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Short- and Long-term Toxicity of Cadmium and Polyaromatic Hydrocarbons on Zebra Mussel *Dreissena polymorpha* (Pallas, 1771) (Bivalvia: Dreissenidae)

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Abstract: This study was designed to examine the possible negative effects, which cadmium (Cd) and polyaromatic hydrocarbons (PAHs) could have on the lysosomal membrane stability in haemocytes of the invasive mollusk zebra mussel (*Dreissena polymorpha*) by applying the neutral red retention assay (NRRA). The mussels were exposed to different concentrations of Cd and PAHs in laboratory conditions for 96 hours (acute exposure) and 31 days (chronic exposure). These are considered as priority substances in surface waters according to Directive 2008/105/EC. We found lysosomal membrane destabilisation in all mussels treated with Cd and PAHs, including concentrations, which were lower than the allowable ones. In addition, we determined a trend of lower retention time in the mussels treated with Cd as compared to the ones treated with PAHs, although these differences were not significant (p>0.05). Our results confirmed that the neutral red retention assay could be used as a cheap, fast and reliable biomarker for Cd and PAHs effects on freshwater mollusks and that zebra mussel could be suggested as a freshwater bioindicator for water contamination. However, further studies are required in order to better understand the negative effects of Cd and PAHs on this bivalve species.

Key words: mussels, Cd, PAH, lysosomes, haemocytes

Introduction

Cadmium (Cd) is non-essential and one of the most toxic metals and poses a serious threat to aquatic organisms (McGeer et al. 2011) because of its persistent nature and slow elimination from the environmental compartments (Sandhu et al. 2014). It is released naturally from weathering of minerals, forest fires and volcanic emission; however most water Cd contamination results from anthropogenic sources, such as mining, smelting and refining sulfide ores of zinc, coal combustion, electroplating processes, iron and steel production, pigment, fertilizer and pesticide use (Garcia-Santos et al. 2013). All the cadmium ultimately deposits into the aquatic systems, and then creates potential hazards to aquatic organisms (Rana 2014). Polycyclic aromatic hydrocarbons (PAHs) are known to result from the incomplete combustion of organic matter, especially fossil fuels (pyrolytic origin), from the discharge of petroleum and its products (petrogenic origin), as well as from the post-depositional transformation of biogenic precedents (diagenetic origin). PAHs can also be produced in nature as a result of forest fires and volcanoes, or can be derived from biogenic precursors (Ya et al. 2017). Under this consideration, the United States Environmental Protection Agency (USEPA) classified 16 of them as priority pollutants

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(Qiao et al. 2006). Therefore, integration of chemical analyses with biomarker responses in organisms is recommended for monitoring anthropogenic activities, which could lead to PAHs contamination (Hylland et al. 2008).

Sediment-associated organisms, such as marine bivalves, have been used to monitor the health of aquatic systems as they are sessile filter feeders, able to accumulate and tolerate high concentrations of pollutants, present across a very wide geographical area, easy to collect, having a suitable size for chemical analyses and particularly abundant in coastal and estuarine waters (Goldberg 1986). Furthermore, mussels have the ability to accumulate organic pollutants, especially PAHs and are, therefore, currently used to monitor PAHs contamination in aquatic environments (Cappello et al. 2013). *Mytilus* sp. have been widely used since the 90^{s} and have been shown to be one of the most successful model organisms for time-integrated responses to complex mixtures of pollutants (UNEP/RAMOGE 1999). The zebra mussel, *Dreissena polymorpha* (Pallas, 1771) can be used as a freshwater substitute of *Mytilus* sp. (Lepom et al. 2012, Binelli et al. 2015). The lysosomes in the mussel cells function as a 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 (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 (Viarengo et al. 1987).

