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Single and combined effects of dimethoate and malathion on oxidative stress biomarkers in the non-target freshwater mussel *Dreissena polymorpha*

by

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Abstract

This study evaluates the single and combined effects of dimethoate (DI) and malathion (MA) on oxidative stress biomarkers in the freshwater mussel *Dreissena polymorpha*, estimating the potential harm of these pesticides on aquatic ecosystems. Superoxide dismutase (SOD) and catalase (CAT) activities, and glutathione (GSH) and lipid peroxidation (TBARS) levels were determined in *D. polymorpha* exposed to sublethal concentrations of DI, MA and a combination of the two during 24 and 96 h periods. The results showed that the GSH levels were decreased but the TBARS levels were increased in all the exposure groups after 24 and 96 h compared to the control. It was observed that SOD activity decreased but CAT activity increased in all the DI exposure groups after 24 h compared to the control. At the end of 96 hours, it was observed that CAT and SOD activities increased again in some exposure groups compared to the control. Exposure time also had an effect on biomarkers in different levels. According to the results, the cytotoxicity of DI and MA combination depended on their concentrations. DI or/and MA, in sub-lethal concentrations, induced oxidative damages in *D. polymorpha*. Combined exposure of the pesticides can alter their toxicity and may be evidence of increased toxicity and oxidative stress.

Key words: Dimethoate, Malathion, combined effects, oxidative stress, *D. polymorpha*

1. Introduction

Pesticides enter aquatic ecosystems through a variety of processes, including direct application, industrial and urban wastewater discharges, and runoff from some other non-point sources (Sharma 1990). The intensive use of organophosphorus pesticides in many fields such as agriculture, medicine and industry can cause many problems in humans and wildlife (Garcia et al. 2003, Kalender et al. 2010, Banaee et al. 2019a). MA is one of the most heavily used organophosphate pesticides against various pests in agriculture, as this insecticide is also considered to be of low toxic value for domestic use (Lasram et al. 2008). Exposure of MA on experimental animals, however, has shown that it can change biochemical and hematological parameters as well as antioxidant enzyme activities in different tissues (Uzun and Kalendar 2011). DI is a dithiophosphate insecticide with wide application on various fruits and vegetables and some other crops (Hassall 1990).

Exposure to chemicals often causes oxidative stress. Oxidative stress induced by overproduction of reactive oxygen species (ROS) is a precursor to many of the problems associated with this organophosphorus exposure and many findings have reported the enhancement of oxidative stress in animals (Hu et al. 1994, Franco et al. 2009). Mixed pesticides can cause significant synergistic effects of toxicity on target species, although they are also effective on non-pest species compared to single pesticides (Bonansea et al. 2017).

In aquatic ecosystems, the organophosphate insecticide dimethoate could increase the environmental risk to nontarget organisms (Acquaroni et al. 2021). Understanding the relationship between pesticides and their toxic effects and accumulation in *D. polymorpha* can provide better insight into the ecological risks from pesticide contamination and is crucial for accurately monitoring and predicting the effects of these pollutants in the aquatic environment (Bashnin et al. 2019). Biomarkers can provide information about the health status of organisms and, therefore, these markers can be used as early warning signals of general or specific stress (Korte et al. 2000). Biochemical and oxidative biomarkers are very useful to detect harmful effects and toxicological mechanisms caused by environmental pollutants (Banaee et al. 2019a). Therefore, the enzymes such as SOD, CAT and GSH can be used as biomarkers in the case of oxidative stress caused by pollutants (Rocher et al. 2006, Acquaroni et al. 2021).

