Environ Sci Pollut Res (2017) 24:21361–21374
DOI 10.1007/s11356-017-9759-x

RESEARCH ARTICLE

Acute toxicity of chlorpyrifos and carbosulfan to glochidia of the freshwater mussel *Hyriopsis bialata* Simpson, 1900

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Received: 7 October 2016 / Accepted: 11 July 2017 / Published online: 25 July 2017

Abstract The acute toxicity of carbosulfan and chlorpyrifos in formulated pesticides to glochidia (larvae) of the freshwater mussel (*Hyriopsis bialata* Simpson, 1900) was evaluated under static conditions in moderately hard dechlorinated tap water. Measured pesticide concentrations were 26 to 34% lower than nominal concentrations; therefore, all results are expressed in terms of measured active ingredient. Carbosulfan was relatively non-toxic to the mussel larvae with median effective concentrations (EC_{50}) of carbosulfan at 24 and 48 h greater than 0.10 mg/L. The EC_{50}s of chlorpyrifos at 24 and 48 h were 0.083 and 0.078 mg/L, respectively (measured concentrations). The 48-h EC_{50} of a combined exposure to a mixture of chlorpyrifos and carbosulfan at a constant ratio of 2.9:1 was 0.0142:0.049 mg CP:CB/L. In a separate experiment, the effect of water hardness on carbosulfan, chlorpyrifos, or a combined exposure was assessed using glochidia exposed to either soft, moderately hard, or hard reconstituted water. There was no effect of water hardness on the survival of glochidia after 24- or 48-h exposure to carbosulfan. The chlorpyrifos 48-h EC_{50}s in soft water, moderately hard water, and hard water were 0.18, 0.11, and 0.16 mg/L, respectively. The data indicate that the lowest water hardness resulted in the highest survival of glochidia, whereas an increase to moderate water hardness resulted in significantly decreased survival of glochidia ($F = 15.5$, $P < 0.05$). The EC_{50}s of a combined exposure at 48 h in soft water, moderately hard water, and hard water were 0.124:0.044, 0.132:0.047, and 0.064:0.022 mg CP:CB/L, respectively. The data indicate that the combined toxicity was lowest at low and moderate water hardness, whereas an increase to high water hardness resulted in a significantly decreased survival of glochidia. After 48 h, the toxicity of the combined chlorpyrifos and carbosulfan exposure in soft and hard water was greater than that of chlorpyrifos alone.

Keywords Freshwater mussels · Glochidia · Carbosulfan · Chlorpyrifos · Hardness

Responsible editor: Cinta Porte

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Introduction

Freshwater mussels *Hyriopsis bialata* Simpson, 1900 belong to the family Unionidae and are widely distributed across large areas of Thailand such as the Mekong River, the Mun River, and the Chi River (Brandt 1974; Kovitvadhi and Kovitvadhi 2002). *H. bialata* is an economically important freshwater pearl mussel in Thailand. The nacreous shell is used for making pearl-inlaid furniture, ornaments, kitchen utensils, and souvenirs. The meat is also a source of protein for humans and animals (Kovitvadhi et al. 1999; Meechonkit et al. 2010; Yeemin 1997). A remarkable difference of *H. bialata* from other species of Thai unionideans (freshwater mussels) is that it has fast growth and the ability to reproduce through the year (Chatchavalvanich et al. 2006). At present, populations of *H. bialata* have declined due to excessive harvesting, increased

commercial use, and water pollution (Chatchavalvanich et al. 2006; Kovitvadhi et al. 2006; Supannapong et al. 2008). The life cycle of freshwater pearl mussels is quite complex. Fertilized eggs develop into glochidia larvae in the gills of the female mussels. When the mature glochidia are released into the water column, they must attach to a suitable host fish for transformation into juveniles (Pennak 1989; Watters 2007). In this situation, the released glochidia may be exposed to aquatic contaminants (Jacobson et al. 1997).

The early life stages of freshwater mussels (glochidia and juveniles) have been reported to be highly sensitive to contaminants such as metals (Wang et al. 2007a, b, 2009), ammonia (USEPA 2013), and some organics, including pulp and paper mill effluents, aromatic hydrocarbons (McKinney and Wade 1996; Weinstein 2001), and some pesticides (Conners and Black 2004) compared to other aquatic invertebrates (including amphipods, chironomids, and cladocerans) and vertebrates (fish and amphibians). This suggests that the early life stages of freshwater mussels are a good indicator organism for aquatic environmental health.

Chlorpyrifos (*O*-diethyl *O*-(3,5,6-trichloro-2-pyridyl) phosphorothioate) is a non-systemic organophosphorus insecticide. The World Health Organization (WHO) classifies chlorpyrifos as a category 2 (moderately hazardous) pesticide. Chlorpyrifos is the active ingredient of many commercial insecticides such as Lorsban 20EC®, Lorsban 40EC®, Pyrenex®, and Chloridin® in dry and wet formulations (WHO 2002). Chlorpyrifos is used in agriculture to control pests, and indoors to control mosquitoes and fire ants. The rate of hydrolysis of chlorpyrifos increases with temperature and alkalinity (John et al. 1999). Chlorpyrifos residuals were 6.73 μg/L in water 24 h after application of 1 g CP/L to a bean plantation (Hongtrakoon et al. 2007) indicating that freshwater mussels and their larvae could experience significant pesticide exposures through runoff from agricultural lands to adjacent waterways. Chlorpyrifos is highly toxic to fish and aquatic invertebrates (Kamrin 1997).

Carbosulfan (2,3-dihydro-2,2-dimethylbenzofuran-7-yl (dibutylaminothio) methylcarbamate) is a benzofuranyl methyl carbamate pesticide used to control insects, mites, and nematodes by soil, foliar, and seed treatment applications, mainly on potatoes, sugar beet, rice, maize, and citrus. Carbosulfan is the active ingredient of many commercial insecticides such as Posse®, Marshal®, and Advantage® in dry granule and wet formulations (FAO/WHO 2000). Carbosulfan is classified by WHO as a category 2 (moderately hazardous) pesticide. Carbosulfan is banned in Europe, but it is still used widely in other countries such as Mexico, Brazil (Capkin and Altinok 2013), and Thailand. The solubility of carbosulfan is 0.3 mg/L at 25 °C (Alvarez 1995) in water, but it is readily soluble in many organic solvents (EFSA 2009). Carbosulfan has a low persistence in the environment, but it can persist longer in a high-pH environment (Ramanand et al. 1991). Also carbosulfan degrades readily to carbofuran—a pesticide in its own right (Tejada and Magallona 1985). Environmental concentrations of carbosulfan range between 0.64 and 29 μg/L in surface and ground water (Leppert et al. 1983; Sao et al. 2008). The maximum concentrations measured in rice paddy water were higher than the accepted value for pesticide residues in drinkable water (0.10 μg/L) (EFSA 2009). In a different study, carbosulfan was not detected in samples of paddy water and soil solution after its application, but the metabolite carbofuran persisted longer in a simulated lake ecosystem, and accumulated in fish and snails (Tejada and Magallona 1985). Carbosulfan is classified as highly acutely toxic to fish and aquatic invertebrates (Dobsikova 2003). It was reported for *Daphnia magna* that the 48-h median effective concentration (EC_{50}) of carbosulfan was 1.5 μg/L, while the 48-h EC_{50} for carbofuran was 18.7 μg/L (PPDB 2014).

Water hardness is composed primarily of the cations calcium and magnesium, but includes other divalent metals such as iron, strontium, and manganese. Hardness concentrations are expressed as equivalent concentrations of $CaCO_3$ in milligram/liter. The hardness of water significantly affects metal toxicity such as copper, chromium, and zinc. The toxicity of some metals is reduced by increased water hardness. In contrast, hardness generally has little or no effect on the toxicity of organic chemicals including pesticides such as endosulfan (Capkin et al. 2006; Pickering and Henderson 1966). However, in an early study of pesticide toxicity to fish, water hardness did affect the survival of fish exposed to carbamate pesticides but did not affect toxicity of organophosphate pesticides (not including chlorpyrifos) (Pickering and Henderson 1966).