Yancheva et al. (2016) published preliminary data on the effects of Cd, Ni, Pb and chlorpyrifos on zebra mussel, which directed us to an expanded, additional comparative study on the effect of both inorganic and organic toxicants on this species. Therefore, the first objective in the present experiment was to study the negative impact, which Cd and PAHs could possibly have on the lysosomal membrane stability in haemocytes of zebra mussel (*Dreissena polymorpha*) by applying the neutral red retention assay (NRRA). The mussels were exposed to different concentrations of Cd and PAHs based on their permissible levels in inland waters according to the EU legislation in laboratory conditions for 96 hours and 31 days. These toxicants are considered as priority substances in surface waters according to Directive 2008/105/EC of the European parliament and the Council. The second objective was to determine, which one, Cd or PAHs, could have more severe effects on the lysosomes.

Materials and Methods

About 150 specimens of the same size-group (mean length 2.5 cm ± 0.5) were hand-collected in the autumn of 2016 from a small lake used for sport fishing near Plovdiv City, Bulgaria (42° 30*'* 07.81*"* N, 24° 80*'* 49.75*"* E). After transportation on the same day the mussels were moved in a 25 L glass aquarium with chlorine-free tap water (by evaporation) to acclimatise 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. After the acclimatisation the mussels were divided into seven groups (n = 20 in each experimental tank and n = 20 for control). The mussels were treated with different concentrations of cadmium nitrate tetrahydrate ($Cd(NO_3)2.4H_2O$), dissolved in water, as well as with a mixture of 16 different organic compounds, dissolved in cyclohexane for a total period of 96 hours (acute exposure) and 31 days (chronic exposure). Their permissible levels in inland waters according to the law are presented in Table 1.

The toxicant concentrations were prepared as 50% above and 50% below the maximum allowable concentration (MAC) set by the national law for 25 L tanks. Therefore, in the current experiment 2 µg Cd (AA, 100%), 1 µg Cd (50% below MAC) and 4 µg Cd (50% above MAC), as well as 2 µg PAHs (AA, 100%), 1 µg PAHs (50% below MAC) and 3 µg PAHs (50% above MAC) were applied. The physical and chemical characteristics of both the control and contaminated water, such as pH, conductivity, temperature and oxygen level, were measured once on the 24th, 48th, 72nd and 96th hour, as well as on the 31st day with a combined field-meter.

The analytical procedure of neutral red retention time (NRRT) assay was adapted from 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 (Rashid et al. 1991). Over 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. About 0.5 mL haemolymph was withdrawn from the posterior adductor muscle of five mussels from each experimental tank and treated as described by Molnar & Fong (2012). In order to maintain the haemocytes alive a calcium/magnesium free physiological saline was prepared: 0.595 g/L HEPES, 3.215 g/L NaCl, 0.185 g/L KCl, 500 ml distilled H_2O, pH adjusted to 7.3.

Table 1. List of PAHs used in the present study (n=16)

Name of substance	Annual average (AA), inland surface waters, µg/L	Maximum allowable concentration (MAC), inland surface waters, µg/L
Cd	0.08	0.45
Acenaphthene*	3.8	50
Acenaphthylene*	0.64	not applicable
Anthracene	0.1	0.1
Benz(a)anthracene*	0.01	not applicable
Benzo(b)fluoranthene	**	0.017
Benzo(k)fluoranthene	**	0.017
Benzo(g,h,i)-perylene	**	0.017
Benzo(a)pyrene	$1,7 \times 10^{-4}$	0.27
Dibenzo(a,h)anthracene*	0.02	not applicable
Fluoranthene	0.0063	0.12
Fluorene*	2,5	not applicable
Indeno(1,2,3-cd)pyren	**	not applicable
Naphthalene	2	130
Phenanthrene*	1.3	not applicable
Pyrene*	0.012	not applicable
Chrysene	0.02	not applicable

* No data for annual average (AA) and maximum allowable concentration (MAC) in inland surface waters (µg/L) according to the Bulgarian legislation based on the environmental quality standards as defined in Directive 2008/105/EC;

** For the group of priority substances of polyaromatic hydrocarbons (PAH) (No 28), the biota EQS and corresponding AA-EQS in water refer to the concentration of benzo(a)pyrene, on the toxicity, of which they are based. Benzo(a) pyrene can be considered as a marker for the other PAHs, hence only benzo(a)pyrene needs to be monitored for comparison with the biota EQS or the corresponding AAEQS in water.

