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Comparative analysis of toxicological and biochemical characteristics of round goby *Neogobius melanostomus* (Pallas, 1814) from different areas of the Sea of Azov

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Chemical elements are the major pollutants for many reservoirs. They are characterized by high accumulative ability and even in trace amounts affect physiological and biochemical status of fish. In this study we investigated toxicological (Hg, Pb, Cd, As, Fe, Zn and Cu concentrations) and biochemical (level of oxidized proteins and lipid peroxidation, antioxidant enzyme activities (superoxide dismutase (SOD), catalase (CAT)), cholinesterase (ChE), alanine aminotransferase (ALT), aspartate aminotransferase (AST) activities and albumin content) characteristics in the liver of round goby *Neogobius melanostomus* inhabiting two areas of the Sea of Azov (Belosaraiskaya Spit (BS)) and the eastern part of the Taganrog Bay (ETB)) with different levels of pollution and sediment granulometric parameters. Hg, Pb and Fe concentrations were detected significantly higher in the liver of fish from ETB compared to BS (p<0.05–0.01). SOD activity was considerably lower, while CAT activity was recorded higher in fish from ETB compared to BS (p<0.01). Levels of oxidized proteins and lipid peroxidation as well as albumin content, ALT and ChE activities in individuals from ETB considerably exceeded the corresponding values in BS (p<0.05–0.01). Possible mechanisms of *Neogobius melanostomus* biochemical response to chemical element content in the liver are discussed. The results obtained can be applicable for assessing the quality of water bodies contaminated by the abovementioned chemical elements.

Keywords: *Neogobius melanostomus*, toxicological and biochemical characteristics, pollution, the Sea of Azov.

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Сравнительный анализ токсикологических и биохимических показателей бычка-кругляка *Neogobius melanostomus* (Pallas, 1814) из разных районов Азовского моря

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Химические элементы являются приоритетными загрязнителями для многих водоёмов. Они характеризуются высокой способностью к накоплению и даже в следовых количествах влияют на физиологический и биохимический статус рыб. В данной работе мы исследовали токсикологические (концентрация Hg, Pb, Cd, As, Fe, Zn и Cu) и биохимические (уровень окислительной модификации белков и перекисного окисления липидов, активность антиоксидантных ферментов (супероксиддисмутазы (СОД), каталазы (КАТ)), холинэстеразы (ХЭ), аланинаминотрансферазы (АЛТ), аспартатаминотрансферазы (АСТ) и содержание альбумина) параметры в печени бычка-кругляка *Neogobius melanostomus*, обитающего в двух районах Азовского моря (Белосарайская коса (БК) и восточная часть Таганрогского залива (ВТЗ)) с разным уровнем загрязнения и гранулометрическими параметрами донных отложений. Концентрации Hg, Pb и Fe в печени рыб из ВТЗ были значительно выше, чем из БК (p<0,05–0,01). Активность СОД была значительно ниже, а активность КАТ – выше у рыб из ВТЗ по сравнению с БК (p<0,01). Уровень окислительной модификации белков и перекисного окисления липидов, а также содержание альбумина, активность АЛТ и ХЭ у особей из ВТЗ значительно превышали соответствующие значения у рыб из БК (p<0,05–0,01). Обсуждаются возможные механизмы биохимического ответа бычка-кругляка на содержание химических элементов в печени. Полученные результаты могут быть использованы для оценки качества водоёмов, загрязнённых вышеперечисленными химическими элементами.

Ключевые слова: бычок-кругляк, токсикологические и биохимические показатели, загрязнение, Азовское море.

Currently, regular monitoring of water bodies polluted by chemical elements (CE) plays a key role under conditions of large anthropogenic pressure on the hydrosphere. Having high bioaccumulation ability, CE are accumulated rapidly in hydrobionts, and even their trace levels are capable to affect physiological and biochemical status of fish [1–4]. The toxic effect of thiol poisons (Pb, Hg, Cd, As) appears in blocking of various biochemical reactions by binding to sulfhydryl groups of protein molecules or displacing essential metals from the active enzyme centers [3, 5]. The action of elements with variable valence (Fe, Cu) is realized through the capacity to enter into Fenton and Haber-Weiss reactions. The hydroxyl radical (OH·) formed as a result of the reactions initiates metal-catalyzed protein oxidation and lipid peroxidation (LPO) [3]. Thus, CE accumulation in hydrobiont tissues leads to a wide scope of negative biochemical changes, i.e. inhibition of the individual enzyme activities and enzymatic complexes, damage of membrane structures, displacement of prooxidant–antioxidant reactions towards free radical oxidation (FRO) of biomolecules, changes in protein metabolism, etc. [1–4] and, accordingly, the development of pathological changes in the organism at higher levels of biological organization (tissue, organ, organism) [6–8]. Such transformations considerably worsen the quality of fish products, result in degrade the biological resources in the water bodies [6, 8, 9]. In this regard, to develop a system for assessing the quality of reservoirs contaminated by CE and conducting the regular field studies are of great importance. It permits to estimate the CE effect in fish tissues based on the set of biochemical characteristics under natural conditions.

