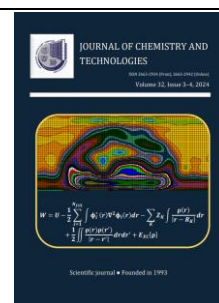




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STUDY ON TNT TOXIC EFFECTS ON THE FUNCTIONAL STATE OF HYDROBIONTS IN THE MODEL CONTAMINATED WATER POND

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Abstract

TNT (2,4,6-trinitrotoluene) and its derivatives make a significant contribution to the pollution of the water environment caused by hostilities in Ukraine, but the effects of TNT on hydrobionts have been little studied. In a model experiment, the effect of TNT in concentrations of 10–75 mg/L on the mortality rate of *Daphnia magna* crustaceans was observed during 96 hours, and $LD_{50} = 41.8$ mg/L was calculated. Impact of TNT on *Carassius gibelio* fish metabolic processes and red blood cells characteristics were studied during chronic (21 days at a dose of 5 mg/L) and acute (eight hours at a dose of 35 mg/L) experiments. A sharp increase in lipid peroxidation and glutathione S-transferase activity were recorded in the blood and spleen of *Carassius gibelio* during the chronic TNT action indicating the state of power oxidative stress and detoxification mechanisms activation. Both chronic and acute exposure to TNT caused an increase in the destructive changes in red blood cells of *Carassius gibelio* as compared to control, including size and shape variation, decreased hemoglobin content, nuclear shift, and cell division disruption. Cytoplasmic vacuolization in fish erythrocytes was detected under the influence of both TNT concentrations, while karyolysis only under acute exposure. The obtained data testify to the severe pathological consequences of 2,4,6-trinitrotoluene impact on the viability and functional state of aquatic organisms under the model conditions and dictate the need to conduct similar studies in the natural polluted aquatic ecosystems to identify ways of their restoration.

Keywords: TNT; aquatic organisms; toxicity; GST; POL; erythrocytes pathologies.

ДОСЛІДЖЕННЯ ТОКСИЧНОГО ВПЛИВУ ТРОТИЛУ НА ФУНКЦІОНАЛЬНИЙ СТАН ГІДРОБІОНТІВ У МОДЕЛЬНІЙ ЗАБРУДНЕНІЙ ВОДОЙМІ

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Анотація

У статті описано математичну модель газової хроматографії як набір концентраційних хвиль, що Тротил (2,4,6-тринітротолуол; ТНТ) і його похідні роблять вагомий внесок у забруднення водного середовища, спричинене бойовими діями в Україні, проте ефекти дії тротилу на організми гідробіонтів мало досліджені. У модельному експерименті вплив тротилу в концентраціях 10–75 мг/л на смертність ракоподібних *Daphnia magna* спостерігали протягом 96 годин і розрахували $LD_{50} = 41,8$ мг/л. Вплив тротилу на метаболічні процеси риб *Carassius gibelio* і характеристики еритроцитів риб досліджували під час хронічного (21 день при дозі 5 мг/л) і гострого (8 годин при дозі 35 мг/л) експериментів. У крові та селезінці *Carassius gibelio* за хронічного впливу ТНТ зареєстровано різке підвищення рівня перекісного окислення ліпідів та активності глутатіон-S-трансферази, що свідчить про стан потужного оксидативного стресу та активацію механізмів детоксикації. Як хронічний, так і гострий вплив тротилу викликав збільшення деструктивних змін в еритроцитах *Carassius gibelio* порівняно з контролем, включаючи зміну розміру та форми, зниження вмісту гемоглобіну, зсув ядра і порушення поділу клітин. Вакуолізація цитоплазми в еритроцитах риб виявлена за впливу обох концентрацій ТНТ, а каріолізис тільки при гострій дії. Отримані дані свідчать про важкі патологічні наслідки впливу 2,4,6-тринітротолуолу на життєздатність і функціональний стан водних організмів за модельних умов та диктують необхідність проведення подібних досліджень у природних забруднених водних екосистемах для виявлення шляхів їх відновлення.

