PERIODICUM BIOLOGORUM
VOL. 119, No 4, 229–237, 2017
DOI: 10.18054/pb.v119i4.4715

UDC 57:61
CODEN PDBIAD
ISSN 0031-5362

original research article

Lysosomal membrane stability and respiration rate in zebra mussel (*Dreissena polymorpha* Pallas, 1771) as biomarkers for *ex situ* heavy metal exposure

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Keywords: *Dreissena polymorpha*, Heavy metals; Lysosomes; Neutral red retention; Respiration rate

Abbreviations: AA – annual average concentration, MAC – maximum allowable concentration, NRRA – neutral red retention assay, NRRT – neutral red retention time.

Received November 08, 2016.
Revised October 11, 2017.
Accepted December 27, 2017.

Abstract

Background and purpose: *In the current study we aimed to investigate the lysosomal membrane stability in haemocytes of the invasive mollusk zebra mussel (*Dreissena polymorpha *Pallas, 1771) by applying the neutral red retention assay (NRRA), as well as changes in the respiration rate and survival under acute heavy metal exposure.*

Materials and methods: *The mussels were treated with different decreasing concentrations of nickel (Ni) and lead (Pb) in laboratory conditions for a total acute period of 72 hours. These metals are considered as priority substances in surface waters according to Directive 2013/39/EU of the European parliament and of the Council amending Directives 2000/60/EC and 2008/105/EC as regards priority substances in the field of water policy. The metal concentrations were prepared as 75, 50 and 25 % of the maximum allowable concentrations (100% MAC) set by law.*

Results and conclusions: *In sum, after 24 and 72 h we found that the lysosomes in the mussels exposed to all Ni and Pb concentrations, including the ones below the MAC (75 and 50 % from MAC) retained the dye shorter than the accepted minimum of 90 min. In addition, the respiration rate increased in a dose-dependent manner. Overall, we can conclude that the acute metal exposure lead to destabilization of the lysosomal membrane stability and changes in the respiration rate of zebra mussel, therefore altered physiological functions. We consider that such experiments could be successfully applied in risk assessment and monitoring programs on metal-contaminated aquatic systems, and the obtained results could be used in the field of water policy, respectively.*

INTRODUCTION

In recent years, heavy metal pollution in the environment and food has been considered as a global issue *(1,2).* Large quantities of heavy metals have been released into aquatic systems, both fresh and marine worldwide due to a global rapid population growth and intensive domestic activities, as well as expanding industrial and agricultural production *(3,4).* Thus, heavy metals have severely deteriorated the aquatic ecosystems because of their toxicity, abundance, persistence, and subsequent bioaccumulation and biomagnification *(5).*

To monitor the health of aquatic systems, sentinel organisms such as mussels (bivalves) have been proposed to be suitable "biomonitors" of pollution as they accumulate heavy metals in their tissues and shells

(6,7). Their filtering habits, low metabolism and ability to bioaccumulate pollutants make them an excellent choice to assess their bioavailability and effects *(8-10).* Mussels are also sessile, sedentary, have a reasonable size, they are worldwide distributed and often found in large amounts *(11-13).*

Marine mussels such as *Mytilus* sp., have been most often addressed as sensitive indicators for monitoring toxic substances in water *(14-17).* However, other bivalve species belonging to different taxa, such as *Dreissena polymorpha* have also been successfully used in ecotoxicological studies and monitoring programs *(18-27).*

The immune defence of mussels is comprised of cell-mediated and humoral mechanisms, in which the haemocytes or blood cells play a key role *(28).* They are also involved in the detoxification process through the accumulation of metallic and organic xenobiotics in their well-developed endolysosomal system *(29).* Lysosomes are highly conserved multi-functional cellular organelles present in almost all cells of eukaryotic organisms, from yeast to humans. Their function in the cellular economy includes the degradation of redundant or damaged organelles (e.g., mitochondria and endoplasmic reticulum) and longer lived proteins as part of the autophagic cellular turnover *(30).* Lysosomes are also involved in normal physiological responses such as the digestion of materials ingested by endocytosis and phagocytosis (i.e., intracellular digestion) *(30,31).* Moreover, the lysosomal system, which is remarkably well-developed in mussel haemocytes is well-known as a target site for toxic metals and organic chemicals, due to its ability to accumulate them *(32-36).* As a consequence, the 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 hydrolitic enzymes into the cytoplasm with subsequent enhanced protein catabolism up to the autophagic conditions indicating a stress syndrome *(34).*

