Research Article

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Ex situ Effects of Chlorpyrifos on the Lysosomal Membrane Stability and Respiration Rate in Zebra Mussel, *Dreissena polymorpha* (Pallas, 1771)

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Abstract: The present study examines the lysosomal membrane stability in haemocytes of Zebra mussel (*Dreissena polymorpha*) by applying the neutral red retention assay (NRRA) as well as the respiration rate and survival under acute pesticide exposure. The mussels were treated with different concentrations of chlorpyrifos in laboratory conditions for a total acute exposure period of 72 hours. The pesticide concentrations were prepared as 50 and 30% of the maximum permissible level (100%) set by the national and EU legislation. We found that destabilization of the lysosomal membrane stability occurred at all tested concentrations and the respiration rate was time- and dose-dependent. Overall, we consider that the results from such experiments can be successfully applied in risk assessment, monitoring programs and water policy, and the use of pesticides such as chlorpyrifos should be controlled very cautiously in plant protection and agriculture.

Key words: *Dreissena polymorpha*, pesticide, lysosomes, neutral red retention, respiration rate

Introduction

Pesticides have been widely applied to protect agricultural crops since the 1940s, and since then, their use has increased steadily (Grung et al. 2015). The organochlorine pesticides (OCPs) became the dominant pesticides after the Second World War. With the publishing of "Silent Spring" by Rachel Carson in 1962, a wider audience was warned of the environmental effects of the widespread use of pesticides. Since 1970s, after evidence of their toxicity, persistence and bioaccumulation in environmental matrices organochlorine pesticide production, usage and disposal have been regulated or prohibited in most of the developed countries (Jan et al. 2009). Therefore, substitutes such as organophosphorus pesticides (OPPs) are being used in large amounts in the European Union (EU) and the USA (Serrano et al. 1997).

Chlorpyrifos (CPF, O,O-diethyl O-3,5,6-trichloro-2-pyridyl phosphorothionate) is an organophosphate insecticide widely used to control a large variety of pests (e.g., Coleoptera, Diptera, Homoptera and Lepidoptera) in agricultural and animal farms, which has led to serious environmental problems (Fu et al. 2013). According to Wang et al. (2013), it is the most common agrochemical of aquatic environment. The United States Environmental Protection Agency classifies CPF as a moderately toxic compound (Li et al. 2015). CPF is estrogenic and alters embryonic hatching, cell proliferation and apoptosis in Zebra fish (Yu et al. 2015); it affects the sex steroid production and thyroid follicular development in adult and larval lake sturgeon (Brandt et al. 2015); the acetylcho-

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linesterase activity in climbing perch and Zebra fish (Nguyen et al. 2015) as well as the activity of glucose-6-phosphate dehydrogenase in rainbow trout (Topal et al. 2014); it causes oxidative stress in common carp and Zebra fish (Chen et al. 2015); it impacts the protein synthesis in Zebra fish (Liu et al. 2015) and DNA and RNA synthesis in common carp (Wang et al. 2014), and causes histopathological alterations in common carp (Xing et al. 2012). The acute lethality (96-h LC_{50}) of CPF to aquatic invertebrates ranges from 0.25 μg/L for the mayfly (Ephemeroptera) to more than 806 μg/L for the snail (Gastropoda) (Barron & Woodburn 1995). However, the ecotoxicological data on the effects of CPF on invertebrates and especially on freshwater mussels is much less studied compared with its effect on vertebrates (Cacciatore et al. 2015, Xing et al. 2015, Ventura et al. 2016).