Ключові слова: ТНТ; водні організми; токсичність; GST; ПОЛ; патології еритроцитів.

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Introduction

The use of explosives is accompanied by a large-scale release of heavy metals and nitrogen-containing compounds, in particular TNT and its transformation products [1], which occurs during hostilities on a large territory of Ukraine. One of the dangerous consequences is the degradation of aquatic ecosystems, which often become the final recipients of pollutants [2]. The vulnerability of aquatic ecosystems is illustrated by Russian troops undermining the Oskil dam (in 2022) resulted in almost complete shallowing of the reservoir, the destruction of valuable fish species and other aquatic life [3], and the Kakhovka dam (in 2023), which caused chemical pollution and habitat destruction, the restoration of which requires US\$59.5 million [4], and the war-tainted aquatic ecosystems of the Kyiv region including water ecosystems of the Moschun [5; 6].

Ecotoxicological threats are even carried by submerged munitions from the time of the First and Second World Wars, which currently hinder human activities in the oceans, including fishing

and shipping or the construction of pipelines [7; 8]. Long-term danger of TNT and products of its transformation for aquatic ecosystems was established by the study of biota in the Baltic Sea coastal waters near the relict munitions dumpsite, where one or more compounds were detected in 98 % of organisms (plankton, macroalgae, tunicate, sponge, mollusk, echinoderm, polychaeta, anemone, crustacea, fish), including industrially important fish species [9], as well as and blue mussels in which TNT metabolite was detected in a quantity of 250 ng/g [10].

TNT (2,4,6-trinitrotoluene) is considered the most widely used nitroaromatic compound for munitions production which has toxic effects on biota [11]. The relatively high solubility of TNT in water in the range of 70–120 mg/L provokes its absorption and accumulation by aquatic organisms [12]. The observation of biota from the munition's dumpsite [9] has proven that TNT and its transformation product compounds ADNT and DANT were detected more frequently and at higher concentrations, indicating the greatest polluting potential of these compounds (Fig. 1).

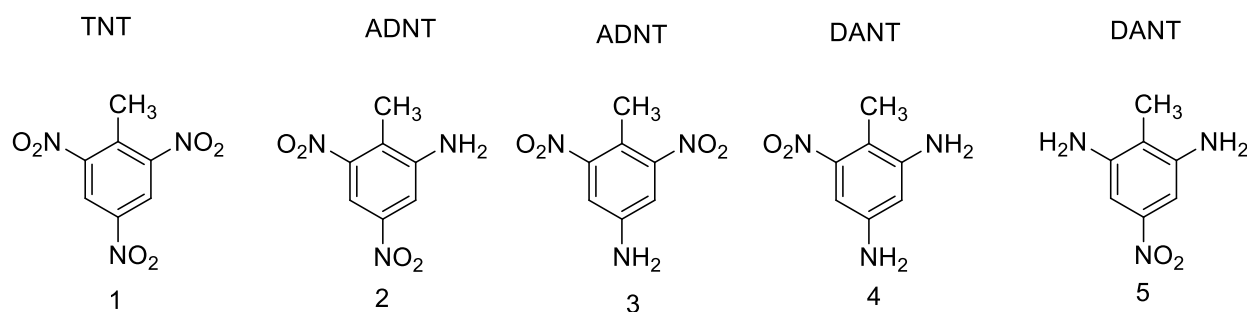


Fig. 1. Explosive substances with the greatest potential accumulation in biota according to [9]: 2,4,6-trinitrotoluene (1); 2-amino-4,6-dinitrotoluene (2); 4-amino-2,6-dinitrotoluene (3); 2,4-diamino-6-nitrotoluene (4); 2,6-diamino-4-nitrotoluene (5)

