 2 times of LC50 for cadmium in group G2, whereas the highest concentration was set at 2 times of LC50 for permethrin and 6 times of LC50 for cadmium in G3. A dilution factor of 1.83 was also used in groups G2 and G3 to generate a series of five concentrations (Table 1 and Fig. S1).

Appropriate amounts of permethrin and $CdCl_2$ were spiked into control sediment with acetone (100 μL/600 g wet sediment) and MiliQ water as carriers, respectively. A solvent control was also prepared by adding the same amount of acetone into control sediment. After spiking, the sediments were thoroughly mixed for 2 h using a stainless steel paddle which was driven by an overhead motor, and then aged at 4 °C in the darkness for 28 days.

After 28-d aging, the sediments were homogenized again and 10-d sediment toxicity tests were conducted in six replicates with *C. dilutus* following the standard methods (USEPA, 2000) with some modifications. Solvent and negative controls were included in the testing. In brief, 70 g of wet sediment was put into a 400-mL beaker and then 250 mL of reconstituted water prepared according to the USEPA protocol (2000) was added as overlying water. After the sediment was settled overnight, ten 3rd instar midge larvae were randomly introduced into each beaker. The tests were carried out at 23 ± 1 °C with a light: dark photoperiod of 16:8 h, and pH, conductivity, temperature and dissolved oxygen of overlying water were monitored every day. Ammonia was measured at the beginning and end of the testing. Approximately 150 mL of overlying water was renewed twice daily using an automatic water exchange system. The midges were fed with 6 mg of grinded fish food daily. At the termination of 10-d exposure, *C. dilutus* were removed from the sediment using a 500-μm sieve. Surviving organisms were counted, rinsed, blotted dry, and stored at −20 °C for measuring the activities of metabolic enzymes.

2.3. Sediment analysis

Du et al. (2013) reported that the degradation of permethrin in sediment was negligible during 10-d toxicity tests. We also found no significant difference between cadmium concentrations at the beginning and end of 10-d testing in our pilot experiments. Thus, concentrations of permethrin and cadmium in sediment which were analyzed at the beginning of toxicity tests (after 28-d aging) were used for all calculations.

Permethrin was extracted from the sediments by sonication (Li et al., 2010), purified with solid phase extraction cartridges packed with primary secondary amine/graphitized carbon black, and finally quantified by Shimadzu QP-2010 plus series gas chromatography/mass spectrometry (GC/MS). Cadmium in sediment was

Table 1
Nominal and measured concentrations of permethrin and cadmium in sediment (C_s) in 10-d joint toxicity tests with *Chironomus dilutus*. The mortality, combination index (CI) values and the type of interaction between permethrin and cadmium are shown as well. Data are shown as mean ± standard deviation (permethrin analysis and toxicity tests were conducted in three and six replicates, respectively).

