Slov Vet Res
DOI 10.26873/SVR-1836-2025
UDC 504:574.2:546.66/.79:577.15
Pages: 15–22

Original Research Article

Determination of Oxidative Stress Responses Induced by the Combination of 4 Different Rare Earth Elements in *Dreissena polymorpha*

Key words

antioxidant enzymes;
Dreissena polymorpha;
oxidative stress;
rare earth element;
thiobarbituric acid reactive substances

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Abstract: Rare Earth Elements (REE), whose usage areas are increasing day by day, are increasing in the amount of mixing with the environment, causing changes in antioxidant enzyme activities by causing oxidative stress in living organisms. In this study, it was aimed to examine the oxidative stress responses induced by the mixture of 4 different REEs (terbium, gadolinium, Lanthanum, Praseodymium) in *Dreissena polymorpha*. For this purpose, sublethal concentration values were determined by literature review. Experimental application was carried out within 24 and 96 hours. In the analyzes performed to determine biomarker responses, samples taken from living organisms were weighed and homogenization processes were performed for the analysis of samples taken from the experimental groups, including the control group. After homogenization, samples were centrifuged at 4.000 rpm for 15 minutes. Supernatants were kept at -86 °C until measurements were made. Superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) activities, and glutathione (GSH) and thiobarbituric acid reactive substances (TBARS) levels were determined using ELISA kits. Statistical analyses were performed using SPSS. One-way ANOVA (Duncan's multiple range test; $p < 0.05$) was used for comparison of measured parameters among groups. As a result of the application, a decrease in CAT activity and GSH level and an increase in TBARS levels were observed after 96 hours compared to the control group, while no statistically significant difference was detected in SOD and GPx activities.

Received: 9 August 2023
Accepted: 1 October 2025

Introduction

The rare earth elements (REE) are a group of 17 elements in the periodic table, including scandium and yttrium with atomic numbers 21 and 39, and the lanthanides (Ln) with atomic numbers 57-71 (1). REEs are generally soft, pliable and easily workable. The reason why we call REEs that generally coexist in the earth's crust as "rare" is not because of their low amount in the ore, but because of the difficulty of processing these elements, separating them from each other selectively by enrichment methods and obtaining them in pure form (1). REEs occur together in minerals due to their chemical similarity. Some REEs have unique magnetic, phosphorescent, catalytic and electrical properties that make them highly valuable in industrial applications and some manufactured products. They are elements that have become extremely important for technology due to these features (2). These elements have an extremely important place in a wide range of technological fields, from mobile phones to televisions, from LED light bulbs to wind turbines. The estimated average REE concentration in the earth's crust is between about 130 mg/g^{-1} and 240 mg/g^{-1}, which is actually quite high compared to other commonly used elements (3).

Gadolinium (Gd) possesses remarkable metallurgical qualities, such that as little as 1% gadolinium can greatly improve iron, chromium, and related metals' machinability

and resistance to oxidation at high temperatures. Gd absorbs neutrons as a metal or salt and is thus occasionally used for shielding in radiography and nuclear reactors in neutron radiography. Terbium (Tb) is employed in solid-state electronics as calcium fluoride, calcium tungstate, and as a crystal stabilizer in high-temperature fuel cells. Terbium is utilized in actuators, maritime sonar systems, and sensors as a component of Terphenol-D (an alloy that expands and contracts more than any other alloy when subjected to magnetic fields). Catalysts, glass additives, carbon lamps for studio lights and projectors, ignition components in lighters and torches, electron cathodes, scintillators, and gas tungsten welding electrodes are just a few of the applications for lanthanum (La) compounds. Lanthanum carbonate is used as a phosphate binder in kidney failure patients who have excessive phosphate levels in their blood. Praseodymium (Pr), like other lanthanide trichlorides, can be anhydrous or hydrated. When exposed to moist air, it rapidly absorbs water and forms a light green heptahydrate (4).

Abiotic variations in the aquatic environment, such as emerging pollutants and those due to climate change, are stress factors that affect the resilience of organisms. These pollution factors consist of greenhouse gas emissions such as carbon dioxide (CO_2), methane (CH_4) and ozone (O_3), mixing with the atmosphere due to the burning of fossil fuels, industrial industrial wastes, household and agricultural wastes and wastes such as REE. The atmospheric layer generated by excessive concentrations of these gases absorbs and traps solar energy as heat, raising world average temperatures (5). The high amount of REEs in the environment and the increase in the concentration level taken into the living organism cause toxic effects in the living body. Depending on the organisms exposed to REE concentrations, toxic effects are known to produce growth-stimulating effects (6).

