

Effect of prometryne on early life stages of marbled crayfish (*Procambarus fallax f. virginalis*)

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Abstract

OBJECTIVES: The aim of this study is to assess the toxicity of prometryne in early life stages of marbled crayfish (*Procambarus fallax f. virginalis*) on the basis of mortality, early ontogeny, growth rate, and histopathology during and at the end of the test.

DESIGN: The early life stages of marbled crayfish were exposed to prometryne at four concentrations, 0.51, (reported concentration in Czech rivers), 144, 1 440, and 4 320 µg.l⁻¹ for 53 days and compared to crayfish in a non-treated control group.

RESULTS: Prometryne in concentration 144, 1 444 and 4 320 µg.l⁻¹ caused decrease of weight and specific growth rates of crayfish. Crayfish exposed the highest concentration 4 320 µg.l⁻¹ showed delay in ontogeny development. All crayfish groups exposed to prometryne showed histopathological changes in gill. On the basis of histopathological changes the values of LOEC=0.51 µg.l⁻¹ and NOEC=for 0.10 µg.l⁻¹ of prometryne for marbled crayfish juveniles was estimated.

CONCLUSIONS: Chronic exposure of prometryne on early life stages of crayfish has affected their mortality, growth rate, and histology. Some of the changes were observed only at higher exposures (144, 1 444 and 4 320 µg.l⁻¹), but histopathological changes in gills were observed also in crayfish exposed to the real environmental concentration in Czech rivers (i.e. 0.51 µg.l⁻¹), which is about 9 times lower than maximal concentration (4.40 µg.l⁻¹) reported in surface waters of Greece. Concentrations of prometryne in World rivers have been reported to generally vary in the range of 0.1–4.40 µg.l⁻¹.

Abbreviations:

ANOVA	- analysis of variance
ANC _{4,5}	- acid neutralization capacity
COD	- chemical oxygen demand
LC50	- lethal concentration
LOEC	- lowest observed effect concentration
NOEC	- no observed effect concentration
OECD	- Organization for Economic Cooperation and Development
SGR	- specific growth rate

INTRODUCTION

Environmental pollution caused by pesticides, especially in aquatic ecosystems, has become a serious problem. Many water ecosystems are contaminated with industrial, domestic, and agricultural chemicals such as pesticides, which are ubiquitous and can spread globally as well as regionally (Flynn & Spellman 2009). Pesticides are liable to affect non-target organisms, including fish, and crayfish leading to dramatic ecological changes in the aquatic environment (Velisek *et al.* 2012a, 2013; Stara *et al.* 2012, 2013, 2014).

Prometryne (2,4-bis (isopropylamino)-6-methylthio-s-triazine) was registered in the United States in 1964 as herbicide for several crops, making it a pioneer herbicide in the thiomethyl triazine class of chemistry (LeBaron *et al.* 2008). Prometryne is classified as a selective herbicide of the S-triazine chemical family. Prometryne has a soil half-life of 60 days and persists for up to 90 days. Following multiple annual applications of the herbicide, prometryne activity can persist for 12–18 months after the final application. Half-life in water is 500 days (US EPA 1996). Prometryne application is not permitted in the Europe Union, but is widely used in China (Zhou *et al.* 2009), Australia, Canada, New Zealand, South Africa, and the United States (Kegley *et al.* 2010). Prometryne has been banned in Europe since 2004, it still can be found in waters. In surface waters of Europe, prometryne has been detected at concentrations ranging from 0.190 to 4.40 $\mu\text{g.l}^{-1}$ (Vryzas *et al.* 2011) and in ground water at concentrations exceeding 1 $\mu\text{g.l}^{-1}$ (Papadopoulou-Mourkidou *et al.* 2004).

