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Toxicity assessment of advanced biological wastewater treatment plant effluent by integrated biomarker response in zebra mussels (*Dreissena polymorpha*)

by

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Abstract

In this study, zebra mussels (*Dreissena polymorpha*) were exposed to advanced biological wastewater treatment plant effluent (ABWTPE) for 96 h. At the end of the 96th hour, antioxidant parameters such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GP_x), glutathione S-transferase (GST), malondialdehyde (MDA) and glutathione (GSH) were examined. The objective of the study was to identify biomarkers that are useful for assessing the potential toxic effects of ABWTPE in freshwater environments. We observed an increase in GP_x, SOD activity and MDA levels, and a decrease in CAT, GST activity and GSH levels. The results obtained in our study showed that the measured biochemical parameters (GSH, MDA, SOD, CAT, GP_x and GST) are useful biomarkers in determining the possible toxicity of ABWTPE in aquatic environments.

Key words: Antioxidant Defense System, ABWTPE, *Dreissena polymorpha*

1. Introduction

Wastewater treatment plants produce environmentally safe liquid waste or solid waste suitable for reuse or disposal. It is possible to reuse wastewater for irrigation or drinking water using advanced treatment technologies (Al-Shahwan 2016). Municipal wastewater treatment plants contain a wide range of pollutants (Metcalfe et al. 2010). Wastewater effluent is a complex mixture containing many different contaminants (Bolong et al. 2009). Effluent from municipal wastewater treatment plants is usually discharged directly into receiving environments such as rivers, lakes or the sea, and aquatic organisms and even humans are exposed to these waters (Samanta et al. 2018). Although this treatment technology can effectively remove nitrogen, phosphorus, ammonia, nitrogen and other pollutants from sewage, wastewater discharged from treatment plants still contains some pollutants such as surfactants, chlorinated hydrocarbons, heavy metals, and endocrine disruptors (Wan et al. 2015). Qualitative and quantitative measurements of pollutants can be performed by chemical analysis, but it is not possible to measure all pollutants in wastewater. In addition, it is not possible to assess the synergistic/antagonistic effects of pollutant mixtures under real field conditions using chemical analysis alone (Kerambrun et al. 2011). Therefore, an alternative monitoring technique involving biochemical biomarkers can be used to monitor water quality in freshwater ecosystems (Van der Oost et al. 1996). Recently, biomarkers have been widely used in monitoring studies due to their sensitivity to pollutants (MAP 2005; WGBEC 2007). Biomarkers are defined as biological measurements of changes at the biochemical, cellular or molecular level occurring in an organism as a result of interactions between the organism and environmental factors (Duarte et al. 2017). Biochemical biomarkers used in ecotoxicology studies act as early warning signals (Gagne et al. 2014).

Exposure to toxic substances is known to cause cell damage with increasing reactive oxygen species (ROS) (Bhattacharya et al. 2021; Chang et al. 2020). The increase in ROS levels causes the formation of MDA and triggers the cell membrane deformation. ROS are also known to cause serious damage to major biomolecules such as DNA, amino acids, and fatty acids (Chatterjee et al. 2021). Oxidative stress occurs with the disruption of the balance between antioxidant enzymes such as CAT, SOD, GPx, GST and ROS (Bhattacharya et al. 2021). SOD catalyzes the superoxide radical to hydrogen peroxide (Manduzio et al. 2003). CAT reduces the hydrogen peroxide to water and protects the cell from hydrogen peroxide-induced damage (Meng et al. 2011). The GPx enzyme plays a role in detoxification by catalyzing the reaction between pollutants and GSH (Dong 2006). GSH can act as a free radical scavenger by reacting with many radicals such as singlet O_2, superoxide and hydroxyl radicals, and provides stabilization of the membrane structure by removing acyl peroxides formed in lipid peroxidation reactions (Price et al. 1990). GSH also serves as a substrate for the GST enzyme. GST is a phase II biotransformation enzyme used as an indicator of exposure to xenobiotics (Gunderson et al. 2016). When the levels of free radicals increase, they damage the lipids in the cell membrane and cause lipid peroxidation. MDA is formed as a result of lipid peroxidation. MDA is used as an indicator of oxidative damage to lipids (Kasai 1997). The zebra mussel *D. polymorpha* was used as a model organism. It is an invasive species that is widely used in biological monitoring of environmental quality in North America and Europe (Binelli et al. 2015). In this study, *D. polymorpha* was exposed to effluent obtained from the Mardin Kızıltepe Advanced Biological Wastewater Treatment Plant at different concentrations for 96 h under laboratory conditions. Changes in biochemical parameters such as MDA and GSH levels and SOD, CAT, GPx and GST activity in *D. polymorpha* were examined, and the treatment efficiency of the wastewater treatment plant was evaluated.

