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Influence of 2,4,6-trinitrotoluene on hemolymph of *Procambarus virginalis* (Decapoda, Cambaridae)

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2,4,6-trinitrotoluene (TNT) is a xenobiotic that exerts toxic effects on all forms of life. Due to active military operations, uncontrolled amounts of this compound are released into ecosystems, making it essential to investigate the potential adaptive responses of tissues and organs. The reaction of hemolymph cells of marble crayfish *Procambarus virginalis* (Lyko, 2017) to the effects of 2,4,6-trinitrotoluene (TNT) was studied in a chronic model experiment. The relevance of the topic is due to the toxic load of explosives on aquatic ecosystems of Ukraine as a result of military operations. Exposure to TNT at concentrations of 25 and 50 mg/L caused significant morphological changes in the immune system cells of the crustaceans. When exposed to 25 mg/L of TNT for 4 weeks, an increase in the average area of hyalinocytes by 79% was observed (from 145.3 μm² in the control to 260.1 μm²), which may indicate the activation of the functional state of hemocytes and the mobilization of the immune response. Such an adaptive reaction indicates a possible increase in the phagocytic or secretory activity of cells in response to a sublethal concentration of the toxicant. It was found that under the influence of a lower concentration, the area of hyalinocytes was 1.79 times larger than in the control, which indicates an active immune response. However, under the influence of a higher concentration, the area of the same cells decreased by 1.25 times, and the area of blasts – by 1.44 times compared to the control, which may indicate an intensive inhibition of hematopoiesis. Blasts were especially sensitive to a high concentration of TNT – their area decreased by 30.6% (from 85.8 to 59.6 μm²). This indicates the inhibition of the maturation processes of young cells or the death of cell precursors under conditions of chronic toxic stress. The application of morphometric analysis of hemolymph cells as a sensitive biomarker allows to assess both adaptive and destructive changes in invertebrates under the influence of xenobiotics. The obtained data can be used for biomonitoring the condition of water bodies contaminated with warfare-related substances.

Keywords: crayfish; hydrobionts physiology; aquatic ecology; toxicity; anthropogenic pollution; bioindication.

Introduction

2,4,6-trinitrotoluene (TNT) is a known xenobiotic that's toxic to all living organisms, including aquatic invertebrates (Strehse et al., 2020). Researchers have noted both acute and chronic toxicity of TNT for various species of organisms: in particular, a negative impact has been established on aquatic plants, crustaceans (shrimp, amphipods, daphniids, copepods), corals, bivalve mollusks, and fish (Nipper et al., 2001; Ownby et al., 2005; Rosen & Lotufo, 2007; Ek et al., 2008; Lotufo et al., 2016; Sharamok et al., 2024).

Even at low concentrations, TNT can exhibit genotoxic effects – for example, in experiments with fish embryos (*Danio rerio* Hamilton, 1822; Cypriniformes, Danionidae), it has been confirmed that TNT causes DNA damage and developmental anomalies (Koske et al., 2019).

Aquatic invertebrates are particularly vulnerable to TNT due to the constant contact of their body and gill epithelium with contaminated water or bottom sediments. Studies on model invertebrates, such as earthworms (as a model of organisms that contact water in the soil), demonstrate clear signs of TNT immunotoxicity: even sublethal doses lead to DNA damage in the immune cells of the worm (coelomocytes) and disruption of lysosomal function, which was manifested by a decrease in the retention time of neutral red dye – a marker of lysosomal membrane stability. At the same time, a dose-dependent pattern was observed: low doses caused reversible damage (with activation of repair), while high doses resulted in persistent damage without recovery. This indicates that the immune system of invertebrates can be suppressed by excessive amounts of TNT – cells don't have time to recover and die (Fuchs et al., 2011).

In the study of mussels (*Mytilus* spp.), researchers detected the induction of protective genes under the influence of TNT, which can be considered as biomarker of toxicity. In particular, the expression of the carbonyl reductase gene significantly increased in mussel tissues after three weeks of exposure to TNT at relatively low concentrations (Strehse et al., 2017; Adomako-Bonsu et al., 2024).