Moreover, once in the aquatic environment, TNT is able to migrate through food chains, which poses a significant threat to the ecosystem in general and the health of seafood consumers in particular [13]. In order to avoid or mitigate environmental risks caused by explosives, complex studies of the impact of nitroaromatic compounds on the environment are being conducted [14; 15], new methods of detecting TNT and its metabolites in environmental objects are being developed [16; 17], and effective adsorption, chemical, electrochemical, photochemical methods for removing TNT and other nitroarenes from wastewater, soil, and sea water are proposed [18; 19]. The study of the interaction of 2,4,6-trinitrotoluene with biological membranes showed that its molecules, after

penetrating the membrane, can show a synergistic effect, contributing to the absorption, penetration and bioaccumulation of subsequent TNT molecules [20].

The ability of hydrobionts to biodegrade 2,4,6-trinitrotoluene molecules was found in freshwater algae [21] and channel catfish [22], as well as in rainbow trout [23] and European eel [24], which metabolized TNT to 2-amino-4,6-dinitrotoluene (2-ADNT) or 4-amino-2,6-dinitrotoluene (4-ADNT). Although the direct effect of TNT on fish organisms is poorly studied, the toxicological aspects of various xenobiotics action indicate oxidative stress [25; 26] and activation of the antioxidant and detox system of fish [27; 28] as a universal reaction. The intervention of toxicants disrupts the redox balance of cells in favor of pro-

oxidants, which provokes excessive formation of reactive oxygen species (ROS), which are one of the most common oxidants in cells [29]. In turn, ROS (hydroxyl and hydroperoxyl radicals) can interact with polyunsaturated fatty acids triggering the sequential formation of lipid radical (L·), lipid peroxide radical (LOO·), and lipid hydroperoxide (LOOH). Such a condition, known as oxidative stress, poses a great threat to biomolecules, including unsaturated lipids contained in the lipid layers of cell membranes [30].

The extreme sensitivity of lipids to oxidant attacks causes lipid peroxidation (LPO) with the formation of derivatives, in particular, polyunsaturated fatty acids form malondialdehyde (MDA), 4-hydroxy-2-nonenal and F2-isoprostane [31], which serve as markers of lipid peroxidation [32]. In turn, MDA is chemically active and can react with DNA fragments and amino acids in proteins.

One of the fish detox mechanisms is provided by glutathione S-transferase (GST) enzymes capable of deactivating various toxic molecules by conjugating them with reduced glutathione [33; 23]. Activation of GST under the action of 2,4,6-trinitrotoluene was observed in the liver of the European eel both at the level of gene expression and at the level of enzymatic activity [34], but little is known about the functioning of glutathione S-transferase under the influence of TNT in the tissues of other fish species. The aim of the work was to find out the toxicity degree of 2,4,6-trinitrotoluene for hydrobionts and to reveal the TNT effects on hematological parameters of fish blood, lipid peroxidation processes and detoxification mechanisms functioning in fish body under the chronic and acute influence of TNT.

Experimental

The study was conducted in May 2024 in the laboratories of Oles Honchar Dnipro National University (Dnipro city, Ukraine). TNT in the bottom soil of natural reservoir of the Dnieper region was detected by the formation of red-colored Janowsky complex [35]. Briefly, 20 g of soil was crushed and extracted with 100 mL of acetone. After sedimentation, the supernatant was filtered, evaporated using IKA® RV 10 (Germany) rotary evaporator, followed by precipitate dissolving in 10 mL of acetone. Further, 2 mL of studied solution was mixed with 2 mL of 5 % KOH, and absorption was measured at 540 nm. Using the calibration curve in the range 1–100 mg/L (y

$= 0.0031x$, $R^2 = 0.9846$), TNT soil content was calculated (Table 1).

