Acute toxicity of triazine pesticides to juvenile signal crayfish (*Pacifastacus leniusculus*)

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Abstract

OBJECTIVES: Crustacea are at high risk of toxic effects of agricultural pesticides. Currently, many questions regarding the toxicity of triazine herbicides to crayfish remain unresolved. The aim of this research was to evaluate the acute toxicity of atrazine, hexazinone, metribuzine, prometryne, simazine, and terbutryne to juvenile signal crayfish (*Pacifastacus leniusculus*).

DESIGN: Acute toxicity tests were performed in accordance with standardized guidelines for testing of chemicals, OECD no. 203, using a semistatic test system. Signal crayfish juveniles (n=672) of 49.0–81.5 mg weight and 12.8–16.0 mm total length were used for the bioassay. Mortalities were recorded daily to 96 h. Each pesticide was tested at concentrations of 1, 10, 40, 70, and 100 $mg.l^{-1}$. Percent mortalities were analyzed by linear regression, and median lethal concentration (LC50) values were computed using probit analysis EKO-TOX 5.2 software.

RESULTS: 96hLC50 values for juvenile signal crayfish were 12.1 $mg.l^{-1}$ for atrazine, 13.9 $mg.l^{-1}$ for terbutryne, 14.4 $mg.l^{-1}$ for prometryne, 19.5 $mg.l^{-1}$ for hexazinone, 30.6 $mg.l^{-1}$ for metribuzine, and 77.9 $mg.l^{-1}$ for simazine. Atrazine showed the greatest toxicity to signal crayfish.

CONCLUSIONS: The present study demonstrated that triazines are toxic to signal crayfish. Signal crayfish is more sensitive than the fish for atrazine, hexazine, metribuzine, and for these triazines signal crayfish can be used as a bio-indicator of environmental contamination.

Abbreviations:

$ANC_{4.5}$	- acid neutralization capacity
CODMn	- chemical oxygen demand
DMSO	- dimethyl sulphoxide
LC	- lethal concentration
OECD	- Organization for Economic Cooperation and Development

ORIGINAL ARTICLE

INTRODUCTION

Many freshwater ecosystems are contaminated with industrial, domestic, and agricultural chemicals such as herbicides, which are ubiquitous and can spread globally as well as regionally (Flynn & Spellman 2009). Surface waters in agricultural areas are vulnerable to pesticide contamination. The efficacy of the pesticides is reflected in high levels of acute toxicity to target and non-target organisms (LeBlanc *et al.* 1997). Herbicides were first mass-produced in the early 1950s for the control of weeds in agriculture, silviculture, rights-of-way, and turf lawns (Gianessi & Reigner 2007). According to the comprehensive worldwide literature database Quested, herbicides are the most frequently detected chemical pollutants in water.

The triazine family of herbicides, introduced in 1950s, represents one of the largest classes of agrochemicals produced, and they are among the most extensively used herbicides. Triazines are highly toxic and frequently appear in natural watercourses (Graymore *et al.* 2001; Hildebrandt *et al.* 2008). All triazine herbicides are considered moderately persistent in water and mobile in soil (Hartley & Kidd 1987). The physiochemical properties of triazines make them susceptible to leaching into ground water and runoff from the site of application to surface waters particularly during heavy rains (Ghosh & Philip 2006). Triazines are low soluble in water (only units of $mg.l^{-1}$) and have only moderate potential for soil sorption, but persistence in soil is high (up to 3 years). These properties of triaznes resulted in the contamination of surface and ground waters. (John & Walther 2003). While the direct effect of triazine application is the elimination of macrophytes, non-target organisms such as fish may also be affected through loss of food supply and habitat degradation (Ervnest 2004). Triazines are liable to affect non-target organisms, including fish, crayfish, and micro-algae, leading to dramatic ecological changes in the aquatic environment (Stara *et al.* 2012; Velisek *et al.* 2012). Simazine, atrazine, and metribuzine are among 67 chemicals listed as suspected endocrine disrupters by the Japan Environment Agency in 1998 (Okihashi *et al.* 2000), while s-triazine is considered a priority hazardous substance on list of priority pollutants of the EU Water Framework Directive (2000/60/EC).

