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EFFECTS OF CADMIUM (Cd) ON THE LYSOSOMAL MEMBRANE STABILITY AND RESPIRATION RATE OF TWO FRESHWATER MOLLUSKS UNDER *ex situ* EXPOSURE: PRELIMINARY DATA

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ABSTRACT. *The aim in the present study was to give some preliminary data on the effects of Cd, which is considered as priority toxic substance in surface waters according to Directive 2008/105/EO (2008) on the lysosomal membrane stability and respiration rate in two invasive and resilient to changes in the surrounding media freshwater mollusks − Chinese pond mussel (Synanodonta woodiana) and zebra mussel (Dreissena polymorpha) in laboratory conditions for 72 hours. Significant decrease in the lysosomal destabilization indices with lower retention time and increase in the respiration rate index were observed in the treated with Cd mussels, compared with the control. In general, the tested species proved to be sensitive to Cd exposure in terms of the two studied biomarkers.*

KEY WORDS: *cadmium, lysosomal membrane stability, respirationy rate, Dreissena polymorpha, Synanodonta woodiana, ex situ exposure.*

Environmental contamination by metals among other chemicals, involves great health risks for all living organisms, humans and wildlife (Klaassen 2013). Heavy metals are toxic, non-biodegradable and persistent environmental contaminants (Has-Schön et al. 2006). One of the most common pollutants is Cd, which is used principally in the production of stabilizers and pigments in plastic, in the electroplating industry and is also

released as a by-product from other anthropogenic activities including mining, metal industries and agriculture (Clark 1989). To monitor the health of coastal systems, sentinel organisms such as mussels (bivalves) have been identified as suitable candidates to indicate levels of contaminants in the aquatic environment and as such, have been proposed to be suitable "biomonitors" of pollution (Naimo 1995, Camusso et al. 2001, Besada et al. 2011).

The main aim in the present study was to give some preliminary data on the effects of Cd, which is considered as priority toxic substance in surface waters according to Directive 2008/105/EO (2008) on the lysosomal membrane stability and respiratory rate in the Chinese pond mussel (*Synanodonta woodiana*) and zebra mussel (*Dreissena polymorpha*) in laboratory conditions, and see if there are any differences between the two species.

Ten specimens of the Chinese pond mussel of the same size-group (mean length 22.5 cm ± 5.5) and 10 specimens of zebra mussel, also of the same size-group (mean length 2.5 cm ± 0.5) were used in the experiment. All animals were collected in the spring of 2015 from one of the basins at the Institute of Fisheries and Aquaculture in Plovdiv, Bulgaria where fish are usually reared under strict toxicant-free conditions. After transportation the mussels were acclimatized for a week. The water was kept oxygen saturated, the animals were maintained under a natural light/dark cycle (12:12 hours) and they were not fed prior or during the experiment.

After acclimatization the mussels were divided into two groups in 50 L tanks – control, untreated and test variants, which were treated with Cd (soluble) for 72 hours. The metal concentration represented the maximum permissible levels set by the national and EU law. According to the Bulgarian legislation based on the EU Directive, the maximum permissible concentration of Cd in inland surface waters is 0.45 µg/L. This means that for 50 L tanks in our experiment 22.5 µg were used. No mussel mortality was recorded during the exposure period.

The physico-chemical characteristics of the aquarium water (pH, temperature, conductivity and oxygen level), were measured once at the start of the experiment (0 h), as well as on the 24and 72 hour according to a standard procedure (APHA 2005).

The analytical procedure for the neutral red retention time (NRRT) assay was adapted from Lowe & Pipe (1994) and Lowe et al. (1995). Haemolymph (about 0.5

mL) was withdrawn from the posterior adductor muscle from both freshwater mussels using a 2.5 mL syringe containing an equal volume of Calcium-Magnesium free physiological solution (CMFS: 4.77 g/L HEPES, 25.48 g/L NaCl, 0.75 g/L KCl, 1000 mL DI H_2O) as described by Molnar & Fong (2012) with slight modifications in order to obtain a 50/50 of cell/physiological solution. The suspension of 40 µl was spread onto the center of microscope slides, transferred to a lightproof humidity chamber and allowed to attach. The slides were removed individually from the chamber and the excess suspension was carefully tipped off on a paper towel. Thereafter, 40 µL of a working solution (10 µL into 5 mL physiological solution from a stock solution of 4 mg Neutral Red (C.I. 50040 Sigma) in 1 mL of dimethyl sulfoxide) was added to the cell monolayer and 22x22 mm cover slip was placed on the top of each slide. After 15 min. incubation, the slides were examined systematically using light microscopy. 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 mussel. Following a further 15 min., each preparation was observed at 30 min. intervals until a total time of 180 min.

