Peculiarities of the prolonged effect of a high concentration of ammonium on the physiological and biochemical state of *Carassius auratus* (L.)

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Abstract. The prolonged influence of a high concentration of inorganic nitrogen compounds on the morphological, physiological, and biochemical indices of *Carassius auratus* (L.) was studied in terms of its dimensional-mass indices, weight-length factor, methemoglobin content in fish blood, in terms of the activity of a set of enzymes, and in terms of the content of energetic compounds (lipids, protein, and glycogen). In the gills of fish specimens taken from the polluted pond compared to those taken from the control pond, the activity of succinate dehydrogenase was higher by a factor of 4.8, whereas the activity of lactate dehydrogenase – by a factor of 7.1. The activity of alkaline phosphatase in their liver was higher by a factor of 3.4, which can be conditioned by the increase in the intensity of fish energetic metabolism. Therefore, the rate of linear-mass growth of fish specimens from the pond polluted by inorganic nitrogen compounds decreased. The obtained results suggest that the communities of *C. auratus* continue to occur and reproduce in the polluted water body, which can be indicative of its capability to adapt to atypical ecological conditions.
Key Words: adaptation, enzyme activity, malone dialdehyde, methemoglobin, pollution.

Introduction. Organism resistance and its adaptation to changes in the environmental conditions are among the main problems of biology. Despite a wide variety of types, levels, and mechanisms of adaptation, they can be considered as an intermediate process conditioned by changes in the environmental conditions overall or by changes in some environmental factors. The process of adaptation is aimed at going from one stable state to another (Nemova & Vysotskaya 2004). Adaptation makes it possible to survive and reproduce under unfavorable conditions. The life of hydrobionts depends to a large extent on the chemical composition of the water. Thus, aquatic ecosystems, communities, and organisms are very sensitive to changes in hydrochemical indices. The quality of transformations of biochemical mechanisms increases with the increase of the organism adaptation period.

The process of urbanization and the development of agriculture result in the increase in anthropogenic contamination of various ecosystems. Intensive contamination of water bodies by agricultural and house-hold sewage results in changes in the chemical composition of water. This, in its turn, influences the species composition, the pattern of distribution, and metabolism of fish.

The processes of animal adaptation are most distinct under atypical ecological conditions. It has been known that each species is characterized by a peculiar range of tolerance. An excess of this range results in irreversible changes in the normal functioning of organisms. The study of transformations in metabolic processes aimed at adaptation to toxic contamination is of considerable importance. Only those species, which can adapt to unfavorable conditions can survive and reproduce under conditions of heavy pollution. Prolonged (chronic) contamination results in changes in the process of metabolism. It has been known (Harris et al 1998) that ammonium can exert an adverse effect on the physiological state of fish. The water bodies of the Oleksandriya Park (the town of Bila Tserkva, Ukraine) can serve as an example of the prolonged effect of the contamination by nitrogen compounds. A high concentration of nitrogen compounds (significantly exceeding reference ones) is registered in these water bodies for 18–20 years. Consequently, hydrochemical and hydrobiological characteristics of the studied water bodies essentially changed (Shevchenko et al 2018; Klochenko et al 2019), whereas the species richness of fish fauna decreased. In this case, sensitive species gave way to tolerant ones.

The objective of the present work was to study the prolonged influence of a high concentration of nitrogen compounds on the biochemical indices of *Carassius auratus* (Linnaeus, 1758). Results focus on the activity of a set of enzymes, and in terms of the content of energetic compounds.

Material and Method

Study area. Investigations were carried out in July 2018 within the territory of the Bila Tserkva Experimental Station of the Institute of Hydrobiology of the National Academy of Sciences of Ukraine located in the Oleksandriya Park (the town of Bila Tserkva, Ukraine) (49º47´56´´N 30º06´55´´E) (Figure 1). Samples were taken from two ponds with significantly different concentrations of inorganic nitrogen compounds (Table 1 and Table 2). Their brief characteristics are given in Table 2.