Organisms in aquatic systems are subject to multigenerational exposure to pollutants throughout the lifecycle. For most pesticides, we have little data on the concentrations that are toxic to crayfish. Given the thousands of toxicology studies that have been conducted over the past several decades, the lack of data on crayfish is surprising. In aquatic systems, tests typically focus on fish and aquatic invertebrates such as daphnia and algae. However, the precise mechanisms of toxicity of these herbicides in other non-target organisms, such as crayfish, are unknown. Crayfish are the largest and among the longest-lived freshwater invertebrates (Johnston & Robson 2009) and are often considered keystone species (Dorn & Wojdak 2004) or ecosystem engineers (Edwards *et al.* 2009), due to their prominent role in physical and biological modification of ecosystems. Many crayfish fulfil the criteria for surrogate species with respect to environmental monitoring and conservation biology (Caro & O'Doherty 1999). Crayfish have been used as an environmental health indicator species, population indicator species, biodiversity indicator species, umbrella species, and flagship species (Fureder & Reynolds 2003).

This study investigated the acute toxicity (96hLC50) of six triazine pesticides to juvenile signal crayfish. Acute toxicity tests provide a basis for understanding the damaging effects of chemicals in organisms. This is the first study reporting toxicity endpoints for triazine pesticides in crayfish. The relative sensitivity of signal crayfish is also assessed by comparing toxicity data with those obtained for other aquatic organisms.

The results of this study not only provide information on the acute toxicity of six triazines to signal crayfish**,** but also contribute to available information on the choice of a crustacean species as a bio-indicator for toxicity testing.

MATERIALS AND METHODS

Chemicals

The triazine pesticides atrazine (chemical purity 98.9%), hexazinone (chemical purity 99.9%), metribuzine (chemical purity 99.5%), prometryne (chemical purity 99.3%), simazine (chemical purity 99.5%), and terbutryne (chemical purity 99.2%) along with other chemicals used were purchased from Sigma-Aldrich (USA).

Experimental animals

Juvenile signal crayfish (n=672) were reared on chironomids and pond zooplankton fed *ad libitum* to the 5^{th}–8^{th} stage (49.0–81.5 mg weight and 12.8–16.0 mm total length). Crayfish were transferred to aquaria with plastic spirals as shelters to limit cannibalism and allowed to acclimatize for 72 h.

Water parameters

The physicochemical parameters of the water in all aquaria were acid neutralization capacity, $ANC_{4.5}$ 1.00 $mmol.l^{-1}$; total ammonia 0.01 $mg.l^{-1}$; NO_3^- 2.92 $mg.l^{-1}$; NO_2^- 0.0001 $mg.l^{-1}$; PO_4^{3-} 0.003 $mg.l^{-1}$; pH 7.50–7.82; sum of Ca^{2+} + Mg^{2+} 7.0 $mg.l^{-1}$; and chemical oxygen demand, COD_{Mn} 0.6 $mg.l^{-1}$. Water temperature was 18.1–19.6 °C and oxygen saturation 95–100%. The test baths were gently aerated on a continual basis. Oxygen saturation, pH, and temperature were monitored daily. The pesticide concentrations were verified daily by high performance liquid chromatography using the method of Katsumata *et al.* (2005). Measured values did not differ from the value stated for test purposes by more than 5%.

Experimental design

Acute toxicity tests were performed in accordance with OECD guidelines for testing of chemicals, OECD no. 203. Acute lethal toxicity of the triazine pesticides was assessed by the determination of 24 h, 48 h, 72 h, and 96 h median lethal concentration (LC50) values for signal crayfish. The acute lethal concentrations (LC) for signal crayfish were determined in the laboratory using semi-static methods. The test solutions were renewed every 48 h during the test (static-renewal). Natural signal crayfish aggression and cannibalism was minimized during the toxicity experiment by housing individuals separately in clear plastic boxes. Each box, 40 mm in height, was divided into chambers with an area of 45 × 30 mm. This system is often used during growth as well as toxicity tests in crayfish (Kouba *et al.* 2010; Gonzalo & Camargo 2012). All acute toxicity tests (n=7) were conducted in triplicate using 20 l glass aquaria containing 5 l water with respective solutions. Each pesticide was tested at concentrations of 1, 10, 40, 70, and 100 mg.l^{-1}, and two additional groups, one in the dilution water only and another in the dilution water with 0.01% dimethyl sulphoxide (DMSO), were used as controls. Stock solutions were prepared with DMSO (99.0% purity), because triazine herbicides exhibit relatively low water solubility. The maximum amount of DMSO added, in all experiments, was less than 0.01% (v/v). Prior to the acute toxicity tests, the solution of DMSO in water was tested to confirm that the 0.01% (v/v) DMSO solution did not show toxic effects to crayfish.