The objective of this study was to evaluate treatment efficiency of the Mardin Kızıltepe Advanced Biological Wastewater Treatment Plant using biochemical biomarkers of the zebra mussel *D. polymorpha*, one of the most useful biological models in freshwater ecotoxicology.

2. Materials and methods

2.1. Water analysis

The effluent used in this study was obtained from the Kızıltepe Wastewater Treatment Plant in Mardin, Turkey. The treatment process at this plant involved biological removal of nitrogen, phosphorus and carbon based on anaerobic (oxygen-free) sludge digestion and a simultaneous denitrification process. The treatment plant also had an ultraviolet disinfection unit. Five liters of effluent were provided and stored in a refrigerator at 4°C. Samples were collected in June 2022. Approximately 10 l of water were collected from a location (38°48′09″N; 38°43′53″E) with a depth of 10 cm on the Munzur River, Tunceli, Turkey, to be used in the control group as unpolluted reference water.

Standard methods were used to analyze COD, BOD, SS, TN, TP, and pH parameters. COD measurements were carried out according to the open reflux method (5220-B Open Reflux Method) on samples collected from both the pilot scale reactor and the treatment plant. TN measurements were also made using the fractionation and distillation method (4500-Norg B. Macro-Kjeldahl Method). The stannous chloride method (4500-P D. Stannous Chloride Method) was used for phosphorus measurements, while PO43-P measurements were made by direct staining, and TP measurements were made by staining after the acid degradation process. SS analyses were performed by the gravimetric method (2540 D; total suspended solids).

Quality parameters of the effluent from the wastewater treatment plant and reference water are presented in Table1.

Table 1

ABWTPE quality parameters

Parameters	Unit	ABWTPE*
Chemical Oxygen Demand (COD)	mg l^{-1}	10.60 ± 1.22
Biochemical Oxygen Demand (BOD)		8.78 ± 0.88
Suspended Solids (SS)		6.56 ± 0.32
Total Nitrogen (TN)		10.85 ± 0.55
Total Phosphorus (TP)		1.50 ± 0.02
pH		7.85 ± 0.31

*means ± standard error as ANOVA results

2.2. Exposure of *D. polymorpha* to ABWWTPE

D. polymorpha model organisms were obtained from the Munzur River. They were placed in ventilated plastic containers. They adapted to environmental conditions in aquaculture laboratories of Munzur University at 18 ± 0.5°C, exposed to a 12 h light/12 h dark cycle for one month. They were fed plankton.

Three experimental groups were designed: 1) a control group (unpolluted reference water); 2) a group exposed to wastewater treatment plant effluent (A); and 3) a group exposed to 1/2 diluted wastewater treatment plant effluent (B). The experiments involved examining the exposure of three groups of *D. polymorpha* (10 individuals in each group) to wastewater samples for 24 h and 96 h.

2.3. Biochemical Analysis

Individuals of the test organisms were weighed and 1/5 w/v PBS buffer (phosphate buffered salt solution) was added and homogenized with ice using a homogenizer. Homogenized samples were centrifuged at 17,000 rpm in a cooled centrifuge for 15 min and the resulting supernatants were kept in a deep freezer at –86°C until analysis.

ELISA kits used for SOD, CAT, GP_X and GSH biochemical assays were purchased from Cayman Chemical Company with catalog numbers 706002, 702002, 703102, 703002 and 707002, respectively.

The superoxide dismutase assay kit utilizes a tetrazolium salt to detect superoxide radicals generated by xanthine oxidase and hypoxanthine. One unit of SOD is defined as the amount of enzyme needed to exhibit 50% dismutation of a superoxide radical, measured as the change in absorbance per minute at 25°C and pH 8.0.

The Catalase Assay Kit utilizes the peroxidatic function of CAT to determine enzyme activity. The method is based on the reaction of the enzyme with methanol in the presence of an optimal concentration of H_2O_2.

The GP_X assay measures GP_X activity indirectly through a coupled reaction with glutathione reductase (GR). Oxidized glutathione (GSSG), produced during the reduction of hydroperoxide by GPX, is recycled to its reduced state by GR and NADPH.

