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Histochemical and Biochemical Alterations in Zebra Mussel *Dreissena polymoprha* (Pallas, 1771) after Cadmium and Polyaromatic Hydrocarbons Exposure

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Abstract: The present study was developed to examine the possible harmful effects, which cadmium (Cd) and polyaromatic hydrocarbons (PAHs) could cause to the gills and digestive gland of the zebra mussel *Dreissena polymorpha* (Pallas, 1771). For this purpose, we explored for the first time their histochemical and biochemical alterations by applying the Periodic-Schiff staining method (PAS) and analysing the catalase (CAT) and cholinesterase (ChE) activity. 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 2013/39/EU. Moreover, the enzymatic measurements are included as biomarkers for biota in the EU Water Framework Directive, the Marine Strategy Framework Directive and in the mussel component of the International Council for the Exploration of the Sea/Oslo-Paris convention for the Protection of the Marine Environment of the North-East Atlantic (ICES/OSPAR) integrated monitoring framework. Based on our results, we also proposed the PAS reaction as an easy, fast, low-cost and trustworthy biological tool, which could be used for biota in monitoring programs. Overall, we found alterations both in the gill structure and enzymatic activity in the digestive gland at all tested concentrations, including the one below the allowable concentration according to the EU legislation. These results confirmed the toxicity of Cd and PAHs. Furthermore, Cd was more toxic compared to PAHs in terms of the studied parameters.

Key words: Cd, PAHs, Zebra mussel, PAS-reaction, Catalase, Cholinesterase

Introduction

Cadmium (Cd, atomic number 48) is a toxic heavy metal, which is regularly released in the environment by volcanic eruptions, forest fires and anthropogenic activities such as agriculture, battery manufacturing, mining and smelting of metals, e.g. iron and nickel (Tabelin et al. 2018). Cd is carcinogenic, inhibits the DNA reparation and induces oxidative stress in various species (Maar et al. 2018). Furthermore, the elimination of Cd is very slow (the elimination half-life is up to 20 years) and depends on the source (soil, water, air, food) and the target organ (Keil et al. 2011).

Polyaromatic hydrocarbons (PAHs) are widespread organic contaminants, which are hyper-toxic and carcinogenic; over the last 30 years, they have originated mainly from anthropogenic processes in

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urbanised or industrialised regions such as vehicle exhausts and coal combustion (Kang et al. 2019). PAHs accumulate in the aquatic environment and are difficult to be biodegraded. Due to their low solubility and high hydrophobicity, PAHs are adsorbed in the aquatic ecosystem onto suspended particles and finally accumulate in sediments instead of dissolving in the water (Zhang et al. 2016). They have been shown to constitute a major threat to wildlife, including invertebrates such as mussels (Oliva et al. 2017, Waszak et al. 2019).

In the field of ecotoxicology, molluscs (in particular bivalves) are considered suitable organisms for monitoring purposes on account of their sessile status and filter-feeding behaviour (Widdows & Donkin 1992). This kind of monitoring was first applied in a marine environment using the blue mussel *Mytilus edulis* (Goldberg et al. 1978) but later in freshwater environments based on the zebra mussel *Dreissena polymorpha* (Bias & Karbe 1985, De Kock & Bowmer 1993).

Following our previous studies (Yancheva et al. 2017, 2018, 2019), we conducted this study in order to deepen our knowledge on the negative effects of trace metals and organic contaminants on zebra mussels. For this purpose, we studied for the first time the gill histological structure (PAS-reaction) and the enzymatic activity (CAT, ChE) in the digestive gland of zebra mussels exposed to Cd and PAHs for 96 h and 31 days.

Materials and Methods

Experimental exposure

The field collection and the experimental work (preparation of environmentally relevant concentrations of Cd and PAHs – a mixture of 16 different substances, based on the EU legislation, exposure period and measurements of basic physical properties of water – electrical conductivity, oxygen concentration, pH, temperature) were performed as explained in details in Yancheva et al. (2018, 2019).