The neutral red stock was prepared by dissolving 4 mg of neutral red into 2 mL DMSO and. 10 µL of the stock solution was made up to 5 mL with the physiological saline. Then 40 µL of the haemolymph was applied on microscopic slides, which were contained in a light proof humidity chamber, then treated with 40 µL of the neutral red and covered with cover slips. The slides were examined using a light microscope (Leica DM500). The time, at which in more than 50% of the lysosomes neutral red leaked out in the cytosol was considered the end point parameter.

The average percent of cells with destabilised lysosomes was calculated for each experimental mussel and thereafter for each toxicant group. The average differences between the groups were analysed using Student t-test ($p<0.05$) using STATISTICA software, version 7.0 for Windows. The water physical and chemical data were analysed in the same way.

Results

Short-term exposure to Cd and PAHs

The physical and chemical properties of the water from each experimental tank are presented in Table 2. In general, the values were similar for the studied test period (96 hours). The conducted statistical analysis did not show significant differences between the values of the control and test water on the 24th, 48th, 72nd and 96th hour ($p>0.05$). Thus, we consider that the changes, which were observed in the lysosomal membrane stability were not due to changes in the environmental conditions.

The summarised average results on the lysosomal membrane stability of the control mussels and those subjected to the action of the tested toxicants on the 24th, 48th, 72nd and 96th hour are presented in Fig. 1. The average retention time of the control mussels on the 24th hour was 90 min. This time represents the accepted minimum, which shows lysosomal stability. Therefore, the lysososmes were considered as healthy and non-stressed. On the other hand, on the 24th hour significant decreases in the indices of lysosomal membrane stability were registered in the mussels treated with Cd. It was observed that the lysosomal membrane stability changed when increasing its concentration. The lowest retention time was 15 min in the mussels exposed to the highest Cd concentrations – 4 µg Cd (50% AA). The mussels exposed to Cd concentrations of 2 µg (AA according to the Bulgarian legislation) retained the dye 27 min, while those exposed to Cd concentrations of 1 µg (50% below AA) 30 min, respectively. The statistical analysis demonstrated a significant difference ($p<0.05$) between the retention time of the dye on the 24th hour

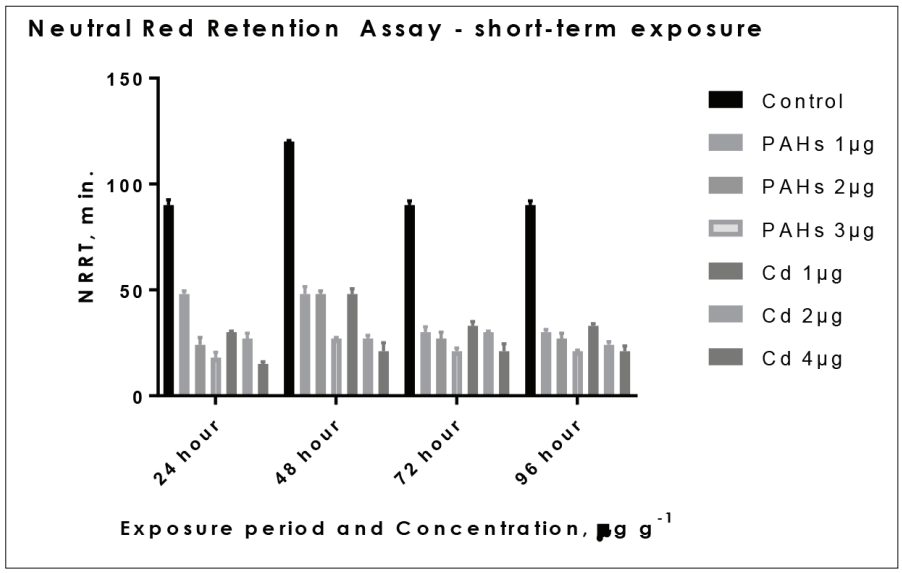

Fig. 1. Average results on the lysosomal membrane stability in control and experimental groups of zebra mussel exposed to short-term Cd and PAHs effects.