Changes in MDA levels were measured spectrophotometrically according to a method developed by Placer et al. (1966). MDA, which can be measured by thiobarbituric acid (TBA), is produced through the peroxidation of fatty acids containing three or more double bonds. MDA, the end product of fatty acid peroxidation, reacts with TBA to form a pink complex. The resulting pink color was read at 532 nm. Accordingly, 0.25 ml of the obtained homogenates were taken and 2.25 ml of the color reagent (TBA and 10% trichloroacetic acid) were added.

2.4. Statistical analysis

Statistical software SPSS 24.0 was used in the present study. Duncan's Multiple Range Test was used to determine changes in the control group and wastewater exposure groups. The differences between the groups were determined using two-way analysis of variance (ANOVA) and an independent *t*-test.

3. Results

The increase in MDA levels was statistically significant in A (effluent exposure) and B (1/2 diluted effluent exposure) groups after 24 h and 96 h compared to the control group ($p < 0.05$). It was observed that MDA levels were higher in group A compared to group B ($p < 0.05$). It was found

that different exposure durations did not cause a statistically significant difference in the control, A and B groups ($p > 0.05$; Fig. 3.1).

The decrease in GSH levels was statistically significant in groups A and B after 24 h and 96 h compared to the control group ($p < 0.05$). When groups A and B were compared, it was observed that GSH levels increased in group B at 24 h ($p < 0.05$). A statistically significant difference was found in group B at 24 h ($p < 0.05$; Fig. 1).

The increase in SOD activity was statistically significant in groups A and B after 24 h and 96 h compared to the control group ($p < 0.05$). It was observed that SOD activity was higher in group B compared to group A ($p < 0.05$). Different exposure durations were found to cause a statistically significant difference in the control and B groups ($p < 0.05$; Fig. 1).

The decrease in CAT activity was statistically significant in groups A and B after 24 h and 96 h compared to the control group ($p < 0.05$). There were no statistical differences between groups A and B ($p > 0.05$). Different exposure durations were found to result in a statistically significant difference in group B ($p < 0.05$; Fig. 1).

The increase in GP_X activity was statistically significant in groups A and B after 24 h and 96 h compared to the control group ($p < 0.05$). When groups A and B were compared, it was observed that GP_X activity decreased in group B at 96 h ($p < 0.05$).