Figure 1. Map of the ponds of the Oleksandriya Dendrological Park: 1 – control pond; 2 – polluted pond (map generated using Google Maps).

Table 1

Concentration of inorganic nitrogen compounds in the studied ponds in different years

	2000 – 2016		*2018*	
Nitrogen compounds	*Control pond*	*Polluted pond*	*Control pond*	*Polluted pond*
NH_4^+ – N, mg·L^{-1}	0.050–0.280	35.09–286.28	0.100–0.276	10.28–65.12
NO_2^- – N, mg·L^{-1}	0.015–0.059	0.32–4.86	0.006–0.059	2.43–4.03
NO_3^- – N, mg·L^{-1}	0.180–0.340	17.35–100.35	0.08–0.190	110.16–185.20

Investigation of environmental parameters. All samples taken from the studied ponds were analyzed for ammonium (NH_4^+-N), nitrite (NO_2^--N), and nitrate (NO_3^--N). The content of inorganic nitrogen compounds was determined by colorimetric method using the KFK-2 MP photoelectric colorimeter (Russian Federation), dissolved oxygen content – by the Winkler method (Arsan et al 2006). The value of pH was defined using the pH-009 (1) pH-meter (Russian Federation).

Table 2

Average values of physio-chemical variables in the studied ponds during the period of investigations, July 2018

Variables	*Control pond*	*Polluted pond*
NH_4^+ – N, mg·L^{-1}	0.187	21.650
NO_2^- – N, mg·L^{-1}	0.028	3.090
NO_3^- – N, mg·L^{-1}	0.133	149.4
O_2, N, mg·L^{-1}	7.70	6.42
pH	8.0	7.6
Water temperature, ^{o}C	21.8	20.3

Laboratory analysis. Overall, six specimens of *Carassius auratus* taken from each pond were analyzed. Experimental samples of fish tissues for biochemical tests were taken from the liver, gills, and muscles. The tissues (100–200 mg) were homogenized in 0.2 M KCL (5 ml) in the Rannie/Caulia homogenizer (Germany). Then, the homogenate was centrifuged using the OP H-8, 97-00027 centrifuge (Russian Federation) at 3000 rpm for 15 min. The activity of lactate dehydrogenase (LDH) was determined by the method of Prokhorova (1982): 3 ml of phosphate buffer (pH 7.5) with pyruvate (0.06 mg) and NADH (0.3 mg) were added to 0.1 ml of enzyme extract. Absorbance was measured at 340 nm with the UNICO UV-2800 spectrophotometer (China) every 30 seconds for 3 minutes.

The activity of succinate dehydrogenase (SDH) was defined by the ferricyanide method (Asatiani 1965): 0.5 ml of enzyme extract was added to 1.4 ml of phosphate buffer (pH 7.8) with succinic acid (1 mg), ethylenediaminetetraacetic acid (EDTA) (0.005 mg), and sodium aside (1.0 mg). The reaction mixture was incubated for 15 min at 25^{o}C. Then 0.1 mg of 25 mM potassium ferricyanide was added and the reaction mixture was incubated for 15 min at 25^{o}C in the TC-80 M-2 thermostat (Russian Federation). The reaction was stopped by the addition of 2 ml of 20% tricyclic antidepressant (TCA). Absorbance was measured at 420 nm with the UNICO UV-2800 spectrophotometer (China).