Histochemical technique

The histochemical analysis was carried out in the laboratory of the Department of Developmental Biology, Section of Histology and Embryology, Faculty of Biology, Plovdiv University. Cryostat (Leica, CM 1520, Germany) was used to cut the samples. Multiple zebra mussel gill sections (6 µm) of each specimen were prepared according to a standard PAS methodology (McManus 1948) as previously described in Georgieva et al. (2013) and Yancheva et al. (2019) and studied by light microscope (Leica DM 2000 LED, Germany). The gill histochemical alterations of all specimens, including controls, were appraised individually and semi-quantitatively using the grading system of Bernet et al. (1999), which was adopted for the purposes of this study. A positive PAS reaction was presented in purple-magenta staining. The histochemical changes were evaluated and presented as an average value. Each grade represented specific histochemical characteristics and was categorised as follows: (0) – negative reaction of histochemical staining; (1) – very weak positive reaction of histochemical staining; (2) – weak positive reaction of histochemical staining; (3) – moderate positive reaction of histochemical staining; (4) – strong positive reaction of histochemical staining.

Biochemical technique

The biochemical analyses were carried out at the Technological Centre in Plovdiv University, Bulgaria. All the chemicals of analytical grade were purchased from Merck (Germany). The digestive glands (pooled wet mass of every three individuals) were rapidly thawed on ice and manually homogenised, using a Potter Elvehjem homogeniser fitted with a Teflon pestle (Thomas Scientific, USA) in chilled phosphate buffer (50 mM, 300 mM NaCl, pH 7.4). Homogenates were centrifuged at 9000 rpm for 15 min in a cooling centrifuge (MPW 351 R, Poland) at 4°C. Supernatant fractions were aliquoted, transferred in new Eppendorf tubes and stored at –80°C for enzyme assays. All biochemical assays were measured spectrophotometrically (Beckman Coulter Spectrophotometer DU 800, USA) at 25°C.

Catalase activity, cholinesterase activity and protein levels

The catalase (CAT EC 1.11.1.6) activity was measured by the decrease in absorbance at 240 nm by H_2O_2 decomposition according to Aebi (1984). The cholinesterase (ChE EC 3.1.1.8) activity was measured by the decrease in absorbance at 405 nm according to Burtis-Ashwood (1999). The total protein levels were measured using the Bradford (1976) method with Coomassie Brilliant Blue G-250 using bovine serum albumin as standard. The absorbance was detected at 595 nm and expressed as milligram protein per millilitre homogenate. The enzyme activity was expressed in international units per milligram of protein (U/mg protein).

Statistical analyses

The statistical analyses were conducted using the program Graph Pad Prism 7 for Windows (GraphPad Software, San Diego, CA, USA). The raw data of the basic water physical properties, histochemical scores and enzymatic activity results were tested for normal distribution with the D'Agostino-Pearson normality test. The differences between the variables were tested using Student's T-test with 95% confidence interval. The results were reported as average ± SD.

Results

Basic physical properties of water

The results for the measured basic physical properties during the short- and long-term experiment were already presented (see Yancheva et al. 2019). As discussed before, the values were close for the entire period of the experiment. Thus, we consider that the alterations in the gill structure and enzymatic activity in the digestive gland of zebra mussels were not due to changes in the abiotic factors.

Periodic-Schiff reaction (PAS)

The results of the histochemical study are presented in Tables 1 and 2, respectively for Cd – short-term experiment (96 hours) and long-term experiment (31 days), and PAHs – short-term experiment (96 hours) and long-term experiment (31 days). In the control group, we found a strongly positive response (Fig. 1), which was expressed in intense purple magenta staining of the cells as opposed to those treated with the tested toxicants for the exposure period.

