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THESIS

ACUTE TOXICITY OF CARBOSULFAN AND CHLORPYRIFOS TO GLOCHIDIA OF FRESHWATER MUSSEL *Hyriopsis bialata* Simpson, 1900

AKKARASIRI SANGSAWANG

A Thesis Submitted in Partial Fulfillment of
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Akkarasiri Sangsawang 2014: Acute Toxicity of Carbosulfan and Chlorpyrifos to Glochidia of Freshwater Mussel *Hyriopsis bialata* Simpson, 1900. Master of Science (Biology), Major Field: Biology, Department of Zoology. Thesis Advisor: Associate Professor Uthaiwan Kovitvadhi, Ph.D. 83 pages.

The acute toxicity of a carbosulfan and chlorpyrifos to glochidia of the freshwater mussel (*Hyriopsis bialata* Simpson, 1900) was evaluated under static conditions in dechlorinated tap water. The median effective concentrations (EC_{50}) of carbosulfan at 24 and 48 h were greater than 0.10 mg/L. The no observed effect concentration (NOEC) was 0.10 mg/L at 48 h and the lowest observed effect concentration (LOEC) was greater than 0.10 mg/L at 48 h. The EC_{50} of chlorpyrifos at 24 and 48 h were 0.083 (0.079-0.087) and 0.078 (0.062-0.092) mg/L, respectively. The NOEC was 0.05 mg/L at 48 h while the LOEC was 0.07 mg/L at 48 h. The EC_{50} of a combined exposure to carbosulfan and chlorpyrifos (expressed as chlorpyrifos concentration) at 48 h was 0.15 mg/L. In a separate experiment the effect of water hardness on carbosulfan, chlorpyrifos, and a combined exposure to carbosulfan and chlorpyrifos were assessed using glochidia exposed to either standard reconstituted soft water, moderately-hard water, or hard water. There was no effect of the water hardness on the survival of glochidia at 24 and 48 h during carbosulfan toxicity test. The chlorpyrifos 48 h EC_{50}s 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. The result indicates 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 (P<0.05). In addition, the EC_{50}s of a combined exposure to carbosulfan and chlorpyrifos at 48 h in soft water, moderately-hard water, and hard water were 0.13 (0.12-0.14), 0.14 (0.13-0.16), and 0.067 (0.060-0.086) mg/L, respectively. The result indicates that the combined toxicity was lowest at low and moderate water hardness, whereas an increase to hard water hardness resulted in a decreased survival of glochidia.

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Akkarasiri Sangsawang

November, 2014

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LIST OF ABBREVIATIONS

ASTM	=	American Society for Testing Material
bw	=	body weight
EC_{50}	=	Median effective concentration
FAO	=	Food and Agriculture Organization
h	=	hour
l	=	litre
LC_{50}	=	Median lethal concentration
mg	=	milligram
NOEC	=	No observed effect concentration
LOEC	=	Lowest observed effect concentration
WHO	=	World Health Organization
95%CI	=	95% confidence intervals

ACUTE TOXICITY OF CARBOSULFAN AND CHLORPYRIFOS TO GLOCHIDIA OF FRESHWATER MUSSEL *Hyriopsis bialata* Simpson, 1900

INTRODUCTION

Pesticides are beneficial for agricultural economy, but they are a major group of environmental contaminants throughout the world. Residue or runoff of pesticides from agricultural fields may easily contaminate the river, resulting in serious damage to non-target species, including mussel. Carbosulfan and chlorpyrifos are broad-spectrum carbamate (CB) and organophosphate (OP) pesticides, respectively, that are widely used in agricultural and urban practices in Thailand. Carbosulfan is classified by WHO as in a category 2 (moderately hazardous) pesticide that is used to control insects, mites and nematodes in soil or on foliage. Chlorpyrifos is the most intensively used organophosphate insecticide (EPA toxicity class II) in agriculture. Chlorpyrifos insecticide was mostly imported to Thailand in year 2012 for control of crop pests in sucking and biting group. The continuous mistake of these pesticides using and the paucity of using knowledge are resulting in contaminants accumulation in freshwater ecosystem and aquatic organisms can be affected. In recently, freshwater mussels (Family Unionidae) are among the widespread decline in many countries around the world and pesticides are one of many risk factors involved in decline of this family.

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). The early stages of freshwater mussels (glochidia and juveniles) are relatively tolerant of exposure to some pesticides and other organic compounds (Keller *et al*., 2005). In contrast, they have been reported to be highly sensitive to contaminants such as metals, ammonia, and some organics, including pulp and paper mill effluents, aromatic hydrocarbons, and some pesticides, compared to other aquatic invertebrates such as amphipods, chironomids, and cladocerans, and vertebrates such

as fish and amphibians. This suggests that the early life stages of freshwater mussels are a good indicator organism for aquatic environment health.

However, water quality also has influence on the toxicity of environmental contaminants. Thus, the objective of this study was to determine the acute toxicity of carbosulfan and chlorpyrifos to glochidia of the freshwater mussel *H. bialata* and then assess the effect of water hardness on the toxicity of carbosulfan and chlorpyrifos to glochidia. In addition, a combination of these insecticides was tested in order to evaluate interactive toxicity to *H. bialata* glochidia.

OBJECTIVES

1. To determine a suitable temperature and survival time for standard toxicity tests of *Hyriopsis bialata* glochidia

2. To evaluate the acute toxicity of carbosulfan, chlorpyrifos, and a combined exposure to carbosulfan and chlorpyrifos to glochidia

3. To examine the effect of water hardness on the toxicity of carbosulfan, chlorpyrifos, and a combined exposure to carbosulfan and chlorpyrifos to glochidia

4. To determine the characteristics of glochidia after exposure to carbosulfan and chlorpyrifos

LITERATURE REVIEW

1. *Hyriopsis bialata* Simpson, 1900

1.1 Taxonomy

Hyriopsis bialata Simpson, 1900 is a freshwater mussel. The taxonomic classification according to Graf and Cummings (2007) is as follows:-

Kingdom Animalia
Phylum Mollusca
Class Bivalvia
Order Unionoida
Family Unionidae
Genus *Hyriopsis*
Species *Hyriopsis bialata*

1.2 Morphology

An adult of *H. bialata* has a medium-sized shell (average length 84.6±14.4 mm; n=35) with an ovate structure and valves of equal size. The dorsal shell has two wings, with the posterior wing larger, sharper and more prominent than the anterior. These two wings are joined by ligaments. The color of the periostracum usually includes some combination of green, brown, or black but the juveniles are usually lighter and shinier than adults and turning to brownish or black with age. Growth lines are prominent and heavy causing shell surface to be roughened. There are two types of teeth on the hinge that create a strong and sturdy connection between the valves. The lateral teeth are thin elongate structures parallel to the hinge ligaments while the pseudocardinal teeth are much shorter than the lateral teeth and are located on the anterior of the shell. The beak cavity is rather shallow and not prominent. The umbones are located dorsally toward the anterior of the shell. There are three anterior and two posterior muscle scars (Kovitvadhi, 2014a).

Freshwater mussel shells have been extensively used as indicator to assess environmental contamination including radionucleotides and some metals that are highly concentrated in the outer periostracal layer of the shell (Widdows and Donkin, 1992; Livingstone and Pipe, 1992).

Figure 1 Shell morphology of *Hyriopsis bialata*. Scale bar = 2 cm

Source: Kovitvadhi (2014a)

The juveniles have a soft periostracum under the glochidial valves. The shell of early juvenile was so thin that the internal organs, e.g. the foot, gill, intestine, stomach, heart, and bundle of muscle, could be seen clearly under a microscope. In addition, the incurrent and excurrent siphons first appear in this stage. The new shell growth is marked by addition of parallel, slender lines initially in anterior region. The shell thickness is increased by additional calcium deposition (Uthaiwan *et al*., 2001).

Mature glochidia of *H. bialata* was observed using scanning electron microscopy (SEM), the shell of the glochidia has semi-oval, equivalve shells with an equilateral valve shape and hookless shells. The internal surface of glochidia valves contained numerous pores while the external surface was covered with keratin fibers and numerous pits (Panha and Eongprakornkeaw, 1995; Uthaiwan *et al*., 2001). The complete perforation of shell on both sides indicated that the encysted glochidia may use the perforation of shell to exchange gases and obtain nutrients from host tissues as they undergo transformation (Coker *et al*., 1921).

The average size of the glochidia valves (n=50) was 190 ± 0.02 µm in length, 230± 0.02 µm in height, and 61± 0.86 µm in width. The valves were joined by straight hinge with a muscle bundle called the larval adductor extended transversely between the valves. There were two layers of larval mantle cells lining the internal glochidial shell surface and a mantle cavity was between the inner mantle cells of each valve. There were a few sensory hair cells on the larval mantle. There were two types of larval thread of the mature glochidia of *H. bialata,* internal and external larval threads. The external larval thread rising from the thread gland was long (up to 2 mm externally), sticky, and pliable, and could attach itself to rough surface organs such as the fin or gill of host fish. The internal larval thread, on the other hand, was located around the larval adductor muscle (Chumnanpuen, 2006; Chumnanpuen *et al*., 2011).

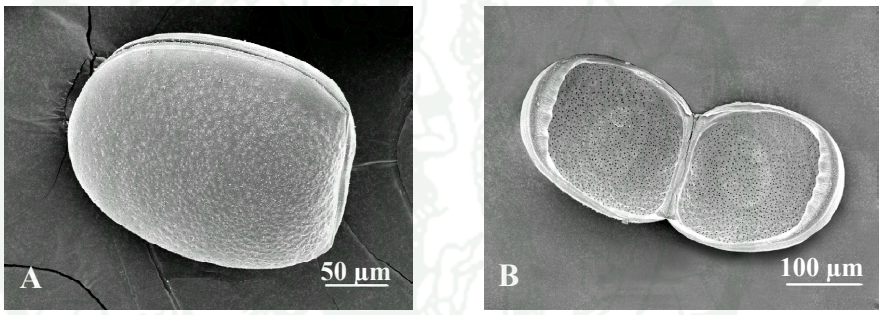

Figure 2 Ultrastructures observed in mature glochidia *Hyriopsis bialata* using scanning electron microscopy (SEM)**:** mature glochidia (A) and the internal surface of glochidia valves showing hookless equivalve shell (B).

Source: Areekijseree (2003)

1.3 Life cycle

Hyriopsis bialata are dioecious and have a complex mode of reproduction. Freshwater mussels have spawning season that varies with species, *H. bialata* can spawn all-year-round (Jindamonkol *et al*., 2003) while other species such as *Chamberlainia hainesiana* and *Hyriopsis myersiana* have spawning season from October to May and the peak of the spawning season is from December to January (Nagachinta and Meejui, 1998).

The beginning of the reproductive process commences when males release spermatozoa through a genital pore and then into the water column via the exhalent siphon and females collect them by taking water in the inhalent siphon. Fertilization occurs internally in the suprabranchial chamber or water tubes in the specialized female brood pouch located in the outer gill. Developing embryos are brooded within the marsupial or brood chamber and develop into the first larval phase called glochidium (plural: glochidia, Uthaiwan *et al*., 2001b). The duration of embryo development varies by species and environmental conditions such as water temperature. Jindamonkol *et al.* (2003) reported that the duration for zygotes of *H. bialata* to develop into mature glochidia was 6-10 days.

H. bialata glochidia are released from the suprabranchial chamber through the exhalent siphon into the water. Glochidia are external parasites and will attach to the skin, fin or gill of a host fish using spines. Once encapsulated, glochidia receive nutrients through pores and the microvillae of the mantle or the mushroom body. During this period, most glochidia do not increase in size. Metamorphosis from the glochidium to the juvenile mussel and the duration of the parasitic phase varies with both the mussel and host species and also depends on water temperature. The newly transformed juvenile drops off the host fish and burrows into the sediment as free-living animal (Kovitvadhi and Kovitvadhi, 2010). The life cycle of freshwater mussels is summarized in Figure 3.

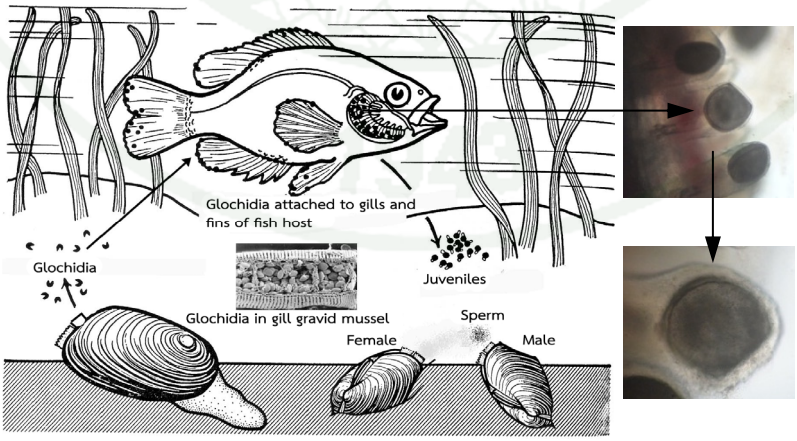

Figure 3 Life cycle of a freshwater mussel

Source: Kovitvadhi (2014b)

1.4 Ecology and distribution

Freshwater mussels are important to aquatic ecosystems because of their effect on food webs, water quality, nutrient cycling, and habitat quality. Most freshwater mussels are confined to borrowing only to the posterior edge of the shell and so burial depth does not become a buffer from exposure to chemicals, temperature extremes, or predators. *H. bialata* is widely distributed in large areas of Thailand such as the Mekong River, the Mun River, the Chi River, the Choen River, the Songkram River, canals in the Garden Palace of Bang Pa-In; Bung Boraphet; Ban La Po, Pisanulok Province and the Pong River, Kon Kaen Province (Brandt, 1974; Kovitvadhi and Kovitvadhi, 2002).

1.5 Sensitivity to Toxic Contaminants

Freshwater mussels exhibit a variety of sensitivities to toxic contaminants based on species, life stages (glochidium, juvenile, or adult), and environmental conditions. Keller *et al*. (2005) indicated that early life stages of freshwater mussels (glochidia and juveniles) tended to be less sensitive in exposure to some pesticides and other organic compounds. In contrast, they have been reported to be highly sensitive to contaminants such as metals, ammonia, and some organic compounds including those in pulp and paper mill effluent and aromatic hydrocarbons, especially when compared to other aquatic invertebrates such as amphipods, chironomids, and cladocerans, fish or amphibians. These results indicated that guidelines such as U.S. Environmental Protection Agency Water Quality Criteria (WQC) for protection of aquatic organisms may not be adequately protective of the early life stages of freshwater mussels, unless they are included in the supporting data.

The tolerance of freshwater mussels to pesticides currently in use depends on the species of mussel, identity of the contaminant, and duration of exposure. The results of acute toxicity tests of chemicals on glochidia of various species of freshwater mussel are summarized in Table 1.