Round goby *Neogobius melanostomus* (Pallas, 1814) was selected as a bioindicator species. It is sedentary, demersal fish globally distributed in many reservoirs [10–12]. The oxidative stress parameters (the level of oxidized proteins (OP) and LPO), the activity of antioxidant enzymes (superoxide dismutase (SOD), catalase (CAT)), aminotransferases, cholinesterase (ChE) activities and albumin content are used as the informative biomarkers.

Thus, the aim of the study was to research the set of toxicological and biochemical characteristics in the liver of round goby *Neogobius melanostomus* inhabiting two areas of the Sea of Azov.

Material and methods of research

Fish specimens were caught by using bottom trawling surveys of fishing vessel in the Sea of Azov in October 2019. The observational trawling lasted 30 minutes at a speed 1.5 m/s.

The researches were conducted in two areas of the Taganrog Bay in shallow waters with the specific granulometric composition of bottom sediments [13, 14] and different levels of CE contamination [15–18] (Figure).

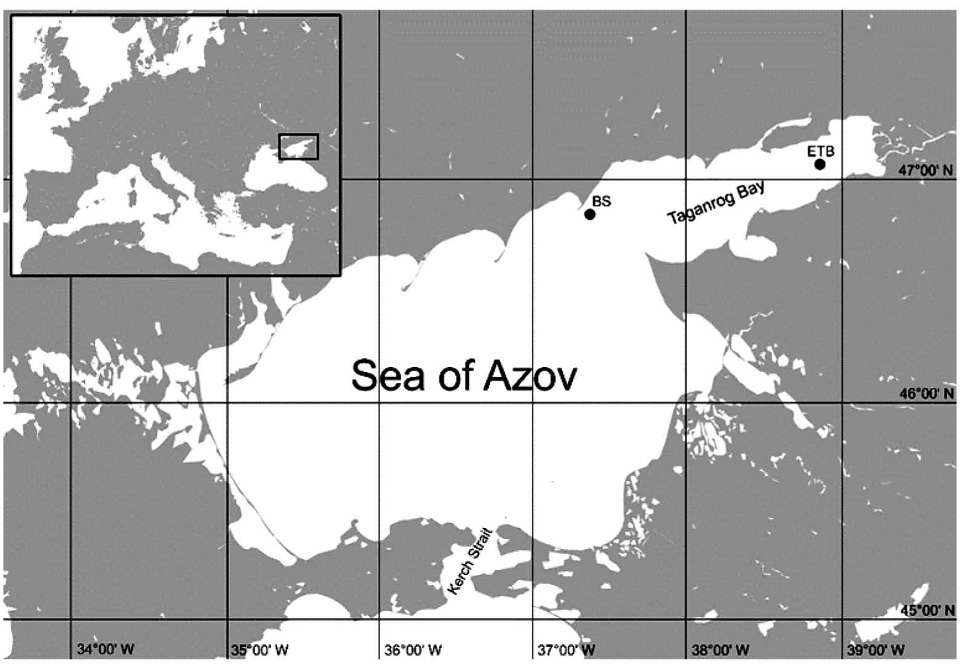

Fig. Sampling areas in the Sea of Azov:
Belosaraiskaya Spit (BS), eastern part of the Taganrog Bay (ETB)

Area 1 (46º47'07" N; 37º23'58" E) is located in the northern part of the mouth of Taganrog Bay (Belosaraiskaya Spit (BS)). The site is characterized by mixed (aleuritic-silty-sandy) type of bottom sediments. The main sources of pollution are navigation, wastewaters and industrial effluents [16].

Area 2 (N 47º02'4"; E 38º49'9") is situated in the eastern part of Taganrog Bay (ETB). Clay silts with the fine-grained fraction content (<0.01 mm) are found dominant in the composition of bottom sediments representing more than 70% [16]. The main pollution sources are runoff from Don, Kalmius, Mius, Yeya and Big Turtle rivers, discharges from Taganrog industries, sewages from Taganrog Metallurgical Plant, navigation and also dredging in channels, etc. [17, 18].