In the mussels treated with Cd for 96 hours, we observed a decrease in the staining intensity and the amount of glycogen, with an increase in the applied concentrations. The strongest positive reaction of histochemical staining was observed in the control and the weakest in the group exposed to 4 μg Cd (Table 1). During the short-term exposure to PAHs, we also observed a decrease in the amount of glycogen, which was determined as moderate. In addition, the PAS-reaction was scored as a weak positive reaction of histochemical staining at all three PAHs concentrations (Table 2). Despite of the trend for Cd, which was dose-dependent, the PAS-reaction in the mussels treated with PAHs for 96 hours was kept constant (Fig. 1, Tables 1 and 2). In the Cd-exposed mussels, we again observed a general tendency of decreasing intensity of histochemical staining and the amount of glycogen, compared to an increase in the applied concentrations after 31 days. We found a similar tendency of decrease in the amount of glycogen relative to the exposure period, i.e. on the 31st day of the experiment we found a very weak positive reaction of histochemical staining as compared to the results from the 96th hour (Table 1 and Table 2). Furthermore, a related point to consider was that the amount of glycogen after exposure to Cd for 31 days was lower, but kept constant at all three tested Cd concentrations (Table 2). We observed a decrease in the amount of glycogen in the mussels treated with PAHs compared to the control group in both short-term and long-term experiment. Similarly to the results for the 96th hour, the degree of histochemical staining on day 31 remained constant at all tested concentrations of PAHs, but it was scored as very weak positive reaction (Table 2). From the obtained results, it could be stated that in the case of Cd, there was a more expressed negative effect on the studied parameter, since we found a less positive PAS response for the exposure period (96 hours and 31 days) and the applied concentrations.

Catalase and cholinesterase activity

At the 24th hour, the highest CAT activity of 26 U/mg protein was observed at the highest Cd concentration and the lowest of 19 U/mg protein – in the control individuals (Table 3), indicating the negative effect of the studied metal. At the 48th hour of the experiment, we observed higher values of the CAT enzyme activity for each of the tested concentrations. The tendency of the highest CAT activity at 4 μg Cd – 29.5 U/mg protein remained, which confirms the toxic effect of the applied heavy metal. It can be seen from Table 3 that with increasing the exposure period, the enzyme activity in the mussel tissues increased significantly after 72 hours of exposure. At the 96th hour, the CAT enzyme activity values were highest for each of the tested Cd concentrations compared to the 24th, 48th and 72nd hour. Therefore, we found that the rate of changes in enzyme activity was directly proportional to the applied concentrations of Cd, as well as the exposure time (Table 3). The results for the enzyme activity of CAT on day 31 showed the same tendency, i.e. an increase in the activity resulting from the toxic action of Cd and the activation of the antioxidant protection of the organism. Again, we found that the rate of changes in the CAT activity was dependent on the toxicant concentrations, as well as the exposure time. In addition, the CAT activity at day 31 was higher than the one measured during the short-term experiment. The t-test showed significant differences in the CAT activity treated with all tested heavy metal concentrations between the 31st day and the 24th hour, similarly to the results on the activity between the 24th and 96th hours.

At the 24th hour in the PAHs-exposed mussels, we observed the highest enzyme activity of 27.75 U/mg protein at 3 μg of PAHs (Table 4). As the exposure period increased, we found that the CAT ac-