Oxidative stress occurs when reactive oxygen species (ROS) accumulate excessively. It regulates the physiological and molecular reactions that occur in response to almost all biotic and abiotic stimuli (7). A number of independent investigations in both plant and animal models have revealed the role of various REEs in the redox imbalance that leads to oxidative stress, and several REEs have been reported to produce oxidative stress (8, 9, 10). Aerobic organisms have a built-in antioxidant defense mechanism that includes both enzymatic and non-enzymatic compounds that can mitigate the damaging effects of ROS. Superoxide dismutase (SOD), catalase (CAT), and glutathione-S-transferase (GSH) are among the antioxidant enzymes that have been widely used as effective biomarkers of environmental contamination in aquatic organisms and have been identified as effective protective barriers against ROS formation (11). Various studies have employed lipid peroxidation, measured as thiobarbituric acid reactive substances (TBARS), as a marker of oxidative stress in response to various environmental pollutants (12, 13, 14, 15). Depletion causes a redox imbalance and a decrease in the ability to cope with organic xenobiotics processed by glutathione S-transferase (GST) and glutathione peroxidases (GPx).

Mussels are particularly vulnerable to the elements present in the water as they are organisms with a sedentary filter feeding character. *Dreissena polymorpha* is vulnerable to environmental change and pollution because of its tendency to accumulate contaminants (16) and vast geographic range. *D. polymorpha* is commonly utilized in ecotoxicological tests due to its strong oxidative defense and relatively high tolerance to xenobiotics (17, 18).

The purpose of this study is to assess SOD, CAT and GPx activities, as well as TBARS and GSH levels, in order to determine the oxidative stress responses generated by a mixture of Tb, Gd, La, and Pr REEs on *D. polymorpha*.

Material methods

Supply of Living Organisms and Adaptation to the Laboratory Environment

D. polymorpha individuals were collected from the Euphrates (38º 48' 25" N, 38º 43' 51" E) (Figure 1). The organisms were quickly brought to the laboratory in plastic bottles. It was fed with plankton in a 12:12 hour light:dark cycle to design an environment similar to natural living conditions in a 20 L aerated aquarium in a room kept at 18 °C for 30 days before being used in the experiments. Organisms at similar developmental stages were selected for the study, were not fed during the experimental study and were checked every 24 hours.

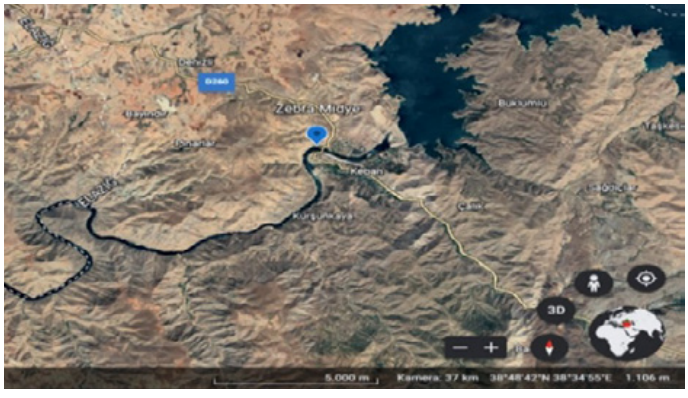

Figure 1: The area where the model organism *D. Polymorpha* was supplied

Chemical Substance Supply

Tb, Gd, Ln, Pr REEs used in the study were obtained from the commercial company Bostonchem with 99.99% purity.

Determination of Subletal Concentrations

As in all toxicological studies, the application concentrations determined in our Mix REE application study, taking into account the release rates to the environment, and after the literature review, the application concentrations were determined compared to the values in this range.

Experiment Design

Eight healthy models of similar size were placed in glass aquariums, each consisting of 2 liters. The O_2 need of living things was provided by air engines. Experimental study consisted of 4 groups, one of which was the control group. Two time slots (24 and 96 hours) were determined for the four groups.

Application concentration was created by mixing La, Gd, La, Pr REEs in equal proportions (1:1:1:1).

