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Bioaccumulation and biochemical effects of diesel on Mediterranean mussel *Mytilus galloprovincialis* Lamarck, 1819

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Petroleum hydrocarbons' pollution of the aquatic environment is a serious ecological problem. This requires a detailed study of their accumulation in the tissues of aquatic organisms and identification of informative biomarkers to assess the animals' functional state under oil pollution. The aim of the work was to study diesel (DF) effect on the bioaccumulation, prooxidant-antioxidant system (level of oxidized proteins and lipid peroxidation, antioxidant enzyme (superoxide dismutase (SOD), catalase (CAT) activities) and aminotransferase (aspartate aminotransferase, alanine aminotransferase) activities in the *Mytilus galloprovincialis* hepatopancreas after five days' experiment. It was shown that hydrocarbon content in mussels' hepatopancreas in the control, I (0.5 mg/L DF) and II (1 mg/L DF) experimental groups were 0.01, 0.10 and 1.43 mg/g dry weight, respectively. The level of lipid peroxidation in I and II experimental groups was significantly higher (+59 % and +95 %, respectively) as compared to the control ($p < 0.05$). The levels of neutral aldehydes from both experimental groups and basic aldehydes from II group significantly increased (+59 %, +47 %, 52 %, respectively) as compared to the control ($p < 0.05$). SOD activity in II group mussels was significantly higher compared to the control (+30 %) and I group (+45 %) ($p < 0.05$). Contrastingly, CAT activity in II group mollusks was significantly lower as compared to the control (–125 %) and I group (–114 %) ($p < 0.05$). Aminotransferase activities did not differ in the control and the experimental groups. Thus, it was determined, that parameters of prooxidant-antioxidant system demonstrate high sensitivity to DF and can be used as biomarkers for assessing the functional state of mollusks in oil polluted environment.

Keywords: diesel, mussel *Mytilus galloprovincialis*, hepatopancreas, parameters of prooxidant-antioxidant system, activity of aminotransferases.

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Биоаккумуляция и биохимические эффекты дизельного топлива на организм средиземноморской мидии *Mytilus galloprovincialis* Lamarck, 1819

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Загрязнение водоёмов нефтяными углеводородами является серьёзной экологической проблемой и требует детального изучения вопросов их накопления в тканях гидробионтов и выявления биомаркеров, информативных для оценки функционального состояния животных в условиях нефтяного загрязнения. Цель работы – изучение влияния дизельного топлива (ДТ) в концентрациях 0,5 и 1 мг/л на биоаккумуляцию, параметры прооксидантно-антиоксидантной системы (уровень окислительной модификации белков и перекисного окисления липидов,

активность антиоксидантных ферментов (супероксиддисмутазы (СОД), каталазы (КАТ)) и активности аминотрансфераз (аспартатаминотрансферазы, аланинаминотрансферазы) в гепатопанкреасе мидии *Mytilus galloprovincialis* после пяти дней экспозиции. Результаты исследований позволили установить, что концентрация углеводородов в гепатопанкреасе моллюсков из контроля, I и II экспериментальных групп составляла 0,01, 0,10 и 1,43 мг/г сухой массы, соответственно. Уровень перекисного окисления липидов был достоверно выше в гепатопанкреасе мидий при концентрациях ДТ 0,5 и 1 мг/л (+59 %, +95 %, соответственно) по сравнению с контрольной группой ($p < 0,05$). Уровень альдегидов нейтрального характера в обеих экспериментальных группах и альдегидов основного характера во II группе был достоверно выше (+59 %, +47 %, 52 %, соответственно) значений соответствующих показателей в контроле ($p < 0,05$). Активность СОД в гепатопанкреасе мидий из II группы была значительно выше по сравнению с контролем (+30 %) и I группой (+45 %) ($p < 0,05$). В то же время активность КАТ была достоверно ниже у моллюсков из II группы по сравнению с аналогичным параметром у особей из контроля (–125 %) и I группы (–114 %) ($p < 0,05$). Активность аминотрансфераз не отличалась в контрольной и опытных группах. Таким образом, установлена высокая чувствительность показателей прооксидантно-антиоксидантной системы гепатопанкреаса *M. galloprovincialis* к нефтяным углеводородам, что позволяет рекомендовать их в качестве релевантных биомаркеров для оценки функционального состояния моллюсков в условиях нефтяного загрязнения.