The objective of this study was to determine the acute toxicity of chlorpyrifos and carbosulfan formulations to glochidia of the freshwater mussel *H. bialata*, and in addition, to assess the effect of water hardness on the toxicity of chlorpyrifos and carbosulfan to glochidia. We also evaluated the toxicity of a mixture of the two pesticides because aquatic organisms may be exposed to multiple products in runoff as a result of agricultural practices where a variety of crops are grown within a catchment.

Materials and methods

Preparation of test organisms

Test organisms were collected and cultured according to Kovitvadhi et al. 2006. Adult *H. bialata* Simpson, 1900 were collected from the Dorm River Basin in Ubon Ratchathani province, Thailand, 2 months before the present study, from a location not thought to be significantly affected by contaminants. Twenty females and ten males (61.95 ± 7.81 g weight, 10.19 ± 0.36 cm length, 4.11 ± 0.35 cm height, and 2.41 ± 0.15 cm width) were cultured together in circle nets

(50 cm in diameter and 50 cm in height) in a water depth of 150 cm in an earthen pond (at pH 7.0–7.2 and temperature 28–30 °C) at the Faculty of Fisheries, Kasetsart University, Bangkok. The mussels filter phytoplankton (their major food source) from the water column in the earthen pond, which is managed to provide sufficient food for mussel growth, normal reproductive activity, and long-term mussel survival. The valves of female mussels were gently opened 5–8 mm with reverse pliers for visual examination of marsupial color, which determined the maturity of the glochidia. Only gravid mussels with completely brown and enlarged marsupia were selected for extraction of glochidia.

To prepare the mussels for collection of glochidia, soil and algae were thoroughly cleaned from the outside of the shells of gravid mussels. The shells were then repeatedly rinsed with dechlorinated tap water. The valves of the female mussels were opened 5–8 mm as for brood pouch inspection, and the mantle, foot, and marsupia were gently rinsed with sterile water. A subsample of mature glochidia were removed from the brood pouch using a sterilized 1-mL syringe with an 18-gauge needle and transferred to a petri dish for examination of glochidia viability by the addition of a saturated sodium chloride (NaCl) solution (24% NaCl) (ASTM 2006). The numbers of opened and closed glochidia were counted before, and then within 1 min of adding 2–3 drops of NaCl solution. Glochidia that closed in response to the NaCl solution were defined as alive (or viable) and the glochidia that were closed before addition of NaCl or that remained open after addition of NaCl were defined as dead (non-viable), (half-closed glochidia were included in the closed glochidia counts). Glochidia from at least three female mussels with an average survival of at least 90% were combined for toxicity testing (ASTM 2006).

Test chemical

A commercial carbosulfan formulation, Posse® (20% *w/v* EC active ingredient), and a commercial chlorpyrifos formulation, Outcide 40® (40% *w/v* EC active ingredient), were purchased from a local agricultural shop. Stock solutions of pesticide formulations were prepared with dechlorinated tap water or reconstituted water at room temperature at concentrations (of active ingredient) of 0.1 mg/L for carbosulfan and 0.36 mg/L for chlorpyrifos, and then further diluted with dechlorinated tap water or reconstituted water to four or five test concentrations.

Acute toxicity test on glochidia

Acute toxicity tests with glochidia were conducted according to standardized guidelines (ASTM (2006) and Clearwater et al. (2014)). Briefly, there were four or five treatments of each test chemical plus a control and three replicates of each treatment. Equal amounts of glochidia (ranging from 300 to 500 glochidia/replicate) were added to 100-mL glass cups with 80 mL test solution. All tests were conducted in an incubator at 25 ± 2 °C under a 16-h light:8-h dark regime. After 6, 24, and 48 h, the viability of exposed glochidia in each treatment was examined under light microscope (×40 magnification) by transferring a 1-mL subsample (containing 100–150 larvae) to a clean Sedgewick-Rafter counting chamber and counting glochidia before and after addition of a saturated NaCl solution (about 2–3 drops). Exposure solutions were not renewed, and glochidia were not fed during the test. Water samples of stock solutions (3 mL each) were taken before starting the tests to determine the concentrations of pesticide.

In the first series of exposures, moderately hard dechlorinated tap water was used as the diluent and control with three replicates per concentration. Nominal concentrations were 0.03, 0.05, 0.10, 0.17, and 0.30 mg/L for carbosulfan; 0.15, 0.21, 0.29, 0.41, 0.57, and 0.80 mg/L for chlorpyrifos; and 0.08:0.02, 0.11:0.03, 0.17:0.05, 0.26:0.07, 0.38:0.10, and 0.58:0.16 mg/L chlorpyrifos:carbosulfan (3.7:1) for the combined exposures.

Effect of hardness on pesticide toxicity

For the evaluation of hardness, reconstituted soft, moderately hard, and hard water were used as the diluents and control treatments with three replicates per concentration. Total hardness was analytically verified by the ethylenediaminetetraacetic acid (EDTA) titration method, where (briefly) 100 mL of sample is titrated with 0.01 M EDTA after addition of 1.0 mL buffer solution and Eriochrome Black T indicator powder (APHA 1989). Nominal concentrations were 0.04, 0.08, 0.15, and 0.30 mg/L for carbosulfan; 0.20, 0.33, 0.53, 0.86, and 1.40 mg/L for chlorpyrifos; and 0.18:0.05, 0.27:0.07, 0.41:0.11, and 0.62:0.17 mg/L chlorpyrifos:carbosulfan (3.7:1) for the combined exposure. The viability of glochidia was assessed at 6, 24, and 48 h after exposure to the test solutions.

Deionized water was used to prepare the reconstituted water which was stored in 6-L plastic containers. Hard reconstituted water based on USEPA 2002 was prepared from reagent grade chemicals as follows: 1.152 g $NaHCO_3$, 0.720 g $CaSO_4{\cdot}2H_2O$, 0.720 g $MgSO_4$, and 0.048 g KCl, then diluted to moderately hard and soft water (ASTM 2003).

Chemical analysis

Technical-grade carbosulfan (98% purity) and technical-grade chlorpyrifos (98.5% purity) used for analytical standards were purchased from Sigma-Aldrich (St. Louis, MO, USA). HPLC-grade acetonitrile and purified water were used to prepare standard solutions and the mobile phase for analysis. A stock solution of carbosulfan and chlorpyrifos was prepared in acetonitrile at a concentration of 1 g/L and diluted to the concentration range of 2.0–8.0 mg/L with acetonitrile. The stock solution of technical-grade pesticide was stored at −20 °C.

The HPLC separation analysis was performed using a mobile phase consisting of acetonitrile:water (80:20 *V*/*V*) with a flow rate of 1.0 mL/min at room temperature. All solutions were filtered through 0.45-μm membranes (Millipore, Bedford, MA, USA) and degassed under vacuum. The sample injection volume was 10 μL and detection was monitored at 205 nm for carbosulfan and 230 nm for chlorpyrifos. The analytical standards were measured in three replicates. The limit of detection was 1.842 mg/L, correlation coefficient (R^2) of 0.996 for carbosulfan and 1.073 mg/L, R^2 of 0.998 for chlorpyrifos which was measured in the stocks of technical-grade pesticide used to make up the exposure solutions. All glassware was rinsed with acetone, cleaned with detergent, and washed with distilled water three times. Extraction efficiency samples were not included in the analysis of the stocks for the exposure solutions.