C1 (Control): The Mix was kept in water taken from the natural environment of the organisms, which was not exposed to any concentration of REE.

C2: Group exposed to 50 mg/L Mix REE concentration (1:1:1:1) at 24 and 96 hours

C3: Group exposed to 100 mg/L Mix REE concentration (1:1:1:1) at 24 and 96 hours

C4: Group exposed to 200 mg/L Mix REE concentration (1:1:1:1) at 24 and 96 hours

In the experimental research, all studies were carried out in triplicate.

Preparation and analysis of oxidative stress parameters in D. polymorpha soft tissue

For measurement of oxidative stress parameters, soft tissue samples were weighed and homogenized by adding 1/5 w/v PBS buffer (phosphate buffered salt solution) and using an iced homogenizer (DAIHAN brand). The homogenized samples were then centrifuged at 4.000 rpm for 15 minutes. Supernatants were maintained at -86 °C until measurements were taken. GSH and TBARS levels, as well as SOD, CAT, and GPx activities, were measured using corresponding ELISA kits. In the investigation, CAYMAN brand GSH (Catalog No 703002), SOD (Catalog No 706002), CAT Catalog No 707002), and GPx Catalog No 703102) were used.

Statistical Analysis (revised)

Statistical analyses were performed separately for each time point (24 h and 96 h) using One-Way ANOVA followed by Duncan's post-hoc test ($p<0.05$) to compare the four experimental groups (Control, 50 mg/L, 100 mg/L, 200 mg/L). Additionally, intra-group comparisons between 24 h and 96 h were conducted using an independent t-test, assuming normal distribution.

Results

TBARS Level

The TBARS assay results reveal a clear concentration- and time-dependent increase in lipid peroxidation in Dreissena polymorpha following exposure to mixed rare earth elements (REEs). At 24 hours, TBARS levels show a gradual elevation with increasing REE concentrations, while at 96 hours, this effect becomes markedly more pronounced particularly in the 200 mg/L group, which exhibits the highest TBARS value (0.137 µM), statistically distinct from all other groups (Figure 2).

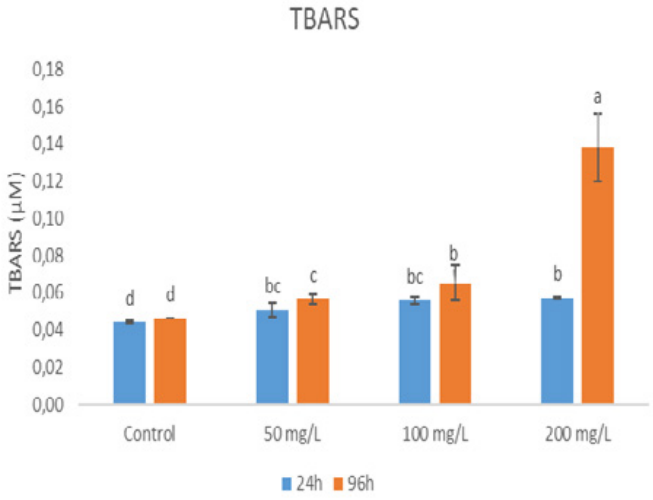

Figure 2: TBARS (µM tissue) levels of *D. polymorpha* exposed to Mix REE. Different letters indicate statistically significant differences ($p<0.05$)

GSH Level

The GSH assay results demonstrate a clear concentration- and time-dependent depletion of intracellular antioxidant capacity in Dreissena polymorpha following exposure to mixed rare earth elements (REEs). At both 24 h and 96 h, GSH levels were highest in the control group, with a progressive decline observed as REE concentration increased (Figure 3).

At 24 h, the control group exhibited a GSH concentration of approximately 53.3 µM, while the 200 mg/L group dropped sharply to 7.6 µM. This trend was similarly evident at 96 h, where the control group maintained relatively high GSH levels (49.3 µM), but the 200 mg/L group declined further to 6.5 µM. These reductions were statistically significant, as indicated by distinct lettering annotations (Figure 3) above the bars ($p < 0.05$).