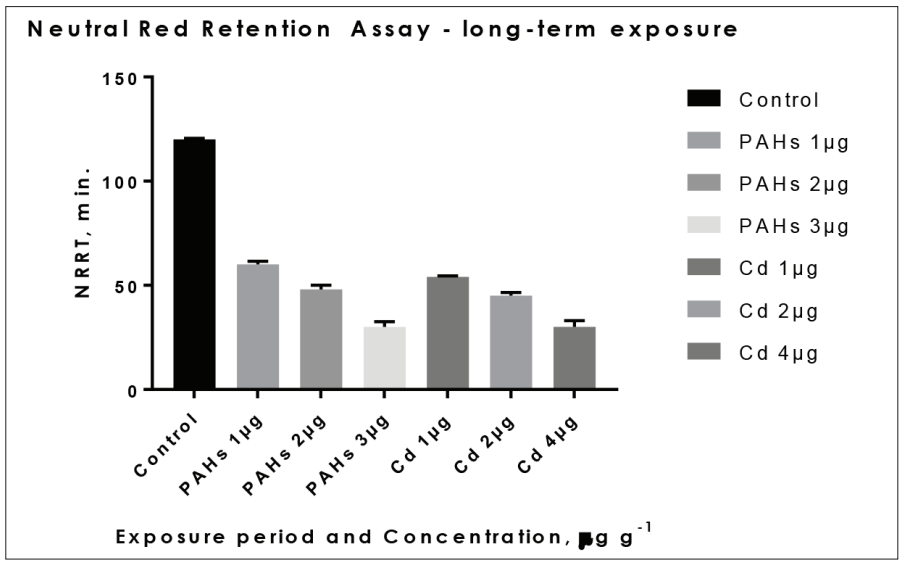

Fig. 2. Average results on the lysosomal membrane stability in control and experimental groups of zebra mussel exposed to long-term Cd and PAHs effects.

in the lysosomes subjected to the action of all Cd concentrations and the control mussels. The average retention time in the control mussels on the 48th hour was longer than in the control mussels on the 24th hour – 120 min. Therefore, the lysosomes were considered also as non-stressed. The trend in the lysosomal stability in the mussels after a 48-hour exposure to Cd was similar to that of the mussels treated with Cd for 24 hours, i.e lysosomal membrane destabilisation was established earlier at higher Cd concentrations. The mussels treated with the highest concentrations of Cd, 4 µg and 2 µg, retained the dye 21 and 27 min, respectively; in those treated with the lowest Cd concentration of 1 µg destabilisation of the lysosomal membranes was registered after 21 min. A significant difference (p<0.05) between the retention time of the dye in the lysosomes exposed to all Cd concentrations and control was proved on the 48th hour. The average retention time of the dye in the lysosomes from the control mussels on the 72nd hour was 90 min, thus we assumed that they were relatively non-stressed. Similarly to the trend, which was observed above, the mussels treated with Cd for 72 hours showed lower retention time at higher concentrations. The mussels exposed to the highest Cd concentrations, 4 µg, retained the dye 21 min (similarly to the mussels examined at the 48th hour). The mussels exposed to Cd concentrations of 2 µg showed lysosomal membrane

destabilisation after 30 min, while those exposed to Cd concentrations of 1 μg – after 33 min. The statistical analysis demonstrated a significant difference (p<0.05) between the retention time of the dye in the lysosomes of control mussels and those exposed to all Cd concentrations on the 72nd hour. The control mussels, dissected on the 96th hour showed stable lysosomal membranes, since destabilisation was not observed earlier than 90 min. On the other hand, significant decreases in the indices of lysosomal membrane stability were registered in the mussels treated with different Cd concentrations. The mussels exposed to the highest Cd concentrations of 4 μg retained the dye 21 min; those treated with 2 μg Cd – 24 min and those treated with the lowest Cd concentration of 1 μg – 33 min.

Comparing the retention time of the dye in the lysosomes treated with different Cd concentrations (100% AA, 50% above AA, 50% below AA) on the 24th, 48th, 72nd and 96th hour, we registered statistically significant differences between the groups subjected to the action of Cd 4 μg and 1 μg on the 24th hour (p<0.05) and Cd 4 μg and 1 μg on the 48th hour (p<0.05).