TNT is metabolized in the body with the formation of reactive intermediates (nitroso- and hydroxylamine derivatives), which initiate redox cycle reactions (Adomako-Bonsu et al., 2024). This leads to excessive production of reactive oxygen species (ROS), which damage cellular macromolecules – DNA, proteins, and lipids. As a result, oxidative stress arises, and the cell's antioxidant systems become depleted. It has been proven that under the influence of TNT, DNA adducts appear in mammalian cells, and the activity of antioxidant enzymes decreases, confirming its cytotoxicity through a prooxidant mechanism (Adomako-Bonsu et al., 2024). Excessive ROS formation can damage cellular membranes and organelles; in particular, in mammalian erythrocytes, TNT induces hemolysis, methemoglobin formation, and a decrease in hematocrit levels (Shinkai et al., 2015). Similarly, in invertebrates, TNT can destabilize lysosomal membranes and disrupt hemocyte functions (Fuchs et al., 2011).

Under the influence of TNT, cells can activate controlled cell death programs (apoptosis) in response to damage. In human liver cell cultures, TNT significantly reduced cell viability and caused DNA strand breaks (Liao et al., 2017). The accumulation of ROS due to TNT exposure triggers intracellular stress signals, including the loss of mitochondrial membrane potential, activation of caspase-9 and -3, and endoplasmic reticulum signaling, indicating apoptosis induction. Scientific studies note that the use of an antioxidant (N-acetylcysteine) reduced DNA damage and cell death under TNT exposure, suggesting that apoptosis was specifically mediated by endoplasmic reticulum stress (Liao et al., 2017).

Although TNT is not a pathogen, the damage it causes to cells and tissues can trigger a nonspecific immune response in invertebrates. Hemocytes respond to stress and damage by activation and migration. Specifically, in crustaceans, rapid release of hemocytes from tissue depots into the circulatory system is observed under any stress

(Zheng et al., 2021). This means that the toxic effect of TNT can cause hemocytosis – a mass release and mobilization of immune cells to eliminate damaged cells and restore homeostasis. Haemolymphocytes in crustaceans are capable of phagocytosis (Bouallegui, 2021), so when cell debris or toxin-induced damage appears, these hemocytes can actively consume it. At the same time, humoral mechanisms in the haemolymph may be activated – for example, the phenoloxidase system, which leads to melanization around damaged areas, isolating the toxin or damage. Therefore, TNT indirectly stimulates the immune system through the induction of damage: in response, crustaceans enhance hemocyte functions (phagocytosis, nodule formation, encapsulation), but at high doses, this protective response may be suppressed due to the mass death of immune cells (Bouallegui, 2021).

The impact of TNT on decapod crustaceans has not been sufficiently studied so far, but by analogy with other species, negative effects of toxic exposure can be expected. It is known that in some species of shrimp and crabs, the toxicity of explosive substances affects growth and survival rates, and changes in hemolymph parameters may also be observed. For example, prolonged exposure to explosives can lead to a decrease in the total number of hemocytes and phenoloxidase activity in crabs, which in turn indicates immune suppression (Bouallegui, 2021).

Material and methods

The model experiment to study the effect of 2,4,6-trinitrotoluene on the hemolymph of marbled crayfish *Procambarus virginalis* (Lyko, 2017) was conducted in three aquariums, each with a volume of 5 L (1 control and 2 experimental). The oxygen content in the water was maintained at a level of 7–9 mg/L using an aerator. The water temperature was regulated by a thermostat, set at +24 °C. TNT was added to the experimental aquariums at concentrations of 25 and 50 mg/L. Each aquarium contained 10 specimens of marbled crayfish, with an average mass of 0.96 ± 0.16 g, corresponding to a single size-weight group. Feeding was done daily with the same amount of food. To prevent cannibalism, special chemically neutral protective shelters were provided for the decapod crustaceans.

The experiment lasted 4 weeks. The effect of TNT on the growth of marbled crayfish was assessed using morphometric measurements and weighing. To evaluate the impact on the physiological processes of the individuals, the hemolymph was studied. Hemolymph was collected by amputating 1/3 of the V pereopod, followed by placing a drop of hemolymph on a microscope slide to prepare a smear. The hemolymph was stained with azure-eosin according to Romanowsky-Giemsa.

To model the relationship between the survival of marbled crayfish (*P. virginalis*) and the concentration of 2,4,6-trinitrotoluene (TNT) in the aquatic environment, a logistic regression model was applied. This method was based on the assumption that the relationship between the toxicant concentration and the probability of survival follows a sigmoidal pattern and can be described by a logistic function:

$$P(x) = \frac{1}{1 + e^{-(a+b*x)}},$$

where $P(x)$ – the predicted probability of survival at TNT concentration x; a, b – model parameters determined empirically.