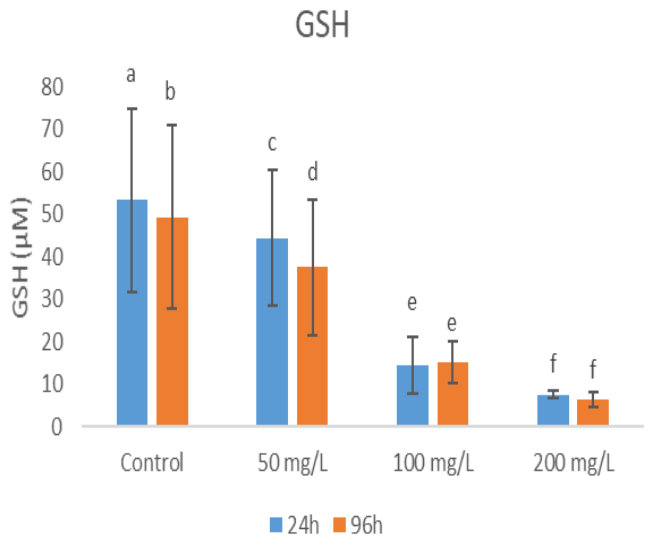

Figure 3: GSH (µM tissue) levels of *D. polymorpha* exposed to Mix REE. Different letters indicate statistically significant differences (p<0.05)

SOD Activity

SOD activities (U/mL tissue) in *D. polymorpha* exposed to different concentrations of Mix REE over time are given in Figure 4. There was no statistically significant difference in SOD activity in the 24 and 96 hour exposure groups compared to the control group (p>0.05).

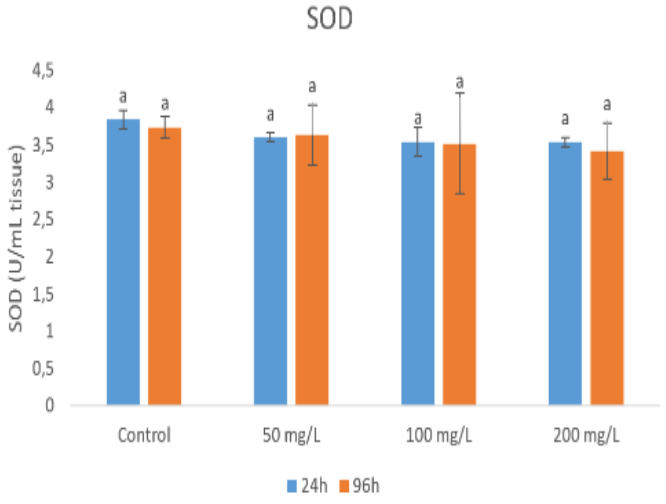

Figure 4: SOD (U/mL tissue) activities of *D. polymorpha* exposed to Mix REE. Different letters indicate statistically significant differences (p<0.05)

CAT Activity

Figure 5 presents CAT activity (nmol/min/mL tissue) in the control and experimental groups exposed to 50 mg/L, 100 mg/L, and 200 mg/L concentrations of the test substance at 24 h and 96 h exposure periods.

CAT activity was highest in the control group at both time points, with no statistically significant difference between 24 h and 96 h values (Figure 5). In the 50 mg/L group, CAT activity at 24 h remained comparable to the control (Figure 5), while 96 h values showed a significant reduction (p<0.05), compared to the control and 24 h values (Figure 5).

A marked and statistically significant decline in CAT activity was observed at 100 mg/L and 200 mg/L for both 24 h and 96 h exposures (p<0.05) compared to control.

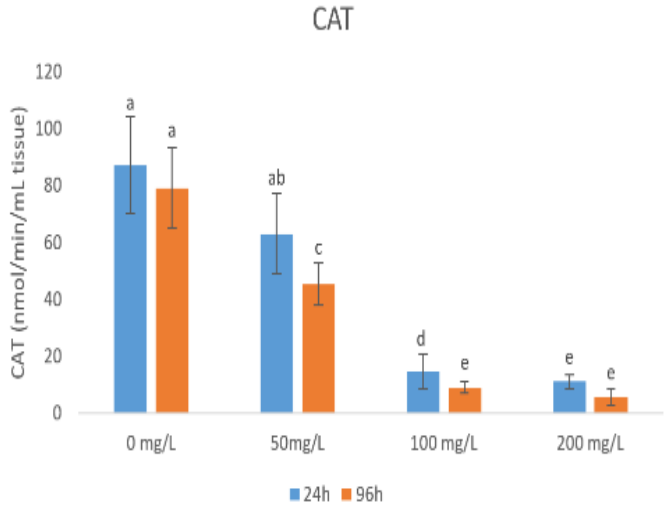

Figure 5: CAT (nmol/min/mL tissue) activities of *D. polymorpha* exposed to Mix REE. Different letters indicate statistically significant differences (p<0.05)