As said before, the average retention time of the control mussels on the 24th hour was 90 min. On the other hand, the mussels exposed to 3 μg PAHs (50% above AA) held the dye only 18 min, those treated with 2 μg PAHs (100% AA), which is the permitted concentrations under the Bulgarian legislation – 24 min and the mussels exposed to 1 μg PAHs (50% below AA) – 48 min. We found a significant difference between the retention time of the dye in the control group and all treated with PAHs concentrations mussels (p<0.05). The average retention time of the dye in the mussels from the control on the 48th hour was 120 min. As for the treated mussels, the retention time of the dye of the lysosomes in the mussels treated with different PAHs concentrations was shorter. The mussels exposed to 3 μg PAHs (50% above AA) held the dye for 27 min, while those exposed to 2 μg and 1 μg PAHs (100% AA and 50% below AA) held the dye equally and little longer – 48 min. A statistically significant difference (p<0.05) between the retention time of the dye in the control group and the groups treated with all PAHs concentrations was found on the 48th hour. The average retention time of the dye in the control mussels on the 72nd hour was 90 min. However, the mussels treated with PAHs showed a similar trend in the dye retention time, which was shorter. Lysosomal destabilisation was registered after 21 min in the mussels treated with 3 μg PAH (50% above AA). In addition, those treated with 2 μg and 1 μg PAH (100% AA and 50% below AA) indicated neutral red leakage into the cytoplasm after 27 and 21 min. A statistically significant difference (p<0.05) was found only between the retention time of the dye of the control mussels and the ones treated with 3 μg PAH (50% above AA). Analogous results were obtained for the mussels on the 96th hour of exposure to PAHs.

Comparing the retention time of the dye of the lysosomes treated with all different PAHs concentrations, which were applied on the 24th, 48th, 72nd and 96th hour, we found statistically significant differences between the groups exposed to 3 μg PAH (50% above AA) and 1 μg PAH (50% below AA) on the 24th hour (p < 0.05), as well as between the groups exposed to 2 μg PAHs (100% AA) and 1 μg PAHs (50% below AA) on the 24th hour (p<0.05).

Comparison between the toxicity of Cd and PAHs on the lysosomal membrane stability – short-term experiment

We could not distinguish a clear trend regarding the toxicity of both pollutants on the lysosomal membrane stability of *Dreissena polymorpha* in the short-term experiment (96 hours). Overall, the retention time of the dye was similar in the groups exposed to Cd and PAHs, i.e a shorter retention time at higher Cd and PAHs concentrations. A longer retention time of the dye was reported only in the mussels exposed to 2 μg Cd (100% AA) on the 24th hour; 2 μg Cd (100% AA) on the 72nd hour and 1 μg Cd (50% below AA) on the 72nd hour as compared to those treated with PAHs. A statistically significant difference (p<0.05),

Table 2. Physical and chemical properties of the water treated with Cd and PAHs – short-term experiment

Test concentrations, 25 l tanks	Temperature, ˚C	pH	Oxygen level, mg/l	Conductivity, μS/cm
2 μg Cd (AA, 100%)	19.9±0.2	7.92±0.07	8.9±0.1	486.5±1.7
1 μg Cd (50% below AA)	20.1±0.08	7.91±0.07	8.8±0.1	487.5±1.8
4 μg Cd (50% above AA)	20.2±0.2	7.9±0.07	9±0.1	488.5±1.1
2 μg PAHs (AA, 100%)	20±0.07	7.89±0.06	9.1±0.1	476.25±0.8
1 μg PAHs (50% below AA)	20.1±0.2	7.84±0.1	9.2±0.1	477.25±0.8
3 μg PAHs (50% above AA)	19.9±0.08	7.9±0.06	8.85±0.06	481±4.9
Control	20.1±0.2	7.68±0.08	8.7±0.2	444.5±9.4

Table 3. Physical and chemical properties of the water treated with Cd and PAHs – long-term experiment