Treatment	Nominal C_s		Measured C_s (μg/g dry weight)		Mortality (%, n = 6)	CI[a]	Interaction[b]
	Permethrin	Cadmium	Permethrin (n = 3)	Cadmium (n = 1)			
G0C1	0.35 LC50[c]	NA[d]	0.113 ± 0.006	NA	25.0 ± 13.8	NA	NA
G0C2	0.65 LC50	NA	0.240 ± 0.009	NA	50.0 ± 19.0	NA	NA
G0C3	1.19 LC50	NA	0.406 ± 0.003	NA	68.3 ± 23.2	NA	NA
G0C4	2.18 LC50	NA	0.605 ± 0.022	NA	88.3 ± 11.7	NA	NA
G0C5	4.00 LC50	NA	1.238 ± 0.047	NA	80.0 ± 11.0	NA	NA
G1C1	0.35 LC50	0.35 LC50[e]	0.0983 ± 0.0025	33.4	15.0 ± 13.8	2.45	Ant
G1C2	0.65 LC50	0.65 LC50	0.231 ± 0.011	59.7	48.3 ± 33.1	1.52	Ant
G1C3	1.19 LC50	1.19 LC50	0.440 ± 0.009	107	48.3 ± 24.8	2.84	Ant
G1C4	2.18 LC50	2.18 LC50	0.743 ± 0.0003	255	90.0 ± 12.7	1.08	Slight Ant
G1C5	4.00 LC50	4.00 LC50	1.424 ± 0.055	420	95.0 ± 5.5	1.15	Slight Ant
G2C1	0.53 LC50	0.18 LC50	0.146 ± 0.012	13.4	21.7 ± 14.7	2.06	Ant
G2C2	0.97 LC50	0.32 LC50	0.278 ± 0.028	27.6	40.0 ± 12.7	1.95	Ant
G2C3	1.79 LC50	0.60 LC50	0.677 ± 0.026	49.5	48.3 ± 16.0	3.50	Strong Ant
G2C4	3.27 LC50	1.09 LC50	0.952 ± 0.011	103	81.7 ± 16.0	1.52	Ant
G2C5	6.00 LC50	2.00 LC50	2.349 ± 0.284	203	90.0 ± 12.7	2.08	Ant
G3C1	0.18 LC50	0.53 LC50	0.0844 ± 0.0037	45.2	8.3 ± 9.8	3.95	Strong Ant
G3C2	0.32 LC50	0.97 LC50	0.191 ± 0.002	88.5	16.7 ± 8.2	4.73	Strong Ant
G3C3	0.60 LC50	1.79 LC50	0.352 ± 0.003	170	38.3 ± 31.3	3.88	Strong Ant
G3C4	1.09 LC50	3.27 LC50	0.415 ± 0.029	323	43.3 ± 16.3	4.99	Strong Ant
G3C5	2.00 LC50	6.00 LC50	0.744 ± 0.028	604	76.7 ± 15.1	3.50	Strong Ant

[a] The values of CI < 1, CI = 1, and CI > 1 indicate synergism (Syn), additivity (Add), and antagonism (Ant), respectively. Computer software CompuSyn was used for the CI simulation.
[b] Degrees of interaction were determined according to Chou and Martin (2005) who recommended that CI values of 1.1–1.2, 1.2–1.45, 1.45–3.3, 3.3–10 and > 10 indicated slight antagonism, moderate antagonism, antagonism, strong antagonism, and very strong antagonism, respectively.
[c] Nominal sediment concentration was set at 0.35 times of the median lethal concentration (LC50) of permethrin (0.554 μg/g dry weight), and thereafter in the same column.
[d] NA = not applicable. The sediments in group G0 were only spiked with permethrin.
[e] Nominal sediment concentration was set at 0.35 times of the LC50 of cadmium (170 μg/g dry weight), and thereafter in the same column.

extracted by microwave digestion and analyzed on an Agilent 7700 inductively coupled plasma/MS (ICP/MS). The details of analytical procedures and information about the quality control are described in the Supplementary Material.

2.4. Measurement of bioavailability of permethrin in sediment

To evaluate the influence of cadmium on the bioavailability of permethrin, the freely dissolved concentrations (C_{free}) of permethrin in sediment porewater in groups G0 and G1 were determined by solid phase microextraction (SPME) (Cui et al., 2013; You et al., 2011). Disposable SPME fibers coated with 10 μm of polydimethylsiloxane (PDMS) with a phase volume of 0.069 mL/cm were used. Before use, the fragile fibers were protected by stainless steel meshes, cleaned by sonication in methanol and water, and air-dried at room temperature. The SPME measurements were conducted in triplicate and 5 cm of fibers were placed into a 15-mL vial which contained 10 g wet sediment and several pieces of copper sheets to eliminate sulfur interference during instrumental analysis. After the vials were gently shaken on a HY-4 shaker (Fuhua Instrument, Jintan, China) for 28 days, the wrapped fibers were retrieved from the sediments, rinsed with water, and dried with tissue paper. The fibers were extracted by sonication with 5 mL of a mixture of hexane and acetone (1:1, v/v) three times. The extracts were combined, concentrated and solvent exchanged to hexane, and then permethrin in the extracts were analyzed using GC–MS. Eventually, C_{free} of permethrin was calculated by dividing the concentrations in SPME fibers by the partition coefficient of permethrin between the fiber and the water (K_{fw}) and a log K_{fw} value of 5.59 was used (You et al., 2007).