GPx Activity

The Figure 6 shows the GPx activity (nmol/min/mL tissue) in the experimental groups exposed to different concentrations of the tested substance (50 mg/L, 100 mg/L, and 200 mg/L) compared to the control, at two exposure durations: 24 hours and 96 hours.

In general, GPx activity remained relatively stable across groups, with no statistically significant reductions observed in the 50 mg/L and 100 mg/L treatments compared to the control at either time point. However, at 200 mg/L, a significant decrease in GPx activity was observed at 96 hours compared to 50 mg/L group (Figure 6).

Discussion

In the literature, there are many scientific studies investigating the effects of pollutants on aquatic organisms with various biomarkers. However, there are very few studies examining the oxidative stress responses by applying REEs to living organisms as a mixture. It is

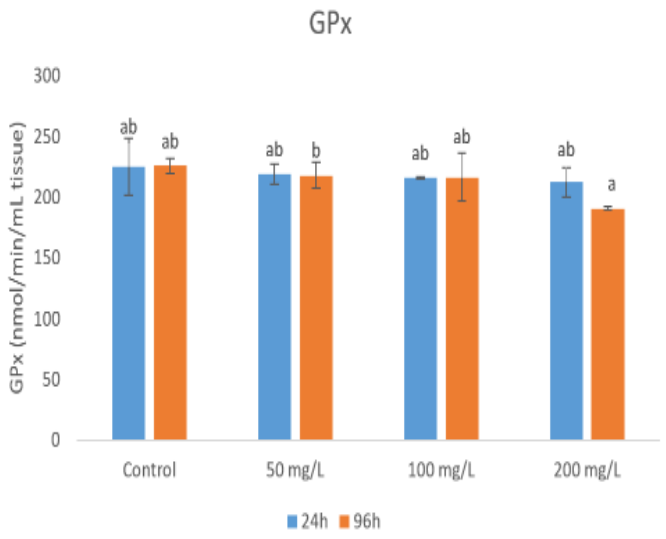

Figure 6: GPx (nmol/min/mL tissue) activities of *D. polymorpha* exposed to Mix REE. Different letters indicate statistically significant differences ($p<0.05$)

thought that this study will contribute to the literature by creating a source for the related field. Hanana et al., (2021), investigated the oxidative stress markers of terbium and praesodymium in *Rainbow trout* and stated that Tb is 2 times more toxic than Pr and plays a role in oxidative stress, calcium binding, hemoprotein activity and protein turnover Tb toxicity (19). Lompré et al. (2021), investigated the effect on the organism by exposing Tb and carbon nanotubes in native *Ruditapes decussatus* and *Ruditapes philippinarum* in their study, and as a result, they observed metabolic deterioration in oysters exposed only to Tb they stated that loss of redox balance and neurotoxicity were proven in this species (20). Freitas et al. (2020), evaluated the metabolic and oxidative stress responses of Dysprosium (Dy) of *Mytilus galloprovincialis* and found that Dy was responsible for the metabolic increase associated with glycogen expenditure of the mussel, activation of antioxidant and biotransformation defenses, and cellular damage with a clear loss of redox balance have emphasized (21). Hanana et al. (2021) evaluated the toxic effects of five mild REE mixtures in *Hydra vulgaris* in their study and stated that it caused a significant early toxic effect in hydra within the first 24 hours, and REE mixture affected hydra reproduction and head regeneration at the level of environmental concentration (22). Kang et al. (2022), investigated the toxic effects of lanthanum (La) and gadolinium (Gd) on zebrafish (*Danio rerio*) in their study and reported that they cause oxidative stress in living things (23).

Exposure to abiotic changes, such as the presence of pollutants, as well as reduced metabolic capacity of organisms can result in excessive production of ROS in bivalves. To prevent lipid membrane damage, organisms can differentiate their antioxidant defenses, namely the activity of SOD, CAT and GPx enzymes (24).

