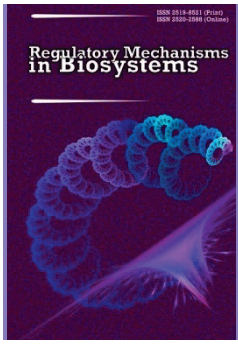

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Simulating 2,4,6-trinitrotoluene (TNT) elimination in a pond inhabited by freshwater algae of the *Rhizoclonium* genus

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Military operations over large areas of Ukraine lead to release of explosives and their derivatives into the environment with subsequent accumulation in natural and artificial water bodies, which unwittingly serve as reservoirs for collecting pollutants from the catchment area. The need to restore aquatic ecosystems dictates the search for efficient, cost-effective and environmentally friendly methods for the elimination of explosives, which corresponds to the processes of biological treatment. In this work, we examined the ability of common freshwater algae of the genus *Rhizoclonium* to detoxify 2,4,6-trinitrotoluene (TNT) under model conditions of water pollution (at a TNT concentration of 100 mg/L). The exposure time of the algae to TNT was 48 hours, during which the content of TNT and nitrites in the aqueous medium was monitored, as well as the content of chlorophyll and the activity of glutathione S-transferase in plant tissues. 2,4,6-trinitrotoluene was extracted from the aqueous medium with toluene, followed by separation in a separatory funnel, removal of residual water with sodium sulfate, and reduction of the extract volume using a rotary evaporator. The decrease in the concentration of TNT, established by GC-MS technique, was 66.4% by the end of the experiment, while the content of nitrites increased almost 15-fold. In the algae cells, a threefold increase in the enzymatic activity was observed already in the second hour of exposure, followed by a gradual decrease and maintenance at a level of 50% of the control until the end of the experiment. The total chlorophyll content increased significantly from the sixth hour of exposure to the end of the experiment due to an increase in the content of chlorophyll *b*. The results obtained indicate the efficient biodegradation process and prospects of using algae of the genus Rhizoclonium for cleaning water bodies contaminated with TNT.

Keywords: *Rhizoclonium*; explosives; 2,4,6-trinitrotoluene; aquatic ecosystems; biodegradation; decontamination.

Introduction

Natural and artificial aquatic systems unwittingly serve as reservoirs for collecting pollutants from their catchment areas (Senze & Kowalska-Góralska, 2019; Martseniuk et al., 2023). The anthropogenic impact including municipal, economic and agricultural activities has led to release of contaminants into water systems, resulting harm to the ecosystem and biotic community (Polechońska & Klink, 2022; Yesipova et al., 2022; Kumar et al., 2023). The conduct of military operations in large areas of Ukraine has caused a sharp increase in pollution and accumulation in the environment of various explosives and their transformation products. Of these, 2,4,6-trinitrotoluene (TNT) has long been popular among other nitroaromatic explosives in both production and use. TNT and its derivatives, such as 2,4-dinitrotoluene (2,4-DNT) and 2,6-dinitrotoluene (2,6-DNT) are used in rockets, projectiles and as intermediates in the production of smokeless powder (Rylott & Bruce, 2019). These compounds are released during the firing of ammunition, through industrial effluents, disposal of ammunition, and open burning. According to Eisentraeger et al. (2007), approximately 1944 TNT-contaminated sites still contain high toxicant levels. TNT presents the greatest concern to surface waters, soils and groundwater, and public health as well due to its mutagenic properties, low mobility and persistent nature. Even at low concentrations, TNT exhibits toxic and mutagenic effects on various organisms, from microbes to humans (Lachance et al., 2004), causing rashes, toxic hepatitis, dermatitis, cyanosis, sneezing, cough, peripheral neuritis, cataracts, muscle pain, aplastic anemia and kidney damage (Kalderis et al., 2008). Xu et al. (2023) emphasized TNT's high water solubility, which provokes its absorbance by aquatic organisms and poses a significant threat to the ecosystem and the health of seafood consumers.