2.5. Measurement of enzyme activities in the midges

Biotransformation potential of permethrin in the midges was assessed by measuring the activities of two metabolic enzymes, including glutathione S-transferase (GST) and carboxylesterase (CarE). The measurements were conducted in triplicate. Five midge larvae were homogenized in 1 mL of 0.1 mol/L cold phosphate buffered solution (PBS, pH 7.4) using a JY92-IIN high intensity ultrasonic processor (Scientz Biotechnology, Ningbo, China) in a 3-s work and 3-s pause mode for 30 s. Then, the homogenates were centrifuged at 10,000 g at 4 °C for 10 min on an ST-16R centrifuge (Thermo Scientific, MA, USA) and the supernatants were decanted for measuring protein contents and enzyme activity.

Protein contents in the extracts were measured using a Bradford method with bovine serum albumin as an external standard (Bradford, 1976). Briefly, the proteins were reacted to a dye (Coomassie Brilliant Blue G250) and protein contents were quantified by measuring absorbance of the blue protein-dye complex at 595 nm using a Varioskan Flash plate-reader (Thermo Scientific, MA, USA).

The activities of GST were determined with 1-chloro-2,4-dinitrobenzene as the substrate following a method proposed by Habig et al. (1974). The reaction system contained 50 μL of extracts, 100 μL of 50 mmol/L PBS solution containing 4 mmol/L 1-chloro-2,4-dinitrobenzene, and 50 μL of 50 mmol/L PBS solution containing 8 mmol/L reduced glutathione (GSH). After the solutions were mixed, change in optical density at 340 nm was immediately recorded for six times with an interval of 0.5 min on Varioskan Flash plate-reader. The GST activity was expressed as the nmol of 1-chloro-2,4-dinitrobenzene-GSH complex formed per mg protein per minute.

The activity of CarE was determined with α-naphthyl acetate as the substrate according to the method of Dary et al. (1990) with some modifications. In brief, 100 μL of 50 mmol/L PBS (pH = 6.0) containing 1.5 mmol/L α-naphthyl acetate was mixed with 50 μL of extracts. The solution was incubated at 37 °C for 5 min, and the reaction was terminated by adding 50 μL of a solution containing a color-developing agent (0.28% Fast Blue B Salt) and an inhibitor

(3.57% sodium dodecyl sulfate). Then, absorbance of α-naphthol-Fast Blue B Salt complex was measured at 535 nm using the plate-reader. The amounts of α-naphthol produced were quantified using external standard curves and CarE activity was expressed as the nmol of α-naphthol formed per mg protein per minute.

2.6. Data analysis and statistical comparison

Joint toxicity of sediment-bound permethrin and cadmium to *C. dilutus* were analyzed using the median-effect equation (Eq. (1)) (Chou and Talalay, 1984). Furthermore, the interaction between permethrin and cadmium was also evaluated using CA and IA models as described in the Supplementary Material.

After logarithmic transformation, Eq. (1) can be rearranged to Eq. (2).

$$\frac{f_a}{1-f_a} = \left(\frac{C}{\mathrm{LC50}}\right)^m \tag{1}$$

$$\log C = \log \mathrm{LC50} + \frac{1}{m}\log\left(\frac{f_a}{1-f_a}\right) \tag{2}$$

Where, f_a is the fraction of *C. dilutus* affected by a test contaminant at a concentration *C*, and *m* is related to the slope of the dose–response curve. The values of $m = , >$ and <1 indicate hyperbolic, sigmoidal, and negative sigmoidal dose–response curves, respectively (Fig. S2).

This method was derived from the mass-action law that took into account both the potency (LC50) and shape (*m*) parameters, and LC50 and *m* values for individual chemicals can be determined from the intercept and slope of the relationship between log*C* and log $f_a/(1\text{-}f_a)$, respectively (Eq. (2)). These two parameters were then used to calculate concentrations of individual contaminants required to provoke various effect levels according to Eq. (1). The goodness of fit of the relationship was expressed by a conformity parameter r^2 (Chou, 2006).