Water analysis

Water chemistry and physical parameters were measured at the beginning and the end of the tests. Dissolved oxygen, pH, conductivity, and temperature were analyzed with a YSI Model 556 MPS (Yellow Spring Instrument, Yellow Spring, OH, USA). Alkalinity (phenolphthalein methyl orange indicator), total hardness (EDTA titration method), ammonia-nitrogen (indophenol method), calcium (EDTA titration method), and silica (molybdosilicate method) were determined prior to the experiment (APHA et al. 1998).

Statistical analysis

Survival (viability) of glochidia was calculated as follows (Eq. 1) (Clearwater et al. 2014):

$$\%OG_i\text{-}\%OG_f = \%S \quad (1)$$

where;

%OG	the percentage of open glochidia
i	initial, before adding NaCl solution
f	final, after adding NaCl solution
%S	the percentage survival of glochidia

In the present study, subsamples of 100–120 glochidia were counted and classified as open or closed, in contrast to the ASTM (2006) method where all of the subsampled glochidia were counted.

The EC_{50} estimates and 95% confidence intervals, the no observed effect concentration (NOEC), and the lowest observed effect concentration (LOEC) values were analyzed based on measured concentrations (nominal concentrations were converted to measured using 25.7% for chlorpyrifos and 32.7% for carbosulfan—further detail is provided below) using the Comprehensive Environmental Toxicity Information System (CETIS v1.7.0.2, Tidepool Scientific Software) software package. Data analysis of each test followed a decision tree included in the software (adapted from USEPA (1994)). Significant differences between means (e.g., controls and test concentrations) were analyzed and ranked by one-way analysis of variance (ANOVA) for NOEC and LOEC values using the average percentage survival of glochidia (data transformed if necessary) followed by appropriate post-hoc tests (e.g., Shapiro-Wilk, Bartlett's, Dunnett's multiple comparison). Point estimates were made using linear interpolation, non-linear regression, or non-parametric analyses as appropriate for the data. Two-way ANOVA was used to compare the average percentage survival of glochidia at different test durations and water hardness followed by Tukey's post-hoc test. The level of statistical significance was set at $P \leq 0.05$. Data are reported as mean ± SD unless stated otherwise.

Results and discussion

Measured concentrations of pesticides

Measured chlorpyrifos and carbosulfan concentrations in the stocks used to make up the exposure solutions were on average 25.7 ± 0.2 and 32.7 ± 11.0% of the nominal concentrations. This result indicates that either the pesticides were degraded rapidly in solution, or that the concentration of the active ingredient in the commercial formulations was lower than expected, or that additional ingredients in the formulated pesticide interfered with the measurement of the active ingredients against technical-grade standards. Extraction efficiencies did not determined the stock solutions. High degradation rates of chlorpyrifos and carbosulfan may have been caused by the relatively high water temperatures typical of Thailand. (Ramanand et al. 1991); Tejada and Magallona (1985) reported that the biodegradation of carbosulfan in water occurs by hydrolysis, with a half-life of 3 days. In this study, the test solutions were prepared immediately before testing, nonetheless some hydrolysis will have occurred during the 48-h test. To fully evaluate the degradation rate, pesticide concentrations would have to be measured at the conclusion of the exposures.

In order to account for low measured concentrations of the pesticides, all of the nominal exposure concentrations and the toxicity endpoints have been converted to measured concentrations of active ingredient. In the first series of exposures when dechlorinated tap water was used as the diluent, measured concentrations (converted from nominals) were 0.01, 0.02, 0.03, 0.06, and 0.10 mg/L for carbosulfan; 0.04, 0.05, 0.07, 0.11, 0.15, and 0.21 mg/L for chlorpyrifos; and 0.019:0.007, 0.029:0.010, 0.044:0.015, 0.066:0.023, 0.099:0.034, and 0.148:0.051 mg/L chlorpyrifos:carbosulfan (2.9:1) for the combined exposures. For the evaluation of hardness, when reconstituted water was used as the diluent,

measured concentrations were 0.01, 0.03, 0.05, and 0.10 mg/L for carbosulfan; 0.05, 0.08, 0.14, 0.22, and 0.36 mg/L for chlorpyrifos; and 0.046:0.016, 0.069:0.023, 0.105:0.036, and 0.159:0.056 mg/L chlorpyrifos:carbosulfan (2.9:1) for the combined exposure. All toxicity endpoints are reported as measured concentrations unless stated otherwise.

Water quality was measured in all of the experiments, and in summary, water temperatures ranged from 24 to 27 °C, dissolved oxygen ranged from 5 to 7 mg/L, pH ranged from 7.4 to 7.9, and conductivity ranged from 367 to 472 μS/cm. In the dechlorinated tap water experiment, hardness was 120–135 mg/L as $CaCO_3$, and alkalinity was 74–108 mg/L as $CaCO_3$. In the reconstituted water experiment, hardness ranged from 42 to 60 mg/L as $CaCO_3$ in soft water, 88–128 mg/L as $CaCO_3$ in moderately hard water, and 168–258 mg/L as $CaCO_3$ in hard water. Except where noted, the percentage survival of glochidia in the control treatments was more than the 90% minimum recommended by ASTM (2006) after 48-h exposure. These water quality data, and control survival rates, indicate that background water quality measured in all of the experiments was unlikely to have a negative effect on the glochidia.

Acute toxicity of chlorpyrifos

In the first chlorpyrifos test in dechlorinated tap water (hardness 120–135 mg/L as $CaCO_3$, alkalinity 74–108 mg/L as $CaCO_3$), the percentage survival of glochidia in the control treatment was more than 90% with the highest percentage survival of 96 ± 1.2% at 6 h. The NOEC of chlorpyrifos was 0.05 mg/L at all exposure times ($P > 0.05$). The LOEC of chlorpyrifos was 0.07 mg/L at 6, 24, and 48 h and the percentage survival of glochidia in this concentration was 9, 31, and 45% lower, respectively, than that in the control treatment ($P < 0.001$). Chlorpyrifos concentrations of 0.15 mg/L decreased glochidia survival to less than 10% after 6-, 24-, and 48-h exposure and were not significantly different to glochidia survival in the highest concentration of chlorpyrifos in this experiment (0.21 mg/L) ($P > 0.05$) (Fig. 1).

The EC_{50} values for chlorpyrifos exposure of *H. bialata* glochidia in dechlorinated tap water were 0.083 (0.079–0.087) mg/L and 0.078 (0.062–0.092) mg/L for 24 and 48 h, respectively (Table 1). This result indicates that *H. bialata* glochidia are more sensitive to chlorpyrifos in the commercial Lorsban 40EC formulation than *Lampsilis siliquoidea* glochidia exposed to either pure chlorpyrifos (48-h EC_{50} 0.4 mg/L), or in the formulation Lorsban® 4-E Insecticide (48-h EC_{50} 0.6 mg/L) (hardness 160–184 mg/L as $CaCO_3$, temperature 20.7–21.7 °C) (Bringolf et al. 2007a). Comparison with Bringolf et al. (2007a) suggests that high water hardness may have decreased the toxicity of chlorpyrifos when compared to the initial test in this study (hardness 120–135 mg/L as $CaCO_3$, temperature 24–27 °C). In contrast, both mussel species are markedly less sensitive to chlorpyrifos than other aquatic invertebrates such as *D. magna* (48-h LC_{50} 1.7 μg/L) and the Korean shrimp (*Palaemon macrodactylus*) (48-h LC_{50} 0.05 μg/L) (Tomlin 2006). In Thailand, chlorpyrifos residuals of 6.73 μg/L have been reported in water 24 h after application of 1 g CP/L (50-mL formulation/20 L, Lorsban 40EC *W/V* EC) (Hongtrakoon et al. 2007). The residual concentrations of chlorpyrifos found in water were lower than the EC_{50} values for 24 and 48 h in this study. Thus, application of chlorpyrifos at that rate may not cause acute toxicity to glochidia. On the other hand, chlorpyrifos may degrade more slowly when applied in a formulated product and we also note that chlorpyrifos has a log K_{ow} of 4.7–5.3 which indicates that it may bioaccumulate in aquatic organisms. Chlorpyrifos at environmentally relevant concentrations (0.05 μg/L) caused oxidative stress and lysosomal abnormalities on the Mediterranean mussel *Mytilus galloprovinciallis* (Patetsini et al. 2013). However, there is a lack of studies on the effects of these pesticides and other pollutants on tropical species, and more data is needed for comprehensive risk assessment of these substances in tropical regions.