Table 1

Detection of TNT in the bottom soil of a natural reservoir			
Indicators	Bottom soil samples		
	I	II	III
Adsorption, D_{540}	0.003	0.004	0.004
TNT content, mg/kg	0.048	0.065	0.065
Average, mg/kg	0.059 ± 0.011		

The toxicity of TNT was studied using as test objects the hydrobionts provided by the scientific complex "Aquarium". The effects of TNT on the known bioindicator freshwater crustacean *Daphnia magna* was determined according to [36], taking 10 samples of crustaceans in three replicates for each concentration of TNT. The effect of 2,4,6-trinitrotoluene on hematological characteristics of fish blood and metabolic processes in fish tissues was studied on five-year-old Prussian Carp (*Carassius gibelio* Bloch), since this fish species has a wide distribution area and is found in almost all reservoirs of the region. Fish (four specimens for chronic and acute experiments and control as well) were kept in settled tap water in aerated aquariums for 10 days before the experiments and received food both during the acclimatization and experiment.

2,4,6-trinitrotoluene was produced in analytical quantities according to [37], and its identity was confirmed by gas chromatography-mass spectrometry. Water pollution was simulated by adding TNT at final concentrations of 10, 25, 50, and 75 mg/L to reservoirs with *Daphnia magna*, and 5 mg/L (chronic experiment) and 35 mg/L (acute experiment) to aquaria with *Carassius gibelio* fish. *Daphnia magna* were exposed to TNT for 96 hours with counting the number of live samples at 12, 24, 48, 72, and 96 hours. The duration of chronic fish treatment with 2,4,6-trinitrotoluene was 21 days, and the duration of acute experiment was eight hours, followed by the selection of tissues samples for hematological and biochemical studies.

Fish blood was taken from the tail vein; histological preparations were made by staining blood smears as described previously [38]. The samples were analyzed using a microscope "Ulab XY-B2TLED" (magnification 400) and Sigeta M3CMOS FMA050 16.0 camera, processing 10 preparations (at least 1000 cells) for each experiment. Abnormal forms of erythrocytes were determined according to [39], indicating the number of pathological red blood cells as a

percentage of the total cell number on the preparation.

The intensity of lipid peroxidation (LPO) in fish tissues was evaluated according to [32] (Garsia) by the content of MDA at 532 nm after reaction with thiobarbituric acid, and was expressed as nmol MDA g⁻¹ spleen tissue or nmol MDA mL⁻¹ blood.

The activity of glutathione-S-transferase was determined by the method [40] at 340 nm with 2,4-dinitrochlorobenzene (DNCB) as a substrate, and expressed as μM DNCB sec⁻¹g⁻¹ spleen tissue or as μM DNCB sec⁻¹mL⁻¹ fish blood.

All measurements were repeated at least three times. Study results proceeding was carried out with Statistica 7.1 StatSoft statistical software. One-way ANOVA was used to analyze all data; the results were represented as mean value ± SD ($\bar{x} \pm SD$), and the mean values were compared using Tukey's HSD. Differences were considered significant at $p \leq 0.05$.

Bioethical standards were not violated during the research, which was performed in accordance

with the Regulations on the Ethics Committee (Bioethics) in Ukraine [41].

Results and Discussion

In the course of determining toxicity of 2,4,6-trinitrotoluene in the concentration range of 10–75 mg/L for hydrobionts using *Daphnia magna* as a test object, a dose-dependent effect of TNT on the level of mortality of test organisms was established (Fig. 1).

A decrease in the number of live *Daphnia magna* at the lowest concentration of 2,4,6-trinitrotoluene was observed after 72 hours of exposure and reached 86.7 % of the control at the end of the experiment. At the highest dose of TNT, the number of live *Daphnia magna* was significantly reduced after 12 hours of exposure, being only 5 % of the control at the end of the experiment. The obtained data indicate the toxicity of TNT in the investigated concentration range for aquatic organisms and characterize *Daphnia magna* as an acceptable test object for monitoring water contamination with TNT.