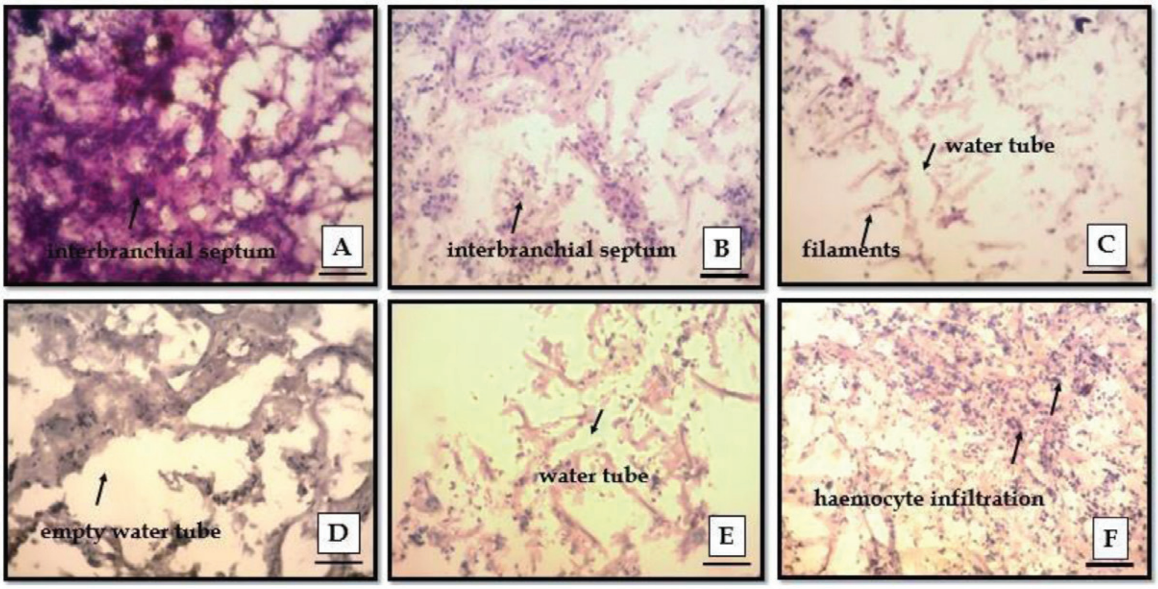

Fig. 1. PAS-reaction in the gills of the zebra mussel exposed to treatment for 96 hours. A. Control. B. Cd, 2 µg. C. Cd, 4 µg. D. PAHs, 1 µg. E. PAHs, 2 µg. F. PAHs, 3 µg.

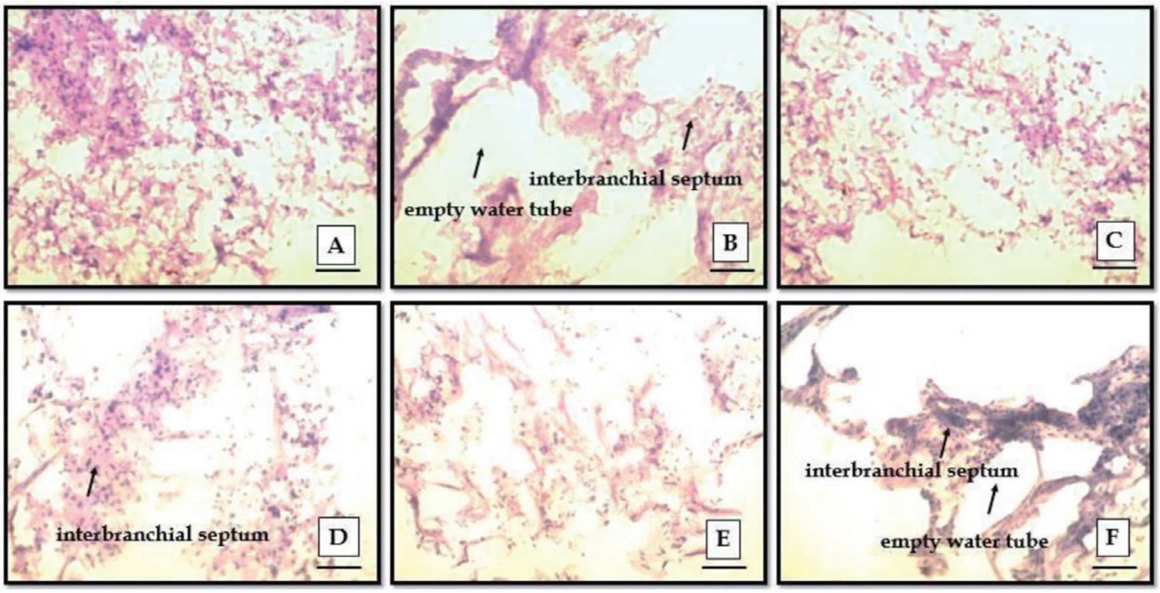

Fig. 2. PAS-reaction in the gills of the zebra mussel exposed to treatment for 31 days. A. Cd, 1 µg. B. Cd, 2 µg. C. Cd, 4 µg. D. PAHs, 1 µg. E. PAHs, 2 µg. F. PAHs, 3 µg.