The SOD catalyzes the superoxide dismutation into O_2 and H_2O_2, which in turn is further reduced by CAT to H_2O and O_2. GSH is a vital antioxidant that acts as a direct scavenger of oxidants as well as being a substrate for antioxidant enzymes (Ferrari et al. 2007). Pesticides, metals and other xenobiotics cause lipid peroxidation, which is considered as an important indicator of oxidative damage of cell membrane components, known as the first step in cell membrane damage (Gamble et al. 1995, Serdar et al. 2018). The zebra mussel *D. polymorpha* is considered an invasive species found from the Caspian Sea to European and North American rivers and lakes. Due to its moderate sensitivity against anthropogenic pollutants, *D. polymorpha* established sustainable populations also in urban watercourses (Horgan et al. 1997).

This study was conducted to determine the sublethal effects of exposure, both single and combined, of the two insecticides DI and MA on oxidative stress in *D. polymorpha* and to evaluate its underlying mechanism.

2. Materials and methods

2.1 Test organisms

The *D. polymorpha* individuals used in the study were obtained from Keban Dam Lake in Elazig, Turkey (38°48'09" N, 38°43'53" E). *D. polymorpha* was collected by hand and brought alive to Munzur University, Faculty of Fisheries, Aquatic Toxicology Research Laboratory in plastic containers with air reinforcement.

2.2 Adaptation of the test organism to laboratory conditions

The living *D. polymorpha* samples brought to the laboratory were placed in prepared aquariums. The photoperiod with 14 hours of light and 10 hours of darkness was applied in laboratory lighting. The ambient temperature was kept constant by adjusting to 18°C during both the adaptation and test stages, thanks to the thermostat air conditioner. Cultured phytoplanktons were used to feed the *D. polymorpha*. In order to meet the oxygen need in stock aquariums, an air motor and external filter were used in the aquarium (Serdar 2021).

2.3 Determination of LC50 value and experimental design

DI was obtained from the local market, in the form of Polygor EC (purity 25%, dissolved in 75% acetone). MA was obtained from the local market, in the form of 65% malathion EC. Each pesticide was prepared

from a stock solution weighed in a volumetric flask containing distilled water. Dilutions of the defined stock solution were used for the tests described below. The stock solutions were renewed every 12 h. The lethal concentration-50 (LC_{50}) value of the DI and MA was determined after 96 hours of exposure. For this, first of all the death range was determined and then the static test was conducted (APHA, 1998). The LC_{50} values of the DI and MA were measured as 120.14 ± 6.26 and 52.06 ± 5.33 mg l^{-1} respectively. The LC_{50} value of the combined exposure of the two insecticides was determined to be 39.91 ± 2.47 mg l^{-1}. Three subletal doses of the DI, MA and combined exposure of the two insecticides (1/16, 1/8 and 1/4 ratio of the LC_{50} value) were applied to the *D. polymorpha* for 24 and 96 h. The *D. polymorpha* (n: 10 for each group) were exposed to synthetic solutions containing DI and MA for 24 hours and 96 hours. Accordingly, the following ten experimental groups were designed: Group A (containing 3.25 mg l^{-1} MA); Group B (containing 6.50 mg l^{-1} MA); Group C (containing 13.00 mg l^{-1} MA); Group D (containing 7.50 mg l^{-1} DI); Group E (containing 15,00 mg l^{-1} DI); Group F (containing 30.00 mg l^{-1} DI); Group G (containing 2.5 mg l^{-1} DI + containing 2.5 mg l^{-1} MA); Group H (containing 5 mg l^{-1} DI + containing 5 mg l^{-1} MA); Group I (containing 10 mg l^{-1} DI + containing 10 mg l^{-1} MA); the Control Group (not containing DI and MA).

2.4 Dissection procedures and preparation of supernatants

The test organism individuals were opened with a scalpel and the dissection process was performed on each of them. 0.5 g of the organism was weighed and homogenized using a homogenizer with ice, adding a PBS buffer (phosphate buffered salt solution) at a ratio of 1/5 w/v. These homogenized samples were then centrifuged in a refrigerated centrifuge at 17.000 rpm for 15 minutes and the supernatants obtained were stored in a -86°C deep freezer until the measurement process was completed (Serdar 2021).