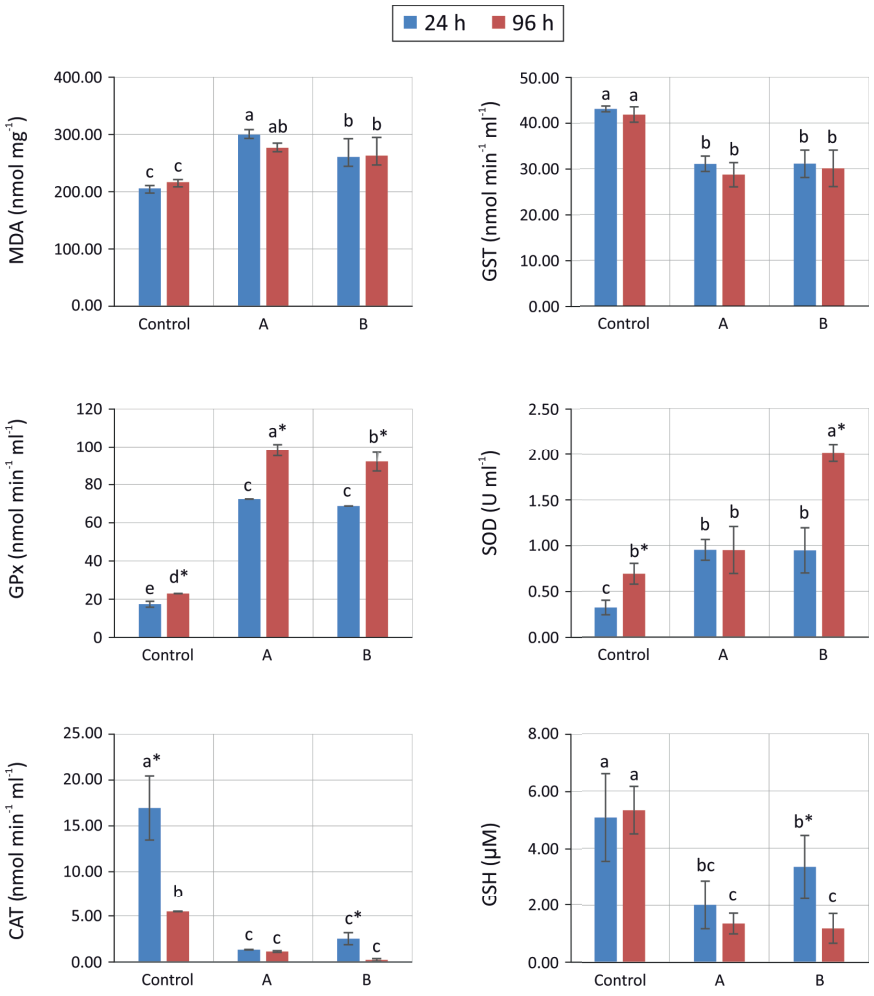

Figure 1

Changes in MDA, GSH levels and SOD, CAT, GP_X, GST activity in *D. polymorpha* exposed to different concentrations of ABWTPE for 24 and 96 h. Different letters (a, b, c, d) on the whiskers show statistical differences between the treatment groups for the same exposure duration ($^{abc}p < 0.05$), calculated according to Duncan's multiple range test. The * symbol on the bar indicates statistical differences between different exposure durations (24 h and 96 h) in the same administration group, calculated according to independent *t*-test.

Different exposure durations were found to cause a statistically significant difference in the control, A and B groups ($p < 0.05$; Fig. 1).

The decrease in GST activity was statistically significant in groups A and B after 24 h and 96 h compared to the control group ($p < 0.05$). There were no statistical differences between groups A and B ($p > 0.05$). Different exposure durations did not cause a statistically significant difference in the control, A and B groups ($p > 0.05$; Fig. 1).

4. Discussion

The present study assessed the biochemical responses of *D. polymorpha* exposed to ABWTPE from the Kızıltepe Advanced Wastewater Treatment Plant. The treatment efficiency of the Mardin Wastewater Treatment Plant was evaluated based on changes in parameters such as MDA, GSH, SOD, CAT, GST and GP_X in *D. polymorpha*.

Yildirim et al. (2020) investigated changes in biochemical biomarkers in *Astacus leptodactylus* exposed to the effluent from the Elazig wastewater treatment plant. They found that SOD activity and MDA levels increased at the end of the 24th and 96th hour, while the CAT activity decreased at the end of the 24th hour, but increased at the 96th hour. GSH levels were found to decrease compared to the control group. In the study conducted by Chang et al. (2020), oxidative damage occurred in the crucian carp (*Carassius auratus*) exposed to increasing concentrations of wastewater from a municipal wastewater treatment plant for 15 days. Increased MDA content and SOD, CAT, GP_X activities were observed, as well as the inhibition of GSH content. In the current study, however, we detected reduced CAT activity. Tetreault et al. (2021) reported induction of antioxidant enzymes and ROS activity in two fish species exposed to wastewater. Karataş et al. (2009) found a decrease in the amount of antioxidant vitamins (A, E and C) and selenium (Se), reduced glutathione (GSH)/oxidized glutathione (GSSG) ratio in *Lemna gibba* L. plants exposed to effluent, and an increase in the amount of MDA (an indicator of lipid peroxidation). In parallel with this study, we also obtained a decrease in GSH levels and an increase in MDA levels. Chang et al. (2019) investigated biochemical responses in the antioxidant defense system in *Carassius auratus*, which was exposed to wastewater from a treatment plant. They found that MDA levels and GP_X activity showed an increasing trend, while the GSH content decreased significantly. Although SOD activity was quite high in 5% and 10% treatment groups, it was found to be significantly lower in 40% and 50% treatment groups. CAT activity was found to have an upward trend in 20%, 30% and 40% groups. In our study, we found an increase in SOD and GP_X activity and MDA levels, but CAT activity tended to decrease, in contrast to Chang et al. (2019). Tatar et al. (2018) assessed the quality of the effluent by evaluating control MDA and GSH levels, and SOD, CAT and GP_X activity. The MDA level and SOD activity in *Gammarus pulex* varied depending on the exposure duration. GSH levels and GP_X activity increased, while CAT activity decreased compared to the control group at the end of the 96th hour in *G. pulex* exposed to wastewater from a treatment plant. Their study and our study show similar results, despite the use of different model organisms. Ahmad et al. (2000) reported that various tissues in the freshwater fish *Channa punctatus* were damaged as a result of short- and long-term exposure to paper mill effluent. They observed a time-dependent increase in GP_X and GST activity and GSH levels, resulting in a decrease in CAT activity in the liver. Similar to Ahmad et al. (2000), we found in our study that GPX and CAT activity increased, while GSH levels decreased.