Table 1 Summary of previous acute toxicity tests on glochidia of different species of freshwater mussel

Test chemicals	Species	Time (h)	End point	Values	Units	References
Organic Compound						
Fluoranthene	*Utterbackia imbecillis*	24	LC_{50}	2.45	µg/L	Weinstein, 2001
Pesticides						
Carbaryl (Sevin®)	*Utterbackia imbecillis*	24	LC_{50}	7.9	mg/L	Conners and Black, 2004
Diazinon (Diazinon®)		24		19.4	mg/L	
Glyphosate (Roundup®)		24		18.3	mg/L	
Atrazine (Atrazine4L®)		24		241.3	mg/L	
MON 0818 (surfactant of glyphosate)	*Lampsilis siliquoidea*	48	EC_{50}	0.5	mg/L	Bringolf *et al.,* 2007b
Roundup®		48	EC_{50}	2.9	mg/L	
Glyphosate		48	EC_{50}	>200	mg/L	
Atrazine	*Lampsilis siliquoidea*	48	EC_{50}	>30	mg/L	Bringolf *et al.,* 2007c
Aatrex 4L®		48	EC_{50}	>30	mg/L	
Chlorpyrifos		48	EC_{50}	0.43	mg/L	
Lorsban®		48	EC_{50}	0.60	mg/L	
Permethrin		48	EC_{50}	>0.2	mg/L	
Mosquito-B-Gone®		48	EC_{50}	>0.2	mg/L	
Atrazine	*Eilliptio complanata*	48	EC_{50}	>30	mg/L	Bringolf *et al.,* 2007a
Permethrin	*Lampsilis fasciola*	48	EC_{50}	>0.2	mg/L	
Fipronil	*Lampsilis siliquoidea*	48	EC_{50}	>0.2	mg/L	
Pendimethalin	*Villosa constrita*	48	EC_{50}	>0.3	mg/L	
Chlorothalonil	*Villosa delumbis*	48	EC_{50}	0.04	mg/L	
Propiconazole		48	EC_{50}	19.21	mg/L	
Pyraclostrobin		48	EC_{50}	0.08	mg/L	
Synthetic Musks						
AHTN	*Lampsilis cardium*	24	LC_{50}	0.454-0.85	mg/L	Gooding *et al*., 2006
HHCB		24	LC_{50}	1.00->1.75	mg/L	

Table 1 (Continued)

Test chemicals	Species	Time (h)	End point	Values	Units	References
Inorganic compound						
Copper (as $CuSO_4 \cdot 7H_2O$)	*Lampsilis fasciola*	48	LC_{50}	40	µg Cu/L	Jacobson *et al.*, 1997
	Villosa iris	48	LC_{50}	46-66	µg Cu/L	
	Medionidus conradicus	48	LC_{50}	16	µg Cu/L	
	Actinonaias pectorosa	48	LC_{50}	51	µg Cu/L	
	Pyganodon grandis					
Copper	*Utterbackia imbecillis*	24	LC_{50}	37.4	µg Cu/L	Conners and Black, 2004
Mercury (as $HgCl_2$)	*Villosa iris*	24	LC_{50}	>107	µg Hg/L	Valenti *et al*., 2005
Chlorine (as $Ca(OCl)_2$)	*Villosa iris*	24	LC_{50}	220	µg TRC/L	Valenti *et al*., 2006
		48	LC_{50}	260	µg TRC/L	
	Lampsilis fasciola	24	LC_{50}	145	µg TRC/L	
		48	LC_{50}	80	µg TRC/L	
	Epioblasma capsaeformis	24	LC_{50}	107	µg TRC/L	
	Epioblasma brevidens	24	LC_{50}	70	µg TRC/L	
	Alasmidonta heterodon	24	LC_{50}	107	µg TRC/L	
		48	LC_{50}	95	µg TRC/L	
Copper (as $CuSO_4$)	*Alasmidonta heterodon*	24	EC_{50}	39	µg Cu/L	Wang *et al*., 2007
	Actinonaias ligamentina	48	EC_{50}	23	µg Cu/L	
Ammonia (as NH_4Cl)	*Epioblasma capsaeformis*	24	EC_{50}	10	mg N/L	
	Venusstaconcha ellipsiformis	48	EC_{50}	7.8	mg N/L	
Chlorine (as NaOCl)	*Villosa iris*	24	EC_{50}	83	µg/L	
	Lampsilis fasciola	48	EC_{50}	63	µg/L	
	Lampsilis siliquoidea					
	Lampsilis rafinesqueana					
	Lampsilis abrupt					
	Potamilus ohiensis					
	Leptodea leptodon					

Table 1 (Continued)

Test chemicals	Species	Time (h)	End point	Values	Units	References
Copper (as $CuSO_4 \cdot 5H_2O$)	*Epioblasma torulosa rangiana*	24	EC_{50}	7-36	µg Cu/L	Gillis *et al*., 2008
	Epioblasma triquetra *Lampsilis fasciola* *Obovaria subrotunda* *Ptychobranchus fasciolaris* *Villosa fabalis* *Actinonaias ligamentina* *Lampsilis siliquoidea* *Ligumia recta*	48	EC_{50}	4-21	µg Cu/L	
Copper (as $CuSO_4 \cdot 5H_2O$)	*Lampsilis siliquoidea*	24	EC_{50}	36-150	µg Cu/L	Gillis *et al*., 2010
Sodium chloride (NaCl)	*Lampsilis siliquoidea* *Lampsilis cardium* *Lampsilis fasciola* *Epioblasma torulosa rangiana*	24	EC_{50}	113-1430	mg Cl/L	Gillis *et al*., 2011
Copper (as $Cu(NO_3)_2 \cdot 3H_2O$)	*Echyridella menziesii*	48	EC_{50}	1.7-3.4	µg/L	Clearwater *et al.,* 2014
Zinc (as $ZnSO_4 \cdot 7H_2O$)	*Echyridella menziesii*	48	EC_{50}	229-337	µg/L	
Total ammonia nitrogen (TAN as NH_4Cl)	*Echyridella menziesii*	48	EC_{50}	12-15	mg TAN/L	

2. Carbosulfan

2.1 General information

Carbosulfan is a benzofuranyl methyl carbamate pesticide. The World Health Organization (WHO) classifies carbosulfan as a category 2 (moderately hazardous) pesticide. Carbosulfan is banned in Europe, but it is still used widely in other countries such as Mexico and Brazil (Capkin and Altinok, 2013) and Thailand.

2.2 Physical and chemical properties

The chemical name of carbosulfan is 2, 3-dihydro-2, 2-dimethylbenzofuran-7-yl (dibutylaminothio) methylcarbamate. The molecular formula is $C_{20}H_{32}N_2O_3S$ and the relative molecular mass is 380.5. The chemical abstract service (CAS) registration number and development codes are 55285-14-8 and FMC 35001. Technical-grade carbosulfan is an orange to yellow coloured, viscous liquid. 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 such as xylene, hexane, chloroform, dichloromethane, methanol, and ethanol. The chemical structure of carbosulfan is shown in Figure 4.

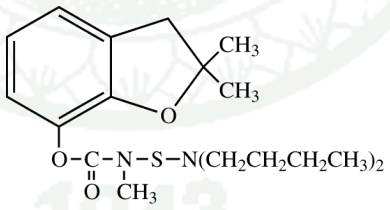

Figure 4 Chemical structure of carbosulfan

2.3 Uses

Carbosulfan is a broad-spectrum 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 available as emulsifiable concentrates, dusts and granular formulations. Cabosulfan is the active ingredient of

many commercial insecticides such as Posse®, Marshal®, and Advantage® in dry granules and wet formulations.

2.4 Biodegradation of carbosulfan

Carbosulfan are has a low persistence in the environment but it can persist longer in a low pH environment (Ramanand *et al*., 1991). Environmental concentrations of carbosulfan range between 0.64 μg/L and 29 μg/L (Leppert *et al*., 1983 and Sao *et al*., 2008). The pesticides persistence in an environmental compartment (e.g., soil) is known as the pesticides half-life. The haft-life is a measure of the time required for the pesticide concentration to be reduced to half the original value. The pesticide concentration is reduced by biological or chemical degradation processes. Luís Pedro de Melo Plese *et al*. (2005) reported a carbosulfan half-life in rice paddy water of 1 day (27 hours). However, the maximum concentration in the paddy water was 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 (Tejada and Magallona, 1985).

The main degradation mechanisms are based on hydrolysis and oxidation. Carbosulfan breaks down into carbofuran, 3-hydroxy carbofuran and 3-keto carbofuran. The first degradation product, carbofuran is more toxic than carbosulfan, so it represents a great risk to the humans, and 3-hydroxy carbofuran also has adverse effects. Carbosulfan has a temporary acceptable daily intake (ADI) of 0–0.01 mg/kgbw and a maximum residue limit (MRL) of 0.05 mg/kg (EFSA, 2009) for the sum of carbosulfan, carbofuran and 3-hydroxy carbofuran in citrus fruits. In the environment, the important degradation pathways are photolysis, hydrolysis and microbial transformation of carbosulfan. The degradation of carbosulfan is increased at high temperatures. Carbosulfan has low mobility in soil and degrades to dimethylamine which is a major non-carbamate product. The major product of carbosulfan in water is carbofuran under acidic conditions and 7-phenol under basic conditions. The metabolic pathways of carbosulfan in oranges are shown in Figure 5.

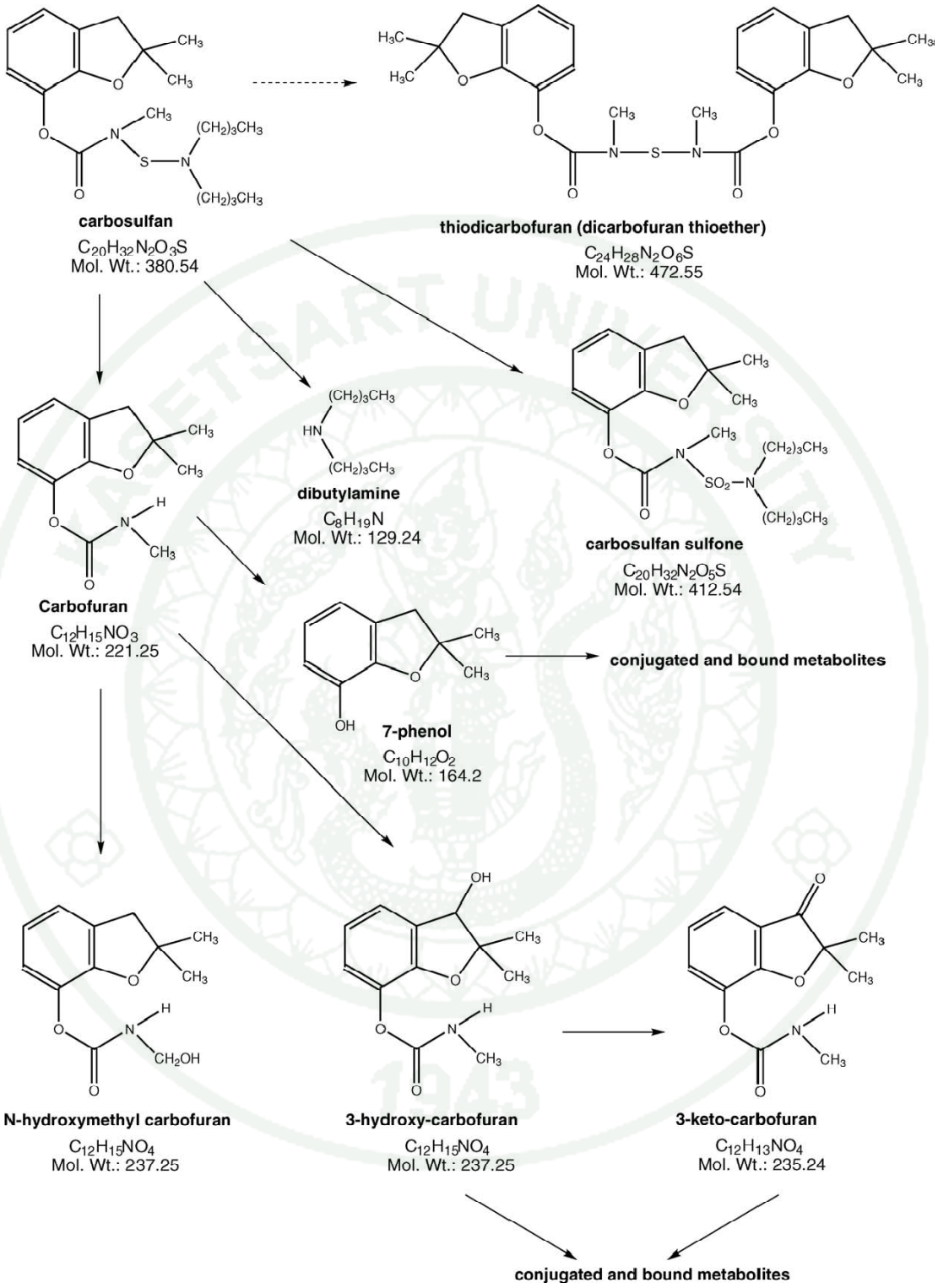

Figure 5 Proposed metabolic pathways of carbosulfan in oranges

Source: Soler *et al.* (2006)

2.5 Toxicology and ecotoxicology

Carbosulfan is classified as highly acutely toxicity to fish and aquatic invertebrates. The bioconcentration factor (BCF) of carbosulfan in fish is 990 (PPDB, 2014). The BCF depends on the species, exposure concentrations, and exposure conditions. Carbosulfan has a lower toxicity to aquatic organisms than its metabolite, carbofuran. Dobšíková (2003) reported for *Daphnia magna* that the 48 h EC_{50} of carbofuran was 0.0187 mg/L, while the 48 h EC_{50} for carbosulfan was 1.5 mg/L (PPDB, 2014). There are no other peer reviewed published studies that report the toxicity of carbosulfan to freshwater mussels especially at the larval stage. Data on the acute toxicity of carbosulfan to other aquatic invertebrates is limited that is shown in Table 2.

Table 2 The acute toxicity of carbosulfan to aquatic organisms

Species	48-h LC_{50} (mg/L)	References
Fish		
Tilapia nilotica	0.17	Tarzwell and Henderson, 1960
(Nile tilapia)	(-)	
Anguilla japonica	1.3	Yokoyama *et al*., 1988
(Japanese eel)	(-)	
Schilbe mystus	0.14	Yameogo *et al*., 1991
(Butter barbel) 50 mm	(0.105-0.162)	
Oncorhynchus mykiss	0.432	Boran *et al*., 2007
(rainbow trout) juvenile	(0.346-0.534)	
Poecilia reticulatae	0.151	Boran *et al*., 2007
(guppy) juvenile	(-)	
Channa punctatus	0.295	Nwani *et al*., 2010
(Bloch) 11cm	(0.264-0.325)	
Aquatic invertebrates		
Daphnia magna	1.5	PPDB, 2014
(water flea)	(-)	

3. Chlorpyrifos

3.1 General information

Chlorpyrifos is a non-systemic organophosphorus insecticide. Chlorpyrifos affects the nervous system by inhibiting the activity of the acetylcholinesterase enzyme. Chlorpyrifos has many negative effects on humans such as nausea, dizziness, confusion, and at very high exposures respiratory paralysis and death (U. S. EPA, 2002). The WHO classifies chlorpyrifos as a category 2 (moderately hazardous) pesticide. Chlorpyrifos is used in agriculture to control pests and indoors to control mosquitoes and fire ants. In Thailand, chlorpyrifos was classified in the red list group in 2000.

3.2 Physical and chemical properties

The chemical name of chlorpyrifos is O, O-diethyl O-(3,5,6-trichloro-2-pyridyl) phosphorothioate. The molecular formula is $C_9H_{11}C_{l3}NO_3PS$ and the relative molecular mass is 350.57. Chlorpyrifos is a clear to white, crystalline solid pesticide with a mild mercaptan odor (U. S. EPA, 2002). The solubility of chlorpyrifos in water is 1.4 mg/L at 25 °C. The chemical abstract service (CAS) registration number is 2921-88-2. The chemical structure of chlorpyrifos is shown in Figure 6.

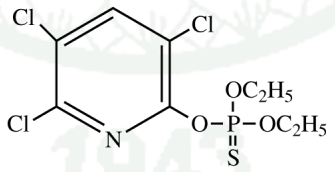

Figure 6 Chemical structure of chlorpyrifos

3.3 Uses

Formulations of chlorpyrifos include emulsifiable concentrate, dust, pellet, and spray. Chlorpyrifos is the active ingredient of many commercial insecticides such as Lorsban20EC®, Lorsban40EC®, Pyrenex®, and Chloridin®. Chlorpyrifos is one of the most widely used organophosphate insecticides which does not adsorb to plant

tissue. Chlorpyrifos is used for control of crop pests in the sucking and biting insect groups such as Coleoptera, Diptera, Homoptera and Lepidoptera found in soil or on foliage, household pests (Blattellidae, Muscidae, Isoptera), mosquitoes (larvae and adults), and pests in animal houses. Chlorpyrifos acts as a cholinesterase inhibitor, with contact, stomach and respiratory action (WHO, 2002).

3.4 Biodegradation of chlorpyrifos

In the environment, chlorpyrifos degrades under abiotic and biotic transformation process. The major pathway of degradation begins with cleavage of the phosphorus ester bond which is in soil, water plant, and animals (Rack, 1993). The primary degradation product is 3, 5, 6-trichloro-2-pyridinol (TCP). TCP is broken down to organochlorine compounds and carbon by microbes and other organisms.

The important degradation processes are hydrolysis and oxidation. The major hydrolysis product is the trichloropyridinol. The hydrolysis of chlorpyrifos increases under alkaline conditions and is strongly influenced by pH. The primary oxidation product is chlorpyrifos oxon which degraded further to trichloropyridinol and diethylphosphate.