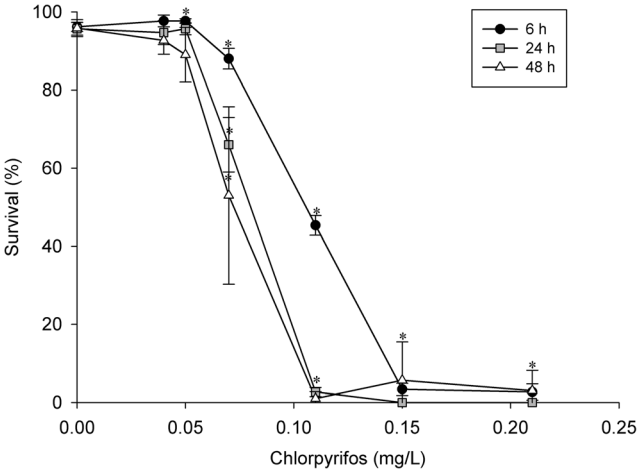

Fig. 1 Percentage survival of *H. bialata* glochidia after exposure to chlorpyrifos (measured concentrations) for 6, 24, and 48 h. Data are presented as the mean ± standard deviation ($n = 3$). The *asterisk* denotes significant differences from the control ($P \leq 0.05$)

Glochidia in the control treatment of the chlorpyrifos exposure showed normal behavior such as snapping 2–3 times within 1 min and, after 48 h, discharge of the larval thread outside the larval valves. The normal glochidia prior to addition of NaCl solution are shown in Fig. 6a. After adding NaCl solution, glochidia responded by closing their valves within a few seconds (Fig. 6b). Afterwards, the larval adductor muscle was prominent within the closed valves. After 24-h exposure to chlorpyrifos (0.21 mg/L), some glochidia showed damage to the larval adductor muscle and mantle tissue (Fig. 6c). These larvae did not respond after addition of 24% NaCl solution so they were determined to be non-viable (Fig. 6d).

Table 1 The toxicity end points for *Hyriopsis bialata* glochidia exposed to carbosulfan (CB), chlorpyrifos (CP), or a combination at a ratio of 2.9:1 (CP:CB), expressed as measured concentrations. *CI* confidence interval, *NA* not available.

Chemical	Hardness	Time (h)	NOEC (mg/L)	LOEC (mg/L)	EC_{50} (mg CP/L) (95% CI)	EC_{50} (mg CB/L) (95% CI)
CB	Dechlorinated tap water (moderate)	6	0.10	>0.10	–	>0.10
		24	0.10	>0.10	–	>0.10
		48	0.10[(1)]	>0.10[(1)]	–	>0.10[(1)]
CP	Dechlorinated tap water (moderate)	6	0.05	0.07	0.10 (0.10–0.11)[(2)]	–
		24	0.05	0.07	0.08 (0.08–0.09)[b]	–
		48	0.05	0.07	0.08 (0.06–0.09)[b]	–
CP:CB	Dechlorinated tap water (moderate)	6	0.15:0.02[(3)]	>0.15:0.03[(3)]	>0.15	>0.05
		24	0.07:0.02[(3)]	0.10:0.03[(3)]	0.19 (0.16–0.22)[(3)]	>0.05
		48	0.04:<0.01[(3)]	0.07:0.01[(3)]	0.14 (0.13–0.15)[(3)]	0.05 (0.04–NA)
CB	Soft	6	0.10	>0.10	–	>0.10
		24	0.10	>0.10	–	>0.10
		48	0.10	>0.10	–	>0.10
	Moderately hard	6	0.10	>0.10	–	>0.10
		24	0.10	>0.10	–	>0.10
		48	0.10	>0.10	–	>0.10
	Hard	6	0.10	>0.10	–	>0.10
		24	0.10	>0.10	–	>0.10
		48	0.10	>0.10	–	>0.10
CP	Soft	6	0.14	0.22	>0.36[a]	–
		24	0.08	0.14	0.25 (0.23–0.26)[b]	–
		48	0.14	0.22	0.18 (0.17–0.19)[b]	–
	Moderately hard	6	0.05	0.08	0.21 (0.19–0.24)[a]	–
		24	0.05	0.08	0.17 (0.15–0.18)[b]	–
		48	0.08	0.14	0.11 (0.10–0.12)[c]	–
	Hard	6	0.05	0.08	0.24 (0.20–0.28)[a]	–
		24	0.05	0.08	0.18 (0.18–0.20)[b]	–
		48	0.08	0.14	0.16 (0.11–0.19)[c]	–
CP:CB	Soft	6	0.07:0.02	0.11:0.04	>0.16	>0.06
		24	0.11:0.04	0.16:0.06	0.16 (0.15–0.16)	0.05 (0.05–NA)
		48	0.05:0.02	0.07:0.02	0.12 (0.12–0.13)	0.04 (0.04–0.05)
	Moderately hard	6	0.16:0.06[(3)]	>0.16:>0.06[(3)]	>0.16	>0.06
		24	0.11:0.04[(3)]	0.16:0.06[(3)]	0.18 (0.10–0.32)	>0.06
		48	0.05:0.02[(3)]	0.07:0.02[(3)]	0.13 (0.12–0.14)	0.05 (0.04–0.05)
	Hard	6	0.05:0.02[(3)]	0.07:0.02[(3)]	>0.16	>0.06
		24	<0.05:<0.02[(3)]	0.05:0.02[(3)]	0.08 (0.08–0.09)	0.03 (0.03–0.03)
		48	<0.05:<0.02[(3)]	0.05:0.02[(3)]	0.06 (0.06–0.07)	0.02 (0.02–0.03)

[(1)] Control survival <90%

[(2)] Treatments not sharing the same superscript letters were significantly different (Tukey's test, $\alpha = 0.05$)

[(3)] Expressed as CP:CB

After 48-h exposure to chlorpyrifos (0.21 mg/L), some of the glochidia still showed normal behavior such as snapping prior to addition of NaCl, and rapidly closing their valves after addition of NaCl, but they snapped more slowly than in the control group. However, most showed damage to the larval adductor muscle and mantle tissue (Fig. 6e) and few of them responded after adding NaCl solution (Fig. 6f). All glochidia at 48-h exposure to the highest chlorpyrifos concentration (0.36 mg/L) did not respond after addition of NaCl solution. These results demonstrate that *H. bialata* glochidia behavior and changes in muscle and mantle characteristics are sensitive indicators of chlorpyrifos exposure.

Acute toxicity of carbosulfan

Survival of glochidia in the 48-h control treatment for the carbosulfan treatment in dechlorinated tap water was less than the 90% minimum recommended by ASTM (2006) after 48-h exposure (Fig. 2); however, carbosulfan was still relatively non-toxic to the glochidia. Control survival was greater than 90% for the 6- and 24-h exposures.