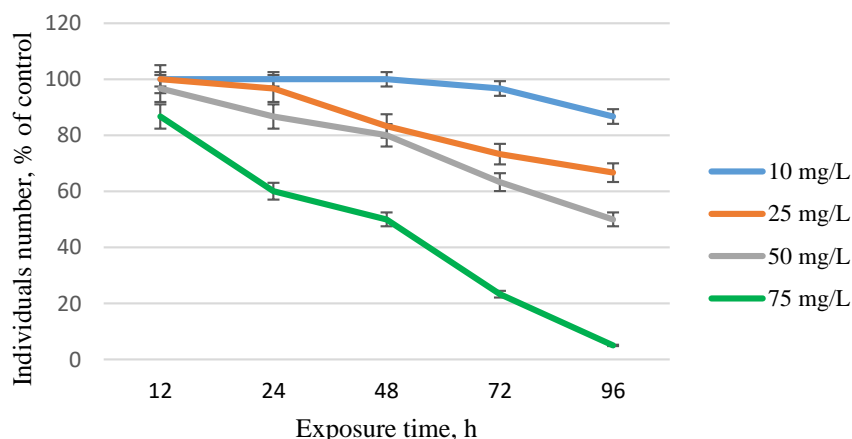


Fig. 1. The dynamic of mortality of *Daphnia magna* depending on TNT concentration and exposure time in the model contaminated water reservoir

The dependence of the number of live *Daphnia magna* specimens on the concentration of TNT in the reservoir had the form of the equation: $y = -9.1955x + 99.997$ ($R^2 = 0.9745$), according to which $LD_{50} = 41.8$ mg/L was calculated.

Taking into account the decrease in the viability of *Daphnia magna* even at the lowest (10 mg/L) 2,4,6-trinitrotoluene content in the aquatic environment, TNT concentrations of 5 mg/L and 35 mg/L were chosen for the investigation of chronic and acute toxic effects on *Carassius gibelio* fish. According to the results of chronic (for 21 days) action of TNT on *Carassius gibelio*, a sharp increase in the intensity of lipid peroxidation and

activity of glutathione-S-transferase in the both fish blood and spleen was recorded (Fig. 2).

The activation of glutathione S-transferase detected in the blood and spleen of *Carassius gibelio* fish is consistent with reported data on other fish tissues during exposure to 2,4,6-trinitrotoluene. In European eel (*Anguilla anguilla*) liver, under TNT concentrations of 0.5, 1.0, and 2.5 mg/L, the expression of the GST gene increased after 24 hours, and a significant increase in GST activity was observed both after 6 and after 24 hours only at the highest concentration of TNT [24].

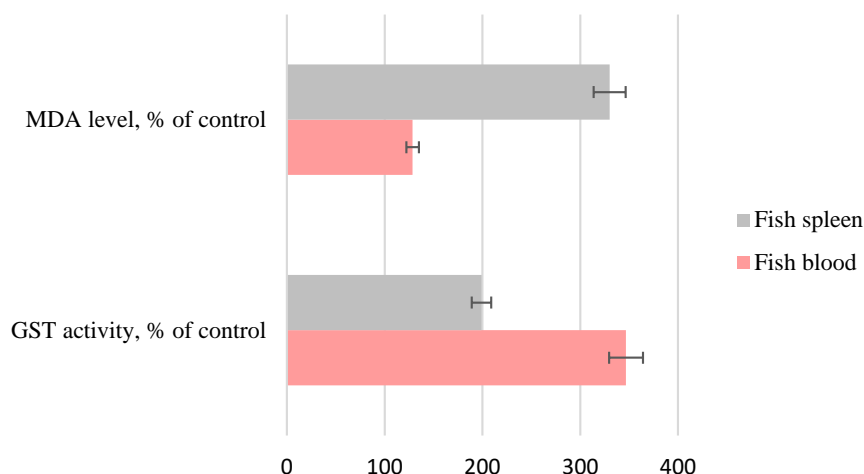


Fig. 2. Intensity of LPO and GST activity level (% of control) in the blood and spleen of *Carassius gibelio* fish after 21 days of treatment with TNT at a concentration of 5 mg/L

Increased activity of glutathione S-transferase was also detected in the bile of rainbow trout (*Oncorhynchus mykiss*) 72 hours after intraperitoneal injection with TNT at doses of 100–400 mg/kg body weight [23].

