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Heavy metal effects on the lysosomal membrane stability and respiratory rate in Chinese Pond Mussel (*Sinanodonta woodiana*) under *ex situ* exposure: preliminary data

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Abstract. The Chinese pond mussel (*Sinanodonta woodiana*) is a unionid mussel, which is known to accumulate heavy metals, making it useful for biomonitoring. The current preliminary research aimed to study the lysosomal membrane stability in heamocytes of *Sinanodonta woodiana* by applying the neutral red retention assay (NRR), as well as changes in the respiratory rate under acute metal exposure. The mussels were treated with different concentrations of Ni and Pb in laboratory conditions for 72 h. After the 72nd h exposure to Ni and Pb the lysosomes retained the dye between 30 to 60 minutes in the mussels exposed to the higher concentrations. The respiratory rate was measured at the 24th and 72nd hour and it increased in a dose-dependent manner. We can conclude that the acute metal exposure, including all metal concentrations below the allowable concentrations, lead to destabilization of the lysosomal membrane stability and changes in the respiratory rate.

Key words: lysosomal membrane stability, respiratory rate, *Sinanodonta woodiana*, heavy metals, *ex situ* exposure.

The U.S. Environmental Protection Agency (EPA) classifies freshwater mussels as biomonitors, because they react acutely to changes in the surrounding environment. In general, mussels are widely used as bioindicators of aquatic pollutants in freshwater, marine and estuarine ecosystems and as a direct measure of toxicant bioavailability because they are filter feeders, sedentary, long-lived, widely distributed, often available in high numbers, easily accessible and tolerant of high trace metal concentrations (Bolognesi et al., 2006, Bellotto & Miekeley 2007, Chandurvelan et al. 2015). The lysosomes in the mussel' cells function as the central site for sequestration and accumulation of toxic metals and organic xenobiotics, but they also play a key role in detoxification processes and further excretion of these compounds (Moore 1985, Viarengo et al. 1987, Domouhtsidou & Dimitriadis 2001). Thus, the measurement of lysosomal membrane stability was proposed as a rational biomarker of general stress in aquatic bivalves, both in laboratory and in vivo studies (Moore 1985, Viarengo et al. 1987). The rate of respiration reflects the metabolic activities of animals and the responses due to changes in the surrounding environment are an indicator of adjustment capacity of the organism (Kumar et al. 2012). Bivalve mollusks 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).

The Chinese pond mussel *Sinanodonta woodiana* (Lea, 1834) (Bivalvia: Unionida: Unionidae) originates from Eastern Asia (Watters 1997). It is probably the unionoid that has been most widely introduced outside its natural distribution area. In Europe, it was first recorded in Romania in 1979 in fish farms (Sárkány-Kiss 1986, Watters 1997) and later in other countries. The species has also spread dense populations among many Bulgarian freshwater ecosystems and today it is one of the main invasive alien species among the mollusks.

Our main aim in the present study was to study the effects of decreasing concentrations of Ni and Pb, which are considered as priority toxic substances (Directive 2013/39/EU) on Chinese Pond Mussel (*Sinanodonta woodiana*) in laboratory conditions (*ex situ*) and see if two biomarkers for metal contamination - the lysosomal membrane stability and respiratory rate, can be successfully applied for this particular species as such data is relatively limited.

Ten specimens of the same size-group - mean length 22.5 cm (1SD: 5.5) were hand collected in the spring of 2015 from one of the basins at the Institute of Fisheries and Aquaculture in Plovdiv (Bulgaria), where the fish are usually reared under strict toxicant-free conditions. The mussels were transported quickly to the laboratory and moved in 50 L glass aquaria with chlorine-free tap water (by evaporation) to acclimatize for a week. During the whole period of study the mussels were not fed. The water was kept oxygen saturated. During the entire duration of the experiment the mussels were maintained under a natural light/darkness cycle. After acclimatization the mussels were divided into eight groups (n = 1 in each experimental tank and n = 2 for control) and treated with different soluble concentrations of Ni and Pb for 72 hours. The metal concentrations were prepared as 75, 50 and 25% of the maximum permissible levels set by the Bulgarian law which is based on the European legislation regarding water quality (Directive 2013/39/EU). The maximum permissible concentration (MPC) of Ni in surface waters is 20 µg/L and of Pb – 7.2 µg/L. Thus, for 50 L tanks in the current experiment, 750 µg/L (75%), 500 µg/L (50%), 250 µg/L 1 (25%) Ni and 270 µg/L (75%), 180 µg/L (50%) and 70 µg/L (25%) Pb were used. No mussel mortality was recorded during the exposure period.