To measure the respiration rate at the given time the Chinese pond mussels were transferred in 6 L tanks filled with water from the test tanks and the zebra mussels in 1.2 L tanks, respectively. The 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. Then they were left for 1 hour, when 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 hour, following Tsekov (1989): $I = Q_2/G$, where I – respiratory rate index; G – weight of the animals, in grams, Q_2 – oxygen consumed by the animals between the two measurements (the difference between the oxygen levels before and after the 1-hour $Q_2 = Q\text{-}Q_1$ hour). Q is calculated by the following formula: $Q = V \times q$, where: Q – total oxygen level in the tank; V – volume of the water in the tank, in liters; q – level of dissolved oxygen in 1 liter of water (mg/L).

The physico-chemical properties of the water showed relatively constant values in the control and experimental tanks. These for the control groups were as follows: pH – 8.1±0.5; conductivity – 435±1.5 µS/cm, temperature – 21.5±2 ˚C and oxygen level – 6.8±0.7 mg/L, and these for the

experimental tank - pH – 7.9±0.5; conductivity – 465±3.5 µS/cm, temperature – 20.5±0.5 ˚C and oxygen level – 6.5±0.7 mg/L, respectively.

Neutral Red Retention Assay. The results on lysosomal membrane stability of both, the control and exposed mussels to Cd for 72 hours are presented in Fig. 1 and 2.

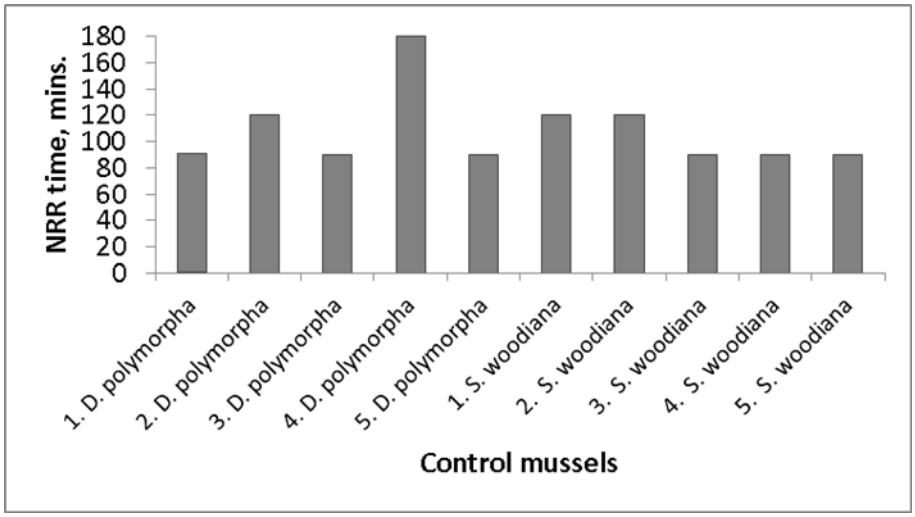

Figure 1. Neutral red retention time for the control mussels.

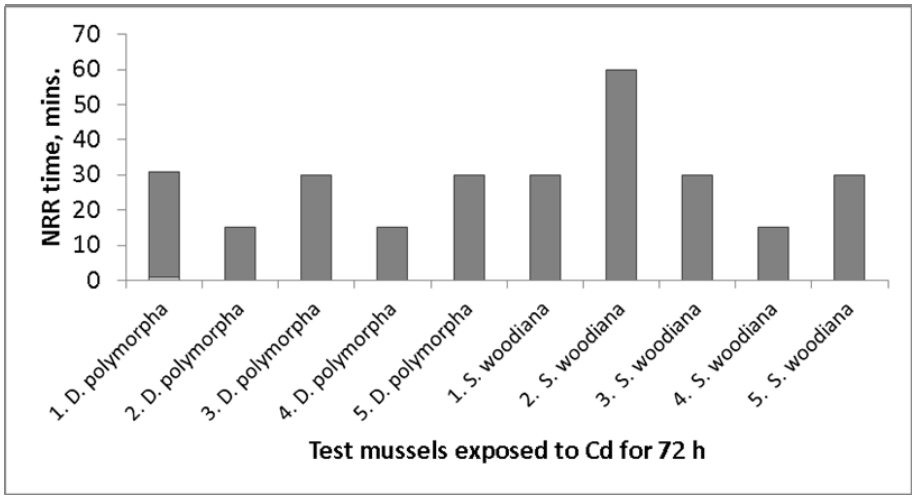

Figure 2. Neutral red retention time for the mussels exposed to Cd for 72 hours.