Table 1. Intensity of PAS-reaction in the gills of the zebra mussel after treatment with Cd for 96 hours and 31 days.

Concentrations of Cd µg, 96 hours				
Intensity of PAS-reaction	Control	1 µg	2 µg	4 µg
	3	2	2	1
Concentrations of Cd µg, 31 days				
Intensity of PAS-reaction	Control	1 µg	2 µg	4 µg
	3	1	1	1

Table 2. Intensity of PAS-reaction in the gills of the zebra mussel after treatment with PAHs for 96 hours and 31 days.

Concentrations of PAHs µg, 96 hours				
Intensity of PAS-reaction	Control	1 µg	2 µg	3 µg
	3	2	2	2
Concentrations of PAHs µg, 31 days				
Intensity of PAS-reaction	Control	1 µg	2 µg	3 µg
	3	1	1	1

Table 3. Catalase (CAT) activity in the digestive gland of the zebra mussel after treatment with Cd for 96 hours and 31 days.

Cd concentrations, 24 hours				
CAT (U/mg protein)	Control	1 µg	2 µg	4 µg
	19±1	23±1.5	25.5±2	26±0.5
Cd concentrations, 48 hours				
CAT (U/mg protein)	Control	1 µg	2 µg	4 µg
	19.5±0.5	26.5±1	29±1	29.5±0.3
Cd concentrations, 72 hours				
CAT (U/mg protein)	Control	1 µg	2 µg	4 µg
	19.5±1.5	32±2	34±0.5	38±2
Cd concentrations, 96 hours				
CAT (U/mg protein)	Control	1 µg	2 µg	4 µg
	19±0.5	38±2	41±1.5	44.5±2.5
Cd concentrations, 31 days				
CAT (U/mg protein)	Control	1 µg	2 µg	4 µg
	21±0.5	41±2	45±1.5	47.5±0.5

Table 4. Catalase (CAT) activity in the digestive gland of the zebra mussel after treatment with PAHs for 96 hours and 31 days.

PAHs concentrations, 24 hours				
CAT (U/mg protein)	Control	1 µg	2 µg	3 µg
	19±0.5	21±1	25±1.3	27.75±0.5
PAHs concentrations, 48 hours				
CAT (U/mg protein)	Control	1 µg	2 µg	3 µg
	19.5±1	25.5±2	27±2.5	31±1.5
PAHs concentrations, 72 hours				
CAT (U/mg protein)	Control	1 µg	2 µg	4 µg
	19.5±0.05	31.5±0.5	37±1	39.5±1.5
PAHs concentrations, 96 hours				
CAT (U/mg protein)	Control	1 µg	2 µg	3 µg
	19.5±2	31.5±1.3	37±0.5	39.5±2
PAHs concentrations, 31 days				
CAT (U/mg protein)	Control	1 µg	2 µg	4 µg
	21±1	41±1.5	45±1.3	47.5±2

Table 5. Cholinesterase (ChE) activity in the digestive gland of the zebra mussel after treatment with Cd for 96 hours and 31 days.

Cd concentrations, 24 hours				
ChE (U/mg protein)	Control	1 µg	2 µg	4 µg
	63±1	35±1	33±1.5	29±0.5
Cd concentrations µg/l, 48 hours				
ChE (U/mg protein)	Control	1 µg	2 µg	4 µg
	45±0.3	17±0.05	15±0.1	10±0.03
Cd concentrations, 72 hours				
ChE (U/mg protein)	Control	1 µg	2 µg	4 µg
	55±1.5	16.5±0.1	13.5±0.02	9.3±0.5
Cd concentrations, 96 hours				
ChE (U/mg protein)	Control	1µg	2 µg	4 µg
	35±0.5	4.5±0.1	3±0.03	2.9±0.05
Cd concentrations, 31 days				
ChE (U/mg protein)	Control	1 µg	2 µg	4 µg
	38.5±0.5	34±2.5	29±1.3	19±0.5

Table 6. Cholinesterase activity (ChE) in the digestive gland of the zebra mussel after treatment with PAHs for 96 hours and 31 days.