Crayfish are important benthic invertebrates in the ecosystem, and they are considered an appropriate model organism (Monot 1995; Buric *et al.* 2013). Although the effects of sub-chronic exposure on oxidative stress and antioxidative enzymes of adult crayfish to prometryne have been well-documented in study Stara *et al.* (2014), there is a dearth of data on the sub-chronic toxicity of prometryne at environmentally realistic concentrations in early life stages of crayfish. Native species of crayfish are endangered and protected in the Czech Republic (Kozak *et al.* 2011). For this reason, we have chosen the invasive marbled crayfish (*Procambarus fallax f. virginalis*), which became established in Europe (Kouba *et al.* 2014), as a model species for this study. The marbled crayfish, which was discovered in the mid-1990s, meets researchers' demands for a vigorous, genetically identical and eurytopic laboratory model very well. Its most prominent advantages are production of high numbers of genetically identical offspring, stepwise alteration of the phenotype by moulting, complex morphology and behaviour (Vogt 2008). The aim of the present study was to describe lethal and sub-lethal effects of prometryne on early life stage of non-target aquatic organism, the marbled crayfish using a 53 day toxicity test.

MATERIALS AND METHODS

Experimental animals

Eggs from single marbled crayfish (*Procambarus fallax f. virginalis*) female (carapace length 31.22 mm, postorbital carapace length 23.62 mm, and weight 9.19 g) were gently stripped with tweezers from pleopods. Female originated from own laboratory culture.

Experimental protocol

Three hundred eggs (mean weight 2.27 mg) in IX–X stage of embryonic development were put into petri dishes with tap water. From petri dishes were randomly transferred separately into plastic macroplates containing one of four experimental solutions of prometryne (Sigma Aldrich, Czech Republic, and chemical purity 99.3%) and water which served as a control. Each trial comprised 60 eggs held as single individuals to eliminate the transfer of fungal infection between incubated eggs. The concentrations were marked as follows: 0.51 $\mu\text{g.l}^{-1}$ (reported environmental concentration in Czech rivers – group 1 – E1), 140 $\mu\text{g.l}^{-1}$ (group 2 – E2), 1440 $\mu\text{g.l}^{-1}$ (group 3 – E3), and 4320 $\mu\text{g.l}^{-1}$ (group 4 – E4). The prometryne concentrations of 140 $\mu\text{g.l}^{-1}$, 1440 $\mu\text{g.l}^{-1}$, and 4320 $\mu\text{g.l}^{-1}$ corresponded to the 1% of 96 hour half lethal concentration (96h LC50), 10% 96h LC50 and 30% 96h LC50 for juvenile signal crayfish (*Pacifastacus leniusculus*) (Velisek *et al.* 2013).

Water parameters

Water quality parameters were as follows: temperature 22.8±1.5 °C, dissolved oxygen >60%, pH 7.5–8.0, ANC_{4.5} 0.91 mmol.l⁻¹, COD_{Mn} 0.31 mg.l⁻¹, total ammonia 0.01 mg.l⁻¹, NO₂⁻ 0.02 mg.l⁻¹, NO₃⁻ 4.38 mg.l⁻¹, and sum of Ca²⁺+Mg²⁺ 32.22 mg.l⁻¹. Temperature was measured hourly using Minikin loggers (Environmental Measuring Systems, Brno, Czech Republic). To ensure agreement between nominal and actual compound concentrations, water was analysed during the experimental period by liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Barcelo & Hennion 1997). The values measured did not differ from the value stated for test purposes by more than 10 %.