The stability of the lysosomal membranes is evaluated using the lysosomal membrane stability test (LMS) of the digestive gland *(37,38),* as well as *in vitro* using the neutral red retention assay (NRR) of the haemocyte lysosomes. NRR measures the lysosomal content efflux into the cytosol, which in stressed mussels, reflects a physiological process after membrane damage and comparatively measures the capacity of cellular processes to adapt to stress conditions *(39).*

In environmental risk assessment, the use of a battery of biomarkers is strongly recommended *(40-42)* because a comprehensive and integrated view of the biological responses of aquatic organisms, even where the levels of contaminants are not particularly high can be studied *(43).* In this sense, the destabilization of lysosomal membranes is an established indicator for toxically induced adverse effects in fish and shellfish *(44,45).* Lysosome enlargement, as well as lysosome number increase, are further morphological alterations of the lysosomal system basically due to the increase in protein turnover, which in molluscs have been considered as reliable signals of cellular stress and damage, resulting from enhanced autophagy additionally as a response to external stimuli such as contaminants *(46-49).* The rate of respiration reflects the metabolic activities of animals and the responses due to changes in the surrounding environment could also be an indicator of adjustment capacity of the organism. Furthermore, bivalve molluscs reflect immediate responses to toxic substances present in the surrounding water by changes in their physiological responses *(48).*

The main goal of the current research was to study how nickel (Ni) and lead (Pb), which are considered as priority substances according to Directive 2013/39/EU of the European parliament and the Council could possibly impact the physiology of relatively resilient and invasive zebra mussel (*Dreissena polymorpha* Pallas*, 1771*) under *ex situ* laboratory conditions. Thus, we aimed to study two biomarker responses: 1) Lysosomal membrane stability by applying the neutral red retention assay and 2) Respiration rate index. These particular methods were chosen as they are simple, easy to perform and quite inexpensive.

MATERIALS AND METHODS

Experimental animals

About 230 specimens of the same size-group (mean length 2.5 cm ± 0.5) were hand collected in the spring of 2015 from "Ovcharitsa" Reservoir, located in southeastern Bulgaria. The mussels were placed in 10 l containers filled with the reservoir water and transported quickly to the laboratory on the same day. After transportation the mussels were moved in 100 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). They were not fed.

Experimental exposure

After acclimatization the mussels were divided into eight groups (n = 25 in each experimental 50 l tank). The mussels were treated with different soluble in water concentrations of $Ni(NO_3)_2$ and and $Pb(NO_3)_2$ for 72 hours. The metal concentrations were prepared as 75, 50 and 25 % of MAC (100 %) set by law. According to the Bulgarian legislation based on Directive 2013/39/EU *(50),* MAC of Ni in inland surface waters is 20 μg l^{-1} (100%) and that of Pb – 14 μg l^{-1} (100%), respectively. Therefore, 750 μg (75 % from MAC), 500 μg (50 %), 250 μg (25 %) Ni, as well as 270 μg (75 %), 180 μg (50 %) and 70 μg (25 %) Pb were applied for 50 l tanks in the current experiment. No mussel mortality was recorded during the exposure period.

The physico-chemical characteristics of the aquarium water such as: pH, temperature, oxygen level and conductivity were measured once at the start of the experiment,

as well as on the 24th and 72nd hour according to a standard procedure with a combined portable field-meter (WTW, Germany).