The carbosulfan EC_{50}s at the exposure times of 6, 24, and 48 h could not be calculated in the acute toxicity test because the mean percentage mortality of glochidia was less than 50% in all treatments (Fig. 2). Thus, carbosulfan toxicity was greater than the 0.1 mg/L (Table 1) water solubility limit of carbosulfan at 20 °C (PPDB 2014) and the higher solubility limit of 0.3 mg/L at 25 °C (Alvarez 1995). These results indicated that *H. bialata* glochidia are not sensitive to the carbosulfan formulation used in this study.

When carbosulfan solutions were prepared for rangefinder tests, concentrations greater than 0.3 mg/L decreased survival of glochidia, which may have been the result of poor solubility of carbosulfan. On the other hand, the water solubility limit of carbosulfan may have less relevance when carbosulfan is applied in a formulated product that is likely to have higher solubility than the active ingredient alone.

Bringolf et al. (2007b) reported that the EC_{50}s at the exposure times of 24 and 48 h of glyphosate (technical-grade) and Aqua Star® (glyphosate formulation) for *L. siliquoidea* glochidia were greater than 200 and 150 mg a.e./L, respectively, because of water solubility limits for technical-grade glyphosate. These results suggested that technical-grade glyphosate and Aqua Star® are not acutely toxic to *L. siliquoidea* glochidia. Also, Bringolf et al. (2007a) reported permethrin and atrazine EC_{50}s greater than 0.2 and 30 mg/L, respectively, for *L. siliquoidea* glochidia, which indicated this glochidia species was not acutely sensitive to atrazine or permethrin or their formulations. To our knowledge, there are no peer-reviewed published studies that report the toxicity of carbosulfan to freshwater mussels especially at the larval stage, so the results of the EC_{50} values in this study could not be compared directly to any previous studies on similar species. Moreover, data on the acute toxicity of carbosulfan to other aquatic invertebrates is limited. The 48-h LC_{50} for carbosulfan exposure of *D. magna* is 1.5 μg/L (PPDB 2014), the 48-h LC_{50} of the Nile tilapia (*Tilapia nilotica*) is 0.17 mg/L, and the 24-h LC_{50} for electric fish (*Pollimyrus isidori*) is 0.081 mg/L (Tarzwell and Henderson 1960; Yameogo et al. 1991; Yokoyama et al. 1988). When results of this study are compared with those previous studies, it indicates that the glochidia of freshwater mussels are less sensitive to carbosulfan (48-h LC_{50} greater than 0.1 mg/L) than several other aquatic organisms used for toxicity testing.

Carbosulfan concentrations in surface and ground water are generally 30 μg/L or lower (Sao et al. 2008). This study suggested that carbosulfan toxicity occurs at concentrations greater than 0.1 mg/L so there is minimal risk of adverse effects on *H. bialata* glochidia from the formulation used in the present study when it occurs as a single pesticide exposure.

In this study, glochidia in the control treatment of the carbosulfan exposure showed normal behavior such as snapping 2–3 times within 1 min and discharge of the larval thread outside the valves—these behaviors were also found in all carbosulfan concentrations (Fig. 7a, c, e). After adding NaCl solution, glochidia responded by closing their valves within a few seconds after which the larval adductor muscle was condensed (Fig. 7b, d). The condensing of the larval adductor muscle could indicate an opportunity for further glochidia development, whereas the glochidia adductor muscle in the highest carbosulfan concentration (0.1 mg/L) at 48 h was less well-defined than adductor muscle at 0 and 24 h (Fig. 7f). In a study of larval development in *H. bialata*, normally developed mature glochidia had valves joined by a straight hinge with the larval adductor muscle extended transversely between the valves (Chumnanpuen et al. 2011). There were two layers of larval mantle cells lining the internal glochidial shell surface, and a mantle cavity was present between the inner mantle cells of each valve (Chumnanpuen et al. 2011).

Acute toxicity of combined chlorpyrifos and carbosulfan

The combination of carbosulfan and chlorpyrifos was more toxic when the exposure time was increased to 48 h compared with toxicity at 6 and 24 h (Fig. 3). The 48-h EC_{50} (Table 1) for chlorpyrifos (0.14 mg/L) and carbosulfan (0.05 mg/L) obtained from the mixed exposure tests was higher for chlorpyrifos and lower for carbosulfan than the 48-h EC_{50} values from the single exposure tests in dechlorinated water for carbosulfan (>0.1 mg/L) and chlorpyrifos (0.08 mg/L). This indicates that the combined exposure to two pesticides that inhibit acetylcholinesterase was less toxic to the glochidia in terms of chlorpyrifos toxicity at moderate hardness. The same trend was observed in the moderately hard reconstituted water exposures (discussed in the next section). Insecticide formulations were used in these tests so the decrease in chlorpyrifos toxicity may result from interactions among inert and/or active ingredients (Georg et al. 1988).

The effect of water hardness on chlorpyrifos toxicity

The effect of water hardness on chlorpyrifos toxicity was assessed using a single batch of glochidia (i.e., >90% viable glochidia from at least three female mussels) exposed concurrently in standard reconstituted soft water (42–60 mg/L as $CaCO_3$), moderately hard water (88–128 mg/L as $CaCO_3$), or hard water (168–258 mg/L as $CaCO_3$). The use of a single batch of glochidia ensures that the tests in different water hardness are directly comparable. Alkalinity of the

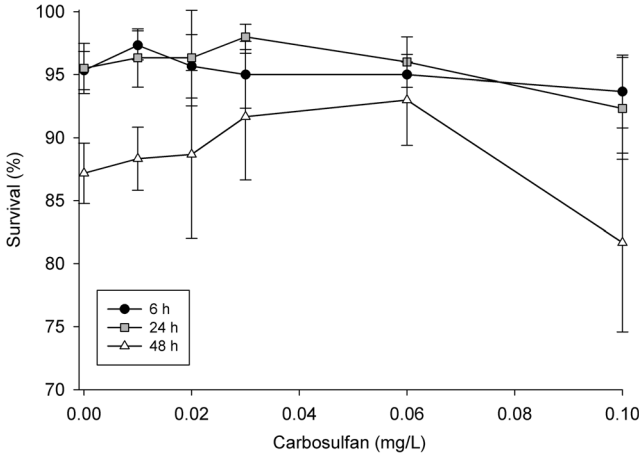

Fig. 2 Percentage survival of *H. bialata* glochidia after exposure to carbosulfan (measured concentrations) for 6, 24, and 48 h. Data are presented as the mean ± standard deviation ($n = 3$)

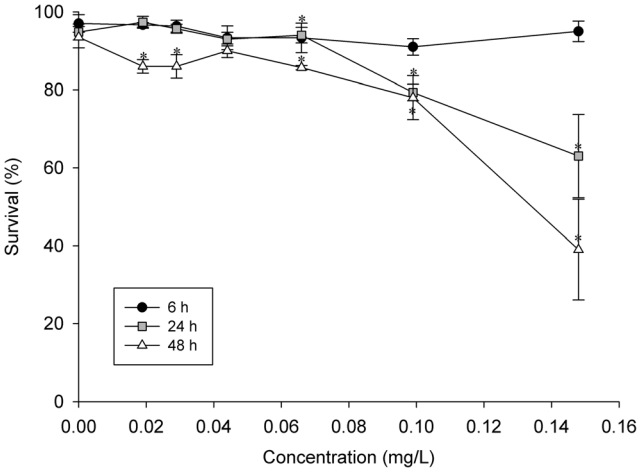

Fig. 3 Percentage survival of *H. bialata* glochidia after exposure to a combination of chlorpyrifos and carbosulfan (2.9:1) for 6, 24, and 48 h in dechlorinated tap water. Data are expressed as measured chlorpyrifos concentrations and presented as the mean ± standard deviation ($n = 3$). The *asterisk* denotes significant differences from the control ($P \leq 0.05$)