Test concentrations, 25 l tanks	Temperature, ˚C	pH	Oxygen level, mg/l	Conductivity, µS/cm
2 µg Cd (50% above AA)	21.4	7.92	9.1	475
1 µg Cd (AA, 100%)	22	7.9	9	478
4 µg Cd (50% below AA)	21.6	7.91	8.9	477
2 µg PAHs (AA, 100%)	21.5	7.87	9.2	479
1 µg PAHs (50% below AA)	22	7.9	8.9	481
3 µg PAHs (50% above AA)	21.7	7.68	8.8	451
Control	20	7.5	8	435

however, was proven for the reported retention time of the dye between the mussels treated with 1 µg PAHs and 1 µg Cd on the 24th hour, as well as for the groups treated with 2 µg PAHs and 1 µg Cd on the 48th hour. The time was longer for the mussels exposed to PAHs as Cd caused destabilisation earlier, probably due to its more toxic influence on the lysososmes.

Long-term exposure to Cd and PAHs

The physical and chemical properties for each experimental tank measured on the 31st day (Table 3) demonstrated, in general, the values close to those measured during the short-term experiment. The data suggested that the physical and chemical properties remained relatively unchanged, which gave us a reason to consider that the observed changes in the lysosomal membrane stability were once again not due to changes in the abiotic factors.

The summarised average results on the lysosomal membrane stability in the mussels treated with different Cd and PAHs concentrations on the 31st day, including the control, are shown in Fig. 2. The neutral red retention time of the control mussels after the long-treatment was 120 min. Therefore, we assumed that they were non-stressed. On the other hand, significant decreases in the lysosomal membrane stability were registered in the treated with Cd mussels. The mussels subjected to the action of 1 µg Cd (50% below AA) held the dye 54 min, those treated with 2 µg Cd (100% AA) – 45 min, while those exposed to 4 µg Cd (50% over AA) showed lysosomal membrane destabilisation after only 30 min. The statistical analysis demonstrated significant differences in the retention time of the dye between the mussels treated with all test Cd concentrations and the control, as well as between the mussels exposed to 1 µg and 2 µg Cd ($p<0.05$).

Significant decreases in the lysosomal membrane stability were also registered in the treated with PAHs mussels. The mussels exposed to 1 µg PAHs (50% below AA) held the dye 60 min, those exposed to 2 µg PAHs (100% AA) – 48 min, while those exposed to 3 µg PAHs (50% over AA) showed destabilisation of the lysosomal membranes after 30 min (similarly to the mussels subjected to the action of the highest Cd concentration). Significant differences in the retention time of the dye were proven between the control mussels and those undergoing all test PAHs concentrations (100% AA), as well as for the mussels treated with 1 µg and 3 µg PAHs ($p<0.05$).

Comparison between the toxicity of Cd and PAHs on the lysosomal membrane stability – long-term experiment

Similarly to the results obtained from the short-term experiment, there was no clear trend regarding the toxicity of Cd and PAHs on the stability of the lysosomal membranes of zebra mussel after the treatment of 31 days (Fig. 2). The overall picture showed shorter retention at higher concentrations of Cd and PAHs. The retention time of the dye was similar in the tested groups, but earlier destabilisation in the lysosomal membranes occurred in the mussel exposed to Cd. However, the differences in the retention time between the mussels exposed to Cd and PAHs on the 31st day were not statistically significant ($p>0.05$).

Figures 1 and 2 show that the lysosomal membranes of the zebra mussels discharged the dye in the cytosol in the first hours of the action of both toxicants. The lowest average time was recorded on the 24th hour in the mussels treated with the highest Cd and PAH concentrations (50% over AA) – 15 and 18 min, respectively. On the other hand, the average retention time of the dye on the 31st day was once again lower in the mussels subjected to the higher toxicant concentrations but was longer compared to those reported on the 24th, 48th, 72nd and 96th hour. The statistical analysis of the results demonstrated significant differences in the retention time of the dye in the mussels exposed to 1 µg Cd on the 31st day and 24th hour, 2 µg Cd on the 31st day and 96th hour and 4 µg Cd on the 31st day and 24th hour ($p<0.05$). Regarding PAHs, the t-test proved significantl differences between the retention time of the dye in the mussels exposed to 1 µg PAH on the 31st day and the 72nd / 96th

hour, as well as 3 μg PAH on the 31st day and the 24th hour (p<0.05). In these cases, the retention time was longer on the 31st day of exposure. Thus, we could hypothesise that this result could indicate some initial form of adaptation of the mussels to live in contaminated aquatic environment.