Neutral red retention assay

The analytical procedure was adapted from Lowe and Pipe *(39)* and Lowe et al. *(44)*. It is based on the use of a cationic probe neutral red, which is taken up into the cells by membrane diffusion where it becomes ion trapped within the lysosomal compartment *(51-54)*. Over the time, the dye tends to leak out of the lysosomes into the cytosol, which is then stained by the dye *(55)*. The exposure to toxic substances damages the lysosomal membrane and hence, increases its permeability. The end point parameter is the time at which a dye loss to the cytosol is evident in 50 % of the granular haemocytes. The rounding up of the cells and enlargement of the lysosomes is also associated with dye loss.

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 (CMFS: 4.77 g l^{-1} HEPES, 25.48 g l^{-1} NaCl, 0.75 g l^{-1} KCl, 1000 ml Di H_2O) as described by Molnar and Fong *(56)* in order to obtain a 50/50 of the 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. Then, 40 µl of the working solution (10 µl into 5 ml physiological saline 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. On the top of each slide a 22x22 mm cover slip was placed. After 15 min. incubation the slides were examined systematically using a light microscope (Nikon, Eclipse E200). The time period between the neutral red (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 neutral red retention time (NRRT) for the mussels. Following a further 15 min., each preparation was observed at 30 min. intervals until a total time of 180 min.

Respiration rate

The respiration rate was measured at the start of the experiment when the toxicants were applied (0 h), at the 24th hour and at the end of the experiment (72nd hour). At the given time 20 mussels were transferred in 1.2 l glass tanks filled with water from the test aquariums. The oxygen levels were measured, using a combined portable field-meter (WTW, Germany). The tanks were then covered with plastic foil in order to eliminate any oxygen transfer. They were left for one hour and thereafter, the oxygen level was measured again. The respiration rate was calculated by determining the difference in the dissolved oxygen levels before and after, following Tsekov *(57)*: $I = Q_2/G$, 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 1 h, $Q_2 = Q\text{-}Q_1$ hour). Q was calculated by the following formula: Q = V x q, where: Q – total oxygen level; V – water volume, in litres; q – level of dissolved oxygen in 1 litre of water (mg l^{-1}).

Statistical analysis

For the statistical processing of the data the software package "Statistica 7.0" (StatSoft Inc. 2004) was used. Student's t-test and one-way analysis of variance (ANOVA) were applied to see if there was a significant difference between the NRRT of the control and mussels examined at the 24th and 72nd hour. In addition, Spearman's correlation analysis was used, when the data is not normally distributed in order to examine the changes of the respiration rate, along with the metal concentrations. Significance level was set to 0.01 and 0.05.

RESULTS AND DISCUSSION

Water properties

The physico-chemical properties of the water showed relatively constant values in all nine experimental tanks (Table 1). These for the control groups were as follows: pH – 8.1; conductivity – 435 µS cm^{-1}, oxygen level – 6.5 mg l^{-1} and temperature – 21.5 °C, respectively. In general, the values between the 24th and 72nd h were similar

Table 1. *Physico-chemical properties in the experimental water tanks, average results at the start of the experiment (0 h), 24th and 72nd h (average ± SD).*

Metal concentration, µg l^{-1}	pH	Conductivity, µS cm^{-1}	Temperature, °C	Oxygen level, mg l^{-1}
Ni 1000	7.98 ± 0.5	492 ± 1.5	21.75 ± 1	6.3 ± 0.5
Ni 750	8.1 ± 0.5	490 ± 1	20.75 ± 1.5	6.3 ± 0.3
Ni 500	8.15 ± 0.5	489.5 ± 0.5	22.75 ± 1.3	6.5 ± 0.5
Ni 250	8.02 ± 0.3	489 ± 0.5	22 ± 0.5	6.6 ± 0.1
Pb 360	8.12 ± 0.5	486.5 ± 0.5	23.5 ± 0.5	6.2 ± 0.5
Pb 270	8.13 ± 0.3	488 ± 0.3	22.75 ± 0.5	6.4 ± 0.5
Pb 180	8.2± 0.5	483.5 ± 0.3	21.75 ± 0.5	6.5 ± 0.3
Pb 70	8.195 ± 0.3	480.5 ± 1.5	21.75 ± 0.3	6.5 ± 0.3

for the studied period. Therefore, we consider that the changes, which we observed in the lysosomal membrane stability and respiration rate were not due to changes in the abiotic factors of the water.