PAHs concentrations, 24 hours				
ChE (U/mg protein)	Control	1 µg	2 µg	3 µg
	52±0.5	78±2	53±1.5	49±0.5
PAHs concentrations, 48 hours				
ChE (U/mg protein)	Control	1 µg	2 µg	3 µg
	40±2	48±2.3	32±1.5	15±0.5
PAH concentrations, 72 hours				
ChE (U/mg protein)	Control	1 µg	2 µg	4 µg
	53±0.3	59±2	39±0.5	13±1.2
PAHs concentrations, 96 hours				
ChE (U/mg protein)	Control	1 µg	2 µg	3 µg
	33±1.5	27±0.3	11±0.05	10±0.5
PAHs concentrations, 31 days				
ChE (U/mg protein)	Control	1 µg	2 µg	4 µg
	44.5±3	70±1.5	45±0.5	24±2.5

tivity also increased similarly to the results for Cd. After 48 hours, in the treated with PAHs mussels the enzyme activity was higher (Table 4). Furthermore, the highest value of 31 U/mg protein was reported for 3 µg PAHs. At the 72nd hour, the CAT activity values were higher than those measured at the 24th and 48th hour. This increase in the enzyme indicated that the activity was negatively affected by the exposure to PAHs. It is worth mentioning that the results for the CAT activity at the 96th hour were identical to those at the 72nd hour. The highest levels of CAT activity in the digestive gland of the zebra mussel were reported after 31 days of exposure to PAHs (Table 4). The results were higher than those during the short-term experiment (24, 48, 72 and 96 hours). Therefore, for PAHs, similarly to Cd, we confirmed that the CAT activity depended on the toxicant concentration and duration of exposure.

The CAT activity generally increased in proportion to the increase in the concentration of both toxicants. The enzyme values were similar for the treated with Cd and PAHs mussels, but it was higher at all Cd concentrations for the entire exposure period compared to PAHs ($P > 0.05$).

At the 24th hour, we observed the highest ChE enzyme activity in the digestive gland of the zebra mussel at the lowest Cd concentration, and respectively, the lowest activity at the highest Cd concentration (4 µg Cd; Table 5). At the 48th hour at each of the tested Cd concentrations, the ChE values were lower compared to values at the 24th hour, maintaining the tendency of enzyme inhibition at the highest toxicant concentration. It is clear from Table 5 that by the 72nd hour the ChE activity decreased at each of the heavy metal concentrations applied, indicating the negative effect of Cd on this enzyme. At the end of the short-term experiment (96 hours), the ChE activity was significantly reduced, respectively at 1, 2 and 4 µg of Cd, indicating an inhibition of the enzyme, being proportional to the duration of exposure. On day 31, we found that the ChE activity was higher than that one measured at the 48th, 72nd and 96th hour during the short-term experiment. The results of the long-term experiment were similar to those reported at the 24th hour (beginning of the experiment). This was the difference between the values of the CAT and ChE enzymes tested at day 31, considering that the reason for the activity of ChE at day 31, being close in value to that at day 24, was likely to be related to adaptation mechanisms of the zebra mussel At the 24th hour the levels of ChE enzyme activity in the digestive gland of the zebra mussel were reduced with increasing the PAHs concentrations (Table 4). In addition, they were higher than those measured for Cd at the 24th hour ($P < 0.05$). The values for ChE activity at the 48th hour were lower for each of the tested concentrations compared to the previous hour ($P < 0.05$). At the 72nd hour we found a slight increase in the ChE activity in the mussels treated with 1 µg and 2 µg PAHs compared to the 48th hour, but the differences were not statistically significant ($P > 0.05$). However, the tendency to inhibit the enzyme activity at the highest toxicant concentration remained. The lowest enzyme activity during the short-term experiment was reported at the 96th hour. Statistically significant differences were demonstrated for the ChE values treated with 1, 2 and 3 µg PAHs between the 24th and 96th hour. On day 31, we found an increase in the ChE activity compared to the results for 48, 72 and 96 hours. Moreover, the enzyme activity was again the lowest at the highest PAHs concentration. We also found that this trend on day 31 was similar to that of Cd, but the ChE values were lower for the mussels treated with PAHs. This is considered to indicate a more expressed negative effect of Cd compared to the organic contaminant on the activity of ChE.