The activity of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) was determined by the method of Reitman and Frankel (1957) using the "AsAT" and "AlAT" standard commercial kits (the "Reagent" PraT, Dnipro, Ukraine). The activity of glutamate dehydrogenase (GDH) was determined following the procedure of Khokhlov et al (1990). The activity of alkaline phosphatase (ALP) was defined using the "Luzhna Fosfataza" standard commercial kit (the Filisit-Diagnostyka, Dnipro, Ukraine). The activity of enzymes was calculated per mg of protein in a sample. Protein content was determined using the Lowry method (Lowry et al 1951). The content of glycogen was defined by the anthrone method (Severin & Solovyev 1989), the content of lipids – using the "Zagalni lipidy" standard commercial kit (the Filisit-Diagnostyka, Dnipro, Ukraine). The content of malone dialdehyde was determined in terms of the reaction with thiobarbituric acid, which at a high temperature in the acidic environment yields colored trimethyne complex. The optical density of the studied samples was measured at 535 and 580 nm with the UNICO UV-2800 spectrophotometer (China).

Methemoglobin content in fish blood was determined by the method of Kanayev et al (1987): fish blood was taken from its heart using a heparinized syringe; 7.3 ml of 0.25% ammonia solution was added to each of two test tubes; 0.2 ml of blood was added to the test tube N 1, 0.2 ml of blood and 2 drops of saturated solution of potassium ferricyanide were added to the test tube N 2. The mixture was incubated for one hour. The optical density of the mixture was measured at 630 nm using the KFK-2 MP photoelectric colorimeter (Russian Federation). The thickness of cuvette walls was 10 mm. The calculation of methemoglobin content in fish blood (%) was carried out by the following formula:

$$X = \left(\frac{0.71 \times E1}{E2} - 0.15 \right) \times 1.61 \times 100$$

where:
X – methemoglobin content in fish blood (%)
E1 – optical density of the solution from test tube N 1
E2 – optical density of the solution from test tube N 2
0.71, 0.15, and 1.61 – calculation factors

The coefficient of condition (*CC*) or weight-length factor by Clark was determined by the formula: $CC = p \times 100/SL^3$, where p – fish mass without organs; SL^3 – fish total length (Anisimova & Lavrovsky 1983).

Statistical analyses. The difference between the average values was determined using the Student T-criterion ($p \leq 0.05$). The obtained data was processed using Statistica 5.5 software. All bioethical norms were observed throughout the experiment.

Results and Discussion. Results of the performed investigations suggest that fish specimens taken from the polluted and clean water bodies significantly differed in their dimensional-mass, physiological, and biochemical indices.

It has been found that fish specimens taken from the polluted pond subjected to the prolonged influence of a high concentration of ammonium (Table 1) were characterized by a less intensive linear and mass growth (Table 3). It is likely that the process of their adaptation to unfavorable conditions consisted in the decrease in growth rate, which was conditioned by physiological and biochemical changes in the organism of *Carassius auratus* occurring for a long time in water bodies with a high content of inorganic nitrogen compounds.

Table 3
Dimensional-mass indices of fish specimens taken from the studied water bodies

Indices	*Control pond*	*Polluted pond*
Fish mass, g	77.9±3.4	24.5±3.1
Fish length, cm	16.0±0.9	12.4±0.5
Weight-length factor by Clark, relative units	1.7±0.7	1.30±0.3

It should be noted that the content of methemoglobin in the blood of the studied groups of fish essentially differed. Thus, in the blood of fish specimens taken from the pond with a high content of inorganic nitrogen was 6.3 times higher than that in the specimens taken from the control water body (Figure 2).

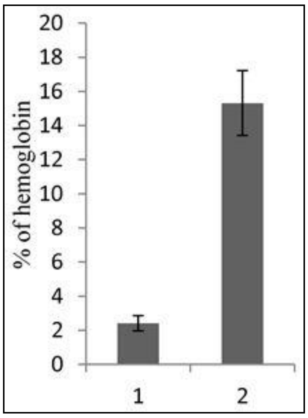

Figure 2. Methemoglobin content in *C. auratus* blood: 1 – control pond; 2 – polluted pond. M±m, n=5.