For a mixture which contained *n* chemicals, the combination index value of the *n* chemicals causing *x*% effect $(\mathrm{CI})_x$ was calculated using Eq. (3). The CI values were indicative of the interaction among the contaminants, with CI<, CI = and CI > 1 suggesting synergistic, additive and antagonistic effects, respectively (Chou and Martin, 2005).

$$(\mathrm{CI})_x = \sum_{j=1}^{n}\frac{C_j}{(C_x)_j} = \sum_{j=1}^{n}\frac{(C_x)_{1-n}\left\{(C)_j \Big/ \sum_1^n (C)\right\}}{(\mathrm{LC50})_j\left\{(f_{ax})_j \Big/ \left[1-(f_{ax})_j\right]\right\}^{1/m_j}} \tag{3}$$

Where, C_j is the concentration of chemical *j* in the *n*-component mixture, and it is the product of $(C_x)_{1\text{-}n}$ and $(C)_j/\sum_1^n(C)$, which present the sum of the concentrations of *n* chemicals causing *x*% effect as a mixture and the proportionality of the concentration of chemical *j* in the *n*-component mixture, respectively. In addition, $(C_x)_j$ is the concentration of chemical *j* that would produce *x*% effect when being applied singly and it can be calculated from the term of $(\mathrm{LC50})_j\{(f_{ax})_j/[1-(f_{ax})_j]\}^{1/m_j}$. The $(\mathrm{LC50})_j$ is the LC50 value of chemical *j*, $(f_{ax})_j$ is *x*% effect caused by chemical *j*, and m_j is related to the slope of the dose–response curve of chemical *j*.

Computer program CompuSyn (http://www.combosyn.com/) was used for calculating the parameters in dose–effect curves (LC50 and *m*) and the CI values. The LC50 values of individual contaminants were estimated using probit analysis with SPSS 19.0 (International Business Machines, New York, USA). The C_{free} of permethrin in sediment and activities of the enzymes in organism among different treatments were statistically compared using student *t*-test with SigmaPlot 12.3 (Systat Software Incorporation, San Jose, CA, USA).

3. Results

3.1. Joint toxicity

The bioassays were conducted using the sediments spiked with mixtures of permethrin and cadmium at concentrations to bracket their respective LC50 values (Table 1). The content of OC in the sediment was 2.26 ± 0.21%. Both nominal and measured concentrations of permethrin and cadmium in sediment are shown in Table 1. A set of quality control samples was also analyzed along with the samples. No target contaminants were detected in the blanks. Control sediment contained no permethrin but 3.3 μg/g dw of cadmium which were much lower than concentrations of cadmium spiked into the sediment. The recovery of the surrogate decachlorobiphenyl in all samples was 80 ± 12%. The recoveries of permethrin in matrix spiked samples and cadmium from a standard reference sediment were 85 ± 7% and 101 ± 1%, respectively.

Throughout the 10-d bioassays, pH (6.93 ± 0.16), conductivity (338 ± 10 μS/cm), temperature (21.8 ± 0.5 °C), and the concentrations of dissolved oxygen (6.15 ± 0.69 mg/L) and ammonia (<0.2 mg/L) in the overlying water all met the requirements of the USEPA guidelines (2000). The survivorship of the midges in negative and solvent controls was 93 ± 8.2% and 95 ± 8.4%, respectively.

The 10-d LC50 values of permethrin and cadmium for *C. dilutus* were 0.235 μg/g dw (0.158–0.310 μg/g dw) and 170 μg/g dw (55.1–477 μg/g dw), respectively. Mixture toxicity tests were performed at multiple dose ratios, including equitoxicity (G1), permethrin-dominated (G2) and cadmium-dominated (G3) groups. As shown in Table 1, the interaction of permethrin and cadmium showed antagonism with CI values larger than 1 for all treatments (1.08–4.99) and the CI values for group G3 were generally higher than those in G1 and G2.

