

Bioaccumulation of Polyaromatic Hydrocarbons and Cadmium and its Toxic Effects on Zebra Mussel *Dreissena polymorpha* (Pallas, 1771) (Bivalvia: Dreissenidae)

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Abstract: The possible negative effects of polyaromatic hydrocarbons (PAHs) and cadmium (Cd) on the lysosomal membrane stability (LMS) in haemocytes of the invasive mollusc zebra mussel (*Dreissena polymorpha*) were studied by applying the neutral red retention time assay (NRRT). In addition, the process of bioaccumulation of PAHs and Cd in the gills of zebra mussel was examined and the bioaccumulation factor (BFA) was calculated. The mussels were exposed to different concentrations of Cd and PAHs in laboratory conditions for 96 hours (acute exposure) and 31 days (chronic exposure). We found higher toxicant concentrations at the 24th h as well as on the 31st day compared to the other tested time periods. These results were linked with the faster lysosomal membrane destabilisation in all mussels treated with Cd and PAHs in the beginning of the experiment.

Key words: bioaccumulation, contamination, Cd, PAHs, zebra mussel, gills, lysosomes

Introduction

Cadmium (Cd), a non-essential metal for biological systems, is potentially toxic at certain levels of bioavailability. Its presence in aquatic environments can result in deleterious effects on aquatic organisms (PEREIRA et al. 2016). Polycyclic Aromatic Hydrocarbons (PAHs) are a class of semi-volatile chemical compounds with at least two fused benzene rings in various arrangements, which mainly originate from petrogenic, biogenic and pyrogenic sources. Among the 16 PAHs listed as priority hazardous pollutants, some of them such as chrysene (Chr) and benzo[a]pyrene (BaP) are potentially carcinogenic to humans according to the International Agency for Research on Cancer (RANJBAR et al. 2019). As aquatic ecosystems are the ultimate receptacle for many pollutants, they can undergo many disturbances. To face this environmental problem, the European Water Framework Directive (DIRECTIVE 2000/60/

EC, WFD) was designed to evaluate, protect and restore aquatic systems (POTET et al. 2018). In addition, the implementation of a biomarker-based assessment of pollution effects induced by contaminants has recently been recommended in the context of the adoptable criteria aimed at the accomplishment of the good environmental status (GES) of marine waters, in compliance with DIRECTIVE 2008/56/EC. Furthermore, biomarkers are based on sub-organism biological responses to pollutant exposure, including responses at the molecular, subcellular, cellular, organ or tissue level. Such biological responses are sensitive, easily detected and they have been regarded as early warning signals of changes that may potentially cause impacts on the whole ecosystem (FU et al. 2017). More specifically, lysosomal alterations are recognised as relevant outputs for the classification of the pollution effects of marine con-

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taminants and to implement the environmental quality standards (EQSs) specified in the Descriptor 8 of the DIRECTIVE 2008/56/EC (MSFD). The assessment of sentinel hydrobiont species may provide valuable information regarding anthropogenic impacts on the aquatic ecosystems.

The zebra mussel *Dreissena polymorpha* (Pallas, 1771) is a widespread invasive bivalve species commonly found in freshwaters of the northern hemisphere and identified by the IUCN as one of the 100 of the World's Worst Invasive Alien Species. It is a sedentary filter-feeder, able to tolerate a wide range of environmental contaminants. The zebra mussel has been used actively as a model organism for freshwaters since the late 1970s (KLIMOVA et al. 2016). Thus, it is currently used in biomonitoring programs to evaluate both presence of contaminants through bioaccumulation measurements and their effects through the use of biomarkers, either in the field or under laboratory conditions (FARIA et al. 2014).

Based on our previous work (YANCHEVA et al. 2018), we undertook this study in order to (1) follow the process of bioaccumulation of environmentally relevant concentrations of PAHs and Cd in gills of zebra mussel, which, to our knowledge, is the first known *ex situ* study with the selected contaminants, applied concentrations and exposure period, and (2) better understand the effects of acute (96 h) and chronic (31 days) exposure of PAHs and Cd on the lysosomal destabilisation in haemocytes of zebra mussel.

Materials and Methods

The field collection of specimens, preparation of environmentally relevant concentrations based on the EU legislation, exposure period and measurement of basic physical and chemical properties (pH, temperature, oxygen concentration, electrical conductivity) of the water were as previously explained in details (YANCHEVA et al. 2018).