Discussion

Periodic acid-Schiff reaction (PAS)

Based on the collected literature on histochemical changes triggered by different toxicants, we can state that the presented histochemical method mainly concerns vertebrates, such as fish and their organs, such as the liver, and to a smaller extent invertebrates, such as mussels and their organs, such as the gills (Drastichová et al. 2005, Wolf & Wolf 2005, Figueiredo-Fernandes et al. 2006, El-Serafy 2009, Singh 2014). Furthermore, the glycogen content in the liver is much greater (about 250–300-fold) than that in the gills, reasonably reflecting the different partitions of the two organs in the energy supply. Generally, the mechanisms of glycogen synthesis and degradation are studied in mammal tissues, including in liver, muscles and other organs (Smythe & Cohen 1991, Bollen et al. 1998). These mechanisms seem to be similar in the gills, which are an energy-consuming organ. Moreover, the mussel gills are attractive models for ecotoxicological studies because the gills are the first uptake site for many toxicants that are present in the aquatic environment and thus, are often affected by exposure to pollutants (Gómez-Mendikute et al. 2005). In addition, the gills have multiple functions including gas exchange, acid–base balance and ionic/osmotic regulation (Evans et al. 2005). In terms of the mechanisms of ionic/osmotic regulation, mitochondrion-rich (MR) cells in gill epithelia are the main sites responsible for the active ion transport functions, which are conducted by various ion transporters and enzymes. These operations are highly energyconsumptive processes (Hirose et al. 2003).

Overall, under the applied Cd and PAHs concentrations, the observed decrease in glycogen in the gills of the zebra mussel suggested an accelerated process of glycogenolysis leading to the production of glucose-6-phosphate and, probably, a release of glucose into the blood. This is another indicator for stress, triggering the depletion of the organism's energy reserves due to the applied toxicants and exposure period. Similarly to Shrivastava (2007), we consider that the changes in glycogen content may indicate negative changes in the carbohydrate metabolism as a result of short- or long-term aquatic pollution. Our results are consistent with the observations of other authors. For example, Mantecca et al. (2006) found that the PAS-activity showed a progressive reduction from the control to the 7th and 14th day groups, the latter being the most affected after paraquat exposure. In addition, in the study of Rajalekshmi & Mohandas (1993), the gill glycogen levels were depleted in the Cu-exposed mussels when compared with the control at all time-periods in all exposures, while in those exposed to mercury (Hg), the depletion was more pronounced at later periods of exposure. These results are in agreement with our findings, i.e. in the mussels exposed to Cd the reduction in the glycogen levels was more evident at the highest concentration. Rajalekshmi & Mohandas (1993) also studied the hepatopancreas, which showed a general trend of depleted levels of glycogen activity in all the heavy metal exposures. We agree with the authors who indicated that hypoxic conditions prevailing in mussels exposed to heavy metals might have resulted in enhanced breakdown of glycogen. This is further suggested by the weakened magenta staining of gills from mussels maintained for a prolonged period of time (31 days), suggesting a consumption of intracellular carbohydrate reserves. In a state of oxidative stress following free radical release as a result of contamination, key survival reactions observed in the organism include a decrease in energy reserves and an increase in antioxidant protection in the gills (Bickler & Buck 2007). In addition, an important key response of the organism to oxidative stress is the regulation of energy metabolism via the pathway of glycolysis and the release of glucose as an easily accessible energy resource (Gruetter 2003). According to Tseng et al. (2007) and Polakof et al. (2012), the decrease in glycogen content is due to the impact of toxicants in the aquatic environment and the induced oxidative stress that triggers need of glucose as an energy source. Moreover, glucose plays a central role in providing energy for metabolism. It is primarily stored in animal tissues as a long branching high-molecular-mass polysaccharide called glycogen (Roach et al. 1998). Glycogen metabolism is the principal energy source in both vertebrates and invertebrates, especially during environmental fluctuations (Karlsson 1979). This observation is also discussed and confirmed by Huang et al. (2015), according to whom glycogen is the major source of energy in the gills when they are exposed to various negative environmental factors.