The model was constructed using survival data from three experimental groups: control (0 mg/L TNT) – 100%, experiment 1 (25 mg/L TNT) – 80%, experiment 2 (50 mg/L TNT) – 60%.

Each individual crayfish was treated as a separate binary observation unit (survived/did not survive), allowing the formation of a dataset suitable for logistic modeling.

During the experiment, the principles of bioethics were followed Regulations on the Ethics Committee (bioethics) (2012). Normative document of the Ministry of Education and Science, Youth and Sport of Ukraine. Order No. 1287. 19.11.2012.

Statistical data was expressed as mean (x) ± standard deviation (SD). For cell areas, a one-way analysis of variance (ANOVA) test was used, considering $P < 0.05$ to be statistically significant.

Results

The highest weight gain of marble crayfish, compared to the beginning of the experiment, occurred in the control group – they increased their mass by 0.574 g. In experimental aquarium 1 (TNT concentration 25 mg/L) an increase of weight was 0.366 g, while in experimental aquarium 2 (TNT concentration 50 mg/L), the increase was 0.245 g.

In the control group (0 mg/L TNT), representing conditionally clean water, 100% survival of crayfish was observed, indicating the absence of toxic effects (Fig. 1).

At a TNT concentration of 25 mg/L, survival decreased to 80%, which corresponds to a moderate toxic effect that does not yet cause mass mortality but may impair physiological functions.

At a concentration of 50 mg/L, survival dropped to 60%, indicating a high level of toxicity, where not only reduced viability is observed but also disruptions in metabolism and growth processes.

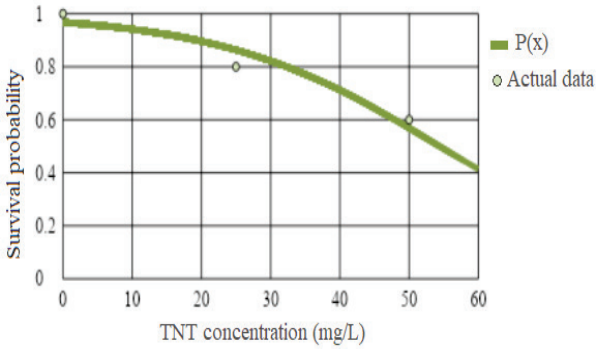

Fig. 1. Probability of crayfish survival at different TNT concentrations

From the logistic model, it is possible to calculate an approximate LC_{50} value, the concentration at which only 50% of the individuals survive. This value means that at a TNT concentration of 54.4 mg/L, half of the marbled crayfish individuals will not survive. LC_{50} is an important toxicological parameter used for risk assessment.

In crayfish exposed to 25 mg/L TNT for 4 weeks, a significant increase in the average area of hyalinocytes was observed: 260.1 ± 13.2 μm² compared to 145.3 ± 55.9 μm² in the control (Table 1). This 1.79 times increase in cell size may be a result of hemocyte activation under the influence of a moderate stressor.

Table 1
Area of hemolymph cells of marble crayfish exposed to TNT (x ± SD, n = 50, duration of experiment – 21 days)

Cell type	Control	TNT concentration, 25 mg/L	TNT concentration, 50 mg/L
Hyalinocytes, μm²	145.3 ± 55.9	260.1 ± 13.2*	116.2 ± 34.4*
Blast, μm²	85.8 ± 16.2	84.0 ± 18.7	59.6 ± 16.0*

Note: * – the difference between the experiment and the control is statistically significant at $P < 0.05$.

Similarly, in crayfish exposed to 25 mg/L TNT, compensatory mechanisms are likely activated, including enhanced metabolism in hyalinocytes, synthesis of protective proteins, and increased activity of antioxidant enzymes. Hyalinocytes may have enlarged due to increased secretory and phagocytic activity, such as consuming peroxidation products or cellular fragments. Additionally, an increased release of young hemocytes from hematopoietic tissues may contribute to the heterogeneity in hyalinocyte size, with activated cells appearing larger. As a result, a low dose of TNT may have stimulated the crayfish immune system, as evidenced by the enlargement of hyalinocytes, indicating their active functional state.