Chlorpyrifos degrades slowly in soil primarily through microbial action and it persists in soil for 60-120 days. Chlorpyrifos is slightly soluble in water which indicates that chlorpyrifos may bioaccumulate in fish and other aquatic organisms (John *et al*., 1999; FAO/WHO, 2000). The resulting environmental transformation products are shown in Figure 7.

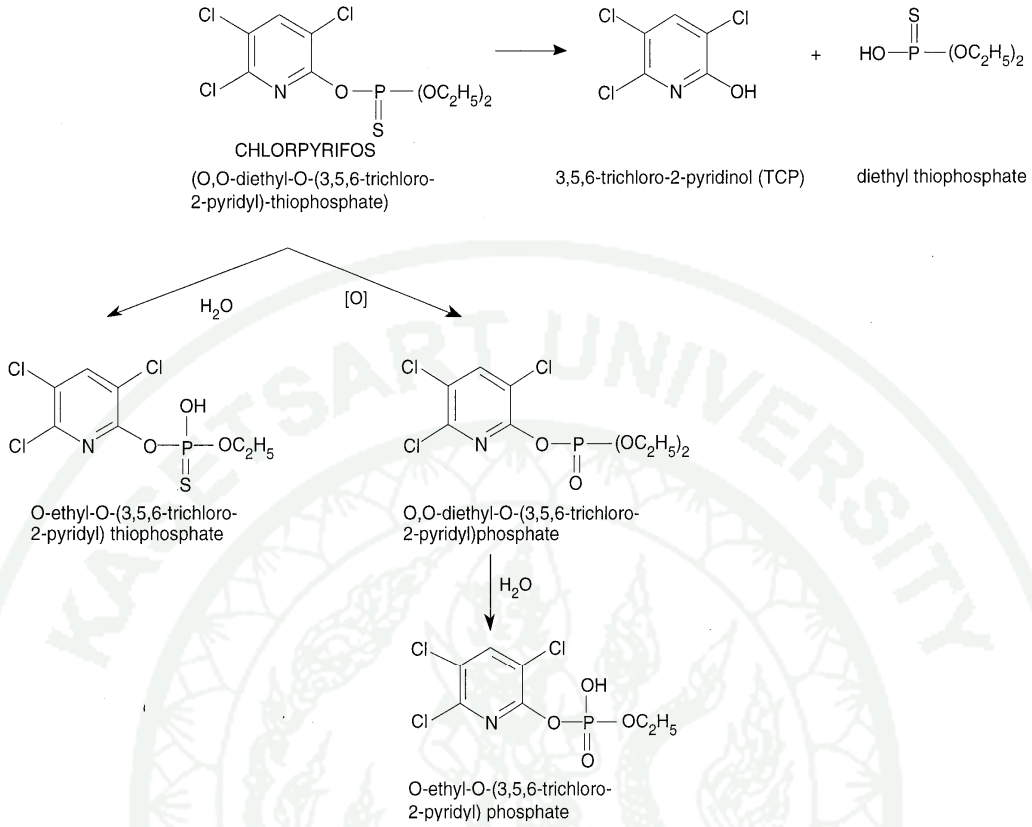

Figure 7 Degradation pathways of chlorpyrifos in the environment

Source: ATSDR (1997)

3.5 Toxicology and ecotoxicology

Chlorpyrifos is highly toxic to fish and aquatic invertebrates (Kamrin, 1997). Bioconcentration factor of chlorpyrifos in invertebrates and fish range from 42 to 5,100 (Mace and Woodburn, 1995). Technical grade chlorpyrifos has similar or greater toxicity than its formulations or emulsifiable concentrate (Jarvinen and Tanner, 1982). The acute toxicity of chlorpyrifos to aquatic organisms is shown in Table 3.

Table 3 The acute toxicity of chlorpyrifos to aquatic organisms

Species	End point	Values (µg/L)	References
Fish			
Oncorhynchus mykiss (rainbow trout) juvenile	96-h LC_{50}	8.0	Holcombe *et al.,* 1982
Pimephales promelas (fathead minnow) 31-32 days old	96-h LC_{50}	203	Holcombe *et al.,* 1982
Pimephales promelas (fathead minnow) larval	96-h LC_{50}	140	Jarvinen and Tanner, 1982
Aquatic invertebrates			
Gammarus lacustris (amphipod) 2 month old ±5 days	96-h LC_{50}	0.11	Sanders, 1969
Daphnia magna (cladoceran) <24 h old	48-h LC_{50}	1.0	Kersting and Wijngaarden, 1992
Daphnia pulex (cladoceran) <24 h old	48-h LC_{50}	0.30	Van and Gerritsen, 1997
Chironomus tentans (midge) fourth instar	96-h EC_{50}	0.51 - 0.75	Pape-Lindstrom and Lydv, 1997
Hyalella azteca (amphipod) juveniles	96-h EC_{50}	138	Brown *et al.*, 1997
Ceriodaphnia dubia (cladoceran) <24 h old	96-h LC_{50}	0.038	CDFG, 1999
Lampsilis siliquoidea (Fatmuket mussel) glochidia	48-h EC_{50}	430	Bringolf *et al.,* 2007c
Daphnia magna (water flea) juvenile, <24 h old	48-h EC_{50}	740 (690-790)	Palma *et al*., 2008
Thamnocephalus Platyurus (Beavertail Fairy Shrimp) Larvae, <24 h old	48-h EC_{50}	530 (260-790)	Palma *et al*., 2008

4. Factors affecting toxicity

The toxicity of pesticides to aquatic organisms may be influenced by exposure conditions, formulation, source and size of fish and water quality. According to Mace and Woodburn (1995) who studied chlorpyrifos toxicity to fish, water hardness and pH has been reported to not influence toxicity because chlorpyrifos is non-polar and non-ionizable. On the other hand, pH and temperature can affect the degradation rate in water, which may influence contaminant exposures in the environment (Racke, 1993).

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 mg/L. 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 has little or no effect on the toxicity of organic chemicals including pesticides such as endosulfan (Pickering and Henderson, 1966; Capkin *et al.,* 2006).

MATERIALS AND METHODS

1. Experimental Preparation

1.1 Preparation of glochidia

1.1.1 Maintenance of adult mussels

Adult *Hyriopsis bialata* Simpson, 1900 were collected from the Dorm River Basin in Ubon Ratchathani province, Thailand. The mussels were transported in chilled cooler to the laboratory of Department of Zoology, Faculty of Science, Kasetsart University, Bangkok. The mussels were sexually identified by microscopic observation of sperms and eggs in fluid aspirated from the gonad. They were cultured in circle nets (50 cm in diameter and 50 cm in height) in water 150 cm deep in an earthen pond at the Faculty of Fisheries, Kasetsart University, Bangkok, Thailand. The mussels were fed with natural food in the pond. 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 (Kovitvadhi *et al*., 2006).

1.1.2 Collecting glochidia

To prepare the mussels for collection of glochidia, soil and algae were thoroughly removed from the outside 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 number of opened and closed glochidia were counted before, and within 1 min of adding 2-3 drops of NaCl solution. Glochidia that closed in response to NaCl solution

were defined as alive (or viable) and the glochidia that closed before addition of NaCl or that remained open after addition of NaCl were defined as dead (nonviable).

1.1.2.1 Determining a suitable temperature and survival time for standard toxicity tests of *H.bialata* glochidia

(1) Preparation of water

Tap water was filtered using 25 μm mesh size filter bag to remove particles and was then stored in tanks. Water was aerated at least 2 days to remove chlorine then filtered with a carbon filter to remove residual chloramines and other chlorinated organic compounds before use.

(2) Experimental design

The experiment consisted of 24 treatments which were combinations of at four water temperatures (15, 20, 25 or 30°C), and six rearing times (0, 1, 6, 12, 24 or 48h); and each treatment had three replicates. Glochidia from several gravid female mussels were pooled and placed in petri dishes (diameter x height, 35 x 10 mm). Each replicate consisted of 50-100 glochidia/dish and 3.5 mL of dechlorinated tap water. Petri dishes were placed in temperature controlled chambers, in the dark. Survival of glochidia was determined under light microscope by adding 2-3 drops of NaCl solution to the glochidia from each the petri dish.

1.2 Preparation of dilution water

Dechlorinated tap water was used as dilution water for the first set of toxicity tests. Reconstituted soft, moderately-hard and hard water were used as dilution water for the remaining tests. Experimental water was made using standard methods (ASTM, 2003) for reconstituted soft water, moderately-hard water, and hard water. Distilled water was used for preparation and stored in 6 L plastic containers. Nominal components of reconstituted water are reported in Table 4.

Table 4 Preparation of reconstituted water for toxicity tests using reagent grade chemicals.

Water	Reagent Added (mg/L)				Hardness
	$NaHCO_3$	$CaSO_4 \cdot 2H_2O$	$MgSO_4$	KCl	(mg/L as $CaCO_3$)
Soft	48.0	30.0	30.0	2.0	40-48
Moderately-hard	96.0	60.0	60.0	4.0	80-100
Hard	192.0	120.0	120.0	8.0	160-180

1.3 Preparation of test solutions for toxicity test

1.3.1 A commercial carbosulfan formulation 20 EC (20% w/v active ingredient) and a commercial chlorpyrifos formulation 40 EC (40% w/v 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 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 4 or 5 test concentrations.

1.4 Preparation of test glassware

The test containers were round with a diameter of 6 cm and 100 cm^3 capacities. All test containers were washed before using then air dried. After each experiment, all containers used were soaked 15 min in tap water, scrubbed with detergent, and rinsed twice with tap water. All glassware was rinsed with acetone and distilled water to avoid pesticide contamination.

2. Experimental procedures

Acute toxicity tests with glochidia were conducted according to standardized guidelines ASTM (2006) and Clearwater *et al*. (2014).

The viability of glochidia isolated from each female mussel was determined before starting a toxicity test by adding a saturated NaCl solution to subsamples of 100-120 glochidia. Numbers of open and closed glochidia were counted before addition of NaCl and within 1 min after addition (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. Equal amounts of glochidia (ranging from 300-500 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 16 h light:8 h dark. After 6, 24, and 48 h, the viability of exposed glochidia in each treatment was examined under light microscope (40x 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.

2.1 Acute toxicity of carbosulfan, chlorpyrifos and a combined exposure to carbosulfan and chlorpyrifos to *H. bialata* glochidia

2.1.1 Range-Finding Test

A range-finding test was conducted to establish the approximate concentration range of pesticides with a test duration of 6 to 48 h that should be used in the definitive test. The concentrations used in a range-finding test were based on the 48 h EC_{50} of chlorpyrifos on *Lampsilis siliquoidea* glochidia (Bringolf *et al*., 2007c).

2.1.2 Definitive Test

Nominal concentrations were 0.01, 0.02, 0.03, 0.06, 0.10 mg/L for carbosulfan; 0.04, 0.05, 0.07, 0.11, 0.15, 0.21 mg/L for chlorpyrifos and 0.019:0.007, 0.029:0.10, 0.044:0.015, 0.66:0.023, 0.099:0.034 and 0.148:0.051 mg/L carbosulfan:chlorpyrifos for the combination of carbosulfan and chlorpyrifos. Dechlorinated tap water was used as the diluent and control with three replicates per

concentration. The viability of glochidia was assessed at 6, 24, and 48 h after exposure of glochidia to the test solutions.

2.2 Examining the effect of water hardness on the toxicity of carbosulfan and chlorpyrifos to *H. bialata* glochidia

2.2.1 Range-Finding Test

A range-finding test was conducted to establish the approximate concentration range of pesticides with a test duration of 6 to 48 h that should be used in the definitive test. The concentrations used in a range-finding test were based on the 48 h EC_{50} of carbosulfan and chlorpyrifos on *H. bialata* glochidia determined in previous tests.

2.2.2 Definitive Test

Nominal concentrations were: 0.01, 0.03, 0.05, 0.10 mg/L for carbosulfan; 0.05, 0.08, 0.14, 0.22, 0.36 mg/L for chlorpyrifos; and 0.016:0.046, 0.023:0.069, 0.036:0.105, 0.056:0.159 mg/L carbosulfan:chlorpyrifos for the combination of carbosulfan and chlorpyrifos. Reconstituted soft, moderately-hard, and hard water were used as the diluents and control treatments with three replicates per concentration. The viability of glochidia was assessed at 6, 24, and 48 h after exposure of glochidia to the test solutions.

3. Water analysis

Water chemistry and physical parameters were measured at the start 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, AWWA, WPCF, 1998).

4. 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 were prepared in acetonitrile at a concentration of 1 mg/L and diluted in 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 of analyses 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.

5. Statistical analysis

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

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

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 and closed, but all of the glochidia in the subsamples were counted in the ASTM (2006) method.

Median effective concentration (EC_{50}) estimates and 95% confidence intervals (CI), the no observed effect concentration (NOEC), and the lowest observed effect concentration (LOEC) values were analysed based on nominal concentrations using the Comprehensive Environmental Toxicity Information System software package (CETIS v1.7.0.2, Tidepool Scientific Software). Survival curves were produced using the statistical analysis component (Regression Wizard) of Sigma Plot v.10.0. Data are reported as mean ± standard deviation (SD) unless stated otherwise.

Significant differences between means were analyzed and ranked by one-way analysis of variance (ANOVA) for EC_{50} values and the average percentage survival of glochidia followed by Tukey's post-hoc test and Duncan's multiple range tests, respectively. Two-way ANOVA was used to compare the average percentage survival of glochidia at different test temperatures and test durations and the effect of water hardness on pesticides toxicity followed by Duncan's multiple range tests and Tukey's post-hoc test, respectively. The level of statistical significance was set at $P \leq 0.05$.

RESULTS AND DISCUSSION

Results

1. Survival of glochidia

1.1 Characteristics of water

All water quality parameters were in the same range for all experiments.

Table 5 The physico-chemical characteristics of dechlorinated tap water used for the experiments

Parameter	Unit	Mean±SD
Temperature	°C	26.8±0.27
pH	-	8.23±0.03
Dissolved oxygen	mg/L O_2	8.90±0.05
Total alkalinity	mg/L $CaCO_3$	73.7±0.80
Free carbondioxide	mg/L CO_2	1.94±0.97
Total hardness	mg/L $CaCO_3$	100.0±3.20
Total ammonia-nitrogen	mg/L NH_3-N	0.32±0.10
Calcium	mg/L $CaCO_3$	68.3±3.00
Silica	mg/L SiO_2	11.6±0.50

1.2 Survival of glochidia at different temperatures and test durations

Survival of glochidia at different test temperatures and test durations was compared using a two-way ANOVA. The average survival of glochidia at different temperatures of 15, 20, 25, and 30ºC was not significantly different (P>0.05). Average survival at 15, 20, 25, and 30ºC was 96.0±3.0, 96.4±2.3, 95.9±3.58 and 96.6±2.14%, respectively. The average survival of glochidia at 0, 1, 6, 12, 24, and 48 h of test duration was significantly different (P<0.01), and was 97.5±1.0, 98.2±1.7, 97.4±2.1,

95.5±2.5, 94.2±2.0, and 93.8±3.9%, respectively. Specifically, the percentage survival at 0, 1 and 6 h was not significantly different (P>0.05) whereas average survival at 12 h was significantly higher than at 24 and 48 h (P<0.05).

The percentage survival of glochidia from the 3 samples of gravid female in the experiment were not significantly different (P>0.05). The highest average survival of glochidia over time was after 1 h with average survival of 98.2±1.7% (Figure 8). The interaction between temperature and time on survival of the *Hyriopsis bialata* glochidia was not significantly different (P>0.05). The results indicated that toxicity test of *H. bialata* could be conducted over a wide range of temperatures from 15 to 30 ºC and that average survival after 48 h was not significantly different so a standard glochidia toxicity test for this species should be 25 ºC in the duration of 2 days.