A two-fold increase in GST activity together with an almost three-fold increase in MDA level in *Carassius gibelio* spleen indicates a high oxidative stress in this tissue, which belongs to the fish hematopoietic system. In the blood of fish, GST activation reached a much higher level than in the spleen, which could determine the lower intensity of LPO processes, because some isoforms of glutathione S-transferase can perform the function of neutralizing ROS and organic peroxides as well [42]. In general, changes in spleen and especially in blood of *Carassius gibelio* due to the chronic exposure to TNT correspond to the signs of systemic oxidative stress in the body of fish [32; 43].

Lipid peroxidation is one of the main causes of cell membrane damage, which triggers an

important mechanism of cellular pathology and leads to many negative effects in different tissues and organs of the fish body. Hematological analysis of blood smears of *Carassius gibelio* revealed quantitative and qualitative variation in erythrocytes, corresponding to the known erythrocyte's changes in a toxic environment [44], which was recognized as an adaptive response to hypoxia. This compensatory reaction is aimed at improving the functional state of fish, when the toxic agent causes general stress, worsens gas exchange, or enhances detoxification pathways [26], which was observed in our experiment in the *Carassius gibelio* blood and spleen under the influence of TNT.

Compared to the control *Carassius gibelio* blood samples, chronic and acute exposure to 2,4,6-trinitrotoluene caused an increase in the number of abnormal erythrocytes. The pathologies concerned both structural disorders of red blood cells and nuclear abnormalities (Table 2).

Table 2

Erythrocyte pathologies in *Carassius gibelio* blood under TNT action

Exposure time	Abnormal forms of red blood cells, % of total number of cells ($\bar{x} \pm SD$)						
	poikilocytosis	anisocytosis	hypochromia	vacuolation	nucleus shift	karyolysis	amitosis
8 hours	4.1 ± 0.48 ^a	29.1 ± 5.18 ^a	14.2 ± 3.85 ^a	7.7 ± 2.30 ^a	8.8 ± 1.66 ^a	2.0 ± 0.01	2.0 ± 0.32 ^a
21 days	9.0 ± 0.93 ^b	7.4 ± 0.52 ^b	2.8 ± 0.45 ^b	1.7 ± 0.55 ^b	3.2 ± 0.70 ^b	not found	1.0 ± 0.01 ^b
Control	1.8 ± 0.49 ^c	2.3 ± 0.42 ^c	1.3 ± 0.04 ^c	not found	2.9 ± 0.74 ^b	not found	0.74 ± 0.17 ^c

Note. Different letters in a column indicate statistically significant differences in mean values according to the Tukey test ($P < 0.05$).

The main structural disorders of *Carassius gibelio* erythrocytes were the changes in the shape of cells (poikilocytosis), their size (anisocytosis), a

decrease in hemoglobin amount in erythrocytes (hypochromia), and vacuolization of red blood cell cytoplasm (Fig. 3).

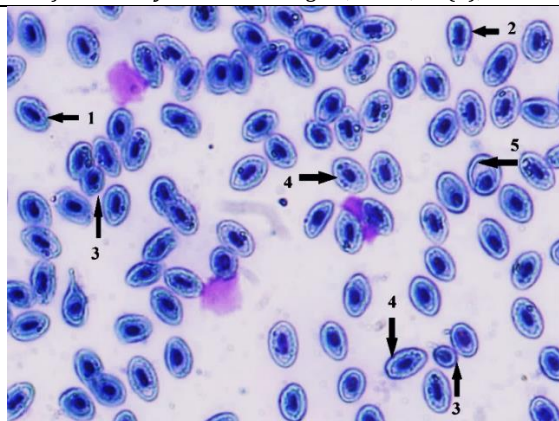


Fig. 3. Alterations in *Carassius gibelio* red blood cells under 2,4,6-trinitrotoluene action: 1 - normal erythrocyte; 2 - poikilocytosis; 3 - anisocytosis; 4 - cytoplasm vacuolation; 5 - hypochromia