Ключевые слова: дизельное топливо, мидия *Mytilus galloprovincialis*, гепатопанкреас, параметры прооксидантно-антиоксидантной системы, активность аминотрансфераз.

Petroleum hydrocarbons (PHs) pollution of the aquatic environment poses a serious ecological problem [1–3]. According to various estimates, approximately from 0.5 to 11 million tons of oil and oil products flow into the World Ocean every year. Oil and its products enter the marine environment through natural seepage from oil reservoirs, extraction, transportation and accidents. They can preserve in the marine environment from a few days to 10 years or more, negatively affecting hydrobionts [4, 5].

The problem of oil pollution in Russia has become particularly relevant and significant after the accident of two tankers in the Kerch Strait in December 2024. According to emergency services, the total volume of oil products spilled was approximately 4000 tons. This accident defined the necessity for long-term regular monitoring of marine coastal ecosystems and studies of the biota's response to oil pollution in the region [6].

Almost all PHs are toxic to aquatic organisms, and some of them can accumulate in tissues and organs and be transmitted through food chains. The level of hydrobionts' intoxication by PHs depends on many factors: the concentration and duration of exposure, environmental temperature, physiological peculiarities of the organisms such as size, age, sex. In this regard a wide range of reactions can be identified at different levels of biological organization: from molecular to population and even ecosystem level [4, 7–13].

Therefore, it is significantly important to study PHs effect on molecular (biochemical) markers of hydrobionts. They reflect the effects of major metabolic processes at the cellular level and are considered to be "early warning" signals that can potentially detect impacts on target biota before they are observed at the population, community, or ecosystem level. Therefore, the use of biomarkers can provide crucial evidence for understanding the relationship between stressors and impacts on coastal resources, as well as for preventing the detrimental effects of pollution on ecosystem structure and functioning. In this regard, the most informative molecular biomarkers are the indicators of the oxidative stress and antioxidant protection, biotransformation enzyme activities as well as the functional state parameters [7, 9, 10, 13–15].

To make the assessment of contaminated marine environment, the bivalves of genus *Mytilus* are most often applied. Their choice is due to abundance, wide geographical distribution, sedentary habits, high survival rate, and toxin resistance to many types of contaminants. Thus, *Mytilus* parameters are commonly used as biomarkers to estimate marine environment polluted with different chemicals [9, 10, 15–18].

In water bodies hydrobionts are generally exposed to multicomponents' PHs including various types of light and heavy fuel. Toxicity effects of these mixtures are usually higher as compared to the individual PHs. Hence, studying the PHs' biological effects in the experiments is of utmost importance for extrapolating the results obtained to the natural habitats of hydrobionts to assess their status as well as the environment state [8–11, 15].

Thus, the aim of the work was to study the bioaccumulation of diesel in hepatopancreas of *Mytilus galloprovincialis* and its effects on some biochemical parameters to determine the mechanisms of the metabolism rearrangement, as well as the adaptive reactions when exposed to the pollutants.

Material and methods of research

Sampling. Mollusks were sampled between the spawning seasons from the collectors in the

mussel farm in Karantinnaya Bay (the Black Sea, Sevastopol) in July.