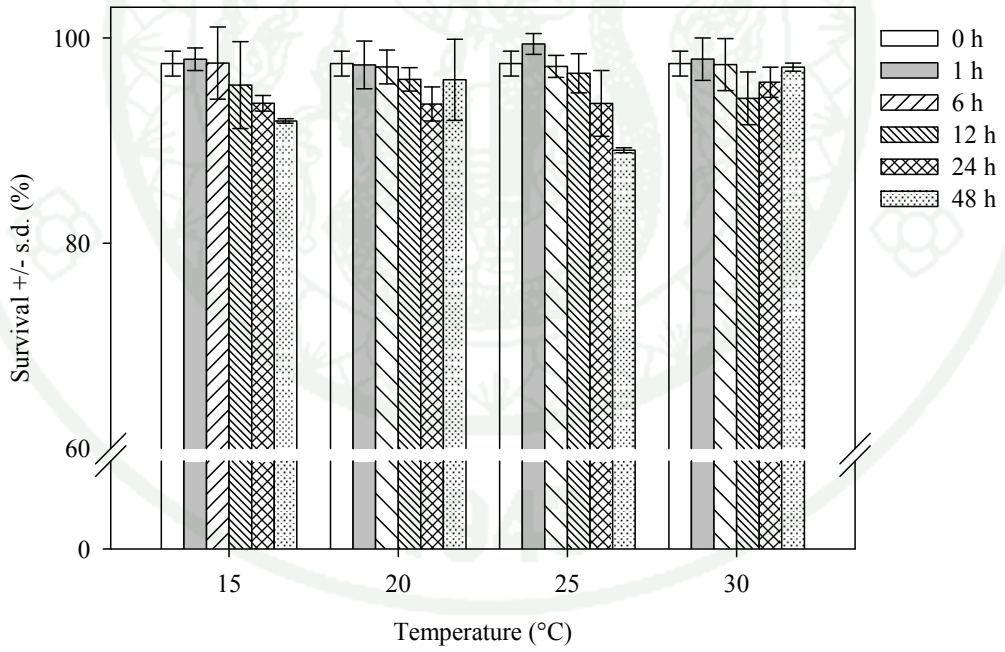

Figure 8 The percentage survival of *Hyriopsis bialata* glochidia at different temperatures (15, 20, 25, and 30 ºC) and test times (0, 1, 6, 12, 24, and 48 h)

2. Acute toxicity tests of glochidia

2.1 Acute toxicity of carbosulfan

Water quality was measured in all of the experiments and, in summary, water temperatures ranged from 25 to 26 °C, dissolved oxygen ranged from 6 to 7 mg/L, pH ranged from 7.3 to 7.8, and conductivity ranged from 373 to 444 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 $CaCO_3$.

The percentage survival of glochidia in the control treatment up to 24 h was greater than the minimum 90% recommended by ASTM (2006). The exception was control treatment survival decreased to 87.7±2.4% after 48 h exposure, but was not significantly different to percentage survival of glochidia in all carbosulfan concentrations. The highest percentage survival of glochidia in the control treatment was 95.5±2.0 % at 24 h. The percentage survival of glochidia exposed to carbosulfan concentrations of 0.01, 0.02, 0.03, 0.06, and 0.10 mg/L for 6, 24, and 48 h were not significantly different to glochidia survival in the control treatment (P>0.05) (Table 6).

Table 6 Percentage survival of *Hyriopsis bialata* glochidia after exposure to carbosulfan in dechlorinated tap water

Carbosulfan (mg/L)	% Average survival[(1)]		
	6 h	24 h	48 h
0.00	95.3±1.5	95.5±2.0	87.7±2.4
0.01	97.3±1.2	96.3±2.3	88.2±2.7
0.02	95.7±2.5	96.2±3.7	88.7±6.7
0.03	95.0±2.6	98.0±0.9	91.7±5.0
0.06	95.0±1.0	96.0±2.0	93.0±3.6
0.10	93.7±2.9	92.4±3.9	81.7±7.1

[(1)] Data are means ± SD (n=3).

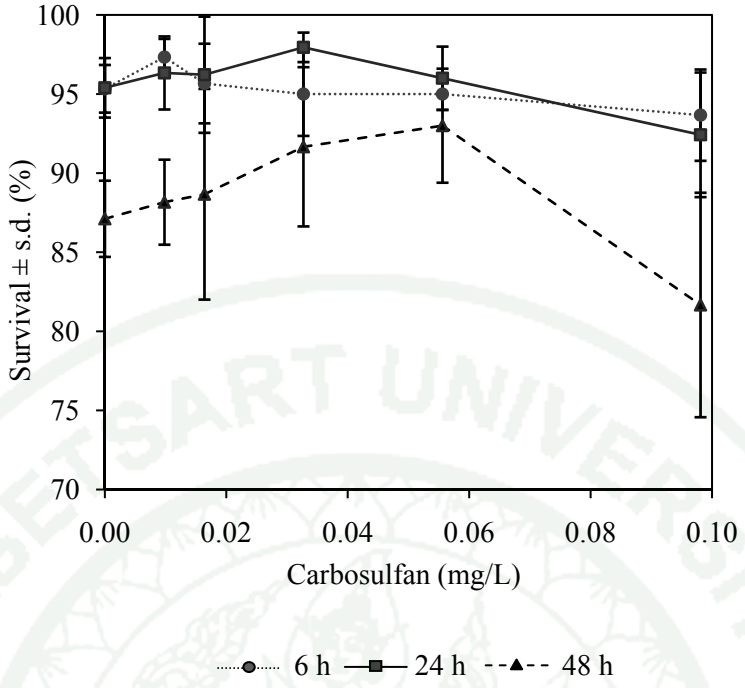

Figure 9 Percentage survival of *Hyriopsis bialata* glochidia after exposure to carbosulfan for 6, 24, and 48 h in dechlorinated tap water

The no observed effect concentration (NOEC), the lowest observed effect concentration (LOEC), and the median effect concentrations (EC_{50}) with 95% confidence interval (CI) of carbosulfan on *H. bialata* glochidia at the exposure times of 6, 24, and 48 h are reported in Table 7. The LOEC and EC_{50} of carbosulfan could not be calculated from the acute toxicity test because the percentage mortality of glochidia was less than 50% in all treatments.

Table 7 The toxicity end points for carbosulfan tested on *Hyriopsis bialata* glochidia in dechlorinated tap water

Time (h)	NOEC (mg/L)	LOEC (mg/L)	EC_{50} (mg/L) (95% confidence interval)
6	0.1	>0.1	>0.1
24	0.1	>0.1	>0.1
48	0.1	>0.1	>0.1

2.2 The effect of water hardness on carbosulfan toxicity

Water quality was measured in all of the experiments and in summary, water temperatures ranged from 24 to 25 °C, dissolved oxygen ranged from 6 to 8 mg/L, pH ranged from 7.2 to 8.0, and conductivity ranged from 152 to 909 µS/cm. Hardness ranged from 42-60 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. Alkalinity of the 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 effect of water hardness on carbosulfan toxicity was assessed using a single batch of glochidia (from three mussels) exposed concurrently in either standard reconstituted soft water, moderately-hard water, or hard water. 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 (Table 8). Glochidia survival was not, however, significantly different across all treatments (P>0.05).

A two-way ANOVA showed that the percentage survival of glochidia exposed to carbosulfan concentrations of 0.01, 0.03, 0.05, and 0.10 mg/L for 6, 24, and 48 h were not significantly different to glochidia survival in the control treatment (P>0.05). There was no significant main effect of hardness, carbosulfan concentrations concentrations, or any interaction between hardness and carbosulfan concentration on the survival of *H. bialata* glochidia after exposures of 6, 24, or 48 h (P>0.05).

Table 8 The effect of water hardness on survival of *Hyriopsis bialata* glochidia after exposure to carbosulfan

Hardness	Carbosulfan (mg/L)	% Average survival[(1)]		
		6 h	24 h	48 h
Soft	0.00	96.0±1.0	94.0±1.7	95.0±1.0
	0.01	96.3±3.1	95.7±2.3	92.6±3.9
	0.03	96.0±2.6	94.6±1.5	94.0±2.6
	0.05	97.0±1.0	95.0±2.6	98.0±0.0
	0.10	94.7±2.1	94.3±0.6	95.0±3.5
Moderately- hard	0.00	98.3±2.9	97.0±2.6	94.7±1.5
	0.01	97.7±1.2	95.3±1.5	90.7±3.2
	0.03	97.0±2.0	97.7±0.6	97.3±1.5
	0.05	96.7±1.5	95.0±0.0	94.3±0.6
	0.10	94.7±3.2	93.7±2.5	96.0±2.6
Hard	0.00	95.0±2.0	98.0±1.0	93.0±2.6
	0.01	93.5±4.0	98.0±1.7	94.3±3.1
	0.03	96.3±0.6	95.3±1.5	93.0±3.0
	0.05	96.0±0.0	95.7±2.1	94.3±0.6
	0.10	95.7±2.1	96.3±3.1	89.3±4.6

(1) Data are means ± SD (n=3).

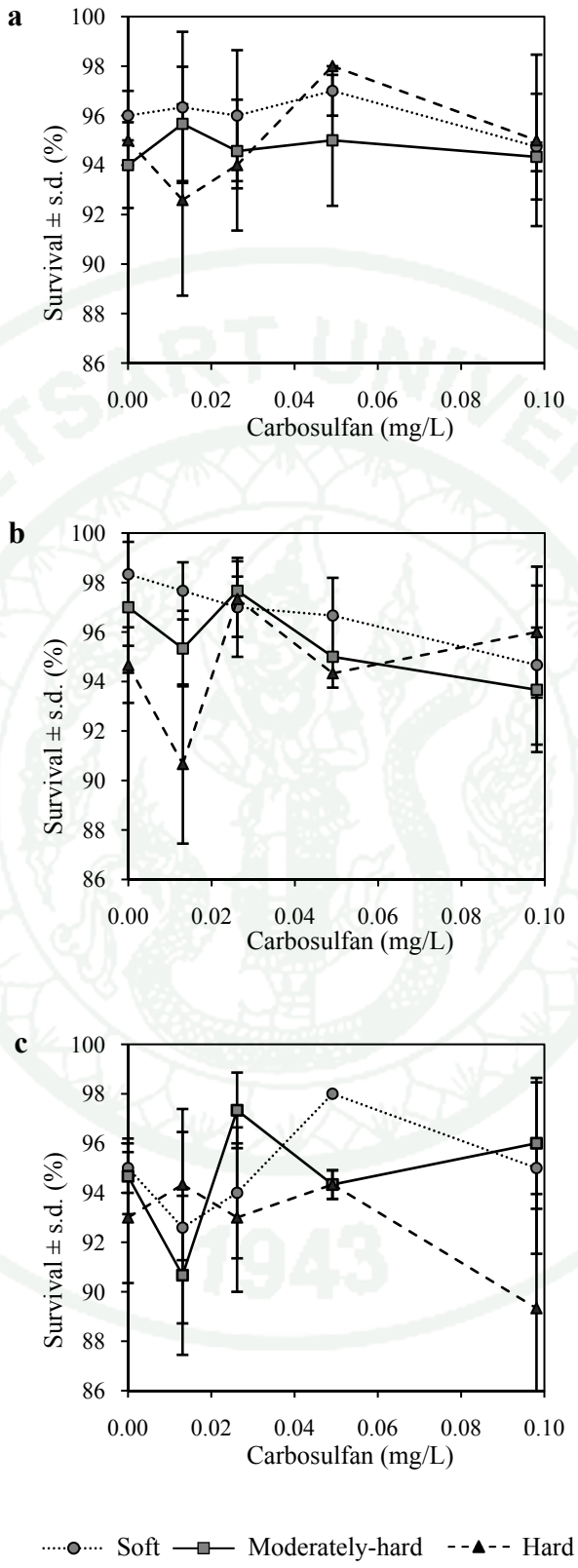

Figure 10 Percentage survival of *Hyriopsis bialata* glochidia after exposure to carbosulfan in either (a) soft, (b) moderately-hard, or (c) hard water

The NOEC, LOEC, and EC_{50}s of carbosulfan for glochidia of *H. bialata* at the exposure times of 6, 24, and 48 h are reported in Table 9. The EC_{50} of carbosulfan could not be calculated in the acute toxicity test because the mortality of glochidia was less than 50% in all treatments. Thus, carbosulfan toxicity to glochidia is occurs at concentrations greater than 0.1 mg/L. At the concentrations tested, carbosulfan EC_{50}s were not significantly different in soft water, moderately-hard water, and hard water (P>0.05).

Table 9 The toxicity end points for carbosulfan tested on *Hyriopsis bialata* glochidia at different water hardness concentrations

Hardness	Time (h)	NOEC (mg/L)	LOEC (mg/L)	EC_{50} (mg/L) (95% confidence interval)
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

2.3 Acute toxicity of chlorpyrifos

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 $CaCO_3$.

The mean survival of glochidia in the control treatment was more than 90% throughout the 48 h test, with the highest mean survival of 96.2±1.2% at 6 h (Table 10). The percentage survival of glochidia in this concentration was 8%, 30%, and 43% lower respectively than 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) (Figure 11).

Table 10 Percentage survival of *Hyriopsis bialata* glochidia after exposure to chlorpyrifos in dechlorinated tap water

Chlorpyrifos (mg/L)	% Average survival[(1)]		
	6 h	24 h	48 h
0.00	96.2±1.2[a(2)]	95.7±1.6[a]	95.8±2.1[a]
0.04	97.7±1.5[a]	94.7±3.1[a]	92.7±3.5[a]
0.05	97.7±0.6[a]	95.7±1.5[a]	89.0±7.0[a]
0.07	88.0±2.6[b]	66.0±7.0[b]	53.0±22.7[b]
0.11	45.3±2.5[c]	2.7±1.2[c]	1.0±1.7[c]
0.15	3.3±1.5[d]	0.0±0.0[c]	5.7±9.8[c]
0.21	2.7±2.1[d]	0.0±0.0[c]	3.0±5.2[c]

(1) Data are means ± SD (n=3).

(2) Treatments with different letters was significantly different (Tukey's test, α = 0.05).

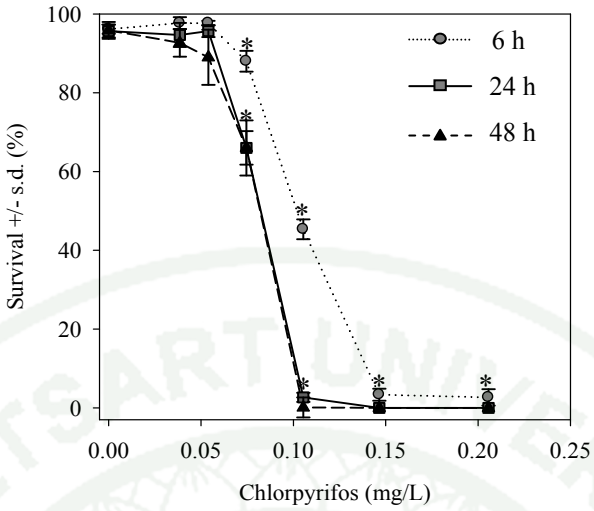

Figure 11 Percentage survival of *Hyriopsis bialata* glochidia after exposure to chlorpyrifos for 6, 24, and 48 h in dechlorinated tap water
* indicates survival was significantly different from controls.

The NOEC, LOEC, and EC_{50} for *H. bialata* glochidia exposed to chlorpyrifos for 6, 24, or 48 h are reported in Table 11. The NOEC of chlorpyrifos was 0.05 mg/L at all exposure indicating that the percentage survival of glochidia was not significantly different to the control treatment (P>0.05). The 6 h EC_{50} was significantly different to 24 h EC_{50} and 48 h EC_{50} (P<0.05), thus, results demonstrated the EC_{50} values of chlorpyrifos on *H. bialata* glochidia showed a progressive decrease when exposure times were increased to 24 h. In addition, chlorpyrifos was more toxic overtime indicating that chlorpyrifos was faster-acting toxicant in dechlorinated tap water.

Table 11 The toxicity end points for chlorpyrifos tested on *Hyriopsis bialata* glochidia in dechlorinated tap water

Time (h)	NOEC (mg/L)	LOEC (mg/L)	EC_{50} (mg/L) (95% confidence interval)
6	0.05	0.07	0.103 (0.100-0.107)[a (1)]
24	0.05	0.07	0.083 (0.079-0.087)[b]
48	0.05	0.07	0.078 (0.062-0.092)[b]

(1) Treatments with different letters was significantly different (Tukey's test, α= 0.05).