It has been known that methemoglobin represents a form of hemoglobin, where the iron is oxidized to Fe(III), which is not capable of transporting oxygen. The formation of methemoglobin in the organism results in hypoxia, including its hemic and histotoxic forms. This, in its turn, can be responsible for a set of significant changes in metabolism both at the enzymatic and at the humoral levels (Cherkesova & Shakhnazarova 2009). Thus, it has been found that the activity of the enzymes of energetic metabolism, including LDH and SDH, in the gills of fish changed. It has been known that SDH is the main enzyme of the Krebs cycle, which is involved as the catalyst in the process of succinate oxidation yielding fumarate (Kumari et al 2011). In this case, LDH is involved in the process of pyruvate conversion into lactate.

The activity of SDH in the gills of fish specimens taken from the polluted water body was 4.8 times higher than that in controls (Figure 3), whereas the activity of LDH was higher by a factor of 7.1. It is likely that an essential increase in the activity of SDH and LDH in the gills of fish was conditioned by the increase in the intensity of energetic metabolism aimed at detoxification and excretion of an excessive amount of ammonium ions and ammonia.

For the most part the intensity of the processes of aerobic metabolism increases under the influence of intensive load on the organism, which is essential to its normal energy supply. Therefore, the number of molecules of adenosine triphosphate (ATP) belonging to the main energy carriers significantly increases. It should be noted that the increase in the activity of the processes of tricarbonic acid cycle was accompanied by the increase in the activity of LDH involved in the process of glycolysis. The obtained results correlate well with literature data, which suggest that the activity of LDH in the gills increases because of an adverse effect of nitrogen compounds on the physiological processes of gas exchange (Arillo et al 1984; Das et al 2004).

Thus, because of a significant dysfunction of the processes of gas exchange in the blood of fish specimens taken from the polluted pond the activity of both aerobic and anaerobic processes aimed at intensive energy supply in response to toxic load increased.

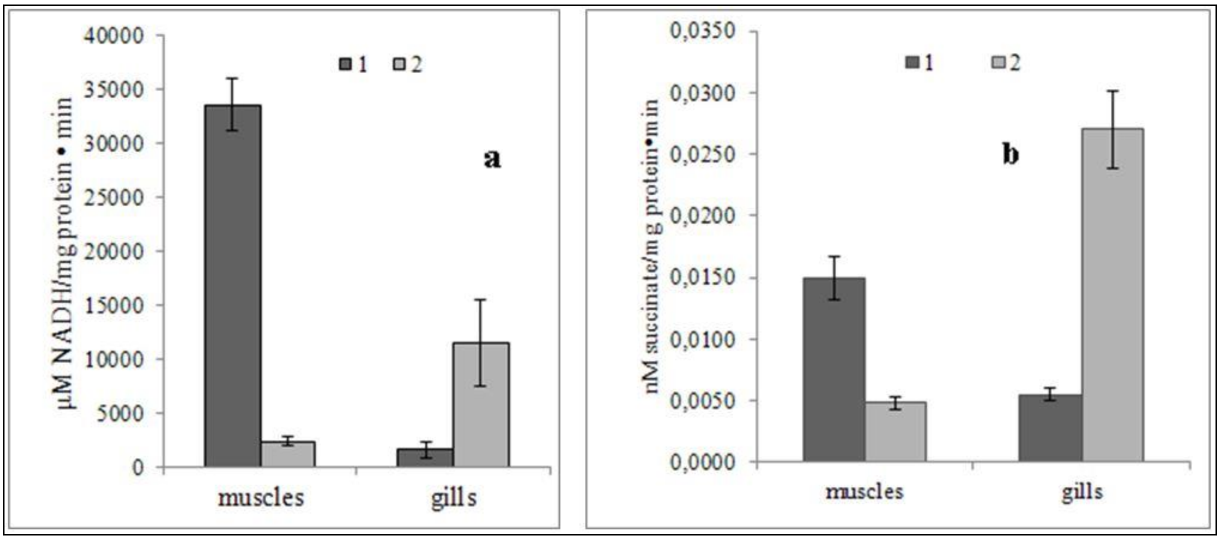

Figure 3. LDH (a) and SDH (b) activity in *C. auratus* tissues: 1 – control pond; 2 – polluted pond. M±m, n=5.