Discussion

Short-term exposure to Cd and PAHs

With regard to the toxicity of Cd, the shortest retention time of the dye was observed at the beginning of the experiment. We assumed that this could be due to stress, which the incorporated toxicant caused on the mussels. They continued to experience stress, as the retention time did not reach the 90-minute stability standard, however it stayed relatively constant. For example, on the 48th hour the mussels exposed to 4 μg Cd showed destabilisation after 48 min and those exposed to 1 μg Cd on the 72nd and 96th hours after 33 min. Probably, in this case and the fact that there were no dead individuals in the course of the whole experiment, we could try to explain the given results with an initial form of acclimatisation to the contaminated aquatic environment.

For the mussels exposed to PAHs the shortest neutral red retention time was also registered in the first hours of the experiment (24th hour), as well as at the highest concentrations (3 μg and 2 μg PAHs). We believe that this could be once again a result of the stress of the toxicant introduction and the following contaminated aquatic environment. On the other hand, the mussels treated with 1 μg PAHs (50% below AA) had a longer retention time of the dye on the 24th and 48th hour (48 min) compared to 72h and 96h (30 min). Thus, we consider that mussels maintained a relatively constant tolerance to PAHs when treated with 1 μg, even though the neutral red retention time was below 90 min. Furthermore, we might consider that the mussels attempted to adapt to the polluted water as stated above. This time was finally reduced from 48 min during the first hours of the experiment to 30 min, at the end of the experiment.

Long-term exposure to Cd and PAHs

After 31 days no mortality was reported for the treated with Cd and PAHs zebra mussels, which supported our conclusion from the short-term experiment that the mussels could have tried to adapt to pollution. In addition, the statistical analysis showed significant differences in the retention time of the dye comparing the short and long-term exposure to Cd and PAHs; it was longer in the mussels observed after 31 days, but still lower than 90 min.

Even though the literature on the effects of heavy metals and different organic contaminants on the lysosomal membrane stability on *Dreissena polymoprha* is relatively limited, we found that our results corresponded to those of other authors and confirmed that the lysosomal stability of the membranes in bivalves and other invertebrates such as gastropods is weakened as a result of contaminated aquatic environment. Consequently, at lower toxicant concentrations the retention time of the dye is longer, and vice versa (Shepard & Bradley 2000, Matozzo et al. 2001, Moschino et al. 2011, Molnar & Fong 2012, Patetsini et al. 2013, Turja et al. 2014, Yancheva et al. 2016 a,b, Yancheva et al. 2017).

Conclusions

Based on the results from the first study with these test concentrations and exposure period, aiming to compare the toxicity of both, inorganic and organic toxicants, it can be concluded that Cd and PAHs have a negative effect on the physiology of *Dreissena polymorpha*, which leads to destabilisation of the cellular organisation. Overall, we found that: (1) the observed changes in the stability of the lysosomal membranes of the mussels were dose-dependent and more pronounced at Cd and PAHs concentrations, which represent 100% AA and 50% above AA; (2) faster cell membrane destabilisation occurs in the first hours (24 h) as compared to the 48th, 72nd, 96th hours and 31 days; (3) changes in the stability of the lysosomal membranes of the mussels subjected to lower concentrations (50% under AA) were also observed, confirming the toxicity of the studied substances; (4) in the treated with Cd mussels the lysosomal membrane stability was impaired faster than with PAHs, although there were no significant differences in the neutral red retention time; (5) the neutral red retention assay confirmed to be an easy to perform and relatively inexpensive, non-specific biomarker in ecotoxicological studies in order to identify the negative effects of different toxicants; (6) *Dreissena polymorpha* could be used as a bioindicator for contaminated freshwater ecosystems, in particular with Cd and PAHs. Last but not least, the results from this experiment can be used as a base in future studies, as well as in monitoring programs dealing with the effects of contamination with inorganic and organic toxicants.

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