Experimental design. One-sized mussels with anaverage shell length of 55.6 ± 0.62 mm were selected for the experiment. Mollusks were placed in glass tanks to acclimatize to the laboratory conditions for 7 days before the experiment at 20–22 °C. The mussels were not fed to avoid the specific effect of food [9]. Diesel concentrations at which DF affect the mussels in tanks were 0.5 mg/L (10 MPC) (I experimental group) and 1 mg/L (20 MPC) (II experimental group). The control samples were kept in clean sea water. Water was changed every 24 h during the exposure. After five days' exposure the samples were taken for chemical and biochemical analyses. Fifteen individuals from each group were taken for biochemical analyses, and 3 samples from each treatment were used for chemical analysis.

To conduct the experiment, DF "Summer", commonly used for marine transport in Sevastopol coastal waters, was added to glass tanks with filtrated marine water. DF concentrations were chosen because in summer in some Sevastopol areas DF contents might reach 0.5 mg/L and was registered even higher [19].

Chemical analysis. We assess the PHs concentration in mussels' hepatopancreas by gas-chromatographic method. All measurements were carried out on the "Kristall 5000.2" (Russia) gas chromatograph with a flame ionization detector in the Scientific and Educational center for collective use "Spectrometry and chromatography" of IBSS. The samples were prepared in accordance with [20]. Hepatopancreas of 3 individuals from each group were homogenized with sodium sulfate and extracted three times with hexane. To separate polar compounds the extracts were passed through Al_2O_3-filled glass column. The samples were concentrated to 1 mL at room temperature. Quantitative determination of the total PHs' content was carried out using the absolute calibration FID method with PHs' mixture prepared by the gravimetric method. To separate the PHs' mixture, standard sample ASTM D2887 Reference Gas Oil (SUPELCO, USA) was used. N-alkanes content was determined by using standard sample of paraffin PHs in hexane with 200 μg/mL explosive concentration of each component (SUPELCO, USA). The PHs and n-alkanes concentrations were calculated by using absolute calibration and percentage normalization. To identify petroleum and biogenic hydrocarbons, the diagnostic index (carbon preference index (CPI)) was applied. For allochthonous biogenic hydrocarbons CPI is more than 1 (CPI > 1). For fossil organic matter, as it undergoes catagenetic transformation, the CPI decreases to 1 and below (CPI ≤ 1) [21].

Biochemical analysis. Since mussel hepatopancreas is the main organ of metabolism and detoxification of a wide range of toxins, it was used for the biochemical analysis. The hepatopancreas of 15 individuals from each group was homogenized and centrifuged at 10 000 g for 15 min at 4 °C in a refrigerated centrifuge MPW-352 (MPW Med. Instruments, Poland). The supernatant obtained by centrifugation was used to determine biochemical parameters.

Superoxide dismutase (SOD) activity was assayed in the nitroblue tetrasolium-phenazine methosulfate – NADH system [22]. Catalase (CAT) activity was measured based on the reaction of interaction between hydroperoxide and molybdite ammonium [23].

The thiobarbituric acid reactive substances (TBARS) concentration was analyzed by the reaction with thiobarbituric acid [24]. The level of oxidized proteins (neutral aldehydes and ketones, basic aldehydes and ketones) was analyzed through the reaction between oxidized protein amino-acid residues and 2,4-dinitrophenylhydrazine [25].

Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities were analyzed through the reaction of oxaloacetate and pyruvate, respectively, with 2,4-dinitrophenylhydrazine [26].

The biochemical parameters were calculated per mg protein. Total soluble protein concentration was quantified by biuret method [27].

Statistical analysis. The results were also processed statistically. Mean values ±SEM (standard error of the mean) were established. The significance of differences between the samples was assessed using Mann-Whitney U-test. The difference was found great at the significance level $p < 0.05$.

Results and discussion

Survivability of mussels. During the experiment mussels demonstrated high resistance while exposed to DF. No mortality was observed among the control and the exposed mussels.

Bioaccumulation. Hydrocarbons' contents in the control, I and II experimental groups were 0.01, 0.10 and 1.43 mg/g dry weight, respectively. The CPI diagnostic index in the control, I and II experimental groups was 1.83, 0.75, 0.78, respectively (Fig. 1).