Dead signal crayfish were collected and recorded and the mortalities expressed as percent mortality. Immobile crayfish were considered dead and removed immediately if they did not respond to probing with a glass rod. Mortality in each trial was recorded every 24 h for 96 h. During the acclimation period, and throughout the duration of the experiment, animals were not fed. No signal crayfish from control groups died during the tests.

Statistical analysis

Data derived from toxicity testing were used to estimate the LC50 of the signal crayfish in the test groups with 95% confidence limits. The maximum likelihood linear regression method with probit analysis was performed using EKO-TOX software, version 5.2 (INGEO Liberec, Czech Republic).

RESULTS

Observations of signal crayfish behaviour were conducted at 12 h intervals during the trial. Crayfish in the two control groups and the 1 mg.l^{-1} concentration of all tested triazines showed normal behaviour throughout the test period. In the aquarium, crayfish usually walked along the wall of chamber, stopping at every corner and inspecting the surroundings using their antennae. Crayfish exposed to higher concentrations of all tested herbicides moved with difficulty and frequently remained at the corners of the chamber. When standing, the crayfish, with its claws and abdomen raised, exhibited a rocking motion or walked in circles in the centre of the chamber. Some crayfish attempted to climb the walls of the chamber. Others settled in the centre of the chamber. After exposure to high concentrations of the triazines, the occurrence and frequency of moving backward increased, together with loss of claws and walking legs. Before death, crayfish lost equilibrium and subsequently, flipped onto their backs.

For all acute toxicity tests, no mortality or sublethal effects were observed among the dilution water and solvent controls. The mortality data for each triazine concentration is shown in Figure 1.

The estimated LC0, LC50, and LC100 for the six triazine herbicides following 24, 48, 72, and 96 h of exposure are summarized in Table 1. The 96hLC50 of triazine pesticides, within 95% confidence limits, were determined: atrazine, 12.1 mg.l^{-1}; terbutryne, 13.9 mg.l^{-1}; prometryne, 14.4 mg.l^{-1}; hexazinone, 19.5 mg.l^{-1}; metribuzine, 30.6 mg.l^{-1}; and simazine, 77.9 mg.l^{-1}. There was no significant difference among replicates.

DISCUSSION

For toxicity tests to be suitable for detecting pesticide pollution in aquatic systems, careful consideration must be given to the selection of test species. Fish and

Tab. 1. Acute toxicity of atrazine, hexazinone, metribuzine, prometryne, simazine, and terbutryne to signal crayfish (*Pacifastacus leniusculus*) juveniles following 24, 48, 72, and 96 h exposure. Lethal concentration (LC) is in mg.l^{-1}.

Triazine	24 h			48 h			72 h			96 h		
	LC0	LC50	LC100	LC0	LC50	LC100	LC0	LC50	LC100	LC0	LC50	LC100
Atrazine	–	–	<1000	8.9	214.5	<1000	9.7	108.4	<1000	9.9	77.9	613.0
Hexazinone	1.0	259.1	<1000	0.8	71.6	<1000	0.8	22.5	641.7	1.0	13.9	200.5
Metribuzine	8.8	95.8	<1000	1.3	37.6	<1000	1.1	17.6	277.2	1.2	14.4	171.5
Prometryne	9.6	76.8	612.5	1.6	17.0	184.3	1.5	14.7	136.2	1.4	12.1	104.5
Simazine	–	–	<1000	0.8	206.3	<1000	0.9	58.7	<1000	1.1	30.6	858.3
Terbutryne	–	–	<1000	0.9	579.3	<1000	0.8	46.2	<1000	1.2	19.5	309.8