Samatha et al. (2018) evaluated the toxic effect of the secondary effluent by using oxidative stress parameters such as CAT and GST activity and lipid peroxidation levels in the freshwater pond carp *Carassius auratus* as a model organism in the Sinchon River. They showed that CAT, GST and lipid peroxidation levels are suitable biomarkers that can be used for assessing effluent quality. Wan et al. (2015) exposed individuals of the marine bivalve *Meretrix meretrix* to 0%, 1%, 5%, 10% and 20% (v/v) treated wastewater for 15 days and examined the activity of SOD, CAT, GP_X, and GR, as well as the content of GSH and MDA as oxidative stress biomarkers. A decrease in GST enzyme and GSH levels was observed as a function of wastewater concentration. Lipid peroxidation damage was not observed in groups exposed to less than 20% wastewater. High amounts of ROS trigger lipid peroxidation, which causes severe oxidative damage to the cell membrane, DNA, amino acids and fatty acids (Chatterjee et al. 2021; Kumari et al. 2014). Similarly, our study found that GSH levels and GST activity decreased as a function of concentration, but unlike the above studies, lipid peroxidation also increased. In the present study, the MDA level increased in *D. polymorpha* exposed to ABWTPE, and this increase is an indicator of oxidative stress. ABWTPE may have induced oxidative stress, possibly leading to excessive ROS production in the model organism (Almeida et al. 2007). After exposure to xenobiotics, changes in biochemical parameters such as GSH, GSSG, GSH/GSSG can be observed as an

adaptation to pollutants. These changes have been proven to play a critical role in maintaining cellular homeostasis (Tatar et al. 2018). Reduced GSH levels are considered a biological biomarker observed in model organisms exposed to stress (Chang et al. 2020). Decreased GSH content was observed in animals exposed to ABWTPE due to excessive use of GSH to cope with stress (Wan et al. 2015). In this study, GSH levels were found to decrease after both periods of exposure to ABWTPE at various concentrations. In this study, it was observed that the GP_X activity increased in groups A and B after the exposure period compared to the control group ($p < 0.05$). GP_X activity in tissues and cells of *D. polymorpha* exposed to ABWTPE is thought to increase to cope with peroxide toxicity (Gill and Tuteja 2010). SOD and CAT, which are antioxidant enzymes, are the first step of the defense system. While SOD converts superoxide radical to hydrogen peroxide, CAT catalyzes hydrogen peroxide into water, protecting cells from possible damage (Chang et al. 2020). In the present study, SOD activity increased in groups A and B after exposure to ABWTPE for 24 h and 96 h compared to the control group ($p < 0.05$). The amount of superoxide radical is thought to increase after exposure to effluent in *D. polymorpha*. It is believed that there may be an increase in SOD activity to catalyze the increased superoxide radical to hydrogen peroxide (Modesto and Martinez 2010; Zheng et al. 2013). In the present study, the reduced enzyme activity of CAT in groups A and B after exposure to ABWTPE for 24 h and 96 h compared to the control group indicated that the detoxification capacity of CAT was surpassed by the amount of hydroperoxide products of lipid peroxidation (Carvalho et al. 2012).

5. Conclusion

The present study demonstrated that ABWTPE could induce oxidative stress in *D. polymorpha*, because the biochemical responses of the antioxidant defense system, namely SOD, CAT, GP_X, GST, GSH and MDA in *D. polymorpha* were significantly altered by ABWTPE over the exposure period. The results showed that SOD, CAT, GP_X, GST, GSH and MDA in *D. polymorpha* can be used as biomarkers to assess water quality of the receiving freshwater environment polluted by ABWTPE due to their sensitive responses to different concentrations of ABWTPE. Although the physicochemical parameters of the effluent are at levels permitted for discharge into the receiving environment under relevant regulations, the biomarker response showed that the treatment efficiency is not adequate in terms of toxicity. According to the results obtained in this study, improvements to the existing treatment processes at the Kızıltepe advanced wastewater treatment plant can be suggested. Furthermore, our findings indicate that changes in these biochemical biomarkers in *D. polymorpha* can be used effectively to assess the wastewater treatment plant efficiency. However, it is recommended that more biochemical parameters be used in future studies.

Ethical approval statement

Animal subjects used in the study were mussels, which are invertebrates and are exempt from this requirement.

Conflict of interest

All authors declare that they have no conflict of interest.

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