3.2. Bioavailability of sediment-bound permethrin

In order to evaluate the influence of cadmium on the bioavailability of permethrin, C_{free} of permethrin in groups G0 and G1 were compared (Fig. 1). The sediments in the two groups were spiked with permethrin at the same concentrations for the corresponding levels, but with and without cadmium in G1 and G0, respectively (Table 1). There was no significant difference between measured sediment concentrations of permethrin at the same nominal levels in groups G0 and G1 ($p > 0.05$). Similarly, the C_{free} of permethrin were not significantly different between the two groups ($p > 0.05$), except that C_{free} of permethrin in G1C3 (with cadmium) was higher than that in G0C3 (permethrin-only) (Fig. 1).

3.3. Activities of metabolic enzymes

The activities of GST and CarE in the midges were determined in groups G0 and G1 (Fig. 2). The higher the permethrin concentrations in sediment, the greater the GST activities in the midges. The activities of GST in the midges exposed at the highest two levels of permethrin-only group (G0C4 and G0C5) were significantly different from the control ($p < 0.05$) (Fig. 2A). Compared to the control, GST activity in the equitoxicity group was significantly enhanced in all five levels ($p < 0.05$), and GST activity in the midges in G1C5 reached 13 times higher than the control (Fig. 2A). Comparably, GST activities in the midges in G1 were significantly higher than those in G0 at the same permethrin concentrations, except for those at the lowest level (C1), implicating the role of cadmium in enhancing GST activity.

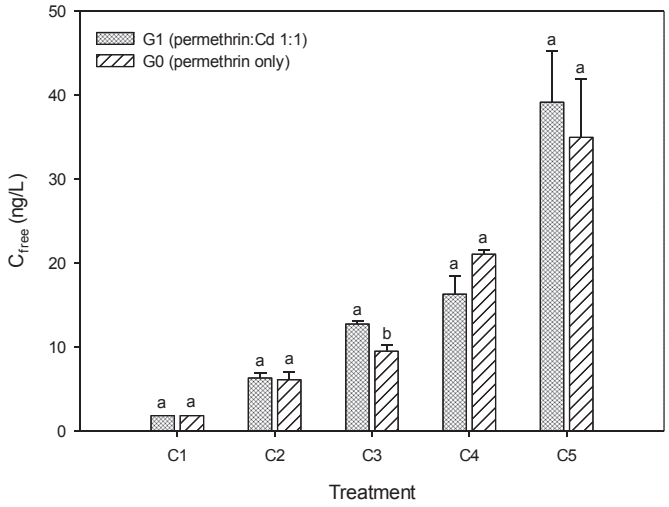

Fig. 1. The freely dissolved concentrations of permethrin in sediment porewater (C_{free}) measured by solid phase microextraction in groups G1 (equitoxicity mixture of permethrin and cadmium) and G0 (permethrin-only). Error bars represent standard deviations (n = 3). Different letters indicate significant difference between the treatments in G1 and G0 with permethrin at the same sediment concentrations (C1–C5) ($p < 0.05$).