The U.S. Environmental Protection Agency (EPA) classifies freshwater mussels as biomonitors because they react to changes in the surrounding environment. As monitoring organisms, they present many advantages because they are sessile and sedentary, have a reasonable size, long-lived, widespread, easily accessible, and often available in high numbers (Farrington et al. 1983, Goldberg 1986). Additionally, since mussels are filter feeders, they bioaccumulate persistent organic pollutants (POPs) and other contaminants in their bodies and shells (Naimo 1995). In comparison to fish and crustaceans, bivalves have a very low level of activity of enzymatic systems capable of metabolizing POPs. Therefore, bivalves are widely used as bioindicators of organic pollution in freshwater, marine and estuarine ecosystems because they are known to provide a time integrated indication of environmental contamination as well as reliable information on the potential impact of seafood consumption on human health (Turja et al. 2015). In this context, *Dreissena polymorpha* (Pallas, 1771) has been successfully used in ecotoxicological studies and monitoring programs (Lepom et al. 2012, Binelli et al. 2015).

The aim of the present work was primarily to study the possible acute effects of decreasing concentrations of chlorpyrifos, which is categorized as a priority substance in surface waters within the European Water Framework Directive for the protection of aquatic ecosystems (Directive 2008/105/EO) on two biomarkers at a cellular and physiological level of organization in Zebra mussel (*Dreissena polymorpha*) – lysosomal membrane stability by applying the neutral red retention assay and respiration rate. These methods were chosen because they are known as reliable, easy to apply, and with comparatively low analytical costs.

Materials and Methods

About 150 specimens of the same size-group (mean length 2.5 cm ± 0.5) were hand collected in the spring of 2015 from Jrebchevo Reservoir (42° 35′ 40.71″ N, 25° 56′49.42″ E), south-eastern Bulgaria, which is considered as relatively uncontaminated water basin. The mussels were placed in 10 L containers filled with the reservoir water and transported quickly to the laboratory at the Department of Ecology and Environmental Conservation, Plovdiv University. After transportation, the mussels were moved in 50 L glass aquaria with chlorine-free tap water (by evaporation) to acclimatize for a week. The water was kept oxygen saturated. During the entire duration of the experiment, the mussels were maintained under a natural light/dark cycle (12:12). After acclimatization, the mussels were divided into 4 groups (n = 30 in each experimental tank and n = 30 for control) in chlorine-free tap water. The mussels were treated with different soluble concentrations of chlorpyrifos, dissolved in cyclohexane for 72 hours. They were prepared as 50 and 30% of the maximum permissible level set by the national and EU law. According to the Bulgarian legislation based on Directive 2008/105/EO, the maximum permissible concentration (MPC) of CPF in surface waters is 0.03 μg/L. Thus, for 50 L tanks in the current experiment, 1.5 μg (100%), 0.75 μg (50%) and 0.45 μg (30%) were applied. Mussel mortality about 1% in the tanks with 100% and 50% pesticide was recorded during the exposure period. Mussels were not fed throughout the experiment. The physico-chemical characteristics of the test water (pH, conductivity, temperature and oxygen level) were measured once in the beginning of the experiment and on the 24th and 72nd h with a combined field-meter.

The Neutral red retention time (NRRT) assay was adapted from Lowe & Pipe (1994). It is based on the use of a cationic probe neutral red, which is taken up into cells by membrane diffusion where it becomes ion trapped within the lysosomal compartment (Rashid et al. 1991). Over the time, the dye tends to leak out of the lysosomes into the cytosol, which is then stained by the dye (Harreus et al. 1997). The exposure to toxic substances damages the lysosomal membrane and hence, increases its permeability. The endpoint parameter is the time at which a dye loss to the cytosol is evident in 50% of the granular haemocytes. Haemolymph (about 0.5 mL) was withdrawn from the posterior adductor muscle of 10 mussels from each experimental tank using a 2.5 mL syringe containing an equal volume of Calcium-Magnesium free physiological solution as described by Molnar

& Fong (2014) in order to obtain a 50/50 of cell/physiological solution. The time period between the NR probe application and the appearance of the first evidence of dye loss from the lysosomes to the cytosol in at least 50% of the examined cells belonging to the granular haemocytes represents the NRR time for the mussels. Following a further 15 min interval, each preparation was observed at 30 min intervals until a total time of 180 min.