2.4 The effect of water hardness on chlorpyrifos toxicity

Water quality was measured in all of the experiments and in summary water temperatures ranged from 23 to 25 °C, dissolved oxygen ranged from 5 to 7 mg/L, pH ranged from 7.6 to 8.7, and conductivity ranged from 151 to 662 µS/cm. In the reconstituted water experiment, hardness ranged from 42-60 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. Alkalinity of the 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 effect of water hardness on chlorpyrifos toxicity was assessed using a single batch of glochidia (from three 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 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 97.3±0.6% in soft water, 97.0±2.0% in moderately-hard water, and 98.0±1.0% in hard water (Table 12). There was no mortality of glochidia greater than 50% after exposure to chlorpyrifos concentrations up to 0.36 mg/L for 6 h in soft water. Glochidia survival decreased to less than 10% after exposure to 0.36 mg/L chlorpyrifos for 24 h in soft water, while in moderately-hard and hard water survival decreased to less than 10% after exposure to 0.22 mg/L chlorpyrifos for 48 h exposure (Figure 12).

The results demonstrated a highly significant effect of hardness on the percentage survival of glochidia after exposure to chlorpyrifos for 6, 24, and 48 h (P<0.001). On the other hand, there was a highly significant main effect of chlorpyrifos concentration (P<0.001), and there was also a significant interaction effect between the hardness and chlorpyrifos concentration on the survival of *H. bialata* glochidia after 6, 24, and 48 h exposure (P<0.001).

Table 12 The effect of water hardness on survival of *Hyriopsis bialata* glochidia after expose to chlorpyrifos

Hardness	Chlorpyrifos (mg/L)	% Average survival[1]		
		6 h	24 h	48 h
Soft	0.00	96.3±2.1[a(2)]	97.0±1.7[a]	91.3±2.3[a]
	0.05	96.3±1.5[a]	95.3±1.2[a]	89.3±2.1[a]
	0.08	97.3±0.6[a]	93.7±0.6[a]	92.0±3.6[a]
	0.14	93.3±2.1[a]	93.0±3.0[a]	91.0±2.6[a]
	0.22	83.7±3.2[b]	60.3±5.1[b]	12.0±7.0[b]
	0.36	52.7±4.5[c]	0.0±0.0[c]	0.0±0.0[c]
Moderately-hard	0.00	97.0±2.0[a]	95.7±2.5[a]	92.3±7.0[a]
	0.05	95.0±2.6[a]	91.7±1.5[ab]	89.7±6.8[a]
	0.08	91.0±2.6[ab]	82.0±6.9[bc]	80.7±5.7[a]
	0.14	81.3±3.8[b]	70.0±4.4[c]	12.7±12.6[b]
	0.22	43.7±6.1[c]	11.3±7.5[d]	0.7±0.6[b]
	0.36	23.3±7.6[d]	0.0±0.0[e]	0.3±0.6[b]
Hard	0.00	98.0±1.0[a]	97.3±1.2[a]	91.3±2.5[a]
	0.05	94.3±1.5[a]	93.0±2.6[a]	82.0±4.4[ab]
	0.08	90.3±3.8[ab]	91.7±1.5[b]	84.7±2.0[ab]
	0.14	81.3±1.5[b]	90.0±1.7[c]	61.3±24.5[b]
	0.22	54.0±8.0[c]	20.7±9.0[d]	3.7±5.5[c]
	0.36	20.7±2.9[d]	3.3±1.2[e]	0.0±0.0[c]

[1] Data are means ± SD (n=3).

[2] Treatments with different letters was significantly different (Tukey's test, $\alpha = 0.05$).

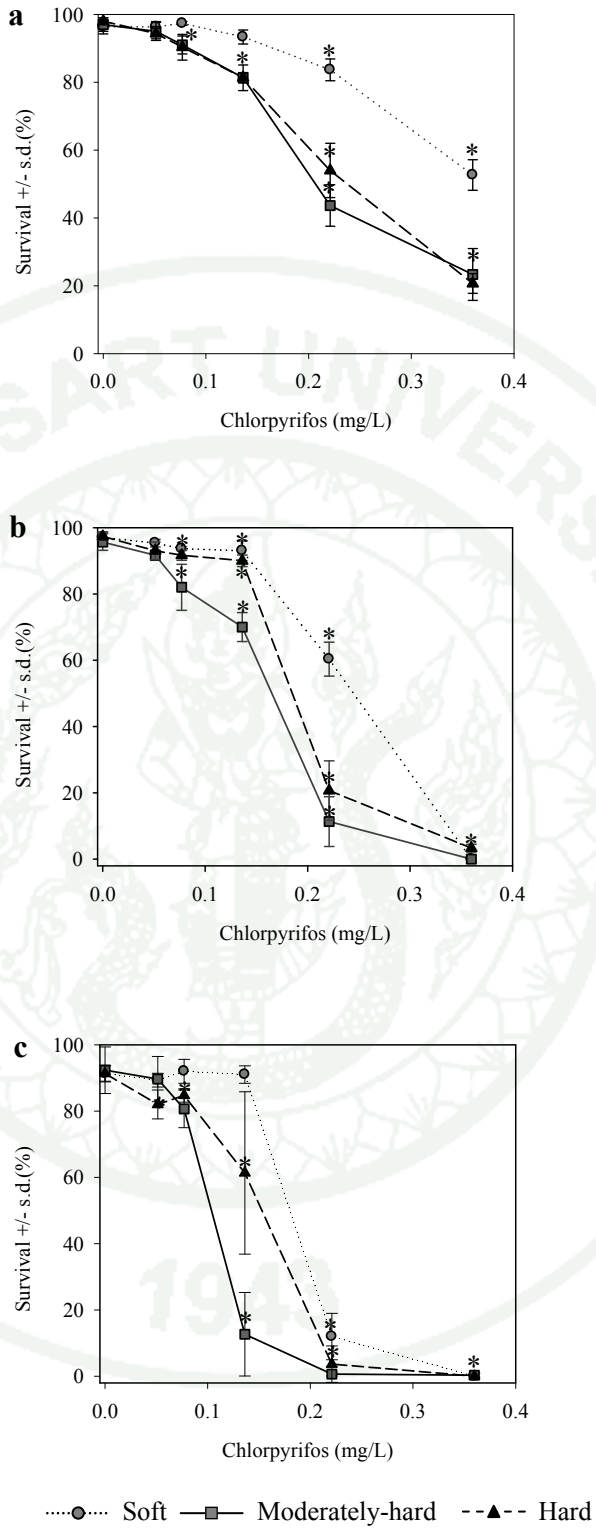

Figure 12 Percentage survival of *Hyriopsis bialata* glochidia after exposure to chlorpyrifos at different water hardness concentrations for either (a) 6, (b) 24, or (c) 48 h. * indicates survival was significantly different from controls.

The NOEC, LOEC, and EC_{50}s for *H. bialata* glochidia exposed to chlorpyrifos for 6, 24, and 48 h are shown in Table 13. In soft water, the chlorpyrifos EC_{50} for 6 h was significantly different to the 24 and 48 h EC_{50} (P<0.05). There was a significant decrease in the chlorpyrifos EC_{50} between the exposure times of 6, 24, and 48 h in moderately-hard water and hard water (P<0.05). 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 13). The results demonstrated 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 (P<0.05) (Figure 13). The results indicated that increasing water hardness from moderate to high hardness had toxic effect on glochidia survival.

Table 13 The toxicity end points for chlorpyrifos tested on *Hyriopsis bialata* glochidia at different water hardness concentrations

Hardness	Time (h)	NOEC (mg/L)	LOEC (mg/L)	EC_{50} (mg/L) (95% confidence interval)
Soft	6	0.14	0.22	>0.36[a(1)]
	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]

(1) Treatments not sharing the same letters was significantly different (Tukey's test, α = 0.05).

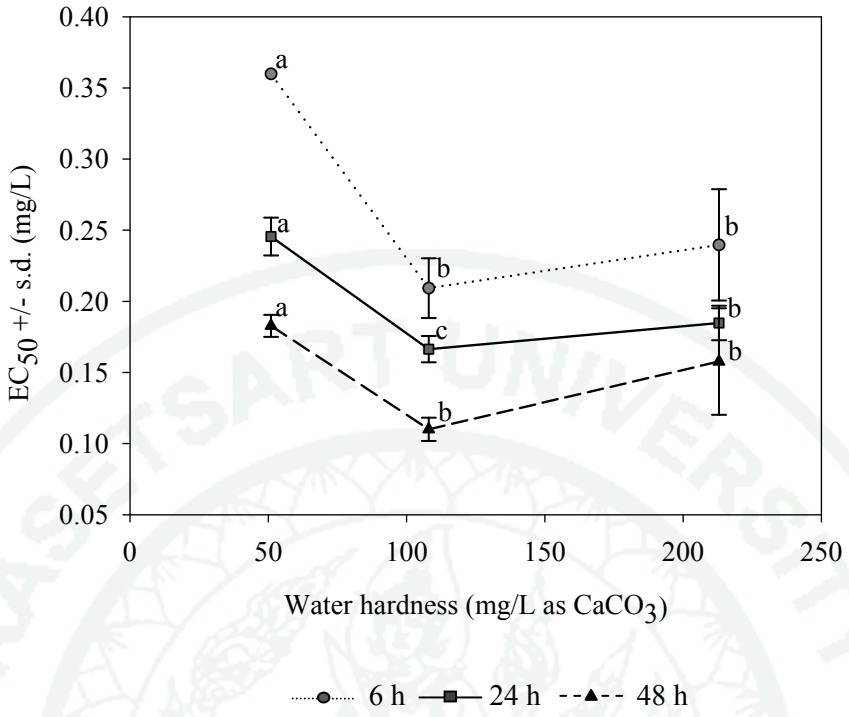

Figure 13 The EC_{50} values after exposure to chlorpyrifos of *Hyriopsis bialata* glochidia at different water hardness concentrations for 6, 24, and 48 h

2.5 Acute toxicity of a combined exposure to carbosulfan and chlorpyrifos in dechlorinated tap water

Water quality was measured in all of these experiments and, in summary, water temperatures ranged from 25 to 27 °C, dissolved oxygen ranged from 6 to 7 mg/L, pH ranged from 7.6 to 8.0, and conductivity ranged from 373 to 497 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 $CaCO_3$.

The survival of glochidia in the control treatments was more than 90% throughout the test with the highest mean survival of 97.0±2.3% at 6 h (Table 14). There was no mortality of glochidia greater than 10% after exposure to any combination of carbosulfan:chlorpyrifos for 6 h and the percentage survival of glochidia exposed to all the combination treatments for 6 h were not significantly different to glochidia in the control treatment (P>0.05). The combination of 0.099

mg/L carbosulfan: 0.034 mg/L chlorpyrifos decreased glochidia survival after 24 h exposure and was highly significantly different to glochidia survival in the control treatment (P<0.001). The lowest percentage survival of glochidia was less than 40% after 48 h exposure in the highest combined concentration in this experiment (Figure 14).

Table 14 Percentage survival of *Hyriopsis bialata* glochidia after exposure to a combination of carbosulfan and chlorpyrifos in dechlorinated tap water

Carbosulfan (mg/L)	Chlorpyrifos (mg/L)	% Average survival[1]		
		6 h	24 h	48 h
0.000	0.000	97.0± 2.3	94.8±2.0^{a}[2]	93.5±2.7^{a}
0.007	0.019	96.7±0.6	97.3±1.5^{a}	86.0±1.7^{ab}
0.010	0.029	96.3±1.5	95.7±1.2^{a}	86.0±3.0^{ab}
0.015	0.044	93.3±3.1	93.0±1.7^{a}	90.0±1.7^{ab}
0.023	0.066	93.3±3.8	94.0±2.0^{a}	85.7±0.6^{ab}
0.034	0.099	91.0±1.7	79.3±2.1^{b}	78.0±4.4^{b}
0.051	0.148	94.0±2.6	65.3±10.7^{c}	39.7±12.9^{c}

[1] Data are means ± SD (n=3).

[2] Treatments with different letters was significantly different (Tukey's test, α = 0.05).

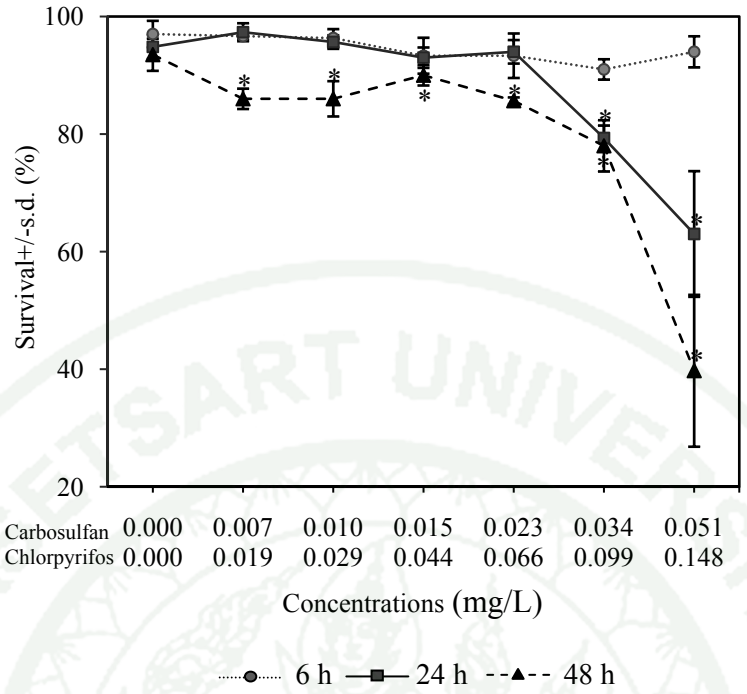

Figure 14 Percentage survival of *Hyriopsis bialata* glochidia after exposure to combination of carbosulfan and chlorpyrifos for 6, 24, and 48 h in dechlorinated tap water. * indicates survival was significantly different from controls.

The NOEC, LOEC, and EC_{50} for *H. bialata* glochidia exposed to a combination of carbosulfan and chlorpyrifos for 6, 24, or 48 h are shown in Table 15 (expressed as chlorpyrifos concentrations). The 6 h EC_{50} and 24 h EC_{50} of the combination of carbosulfan and chlorpyrifos could not be calculated because the mean mortality of glochidia was less than 50% in these treatments. The combination was greater effect with increasing exposure time. Comparison of the single exposure tests in dechlorinated tap water that the EC_{50} for the combination of carbosulfan and chlorpyrifos was less than the 48 h EC_{50} value of carbosulfan (>0.1 mg/L) (Table 7) but it was greater than 48 h EC_{50} value of chlorpyrifos (0.078 mg/L) (Table 11).

Table 15 The toxicity end points[1] for *Hyriopsis bialata* glochidia exposed to a combination of chlorpyrifos and carbosulfan at a ratio of 2.9:1

Time (h)	NOEC (mg/L)	LOEC (mg/L)	EC_{50} (mg/L)[1] (95% confidence interval)
6	0.07	0.1	>0.16
24	0.07	0.1	>0.16
48	<0.02	0.02	0.15 (0.12-NA)[2]

[1] Data were expressed as chlorpyrifos concentrations.

[2] NA = not available

2.6 The effect of water hardness on the acute toxicity of a combination of carbosulfan and chlorpyrifos to *H. bialata* glochidia

Water quality was measured in all of the experiments and, in summary, water temperatures ranged from 24 to 27 °C, dissolved oxygen ranged from 6 to 8 mg/L, pH ranged from 7.7 to 8.4, and conductivity ranged from 131 to 608 µS/cm. In the reconstituted water experiment, hardness ranged from 48-62 mg/L as $CaCO_3$ in soft water, 96-106 mg/L as $CaCO_3$ in moderately-hard water, and 188-220 mg/L as $CaCO_3$ in hard water. Alkalinity of the 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 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. There was no mean mortality of glochidia greater than 50% after exposure to the combination of carbosulfan and chlorpyrifos for 6 h in either soft, moderately-hard, or hard water.

The highest combination concentration of 0.056 mg/L carbosulfan: 0.159 mg/L chlorpyrifos significantly decreased glochidia survival after 24 h exposure

(P<0.001) and the lowest mean survival of glochidia was less than 10% and 30% after 48 h exposure in soft water and moderately-hard water, respectively (P<0.001). On the other hand, the mean survival of glochidia was 0±2.6% after 24h exposure to the same combination concentration in hard water (P<0.001) (Table 16).