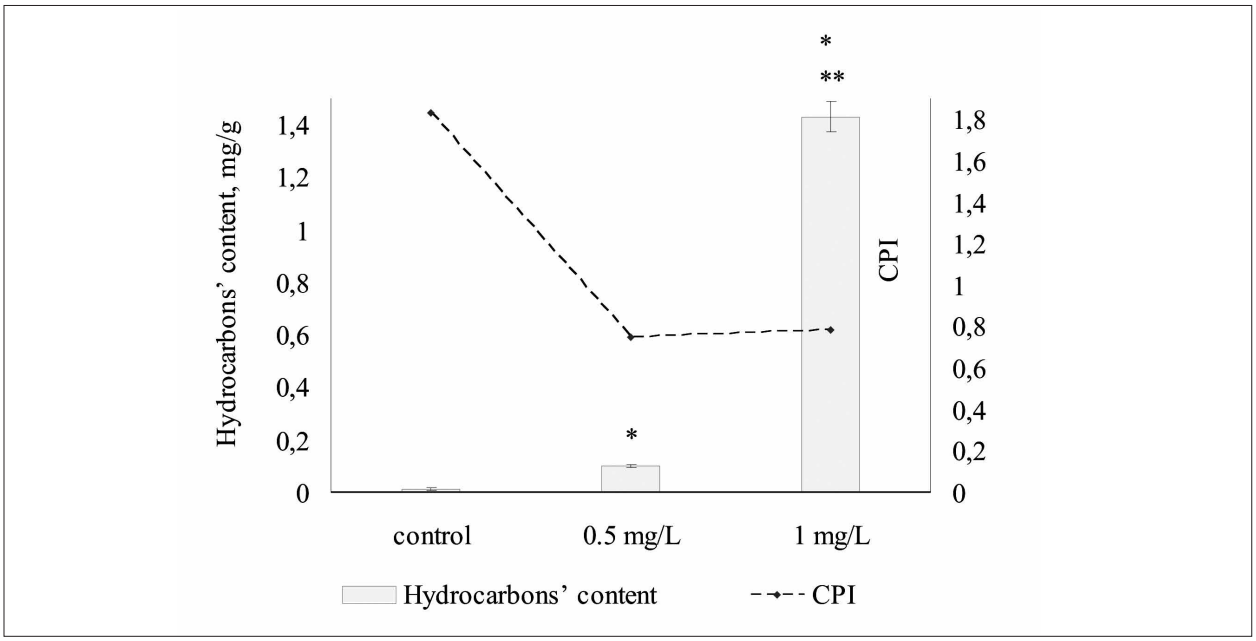

Fig. 1. Hydrocarbons' content in *Mytilus galloprovincialis* hepatopancreas (mg/g dry weight) and CPI index: CPI – carbon preference index; * and ** – statistically significant differences ($p < 0.05$) as compared to the control and to the I experimental group (0.5 mg/L), respectively