The need to restore aquatic ecosystems dictates the search for efficient, cost-effective and environmentally friendly methods for the elimination of explosives, which corresponds to the processes of biological treatment (Lewis et al., 2004; Shemer et al., 2018; Dietz-Vargas et al., 2023). Serrano-González et al. (2018) have proposed combining several kinds of biological TNT treatment: anaerobic, aerobic, and enzymatic processes. From this standpoint, immersed aqueous plants represent a promising group of aquatic organisms effective in the biodegradation of toxic agents (Polechońska et al., 2018). An appealing technique for elimination of heavy metals from wastewater has appeared, bioremediation by algal species, termed as "phycoremediation" (Salama et al., 2019). Different microalgae strains have been explored and employed for the removal of heavy metals, which proves this method to be sustainable, simple, and green (Chugh et al., 2022). Freshwater algae *Chlorella vulgaris* exhibited the dose-dependent effect of biological treatment of a cyanide-contaminated aquatic ecosystem (Liu et al., 2018). As for biodegrading of nitroaromatic explosives, the treatment systems, based on the biological activity of *Pseudomonas* sp. ST53 or the fungus *Phanerochaete chrysosporium* were reported as an effective method for the remediation of contaminated soil and water (Snellinx et al., 2002). Filamentous green algae *Rhizoclonium* spp. (Chlorophyta) are widely distributed worldwide (Leal et al., 2020), and most *Rhizoclonium* strains are found in freshwater environments, with a few species that can tolerate medium-to-high salinity conditions (Saber et al., 2017). These algae have been described as an important source of bioactive molecules (Osuna-Ruiz et al., 2019), and have exhibited antibacterial activity compared to standard antibiotics (Morsi et al., 2023). However, the ability of *Rhizoclonium* spp. to effect biodegradation of toxicants remains unclear. Plants can involve different intracellular mechanisms to accomplish resistance to the stresses or to diminish their negative consequences. Among the enzymatic defense mechanisms, one of the effective ways is functioning of a large family of glutathione S-transferases (EC 2.5.1.18), showing the ability to combat biotic and abiotic stresses (Ding et al., 2017), and to regulate plant growth, development, and detoxification (Wang et al, 2023). Glutathione S-transferases neutralize various reactive molecules by catalyzing their conjugation with reduced glutathione, thus protecting cells from oxidative bursts (Kumar & Trivedi, 2018). The aim of the work was to identify the capability of freshwater algae *Rhizoclonium* sp. to utilize TNT in the aqueous medium and to assess their potential for decontamination of the water bodies.

Materials and methods

A model experiment was conducted in June 2023 under laboratory conditions in the Research Institute of Biology of Oles Honchar Dnipro National University (Dnipro city, Ukraine). Freshwater algae *Rhizoclonium* sp. were provided by the Educational and Scientific Complex "Aquarium" of Oles Honchar Dnipro National University, then kept in the settled tap water before the experiments. For the TNT exposure, plant biomass was taken in the ratio 30 g of alga wet weight per 1 L of aqueous medium.

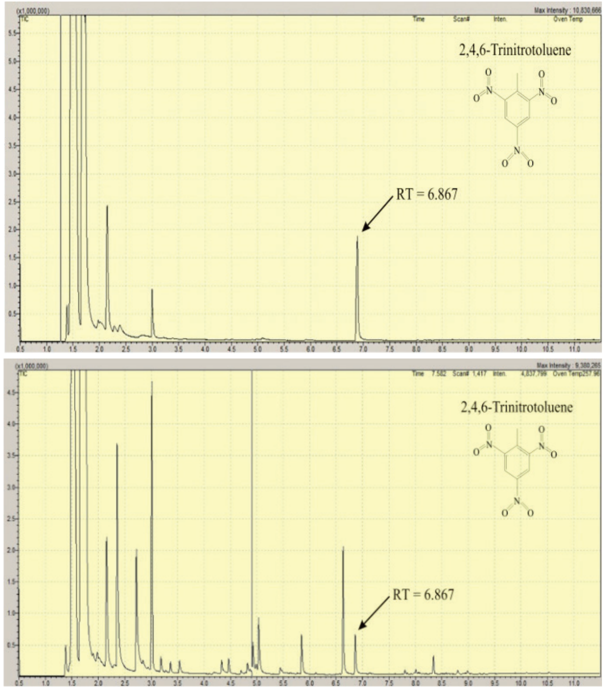
TNT (2,4,6-trinitrotoluene) was prepared in analytical quantities according to the described method (Kröger & Fels, 2000) exclusively for research purposes, and its identity was confirmed by gas chromatography – mass-spectrometry assays. Experimental contamination of aqueous medium was simulated by addition of TNT (100 mg/L) to the aquarium filled with tap water settled for 10 days. TNT from aqueous medium was extracted with toluene (1:1 v/v) during one hour, followed by separation in a separatory funnel, removal of residual water with sodium sulfate, and reduction of the extract volume using a rotary evaporator IKA® RV 10 (Germany).