For the bioaccumulation of PAHs, the samples were transferred to aluminium foil and polyethylene zip-bags and stored in a freezer at -20°C until analysed. The sample preparation was performed using a microwave digestion (Mars 6, Spectrotech, USA) for withdrawing about 0.1 g sample extracted with organic solvent Hexane:Acetone (1:1), oven temperature of 120°C, 25 min. The resulting extract was concentrated, purified on silica gel and concentrated once again. The extracted samples were analysed by mass spectrometry gas chromatography (GC/MS Agilent7890B/5977A/MSD). Certified reference materials were used: BCR 682-70G Mussel tissue,

2974a – Organics in Freeze-Dried Mussel Tissue, NIST; PAH – Dr. Ehrenstorfer. All results showed a good agreement with the standards and recovery ranged between 92 % and 101 % for PAHs. The results were obtained in µg/kg body weight (wet weight). The detection limit of all 16 organic substances in the PAHs mixture for the applied method is presented in Table 1.

For studying the bioaccumulation of Cd, the samples were placed directly in polyethylene zip-bags and stored in a freezer at -20°C until analysed. They were then processed as described by KINGSTON & JASSIE (1988) in closed pressure vessels, program of microwave decomposition: oven temperature of 180°C, 25 min. The obtained extract was quantitatively transferred to 50 ml. The extracted samples were then analysed by an inductively coupled plasma mass spectrometer (ICP MS Agilent 7500ce G3272B, Agilent Technologies, Tokyo, Japan) according to ISO 17294-2:2005. The results were controlled with a certified reference material (Fish

Table 1. Limit of detection and limit of quantitation of the organic substances according to the applied method.

Wet weight, W.W.	Abbreviation	Limit of detection, LOD, µg/kg	Limit of quantitation, LOQ, µg/kg
Acenaphthene	ACE	<3	<10
Acenaphthylene	ACY	<3	<10
Anthracene	ANT	<0.7	<2
Benz[a]anthracene	BaA	<0.3	<1
Benzo[b]fluoranthene	BbF	<0.3	<1
Benzo[k]fluoranthene	BkF	<0.3	<1
Benzo[g,h,i]perylene	BghiP	<1	<3
Benzo[a]pyrene	BaP	<0.3	<1
Dibenzo[a,h]anthracene	DbA	<1	<3
Fluoranthene	FLA	<0.3	<1
Fluorene	FLU	<3	<10
Indeno[1,2,3-cd]pyren	IND	<1	<3
Naphthalene	NAP	<6	<20
Phenanthrene	PHE	<0.7	<2
Pyrene	PYR	<0.3	<1
Chrysene	CHR	<0.3	<1

Limit of Blank (LoB), Limit of Detection (LoD), and Limit of Quantitation (LoQ) are terms used to describe the smallest concentration that can be reliably measured through an analytical procedure (see ARMBRUSTER & PRY 2008).

Protein – DORM-4, National Research Council, Canada) and obtained in mg/kg body weight (wet weight) and converted into µg/kg. All results showed a good agreement with the standards and recovery ranged between 96 % and 105 % for Cd. The detection limit of Cd for the applied method was 0.03 mg/kg (30 µg/kg).

The bioaccumulation factor (BAF) for PAHs and Cd was calculated after NIKANOROV et al. (1985) according to the formula shown below.

$$\text{BAF} = \frac{\text{toxicant concentration in zebra mussel gills}}{\text{toxicant concentrations in water}}$$

For the BAF calculations of PAHs, we took into account the average concentration of all 16 substances determined in the zebra mussel gills according to the concentration in the water (1, 2 and 3 µg, respectively).

The analytical procedure of the NRRT assay was adapted from MARTÍNEZ-GÓMEZ et al. (1995). For each experimental condition, LMS was expressed as destabilisation time (means of min ± SEM) representing the time at which >50% of the lysosomes released the dye into the cytosol.

Data on basic physical properties, toxicant concentrations in the gills and NRRT in the haemocytes were distributed normally and analysed using Graph Pad Prism 7 for Windows (USA). The differences between the variables were tested using Student's

t-test at significance level of 95% ($p < 0.05$). The results were reported as mean ± SD.

Results

Basic properties of water

The results for the measured basic physical properties of each experimental aquarium for the short-term experiment are presented in Table 2. The results of the measured physical parameters on the 31st day of exposure to PAHs and Cd were analogous to the ones for the short-term experiment (Table 3).