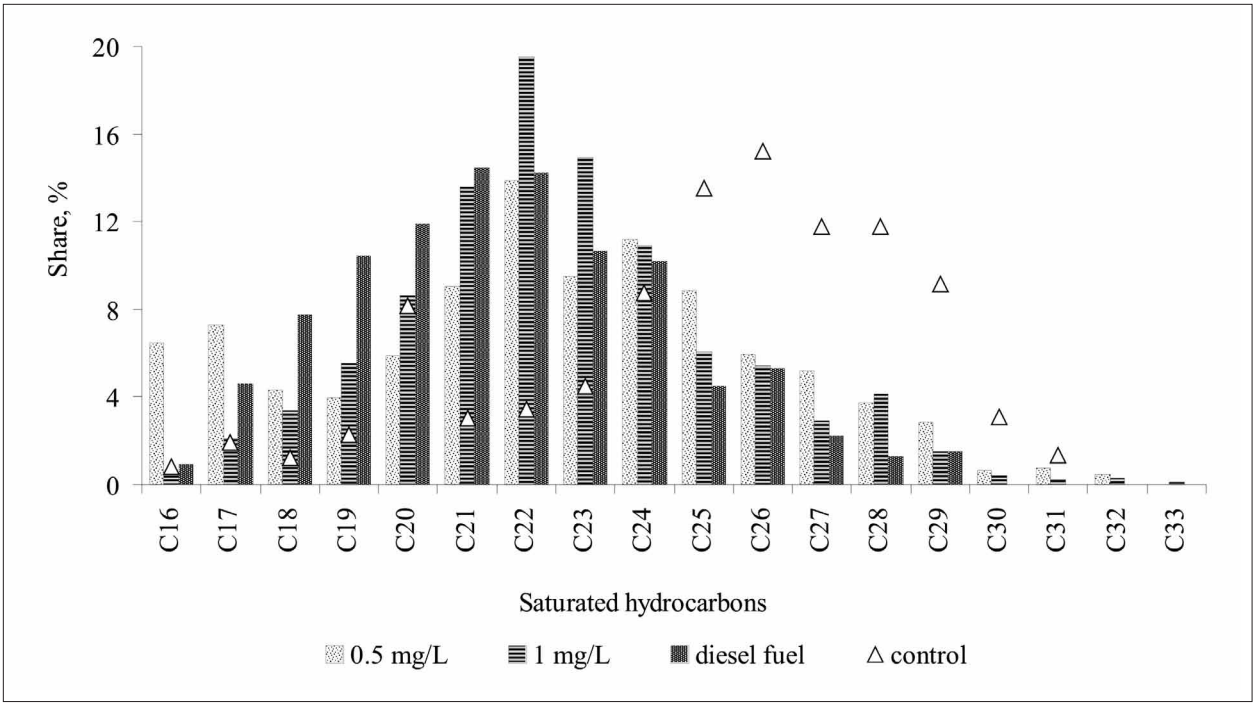

Fig. 2. Composition of n-alkanes in hepatopancreas of *M. galloprovincialis* exposed to diesel

As a result of the chromatographic analysis, the differences in the quantitative content and the qualitative composition of hydrocarbons in the hepatopancreas of mussels from the control and two experimental groups were revealed. In the control group C24-C29 n-alkanes dominated, while in both experimental groups C19-C24 n-alkanes prevailed. C19-C24 n-alkanes predominance in the hepatopancreas of mussels from the experimental groups was found the same as it was in the diesel composition. It indicated PHs accumulation in mollusks during the filtration process (Fig. 2). This fact is confirmed by the diagnostic index values (CPI < 1) in the I (CPI = 0.75) and the II (CPI = 0.78) experimental groups (Fig. 1).

It should be noted that mollusks from the II group have a significant accumulation of hydrocarbons compared to the I group (more than 14 fold). This may be caused by suppression of filtration activity, disruption of biotransformation and elimination of pollutants under high PHs concentrations in water.

Biochemical response. Diesel changed biochemical parameters in the mussel hepatopancreas. TBARS contents at 0.5 and 1 mg/L were significantly higher (+59 %, +95 %, respectively) as compared to the control ($p < 0.05$) (Fig. 3). The levels of neutral aldehydes (D356) in the hepatopancreas from both experimental groups and basic aldehydes (D430) in specimens from the II group significantly increased (+59 %, +47 %, 52 %, respectively) as compared to the control ($p < 0.05$). No significant differences in neutral (D370) and basic ketone (D530) levels between the control and the experimental groups were found (Fig. 3). SOD activity in mussels from the II group was significantly higher compared to the specimens from the control (+30 %) and the I group (+45 %) ($p < 0.05$). Contrastingly, CAT activity in mollusks from the II group was significantly lower as compared to the control (–125 %) and the I group (–114 %) ($p < 0.05$) (Fig. 3). However, aminotransferase (ALT and AST) activities in the hepatopancreas did not differ in the control and the experimental groups (Fig. 3).