GC-MS analyses were carried out using Shimadzu-GC-MS (QP 2020 El, Japan) equipped with Rxi®-5 ms column (30 m × 0.25 mm, film thickness 0.25 µm; 5% diphenyl/95% dimethyl polysiloxane as a fixed liquid phase). The oven temperature increased from 100 °C (with 2 min initial hold) to 300 °C and kept constant for two min. The carrier gas was helium, column flow was 1.2 mL/min. Injector temperature was 280 °C; sample volume 1 µL. The identification of TNT was achieved based on Mass Spectrum Library 2014 for GC-MS by their mass spectra comparison with those in the National Institute of Standards and Technology (NIST14.lib) spectral database. TNT content was estimated using the corresponding peak area and the calibration graph prepared in the TNT concentration range 0.5–5.0 µg/µL. Finally, the component detected with the retention time (RT) of 6.867 min was identified as 2,4,6-trinitrotoluene in both standard (Fig. 1a) and experimental samples (Fig. 1b).

Concentration of nitrites in the experimental aqueous medium was measured using a set of express tests for rapid determination of water quality Testlab (JBL, Germany).

Glutathione S-transferase (GST) activity in the algal tissues was detected by spectrophotometric method described by Habig & Jakoby (1981) with 2,4-dinitrochlorobenzene (DNCB) as substrate. The enzymetic activity was expressed in the µM of DNCB converted by GST in one second (µkat/g of algal tissues).

Chlorophyll content (Chl a, Chl b) and the amount of pigments (Chl a + Chl b) in *Rhizoclonium* sp. wet mass were determined by the spectrophotometric method (Wintermans & De Mots, 1965), measuring optical density of the algae ethanolic extracts at wavelengths of 649 and 665 nm. The results were expressed as mg/g of plant material. All assays were repeated at least three times. Study results processing 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 ± standard deviation (x ± SD), and the mean values were compared using Tukey's HSD. Differences were considered significant at $P < 0.05$.



a
b

Fig. 1. GC-MS determination of 2,4,6-trinitrotoluene (TNT): *a* – standard solution (5 µg/µL), *b* – extract from the experimental aqueous medium

Results

The extracted amount of 2,4,6-trinitrotoluene (TNT) from the model contaminated aqueous medium reached 83.04 mg/L before the alga inhabitation, and that TNT concentration was considered as initial (0 h of the experiment) followed by sampling at 1, 2, 3, 4, 24, and 48 h. Further measurements revealed the gradual decrease in TNT content (Fig. 2) up to the undetectable level (equal or less than 12.5 mg/L in aqueous medium). Thus, the efficiency of TNT removal in the model polluted reservoir, inhabited with *Rhizoclonium* sp. (30 g of algal wet mass per one liter), was 66.4% of the extractable amount of 2,4,6-trinitrotoluene.