The most striking changes were related to the morphological parameters of *Carassius gibelio* erythrocytes, including an increase in the number of anisocytes (12.7 and 3.2 times higher than control, under acute and chronic TNT exposure respectively) and poikilocytes (three and five times higher than control). Structural abnormalities of erythrocytes, detected in *Carassius gibelio* under the influence of 2,4,6-trinitrotoluene, arise as one of the first biomarkers of various toxicants action on fish organisms which were considered as pathological symptoms [45]. In particular, different morphological disorders of erythrocytes in peripheral fish blood were found in *Carassius gibelio* from the most radionuclide-contaminated water bodies of the Chernobyl Exclusion Zone [46].

Anisocytosis of erythrocytes can occur due to the effects of toxic agents, as well as due to insufficiency of hematopoietic organs and anemia accompanied by oxygen starvation [47]. Poikilocytosis of erythrocytes is considered a degenerative phenomenon that occurs as a result of inhibition of the process of formation of red blood cells due to a negative effect on the hematopoietic organ or on blood cells directly [48; 49]. Based on our results, it appears that TNT-induced processes in *Carassius gibelio* red blood cells which lead to changes in the erythrocytes size are weakened during chronic exposure, while processes affecting the erythrocytes shape are significantly enhanced. Our findings are consistent with the data [50] that for the fish organism, poikilocytosis can be a compensatory phenomenon that serves to increase the surface of red blood cells.

Vacuolization of the erythrocyte's cytoplasm, absent in *Carassius gibelio* control samples, was 4.5 times higher during acute exposure to TNT

than during chronic exposure. Such a pathology occurs due to swelling of mitochondria and destruction of cell organelle membranes under the action of toxicants [26]. Hypochromia, characterized by the pale coloration of erythrocytes with areas of unstained cytoplasm, increased in the blood of *Carassius gibelio* under the acute and chronic effects of TNT by 10.9 and 2.2 times, respectively, which according to [47] may be a consequence of inhibition of the erythrocyte formation process.

In addition, hematological analysis revealed various nuclear pathologies in *Carassius gibelio* erythrocytes under the influence of TNT, including karyolysis, nuclear displacement and micronuclei induction, as well as amitosis, i.e. disorders of erythrocytes mitotic division (Fig. 4).

Nucleus shift in erythrocytes of *Carassius gibelio* was indicative under acute TNT exposure (three times higher than control), but did not differ from control under chronic exposure. Denucleated erythrocytes were found only under acute action of 2,4,6-trinitrotoluene, while cell division disorders were enhanced under high and low concentrations of the toxicant (by 2.7 and 1.4 times, respectively).

Nuclear deformations in red blood cells of fish are considered as an indicator of cytotoxicity and genotoxicity of xenobiotics [26]. In particular, micronuclei were identified in the erythrocytes of *Labeo rohita* fish under the influence of an azo dye and defined as severe nuclear abnormalities [45]. The action of heavy metal mixtures provoked micronuclei formation in the erythrocytes of *Channa punctatus* fish [51], as well as micronuclei and other nuclear abnormalities (vacuolated and deformed nuclei, karyolysis) in two different Nile tilapia species [52], which was evaluated as the genotoxic effects.