Experimental protocol

The macroplates were placed in a laboratory (open-air conditions) with the light exposure (11:13 h light: dark). The exposure water for each treatment was renewed three times weekly until the third developmental stage (24 days) was reached. Water was gently by drained from each chamber, then a new solution was slowly added to prevent disturbance. Observations of survival were made daily and dead eggs were removed. From the third development stage, juveniles were kept individually in small boxes made from clear plastic to prevent cannibalism. Each box, 40 mm in height, was divided into 18 separated chambers, with the bottom area of each individual chamber 45 × 30 mm (Kozak *et al.* 2009)

was placed in an aquaria containing 20 l of respective solutions. Animals were fed by freshly hatched, tap-water-rinsed brine shrimp (*Artemia salina*) nauplii *ad libitum* one time daily. The nauplii were rinsed with tap water to avoid contaminating the exposure water with chloride. During and at the end of the experiment, early development stages were observed to monitor development, occurrence of morphological anomalies and body weight. Determination of developmental periods and stages followed Vogt *et al.* (2004), who subdivided into embryonic, juvenile, adolescent and adult phases. The embryonic period starts with oviposition and ends 17–28 days later with hatching. The juvenile phase includes approximately seven to eight stages, which are characterized by a spotted pigmentation pattern. The seven to 10 adolescent stages are increasingly marbled and have clearly visible female characters.

Weight, to 0.1 mg, was measured by using a Mettler-Toledo analytical balance after removing excess water on filter paper. The mean specific growth rate (SGR) for crayfish in each of the experimental groups was calculated for the period beginning at day 24 (the first sampling time) and ending at day 53 (end of the trial) using the method described by Kroupova *et al.* (2010).

The inhibition of specific growth rate in each experimental group was calculated using the following formula according to OECD number 215 (OECD 2000):

$$I_x[\%] = [SGR_x(\text{control}) - SGR_x(\text{group})] / [SGR_x(\text{control})] \times 100$$

where is I_x = inhibition of specific growth in selected experimental group of crayfish after x days of exposure, $SGR_x(\text{control})$ = mean specific growth rate in the control group, $SGR_x(\text{group})$ = mean specific growth rate in selected experimental group of crayfish.

Evaluation of 53 day LC50, LOEC, and NOEC

For the evaluation of 53 day LC50 values, a probit analysis was used based on mortality at different prometryne concentrations. For the evaluation of LOEC and NOEC values, the probit analysis was based on histopathological changes in tissues at different prometryne concentrations and the EKOTOX 5.1 software (INGEO Liberec, Czech Republic) was used.

Histopathology examination

Histopathology was evaluated in groups E1–E3 and control at the end of the experiment (day 53), the group E4 was not sampled for histology because 30 days all juveniles died. Ten whole crayfish from each group were placed in 10% buffered formalin, prepared with standard histological techniques, and stained with haematoxylin and eosin, examined by light microscopy, and photographed using a digital camera.

Statistical analysis

One-way ANOVA was conducted to compare differences among the test groups using the software program Statistica version 12.0 for Windows (StatSoft).

RESULTS

Accumulated mortality

Significant ($p < 0.01$) differences in total accumulated mortality were found in crayfish exposed to the three highest prometryne concentrations, compared with controls (Figure 1). Prometryne in concentration $4320 \mu\text{g.l}^{-1}$ caused 100% mortality of crayfish. Massive mortality in this group occurred on day 15 and 30 (developmental stage 2 and 3). Based on accumulated mortality in the experimental groups, values of lethal concentrations of prometryne was estimated at 53 day $LC_{50} = 40 \mu\text{g.l}^{-1}$.

Growth parameters

Mean body weight of crayfish related to prometryne concentration in water is depicted in Figure 2. No significantly negative effects on body weight and specific growth rate were observed when environmental concentration of prometryne (E1; $0.51 \mu\text{g.l}^{-1}$) was compared with the control. From 24 days of exposure, crayfish exposed to the highest tested groups E4 – $4320 \mu\text{g.l}^{-1}$ prometryne showed significantly ($p < 0.01$) lower mass compared with control. Beginning on day 38 of expo-