At a TNT concentration of 50 mg/L, the effect was opposite — the average area of hyalinocytes decreased to 116.2 ± 34.4 μm², which is lower than the control values (Fig. 2). In crayfish from the control group, the blast cell area was 85.8 ± 16.2 μm², while at a TNT concentration of 50 mg/L, it decreased to 59.6 ± 16.0 μm². This indi-

cates a negative impact of the high concentration, where severe toxic stress exceeded the adaptive capacity of the cells.

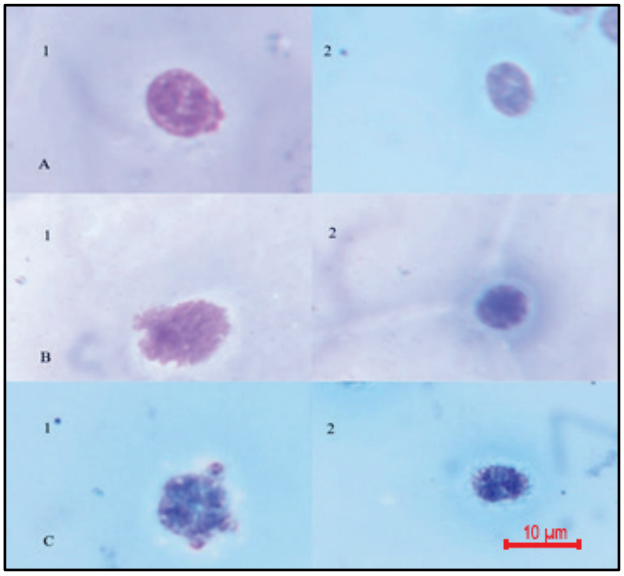

Fig. 2. The hemolymph cells of *P. virginalis*: *A* – control; *B* – experiment with a concentration of 25 mg/L; *C* – experiment with a concentration of 50 mg/L; *1* – hyalinocyte, *2* – blast

Discussion

The logistic model, constructed based on the obtained experimental data, reflects a typical dose-dependent response of a living organism to a toxic agent – in our case, the effect of dissolved TNT in water on the survival of marbled crayfish (Fig. 1). The sigmoidal shape of the curve is characteristic of many biological systems, where low concentrations of the toxicant cause little or no effect, followed by a phase of rapid decline in survival, and at high concentrations – a plateau of minimal viability, corresponding to near-complete lethality.

Although the obtained results are not statistically significant according to ANOVA ($P = 0.84$), they reveal a clear biological trend toward a dose-dependent toxic effect of TNT. It is important to note that survival of the crayfish appears to be a more sensitive indicator of toxicity in this experiment than changes in body mass.

TNT at concentrations of 25 and 50 mg/L reduces both weight gain and survival, triggering a stress response in the organism. Even in the absence of statistically significant differences in body mass, mortality rates indicate the potential hazard of chronic TNT exposure to invertebrates. Marbled crayfish may serve as bioindicators for assessing the toxicity of explosive compounds in aquatic environments.

Moderate, sublethal doses of the toxicant often induce an adaptive response or a hormesis effect, where minor damage stimulates the organism's defense systems. In particular, experiments with earthworms have shown that low concentrations of TNT initially increase the level of DNA damage, but after some time, the organism activates repair systems, and the extent of damage decreases to the control level (Fuchs et al., 2011).

The accumulation of toxic TNT metabolites at 50 mg/L may have caused damage to the hyalinocytes themselves. It is known that under intense endoplasmic reticulum stress induced by reactive oxygen species (ROS), cells undergo apoptosis, which is characterized by cell shrinkage and nuclear fragmentation (Liao et al., 2017). Therefore, the reduction in hyalinocyte size may indicate that some of these cells underwent apoptotic changes (shrinking before death) or were replaced by smaller cells. Moreover, prolonged exposure to a high TNT dose may have led to the depletion of the population of large, mature hyalinocytes, leaving primarily smaller and less differentiated cells. Parallels can be drawn with studies on worms: at high TNT concentrations, the level of DNA damage in immune cells remained persistently high, with no possibility of recovery (Fuchs et al., 2011).