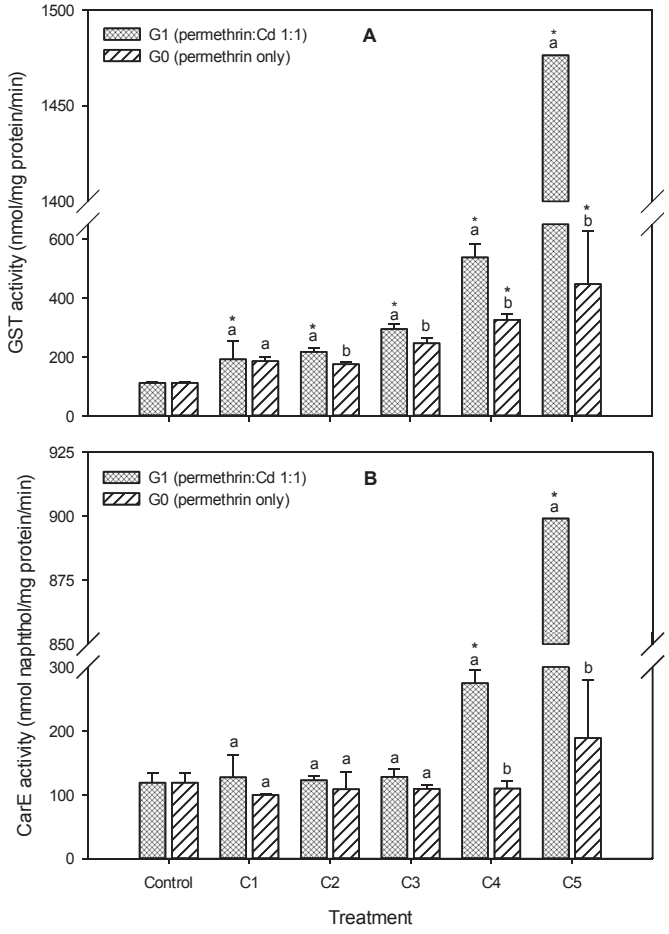

Fig. 2. The activities of glutathione S-transferase (GST) (A) and carboxylesterase (CarE) (B) in *Chironomus dilutus* after 10-d exposure to permethrin (G0) or a mixture of permethrin and cadmium (G1) in sediment. Error bars represent standard deviations (n = 3). Asterisks indicate significant difference of test treatments compared to the control, and different letters signify significant difference between the treatments in G1 and G0 with permethrin at the same sediment concentrations (C1–C5) ($p < 0.05$).

The activity of CarE in the midges in G0 was not significantly different from the control although CarE activity was slightly increased at the highest level G0C5 (Fig. 2B). In comparison, CarE activities in the midges in G1C4 and G1C5 were significantly higher than the control ($p < 0.05$) (Fig. 2B). In addition, CarE activities in the midges in G1C4 and G1C5 were also significantly higher than their counterparts in permethrin-only treatments (G0C4 and G0C5). The difference in CarE activities between G1 and G0 was also caused by the addition of cadmium, in accordance with GST activities.

4. Discussion

4.1. Antagonism between permethrin and cadmium

The challenge in model selection limited the use of CA and IA models for evaluating joint toxicity. It was hard to determine which model was more appropriate because there was no agreement how strict the requirements for similarity of action have to be to employ CA model (Grimme et al., 1996) and modes of action for many contaminants are still unclear. Alternatively, ME/CI-isobologram equation provides a way to estimate the interaction among contaminants without knowing their mode of actions. The equation also predicts the interaction at various effect levels (f_a) and thus provides a more accurate prediction of the joint toxicity compared to traditional CA and IA models (Chen et al., 2015). The CI values shown in Table 1 showed permethrin and cadmium interacted antagonistically to *C. dilutus* in all test combinations. The antagonism was also confirmed by the assessments using CA and IA models (Table S1).

Antagonism between organics and metals has been previously reported (Mehler et al., 2011b; van der Geest et al., 2000), but it is not always the case. Synergistic lethality to marine microcrustacean *Tigriopus brevicornis* was reported when binary mixtures of a metal (arsenic, copper or cadmium) and a pesticide (carbofuran or dichlorvos) were applied (Forget et al., 1999). Stereoisomerism also affected the interaction. Zhang et al. (2014) noted that the joint toxicity of binary mixtures of *p*-chlorobenzene and a metal (copper, cadmium or chromium) was antagonism, yet *o*-chlorobenzene showed synergism with the same metals. Overall, the interaction between organics and metals were complicated and different mixture components may generate different interactions.