Bioaccumulation of PAHs and Cd

The control results for both short and long-term experiments were below the detection limit of the instrument. Tables 4, 5 and 6 present the results for the bioaccumulated PAHs at a concentration of 1 µg (50% below AA-EQS for inland surface waters), 2 µg (AA-EQS for inland surface waters), 3 µg (50% above AA-EQS for inland surface waters) in the zebra mussel gills at 24th, 48th, 72th and 96th h (short-term experiment). Table 7 presents the results for the bioaccumulation of the applied PAHs concentrations on the 31st day of exposure (long-term experiment). The results for the long-term experiment confirmed that each of the 16 substances composing the PAHs mixture had a different degree of bioaccumulation in the gills of zebra mussels and in general, the trend showed

Table 2. Average values of basic physical and chemical properties of water (short-term experiment).

Tested concentrations, 25 l water tanks	Temperature, °C	pH	Oxygen level, mg/L	Conductivity, µS/cm
PAHs 1 µg (50% below AA-EQS for inland surface waters)	25.2±0.2	7.95±0.07	6.2±0.1	481.2±1.5
PAHs 2 µg (AA-EQS for inland surface waters)	24.9±0.06	7.97±0.07	5.96±0.1	436±1.1
PAHs 3 µg (50% above AA-EQS for inland surface waters)	24.7±0.2	8.02±0.02	6.2±0.1	450.7±0.05
Cd 1 µg (50% below AA-EQS for inland surface waters)	25±0	7.91±0.05	6.4±0.1	477.25±0.8
Cd 2 µg (AA-EQS for inland surface waters)	24.7±0.2	7.84±0.1	7.7±0.08	394.5±0.6
Cd 4 µg (50% above AA-EQS for inland surface waters)	25.2±0.05	7.9±0.05	7.36±0.07	466±0.5
Control	23.5±2	7.6 ±0.5	6.9±0.5	510±1.5

Table 3. Average values of basic physical and chemical properties of water (long-term experiment).

Tested concentrations, 25 l water tanks	Temperature, °C	pH	Oxygen level, mg/l	Conductivity, µS/cm
PAHs 1 µg (50% below AA-EQS for inland surface waters)	28.5±1.2	8.03±0.3	7.4±2	612±1.5
PAHs 2 µg (AA-EQS for inland surface waters)	28.6±0.5	8.07±2	7.4±0.5	641±2
PAHs 3 µg (50% above AA-EQS for inland surface waters)	28.5±1.7	8.2±1	7.5±0.5	873±2.5
Cd 1 µg (50% below AA-EQS for inland surface waters)	27.5±0.3	7.95±0.05	7.5±1.3	682±1
Cd 2 µg (AA-EQS for inland surface waters)	27.4±2	7.88±1	7.4±1	574±0.5
Cd 4 µg (50% above AA-EQS for inland surface waters)	28±1.5	7.94±0.3	7.4±0.5	588±3.5
Control	27.6±0.5	7.69±0.5	7.6±0.3	597±2.5

Table 4. Average bioaccumulation concentration of PAHs 1 µg (50% below AA-EQS for inland surface waters) in zebra mussel gills (n=10), short-term experiment, µg/kg.

PAHs 1 µg (50% below AA-EQS for inland surface waters)	24 h	48 h	72 h	96 h
NAP	0.78±0.01	<i>0.17[†]±0.003</i>	0.64±0.02	0.59±0.03
ACY	0.49±0.1	0.41±0.1	0.45±0.01	<i>0.01[†]±0.003</i>
ACE	0.52±0.05	0.42±0.02	0.51±0.005	<i>0.05[†]±0.001</i>
FLU	<i>0.35±0.05</i>	0.51±0.1	0.64±0.03	0.60±0.05
PHE	0.95±0.01	0.90±0.05	<i>0.77±0.01</i>	0.85±0.01
ANT	<i>0.32±0.01</i>	0.51±0.1	0.60±0.05	0.50±0.03
FLA	0.62±0.05	0.62±0.03	0.83±0.3	<i>0.52±0.1</i>
PYR	<i>0.39±0.1</i>	0.49±0.1	0.86±0.05	0.61±0.05
BaA	0,66±0,1	0.88±0.04	<i>0.32±0,02</i>	0.81±0.1
CHR	0,84±0,03	0.65±0.02	<i>0.55±0.05</i>	0.74±0.01
BbF	0.97±0.05	<i>0.53±0.05</i>	0.69±0.1	0.70±0.05
BkF	0.47±0.02	<i>0.45±0.03</i>	0.55±0.03	0.64±0.04
BaP	0.60±0.04	0.55±0.05	<i>0.44±0.03</i>	0.46±0.01
IND	0.78±0.01	0.95±0.1	0.67±0.01	<i>0.53±0.05</i>
DbA	0.95±0.05	0.98±0.01	<i>0.91±0.2</i>	1.01±0.5
BghiP	0.38±0.01	0.35±0.03	<i>0.27±0.002</i>	0.39±0.02