The physio-chemical characteristics of the aquarium water such as: pH, temperature and conductivity were measured once on the 24th and 72nd hour according to a standard procedure with a combined field-meter (APHA 2005).

The neutral red retention assay was adapted from the method of Lowe & Pipe (1994) and Lowe et al. (1995). 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 (Koenig 1962, Nemes et al. 1979, 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. 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.

The respiratory rate was measured at the beginning of the experiment (0-hour), at the 24th hour and at the end of the experiment (72nd hour) and calculated, following Tsekov (1989):

$I = Q_2/G$,

where I – respiratory rate index; G – weight of the mussels in grams; Q_2 – oxygen consumed by the mussels between the two

Table 1. Index of respiratory rate of the Chinese pond mussel (*Sinanodonta woodiana*) exposed to different Ni and Pb concentrations, at the beginning of the experiment (0 hour), at the 24th hour and at the end of the experiment (72nd hour) (explanations are in the text).

Test variant, µg/L	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 hour)								
Control	6	359.6	9.5	57.0	7.6	45.6	11.4	0.032
Pb-70	6	602.8	8.8	52.8	7.2	43.2	9.6	0.016
Pb-180	6	305.2	9.9	59.4	8.2	49.2	10.2	0.033
Pb-270	6	283.0	10.1	60.6	8.7	52.2	8.4	0.030
Pb-360	6	539.0	9.1	54.6	7.1	42.6	12.0	0.022
Ni-250	6	592.8	8.5	51.0	7.5	45.0	6.0	0.010
Ni-500	6	243.8	8.7	52.2	7.9	47.4	4.8	0.020
Ni-750	6	382.0	8.7	52.2	7.4	44.4	7.8	0.020
Ni-1000	6	493.7	8.6	51.6	7.4	44.4	7.2	0.015
24th hour								
Control	6	359.6	9.1	54.6	8.6	51.6	3.0	0.008
Pb-70	6	602.8	8.8	52.8	7.7	46.2	6.6	0.011
Pb-180	6	305.2	8.8	52.8	7.9	47.4	5.4	0.018
Pb-270	6	283.0	8.2	49.2	6.9	41.4	7.8	0.028
Pb-360	6	539.0	8.8	52.8	6.3	37.8	15.0	0,028
Ni-250	6	592.8	9.1	54.6	8.0	48.0	6.6	0.011
Ni-500	6	243.8	8.8	52.8	7.9	47.4	5.4	0.022
Ni-750	6	382.0	8.7	52.2	6.9	41.4	10.8	0.028
Ni-1000	6	493.7	8,7	52,20	6,4	38,40	13,80	0,028
72nd hour								
Control	6	359.6	8.6	51.6	7.6	45.6	6.0	0.017
Pb-70	6	602.8	8.3	49.8	8.1	48.6	1.2	0.002
Pb-180	6	305.2	8.6	51.6	7.7	46.2	5.4	0.018
Pb-270	6	283.0	8.9	53.4	7.6	45.6	7.8	0.028
Pb-360	6	539.0	8.9	53.4	7.5	45.0	8.4	0.016
Ni-250	6	592.8	8.2	49.2	7.9	47.4	1.8	0.003
Ni-500	6	243.8	8.5	51.0	7.6	45.6	5.4	0.022
Ni-750	6	382.0	8.9	53.4	7.0	42	11.4	0.030
Ni-1000	6	493.7	8.4	50.4	7.5	45	5.4	0.011

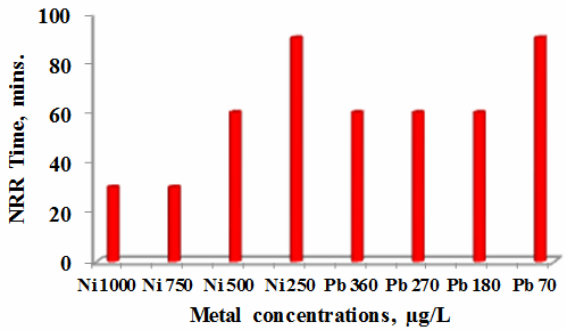

Figure 1. Neutral red retention time in the Chinese pond mussel (*Sinanodonta woodiana*) exposed to different Ni and Pb concentrations, representing 75, 50 and 25% of the Bulgarian maximum permissible levels.

measurements (the difference between the oxygen levels before and after the 1-hour $Q_2 = Q\text{-}Q_{1hour}$). 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 litres); q –level of dissolved oxygen in 1 litre of water (mg/L).