Neutral red retention time

The results on lysosomal membrane stability of the control and exposed mussels to Ni and Pb after the 24th and 72nd hour are presented in Fig. 1 and 2. The average NRRT of the dye in the mussels from the control groups (0, 24th and 72nd hour) was 106 min., with a maximum average time of 110 min. for the mussels examined at the beginning of the experiment. There was no significant difference between the NRRT for the controls ($p > 0.05$). Overall, the lysosomes of control mussels could be considered as healthy and non-stressed; with no destabilized lysosomes since destabilization of the lysosomal membrane stability was not observed before the 90th min.

On the other hand, significant reductions in the indices of the lysosomal membrane stability were registered in the treated with heavy metals mussels after 24 and 72 hours. We found that the lysosomal membrane stability changed with the concentration of both metals. The lowest NRRT was measured in the mussels exposed to the highest heavy metals concentrations, which represent MAC in inland surface waters according to the national and EU legislation.

For example, the mussels subjected to the action of 1000 μg Ni for 24 h had an average NRRT ofonly 48 min. The mussels treated with 750 and 500 μg Ni retained the dye little longer – 78 and 90 min., while those treated with 250 μg Ni showed lysosomal destabilization on the 102nd min. The statistical analysis of the data demonstrated a significant difference ($p = 0.01$) between the NRR time of the dye in the lysosomes of mussels exposed to 1000 μg Ni and the control. Statistically significant differences were not found among the mussels exposed to the other Ni concentrations and the control ($p > 0.05$). Similarly, the mussels treated with Pb for 24 hours showed an analogous trend in retaining of the dye – a shorter time at the higher heavy metal concentrations and a longer time at the lower concentrations. The mussels treated with Pb concentration of 360 μg had an average NRRT only 42 min. At concentration of 270, 180 and 70 μg the lysosomal destabilization was recorded respectively at the 60th, 66th and 72nd min. The statistical analysis demonstrated significant differences between the NRRT of the dye in the mussel lysosomes from the control group and all Pb concentrations ($p < 0.05$). After the 72nd h the mussels exposed to Ni concentration of 1000 μg showed leaking of the dye from the lysosomes in the cytosol just after 30 min. The mussels treated with 750, 500 and 250 μg held the dye 42, 54 and 72 min., respectively. The statistical data processing proved significant differences ($p < 0.05$) between the NRRT of the mussels exposed to all Ni concentrations and the control. The picture for the mussels after 72 h exposure to Pb was similar to that of Ni, i.e. at the higher metal concentrations destabilization of the lysosomal membranes was established earlier. The mussel lysosomes exposed to Pb concentration of 360 μg held the dye only 30 min. and those exposed to 270, 180 and 70 μg – 36, 54 and 66 min., respectively, which also confirmed the disturbances, occurring in the the cell organelles. The applied t-test proved significant statistical differences between the NRRT of the dye in the mussel lysosomes from the control group and all four Pb concentrations ($p < 0.05$). We found statistically significant dif-