Bold – highest concentration for the exposure period, *Italic* – lowest concentration for the exposure period, † – significantly different (p<0.05)

that the substances accumulated the most at the highest concentrations of 3 µg, with very few exceptions (Table 7). Table 8 presents the results for Cd bioaccumulation in the zebra mussel gills for the applied metal concentrations and exposure period (short and long-term experiments). In addition, similarly to the trend for PAHs, the highest Cd concentrations bioaccumulated in the gills were determined for the highest applied concentration. As for the 31st day, the results were close between the tested concentrations but increased between the tested time, probably because of the chronic effect of the heavy metal.

Factor of Bioaccumulation

In general, the BFA calculations showed how many times more the concentration of the toxicant in the zebra mussel gills was compared to that in the water (Table 9). Overall, the BAF for PAHs during the short-term experiment was low, whereas the BAF for Cd was several times higher; the BAF for PAHs during the long-term experiment was found to be also low, whereas the one for Cd confirmed its high

Table 5. Average bioaccumulation concentration of PAHs 2 µg (AA-EQS for inland surface waters) in zebra mussel gills (n=10), short-term experiment, µg/kg.

PAHs 2 µg (AA-EQS for inland surface waters)	24 h	48 h	72 h	96 h
NAP	0.60±0.2	<i>0.33[†]±0.04</i>	1.09±0.5	1.30±0.3
ACY	0.46±0.05	0.49±0.02	0.45±0.003	<i>0.43±0.05</i>
ACE	0.52±0.1	<i>0.49±0.005</i>	0.52±0.05	0.56±0.01
FLU	0.77±0.03	<i>0.51±0.03</i>	0.71±0.02	0.57±0.01
PHE	2.32±0.5	<i>0.72[†]±0.01</i>	1.17±0.5	1.28±0.3
ANT	0.31±0.01	<i>0.11[†]±0.005</i>	0.21±0.002	0.57±0.02
FLA	0.65±0.01	<i>0.44±0.03</i>	0.73±0.05	0.51±0.3
PYR	0.63±0.1	0.58±0.1	0.80±	<i>0.54±0.05</i>
BaA	1.00±0.5	0.35±0.02	0.82±0.05	<i>0.34±0.05</i>
CHR	1.09±0.3	<i>0.82±0.03</i>	1.18±0.5	0.94±0.03
BbF	1,54±0,05	1.63±0.05	1.38±0.03	<i>0.94±0.5</i>
BkF	0.46±0.05	0.56±0.01	0.47±0.01	<i>0.42±0.03</i>
BaP	0.57±0.03	0.62±0.02	<i>0.50±0.005</i>	0.60±0.01
IND	<i>0.98±0.001</i>	1.07±0.005	1.61±0.03	1.21±0.01
DbA	<i>0.62±0.3</i>	1.09±0.05	1.16±0.01	1.08±0.001
BghiP	<i>0.58±0.05</i>	0.85±0.03	1.22±0.05	1.01±0.01

Bold – highest concentration for the exposure period, *Italic* – lowest concentration for the exposure period, † – significantly different (p<0.05)

bioaccumulation ability. We also found more significant differences in the BFA values between the tested concentrations of the toxicants for the respective hour than when comparing the respective concentration between the tested hours.

Neutral red retention assay

The observed alterations in the stability of lysosomal membranes were dose-dependent and more pronounced at AA-EQS and 50% above AA-EQS for inland surface waters exposure (Fig. 1). Alterations in the membrane stability of the lysosomes subjected to lower contaminant concentrations (50% below AA) were also established, indicating the toxicity of PAHs and Cd. In general, the NRRT of the dye was similar and the overall trend showed shorter retention at higher concentrations for both PAHs and Cd. However, in the mussels treated with Cd, the LMS was impaired faster than the ones treated with PAHs, although no significant differences in the NRRT between the tested groups were reported (p>0.05). There were also no statistically significant

Table 6. Average bioaccumulation concentration of PAHs 3 µg (50% above AA-EQS for inland surface waters) in zebra mussel gills (n=10), short-term experiment, µg/kg.