reconstituted water was approximately 31 mg/L as $CaCO_3$ in soft water, 60 mg/L as $CaCO_3$ in moderately hard water, and 115 mg/L as $CaCO_3$ in hard water. The mean survival of glochidia in the control treatments was greater than 90% during the test with the highest mean survival in each water hardness at 6 h of 96.3 ± 2.1% in soft water, 97.0 ± 2.0% in moderately hard water, and 98.0 ± 1.0% in hard water. The results demonstrated a significant effect of hardness on the percent survival of glochidia after exposure to chlorpyrifos for 6 ($F = 82.3$, $P < 0.001$), 24 ($F = 66.1$, $P < 0.001$), and 48 h ($F = 21.7$, $P < 0.001$). The chlorpyrifos EC_{50}s for 48 h in soft water, moderately hard water, and hard water were 0.18 (0.17–0.19), 0.11 (0.10–0.12), and 0.16 (0.11–0.19) mg/L, respectively (Table 1). This result demonstrated that the lowest water hardness resulted in the highest survival of glochidia, whereas an increase to moderate water hardness resulted in decreased survival of glochidia (Fig. 4). Exposure to chlorpyrifos in high hardness water resulted in intermediate but variable survival rates that were not significantly different to those at moderate hardness. Comparison with the first experiment in dechlorinated tap water indicates that 48-h EC_{50} of 0.08 mg/L (hardness 120–135 mg/L as $CaCO_3$, temperature 25–26 °C) was similar to the 48-h EC_{50} of 0.11 mg/L in moderately hard water in the second experiment which suggested that the same levels of water hardness cause a similar effect of chlorpyrifos on glochidia. Chlorpyrifos was more toxic in the first experiment which indicates that chlorpyrifos may be faster acting in the dechlorinated tap water that contained unknown concentrations of various ions compared with that of the reconstituted water used in the second test. Alternatively, the batch of larvae in the first experiment may have been more sensitive than those in the reconstituted water experiment, however we did not conduct tests on a standard toxicant in parallel with those on the pesticides. In an early study of pesticide toxicity to fish, water hardness did not affect survival of fish exposed to organophosphate pesticides (not including chlorpyrifos), but did affect toxicity of carbamate pesticides (Pickering and Henderson 1966). Increased water hardness generally decreases the toxicity of metals such as copper, cadmium, and zinc but these have different mechanisms of toxicity (inhibition of ion uptake) to that of chlorpyrifos and carbosulfan (acetylcholinesterase inhibition).

Other aspects of water chemistry such as temperature, pH, and alkalinity influence chlorpyrifos toxicity in aquatic environments. For example, the rate of chlorpyrifos hydrolysis is enhanced in alkaline conditions causing shorter half-lives in water (John et al. 1999). Both pH and alkalinity are related to water hardness, so we would expect that increased hardness

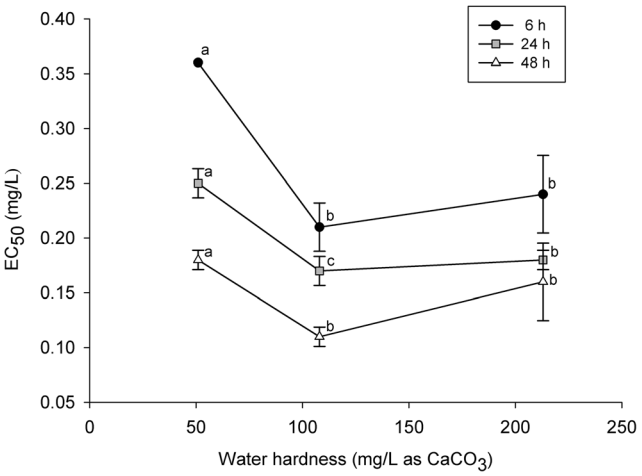

Fig. 4 The EC_{50} values (measured chlorpyrifos concentrations) after exposure to chlorpyrifos to *H. bialata* glochidia at different hardness levels. Data are presented as the mean ± 95% confidence limits ($n = 3$). Treatments not sharing *the same letters* were significantly different (Tukey's test, $\alpha = 0.05$)

may result in increased hydrolysis of chlorpyrifos thus indirectly causing reduced toxicity. Alternatively, chlorpyrifos may degrade to metabolites that are toxic to the glochidia, thus providing an indirect mechanism for increased toxicity as hardness increases.

As for our tests in dechlorinated tap water, these results indicate *H. bialata* glochidia are more sensitive to chlorpyrifos in this formulation (Outcide 40) than *L. siliquoidea* glochidia (48-h EC_{50} 0.6 mg/L) exposed to pure chlorpyrifos or Lorsban® 4-E (hardness 160–184 mg/L as $CaCO_3$, temperature 20.7–21.7 °C) (Bringolf et al. 2007a). In contrast, *H. bialata* glochidia are less sensitive to chlorpyrifos than other aquatic invertebrates. However, the chlorpyrifos concentration residues in the aquatic environment that have been reported in Thailand are lower than the chlorpyrifos EC_{50} values in this study by about 10-fold. Thus, under similar application regimes, chlorpyrifos may not cause acute toxicity to glochidia. Alternatively, application of chlorpyrifos in a different formulation may result in increased toxicity, or a different application rate may increase residual concentrations in the environment. Other research have indicated formulated pesticides can be more toxic to aquatic organisms than preparations of the active ingredient alone (Beggel et al. 2010).

The effect of water hardness on carbosulfan toxicity

The percentage survival of glochidia in the control treatments was more than 90% throughout the test, with the highest percentage survival of 98.3 ± 2.9% at 6 h in moderately hard water. On the other hand, the lowest percentage survival was 89.3 ± 4.6% after exposure to 0.10 mg/L carbosulfan for 48 h in hard water. Glochidia survival was not, however, significantly different across all treatments ($P > 0.05$). The carbosulfan EC_{50}s at the exposure times of 6, 24, and 48 h could not be calculated in the acute toxicity test because the percent survival of glochidia exposed to carbosulfan concentrations for 6, 24, and 48 h was not significantly different from glochidia survival in the control treatment ($P > 0.05$) and the percentage mortality of glochidia was less than 50% in all treatments. In addition, there was no effect of water hardness on the toxicity of carbosulfan. Therefore, our data indicate that *H. bialata* glochidia are relatively insensitive to the carbosulfan formulation used in this study.

In an early study of carbamate pesticides (that did not include carbosulfan), increased hardness increased the toxicity of one pesticide (fermate) to fathead minnows *Pimephales promelas* but decreased the toxicity of another (cumate) (Capkin and Altinok 2013; Pickering and Henderson 1966). In this study, an increase from soft water (42 mg/L as $CaCO_3$) to high water hardness (168–258 mg/L as $CaCO_3$) did not affect survival of *H. bialata* glochidia exposed to carbosulfan.

The effect of water hardness on the toxicity of combined chlorpyrifos and carbosulfan exposure

The percentage survival of glochidia in the control treatments was more than 90% during the test with the highest percentage survival of 96% at 6 h in soft water, 97% at 48 h in moderately hard water, and 98.7% at 24 h in hard water. The 48-h EC_{50}s for a combination of chlorpyrifos and carbosulfan in soft water, moderately hard water, and hard water were 0.12:0.04, 0.13:0.05, and 0.06:0.02 mg CP:CB/L, respectively (Table 1). These results demonstrated that the lowest to moderate water hardness resulted in the highest survival of glochidia, whereas an increase to high hardness resulted in significantly decreased survival of glochidia (Fig. 5) ($F = 43.3$, $P < 0.001$ for carbosulfan, $F = 48.0$, $P < 0.001$ for chlorpyrifos). In comparison, the 48-h EC_{50}s for the single exposure tests of chlorpyrifos demonstrated that the lowest water hardness resulted in the highest survival of glochidia, whereas moderate and hard water hardness resulted in significantly decreased survival of glochidia ($F = 15.5$, $P < 0.05$). The 48-h EC_{50} value (0.06 mg/L) obtained for chlorpyrifos from the mixed exposure in hard water was lower than EC_{50} values from the single exposure test for chlorpyrifos exposure in hard water (0.16 mg/L) by 2.7-fold. This suggests that the combination of chlorpyrifos and carbosulfan in reconstituted hard water is markedly more toxic than the single exposure to chlorpyrifos in hard water, despite the relatively low toxicity of carbosulfan alone.