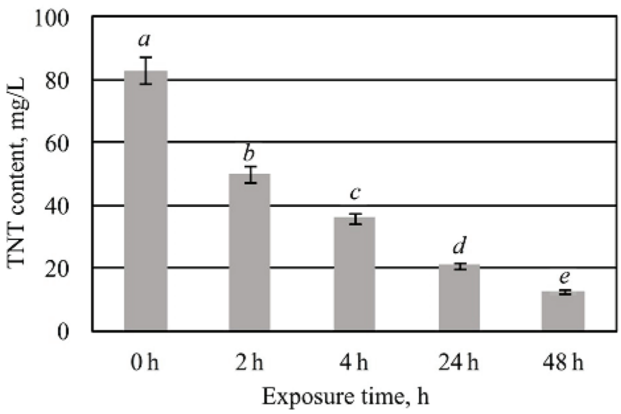


Fig. 2. Dynamics of 2,4,6-trinitrotoluene (TNT) content (mg/L) in the model aqueous medium inhabited by *Rhizoclonium* sp.: 30 g of algal biomass/L; mean value ± SD, n = 5

Content of nitrites in the TNT-polluted aqueous medium inhabited by algae, demonstrated the tendency to gradual growth during the experimental period from 0.05 to 0.75 mg/L. At the same time, negligible increase in the nitrites' content was revealed in the reference aqueous medium without algae, which served as control elimination of TNT due to photolysis and other losses (Fig. 3).

Chlorophyll content in the algal cells was found to be altered in comparison with control (0 h of exposure) only after 6 h of the exposure to 2,4,6-trinitrotoluene in the model experiment (Fig. 4). The content of chlorophyll *a* increased insignificantly up to 24 h of exposure, but at 48 h was slightly lower than control level. In contrast, chlorophyll *b* content showed the great growth up to 58.9%, 84.2%, and 112.4% above control level at 6, 24, and 48 h respectively. The ratio of pigments Chl *a*/Chl *b* gradually declined from point 1.09 in the control samples to 0.74, 0.61, and 0.51, respectively, at 6, 24, and 48 h. The total amount of chlorophyll during the experiment exceeded the control level by 30.8%, 40.0%, and 50.8%, respectively, at 6, 24, and 48 h.

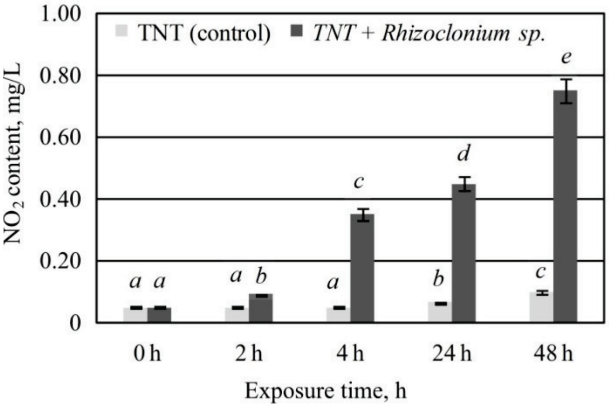


Fig. 3. Dynamics of nitrites' content (mg/L) in the model TNT-contaminated (100 mg/L) aqueous medium: gray bar – inhabited by Rhizoclonium sp.; black bar – without *Rhizoclonium* sp.: mean value ± SD, n = 5

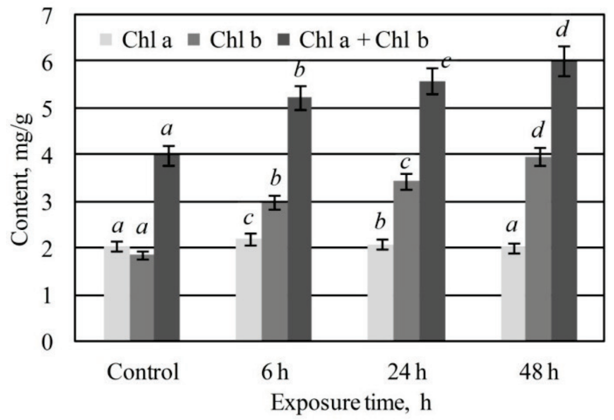


Fig. 4. Altering the photosynthetic pigments content (mg/g) in *Rhizoclonium* sp. tissues due to TNT action in the model experiment: mean value ± SD, n = 5

Activity of glutathione S-transferase in the algal tissues exhibited a significant change during the experiment, reaching 196.4% of the control level just at one hour of exposure to TNT (Fig. 5).