The physio-chemical properties of the water showed relatively constant values in all eight experimental tanks and they were close to the ones for the control groups: pH – 8.1; conductivity – 435 µS/cm, temperature – 21.5˚C and oxygen level – 6.5±1.5 mg/L, respectively. The results on lysosomal membrane stability on the exposed to Ni and Pb mussels are presented in Fig. 1. The average retention time for the control group was 105 min, with minimum of 90 min and maximum of 180 min. Thus, the control animals did not show any destabilized lysosomes and were considered as healthy and no stressed. However, significant decreases in the lysosomal destabilization indices with lower retention time were observed in the treated with heavy metals mussels. In general, the lysosomal membrane stability changed with the decreasing metal concentrations, but the lowest NRR-times and the highest lysosomal damage were detected in mussels exposed to the highest metal concentrations, which represent the maximum permissible levels according to the Bulgarian legislation. In addition, destabilized lysosomal membrane stability was also observed in the mussels treated with 75% and 50% of the allowable limit for Ni and Pb.

The results from the respiratory rate measurements are presented in Table 1. At the beginning of the experiment (0 hour) there was no visible increase of the respiratory rate and the heavy metal concentrations for both metals. After 24 hours of exposure we recorded a visible increase of the respiration rate with the heavy metals concentration as all indices were higher than the control. At the end of the experiment, after the 72nd hour this pattern changed and was close to the one we observed at the beginning of the experiment.

The results from our experiment confirm the results of other authors (Moore et al. 2004, Regoli et al. 2004, Marigómez et al. 2005, Nigro et al. 2006) that the destabiliza-

tion of lysosomal membrane stability in mussel haemocytes is a sensitive biomarker of aquatic contamination and it is strongly correlated to the metal species and concentration (Molnar & Fong 2012). However, it is a non-specific biomarker as different pollutants or environmental stressors can cause lysosmal damage (Viarengo & Cenessi 1991). We think that our results on *Sinanodonta woodiana* show that although more resistant to changes of abiotic and biotic factors, i.e elevated temperature, rapid salinity change, starvation or reproduction, as well as with wide ecological plasticity, invasive species are also sensitive to aquatic contamination. Thus, they could be successfully applied in monitoring programs on contaminated with different toxicant aquatic ecosystems or in studies on the sensitivity of both, native and alien species. Moreover, we recorded lysosomal damage not only in the mussels exposed to the highest concentrations, but also in the mussels exposed to the concentrations, which represent 75% and 50% of the allowable levels according to the Bulgarian law as described above. From our perspective, this is an interesting result, which might be taken into account in terms of water policy and legislation. In this sense, we consider that not the metal concentration, but the toxic properties of the metal need to be considered when establishing national regulations in this field.

It is known that most bivalve mollusks reflect immediate responses to toxic substances present in surrounding water by changes in physiological responses (Basha et al. 1988). In most cases the respiratory 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, cause by the toxic exposure. It seems that in our case with *Sinanodonta woodiana*, the mussels reacted by increasing their respiratory rate with the increase of the metal concentrations after the 24th hour of exposure to Ni and Pb. After the 72nd hour of exposure it seems that the initial stress, caused by the pollutants is on some level overcome, which means that the mussels probably tried to adapt to the new contaminated medium. This most likely could be accepted as some defense-mechanism, which on the other hand was not observed in terms of the lysosomal membrane stability.

Overall, we consider that the two used biomarkers in our study – neutral red retention assay and respiratory rate can be successfully applied on *Sinanodonta woodiana,* which can be also used as a sensitive to contamination species in similar studies. However, we suggest that further detailed research in this particular area should be carried out.

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