In the muscles of fish specimens taken from the polluted pond, the activity of LDH and SDH was 13.8 and 3.1 times lower than that in fish specimens taken from the control water body. A decrease in the activity of these enzymes in the muscles of fish specimens taken from the polluted water body can be indicative of the decrease in the intensity of metabolic processes. Literature data suggest that intensive linear growth of fish tissues is accompanied by the increase in the activity of LDH. This index is widely used in assessing the physiological state of fish (Yang & Somero 1996). Thus, it might be supposed that energy redistribution registered in fish taken from the polluted water body was aimed at maintenance of homeostasis. The increase in energy expenditure for the processes of gas exchange and detoxification was accompanied by a decrease in energy expenditure for the processes of linear growth. The indices of linear-mass growth of fish specimens taken from the polluted water body were essentially lower than those in fish from the control water body (Table 3).

The activity of alkaline phosphatase (ALP) in fish from the polluted and control water bodies also differed. It has been found that the activity of ALP in the liver of fish taken from the polluted water body was 3.4 times higher than that in controls (Figure 4).

Table 3

Dimensional-mass indices of fish specimens taken from the studied water bodies

Indices	*Control pond*	*Polluted pond*
Fish mass, g	77.9±3.4	24.5±3.1
Fish length, cm	16.0±0.9	12.4±0.5
Weight-length factor by Clark, relative units	1.7±0.7	1.30±0.3

It has been known that the biochemical role of alkaline phosphatase consists in the process of dephosphorylization of chemical compounds (Wala et al 2014; Hussian et al 2016). Thus, the increase in the activity of this enzyme is indicative of the response of fish organism to the influence of toxicants, which can be considered as adaptation. This phenomenon can be conditioned by the increase in the intensity of energetic processes in fish gills (the increase in the activity of LDH and SDH) under the influence of excessive concentrations of ammonium. Intensive ATP synthesis in fish gills because of aerobic and anaerobic processes is accompanied by the increase in the intensity of their hydrolysis. Consequently, a large portion of energy is released for inhibiting an adverse effect of the compounds of inorganic nitrogen on fish organism. The obtained results correlate well with literature data, which suggest that the increase in the activity of alkaline phosphatase in tissues probably supplies metabolic processes with phosphate radicals involved in the processes of detoxification of fish tissues (Fatma & Gad 2008; Moraes et al 2016).

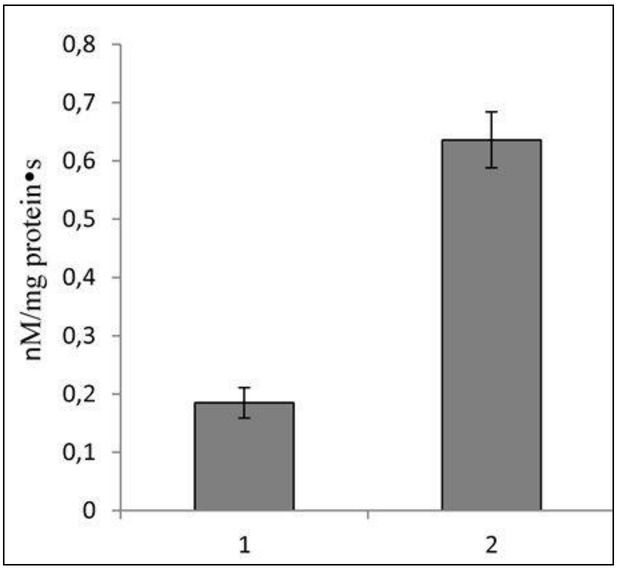

Figure 4. Alkaline phosphatase activity in *C. auratus* liver: 1 – control pond; 2 – polluted pond. M±m, n=5.