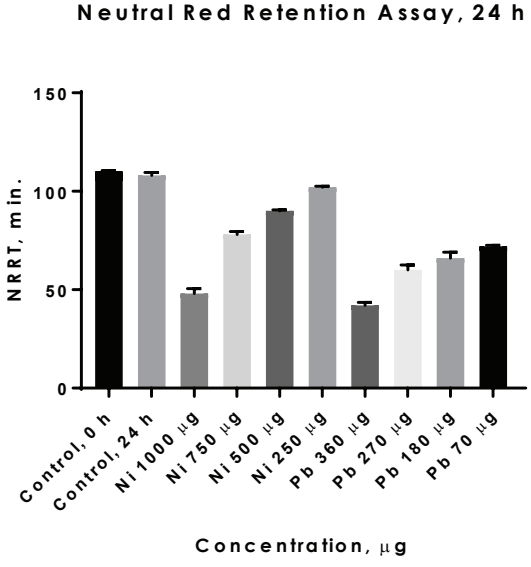

Figure 1. *Average neutral red retention time in zebra mussels exposed to Ni and Pb for 24 h (whiskers representing standard deviation).*

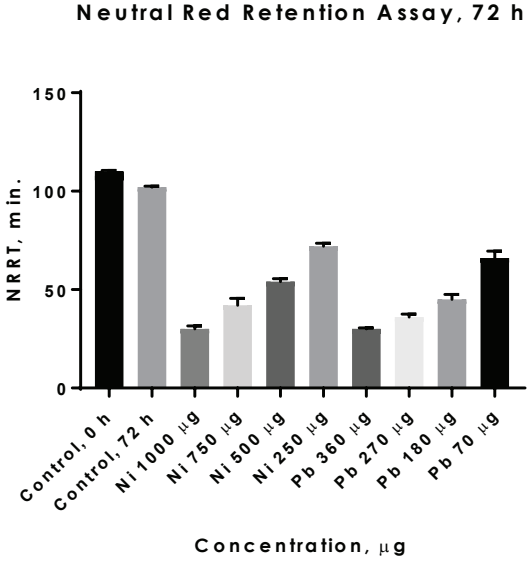

Figure 2. *Average neutral red retention time in zebra mussels exposed to Ni and Pb for 72 h (whiskers representing standard deviation).*

ferences between the groups exposed to 750 and 250 µg Ni (p = 0.033; p = 0.034) comparing the NRRT of the dye in the lysosomes of the mussels treated with Ni on the 24th and 72nd hour). None were found for the mussels exposed to Pb on the 24th and 72nd h (p > 0.05).

Overall, in the international scientific literature more data is available on the effects of various organic pollutants (PAHs, PCBs, pharmaceutical products and oil derivatives) on the lysosomal membrane stability of different invertebrate species such as mussels, snails, etc. (27, 58-63) compared with heavy metals (26,55,64). On the basis of the collected literature regarding the effects of heavy metals on the lysosomal membrane stability of mussels (and other mollusc species) we found that our results are similar with those of other authors and confirm that the lysosomal stability weakens with the increasing toxicant concentrations, and vice versa – the retention time of the dye is longer for the lower concentrations..

For example, Molnar and Fong *(56)* applied the neutral red retention assay to investigate the effects of Cd and Cu on two species of freshwater molluscs (*Elliptio complanata* and *Helisoma trivolvis*). The authors found that the mussels and snails exposed to heavy metals showed a significant increase in the percentage of destabilized lysosomes compared to the control, as well as after 7 and 14 days after exposure for all the concentrations, which were applied. Scott-Fordsmand et al. *(65)* also applied the same method to study the effects of different Ni concentrations on *Eisenia veneta* and established a similar trend. In their research Svendsen and Weeks *(66)* studied Cu toxicity on *Viviparu scontectus* at concentrations of 31 to 100 µg l^{-1}. The authors found that all tested concentrations resulted in a significantly shorter NRR time in comparison with the control. Shepard and Bradley *(67)* demonstrated that the lysosomal destabilization in *Mytilus edulis* was dose-dependent; as such occurred only after 24 h exposure of 20-80 µg l^{-1} Cu. Nicholson *(68)* found that the NRRT decreased with the increasing Cu concentrations in the mussel *Perna viridis*. Such a conclusion was also made by Matozzo et al. *(69)* for *Tapes philippinarum* after exposure to Cu and Cd at concentrations of 10-110 µg l^{-1}. These authors also demonstrated that Cd had more pronounced toxicity on the lysosomes in this species compared to Cu.