PAHs 3 µg (50% above AA-EQS for inland surface waters)	24 h	48 h	72 h	96 h
NAP	0.53±0.1	<i>0.45±0.1</i>	0.46±0.05	0.49±0.03
ACY	1.22[†]±0.5	0.89±0.05	<i>0.81±0.3</i>	<i>0.81±0.05</i>
ACE	1.58±0.5	1.41±0.3	<i>1.27±0.5</i>	1.37±0.1
FLU	1.38±0.3	1.46±0.5	1.40±0.5	<i>1.37±0.3</i>
PHE	1.39±0.5	<i>1.32±0.5</i>	1.64±0.05	1.70±0.1
ANT	<i>0.64±0.03</i>	0.66±0.1	0.65±0.05	0.95±0.03
FLA	<i>0.74±0.5</i>	1.13±0.5	1.25±0.5	1.13±0.05
PYR	<i>0.87±0.5</i>	1.08±0.5	1.15±0.3	1.06±0.3
BaA	1.03±0.5	0.98±0.3	1.30±1	<i>0.81±0.5</i>
CHR	1.36±0.5	<i>1.01±0.3</i>	1.75±0.5	1.17±0.1
BbF	<i>0.95±0.5</i>	1.18±0.5	<i>0.95±0.2</i>	1.05±0.4
BkF	<i>0.39±0.05</i>	0.56±0.03	0.53±0.1	0.45±0.03
BaP	0.50±0.05	<i>0.44±0.03</i>	0.46±0.05	0.60±0.2
IND	1.64±0.1	2.05[†]±0.5	1.65±0.03	<i>1.21±0.01</i>
DbA	2.20±0.5	2.68±0.3	2.60±0.5	<i>2.07±0.1</i>
BghiP	0.50±0.02	0.48±0.1	<i>0.45±0.05</i>	0.48±0.2

Bold – highest concentration for the exposure period, *Italic* – lowest concentration for the exposure period, † – significantly different (p<0.05)

differences in the observed NRRT of the dye between the groups subjected to PAHs and Cd during the short and long-term experiments ($p>0.05$). Such were found only between the control and the mussels exposed to the tested contaminants. The NRRT was slightly higher on the 31st day, however it was still lower than the accepted minimum of 90 mins.

Discussion

Basic physical properties of water

Overall, the values were similar for the whole study period and the data showed that the physical parameters remained relatively unchanged during the entire experiment. This gives us a reason to consider that the observed changes in the LMS in zebra mussels were not due to changes in the abiotic factors.

Bioaccumulation of PAHs and Cd

Based on our results, we can summarise that with increasing the concentrations of the respective toxicant in the water, its bioaccumulation in the zebra mussel gills also increased.

As for the exposure time, higher toxicant concentrations were generally accumulated at the 24th h, although the values for the different organic substances varied between the hours and the applied concentrations. We consider that this might be due to the stress reaction of the organism from incorporating the toxicants at the beginning of the experi-

Table 7. Average bioaccumulation concentration of PAHs in zebra mussel gills (n=10), long-term experiment, µg/kg.

31 d	PAHs 1 µg (50% below AA-EQS for inland surface waters)	PAHs 2 µg (AA-EQS for inland surface waters)	PAHs 3 µg (50% above AA-EQS for inland surface waters)
NAP	<i>0.96[†]±0.03</i>	1.77±0.05	2.4[†]±0.5
ACY	0.98±0.005	<i>0.68±0.5</i>	1.04±0.01
ACE	0.82±0.5	<i>0.36[†]±0.05</i>	1.96±0.5
FLU	0.48±0.03	<i>0.42±0.01</i>	1.45[†]±0.5
PHE	<i>0.97±0.5</i>	1.45±0.03	1.61±0.03
ANT	<i>0.69±0.05</i>	0.72±0.05	0.84±0.04
FLA	<i>0.85±0.5</i>	1.05±0.1	1.8±0.05
PYR	<i>0.87[†]±0.03</i>	1.54±0.05	2.36[†]±0.5
BaA	<i>0.69±0.5</i>	1.57±0.05	1.38±0.01
CHR	<i>0.75±0.03</i>	1.13±0.05	1.63[†]±0.5
BbF	<i>0.78±0.5</i>	1.6±0.03	1.23±0.1
BkF	<i>0.43±0.05</i>	0.48±0.05	0.6±0.03
BaP	0.75±0.01	<i>0.52±0.05</i>	0.81±0.3
IND	<i>0.67±0.05</i>	1.73±0.5	1.85±0.1
DbA	<i>0.99[†]±0.4</i>	1.62±0.5	2.5[†]±1
BghiP	<i>0.45±0.1</i>	1.3±0.5	0.49±0.5

Bold – highest concentration for the exposure period, *Italic* – lowest concentration for the exposure period, † – significantly different (p<0.05)

Table 8. Average bioaccumulation concentration of Cd in zebra mussel gills (n=10), short and long-term experiments, µg/kg.