Catalase and cholinesterase activity

The CAT activity generally increased in proportion to the increase in the concentration of both toxicants. The enzyme values were similar for the treated with Cd and PAHs mussels, but it was higher at all Cd concentrations for the entire exposure period compared to PAHs ($P > 0.05$). According to Clasen et al. (2018), CAT is a major antioxidant enzyme found in almost all aerobic organisms. The enzyme activity varies across tissues, being higher in organs with higher oxidative potential. Overall, increasing the levels of the CAT enzyme under the action of toxicants during short-term and chronic exposures is likely to demonstrate the presence of oxidative stress in the organism and could be a response to increased generation of oxygen radicals. We found such an increase in our study and our results are in line with those of Nunes et al. (2018) who have worked with various aquatic organisms, including mussels. In conclusion, we, similarly to Jourmi et al. (2015), Chen et al. (2018), Liang et al. (2019) and Brand et al. (2019), consider that a change in the activity of antioxidant enzymes (e.g. CAT) can be considered a biomarker for the negative effects of inorganic and organic toxicants. In the present study, this is also probably a result of the bioaccumulation of both toxicants found in our previous study (Yancheva et al. 2019) and the attempts of zebra mussel to prevent the negative effects of Cd and PAH by activating its antioxidant protection.

Overall, we monitored a decrease in the ChE activity compared to the control, confirming inhibition of the enzyme in zebra mussels exposed to both toxicants. We found that the heavy metal affects more negatively ChE than the organic pollutant ($P > 0.05$), which is in line with our previous results on the effects of Cd and PAHs on the lysosomal membrane stability of zebra mussels (Yancheva et al. 2018). Changes in the ChE activity and choline content indicate inhibition of the enzyme activity, followed by a concomitant increase in the acetylcholine content in all tissues, mainly when exposed to organic pollutants, such as organophosphorus pesticides (Venkataramudu et al. 2008). Our results correspond with those of other authors (see Choi et al. 2011 and Sabullah et al. 2014) who determined changes in the ChE enzymatic activity upon exposure to heavy metals (Cu, Cr, Hg) in the liver of the cyprinid fish *Puntius javanicus* (Aurivillius, 1916) and the Japanese mussel *Ruditapes philippinarum* (Adams & Reeve, 1850) after exposure to pesticides. In addition, the ChE activity was significantly reduced after 9 days of exposure and, similarly to our long-term experiment, the results were higher on day 15 compared to the start of the experiment, indicating possible adaptive mechanisms of the organisms in a contaminated aquatic environment. According to inhibition of ChE in the adductor muscle of the Antarctic scallop *Adamussium colbecki* (Smith, 1902) was observed when exposed to Cd and organophosphorus pesticides. Both Bonacci et al. (2006) and our results demonstrate that the enzyme activity was higher in the group treated with organic toxicants compared to that of Cd-exposed mussels, which in turn confirms the higher toxicity of the tested heavy metal. Thus, our results suggest that the acetylcholinesterase-like enzyme in the digestive gland of zebra mussels has a lower sensitivity to PAHs and is more inhibited by exposure to Cd.

Conclusions

Overall, we could conclude that at the higher concentrations of Cd and PAHs there was fading in the staining (PAS-reaction) compared to the control, which indicated a depletion of glycogen amount. Such fading was observed in both the short-term and the long-term experiments but the negative effects of Cd were more expressed than that of PAHs on the gill histochemical structure of the zebra mussel. Furthermore, the CAT activity increased and that of ChE decreased compared to the control in the Cd and PAHs-exposed zebra mussels. Such enzymatic changes were measured in both the short-term and long-term experiments but the results showed that the Cd toxicity was more severe compared to the PAHs'. Last but not least, we confirmed that such histochemical and biochemical alterations could be used as biomarkers in environmental monitoring and zebra mussel could be used as a successful freshwater bioindicator.

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