The mechanisms for chemical toxicity of combinations of pesticides are not fully understood. Some theories include an increase in the rate of uptake, the formation of toxic

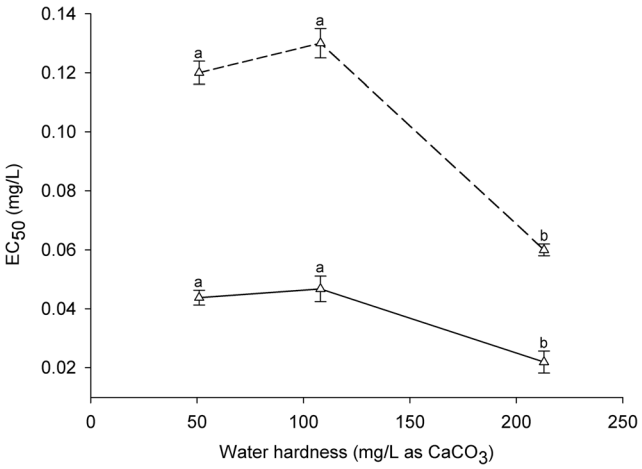

Fig. 5 The effect of water hardness on the EC_{50} values for *H. bialata* glochidia exposed to a combination of chlorpyrifos and carbosulfan for 48 h. Data are measured chlorpyrifos concentrations with a *dashed line* and measured carbosulfan concentrations with a *solid line*. Data from the dechlorinated tap water exposures are also shown with a *solid circle* (chlorpyrifos) or a *solid square* (carbofuran). Data are presented as the mean ± 95% confidence limits ($n = 3$). Treatments with *different letters* are significantly different (Tukey's test, $\alpha = 0.05$)

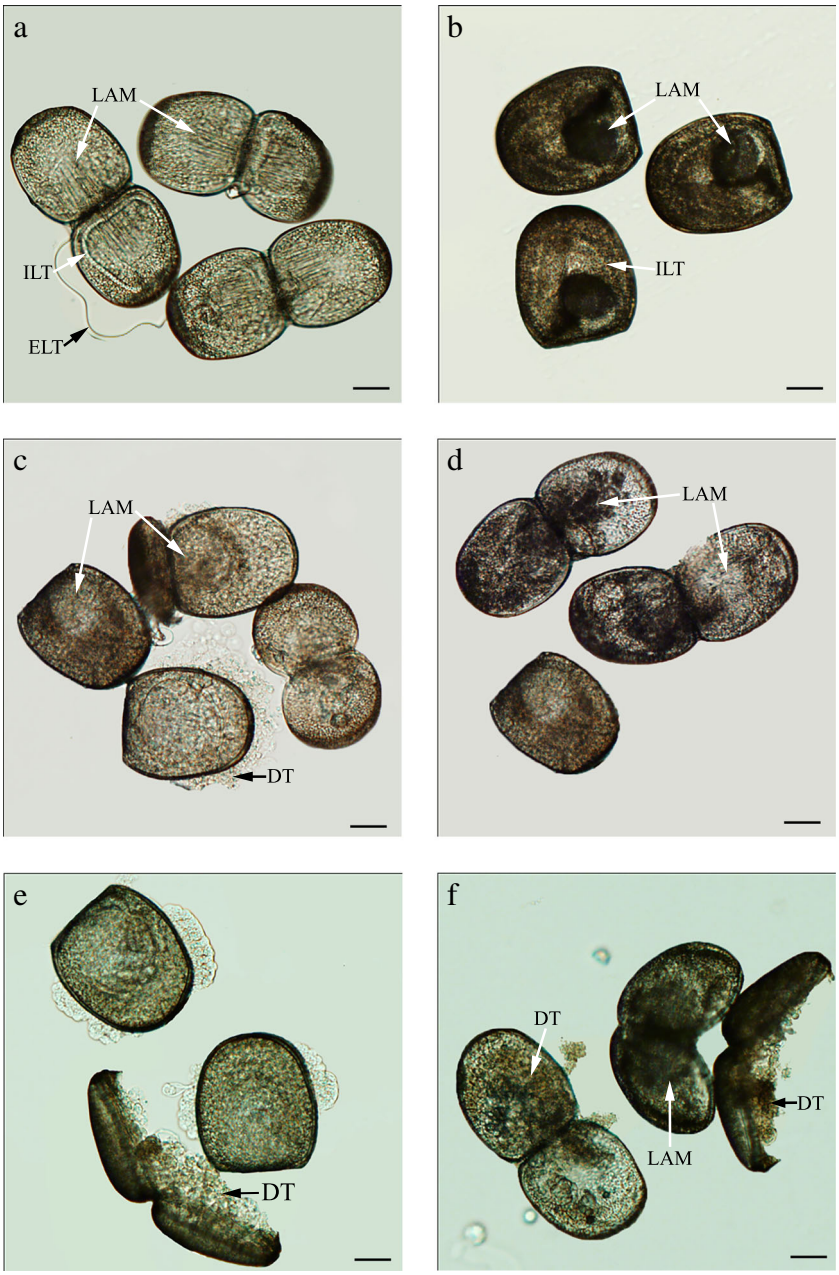

Fig. 6 Characteristics of *H. bialata* glochidia observed after exposure to 0.21 mg/L chlorpyrifos for 0 (**a** and **b**), 24 (**c** and **d**) and 48 h (**e** and **f**). Images (**a**), (**c**), and (**e**) were taken before adding 24% NaCl solution, and images (**b**), (**d**), and (**f**) were taken after adding NaCl solution. *Scale bars* = 50 μm. *ELT* external larval thread, *ILT* internal larval thread, *LAM* larval adductor muscle, *DT* damaged tissue

metabolites, an alteration of toxicant distribution within the organism, or an inhibition of detoxification systems (Marking 1977). Scholz et al. (2006) evaluated the joint toxicity of a simple combination of organophosphate and carbamate insecticides that caused a strictly additive neurotoxicity in salmon. The mechanism of action is similar for organophosphates and carbamates, as they both inhibit acetylcholinesterase, but there is a difference in the duration of inhibition. Organophosphate-induced inhibition is effectively irreversible in some fish species whereas, inhibition by carbamates is reversible (Aldridge and Reiner 1972). In the present study, the combination of chlorpyrifos and carbosulfan was tested in a dose ratio of 2.9:1 (3.7:1 nominal ratio) and the chemical interaction resulted in an increase in acute chlorpyrifos toxicity in low and high hardness water. At moderate hardness, chlorpyrifos toxicity was slightly decreased in the mixed exposure in both the dechlorinated tap water and the reconstituted water exposures.