Joint toxicity of a certain mixture may vary when different endpoints were evaluated. Amorim et al. (2012) assessed joint toxicity of soil-bound dimethoate and cadmium to *Folsomia candida* and found additive effect on the survival level but additive or synergistic effect when reproduction was evaluated at low concentrations. Concentration-dependence of joint toxicity was also observed for other chemicals at various doses. Copper and diazinon showed antagonism on the survival of *Ceriodaphnia dubia* at low effect levels, but additivity at high levels (Banks et al., 2003). The ratio of chemical concentrations also affected the interaction between penta-brominated diphenylether (BDE) and a metal (cadmium or copper) on the lethality to *Daphnia magna* (Tang et al., 2011). Antagonism was found in mixtures of cadmium and penta-BDE (1:3 and 1:1) and copper and penta-BDE (1:3), but interaction in the mixtures of cadmium and penta-BDE (3:1) and copper and penta-BDE (1:1 and 3:1) was additive. Thus, it is imperative to conduct joint toxicity testing at different dose levels and ratios. Most mixture toxicity tests, however, were only designed to make the components equitoxicity.

The joint toxicity was assessed at three ratios of toxicity contribution from permethrin and cadmium (1:1, 3:1 and 1:3 in G1, G2, and G3, respectively). Different from the results reported by

Tang et al. (2011), permethrin and cadmium showed antagonistic lethality to *C. dilutus* at all tested ratios (Table 1). As mentioned above, ME/CI-isobologram equation had the advantage in predicting the interaction at various effect levels and CI values reflected degrees of interactions. Chou and Martin (2005) recommended that CI values of 1.1–1.2, 1.2–1.45, 1.45–3.3, 3.3–10 and > 10 indicated slight antagonism, moderate antagonism, antagonism, strong antagonism, and very strong antagonism, respectively. The mixtures in the equitoxicity group G1 showed antagonism at low dose levels (G1C1 to G1C3), but the degree of antagonism decreased and approached additivity when chemical concentrations increased. In permethrin-dominated group G2, the mixtures showed antagonism at all levels except for G2C3 which showed strong antagonism with a CI of 3.5. Comparatively, cadmium-dominated group G3 showed strong antagonism at all levels. The results suggested that the composition of contaminants in the mixtures affected the degree of interaction, requiring better understanding of the causes of the antagonism.

4.2. Reasons for the antagonistic interaction

Pyrethroid insecticides are highly toxic to benthic invertebrates and have been found as major contributors to acute mortality of *C. dilutus* in aquatic ecosystems (Mehler et al., 2011a). In comparison, lethal concentrations of cadmium to *C. dilutus* (LC50 of 170 μg/g dw) were rarely measured in the field although it was ubiquitous (Li et al., 2007). Permethrin was more risky to the midges than cadmium at environmentally relevant concentrations, and thus the influence of cadmium on the toxicity of permethrin to *C. dilutus* was investigated to elaborate their antagonistic interaction. When studying the interaction between organophosphate and neonicotinamide insecticides, Svendsen et al. (2010) found that alteration in toxicokinetic processes was more important reason for the interactions relative to toxicodynamics. While toxicodynamic alteration may contribute to the antagonism, the current study merely focused on the discrepancy in the bioavailability and biotransformation of permethrin in the midges caused by the addition of cadmium.

Bioavailability. The bioavailability of a chemical affects its bioaccumulation and toxicity in biota and C_{free} of a contaminant quantified by SPME has been proposed as a measure of bioavailability (Cui et al., 2013; You et al., 2011). Components in a mixture may form complexes or compete for binding sites in sediment particles, causing alteration in bioavailability of individual components. In an in vitro bioassay, Chen et al. (2013) suggested that the formation of cadmium–chlorpyrifos complex facilitated the transport of toxicants into Hep G2 cells and provoked synergistic hepatoxicity.

In the current study, the bioavailability of sediment-bound permethrin in G0 and G1 was measured as C_{free} and used to evaluate the impact of altered bioavailability on joint toxicity. As shown in Fig. 1, the addition of cadmium hardly changed the bioavailability of sediment-bound permethrin (C_{free}), which was in accordance with a previous study (Mehler et al., 2011b). Mehler et al. (2011b) found that the presence of lead in sediment did not change the bioavailability of cypermethrin which was measured by 24-h Tenax extraction. The observed antagonism between permethrin and cadmium was not caused by the alteration in bioavailable fraction of permethrin in sediment, and other toxicokinetic processes, such as biotransformation, should be considered.