After being lifted onto the deck, fish specimens were immediately killed by medullar sectioning and subjected to the standard biological analysis. Total length, standard length, total weight, somatic weight (weight without organs), liver weight, age, sex and maturity stages were determined [19]. The age of fish was identified by using otoliths. Fish samples were treated according to CCAC guidelines about care and use of fish in the research, teaching and testing [20] and also the Guide about care and use of the laboratory animals (2011) [21].

Shortly after the biological analysis, the liver was frozen and stored for no more than a month at –22 ºC. The concentration of chemicals and biochemical parameters were studied in the liver of the *Neogobius melanostomus* males (25 specimens) that were dominant in catches in the age group (0+), exposing the II–III stages of maturity.

The chemical elements – mercury (Hg), lead (Pb), cadmium (Cd), arsenic (As), iron (Fe), zinc (Zn) and copper (Cu) were detected in fish liver by inductively coupled plasma mass spectrometer (ICP-MS) PlasmaQuant MS Elite (Analytik Jena AG, Germany) in the Scientific and Educational center for collective use "Spectrometry and chromatography" of IBSS. Tissue samples were obtained from 7 individuals taken from each area and dried to constant weight in a drying cabinet at +105 ºC. A portion of the dried tissue was transferred into the test fluoroplastic tubes for wet ashing using a plastic spatula. Wet ashing of tissue samples was made using chemically pure nitric acid (70%), additionally purified by double distillation without boiling in a DST-1000 acid purification system (Savillex, USA). The measurements were conducted in triplicate.

The fish liver of 10 and 15 individuals from BS and ETB was used for the biochemical studies. The liver was washed several times by cold 0.85% physiological solution, homogenized in potassium-phosphate buffer (50 mM, pH 7.2) with the addition of 1 mM EDTA. Homogenates were centrifuged at 10000 g for 15 min at the temperature of 0–4 ºC in a refrigerated centrifuge MPW-352 (MPW Med. Instruments, Poland). All biochemical parameters were analyzed in the supernatants.

The level of oxidized proteins (OP) was determined by the reaction of interaction of oxidized amino acid residues of protein with 2,4-dinitrophenylhydrazine to form 2,4-dinitrophenylhydrazones [22]. The optical density of the newly formed 2,4-dinitrophenylhydrazones was recorded at the following wavelengths (λ): 356 nm (neutral aldehydes), 370 nm (neutral ketones), 430 nm (basic aldehydes), and 530 nm (basic ketones).

The concentration of lipid peroxidation secondary products – thiobarbituric acid reactive substances (TBARS) was analyzed by the reaction with thiobarbituric acid (λ=352 nm) [23].

Superoxide dismutase (SOD) activity was assayed in the nitroblue tetrasolium-phenazine methosulfate – NADH system (λ=560 nm) [24]. Catalase (CAT) activity was measured by hydroperoxide and molybdite ammonium interaction reaction (λ=410 nm) [25].

Cholinesterase (ChE) activity was detected by hydrolysis of butyrylthiocholin to oil acid and tiocholine using standard assay kits DiaVetTest (Russia) (λ=405 nm).

Activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were analyzed by standard assay kits OLVEX DIAGNOSTICUM (Russia). AST activity was analyzed by oxaloacetate and 2,4-dinitrophenylhydrazine interaction reaction. ALT activity was measured by pyruvate and 2,4-dinitrophenylhydrazine interaction reaction in an alkaline medium (λ=537 nm).

Albumin (Alb) concentration was determined based on the interaction of Alb with bromcresol green reagent using standard assay kits OLVEX DIAGNOSTICUM (Russia) (λ=600 nm).

All measurements were carried out on spectrophotometer SF-2000 (Russia). The biochemical parameters were calculated per mg of protein. Total soluble protein content was quantified on the basis of biuret reaction using standard assay kits OLVEX DIAGNOSTICUM (Russia).

The results were subjected by statistical process. Mean values +/- SEM (standard error of the mean) were calculated. The significance of the difference between the samples was evaluated by applying Mann-Whitney U-test. The difference was found great at the significance level $p \leq 0.05$. The statistical analysis was done by using software programs Past 3 and Microsoft Office Excel 2016.

Results and discussion

The results of elemental analysis demonstrated considerably high Hg, Pb and Fe levels in the liver of individuals from ETB (+91%, +22%, +125%, respectively) as compared to BS (p<0.05–0.01). The content of Cd, As, Cu and Zn did not differ in the liver of fish from two areas (Table 1).