The average retention time for the control Chinese pond mussels was 114 min., with minimum of 90 min and maximum of 180 min. In addition, the average retention time for the control zebra mussels was 102 min., with minimum of 90 min. and maximum of 120 min. Therefore, the control animals did not show any destabilized lysosomes and could be considered as healthy and not stressed. On the other hand, significant decreases in the lysosomal destabilization indices with lower retention time were observed in the treated with Cd mussels. The Chinese pond mussels had an average NRR time of the dye 33 min., while the zebra mussels – only 24 min.

In general, both species proved to be sensitive to Cd exposure in terms of lysosomal membrane stability. Even though, there is limited data on the effects of heavy metals on freshwater bivalves such as the Chinese pond mussel and zebra mussel (most studies deal with *Mytilus sp*. and organic contamination) our results correspond with the ones reported by other authors regarding the heavy metal effects on invertebrates (Svendsen et al. 1995, Shepard & Bradley 2000, Matozzo et al. 2001, Molnar & Fong, 2012).

Respiration rate. The results from the respiration rate measurements are presented in Table 1. At the beginning of the experiment (0 hour) we found that the respiratory rate index in the Chinese pond and zebra mussels from the control was higher than the test concentration of Cd. After 24-hour of exposure approximately 1.33 times of increase of the respiration rate in the zebra mussels was recorded, which remained almost unchanged after the 72-hour of exposure. Both values were much higher compared to the control. For the Chinese pond mussels we recorded 1.83÷2.41 increase in therespiration rate index after 24 and 72-hour of exposure, respectively. Both values were higher than the control once again.

A similar pattern was observed by Nikolov et al. (2009) in zebra mussel treated with zinc after 96-hour exposure. The mussels increased their respiration rate with the heavy metals concentration. A study by Kraak et al. (1994) suggests that the effects of Zn and Pb on the filtration rate of zebra mussel also increase when the exposure time is increased.

Most bivalve mollusks 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 the Chinese pond and zebra mussels in the present experiment − the mussels reacted by increasing their respiration rate in test tanks after the 24-hour of exposure to Cd, and this pattern remained unchanged after the 72-hour of exposure.

Table 1. Index of respiratory rate of zebra mussel (*Dreissena polymorpha*) and Chinese pond mussel (*Synanodonta woodiana*), exposed to cadmium (Cd) at the beginning of the experiment (0 hour), 24-hour and the end of the experiment (72-hour).

Test variant	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)	
Dreissena polymorpha								
			Beginning (0 hour)					
Control	1.2	13.758	8.8	10.56	7.4	8.88	1.68	0.122
Test	1.2	14.395	8.5	10.20	7.6	9.12	1.08	0.075
			24-hour					
Control	1.2	13.467	9.1	10.92	8.7	10.44	0.48	0.036
Test	1.2	13.142	8.5	10.20	7.4	8.88	1.32	0.100
			72-hour					
Control	1.2	12.523	7.7	9.24	7.0	8.40	0.84	0.067
Test	1.2	13.550	8.4	10.08	7.3	8.76	1.32	0.097
Synanodonta woodiana								
			Beginning (0 hour)					
Control	6	359.6	9.5	57.00	7.6	45.60	11.40	0.032
Test	6	495.6	8.7	52.20	7.7	46.20	6.00	0.012
			24-hour					
Control	6	359.6	9.1	54.60	8.6	51.60	3.00	0.008
Test	6	495.6	8.1	48.60	6.3	37.80	10.80	0.022
			72-hour					
Control	6	359.6	8.6	51.6	7.6	45.6	6.0	0.017
Test	6	495.6	8.8	52.8	6.4	38.4	14.4	0.029

In general, both species proved to be sensitive to Cd exposure in terms of lysosomal membrane stability and respiratory rate. We suggest that further detailed research need to be performed in this particular area in order to study the physiological responses of freshwater mollusks, including invasive species to heavy metal intoxication.

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