It has been known that the activity of cell enzymes is one of the main indices of metabolism pattern. Thus, the intensity of metabolic processes in the tissues of *Carassius auratus* under the influence of ammonium ions can be assessed in terms of the activity of the enzymes of constructive metabolism, including aspartate aminotransferase (AST) and alanine aminotransferase (ALT). These enzymes are involved in the process of protein metabolism regulation (Shivakumar 2005). It has been found that under the influence of inorganic nitrogen compounds the activity of AST in fish muscles and liver was respectively 3.3 and 3.5 times lower than that in controls. The same is true of the activity of ALT. In the muscles and liver of fish specimens taken from the polluted water body, its activity was respectively 4.2 and 2.3 times lower than that in controls (Figure 5).

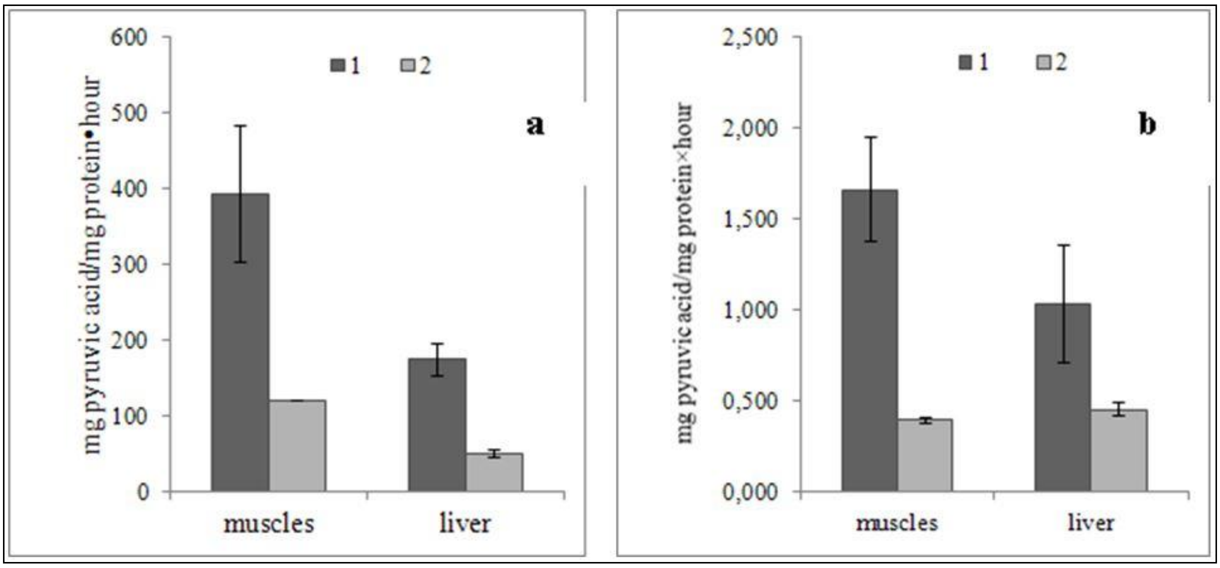

Figure 5. AST (a) and ALT (b) activity in *C. auratus* tissues: 1 – control pond; 2 – polluted pond. M±m, n=5.

Fish samples were taken in summer (July) at the stage of their active growth. Thus, the activity of these enzymes under favorable conditions should be high. Literature data (Kumar et al 2018) suggests that under conditions of acute ammonium toxicity the activity of ALT and AST increased. However, original data suggests that the prolonged effect of nitrogen compounds, unlike its short-term effect, inhibited the processes of constructive metabolism in fish. As it was mentioned above, these factors later resulted in the decrease in the dimensional-mass indices of fish.