This indicates that under strong toxin exposure, reparative and protective processes fail to neutralize the negative effect in time. In crayfish, this manifests as enlarged (activated) hyalinocytes being unable to maintain their size and function, leading to their degradation or replacement by smaller cells. In other words, at 50 mg/L TNT, the damaging effect prevails over the stimulatory one, leading to structural degeneration of hyalinocytes and a reduction in their average size.

Significant change in blast cells area indicates that the high TNT concentration particularly negatively affected young hemocytes. The reduction in blast cell size may suggest that, under TNT exposure, their cell cycle was slowed down, and they remain smaller, failing to mature to normal sizes or suffer from atrophy, leading to apoptosis (Liao et al., 2017).

Chronic exposure to the toxicant could disrupt the normal functioning of the hemopoietic organs of the crayfish, reducing the influx of fully functional new hemocytes into the circulatory system. Likely, in an attempt to compensate for the loss of immune cells, at a TNT concentration of 50 mg/L, the organism massively releases immature small blasts into the hemolymph. It is known that crustaceans have a mechanism for the rapid replenishment of hemocytes by releasing reserve cells from tissues (Zheng et al., 2021). If larger, mature hemocytes die from toxic exposure, less developed cells may replace them, which consequently are smaller in size. This explains why the average size of the blasts decreased – in the bloodstream, smaller precursor cells, released urgently, dominate. This effect is similar to the stress-induced shift of immature leukocytes into the blood of vertebrates during severe intoxication. It shouldn't be ruled out that TNT directly or indirectly damages the niches of hemocyte maturation (e.g., the adipose tissue or other organs of the crayfish). Damage to the support cells or signals required for hemocyte growth may lead to blasts not receiving the proper stimuli for development, remaining smaller. Therefore, the decrease in the area of the blasts at a TNT concentration of 50 mg/L indicates the immunosuppressive effect of the high TNT dose. The toxicant either directly destroys young hemocytes or disrupts their formation, leaving only the most resilient small cells in the hemolymph. This is dangerous for the organism as it limits its ability to adequately function in terms of immune system response and adaptation to stress.

The obtained results regarding the effect of 2,4,6-trinitrotoluene and previous studies on the impact of heavy metals (nickel, lead, manganese) (Naboka et al., 2018) on the hemolymph cells of *P. virginalis* confirm the general patterns of toxic effects of chemical pollutants on aquatic organisms, particularly on the immune-competent cells of invertebrates.

The comparison of experimental data showed that sublethal concentrations of TNT (25 mg/L) and lead ions have a similar biological effect – they cause an increase in the area of hemolymph cells: hemocytes increased in size by 1.79 times (TNT), and blast cells by 1.40 times (Pb). This effect aligns with the widely accepted concept of hormesis, where a small toxic load triggers the activation of immune system cells. Similar responses have been described in the works of Fuchs on earthworms, where sublethal doses of TNT stimulated reparative processes in coelomocytes, as well as in the studies of Strehse, where the expression of protective genes was activated in mollusks under TNT exposure (Strehse et al., 2020; Fuchs et al., 2011).

At the same time, at high concentrations of TNT (50 mg/L) and nickel ions, an opposite trend was observed – a reduction in cell size: the area of hemocytes decreased by 1.25 times (TNT) and by 1.70 times (Ni), while the area of blast cells decreased by 1.40 times (TNT). These results correlate with the studies of Liao, who described apoptosis, DNA damage, and disruption of mitochondrial homeostasis in cells exposed to TNT. Similar effects of morphometric disruption and cell shrinkage were described by Adomako-Bonsu for human liver cells (Liao et al., 2017; Adomako-Bonsu et al., 2024).

The reduction in cell area in experiments with Ni and TNT was also accompanied by morphological signs of degeneration, which aligns with the processes of apoptosis described in the literature. Therefore, the results obtained are consistent with the understanding of the cytotoxic effects of TNT and nickel ions through mechanisms

of oxidative stress, endoplasmic reticulum stress, and disruption of the cell cycle.

Conclusions

Thus, the general pattern observed when comparing the two series of experiments is as follows: moderate toxic load stimulates immune cells, increasing their size (activation phase); high concentrations of toxicants lead to cell death or degeneration, manifested by their reduction in size (suppression phase). These observations are consistent with the universal mechanisms of invertebrate response to xenobiotics: initially, the activation of protective processes, followed by immunosuppression once the adaptive potential is exceeded.

The authors declare no conflict of interest.

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