Belden and Lydy (2000) reported that the presence of atrazine at levels as low as 40 μg/L significantly increased the toxicity of chlorpyrifos, methyl parathion, and diazon whereas atrazine alone was not acutely toxic at 10,000 μg/L, which is near its water solubility limit. They demonstrated that atrazine may synergistically affect chlorpyrifos toxicity in chironomids

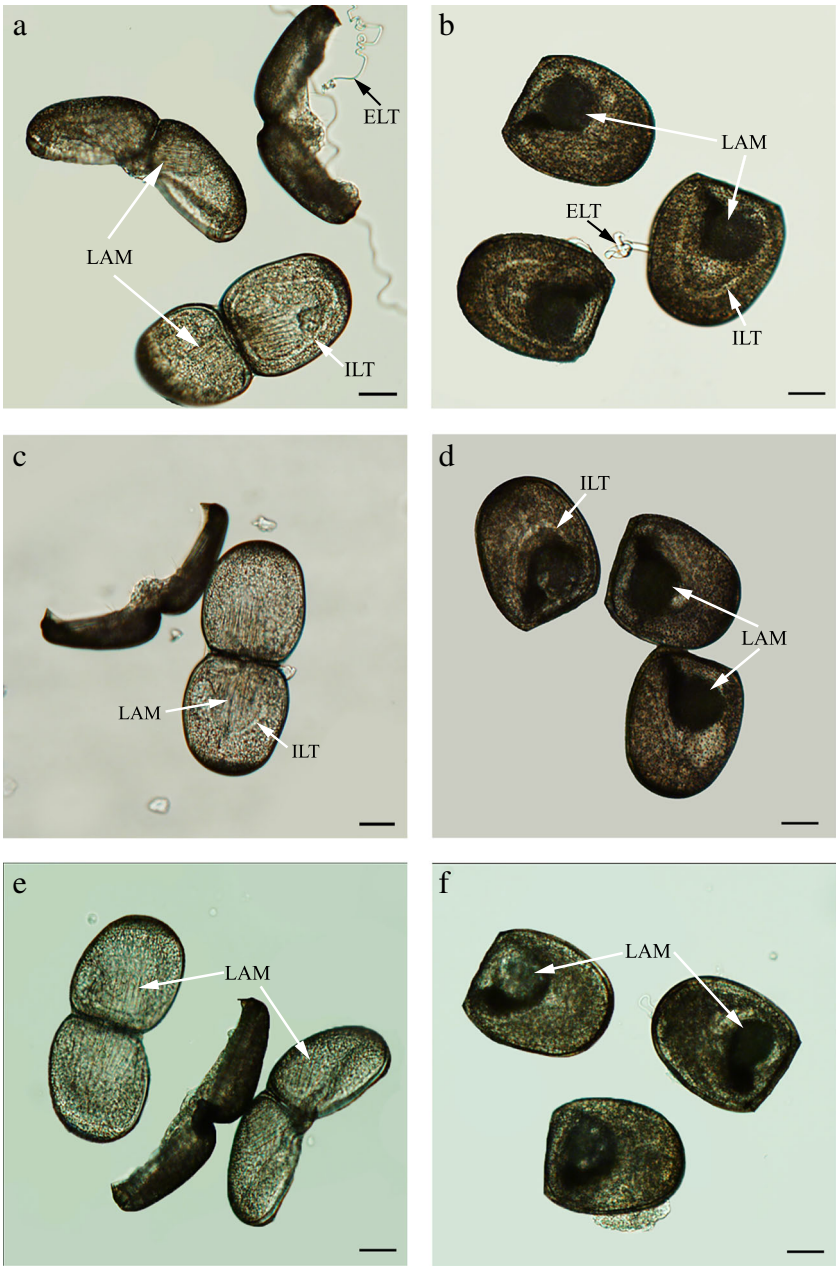

Fig. 7 Characteristics of *H. bialata* glochidia after exposure to 0.1 mg/L carbosulfan for 0 (**a** and **b**), 24 (**c** and **d**), and 48 h (**e** and **f**). Images (**a**), (**c**), and (**f**) were taken before adding 24% NaCl solution and images (**b**), (**d**), and (**f**) were taken after adding NaCl solution. *Scale bars* = 50 μm. *ELT* external larval thread, *ILT* internal larval thread, *LAM* larval adductor muscle

by increasing its bioactivation and the production of toxic metabolites. Similar results were found in a more recent study on another chironomid species and two fish species—and indicated that mixture toxicity of chlorpyrifos and atrazine is specific to both the life stage and species exposed (Tyler Mehler et al. 2008). Synergistic toxicity of chlorpyrifos and another organophosphate azinphos-methyl has been demonstrated in another mollusk, *Planorbarius corneus* (Cacciatore et al. 2013).We are not aware of any other studies that simultaneously examine the effect of a chlorpyrifos mixture and hardness on toxicity to an aquatic organism. In the present study, both pesticides were added as formulations and the presence of additional ingredients may have caused increased toxicity at high hardness or decreased degradation rates. Alternatively, the combination of exposure to an organophosphate and a carbamate pesticide may have caused potentiated toxicity via combined effects on cellular physiology (e.g., Scholz et al. (2006) discussed earlier). Current agricultural practices result in the use of multiple chemicals within a catchment so it is likely that aquatic organisms will be exposed to mixtures of pesticides in runoff. The mussel species in this study, *H. bialata*, is thought to spawn and release glochidia all year round (Kovitvadhi et al. 1999; Chatchavalvanich et al. 2006). Moreover, both pesticides are used for agriculture

throughout the year, so it is likely that *H. bialata* glochidia will be exposed to mixtures of these chemicals (Figs. 6 and 7). Our study indicates that it is difficult to predict the toxicity of combinations of chemicals, therefore it is important to minimize the use of pesticides to not only provide cost-effective outcomes for farmers, but to also reduce the exposure of non-target organisms to multiple pesticides.

Conclusions

In this study, carbosulfan was relatively non-toxic to *H. bialata* glochidia with 48-h EC_{50} > 0.1 mg/L, and there was no measurable effect of water hardness on carbosulfan toxicity. The EC_{50} values for *H. bialata* glochidia exposed to chlorpyrifos in dechlorinated tap water demonstrated that the toxicity of chlorpyrifos progressively increased as exposure times were increased. Low water hardness resulted in the highest survival of glochidia exposed to chlorpyrifos alone, whereas moderate and hard water hardness resulted in significantly decreased survival of glochidia. Our data indicate that water hardness affects glochidia sensitivity to chlorpyrifos in a non-linear fashion. The combination of carbosulfan and chlorpyrifos in moderately hard dechlorinated tap water was more toxic when exposure time was increased to 48 h compared to toxicity at 6 and 24 h. The 48-h EC_{50} values obtained from the combined exposure tests in dechlorinated tap water (0.05 mg/L carbosulfan and 0.14 mg/L chlorpyrifos) were lower than EC_{50} values from the single exposure tests for carbosulfan (>0.1 mg/L) but were greater than the 48-h EC_{50} for chlorpyrifos (0.08 mg/L) alone. Combined exposure to carbosulfan and chlorpyrifos in moderately hard dechlorinated tap water therefore decreased the toxicity of chlorpyrifos.

When the effect of water hardness on the toxicity of a combination of carbosulfan and chlorpyrifos was examined in reconstituted water exposures, the low to moderate water hardness resulted in the highest survival of glochidia, whereas an increase to high water hardness resulted in significantly decreased survival of glochidia. Exposure as a mixture of carbosulfan and chlorpyrifos in hard reconstituted water (carbosulfan 48-h EC_{50} 0.02 mg/L, chlorpyrifos 48-h EC_{50} 0.06 mg/L) therefore increased the toxicity of chlorpyrifos by 2.7-fold relative to chlorpyrifos alone in hard reconstituted water. Our data indicate that *H. bialata* is more sensitive to a combined exposure to the organophosphate chlorpyrifos and the carbamate carbosulfan than would be expected from their toxicity in single exposures, and that high water hardness increases the toxicity of the combined pesticides.

Acknowledgements The research was a part of the project grant provided by the Development and Promotion of Science and Technology Talents Project, Graduate School (Kasetsart University), Department of Zoology (Faculty of Science, Kasetsart University), and the Faculty of Science (Kasetsart University). We thank Mrs. Pakasinee Klaimala, Science Research and Development, Department of Agriculture, Thailand, for her advice on the toxicant database.

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