Table 16 The effect of water hardness on survival of *Hyriopsis bialata* glochidia after expose to a combination of carbosulfan and chlorpyrifos

Hardness	Carbosulfan (mg/L)	Chlorpyrifos (mg/L)	% average survival[(1)]		
			6 h	24 h	48 h
Soft	0.000	0.000	96.0±1.0^{a}	95.0±1.7^{a}	95.0±0.0^{a}
	0.016	0.046	94.0±1.0^{a}	94.1±2.8^{a}	89.7±4.7^{a}
	0.023	0.069	94.3±1.2^{a}	93.1±4.4^{a}	82.8±4.1^{ab}
	0.036	0.105	93.0±1.0^{ab}	91.1±2.9^{a}	74.3±8.1^{b}
	0.056	0.159	89.4±3.7^{b}	43.6±11.1^{b}	8.0±5.6^{c}
Moderately-hard	0.000	0.000	96.7±0.6^{a}	96.7±3.5^{a}	97.0±1.0^{a}
	0.016	0.046	93.3±5.5^{a}	91.8±3.3^{a}	93.0±1.0^{a}
	0.023	0.069	90.7±4.7^{a}	88.1±9.2^{a}	85.2±8.5^{ab}
	0.036	0.105	89.0±2.6^{a}	91.4±7.5^{a}	72.3±2.3^{b}
	0.056	0.159	93.0±3.5^{a}	67.3±3.1^{b}	29.7±11.0^{c}
Hard	0.000	0.000	97.0±0.0^{ab}	98.7±2.3^{a}	96.0±3.5^{a}
	0.016	0.046	98.0±0.0^{a}	91.3±1.2^{b}	80.7±3.2^{b}
	0.023	0.069	88.7±2.1^{bc}	74.3±5.1^{c}	43.0±11.5^{b}
	0.036	0.105	82.7±3.2^{c}	13.7±6.1^{d}	0.0±0.0^{c}
	0.056	0.159	67.3±6.03^{d}	0.0±2.6^{e}	0.0±0.0^{c}

[(1)] Data are means ± SD (n=3).

[(2)] Treatments with different letters was significantly different (Tukey's test, $\alpha = 0.05$).

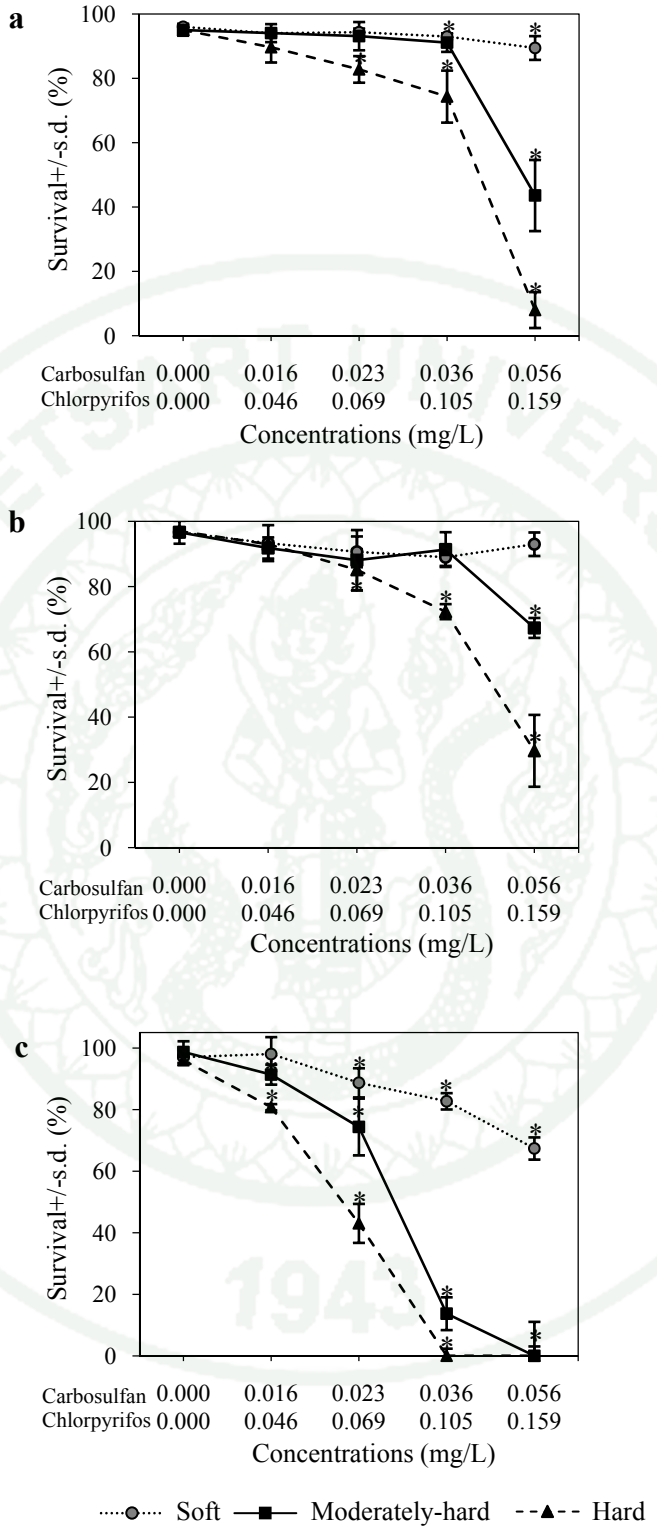

Figure 15 Percentage survival of *Hyriopsis bialata* glochidia after exposure to a combination of carbosulfan and chlorpyrifos at different water hardness concentrations for either (a) soft, (b) moderately-hard, or (c) hard water
* indicates survival was significantly different from controls.

The NOEC, LOEC, and EC_{50} for *H. bialata* glochidia exposure to a combination of carbosulfan and chlorpyrifos on glochidia for 6, 24, or 48 h at different water hardness concentrations are reported in Table 17. The 6 h EC_{50} of the combination of carbosulfan and chlorpyrifos at all water hardnesses could not be calculated from the acute toxicity test results because the percentage of mortality glochidia was less than 50% in these treatments.

The EC_{50}s for the combination of carbosulfan and chlorpyrifos at 48 h in soft, moderately-hard, and hard water were 0.13 (0.12-0.14), 0.14 (0.13-0.16), and 0.067 (0.060-0.086) mg/L, respectively. This result demonstrated that the lowest 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 (P<0.05) (Figure 16).

Table 17 The toxicity end points[(1)] for a combination of carbosulfan and chlorpyrifos at different water hardness concentrations

Hardness	Time (h)	NOEC (mg/L)	LOEC (mg/L)	EC_{50} (mg/L)[(1)] (95% confidence interval)
Soft	6	0.07	0.11	>0.17
	24	0.11	0.17	0.17 (0.14-NA) [(2)]
	48	0.05	0.07	0.13 (0.12-0.14)
Moderately hard	6	0.17	>0.17	>0.17
	24	0.11	0.17	>0.17
	48	0.05	0.07	0.14 (0.13-0.16)
Hard	6	0.05	0.07	>0.17
	24	<0.05	0.05	0.086 (0.082-0.091)
	48	<0.05	0.05	0.067 (0.060-0.086)

(1) Data were expressed as chlorpyrifos concentrations.

(2) NA = not available

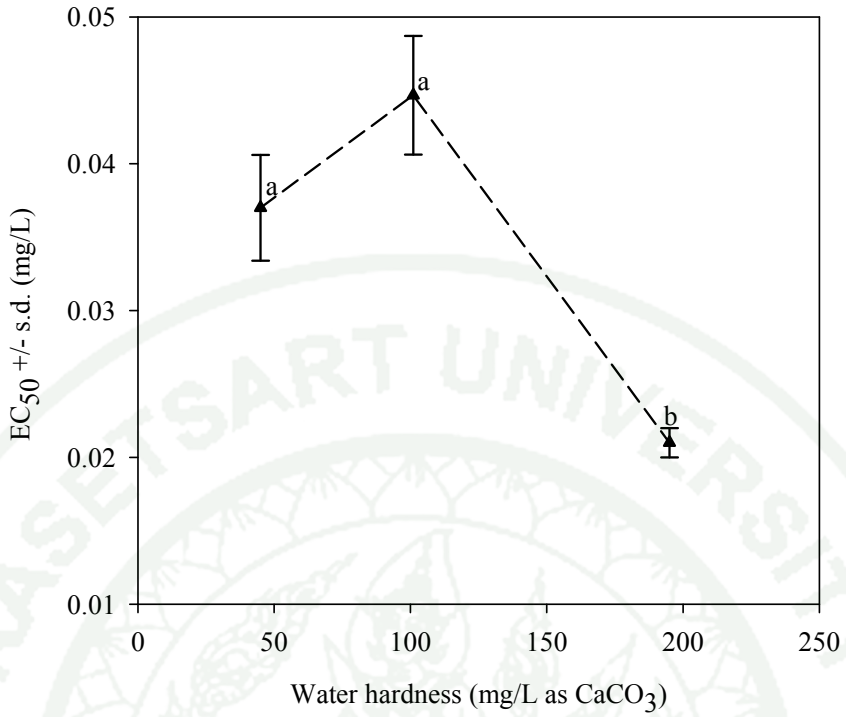

Figure 16 The effect of water hardness on the EC_{50} values for *Hyriopsis bialata* glochida exposed to a combination of carbosulfan and chlorpyrifos for 48 h. Treatments with different letters was significantly different (Tukey's test, $\alpha = 0.05$).

3. Characteristics of glochidia after exposure to carbosulfan and chlorpyrifos

3.1 Acute toxicity of carbosulfan

In this study, glochidia in control treatment showed normal behavior such as snapping 2-3 times within 1 minute and external thread was spread out of body and these also were found in all carbosulfan concentrations (Figure 17A, C, and E). After adding NaCl solution, glochidia responded by quickly snapping and closed their valves within few seconds and larval adductor muscle was condensed (Figure 17B, and D). The condensing of larval adductor muscle could indicate the opportunity of glochidia development further, whereas the glochidia adductor muscle in the highest carbosulfan concentration (0.1 mg/L) at 48 h was softer than adductor muscle at 0 and 24 h (Figure 17F). The normal glochidia in control treatment similarly another study

found that the mature glochidia valves were joined by straight hinge with the larval adductor extended transversely between the valves. There were two layers of larval mantle cells lining the internal glochidial shell surface and a mantle cavity was between the inner mantle cells of each valve (Chumnanpuen *et al*., 2006).

3.2 Acute toxicity of chlorpyrifos

Glochidia in control treatment showed normal behavior such as snapping 2-3 times within 1 minute and thread spread out of body after exposed for 48 h. The normal glochidia before adding NaCl solution were shown in Figure 18A. After adding NaCl solution, glochidia responded by quickly snapping and closed their valves within few seconds and larval adductor muscle was prominent (Figure 18B). After 24 h exposure to chlorpyrifos (0.21 mg/L), some of glochidia showed damaged tissue (Figure 18C) and were not responding after adding 24% NaCl solution that these were determined as non-viability (Figure 18D).

After 48 h exposure chlorpyrifos (0.21 mg/L), some of the glochidia still showed normal behavior such as snapping but they snapped more slowly than in the control group and responded by closed their valves after adding NaCl solution. However, most glochidia showed damaged tissue (Figure 18E) and few of them responded after adding NaCl solution (Figure 18F).

All glochidia at 48 h exposure to highest chlorpyrifos concentration (0.36 mg/L) were no responding after adding NaCl solution. These results demonstrated that glochidia *H. bialata* characteristics are obviously sensitive indicators of chlorpyrifos effect.

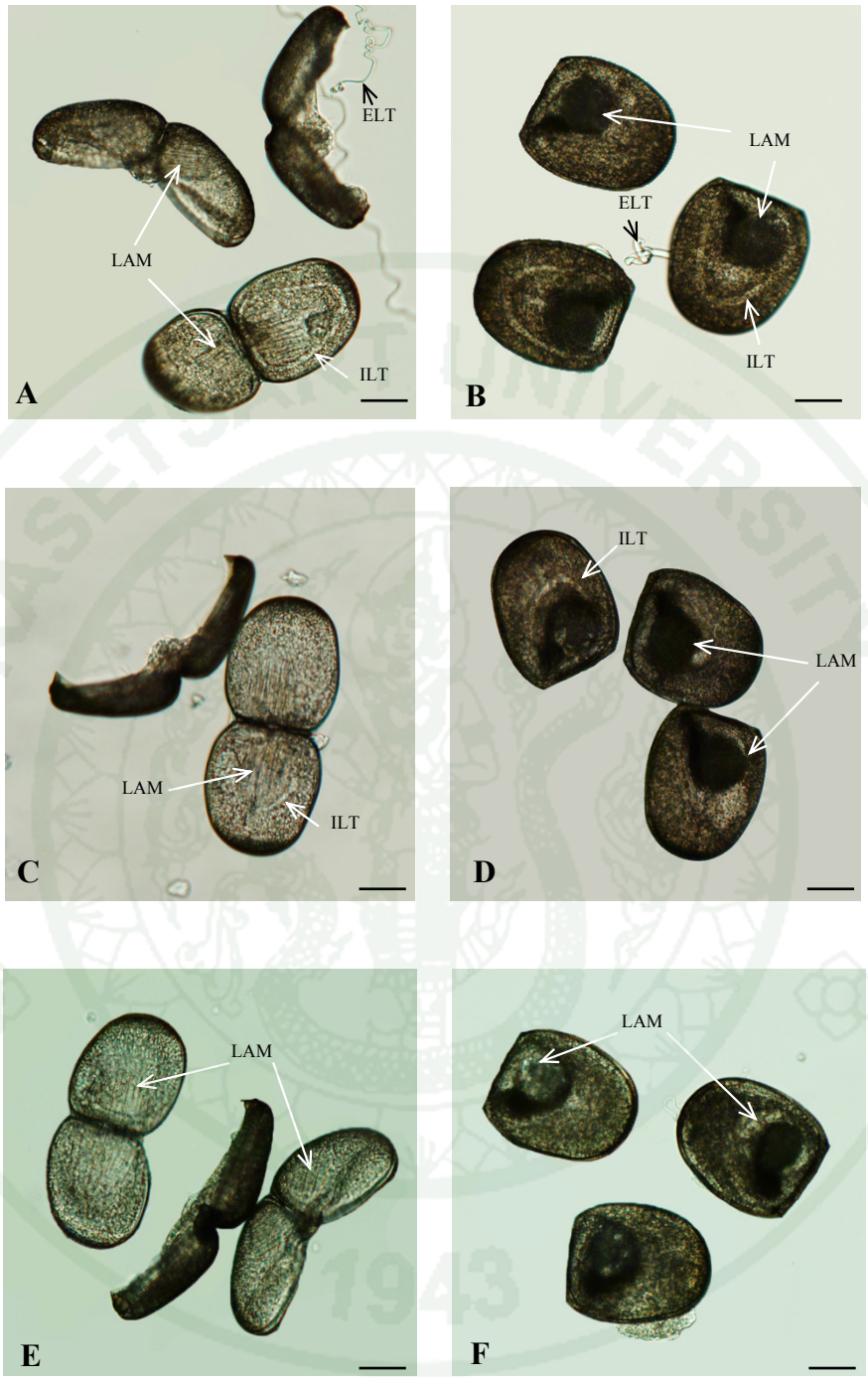

Figure 17 Characteristics of *Hyriopsis bialata* glochidia after exposure to 0.1 mg/L carbosulfan was observed by adding 24% NaCl solution. Before adding NaCl solution for 0 (A), 24 (C) and (E) 48 h and after adding NaCl solution for 0 (B), 24 (D) and (F) 48 h. Scale bars = 50 µm. ELT = external larval thread, ILT = internal larval thread, LAM = larval adductor muscle