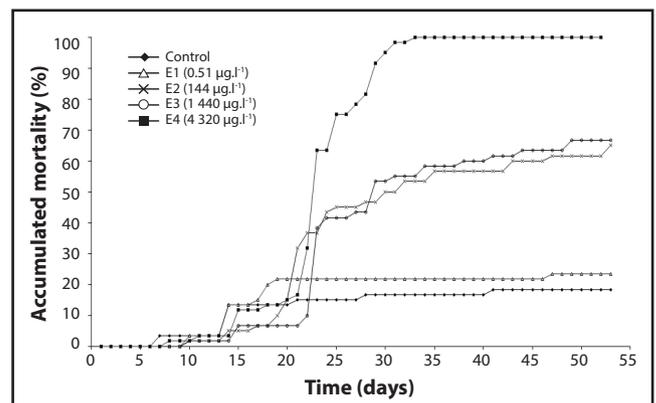


Fig. 1. Accumulated mortality (percentage) of early life stages of marbled crayfish (*Procambarus fallax f. virginalis*) after prometryne exposure.

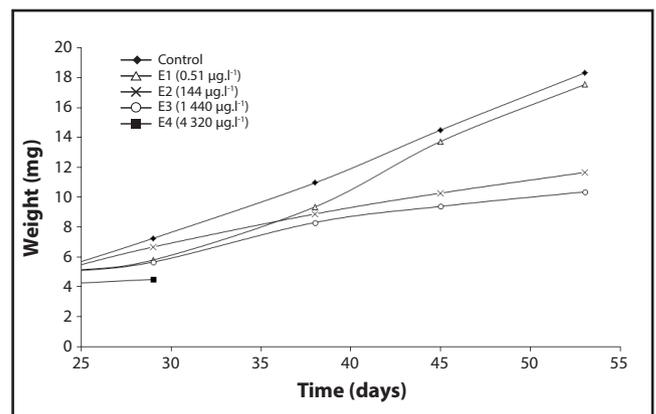


Fig. 2. Mean body weight of early life stages of marbled crayfish (*Procambarus fallax f. virginalis*) after prometryne exposure.

sure, also crayfish exposed to the tested groups E3 – 1440 $\mu\text{g.l}^{-1}$, and E2 – 144 $\mu\text{g.l}^{-1}$ prometryne showed significantly ($p < 0.01$) lower mass compared with control. Specific growth rates and inhibition of growth were calculated for 53 day of exposure and are given in Table 1. Inhibition of growth in the group exposed to prometryne in concentrations 144 (E2) and 1440 $\mu\text{g.l}^{-1}$ (E3) was 34.8 and 42.6 % compared to control, respectively.

Early ontogeny

Only crayfish exposed the highest concentration 4320 $\mu\text{g.l}^{-1}$ showed delay in ontogeny development compared with the control group. Furthermore, no significant differences in the type and occurrence of morphological abnormalities were observed in tested crayfish during the test.

Histopatology

There were no apparent differences in hepatopancreas tissue between control and group E1. The morphology of the hepatopancreas in these two groups was normal, with tubules tightly arranged. Different cell types were both easily recognized and reasonably uniform in shape and size. The marked alterations in hepatopancreas tubules were observed in groups E2 and E3. The pathologies were as follows: tubular dilatation with the predominance of mononuclear cells in interstitial tissue and focal dystrophic tissue destruction with more pronounced changes in group E3 (Figure 3).

Large fragmental alterations with pseudocystical formations in gills of all experimental groups were apparent. The most pronounced changes were represented by dilatations of filaments into pseudocystical structures filled with fine-grained substance. All described changes were more frequent in the group exposed to higher concentration of prometryne (E3).

On the basis of histopathological changes of gills in the experimental groups, values of LOEC = 0.51 $\mu\text{g.l}^{-1}$ and NOEC = 0.10 $\mu\text{g.l}^{-1}$ of prometryne for marbled crayfish juveniles were estimated.