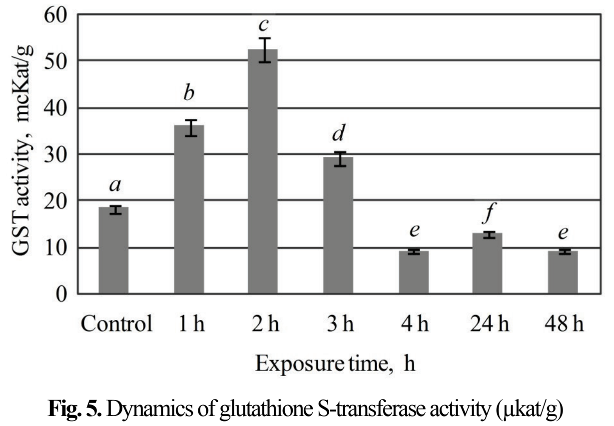


Fig. 5. Dynamics of glutathione S-transferase activity (µkat/g) in *Rhizoclonium* sp. tissues due to TNT action in the model experiment: mean value ± SD, n = 5

Further enzyme activation resulted in 286.9% of the control level at 2 h of exposure. Then, the gradual decrease in GST activity led to 158.8% of the control level at 3h hour, while only 50% of the control level was detected at 4 h. However, TNT-induced inhibition of glutathione S-transferase activity in the tissues of *Rhizoclonium* sp. was not below 50% of control level until the end of the experiment.

Discussion

Algae are the principal primary producers of aquatic ecosystems, but they are obviously forced to have protective mechanisms against the restrictive influence of various chemicals, including explosives, which have been released into the environment. Recent studies have provided a lot of evidence for the detoxification role of algae. As Ankit et al. (2022) have noted, macro- and microalgae can utilize water waste as a source of nutrition and decrease heavy metals and other pollutants by enzymatic and metabolic processes. Subashchandrabose et al. (2013) emphasized the ability of algae to degrade or accumulate various organic pollutants including phenolics, hydrocarbons, pesticides and biphenyls. Kumar et al. (2023) reported the adsorption of methylene blue by *Chlamydomonas* sp. with 96.1% removal efficiency; similarly, Isochrysis galbana demonstrated a nonylphenol accumulation in the cells with 77% removal efficiency indicating the potential of algae as an efficient retrieval system for organic contaminants. The alga *Chlorella vulgaris* showed potential capability for KCN (potassium cyanide) elimination, with the maximal removal rate of 61% (Liu et al., 2018). Seaweed species *Rhizoclonium riparium* manifested strong antioxidant capacity and antimutagenic activity (Osuna-Ruiz, 2019). However, there is a lack of information on the algal capability to biodegrade the explosives.

In our experiment, freshwater algae *Rhizoclonium* sp. during 48 h of the exposure to TNT in concentration 100 mg/L achieved a 66.4% decrease in the t amount of toxicant, which is a comparatively high biodegrading efficiency. For example, Mercimek et al. (2015) documented TNT-degradation ability of *Pseudomonas aeruginosa* after 48 h of exposure with maximal efficiency 46% at TNT initial concentration 75 mg/L, and 59% efficiency at 50 mg/L. The blue-green alga *Spirulina platensis* has high ability to adsorb TNT uptaking about 87% of 2,4,6-trinitrotoluene from polluted water during 15 days (Adamia et al., 2018). Hydroponic poplar (*Populus trichocarpa*) plantlets showed a fast reduction of the initial content of TNT (5 mg/L), reaching insignificant levels after 48 h, according to the analysis of 2,4,6-trinitrotoluene and its metabolites (Brentner et al., 2008).

The significant increase in the nitrites' concentration in the experimental aqueous medium, inhabited by *Rhizoclonium* sp., in contrast with the negligible growth of nitrites' content in the reference medium, can indicate a release of nitro-groups from 2,4,6-trinitrotoluene molecules, which suggests the processes of their biodegradation. Similarly, release of nitrites at level 0.47 mg/L at 72 h of exposure was shown by *P. aeruginosa* examined for the TNT-degrading capability (Mercimek et al., 2015).