Thus, as seen, DF affected the parameters of prooxidant-antioxidant system in the mussel hepatopancreas. However, more significant changes were detected at 1 mg/L concentration due to high level of PHs accumulation.

Prooxidant-antioxidant system (PAS) parameters are widely used as molecular biomarkers in ecotoxicological studies to assess the state of hydrobionts and quality of aquatic environment. A shift in the prooxidant-antioxidant balance towards an increase in free radical processes causes oxidative stress leading to various pathological conditions and diseases. In particular, lipid peroxidation and protein oxidation are the most significant causes of pathologies. Hence, the universal indicator of oxidative stress is an increase in levels of lipid peroxidation products and oxidized proteins in hydrobionts' tissues. Low-molecular-weight parameters and antioxidant enzyme activities are the sensitive indicators of the organisms' state as well as their habitat. Their changes in hydrobionts affected by the anthropogenic factors are registered in many works [8, 11, 14–17, 27, 28].

The increase in the TBARS content and the levels of neutral (D356) and basic aldehydes (D430) in the hepatopancreas of mollusks exposed to DF demonstrated the oxidative stress development due to intensification of lipid peroxidation and protein oxidation. At the same time, an increase in SOD activity in the II group may indicate the compensatory adaptive response to high toxicant concentrations in order to decrease the intensity of free radical oxidation. However, CAT values were found decreased in the hepatopancreas of the II group against the background of increase in SOD activity. This fact may indicate inhibition of the enzyme activity due to high levels of PHs' accumulation, as well as imbalance in the SOD and CAT coordinated work.

CAT and SOD is presented in a functional complex in cells. The joint work of these enzymes reliably protects from the toxic effect of superoxide anion radical (O_2^-) and hydrogen peroxide (H_2O_2) high concentrations. However, under stress factors, including oil pollution, reactive oxygen species are synthesized in large concentrations which leads to the inhibition of antioxidant enzymes, an imbalance in the work of enzymatic complexes, and the oxidation of proteins and lipids. Despite its high specificity SOD can interact with H_2O_2 and acts as a prooxidant, initiating the synthesis of O_2^- and hydroxyl radicals ($OH^\bullet$) when exposed to adverse factors. In this case O_2^- is capable to inhibit CAT, while $OH^\bullet$ initiate the oxidation of proteins and lipids [14, 28, 29].

Available literature data indicate that anthropogenic pollutants under natural and experimental conditions cause changes in the PAS parameters of hydrobionts. At the same time, the PAS reactions are not always clearly expressed and have the same direction. Their vector largely depends on the concentration and characteristics of the acting factor, exposure time, as well as biological features, nutritive status and physiological state of the organism [8, 11, 14, 15, 17, 18, 29]. For example, changes in PAS parameters in the hepatopancreas of Antarctic limpet *Nacella concinna* exposed to DF in concentrations of 0.05 % and 0.1 % were described. SOD, CAT, glutathione-S-transferase and glutathione peroxidase (GPx) activities, as well as lipid peroxidation and protein oxidation levels were studied after 24, 48 and 168 h of exposure. The activity of most enzymes increased with DF exposure and depended on the toxicant concentration. The most pronounced effect was demonstrated by GPx. Its activity significantly increased at the 0.1 % exposure concentration compared to the control. The lipid peroxidation and protein oxidation levels significantly