DISCUSSION

Crayfish can serve as an excellent model species to increase the knowledge-base for invertebrate ecotoxicology (Burggren & McMahon 1983). They are large invertebrates and easily reared in a laboratory setting. This makes them useful not only for toxicity testing, but as an invertebrate model for a variety of physiological experiments. The eggs are very large for a crustacean and can easily be counted and assessed for fertilization status, mortality, etc., thus making them useful for early life stage studies. Studies of the embryonic development of crayfishes are important, not only to increase knowledge of the developmental processes, but also to understand species-specific adaptations and their ecological value in the course of speciation (Meijide & Guerrero 2000). The sensitivity of embryos of the marbled crayfish to pollutant was tested with testosterone (Vogt 2007). Although we have information on the toxicity of the prometryne in adult of crayfish, there is no information on its toxicity in early life stages. This is the first study investigating toxicity of prometryne on early life stage of crayfish.

Mortality, decreased growth rate, and delayed early development are common chronic toxicity responses (Woltering 1984). Prometryne in concentration 4320 $\mu\text{g.l}^{-1}$ caused total mortality of crayfish during 15 and 30 day (stage 2 and 3). The most of the external sense organs become functional in juvenile stage 2, that the digestive system becomes functional in stage 3 (Voght 2008). This is in accordance with data concerning the so called “point of no return” the moment when the juvenile irreversibly lose ability to feed, and die even if provided with food. In marbled crayfish juvenile the point of no return occurs about day 10–22 days (Voght 2008) and starvation-induced mortality occurs after that time.

The present study revealed no significant negative effects of prometryne on growth and mortality in early life stages at the environmental concentration

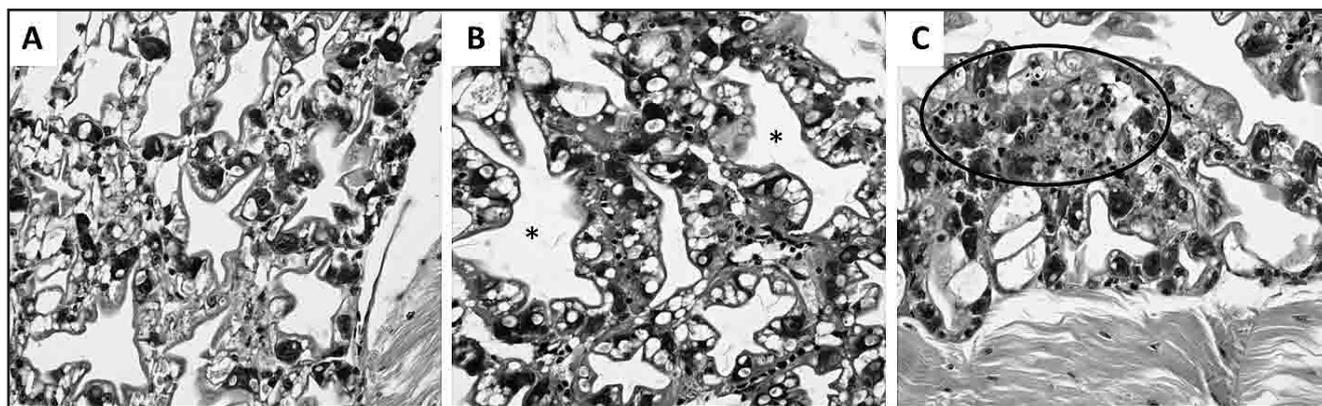


Fig. 3. Transversal sections of juvenile marbled crayfish (*Procambarus fallax f. virginalis*) hepatopancreas. A – control group; B – E2 group exposed to 1% of 96 hour half lethal concentration (96h LC50) of prometryne for 53 days; C – E3 group exposed to 10% of 96h LC50 prometryne for 53 days (400 \times). Asterisks (*) mark noticeable tubular dilatation; ellipse indicates.