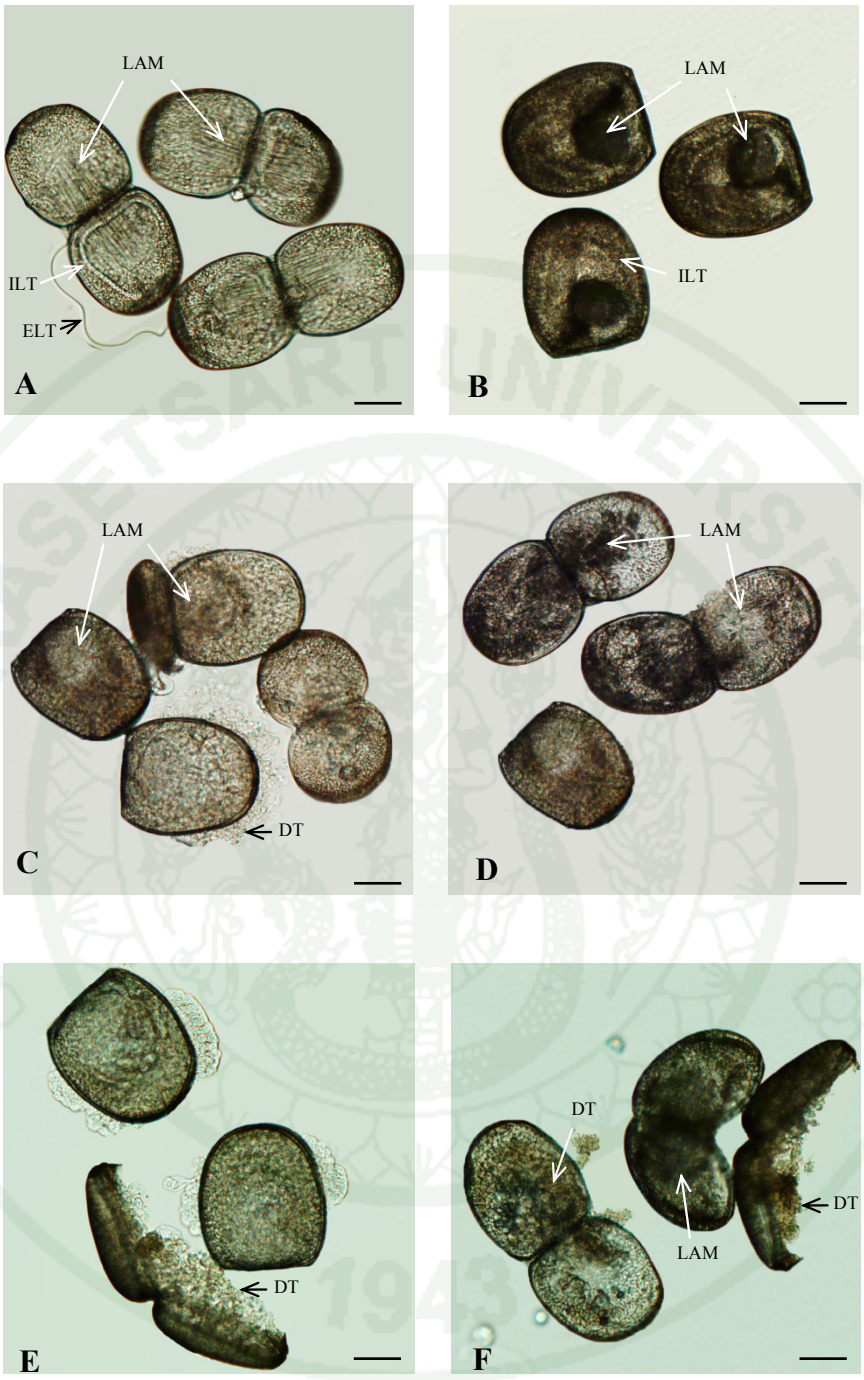

Figure 18 Characteristics of *Hyopsis bialata* glochidia after exposure to 0.21 mg/L chlorpyrifos was observed by adding 24% NaCl solution for 0 (A), 24 (C) and (E) 48 h and after adding NaCl solution for 0 (B), 24 (D) and (F) 48 h. Scale bars = 50 µm. ELT = external larval thread, ILT = internal larval thread, LAM = larval adductor muscle, DT = damaged tissue

Discussion

1. Survival of glochidia

1.1 Survival of glochidia at different temperatures and test durations

The average survival of *Hyriopsis bialata* glochidia at the different temperatures of 15, 20, 25, and 30 ºC was greater than 90% at all times from zero to 48 h and the highest average survival of glochidia over time was after 1 h with average survival of 98%. Zimmerman and Neves (2002) found that the survival of glochidia in the water column after released from the marsupial pouch, and before attachment to the host, was a different period of time depending on the species and water temperature. The survival of *Actinonaias pectorosa* glochidia at 20 ºC was greater than 48 h and the percentage survival was greater than 90%. Similarly, Jacobson (1990) reported the survival of *Villosa nebulosa* glochidia was greater than 90% at 20 ºC in the duration of greater than 2 days. In this study, the average survival of glochidia decreased approximately 40% after 72 h in dechlorinated tap water. The results indicated that toxicity test of *H. bialata* could be conducted over a wide range of temperatures from 15 to 30 ºC. However, a standard glochidia toxicity test for this species should be carried at 25 ºC in the duration of 48 h because it is a practical room temperature in the laboratory and an optimal temperature for the culturing of this species (Kovitvadhi *et al*., 2006).

1.2 Water quality

Water quality was measured in all of the experiments and, in summary, water temperatures ranged from 26.8±0.27 °C, dissolved oxygen (DO) ranged from 8.9±0.05 mg/L, and pH ranged from 8.23±0.03. In the dechlorinated tap water experiment hardness was 100.0±3.20 mg/L as $CaCO_3$, and alkalinity was 73.7±0.80 mg/L as $CaCO_3$. These results indicated that water quality in the tests was nearly the water quality for *H. bialata* in a natural habitat (Kovitvadhi *et al.,* 2005) (Appendix Table 1).

Dissolved oxygen concentrations were always above the 4 mg/L minimum that the ASTM (2006) recommended in the standard guide for conducting laboratory toxicity tests with freshwater mussels. Dissolved oxygen concentrations below 5 mg/L may adversely affect the function and survival of biological communities. Measurement of DO can be used to indicate the degree of pollution by organic matter, the degradation of organic substances by microbial respiration and conversely photosynthesis by algal or plant communities (Chapman, 1996).

The water hardness of the dechlorinated tap water was approximately 100 mg/L which is defined as moderately-hard. Moderately-hard and hard water is considered optimal for survival and growth of aquatic organisms, particularly molluscs, which have high calcium requirements (50-250 mg/L as $CaCO_3$) (WQA, 1996). Thus, the water hardness in the dechlorinated water study was at optimal levels for glochidia and unlikely to negatively affect the test organisms.

Buddensiek (1955) suggested that the temperature of water is an important factor in the survival of the freshwater mussel *Margaritifera margaritifera.* Akiyama and Iwakuma (2007) reported that an increase in water temperature resulted in a decrease in survival of glochidia because of a reduction in their potency to attach to a host. However, the survival of test organisms can be influenced by other water quality parameters.

2. Acute toxicity tests with glochidia

2.1 Acute toxicity of carbosulfan

Water quality parameters, apart from the pesticides, measured in all of the experiments indicated that water quality was unlikely to have a negative effect on the glochidia. In addition, the percentage survival of glochidia in the control treatment was more than the 90% minimum recommended by ASTM (2006) after 48 h exposure.

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 percentage mortality of glochidia was less than 50% in all treatments. Thus, carbosulfan toxicity was greater than the 0.1 mg/L 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 had a resulted in decreased survival of glochidia as shown in the Appendix Figure 1. The results suggested that the carbosulfan toxicity in concentrations of greater than 0.3 mg/L equivocal results that probably because of poor solubility of carbosulfan. On the other hand, the water solubility limit of carbosulfan may have some relevance when carbosulfan is applied in a formulated product that may 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 *Lampsilis 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*. (2007c) 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.

There are no other 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 the same species.

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 of freshwater mussels from the formulation used in the present study.

Data on the acute toxicity of carbosulfan to other aquatic invertebrates is limited, but the 48 h LC_{50} for *Daphnia magna* is 1.5 mg/L (PPDB, 2014). Similarly there is little information on the toxicity of carbosulfan to aquatic vertebrates (see Table 2 of the literature review) and the 48 h LC_{50} for carbosulfan exposure of the Nile tilapia *(Tilapia nilotica)*, Japanese eel *(Anguilla japonica*), and electric fish *(Pollimyrus isidori*) is 0.17, 1.3, and 0.081 mg/L, respectively (Tarzwell and Henderson, 1960; Yokoyama *et al*., 1988; Yameogo *et al*., 1991).

Results of this study compared to these previous studies indicate that the glochidia of freshwater mussels are less sensitive to carbosulfan (48 h LC_{50} greater than 0.1 mg/L) than most other aquatic organisms used for toxicity testing.

2.2 The effect of water hardness on carbosulfan toxicity

Water quality measured in all of these experiments indicated that the basic water quality parameters of DO, pH and temperature were unlikely to have had a negative effect on the glochidia. In addition, the percentage survival of glochidia in the control treatment was more than the 90% minimum recommended by ASTM (2006) after 48 h exposure.

The carbosulfan EC_{50}s at the exposure times of 6, 24, and 48 h were not calculated in the acute toxicity test because the percentage survival of glochidia exposed to carbosulfan concentrations for 6, 24, and 48 h was not significantly different to glochidia survival in the control treatment (P>0.05) and the percentage of mortality of glochidia was less than 50% in all treatments. On the other hand, there was no effect of water hardness on the toxicity of carbosulfan. The results indicated that *H. bialata* glochidia are relatively insensitive to the carbosulfan formulation used in this study.

Measured carbosulfan concentrations in water samples collected at the start of the exposures were on average 33% of the stock solution. This result indicates that carbosulfan was limited by water solubility and reached equilibrium in solution. Tejada and Magallona (1985) and Ramanand *et al.* (1991) 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 may have occurred during the 48 h test thus, to evaluate the degradation rate, carbosulfan concentrations would have to be measured after testing.

The toxicity of a compound to aquatic organisms may be influenced by water quality. According previous studies, the toxicity of some metals is reduced by increased water hardness. In contrast, hardness has little or no effect on the toxicity of organic chemicals including pesticides such as endosulfan (Pickering and Henderson, 1996; Capkin *et al*., 2006). Similarly in this study, an increase from soft water (42 mg/L as $CaCO_{3)}$ to high water hardness (168-258 mg/L as $CaCO_3$) may not affect survival of *H. bialata* glochidia exposed to carbosulfan.

2.3 Acute toxicity of chlorpyrifos

Background water quality measured in all of the experiments indicated that water quality was unlikely to have a negative effect on the glochidia in the chlorpyrifos tests. The percentage survival of glochidia in the control treatment was more than the 90% minimum recommended by ASTM (2006) after 24 h exposure.

The EC_{50} values for chlorpyrifos exposure of *H. bialata* glochidia in dechlorinated tap water were 0.083 (0.079-0.087) and 0.078 (0.062-0.092) mg/L for 24 and 48 h, respectively. This result indicates that *H. bialata* glochidia are more sensitive to chlorpyrifos in the commercial chlorpyrifos formulation than *Lampsilis siliquoidea* glochidia exposed to either pure chlorpyrifos, or in the formulation Lorsban® 4-E Insecticide (48 h EC_{50} 0.60 mg/L, hardness 160-184 mg/L as $CaCO_3$, temperature 20.7-21.7 °C) (Bringolf *et al*., 2007c). In Thailand chlorpyrifos residuals of 6.73 µg/L have been reported in water 24 h after application of 1 g/L chlorpyrifos

(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, therefore, application of chlorpyrifos at that rate is unlikely to cause acute toxicity to glochidia. On the other hand, chlorpyrifos may be more toxic when applied in a formulated product and the fact that chlorpyrifos is only slightly soluble in water indicates that chlorpyrifos may bioaccumulate in fish and other aquatic organisms.

2.4 The effect of water hardness on chlorpyrifos toxicity

The effect of water hardness on chlorpyrifos toxicity was assessed using a single batch of glochidia 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 results demonstrated a highly significant effect of hardness on the percentage survival of glochidia after exposure to chlorpyrifos for 6, 24, and 48 h (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. 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. Exposure to chlorpyrifos in high hardness water resulted in intermediate but highly variable survival rates. Moreover, this result 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 25-26 °C). In contrast, water hardness did not affect survival of fish exposed to endosulfan (Capkin *et al*., 2006). Similarly another study found that the acute toxicity of endosulfan was not influenced by water hardness (Pickering *et al.* 1966). On the other hand, increased water hardness decreases the toxicity of some metals such as copper, chromium, and zinc. Comparison with the first chlorpyrifos experiment in dechlorinated tap water achieved a 48 h EC_{50} of 0.078 mg/L which was similar to the 48 h EC_{50} of 0.11 mg/L at moderately-hard water in the second experiment suggesting that the same levels of water hardness cause a similar effect of chlorpyrifos on glochidia. On the other hand, chlorpyrifos was slightly more toxic in the first experiment indicating that chlorpyrifos may be faster-acting in dechlorinated tap water. The dechlorinated tap water was not analysed in the present study and

contains unknown concentrations of various ions compared with the reconstituted water used in the second test.

Other aspects of water chemistry that are thought to influence the hydrolysis of chlorpyrifos in aquatic environments are temperature, pH, and alkalinity. The rate of chlorpyrifos hydrolysis is enhanced in alkaline conditions and may cause shorter half-lives in water (John *et al*., 1999). Both pH and alkalinity are related to water hardness, so increased hardness may result in increased hydrolysis of chlorpyrifos and thus reduced toxicity of chlorpyrifos.

These results indicate *H. bialata* glochidia are more sensitive to this chlorpyrifos formulation compared to other species of glochidia. In contrast, *H. bialata* glochidia are less sensitive to chlorpyrifos than other aquatic invertebrates. However, the chlorpyrifos residual concentrations in the aquatic environment that have been reported for Thailand are lower than the chlorpyrifos EC_{50} values in this study, thus, under those use regimes it may not cause acute toxicity to glochidia. Alternatively application of chlorpyrifos as a different formulation may increase its toxicity.

2.5 Acute toxicity of a combination of carbosulfan and chlorpyrifos

In both the first carbosulfan test in dechlorinated tap water and the second carbosulfan test in reconstituted water, carbosulfan was not acutely toxic at the highest concentration of carbosulfan in the experiment (0.1 mg/L), which was also the water solubility limit of carbosulfan. The combination of carbosulfan and chlorpyrifos was more toxic when the exposure time was increased to 48 h (0.15 mg/L) compared to toxicity at 6 and 24 h. The 48 h EC_{50} for chlorpyrifos (0.15 mg/L), and carbosulfan (0.05 mg/L) obtained from the mixed exposure tests were lower than the 48 h EC_{50} values from the single exposure tests in dechlorinated water for carbosulfan (>0.1 mg/L) but greater than 48 h EC_{50} for chlorpyrifos (0.078 mg/L), respectively. These comparisons suggest that the presence of carbosulfan decreased the toxicity of chlorpyrifos. Insecticide formulations were used in these tests so the mitigation of

chlorpyrifos toxicity may result from interactions among inert and/or active ingredients (George *et al*., 1988).

The mechanisms for chemical synergy of the combination of pesticides are not fully understood. Some theories include an increase in the rate of uptake, the formation of toxic metabolites, an alteration of distribution within the organism, or an inhibition of detoxification systems (Marking, 1977). In another review, George *et al*., (1988) reported a chemical synergy of the effects of a binary combination of atrazine and alachlor on amphibian larvae that was greater at 96 h than at 24 h. They suggested that the chemical synergy increased with time and temperature. In contrast they found an absence of chemical synergy in rainbow trout exposure to the same combination, but at a lower test water temperature.

Scholz *et al*., (2006) evaluated the joint toxicity of simple combination of organophosphate and carbamate insecticides that caused strictly additive neurotoxicity in salmon. The mechanism of action is similar for organophosphates and carbamates, as they both inhibit acetylcholine esterase, but there is a difference in the duration of inhibition. Organophosphate-induced inhibition is effectively irreversible whereas, inhibition by carbamates is reversible (Aldridge and Reiner, 1972). In this study the combination of carbosulfan and chlorpyrifos was tested in a dose ratio of 1:2.9 (1:4 nominal ratio) combinations and the chemical interaction resulted in a decrease in acute toxicity. Different ratios may have to be investigated in order to see interactive toxicity (antagonism or synergism) or noninteractive toxicity (addition).

2.6 The effect of water hardness on the combination of carbosulfan and chlorpyrifos

Water quality measured in all of the experiments indicated that water quality was unlikely to have a negative effect on the glochidia in all of the tests. Additionally, the percentage survival of glochidia in the control treatment was more than the 90% minimum recommended by ASTM (2006) after 24 h exposure.