Tested concentrations	24 h	48 h	72 h	96 h	31 st day
Cd 1 µg (50% below AA-EQS for inland surface waters)	660±1.2	590±0.5	580±2.5	480±5.5	820±4.5
Cd 2 µg (AA-EQS for inland surface waters)	740±2.5	610±3.5	550±0.5	550±3.5	930±5.1
Cd 4 µg (50% above AA-EQS for inland surface waters)	910±5.5	680±1.5	620±3.3	600±.5	1120[†]±13.5

Bold – highest concentration for the exposure period, *Italic* – lowest concentration for the exposure period, † – significantly different (p<0.05)

Table 9. Average results for BAF of PAHs and Cd in the zebra mussel gills (short and long-term experiments).

Tested concentrations	24 h	48 h	72 h	96 h	31 days
PAHs 1 µg (50% below AA-EQS for inland surface waters)	0.63	0.59	0.60	<i>0.56</i>	0.75
PAHs 2 µg (AA-EQS for inland surface waters)	0.41	<i>0.33</i>	0.43	0.38	0.56
PAHs 3 µg (50% above AA-EQS for inland surface waters)	0.35	0.36	0.37	<i>0.34</i>	0.49
Cd 1 µg (50% below AA-EQS for inland surface waters)	660	590	580	480	820
Cd 2 µg (AA-EQS for inland surface waters)	370	305	275	275	465
Cd 4 µg (50% above AA-EQS for inland surface waters)	228	170	155	150	280

Bold – highest BAF value for the exposure period, *Italic* – lowest BAF value for the exposure period

Neutral Red Retention Assay - short and long-term experiment

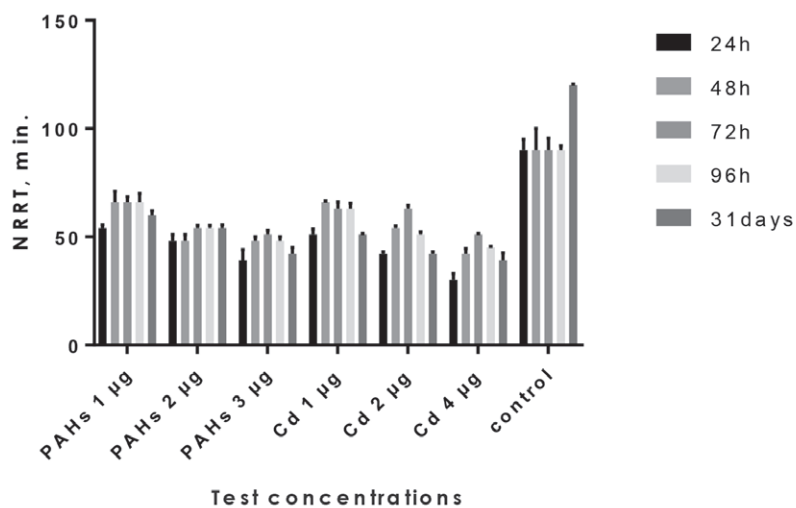


Fig. 1. Average neutral red retention time in hemocytes of zebra mussel (n=5) after exposure to PAHs and Cd (96 h and 31 days).

ment. It is also worth mentioning that overall, there was a difference in the bioaccumulation ability between the substances, but the concentrations of each substance between the different hours were similar, even though some of the highest concentrations were determined at the 24th h of exposure (statistically significant differences are shown in the tables). Regardless of the applied concentrations (1, 2, 3 µg), the organic substances that bioaccumulated at the highest concentrations during the experiment were phenanthrene, chrysene and benzo[b]fluoranthene, as well as acenaphthene and dibenz[a,h]anthracene,

for which a threefold increase was determined at concentration of 3 µg PAHs compared to the results for 1 and 2 µg PAHs. We consider that the obtained results could be associated with the actual toxic potential of these particular organic substances.

Regarding the heavy metal, the Cd bioaccumulation results were several times higher than those of PAHs, irrespective of the concentration and study period, which confirms its high and more toxic bioaccumulation ability compared to PAHs. In general, our results are in agreement with those of other authors', both for PAHs and Cd accumulation regard-

ing the exposure period and determined concentrations (SHEFER et al. 2015; OLIVA et al. 2017).