In the present study we also tested different concentrations of two heavy metals – Ni and Pb, and found that Pb has more negative effects on zebra mussel in terms of lysosomal membrane stability. Although, no significant differences were proven in the NRRT in the lysosomes exposed to all Ni and Pb concentrations on the 24th and 72nd h, our results demonstrated that the NRRT time was shorter for the mussels, exposed to Pb. Therefore, we could consider that Pb has more severe toxicity compared to Ni in terms of faster lysosmal destabilization. Probably the most significant result, which we obtained from this experiment, was that interference occurred at cellular level in the mussels treated with Ni and Pb concentrations lower than the MAC. Similarly, Yancheva et al. (70) studied the effect of different concentrations of Cd and PAH; equaled to MAC according to Directive 2013/39/EU, 50% above and 50% below MAC on zebra mussels, and found similar results – destabilization in the lysosomal membranes occurred even in the groups treated with lower than the permissible concentrations. In this sense, in some western countries the legislation related to water quality uses not only annual average concentrations (AA) or MAC, but also no observed effect and low observed effect concentrations (NOEC and LOEC) – for aquatic organisms such as fish, mollusks, etc.

Although, our data is obtained from a single laboratory study, we consider that these results could be taken into account and serve as a basis for comparison with other results in similar future studies, as well as monitoring programs on freshwater ecosystems under anthropogenic pressure.

Respiration rate

The results from the respiration rate measurements are presented in Table 2. After 24 h of exposure we recorded a very strong, positive (which was statistically significant only for Pb) correlation between the concentration of both metals and the index of respiration rate (Pb – s = 0.97, p = 0.006; Ni – s = 0.85, p = 0.07). At the end of the experiment this correlation remained almost unchanged (Pb – s = 0.96, p = 0.009; Ni – s = 0.82, p = 0.08), which we could link to the toxic effect of Ni and Pb on this studied parameter.

A similar pattern was observed by Nikolov et al. *(71)* in zebra mussel treated with Zn for 96 h. The mussels increased their respiration rate with the heavy metal concentration. A study by Kraak et al. *(72)* suggests that the effects of Zn and Pb on the filtration rate of zebra mussel also increased when the exposure time was increased. It is known that without time for acclimation the mussels typically reduce their clearance rate (volume of water passing through gills per unit time), thus potentially lowering their intake of oxygen *(73).* However, most bivalve molluscs reflect immediate responses to toxic substances present in the surrounding water by changes in the physiological responses *(52).* In most cases the respiration rate increases with the increase of the pollutant concentration and level of toxicity *(51).* Our results are in agreement with Yancheva et al. *(27)* who studied the negative effects of different concentrations of chlorpyrifos on zebra mussels and found that the respiration rate was increased compared to the control group. The same authors also investigated the effects of Cd on two freshwater mollusks *(26)* and concluded that the respiration rate was higher in the Chinese pond mussel and zebra mussel after 24 and 72 h compared to the control. Probably the reason for this is that the organism, triggered by the stress, caused by the

Table 2. *Index of respiration rate of zebra mussel exposed to different Ni and Pb concentrations at the start of the experiment (0 h), 24th and 72nd hour.*