The 48 h EC_{50}s for a combination of carbosulfan and chlorpyrifos in soft water, moderately-hard water, and hard water were (expressed as chlorpyrifos concentrations) 0.13 (0.12-0.14), 0.14 (0.13-0.16), and 0.067 (0.060-0.086) mg/L, respectively. This result demonstrated that the lowest to moderate water hardness resulted in the highest survival of glochidia, whereas an increase to hard water hardness resulted in decreased survival of glochidia. In comparison the single exposure tests for chlorpyrifos 48 h EC_{50}s demonstrated that the lowest and hardest water hardness resulted in the highest survival of glochidia, whereas moderate water hardness resulted in significantly decreased survival of glochidia. However, the 48 h EC_{50} values obtained for chlorpyrifos from the mixed exposure in hard water (0.067 mg/L) were lower than EC_{50} values from the single exposure tests for chlorpyrifos exposure in hard water (0.16mg/L). This suggests that the combination is more toxic than the single exposure tests for chlorpyrifos. 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 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 by increasing its bioactivation.

CONCLUSION

In this study, the acute toxicity of the insecticides carbosulfan and chlorpyrifos and the effect of water hardness on their toxicity to *Hyriopsis bialata* glochidia were assessed and summarized as follows:-

1. The average survival of *H. bialata* glochidia at the different temperatures of 15, 20, 25, and 30 ºC was greater than 90% at all times from zero to 48 h. The results indicated that toxicity test of *H. bialata* could be conducted over a wide range of temperatures from 15 to 30 ºC and a standard glochidia toxicity test for this species should be 25 ºC in the duration of 48 h.

2. The EC_{50} values for carbosulfan exposure of *H. bialata* glochidia in dechlorinated tap or in standard reconstituted water were greater than 0.1 mg/L (the solubility limit of carbosulfan). The result demonstrated that water quality was unlikely to have a negative effect on the glochidia in all of the tests. There was no effect of water hardness on carbosulfan toxicity to *H. bialata* glochidia at 6, 24, or 48 h.

3. The EC_{50} values for *H. bialata* glochidia exposed to chlorpyrifos in dechlorinated tap water were 0.083 (0.079-0.087) and 0.078 (0.062-0.092) mg/L for 24 and 48 h, respectively, thus the toxicity of chlorpyrifos progressively increased when exposure times were increased to 48 h.

4. The 48 h EC_{50}s for chlorpyrifos 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. The result demonstrated that the lowest and highest water hardness resulted in the highest survival of glochidia, whereas moderate water hardness resulted in decreased survival of glochidia. These results indicate that water hardness concentrations affect chlorpyrifos sensitive of glochidia in a non-linear fashion.

5. The combination of carbosulfan and chlorpyrifos was more toxic when exposure time was increased to 48 h (0.15 mg/L) than the toxicity of the combination

for the exposure time at 6 and 24 h. The EC_{50} values obtained from the combined exposure tests in dechlorinated tap water (0.15:0.05 chlorpyrifos:carbosulfan) were lower than EC_{50} values from the single exposure tests for carbosulfan (>0.1 mg/L) but were greater than 48 h EC_{50} chlorpyrifos (0.078 mg/L).

6. The 48 h EC_{50}s for a combination of carbosulfan and chlorpyrifos (expressed as chlorpyrifos concentrations) in soft water, moderately-hard water, and hard water were 0.13 (0.12-0.14), 0.14 (0.13-0.16), and 0.067 (0.060-0.086) mg/L, respectively. The result demonstrated that the lowest to moderate water hardness resulted in the highest survival of glochidia, whereas an increase to hard water hardness resulted in decreased survival of glochidia, and increased toxicity of the combination. These results indicate that water hardness concentrations affect a combination sensitive of glochidia in a non-linear fashion.

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APPENDIX

Appendix Table 1 The physico-chemical characteristics of water in natural habitat for *Hyriopsis bialata*

Parameter	Unit	Mean±SD
Temperature	°C	27.2±0.73
pH	-	7.25±0.23
Dissolved oxygen (DO)	mg/L O_2	5.47±1.13
Total alkalinity	mg/L $CaCO_3$	75.92±4.01
Free carbondioxide	mg/L CO_2	5.58±6.84
Total hardness	mg/L $CaCO_3$	112±48.18
Total ammonia-nitrogen	mg/L NH_3-N	0.29±0.16
Calcium	mg/L $CaCO_3$	75.5±26.23
Silica	mg/L SiO_2	8.58±3.26

Source: Kovitvadhi *et al.* (2005)

Appendix Table 2 Water quality during the acute toxicity test of carbosulfan

Parameter	Time	Carbosulfan (mg/L)					
	(h)	0.00	0.01	0.02	0.03	0.05	0.10
Temperature (°C)	0	25.92	25.17	25.22	25.37	25.12	24.90
	48	25.82	25.45	25.44	25.85	26.05	25.87
pH	0	7.78	7.51	7.49	7.49	7.46	7.55
	48	7.26	7.31	7.35	7.40	7.58	7.66
DO (mg/L)	0	6.28	6.05	6.59	6.57	6.98	6.56
	48	5.50	6.01	6.18	6.00	6.23	6.03
Conductivity (µS/cm)	0	381	376	377	375	373	373
	48	441	440	434	402	424	444

Appendix Table 3 Water quality during the acute toxicity test of chlorpyrifos

Parameter	Time	Chlorpyrifos (mg/L)						
	(h)	0.00	0.04	0.05	0.07	0.11	0.15	0.21
Temperature (°C)	0	25.27	24.52	24.34	24.26	24.12	23.95	23.91
	48	26.75	26.75	26.59	26.50	26.44	26.33	26.28
pH	0	7.88	7.43	7.51	7.55	7.59	7.69	7.62
	48	7.95	7.79	7.82	7.84	7.81	7.82	7.91
DO (mg/L)	0	6.81	7.40	6.77	6.72	6.93	6.32	7.24
	48	6.28	6.58	5.49	6.03	5.77	6.12	6.13
Conductivity (µS/cm)	0	373	372	370	369	367	367	367
	48	472	432	438	413	406	409	415

Appendix Table 4 Water quality during the acute toxicity test of the combination

Parameter	Time	Carbosulfan: Chlorpyrifos (mg/L)						
	(h)	0.00: 0.00	0.019: 0.007	0.029: 0.010	0.044: 0.015	0.066: 0.023	0.099: 0.034	0.148: 0.051
Temperature (°C)	0	27.26	26.61	26.17	26.03	25.89	25.98	25.84
	48	26.17	26.56	25.74	25.68	25.85	25.81	25.47
pH	0	7.66	7.83	7.75	7.74	7.74	7.55	7.6
	48	7.67	7.71	7.68	7.71	7.78	7.93	7.98
DO (mg/L)	0	6.08	6.54	6.87	7.01	6.65	7.17	6.65
	48	5.88	6.19	6.27	6.30	6.63	6.25	6.32
Conductivity (µS/cm)	0	377	395	375	374	373	374	373
	48	422	409	441	440	438	469	497

Appendix Table 5 Water quality during the acute toxicity test of carbosulfan at different water hardness concentrations

	Parameter	Time	Carbosulfan (mg/L)				
		(h)	0.00	0.01	0.03	0.05	0.10
Soft 42-60 mg/L as $CaCO_3$	Temperature (°C)	0	24.16	24.66	24.10	24.46	24.36
		48	25.12	25.00	24.90	24.82	24.59
	pH	0	7.43	7.51	7.33	7.23	7.51
		48	7.76	7.69	7.55	7.48	7.44
	DO (mg/L)	0	5.97	6.69	6.64	6.74	6.45
		48	5.76	6.18	7.22	6.02	7.84
	Conductivity (us)	0	155	156	155	153	152
		48	233	228	183	191	163
Moderately hard 88-128 mg/L as CaCO3	Temperature (°C)	0	24.34	24.25	24.47	24.46	23.85
		48	24.12	24.85	23.76	23.71	23.44
	pH	0	7.56	7.26	7.33	7.56	7.54
		48	7.36	8.04	7.51	7.51	7.53
	DO (mg/L)	0	5.85	6.83	6.99	6.89	6.99
		48	7.95	6.08	8.17	6.22	8.46
	Conductivity (µS/cm)	0	291	290	295	295	291
		48	229	459	228	379	401
Hard 168-258 mg/L as CaCO3	Temperature (°C)	0	24.38	24.13	24.16	23.89	23.62
		48	23.47	23.74	23.97	24.00	24.15
	pH	0	7.59	7.42	7.76	7.76	7.83
		48	7.68	7.89	8.00	8.04	8.02
	DO (mg/L)	0	6.61	6.78	7.27	7.01	6.52
		48	6.42	6.11	5.86	5.30	7.92
	Conductivity (µS/cm)	0	552	542	547	546	514
		48	799	909	848	902	722

Appendix Table 6 Water quality during the acute toxicity test of chlorpyrifos at different water hardness concentrations

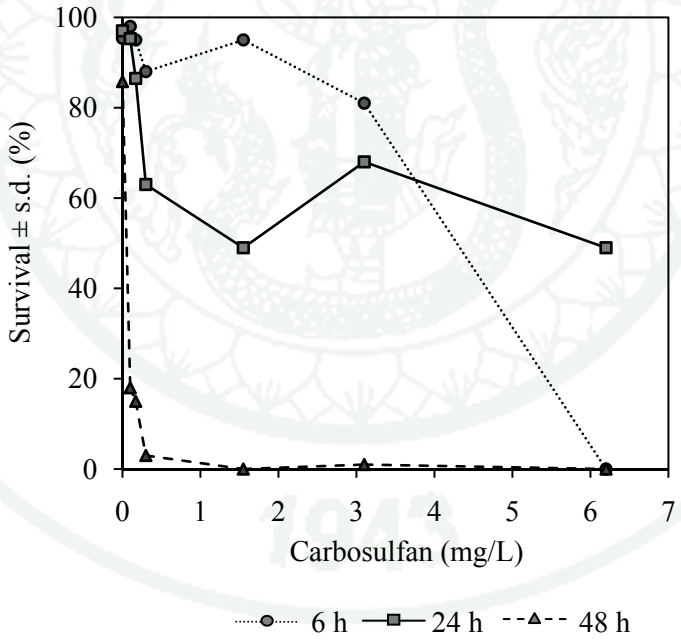
	Parameter	Time	Chlorpyrifos (mg/L)					
		(h)	0.00	0.05	0.08	0.14	0.22	0.36
Soft 42-60 mg/L as $CaCO_3$	Temperature (°C)	0	24.98	23.16	23.10	23.28	23.15	23.28
		48	24.92	24.88	23.31	23.70	24.77	24.72
	pH	0	8.19	7.76	7.65	7.63	7.56	7.59
		48	8.74	8.34	8.21	8.09	8.00	7.99
	DO (mg/L)	0	6.35	6.68	5.84	6.04	6.56	6.07
		48	6.73	5.69	5.72	5.84	5.24	5.89
	Conductivity(µS/cm)	0	155	155	151	152	151	152
		48	166	167	183	183	180	164
Moderately hard 88-128 mg/L as CaCO3	Temperature (°C)	0	24.78	23.46	23.42	23.54	23.48	23.53
		48	24.58	24.72	24.86	24.76	24.77	24.58
	pH	0	8.00	7.59	7.67	7.64	7.7	7.76
		48	8.4	8.05	8.08	8.1	8.06	8.08
	DO (mg/L)	0	6.83	6.32	6.62	6.95	6.62	6.77
		48	6.8	5.76	5.9	5.78	6.18	5.84
	Conductivity (µS/cm)	0	298	286	289	291	290	290
		48	300	304	342	319	316	318
Hard 168-258 mg/L as CaCO3	Temperature (°C)	0	24.54	23.36	23.41	23.24	23.02	23.29
		48	24.85	24.53	24.08	24.19	24.09	23.92
	pH	0	8	7.9	7.94	8.74	8.4	8.34
		48	8.34	8.15	8.28	8.31	8.35	8.34
	DO (mg/L)	0	5.95	6.84	6.76	6.73	6.8	5.89
		48	5.89	5.95	5.83	5.95	5.8	6.05
	Conductivity (µS/cm)	0	555	540	544	540	542	545
		48	555	595	662	639	625	575

Appendix Table 7 Water quality during the acute toxicity test of the combination at different water hardness concentrations

	Parameter	Time (h)	Carbosulfan:Chlorpyrifos (mg/L)				
			0.00: 0.00	0.016: 0.046	0.023: 0.069	0.036: 0.105	0.056: 0.159
Soft 42-46 mg/L as $CaCO_3$	Temperature (°C)	0	24.96	24.22	24.46	24.52	24.61
		48	24.20	26.07	26.20	26.19	26.21
	pH	0	7.92	7.91	7.75	7.74	7.74
		48	8.06	7.72	7.73	7.69	7.67
	DO (mg/L)	0	6.23	6.47	5.76	6.03	6.08
		48	6.80	6.15	6.07	7.69	6.12
	Conductivity (µS/cm)	0	133	131	133	133	134
		48	150	141	144	144	144
Moderately hard 100-102 mg/L as CaCO3	Temperature (°C)	0	25.97	25.79	25.81	25.78	25.97
		48	24.20	25.69	25.47	25.46	25.39
	pH	0	7.90	7.93	8.03	8.05	8.07
		48	8.03	7.95	7.89	7.83	7.85
	DO (mg/L)	0	6.24	6.30	6.21	6.18	6.15
		48	6.39	6.13	6.22	6.09	6.15
	Conductivity (µS/cm)	0	300	303	309	317	309
		48	309	309	326	333	326
Hard 186-202 mg/L as CaCO3	Temperature (°C)	0	25.92	25.93	25.97	26.21	26.13
		48	24.20	26.50	26.20	26.22	26.35
	pH	0	7.96	8.02	7.96	7.93	8.00
		48	7.87	8.42	8.19	8.06	8.04
	DO (mg/L)	0	5.71	5.99	6.33	6.21	6.08
		48	6.42	6.10	6.01	6.53	6.37
	Conductivity (µS/cm)	0	565	576	581	580	578
		48	592	585	601	593	608

Appendix Table 8 Percentage survival of *Hyriopsis bialata* glochidia after exposure to carbosulfan in dechlorinated tap water for the range-finding test

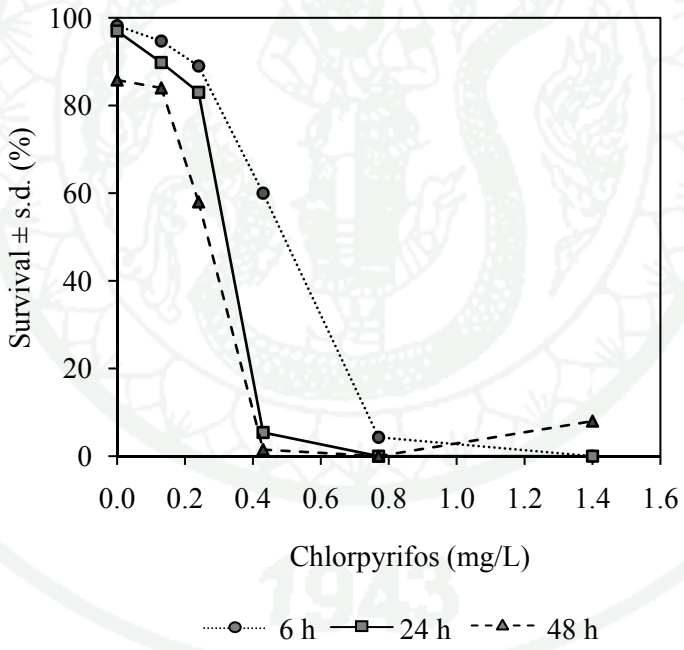
Carbosulfan (mg/L)	% Average survival		
	6 h	24 h	48 h
0.00	95.4	97.0	85.8
0.10	98.0	95.3	18.0
0.17	95.0	86.5	15.0
0.30	88.0	63.0	3.0
1.55	95.0	49.0	0.0
3.10	81.0	68.0	1.0
6.20	0.0	49.0	0.0



Appendix Figure 1 Percentage survival of *Hyriopsis bialata* glochidia after exposure to carbosulfan for 6, 24, and 48 h in dechlorinated tap water for the range-finding test

Appendix Table 9 Percentage survival of *Hyriopsis bialata* glochidia after exposure to chlorpyrifos in dechlorinated tap water for the range-finding test

Chlorpyrifos (mg/L)	% Average survival		
	6 h	24 h	48 h
0.00	98.2	97.0	85.8
0.13	94.7	89.8	84.0
0.24	89.0	83.0	58.0
0.43	60.0	5.4	1.5
0.77	4.3	0.0	0.0
1.40	0.0	0.0	8.0



Appendix Figure 2 Percentage survival of *Hyriopsis bialata* glochidia after exposure to chlorpyrifos for 6, 24, and 48 h in dechlorinated tap water for the range-finding test

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