During the experiment, negative correlation ($r = -0.08$, $P < 0.05$) between TNT concentrations in the aqueous medium and glutathione-S-transferase activity in the algal cells was followed. Glutathione-S-transferase activation, obviously, began in the first minutes of the alga's contact with TNT, exceeding the control level almost twice at the end of the first hour of exposure. The greatest enzyme activity was observed at the second hour of exposure, then gradually declined by the third hour, followed by maintenance at approximately half of control level until the end of the experiment. Taken together, these findings can prove the involvement of GST in detoxication of TNT by enzymatic pathway in the cells of studied *Rhizoclonium* sp. Our results are consistent with the data of Brentner et al. (2008), that conjugation of 2,4,6-trinitrotoluene with reduced glutathione (GSH), catalyzed by glutathione-S-transferase and leading to TNT inactivation, may be a possible catabolic pathway within the plant cells. Additionally, recent studies (Rylott et al., 2015) established that the detoxification of 2,4,6-trinitrotoluene (TNT) in *Arabidopsis thaliana* cells included the formation of TNT-glutathionyl products.

The photosynthetic process at whole and the chlorophyll content as well are very sensitive to environmental stressors (Luo et al., 2017), especially in the immersed aquatic plants. Adaptation of plants includes photosynthetic apparatus rearrangements, which depend on plant genetic characteristics, the initial composition and content of pigments, as well as the regulatory mechanisms of pigment synthesis (Zhang et al., 2016). For example, exposure of the green alga *C. vulgaris* Beijerinck to high dose of indomethacin (10^{-3} M) reduced Chl *a* content by 30% and Chl *b* by 15% (Piotrowska-Niczyporuk, 2008). Significantly different tolerance to acidity of three typical freshwater algae was documented Ma et al. (2022) according to levels of the photosynthesis inhibition, decrease in chlorophyll *a* (Chl *a*) content, and antioxidant enzyme activity. Under the combined stress conditions, the macroalga *Ulva prolifera* showed a tolerance to a wide range of salinity (5–25%) in contrast to the simulated acid rain (pH 4.4), which caused reduction in the content of chlorophyll *a* (Li et al., 2017). In our study, during 48 h under the high TNT concentration, freshwater algae *Rhizoclonium* sp. manifested the maintenance of Chl *a* content, significant increase in Chl *b* and total chlorophyll content. Altered ratio Chl *a*/Chl *b* reflects the enhancement of Chl *b* biosynthesis in the algal cells, which can serve as a compensatory and defense mechanism for the photosynthetic process in adverse conditions.

Conclusion

A model experiment was carried out to characterize the 2,4,6-trinitrotoluene-induced shifts in the state of an aqueous medium inhabited with freshwater algae *Rhizoclonium* sp. In the contaminated medium (TNT in concentration of 100 mg/L), the removal of 2,4,6-trinitrotoluene began at the first hour of exposure and reached 66.4% of the extractable amount of toxicant at 48 hours. In parallel, a great increase in the nitrites' content (by 15 times as compared to initial level) was observed during the experiment, suggesting that the nitrites were produced as a result of possible TNT degradation. In the tissue of *Rhizoclonium* sp., exposure to TNT after 6 h caused a prominent change in the algal photosynthetic apparatus composition, which included increase in both chlorophyll *b* and total chlorophyll content, and decrease in the ratio Chl *a*/Chl *b*. Activity of glutathione-S-transferase in algal cells exhibited a 2.9 times growth already at 2 hours of exposure, but declined up to half of the initial level at 4 h, suggesting the possibility of a GST-catalyzed process of TNT conjugation with reduced glutathione as an effective way of TNT inactivation. Thus, green freshwater algae *Rhizoclonium* sp. were found to be promising in terms of decontamination of the aqueous medium.

Author contributions: conceptualization, N. K., O. M., and T. S.; methodology, N. O., N. Y.; validation, N. K., O. M.; formal analysis, O. M.; investigation, N. K., O. N., V. K., R. M.; resources, O. M., R. M.; data curation, N. K., O. M., N. Y.; writing – original draft preparation, N. K., N. Y.; writing – review and editing, N. K., O. M.; visualization, N. K., O. N., V. K.; supervision, N. K., O. M.; project administration, N. K.; funding acquisition, O. M. All authors have read and agreed to the published version of the manuscript.

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The authors declare no conflicting interests.

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