In the present study, decreased CAT activity was observed in *D. polymorpha* following exposure to Mix REEs. Our results suggest that CAT activity is significantly suppressed at higher concentrations of the substance, regardless of exposure duration. These findings indicate a clear concentration-dependent inhibition of CAT activity, particularly at 100 mg/L and 200 mg/L, pointing to potential oxidative stress and an impaired antioxidant defense system at elevated concentrations. Similarly, Dubé et al., investigated 7 different REE effects in young rainbow (*Oncorhynchus mykiss*) trout and reported reductions in CAT activity. Andrade et al. (2023), examined the oxidative stress results of yttrium (Y) in M. galloprovincialis and observed that CAT activity was inhibited. Figueiredo et al. (2018), evaluated the enzyme activities occurring in lanthanum exposure in Anguilla anguilla and observed reductions in CAT activity. Andrade et al. (2021), in their study, examined the response of *M. galloprovincialis* to lanthanum and reported that the decrease in CAT activity may be caused by pollution. Yang et al. (2016), examined the effects of yttrium on *Microcystis aeruginosa*, and determined reductions in CAT activities as a result of the examination. Figueiredo et al. (2022), In their study, they examined the oxidative stress responses in *Spisula solida*, which they exposed to La, and reported that there was a decrease in CAT activities as a result. Mixed REE exposure may parallel CAT activity reduction, which may reflect higher OH- production leading to CAT inhibition. In fact, the increase in intracellular ROS due to OH^- overproduction is associated with a decrease in CAT expression (30).

Activation of SOD under REE exposure reflects the organism's first enzymatic defense mechanism responsible for the dismutation of oxygen radicals to oxygen and hydrogen peroxide (31). No significant change was observed in SOD activities in *D. polymorpha* individuals exposed to mixed REE. Contrary to our results, Trapasso et al. (2021) who investigated the effect of Gd in *M. galloprovincialis* observed increases in SOD activity. Freitas et al. (2020), study, investigated the toxicological effects of neodymium on *M. galloprovincialis* and stated that SOD activities increased compared to the control. Andrade et al. (2021), their study, examined the response of *M. galloprovincialis* to La and observed decreases in SOD activity. Yang et al. (2016), examined the effects of yttrium on *M. aeruginosa* and found that there were decreases in SOD activities. Figueiredo et al. (2022), examined the oxidative stress responses in *S. solida* exposed to lanthanum in their study and stated that there was a decrease in SOD activity as a result.

Overall, GPx activity remained relatively stable across groups following exposure to Mix REEs. The only significant change was observed at 200 mg/L after 96 h, where GPx activity declined compared with the 50 mg/L group at the same time point. This finding suggests that prolonged exposure to the highest concentration tested may impair antioxidant defense, as reflected by reduced GPx activity.

Freitas et al. (2020), in their study, investigated the toxicological effects of neodymium on *M. galloprovincialis* and stated that no significant difference was observed in the control organisms in the case of GPx, at the highest exposure concentration. Alp et al. (2023), investigated the effects of Tb concentrations on *Lemna minor* and stated that there was an increase in GPx activities as a result. Yang et al. (2016), examined the oxidative stress responses in *S. solida* exposed to lanthanum in their study and observed decreases in GPx levels. Because GSH can be converted to GSSG in the presence of ROS, the GSH/GSSG ratio tends to drop when GSSG increases under stressful situations. Organisms that employ glutathione for redox homoeostasis can produce reduced glutathione, but they are also distinguished by their glutathione recycling capabilities. Glutathione reductase (GRed) is an enzyme that converts oxidized glutathione to its reduced form (34). As a result, the increased GSSG content seen in polluted mussels with decreased GSH/GSSG levels implies that GRed is unable to convert oxidized glutathione to its reduced form. In general, the GSH/GSSG ratio is used to quantify the oxidative stress of organisms exposed to contaminants (35, 36, 37). Freitas et al. (2020), study, they aimed to determine the oxidative damage of neodymium in *M. galloprovincialis* and as a result, they observed significant reductions in GSH levels. Trapasso et al. (2021), investigated the effects of Tb concentrations on *L. minor* and stated that there were decreases in GSH levels as a result. Pinto et al. (2010), study examined the effects of La in *M. galloprovincialis* and stated that there were significant decreases in GSH levels compared to the control. Ippolito et al. (2010), study, examined the oxidative stress responses of La, Ce, Pr, Nd, Gd REEs in *L. minor* and reported that GSH levels decreased depending on time and concentration.