The respiration rate was measured at the beginning of the experiment (0 h), at the 24th h and at the end of the experiment (72nd h). At the given time, 10 mussels were transferred in 1.2 L tanks filled with water from the test aquariums. Oxygen levels were measured, using oximeter "Oxi 315i/SET". The tanks were then covered with plastic foil (water level must be to the edge of the tank) in order any oxygen transfer from the air to be eliminated. The tanks were left for one hour and thereafter the oxygen level was measured once again. The respiration rate was calculated by determining the difference in the dissolved oxygen levels before and after the passed time, following Tsekov (1989): $I = Q_2/G$, where: I – respiration rate index; G – weight of the mussels, in grams, Q_2 – oxygen consumed by the mussels between the two measurements (the difference between the oxygen levels before and after the 1-hour Q_2=Q-Q_1hour). Q is calculated by the following formula: Q = V x q, where: Q – total oxygen level in the tank; V – volume of the water in the tank, in litres; q – level of dissolved oxygen in 1 litre of water (mg/L).

For the statistical processing of the data the software package "Statistica 7.0" was used. T-test was applied to see if there is a significant difference between the NRR time of the control and mussels exposed to CPF on the 24th and 72nd hour. Spearman's correlation analysis was also applied, when the data were not normally distributed (Fowler et al. 1998), in order to examine the changes of the respiratory rate, along with the changes of the pesticide concentrations. Significance level was set to $p \leq 0.05$.

Results and Discussion

The physico-chemical properties of the water showed relatively constant values in all 4 experimental tanks (Table 1). In general, the values between the 0, 24th and 72nd h were similar for the studied period. These for the control groups were as follows: pH – 7.8 ± 0.2, conductivity – 456 µS/cm ± 2.1, temperature – 22 °C ± 0.4 and oxygen level – 6.4 mg/L ± 0.1, respectively. Therefore, we think that the changes, which we observed in the mussels, were not due to changes in the abiotic factors.

The results on lysosomal membrane stability of the control and exposed mussels to CPF after the 24th and 72nd h are presented in Fig. 1. Overall, the control mussels did not show destabilized lysosomes as the average NRR time was 132 min. There was no significant difference between the NRR time of the controls examined on the 0, 24th and 72nd h ($p > 0.05$). However, CPF had a significant effect on the mussels exposed to it for 24 and 72 hours. The mussels treated for 24 h with 1.5 µg (100%) CPF had an average NRR time only 54 min, while those treated with 0.75 µg (50%) and 0.45 µg (30%) CPF – 84 and 102 min, respectively. The statistical analysis showed a significant difference ($p < 0.05$) between the control and mussels exposed to 1.5 µg (100%) and 0.75 µg (50%). Such was also found between the mussels exposed to 1.5 µg (100%) and 0.45 µg (30%) CPF for 24 hours. Overall, there was a dose response of toxicity in terms of the lysosomal membrane stability on Zebra mussels. Similarly, the exposure to CPF for 72 hours had a significant effect on the destabilization of lysosomes. The mussels treated for 72 hours with 1.5 µg (100%) CPF had a shorter average NRR time compared with the ones examine on the 24th h – 39 min, and those treated with 0.75 µg (50%) and 0.45 µg (30%) CPF – 54 and 72 min, respectively. There were significant differences for the NRR time between the controls and all three groups of mussels exposed to CPF on the 72nd hour ($p < 0.05$), but there were no such for the NRR time between the mussels exposed to 1.5 µg (100%), 0.75 µg (50%) and 0.45 µg (30%) CPF. In addition, there was a significant difference found for the NRR time of the dye between the mussels exposed to 0.45 µg (30%) CPF on the 24th and 72nd h.