2.5 Determination of the biochemical response

In this study, the activities of the SOD, CAT and the levels of GSH, TBARS were measured to determine the oxidative biomarker response of the *D. polymorpha*. All the biochemical parameters (SOD, CAT, GSH and TBARS) were measured in a microplate reader using commercial kits purchased from the Cayman Company (catalog number SOD: 706002, CAT: 707002, GSH: 703002, TBARS: 10009055) (Erguven et al. 2022).

2.6 Statistical analysis

SPSS version PASW Statistics 18 was used for the statistical analysis. A one-way ANOVA and Duncan's multiple range tests were applied to determine the statistical differences in the control and all exposure groups (A, B, C, D, E, F, G, H and I) at the same exposure time ($^{abc}p < 0.05$). The two-tailed independent *t*-test was applied in order to compare the differences between the exposure times (24 and 96 h) in the same control and exposure groups ($p < 0.05$).

3. Results

3.1 Acute toxicity (LC_{50}) value

In our study, the average of the LC_{50} value of *D. polymorpha*, exposed to three repetitive DI pesticides, was found as 120.14 ± 6.26 mg l^{-1}. The LC_{50} value of the MA was determined as 52.06 ± 5.33 mg l^{-1}. The LC50 value of the combined exposure of the two insecticides was measured as 39.91 ± 2.47 mg l^{-1}.

3.2 Biochemical Response Parameters

The activities of the SOD, CAT and the levels of GSH, TBARS are given in Table 1.

3.2.1 SOD enzyme Activity

It was determined that the SOD activity decreased in all the groups after 24 h compared to the control ($p < 0.05$). An increase was detected in the combined exposure of the two insecticides groups after 96 h compared to the control ($p < 0.05$). When the SOD enzyme activities were compared at the same exposure time, it was determined that there was a statistically significant change in these activities at the end of 96 hours ($p < 0.05$) (Figure 1).

3.2.2 CAT enzyme Activity

The CAT activity increased in all the DI exposure groups after 24 h compared to the control ($p < 0.05$). There was a decrease in the E and F groups depending on the MA exposure after 24 h compared to the control ($p < 0.05$). It was found that CAT activity increased significantly in the B, C, D, G and H groups after 96 h compared to the control ($p < 0.05$), but there was a statistically insignificant decrease in CAT activity in the A, E, F, I and B groups ($p > 0.05$). Compared to the single exposure, the CAT activity was reduced in the combined exposure after 24 h ($p < 0.05$). There was a

Table 1

Biochemical response parameters

Parameters	Groups	Exposure Time	
		24 h	96 h
GSH (µM)	Control	2.66 ± 0.61	2.95 ± 0.04
	A	1.50 ± 0.62	1.70 ± 0.20
	B	1.37 ± 0.14	1.32 ± 0.36
	C	1.55 ± 0.21	1.24 ± 0.49
	D	1.05 ± 0.32	1.04 ± 0.10
	E	1.43 ± 0.24	0.94 ± 0.01
	F	0.64 ± 0.10	0.80 ± 0.009
	G	1.34 ± 0.46	1.16 ± 0.16
	H	0.68 ± 0.05	0.55 ± 0.05
	I	1.05 ± 0.07	0.5 ± 0.01
CAT (nmol/min/ml)	Control	3.01 ± 0.21	3.64 ± 0.09
	A	6.79 ± 0.42	2.83 ± 0.12
	B	5.49 ± 1.09	7.66 ± 0.73
	C	3.13 ± 0.39	6.57 ± 0.15
	D	3.24 ± 0.90	5.08 ± 0.90
	E	2.36 ± 0.18	2.56 ± 0.21
	F	2.04 ± 0.02	2.22 ± 0.009
	G	2.56 ± 0.04	9.18 ± 0.05
	H	2.68 ± 0.13	5.19 ± 0.006
	I	2.64 ± 0.07	2.47 ± 0.07
SOD (U/ml)	Control	0.034 ± 0.0005	0.0245 ± 0.001
	A	0.0260 ± 0.006	0.0266 ± 0.003
	B	0.0206 ± 0.002	0.0377 ± 0.002
	C	0.0233 ± 0.003	0.0307 ± 0.001
	D	0.0328 ± 0.002	0.0326 ± 0.001
	E	0.0247 ± 0.002	0.0367 ± 0.002
	F	0.0254 ± 0.001	0.0187 ± 0.0006
	G	0.0283 ± 0.004	0.0272 ± 0.0003
	H	0.0309 ± 0.003	0.0287 ± 0.002
	I	0.0317 ± 0.001	0.0220 ± 0.003
TBARS (µM)	Control	4.78 ± 0.59	2.93 ± 0.24
	A	5.43 ± 0.13	6.58 ± 0.26
	B	9.46 ± 0.11	8.85 ± 0.45
	C	7.09 ± 0.70	7.34 ± 0.40
	D	5.83 ± 0.15	7.01 ± 0.23
	E	6.50 ± 0.53	8.49 ± 0.19
	F	14.41 ± 1.73	11.43 ± 1.05
	G	6.13 ± 1.73	12.36 ± 0.08
	H	6.55 ± 0.26	14.40 ± 0.05
	I	15.07 ± 0.65	47.71 ± 0.76