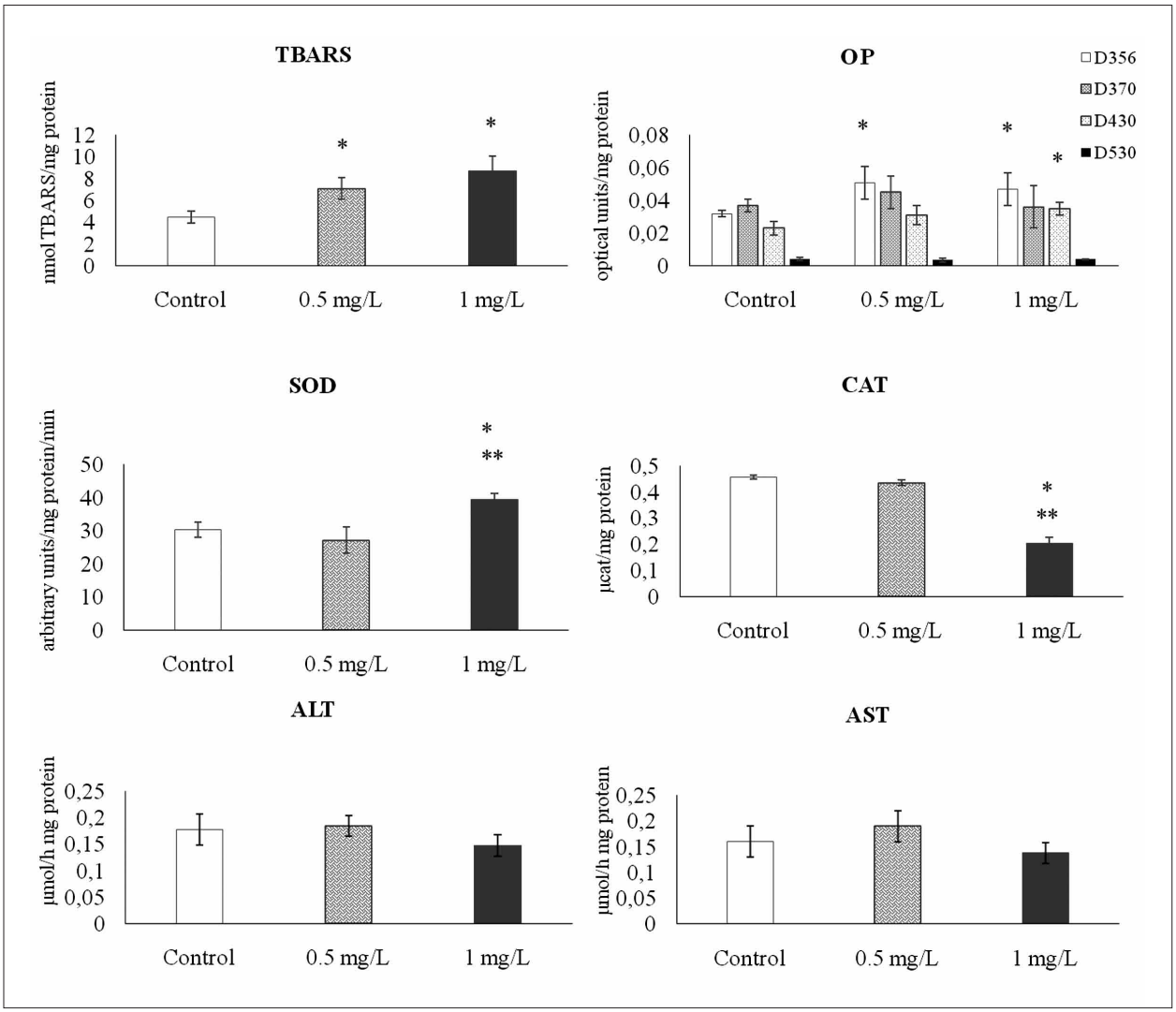

Fig. 3. Biochemical parameters in *M. galloprovincialis* hepatopancreas exposed to diesel: TBARS – thiobarbituric acid reactive substances; OP – oxidized proteins; D356 – neutral aldehydes; D370 – neutral ketones; D430 – basic aldehydes; D530 – basic ketones; SOD – superoxide dismutase; CAT – catalase; ALT – alanine aminotransferase; AST – aspartate aminotransferase; * and ** – statistically significant differences ($p < 0.05$) as compared to the control and to the I experimental group (0.5 mg/L), respectively