ROS can cause membrane lipid peroxidation when they are overproduced and not adequately removed by antioxidant systems. Malondialdehyde (MDA) is one of the most extensively used oxidative stress markers among all the peroxidized compounds produced in the LPO process (24, 40). Our study demonstrated a significant increase in the lipid peroxidation marker TBARS in *D. polymorpha* exposed to Mix REEs compared to the control groups, which is likely attributable to elevated substance concentrations. These findings suggest that higher concentrations of the tested compound induce substantial lipid peroxidation, particularly during prolonged exposure. The observed increase in TBARS levels at 96 hours may reflect cumulative oxidative damage or a delayed response of the antioxidant defense system. Pagano et al. (2016), investigated the impacts of REEs such as Y(III), La(III), Ce(III), Nd(III), Sm(III), Eu(III), and Gd(III) on *Paracentrotus lividus*. They studied and discovered that Ce and Gd enhanced MDA levels, whereas Y(III), La(III), Sm(III), and Nd(III) did not. Trapasso et al. (2021), investigated the effects of Tb concentrations on *L. minor*, and as a result, they observed increases in TBARS levels. Pinto et al. (2019) study, examined the oxidative stress responses of La, Ce, Pr, Nd, Gd REEs in *L. minor* and stated that increases in MDA levels occurred. Yang et al. (2016), examined the effects of yttrium on *M. aeruginosa* and determined increases in MDA levels.

The results of oxidative stress responses in *D. polymorpha* exposed to Mix REE show parallelism with the studies in the literature. It is thought that the use of REEs in combination in experimental practice and the evaluation of their results will contribute to the literature.

Conclusion

According to the literature review and the study results, it is thought that the use of REEs alone or in combination causes environmental pollution and causes oxidative stress in the organism by penetrating living organisms, causing vital damages in the organism. It can be recommended to pay attention to the use of REE and not to release wastes to the environment.

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Determinacija odgovorov na oksidativni stres, ki ga povzroča kombinacija 4 različnih redkih zemljinskih elementov v *Dreissena polymorpha*

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Izvleček: Redki zemeljski elementi (REE), katerih uporaba se iz dneva v dan povečuje, se vse bolj mešajo z okoljem in povzročajo spremembe v aktivnosti antioksidativnih encimov, saj v živih organizmih povzročajo oksidativni stres. V tej študiji smo želeli preučiti odzive na oksidativni stres, ki jih povzroča mešanica 4 različnih REE (Terbij, gadolinija, lantana, praseodima) v *Dreissena polymorpha*. V ta namen so bile na podlagi pregleda literature določene subletalne koncentracije. Eksperimentalna aplikacija je bila izvedena v 24 in 96 urah. V analizah, izvedenih za določitev odzivov biomarkerjev, so bili vzorci, odvzeti iz živih organizmov, stehtani in homogenizirani za analizo vzorcev, odvzetih iz eksperimentalnih skupin, vključno s kontrolno skupino. Po homogenizaciji so bili vzorci 15 minut centrifugirani pri 4000 rpm. Supernatanti so bili shranjeni pri temperaturi –86 °C do izvedbe meritev. Aktivnosti superoksid dismutaze (SOD), katalaze (CAT) in glutation peroksidaze (GPx) ter ravni glutationa (GSH) in s tiopropioninsko kislino reaktivnih snovi (TBARS) so bile določene z uporabo kompletov ELISA. Statistične analize so bile opravljene z uporabo SPSS. Za primerjavo izmerjenih parametrov med skupinami je bila uporabljena enosmerna ANOVA (Duncanov test večkratnega obsega; $p < 0,05$). Kot rezultat uporabe je bilo po 96 urah v primerjavi s kontrolno skupino opazno zmanjšanje aktivnosti CAT in ravni GSH ter povečanje ravni TBARS, medtem ko v aktivnostih SOD in GPx ni bila ugotovljena statistično značilna razlika.

Ključne besede: antioksidativni encimi; *Dreissena polymorpha*; oksidativni stres; redki zemeljski elementi; snovi, ki reagirajo s tiobarbiturno kislino