It has been known that glutamate dehydrogenase (GDH) is involved in the process of nitrogen metabolism regulation, indirectly influencing protein metabolism (Chandrudu & Radhakrishnaiah 2013; Ganesh et al 2006). Detoxification of ammonium compounds, in particular the formation of their complexes with ketoglutaric acid, is one of the functions of GDH (Metwally & Wafeek 2014). It has been known that this enzyme is located mainly in liver tissues. In fish gills it is not abundant. The activity of GDH in gills depends on the amount of ammonia in fish organism (on the processes of ammonia detoxification). However, original investigations have shown that the activity of GDH in fish gills remained unchanged under the influence of an excessive concentration of ammonium ions. The activity of this enzyme in the liver of fish specimens taken from the polluted pond was 6.2 times lower than that in the liver of fish from the control water body (Figure 6). This fact suggests that under the studied conditions GDH was not involved in the process of ammonia and ammonium release from fish organism. During the period of prolonged adaptation to a high concentration of ammonium in the water, other mechanisms of ammonia and ammonium release from fish organism were elaborated. Thus, a decrease in the activity of GDH in liver tissues is indicative of a decrease in the activity of the processes of biosynthesis, primarily of protein synthesis in the organism of fish specimens taken from the polluted water body.

This fact is supported by the decrease in the content of lipids and protein in liver tissues respectively by a factor of 1.48 and 1.45 at a high concentration of ammonium and during the prolonged period of fish adaptation to such conditions.

Also the content of glycogen in fish specimens taken from the pond with a high concentration of nitrogen compounds was higher by a factor of 1.7 (Figure 7). It has been known that the enzymes of transamination, including ALT and AST, are involved in the process of protein and carbohydrate metabolism (Shivakumar 2005; Gabriel et al 2012). Depending on the response of the organism to the influence of some environmental factors, various energy intensive substrata are used for the supply of energetic processes (Grubinko et al 2012; Banaee et al 2015). Thus, for example, aminotransferase activity inhibition results in changes in protein metabolism regulation. In this case, the content of free amino acids, including aspartic acid used as the substratum for gluconeogenesis, increases (Roshchina 2010; Musayev et al 2010; Yhasmine et al 2013; Al-Ghanim 2014). The increase in the content of glycogen in the tissues of fish specimens taken from the polluted pond can be conditioned by the increase in the activity of alkaline phosphatase involved in these processes (El-Naga et al 2005; Sreekala & Zutshi 2010; Shaikila et al 1993). The increase in the content of the products of lipid metabolism can also result in the increase in the content of glucose and glycogen in fish tissues.

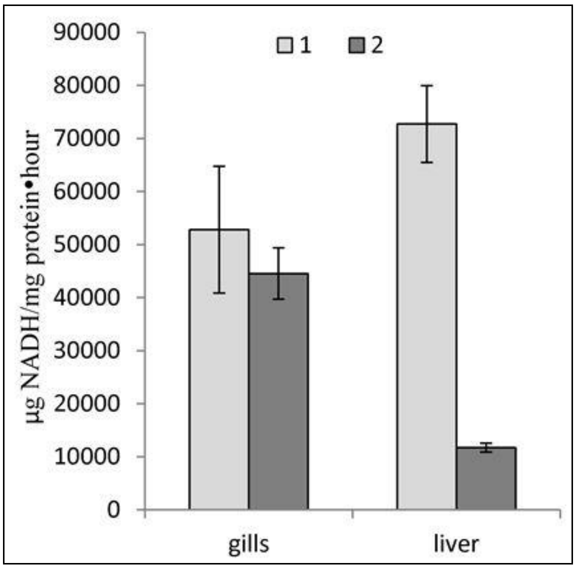

Figure 6. GDH activity in *C. auratus* tissues: 1 – control pond; 2 – polluted pond. M±m, n=5.