Lysosomes are highly conserved multi-functional cellular organelles present in haemocytes, which play a key role in the immune defence of mussels (Cajaraville & Pal 1995). Moreover, the lysosomal system, which is remarkably well-developed in mus-

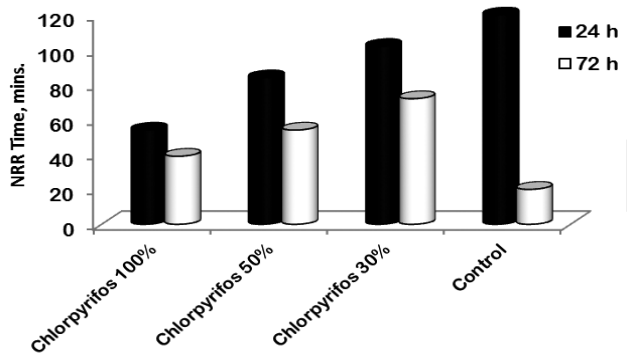

Fig. 1 Average neutral red retention time in Zebra mussel, exposed to chlorpyrifos for 24 and 72 hours as well as in the control group

Table 1. Water physico-chemical properties in the experimental tanks, average results for the 0 h, 24th h and 72nd h

Pesticide concentration	pH	Conductivity, µS/cm	Temperature, °C	Oxygen level, mg/L
Chlorpyrifos 30%	7.88±0.06	462±2.2	21±0.8	6.4±0.3
Chlorpyrifos 50%	7.84±0.09	457±0.8	21.3±0.3	6.5±0.4
Chlorpyrifos 100%	7.85±0.1	457±1.4	21.5±0.4	6.3±0.3

Table 2. Index of respiration rate of Zebra mussel exposed to different chlorpyrifos concentrations at the beginning of the experiment (0 h), 24th h and the end of the experiment (72nd h).

Pesticide concentration	Water volume, L	Weight, g (G)	Total oxygen level (mg/L)					Index of respiratory rate (I)
			Beginning		End		Total	
			q	Q	q_{1h}	Q_{1h}	(Q_2)	
Beginning (0 h)								
Control	1.2	16.73	5.7	6.84	5.6	6.72	0.12	0.007
Chlorpyrifos 30%	1.2	17.30	5.5	6.60	5.4	6.48	0.12	0.007
Chlorpyrifos 50%	1.2	16.95	5.5	6.60	5.4	6.48	0.12	0.007
Chlorpyrifos 100%	1.2	14.82	5.5	6.60	5.3	6.36	0.24	0.016
24th h								
Control	1.2	16.00	5.7	6.84	5.6	6.72	0.12	0.008
Chlorpyrifos 30%	1.2	16.27	5.7	6.84	5.4	6.48	0.36	0.022
Chlorpyrifos 50%	1.2	16.29	5.7	6.84	5.3	6.36	0.48	0.029
Chlorpyrifos 100%	1.2	17.65	5.8	6.96	5.3	6.36	0.60	0.034
72nd h								
Control	1.2	15.44	5.9	7.08	5.7	6.84	0.24	0.016
Chlorpyrifos 30%	1.2	13.99	5.8	6.96	5.6	6.72	0.24	0.017
Chlorpyrifos 50%	1.2	14.08	5.8	6.96	5.5	6.60	0.36	0.026
Chlorpyrifos 100%	1.2	16.08	5.9	7.08	5.5	6.60	0.48	0.030

sel haemocytes, is well-known as a target site for toxic metals and organic chemicals, due to its ability to accumulate them (Moore 1985, 1990, Viarengo et al. 1985). As a consequence, cell health deteriorates after lysosomal damage induced by different contaminants. The damage is mainly due to rapid weakening of the lysosomal membranes, which may release hydrolytic enzymes into the cytoplasm with subsequent enhanced protein catabolism up to the autophagic conditions indicating a stress syndrome (Moore 1985). In this sense, the destabilization of lysosomal membranes is an established indicator for toxically induced adverse effects in fish and shellfish (Lowe & Pipe 1994, Fernley et al. 2000). Our results are in agreement with other studies that report the effects of organic contaminants on the lysosomal membrane stability in molluscs or other invertebrates (Sforzini et al. 2011, Shaw et al. 2011), i.e. the destabilization of lysosomes is dose and time-dependent. However, our study is one of the few that addresses the effects of CPF on lysosomal destabilization in freshwater invertebrates. Probably the most interesting result, which we obtained from this experiment, was that interference occurred at cellular level in the mussels treated with CPF concentrations lower than the allowable levels in surface waters in Bulgaria and EU. This fact shows the great need for further studies on the effects of CPF.