Factor of bioaccumulation

During the short-term experiment, the BFA both for PAHs and Cd showed the highest values at the beginning of the experiment. We believe that this result is linked to the higher bioaccumulation of the toxicants on the 24th h. In addition, on the 31st day we determined higher BFA values comparing the short-term experiment and these results are in line with the highest concentrations found in the zebra mussel gills at the end of the long-term experiment. The BFA values for PAHs were < 1, which determines zebra mussel as “de-concentrator” in relation to organic contaminants, but the BFA values for Cd were > 1, which determines this species as “macro-concentrator” in relation to heavy metals accumulation. MUSA-BANDOWE et al. (2014) determined BFA of 28 organics in different fish species (*Drapane africana*, Osorio 1892, *Cynoglossus senegalensis*, Kaup 1858 and *Pomadysys peroteti*, Cuvier 1830) and contrary to our results, the BFA values were > 1. On the other hand, DAVIES et al. (2006) studied the BFA of three heavy metals: Cr, Cd and Pb in the shells and soft tissues of the mollusk *Tympanotonus fuscatus var radula* (Linnaeus, 1758). The authors found that Cr was the most heavily bioaccumulated metal and the BFA results revealed that the studied heavy metals had a great ability for bioaccumulation. These correspond with our results for Cd in the zebra mussel gills in the present study. We can also say that the BFA values differ for different species, regardless of the toxicant nature (organic, inorganic), which we also confirmed in our study.

Neutral red retention assay

The dye leaked in the cytosol in the hemocytes of zebra mussels treated with both PAHs and Cd during the first hours. This result confirmed our suggestion that the organisms were stressed because of the toxicant application. Moreover, the results for the destabilisation of lysosomes were in line with the higher bioaccumulated PAHs and Cd at the first hours of exposure. Furthermore, we found that after exposure to Cd, the mussels retained the dye shorter. This is worth mentioning because when it comes to toxicity it seems that Cd has more severe effects on zebra mussel, which we also confirmed in our previous study (YANCHEVA et al. 2018). During the long-term study the mussels had longer NRRT, which could be accepted as an initial form of adaptation to survive in contaminated waters. Our results correspond to those of other authors (TURJA et al. 2013, CAPOLUPO

et al. 2016) and we agree that the LMS evaluation is one of the most indicative biomarkers of environmental stress.

Conclusions

On the basis of the obtained results we can conclude that a leading factor in the bioaccumulation of PAHs and Cd in the zebra mussel gills was not only the applied concentration but also the exposure time. We could link this interesting result to the destabilisation of the lysosomal membranes, which was faster at the first hours as also found in previous studies. Moreover, we confirmed that Cd has a more toxic effect on LMS compared to PAHs as demonstrated by the higher BAF, which is an important finding regarding the toxicity of different contaminants in aquatic toxicology. Finally, we confirmed that zebra mussel could be used as a good bioindicator for freshwater contamination and the NRRT assay as a low-cost, fast, sensitive, feasible and more importantly, non-destructive biomarker for organic and heavy metal pollution. Therefore, we suggest zebra mussel as a bioindicator along with the chemical analyses, as well as the NRRT assay as a biological effect-technique at a sub-organism level to answer the challenges of WFD for improved detection of the impact of different contaminants on aquatic organisms.

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References

- NIKANOROV A., JULIDKOV A. & POKARZEVSKII A. 1985. Bio-monitoring of heavy metals in freshwater ecosystems. L. Hydrometeoizdet. pp. 144. (In Russian)
- ARMBRUSTER D. & PRY T. 2008. Limit of blank, limit of detection and limit of quantitation. *Clinical Biochemist Reviews* 29 (1): 49-52.
- CAPOLUPO M., P. VALBONESI, A. KIWAN, S. BURATTI, S. FRANZELITTI & E. FABBRI. 2016. Use of an integrated biomarker-based strategy to evaluate physiological stress responses induced by environmental concentrations of caffeine in the Mediterranean mussel *Mytilus galloprovincialis*. *Science of the Total Environment* 563-564: 538-548.