The increase in the intensity of the processes of lipid peroxidation in the organism is one of the main indices of an adverse effect of the environmental factors. Malone dialdehyde is one of the end products of lipid peroxidation. The accumulation of this compound in the tissues is indicative of the intensity of the process of lipid peroxidation (Osoba 2013).

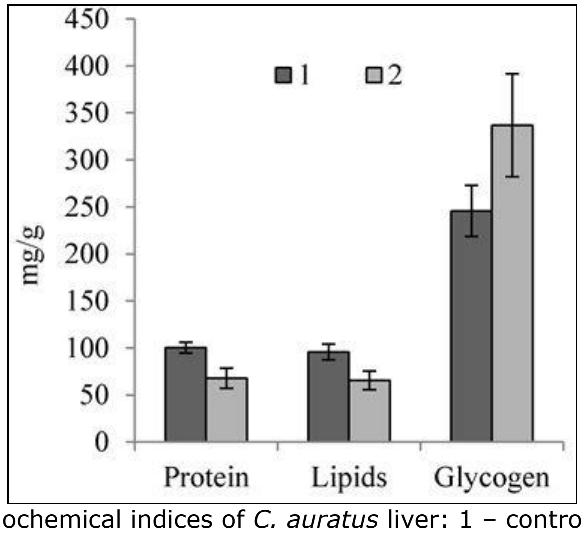

Figure 7. General biochemical indices of *C. auratus* liver: 1 – control pond; 2 – polluted pond. M±m, n=5.

Original investigations suggest that the content of malone dialdehyde in the liver of fish specimens taken from the polluted water body was almost three times higher than that in the liver of fish specimens taken from the control pond (Figure 8). Thus, the increase in the content of malone dialdehyde is indicative of an adverse effect of a high concentration of ammonium even under conditions of the prolonged adaptation of fish to this factor.

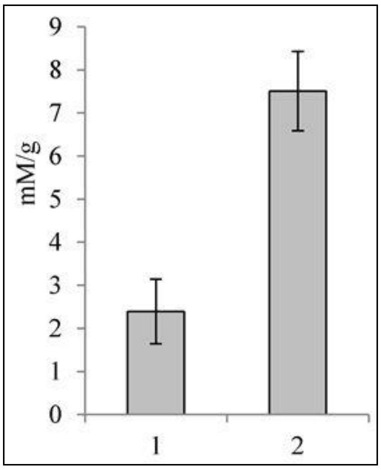

Figure 8. Malone dialdehyde content in *C. auratus* liver: 1 – control pond; 2 – polluted pond. M±m, n=5.

The energetic metabolism in fish increased in response to the influence of a high concentration of nitrogen compounds. In particular, the activity of SDH in fish gills increased by a factor of 4.8, whereas the activity of LDH – by a factor of 7.1.

The intensity of the processes of phosphorylization also increased. The activity of alkaline phosphatase in fish liver increased by a factor of 3.4. This process, as well as the increase in the activity of energetic metabolism, can be aimed at ammonia and ammonium detoxification and their release from fish organism.

It should be noted that lipids and proteins are involved in energy supply of the processes of adaptation. Therefore, their content in fish liver decreased.

As a result of the increase in energy expenditure in response to the influence of a high concentration of ammonium, the rate of fish linear-mass growth decreased.

The obtained results suggest that *Carassius auratus* continues to occur and reproduce in the polluted water body, which can be indicative of its capability to adapt to atypical ecological conditions.

Conclusion. Results of the study have shown that an adverse impact of the compounds of inorganic nitrogen is responsible for the formation of certain adaptations of *Carassius auratus*. The redistribution of the activity of the processes of metabolism in its tissues is one of the main adaptations of this fish species to the toxic influence of the above-mentioned compounds. The increase in the intensity of the processes of lipid peroxidation and the formation of large amounts of methemoglobin in the blood can be considered as fish response to an adverse effect of inorganic nitrogen compounds.

Conflict of Interest. The authors declare that there is no conflict of interest.

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