The results from the respiration rate measurements are presented in Table 2. At the beginning of the experiment (0 h) we found that there was no change in the respiration rate index in Zebra mussel from the control group and the groups treated with 30% and 50% concentrations of CPF. We recorded a 2.82 times increase in the respiration rate of the mussels from the highest pesticide concentration compared with the control. We also registered a moderate, statistically insignificant, correlation between the CPF concentrations and the respiration rate index ($s = 0.77$, $p = 0.5$). After 24 hours of exposure we recorded a 2.75 ÷ 4.25 times increase of the respiration rate in the mussels from the test variants compared with the control. We also recorded a full positive correlation between the chlorpyrifos concentration and the index of respiration rate ($s = 1.00$, $p = 0.08$). At the end of the experiment (72nd h) this correlation remained almost unchanged ($s = 0.98$, $p = 0.02$) but there was only a 1.06 ÷ 1.84 times increase in the respiration rate between the control and the exposed mussels.

The rate of respiration reflects the metabolic activities of animals and the responses due to changes

in the surrounding environment are a good indicator of adjustment capacity of the organism (Kumar et al. 2012). Bivalve molluscs reflect immediate responses to toxic substances present in the surrounding water by changes in their physiological responses (Basha et al. 1988) and histological arrangement (Kumar et al. 2012). It is known that without time for acclimation mussels typically reduce their clearance rate (volume of water passing through gills per unit time), thus potentially lowering their intake of oxygen (Aldridge et al. 1987). However, most bivalve molluscs reflect immediate responses to toxic substances present in the surrounding water by changes in physiological responses (Basha et al. 1988). In most cases the respiration rate increases with the increase of the pollutant concentration and level of toxicity (Kumar et al. 2012). The reason for this is that the organism tries to deliver more oxygen to all tissues and organs triggered by the stress, which is caused by the toxic exposure. This was the case with Zebra mussel in the present experiment, the mussels reacted by increasing their respiration rate with the increase of the pesticide concentrations after the 24th h of exposure and this pattern remained unchanged after the 72nd hour of exposure. As stated by Naimo (1995) analyses of freshwater mussels can indicate metal bioavailability and these organisms may be useful in more sensitive, sublethal toxicity tests. Zebra mussels are sentinels of contaminant bioavailability in the natural water bodies and may be an important link in the trophic transfer of contaminants in the water basin because of their increasing importance in the diets of certain fish and waterfowl (Cope et al. 1999). Most research on the effects of pesticides on freshwater mussels however has concerned bioaccumulation. Experimental studies should incorporate complex measurements that examine the linkages between sublethal toxicity and bioaccumulation in freshwater mussels. Thus, more immediate information on exposure concentrations and physiological activity of freshwater mussels will be needed.

Conclusions

In Bulgaria, POPs pollution including pesticides is a relatively new ecological problem and its effects on biota is poorly studied as there is need for well-trained ecotoxicolgists and lab technicians. Based on the results from the present study we can conclude that CPF has severe effects on the physiology of Zebra mussel. These effects are presented as a destabilized cellular compartment and altered respiratory functions. Furthermore, the negative impact of this pesticide was observed at concentrations lower than the maximum permissible levels, which we think is evidence for its severe toxicity on freshwater mussels. However, we suggest that further detailed research needs to be carried out it this particular area as this experiment was performed only once. Lastly, we consider that such results could be used for better agricultural practices as well as in the field of aquatic legislation and environmental conservation, respectively.

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