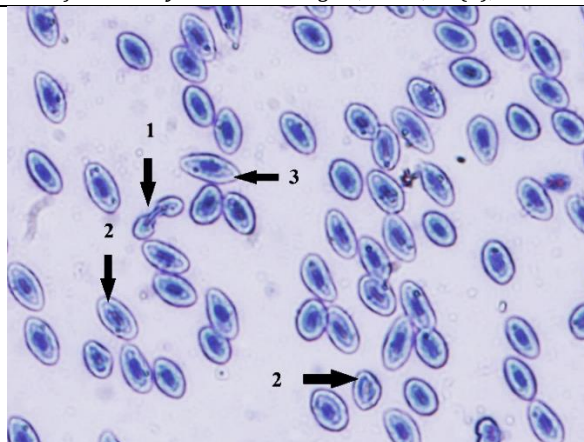


Fig. 4. Nuclear anomalies in *Carassius gibelio* red blood cells under 2,4,6-trinitrotoluene action: 1 - amitosis; 2 - karyolysis; 3 - nucleus shift and micronuclei

An increase in the number of cases of amitosis by almost 15 times was detected in erythrocytes of *Carassius gibelio* from water body contaminated with radionuclides and determined as an indicator of violation of cells genetic structures due to long-term radiation exposure [53]. The formation of denucleated erythrocytes in fish blood is possible in anemias accompanied by oxygen starvation, the consequence of which is the massive pushing out of nuclei from red blood cells and a decrease in the consumption of oxygen carried by the cell [54]. Therefore, the appearance of denucleated red blood cells, noted in *Carassius gibelio* under the acute influence of TNT, is one of the adaptive reactions of the fish organism, which ensures the preservation of a certain oxygen transport level.

Conclusion

The effect of 2,4,6-trinitrotoluene in the concentration range of 10–75 mg/L on the survival of hydrobionts, assessed on the test object *Daphnia magna*, had a dose- and duration-dependent nature, and was correctly described by the regression equation, according to which $LD_{50} = 41.8$ mg/L. The obtained data indicate the toxicity of TNT in the investigated concentration range for aquatic organisms and characterize *Daphnia magna* as an acceptable test object for monitoring water contamination with TNT.

The chronic effect of TNT (5 mg/L for 21 days) caused an increase in the MDA accumulation and GST activity in the blood and spleen of *Carassius gibelio*, which indicates the intensification of lipid peroxidation (LPO) and the activation of detox mechanisms as markers of total oxidative stress in the body of fish. In the blood of *Carassius gibelio*, strong GST activation contributed to the maintenance of lipid peroxidation processes at a relatively low level, while in the spleen of fish,

where GST activation was less significant, a strong increase in LPO level was observed.

Hematological analysis of the blood of *Carassius gibelio*, acutely and chronically exposed to 2,4,6-trinitrotoluene, revealed an increase in the structural abnormalities of red blood cells relative to the control, as well as the appearance of pathologies absent in the control, such as cytoplasmic vacuolation and karyolysis. In general, acute exposure to TNT (35 mg/L for 8 hours) caused a stronger increase in all abnormalities, except the poikilocytosis, which reached a higher level under the chronic TNT influence. Nuclear abnormalities, such as nucleus shift and micronuclei, karyolysis and amitosis, were most clearly manifested under acute exposure to TNT and serve as markers of cytotoxicity and genotoxicity of 2,4,6-trinitrotoluene for *Carassius gibelio*.

Compensatory reactions aimed at improving the functional state of fish under chronic and acute exposure to TNT included the changes in the shape and size of erythrocytes to increase the surface area of cells. The formation of denucleated erythrocytes under the acute TNT influence can contribute to the reduction of oxygen consumption in erythrocytes and support the transport of oxygen in the body of fish.

Sharp changes in the metabolic processes in the blood and spleen of *Carassius gibelio* and related violations of the morphological parameters of erythrocytes, a decrease in the hemoglobin content and nuclear abnormalities in the red blood cells collectively indicate a state of powerful oxidative stress in the body of fish under the simulated effect of 2,4,6-trinitrotoluene. At the same time, the obtained results indicate the need for studies of the toxic effects of TNT and other explosive substances in the natural water

ecosystems of Ukraine, polluted due to prolonged hostilities.

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