Research results of the oxidative stress parameters demonstrated considerably high levels of TBARS, neutral aldehydes (D356) and ketones (D370), basic aldehydes (D430) in the liver of gobies from ETB (+40%, +52%, +67%, +54%, accordingly) (p<0.05). SOD activity was significantly lower, whilst CAT activity was found higher in specimens from ETB (-1353%, +44%, respectively) as compared to BS (p<0.01) (Table 2).

Activity of ALT and ChE as well as albumin concentration were much higher in the liver of ETB individuals (+58%, +206%, +145%, respectively) compared to BS ones (p<0.01). The level of basic ketones (D530) and AST activity did not differ in two areas (Table 2).

The analysis of the ratio between lipid and protein oxidation processes and the antioxidant (AO) enzyme system reactions allows researchers to evaluate the response of organisms to the effect of individual pollutants or their complexes in both experimental and natural conditions. An increase in the AO enzyme activities under constant or low values of oxidative stress parameters (LPO and OP levels) is interpreted as an adaptive response of an organism. An increase in the AO enzyme activities together with the LPO and OP levels is considered as an adaptive compensatory reaction. Reduced or relatively low AO enzyme activities against the background of high LPO and OP levels indicates a shift in prooxidant–antioxidant reactions towards the processes of free-radical oxidation of biomolecules and the development of oxidative stress preceding pathological states in the organisms [26, 27]. In

Table 1

Chemical element concentrations (mg/kg of dry weight) in the liver of *Neogobius melanostomus* (mean ± SEM)

Chemical element	Belosaraiskaya Spit	Eastern part of Taganrog Bay
Hg	0.023±0.005	0.044±0.010*
Pb	0.63±0.0006	0.77±0.06*
Cd	0.18±0.04	0.2±0.03
As	4.4±0.9	2.7±0.6
Fe	117±13	262±29*
Cu	153±20	197±34
Zn	38.7±2.3	44.3±3.3

*Note: * – significant differences between the samples (p<0.05).*

Table 2

Some biochemical parameters in the liver of *Neogobius melanostomus* (mean ± SEM)

Biochemical parameter	Belosaraiskaya Spit	Eastern part of Taganrog Bay
TBARS, nmol TBARS/mg protein	17.5±1.7	24.4±2.3*
D356, optical units/mg protein	0.087±0.011	0.132±0.013*
D370, optical units/mg protein	0.110±0.012	0.184±0.015*
D430, optical units/mg protein	0.068±0.008	0.105±0.009*
D530, optical units/mg protein	0.013±0.003	0.013±0.002
SOD, arbitrary units/mg protein/min	60±13	4.2±0.8*
CAT, mcat/mg protein	0.27±0.04	0.39±0.03*
ALT, µmol/h mg protein	0.14±0.07	0.21±0.02*
AST, µmol/h mg protein	0.032±0.007	0.032±0.004
ChE, µcat/g protein	0.88±0.14	2.69±0.36*
Alb, mg/mg protein	0.311±0.017	0.761±0.094*

*Note: TBARS – thiobarbituric acid reactive substances, D356 – neutral aldehydes, D370 – neutral ketones, D430 – basic aldehydes, D530 – basic ketones, SOD – superoxide dismutase, CAT – catalase, ALT – alanine aminotransferase, AST – aspartate aminotransferase, ChE – cholinesterase, Alb – albumin, * – significant differences between the samples (p<0.05).*

our study, CAT activity and LPO and OP levels were significantly higher and SOD activity was considerably lower in the liver of fish from ETB, where Hg, Pb and Fe concentrations exceeded corresponding values in BS. The results obtained indicate the inhibition of SOD activity due to high CE concentrations in the liver of specimens from ETB and the shift of prooxidant–antioxidant reactions towards free-radical oxidation of proteins and lipids. At the same time, an increase in CAT activity against the background of high values of oxidative stress parameters (LPO and OP levels) in the liver of specimens from ETB is regarded as an adaptive compensatory response at high levels of hydrogen peroxide.

SOD does not belong to SH-containing enzymes [28], therefore, Hg and Pb toxic effect can only manifest in the competitive inhibition of Zn,Cu-SOD as a result of Zn^{2+} replacement with Pb^{2+} or Hg^{2+} [4]. At the same time, higher Fe concentration in the liver of *Neogobius melanostomus* from ETB can initiate metal-catalyzed oxidation of SOD. Oxidation of tyrosine, tryptophan and His61 residues of the SOD active center, deep structural changes and the enzyme inactivation during incubation in Fenton's medium [29].The mechanisms of CE enzymotoxicity are not mutually exclusive and can be manifested simultaneously.