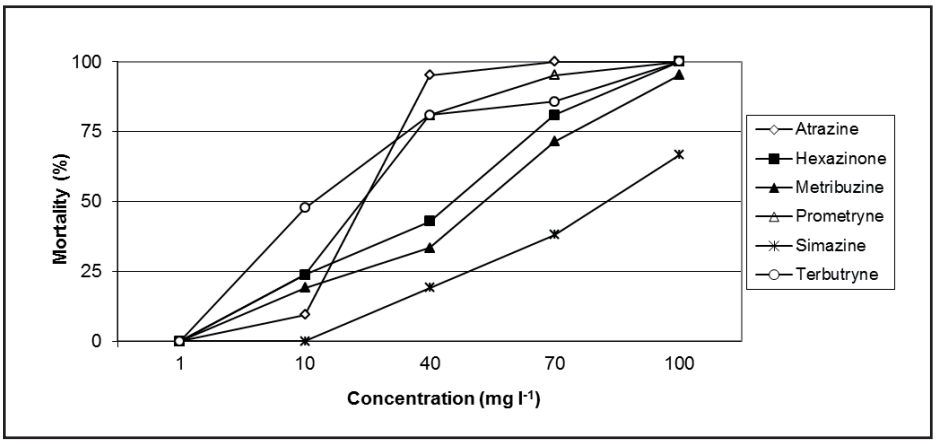

Fig. 1. Mean cumulative mortality of signal crayfish (*Pacifastacus leniusculus*) juveniles at tested concentrations in acute toxicity tests.

Daphnia bioassays are accepted by scientists and regulatory bodies. The considerable time and effort spent in culture and maintenance of test organisms under laboratory conditions increases the complexity of toxicity testing (Rand 1995; Tyagi *et al.* 2007). The ease of culture of the signal crayfish and its reproductive characteristics make this species a candidate for toxicity testing.

Atrazine is one of the most widely used herbicides and, because of its persistence and mobility in soil and water, is considered a common terrestrial and aquatic contaminant (Gianessi & Reigner 2007). Atrazine has been banned in EU, but still is detected in ground water. In our acute toxicity test, the 96hLC50 value for atrazine was 12.1 mg.l^{-1}. A review of toxicity data for fish and crustaceans reported atrazine 96hLC50 values of 0.094 mg.l^{-1} and 0.125 mg.l^{-1} for the calanoid copepods *Acartia tonsa* and *Eurytemora affinis*, respectively (Ward & Ballantine 1985; Forget-Leray *et al.* 2005); 9.0 mg.l^{-1} for grass shrimp (*Palaemonetes pugio*) (Munn *et al.* 2006); 16.0 mg.l^{-1} for perch (*Perca fluviatilis*) (Hartley & Kidd 1987); 18.8 mg.l^{-1} for European whitefish (*Coregonus lavaretus*) (Munn *et al.* 2006); 29.0 mg.l^{-1} for fiddler crab (*Uca pugilator*) (Ward & Ballantine 1985); 76.0 mg.l^{-1} for common carp (*Cyprinus carpio*) (Hartley & Kidd 1987); and 100.0 mg.l^{-1} for crucian carp (*Carassius carassius*) (Bathe *et al.* 1975). These reports indicate that crustaceans are more sensitive to the atrazine than are fish.

Terbutryne degrades slowly, with a half-life of 240 and 180 days in pond and river sediments, respectively. The application of terbutryne has been banned in many countries because it has the potential to bioaccumulate in organisms, but it is still detected in waters (Quednow & Puttmann 2007). The present study found a 96hLC50 value for terbutryne of 13.9 mg.l^{-1}. In acute tests with other aquatic organisms, reported 96hLC50 values were 3.0 mg.l^{-1} for rainbow trout (*Oncorhynchus mykiss*), 4.0 mg.l^{-1} for common carp (Kidd & James 1991), 4.0 mg.l^{-1} for bluegill sunfish (*Lepomis macrochirus*) (Bathe *et al.* 1973) and 5.7 mg.l^{-1} for zebrafish (*Danio rerio*) (Plhalova *et al.* 2009). Signal crayfish were shown to be less sensitive to terbutryne than are these fish species.

Prometryne application is not permitted in Europe, but is widely used in China, Australia, Canada, New Zealand, South Africa, and the United States. We obtained a 96hLC50 value for prometryne of 14.4 mg.l^{-1}. Acute toxicity 96hLC50 has been reported as 2.9 mg.l^{-1} for rainbow trout (Kegley *et al.* 2010), 7.9 mg.l^{-1} for bluegill sunfish, 5.1 mg.l^{-1} for sheepshead minnow (*Cyprinodon variegatus*), and 8.0 mg.l^{-1} for common carp (Popova 1976). Lethal concentrations for the tested fish are lower than observed for signal crayfish.