Test variant	Water volume, L	Weight, g (G)	Total oxygen level (mg L^{-1})					Index of respiration rate (I)
			Beginning		End		Total	
			q	Q	q_{1h}	Q_{1h}	(Q_2)	
Beginning (0 hour)								
Control	1.2	13.76	8.8	10.56	7.4	8.88	1.68	0.12
Pb-70	1.2	12.88	9.7	11.64	7.8	9.36	2.28	0.17
Pb-180	1.2	13.47	8.7	10.44	7.4	8.88	1.56	0.12
Pb-270	1.2	13.14	9.1	10.92	8.0	9.60	1.32	0.1
Pb-360	1.2	13.62	9.4	11.28	8.1	9.72	1.56	0.12
Ni-250	1.2	14.68	9.1	10.92	7.0	8.40	2.52	0.17
Ni-500	1.2	13.46	9.0	10.80	7.2	8.64	2.16	0.16
Ni-750	1.2	13.83	9.2	11.04	7.1	8.52	2.52	0.18
Ni-1000	1.2	13.42	9.7	11.64	7.7	9.24	2.40	0.18
24th hour								
Control	1.2	13.47	9.1	10.92	8.7	10.44	0.48	0.036
Pb-70	1.2	13.25	8.3	9.96	7.7	9.24	0.72	0.054
Pb-180	1.2	12.16	8.8	10.56	7.9	9.48	1.08	0.089
Pb-270	1.2	11.68	9	10.8	8.1	9.72	1.08	0.093
Pb-360	1.2	11.15	8.7	10.44	7.7	9.24	1.20	0.108
Ni-250	1.2	13.02	8.9	10.68	7.8	9.36	1.f	0.101
Ni-500	1.2	12.28	8.9	10.68	7.8	9.36	1.32	0.107
Ni-750	1.2	11.43	8.4	10.08	7.3	8.76	1.32	0.115
Ni-1000	1.2	11.82	8.5	10.2	7.3	8.76	1.44	0.122
72nd hour								
Control	1.2	12.52	7.7	9.24	7.0	8.40	0.84	0.07
Pb-70	1.2	12.07	8.6	10.32	7.7	9.24	1.08	0.09
Pb-180	1.2	12.13	8.5	10.20	7.5	9.00	1.20	0.09
Pb-270	1.2	11.27	8.2	9.84	7.2	8.64	1.20	0.12
Pb-360	1.2	12.39	8.5	10.20	7.3	8.76	1.44	0.12
Ni-250	1.2	13.16	8.4	10.08	7.9	9.48	0.60	0.05
Ni-500	1.2	13.16	8.9	10.68	8.0	9.60	1.08	0.08
Ni-750	1.2	11.88	8.4	10.08	7.5	9.00	1.08	0.09
Ni-1000	1.2	11.99	8.3	9.96	7.3	8.76	1.2	0.1

toxic exposure, tries to deliver more oxygen to all tissues and organs. This was also the case with zebra mussel in the present experiment; the mussels reacted by increasing their respiration rate with the increasing metal concentrations after the 24th h, and this pattern remained unchanged until the 72nd h of exposure. As stated by Naimo *(74)* analyses of freshwater mussels can indicate metal bioavailability and these organisms may be useful in more sensitive, sublethal toxicity tests. However, most studies on the effects of heavy metals on freshwater mussels have concerned bioaccumulation. Experimental research should incorporate complex measurements, which examine the links between sublethal toxicity and bioaccumulation in freshwater mussels. Thus, more immediate information on exposure concentrations and physiological activity of freshwater mussels will be needed.

CONCLUSION

In summary, in Bulgaria, heavy metal pollution remains a problem, particularly in regions with intensive industry. Thus, in 1995 five hot spots were indicated in the country for this type of pollution, which are linked to the activity of metallurgical plants, smelters and refiners. However, not many studies deal with the negative effects of metals on freshwater mussels. Based on the data from

the present experiment we can conclude that Ni and Pb had severe effects on the physiology of zebra mussel, which led to a destabilized cellular compartment and altered respiration functions. We confirmed that neutral red retention assay is a feasible, non-specific biomarker and proved that the respiration rate measurement could also be applied as biomarker for heavy metal contamination in similar studies. Moreover, the negative impact of both heavy metals was observed at concentrations lower than MAC. We suggest that further research in this particular area need to be repeated in order to study in details the heavy metal effects on zebra mussels, which are considered relatively resilient to changes in the surrounding media. The results could be carefully considered in the field of water legislation and conservation, respectively.

Acknowledgments

This study was supported by the NPD – Plovdiv University "Paisii Hilendarski" under Grant No NI15-BF-003, Integrated biological approaches for monitoring priority substances in water.

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