(0.51 $\mu\text{g.l}^{-1}$). Crayfish exposed the highest concentration 4320 $\mu\text{g.l}^{-1}$ showed delay in ontogeny development compared with the control group. Delay in ontogenetic development is described after prometryne exposure in concentrations ranging from 200 to 2000 $\mu\text{g.l}^{-1}$ in early life stages of fish (Velisek *et al.* 2012b). In the present study, crayfish exposed to the 144, 1440 and 4320 $\mu\text{g.l}^{-1}$ prometryne showed significantly lower mean body weight compared with the control. Growth reductions might delay maturation and reproduction as well as increase the susceptibility of young crayfish to predation. Their ability to obtain food and to compete for suitable habitats might also be reduced (Woltering 1984). Velisek *et al.* (2012a,b) reported a significant decrease growth after triazines pesticide (terbutryne and simazine) exposure in common carp (*Cyprinus carpio*). Significant decrease of growth in juvenile Australian red claw crayfish (*Cherax quadricarinatus*) after exposure mixture glyphosate (22.5 mg.l^{-1}) and polyoxyethylenamine (15 mg.l^{-1}) reported Frontera *et al.* (2011).

The effect of chronic exposure to triazine at low concentrations on histopathology of early life stages has not yet been studied. In our study, crayfish exposed to prometryne in all tested concentrations (0.51; 144; 1440 and 4320 $\mu\text{g.l}^{-1}$) resulted in histopathological changes of gills and hepatopancreas. Crustacean gills are a vital organ as they play an important role in diffusion and transport of respiratory gases and regulation of osmotic and ionic balance. Gills are a primary target organ for most pollutant, uptake from water is the most important route, and may be one of the first organs to exhibit symptoms of toxicity (Desouky *et al.* 2013). Changes caused of prometryne in gills may affect osmoregulatory gills function and gases exchange. Observed pathological changes are in accord with those described in Desouky *et al.* (2013) in red swamp crayfish (*Procambarus clarkii*) gills after exposure of organophosphorus insecticide ethion (0.36 mg.l^{-1}). The crustacean hepatopancreas is likely the main organ for the detoxification of pollutants (Vogt 2002) and produces and secretes all digestive enzymes involved in carbohydrate metabolism, production of emulsifiers, excretion, and calcium, and heavy metal storage (Holdich & Reeve 1988). These changes in our experiment are probably due to accumulation of the prometryne in the cells of hepatopancreas or due to increasing the activity of lysosomal enzymes which are capable of destroying cell organelles. Similar histopathological changes in hepatopancreas were also reported in red swamp crayfish after exposure of ethion (Desouky *et al.* 2013); malathion (Garo *et al.* 1998); diazinon (Heiba 1999) and fenitrothion (Aly 2000). On the other hand, Stara *et al.* (2014) found no histopathological changes in hepatopancreas of adult red swamp crayfish after prometryne exposure in concentrations (0.51–1440 $\mu\text{g.l}^{-1}$).

Histopathological changes were used for estimation of NOEC and LOEC. The values for NOEC and

LOEC were estimated at 0.1 and 0.51 $\mu\text{g.l}^{-1}$ prometryne, respectively. It appears that prometryne may be a serious problem for early life stages of crayfish in the wild. Some of the changes were observed only at higher exposures (144, 1440 and 4320 $\mu\text{g.l}^{-1}$), but histopathological changes in gills and hepatopancreas were observed in crayfish exposed the real environmental concentration in Czech rivers (i.e., 0.51 $\mu\text{g.l}^{-1}$), which is about 9 times lower than maximal concentration (4.40 $\mu\text{g.l}^{-1}$) reported in surface waters of Greece (Vryzas *et al.* 2011; Caquet *et al.* 2013).

Histopathological changes in gills and hepatopancreas are potential biomarkers for monitoring residual triazine pesticides in early life stages of crayfish. For detailed elucidation of prometryne effects, further research is necessary. Aquatic environment may be polluted by many substances, the effects of which can be potentiated with combined exposures. This research should be focused not only on the studies of effects of prometryne alone, but in view of possible synergic or potentiation effects.

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