Changes in the parameters of the prooxidant–antioxidant system in fish affecting different levels of pollutants observed in the laboratory and field conditions were reported by many authors. All these alterations depend on chemical properties and concentration of pollutants, taxonomic status of fish, tissue and organ specificity and functions [2, 30–34]. A decrease in SOD and CAT activities and increase in LPO level in gills, liver and spleen of *Synechogobius hasta* were found after 15-days of waterborne Cd exposure at the concentrations of 0.10, 0.17 and 0.29 mg Cd/L [32]. The Cu exposure of 50 g/L and 200 g/L for 96 h increased CAT activity and the level of protein carbonyls, decreased SOD activity in gill, liver, and intestine of killifish *Fundulus heteroclitus* [34]. In the field studies the activity of antioxidant enzymes and oxidative stress parameters vary with the level of complex pollution, including chemical elements. Activity of SOD and CAT, as well as LPO and oxidized protein levels in the liver of *Scorpaena porcus* from the most contaminated areas were significantly higher as compared to other tested sites [2, 30, 33].

Aminotransferase enzymes are considered to be the other important biomarkers recommended for assessing toxic effects on the hydrobionts. The analysis of these parameters in the liver of fish enables to assess the functional state of the organ and the direction of metabolic changes in it. In our studies AST activity did not differ in the liver of *Neogobius melanostomus* from two locations, whilst ALT activity was more than 2 times higher in the liver of fish from ETB. The revealed feature against high concentrations of LPO products and OP in the liver of fish from ETB illustrates an adaptive compensatory increase in ALT activity – a supplier of pyruvate for ensuring the processes of gluconeogenesis and maintaining homeostasis in the liver cells of fish from this location. An increase in the aminotransferase activities and rise of oxidized proteins and pyruvate concentrations were also recorded in the liver of trout *Salmo trutta* and grayling *Thymallus thymallus* exposed to chlor-amine [35].

Albumin is a multifunctional serum protein synthesized in liver. It plays a key role in maintaining colloid osmotic blood pressure and protein reserve, performs antioxidant, transport and detoxification functions in organisms [36, 37]. Transport and detoxification functions are implemented through the ability to connect and transport free radical oxidation products, protein proteolysis products, xenobiotics, including heavy metals, to the site of disposal [36]. The albumin molecule has binding sites for cations of many metals (Cu, Ni, Ca, Mg, Zn, Cd, Hg, Al, Mn, Co, etc.) [38]. Therefore, to assess the organism's response to CE, as well as the protein-synthesizing function of liver, it is important to investigate the albumin content in the liver of fish. Our studies confirmed that albumin concentration was more than 2 times higher in the liver of specimens from ETB. This can be considered as an adaptive response of the organism to higher concentrations of Hg, Pb, and Fe in the liver of fish from ETB compared to BS, and illustrates a significant role of albumin in the detoxification of CE. An adaptive increase in albumin concentration in the liver of *Scorpaena porcus* was found in the most contaminated areas as compared to less polluted sites [33].

The study of ChE activity in liver of fish is suggested for assessing the protein-synthesizing function of the organ [39, 40], in blood serum – for evaluation of the neurotoxicity of the aquatic environment polluted by CE and by pesticides [41, 42]. Our studies show that ChE activity was 3 times higher in the liver of fish from ETB compared to BS which may reflect high intensity of protein-synthetic processes in the liver of fish from ETB. In addition this can be considered as a compensatory response aimed at replenishment of possible ChE "deficiency" in the blood serum and other tissues of fish under high CE and pesticide pollution in this location.

Conclusion

Due to the geographic isolation of Taganrog Bay, the size of the sediment fraction largely determines the formation of zones with different sorption capacity and, accordingly, the ability to accumulate pollutants. ETB area is characterized by fine-grained fractions with a high clay content and pronounced sorption ability, chronic influx of pollutants from river runoff and industrial enterprises. All these lead to pollutant accumulation in sediments, including chemical elements. Comparing the results of toxicological and biochemical studies allowed us to establish higher concentrations of Hg, Pb and Fe in the liver of *Neogobius melanostomus* from ETB which resulted in: inhibition of SOD activity and a shift of the prooxidant–antioxidant balance towards increased peroxidation of proteins and lipids; reorganization of protein metabolism aimed at the energy supply of hepatocytes; increased synthesis of albumin involved in elimination of chemical elements; adaptive compensatory increase of ChE activity.

To conclude, the toxicological and biochemical characteristics of *Neogobius melanostomus* liver demonstrate significant informativeness for assessing the quality of marine water bodies contaminated with chemical elements and thus can be used in biomonitoring programs.

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