- DAVIES A., ALLISON M. & UYI X. 2006. Bioaccumulation of heavy metals in water, sediment and periwinkle (*Tympanotonus fuscatus var radula*) from the Elechi Creek, Niger Delta. *African Journal of Biotechnology* 5 (10): 968-973.
- DIRECTIVE 2000/60/EC of the European Parliament and of the Council of 23 October 2000 establishing a framework for Community action in the field of water policy (European Water Framework Directive).
- DIRECTIVE 2008/56/EC of the European Parliament and of the Council of 17 June 2008 establishing a framework for community action in the field of marine environmental policy (Marine Strategy Framework Directive).
- DIRECTIVE 2013/39/EU of the European Parliament and of the Council of 12 August 2013 amending Directives 2000/60/EC and 2008/105/EC as regards priority substances in the field of water policy.
- FARIA M., OCHOA V., BLÁZQUEZ M., JUAN M. F., LAZZARA R., LACORTE S., SOARES A. M. & BARATA C. 2014. Separating natural from anthropogenic causes of impairment in zebra mussel (*Dreissena polymorpha*) populations living across a pollution gradient. *Aquatic Toxicology* 152: 82-95.
- FU D., BRIDLE A., LEEF M., GAGNON M. M., HASSELL K. L. & NOWAK B. F. 2017. Using a multi-biomarker approach to assess the effects of pollution on sand flathead (*Platycephalus bassensis*) from Port Phillip Bay, Victoria, Australia. *Marine Pollution Bulletin* 119: 211-219.
- ISO 17294-2 (2005-02) Water quality – Application of inductively coupled plasma mass spectrometry (ICP-MS) Part 2: Determination of 62 elements (ISO 17294-2:2003).
- RANJBAR J. A., BAKHTIARI A. R., YAGHOobi Z., YAP C. K., MAISANO M. & CAPPELLO T. 2019. Distributions and compositional patterns of polycyclic aromatic hydrocarbons (PAHs) and their derivatives in three edible fishes from Kharg coral Island, Persian Gulf, Iran. *Chemosphere* 215: 835-845.
- KINGSTON H. & JASSIE L. B. 1988. Monitoring and predicting parameters in microwave dissolution. In: Introduction to microwave sample preparation: Theory and practice. American Chemical Society, Washington D.C. 263 p.
- KLIMOVA Y. S., CHUIKO G. M., GAPEEVA M. V. & PESNYA D. S. 2017. The use of biomarkers of oxidative stress in Zebra mussel *Dreissena polymorpha* (Pallas, 1771) for chronic anthropogenic pollution assessment of the Rybinsk reservoir. *Contemporary Problems of Ecology* 10 (2): 178-183.
- MARTÍNEZ-GÓMEZ C., J. BENEDICTO, J. A. CAMPILLO & M. MOORE. 2008. Application and evaluation of the neutral red retention (NRR) assay for lysosomal stability in mussel populations along the Iberian Mediterranean coast. *Journal of Environmental Monitoring* 10: 490-499.
- MUSA-BANDOWE B., BIGALKEA M., BOAMAH L., NYARKO E., SAALIA F. & WILCKEA W. 2014. Polycyclic aromatic compounds (PAHs and oxygenated PAHs) and trace metals in fish species from Ghana (West Africa): Bioaccumulation and health risk assessment. *Environment International* 65: 135-146.
- OLIVA A., ARIAS A., QUINTAS P., BUZZI N. & MARCOVECCHIO J. 2017. Polycyclic aromatic hydrocarbons in mussels from a South American estuary. *Archives of Environmental Contamination and Toxicology* 72 (4): 540-551.
- PEREIRA L. S., RIBAS J. L. C., VICARI T., SILVA S. B., STIVAL J., BALDAN A. P., VALDEZ DOMINGOS F. X., GRASSI M. T., CESTARI M. M. & SILVA DE ASSIS H. C. 2016. Effects of ecologically relevant concentrations of cadmium in a freshwater fish. *Ecotoxicology and Environmental Safety* 130: 29-36.
- POTET M., GIAMBÉRINI L., PAIN-DEVIN S., LOUIS F., BERTRAND C. & DEVIN S. 2018. Differential tolerance to nickel between *Dreissena polymorpha* and *Dreissena rostriformis bugensis* populations. *Scientific Reports* 8: 700
- SHEFER E., SILVERMAN J. & HERUT B. 2015. Trace metal bioaccumulation in Israeli Mediterranean coastal marine mollusks. *Quaternary International* 390: 44-55.
- TURJA R., HÖHER N., SNOEIJNS P., BARŠIENĖ J., BUTRIMAVIČIENĖ L., KUZNETSOVA T., KHOLODKEVICH S. V., DEVIER M.-H., BUDZINSKI H. & LEHTONEN K. K. 2014. A multibiomarker approach to the assessment of pollution impacts in two Baltic Sea coastal areas in Sweden using caged mussels (*Mytilus trossulus*). *Science of the Total Environment* 473-474: 398-409.
- YANCHEVA V., GEORGIEVA E., STOYANOVA S., TSVETANOVA V., TODOROVA K., MOLLOV I. & VELCHEVA I. 2018. Short- and long-term toxicity of Cadmium and polyaromatic hydrocarbons on zebra mussel *Dreissena polymorpha* (Pallas, 1771) (Bivalvia: Dreissenidae). *Acta Zoologica Bulgarica* 70 (4): 557-564.

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