The average half-life of hexazinone in soils is 90 days, but it can sometimes be found in runoff up to six months after application (Ervnest 2004). In our acute toxicity test the 96hLC50 value for hexazinone was 19.5 mg.l^{-1}. Reported 96 h LC50 values for fish species are 257.0 mg.l^{-1} for rainbow trout, 370.0 mg.l^{-1} for bluegill sunfish (Michael *et al.* 1999), and 236.0–676.0 mg.l^{-1} for Pacific salmonids (Wan *et al.* 1988; Munn *et al.* 2006). The results of the present study indicate that hexazinone is more toxic to signal crayfish than to the studied fish.

Metribuzine is widely used in agriculture and has been found in groundwater (Undabeytia *et al.* 2011). In our acute toxicity test the 96hLC50 value for metribuzine was 30.6 mg.l^{-1}. Reported acute toxicities (96hLC50) are 80.0–100.0 mg.l^{-1} for bluegill sunfish, 42.0–76.0 mg.l^{-1} for rainbow trout, and 140.0 mg.l^{-1} for harlequin rasbora (*Rasbora heteromorpha*) (Worthing & Walker 1987). Munn *et al.* (2006) reported a 96hLC50 value of 92.0 mg.l^{-1} metribuzine for bluegill sunfish. These results show the signal crayfish to be more sensitive to metribuzine than are the tested fish species.

Simazine is the second most commonly detected pesticide in surface and ground waters in the U S, Europe, and Australia, presumably due to its persistence in soil up to 3 years and half-life 50–176 days in water (Inoue *et al.* 2006). The 96LC50 value in the present study for simazine was 77.9 mg.l^{-1}. The 96hLC50 value for rainbow trout is reported as 25.0 mg.l^{-1} (Pesticide Ecotoxicity Database 2000); for fathead minnow (*Pimephales promelas*), 6.4 mg.l^{-1} (Munn & Gilliom

2001); for black bullhead (*Ameiurus melas*), 65.0 mg.l^{-1}; for yellow bullhead (*Ameiurus natalis*), 110.0 mg.l^{-1} (Munn *et al.* 2006). For *Penaeus duorarum* 96hLC50 is reported to be 113.0 mg.l^{-1}, and for *Neopanope texana*, above 1000 mg.l^{-1} (Pesticide Ecotoxicity Database 2000). Simazine is less toxic than other tested triazines, and tested crustaceans have been shown to be less sensitive to simazine than other species. The differential toxicity of simazine can be attributed to differences in susceptibility and tolerance related to its accumulation, biotransformation, and excretion.

According to the categories established in EU legislation, atrazine, hexazinone, metribuzine, prometryne, simazine, and terbutryne can be considered harmful (LC50 in the range of 10–100 mg.l^{-1}) to signal crayfish.

CONCLUSION

The LC50 values estimated in this study were obtained by exposing crayfish under laboratory conditions to concentrations that might represent environmental maximum exposure. Under field conditions, a number of factors, such as biological and chemical removal processes, sediment interaction, and mixing and dilution would likely influence the toxicity of these herbicides. The accelerated expansion of agribusiness and the potential risk of water and aquaculture contamination by xenobiotic agents necessitate the evaluation of herbicide toxicity in species considered sentinel organisms. The investigation of effects of pesticides in organisms is crucial to predicting the impact of these products on non-target species. The present study demonstrated that the triazines are toxic to signal crayfish and that this species can be used as a bio-indicator of environmental contamination by triazine pesticides. The crayfish-specific methodology presented may serve to supplement standard toxicity testing protocols, with macro-invertebrates. In surface waters of Europe and USA triazines were has been recorded at concentrations from hundredths to tens μg.l^{-1}. Environmental concentrations of triazines do not reach lethal concentrations for signal crayfish. Further research is needed to draw more comprehensive conclusions on the use of the signal crayfish as a test organism using sublethal endpoints of trace pesticide toxicity, bioaccumulation, and effects at the molecular level.

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