increased after 168 h exposure. However, both parameters were higher in the group exposed to tAs for *Mytilus edulis* mussels from the Spain coastal waters, no significant differences in SOD activity were found, while increase in lipid peroxidation was registered in areas affected by oil spills compared to relatively clean waters [27]. It was shown that under chronic oil contamination hepatopancreas cells of *Dreissena polymorpha* and *M. galloprovincialis* demonstrated higher sensitivity to the pollutants and higher level of antioxidant protection as compared to gills. It was recorded a significant increase in CAT, SOD, glutathione reductase activities in the hepatopancreas, as well as in the level of lipid peroxidation in the hepatopancreas and gills in both river (*D. polymorpha*) and marine (*M. galloprovincialis*) mollusks after 28 days of exposure with 50 mg/L mazut [11].

AST and ALT are widely distributed in tissues of aquatic animals and are used for diagnostic of pollutant damage in various tissues. Both aminotransferases activities can be measured to assess the levels of contamination in the environment and pollutant toxicity before the negative effect occurrences. The increase in their activities might be due to generation of suitable substrates for gluconeogenesis and/or energy production that is need for the energy supply of aquatic organisms in stress [16, 30–34].

In our studies, ALT and AST activities did not differ in the control and the experiment. It could indicate that the DF in concentrations

of 0.5 mg/L and 1 mg/L didn't affect the main parts of carbohydrate and protein metabolisms and didn't cause additional energy production. However, other studies demonstrated changes in aminotransferase activities in the tissues of hydrobionts exposed to various toxicants. For example, in the muscle and digestive system of the bay scallop *Placopecten magellanicus* exposed to crude oil in concentrations of 2.50, 5.00 and 10.00 mL/L both aminotransferases rose, though such increase was not concentration and time dependent [32]. But more pronounced changes in aminotransferase activity were observed in the tissues of hydrobionts exposed to heavy metals [30, 34]. It was recorded an increase in the ALT and AST activities in the foot, mantle, and gills of a freshwater mussel *Parreysia rugosa* affected by sublethal concentrations of mercuric chloride [34]. Other authors mentioned that AST activity significantly rose in the homogenate, mitochondrial and cytosolic fractions of snails *Helisoma duryi* and *Lymnaea natalensis* with increasing concentrations of copper (0.01, 0.1 mg/L); however, AST activity decreased at 1 mg/L content. Mitochondrial ALT disappeared at copper ion concentrations of approximately 0.2 mg/L for *L. natalensis* and 1 mg/L for *H. duryi*, possibly indicating mitochondrial degeneration [30].

Conclusions

Under the experimental exposure the mussels demonstrated high resistance to DF. No mortality was observed among the control and the treated specimens. However, petroleum hydrocarbons accumulation was registered in hepatopancreas of the experimental mussels. Significant hydrocarbons' accumulation was detected in II experimental group. This could be caused by suppression of filtration activity, disruption of biotransformation and elimination of pollutants contained high concentrations of petroleum hydrocarbons. DF stimulated oxidative stress by shifting the prooxidant-antioxidant balance towards intensification of lipid peroxidation and protein oxidation in both experimental groups. Toxicants caused SOD activation, and CAT inhibition at 1 mg/L concentration. The prooxidant-antioxidant system parameters demonstrated high sensitivity to DF, and can be used as biomarkers for assessing the functional state of mollusks under oil pollution of the environment. However, the patterns of biomarkers' response are rather complicated because of their dependence on the target organ, dose and exposure time. Therefore, all characteristics mentioned should be taken into account in marine monitoring programs worldwide.

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