Metabolic enzyme activities. Invertebrate pests tended to develop resistance to pyrethroids via enhancing capability to transform pyrethroids (Chouaibou et al., 2013). Similarly, enhanced biotransformation of permethrin in the midges may contribute to the observed antagonistic interaction between permethrin and cadmium. The changes in the activities of metabolic enzymes reflected the altered biotransformation capacity (Domingues et al., 2010). In the current study, the activities of GST and CarE in *C. dilutus* were compared in groups G0 and G1 (Fig. 2).

As an important detoxification enzyme in phase II biotransformation, GST plays vital roles in the excretion of xenobiotics from the organisms. GST enzymes catalyze the conjugation of the tripeptide glutathione with compounds containing electrophilic centers and the conjugation products are normally more water soluble than parent compounds, facilitating the excretion of xenobiotics (Hyne and Maher, 2003). Vontas et al. (2001) found elevated GST activity in pyrethroid-resistant insects and concluded that it was an important way for insects to attenuate pyrethroid-induced toxicity. Enhanced GST activity after short-term exposure to pyrethroids was also noted. Velki and Hackenberger (2013) reported that GST activity in *Eisenia andrei* was considerably enhanced in the first 3-d exposure to deltamethrin, and interestingly, the organisms gradually adjusted to the stimuli and GST activity recovered to normal level at 28 d.

Compared to the control, significant increase in GST activity was only observed in the midges exposed to permethrin solely at high concentrations (G0C4 and G0C5), while the mixtures of cadmium and permethrin (G1) significantly increased GST activities in the midges at all levels (Fig. 2A). Comparatively, the addition of cadmium caused GST activity in the midges reached 1.2–3.3 times of their permethrin-only counterparts, implying a significant enhancement of detoxifying capability of the midges when they were exposed to permethrin and cadmium jointly.

The CarE plays a key role in phase I biotransformation of pyrethroids by catalyzing the hydrolysis of the compounds to acids and alcohols through breaking ester bonds. Because the metabolites of pyrethroids were generally less toxic than the parent compounds, CarE was considered as a group of important detoxification enzymes for pyrethroids (Wheelock et al., 2006). As shown in Fig. 2B, CarE activity changed little compared to the control when the midges were exposed to permethrin solely (G0). Conversely, CarE activity considerably increased when the midges were exposed to the mixtures at the highest two concentrations (G1C4 and G1C5), attaining 2.3 and 7.6 times of the control, respectively. The activity of CarE in G1C4 and G1C5 was also significantly higher than their counterparts in G0 and the degree of induction CarE activity increased when cadmium concentrations increased. This partially explained the stronger antagonism observed in cadmium-dominated group than equitoxicity and permethrin-dominated groups. Collectively, enhanced GST and CarE activities by cadmium were supposed to speed up the biotransformation of permethrin in *C. dilutus* and subsequently reduced the toxicity of permethrin. Therefore, enhanced activity of the enzymes was largely attributable to the antagonistic effects of permethrin and cadmium to the midges.

5. Conclusions

When *C. dilutus* larvae were exposed to sediment-bound permethrin and cadmium, antagonistic interaction was noted for midge mortality at different dose ratios. Comparatively, the midges in cadmium-dominated group showed stronger antagonism than equitoxicity and permethrin-dominated groups. The bioavailability of sediment-bound permethrin was not changed by cadmium, but biotransformation capacity of permethrin in the midges was significantly enhanced as indicated by increased activities of GST and CarE, which largely explained the antagonism. Notably, toxicodynamic processes may also contribute the antagonism, however, this is beyond the scope of the current study.

To evaluate joint toxicity between contaminants which

frequently co-occur in the environment, more accurate sediment risk assessment is needed. Li et al. (2013) reported that pyrethroids in sediment samples from Guangzhou, China were the main toxicity contributors to *Hyalella azteca*, yet the traditional toxic unit evaluation considerably overestimated the toxicity. In the study area, metals were also frequently detected (Mehler et al., 2011a), and antagonism of cadmium and permethrin observed in the current study partially explained the overestimation of sediment toxicity to *H. azteca*.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.envpol.2015.09.012.

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