statistically significant increment in CAT activity in the groups A, C, G, and H after 96 h when compared to the shorter exposure times ($p < 0.05$) (Figure 2).

3.2.3 GSH Levels

The GSH levels were decreased in all exposure groups after 24 and 96 h compared to the control ($p < 0.05$). When the exposure times were compared at the

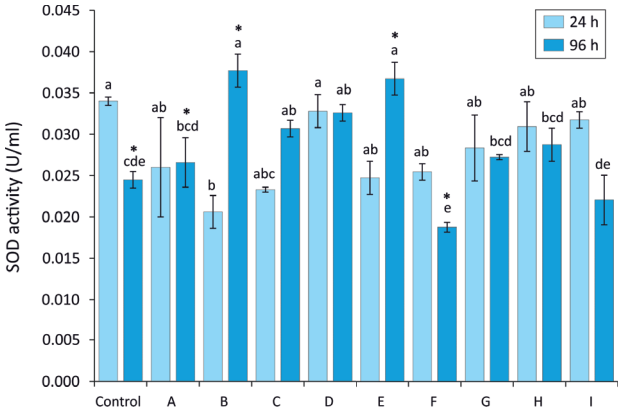

Figure 1

The SOD activities in *D. polymorpha* after single and combined DI and MA exposure during 24 and 96 h. The asterisk (*) on the bar shows statistical differences between different exposure times (24, 96 h) in the same groups according to the independent *t*-test (*$p < 0.05$). Different letters (a, b, c, d, e) on the bar show statistical differences among all groups in the same exposure time according to Duncan's multiple range test ($^{abc}p < 0.05$). Values represent mean ± SE.

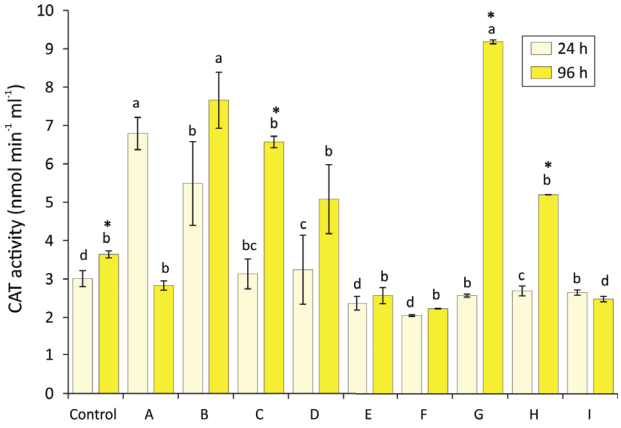

Figure 2

The CAT activities in *D. polymorpha* after single and combined DI and MA exposure during 24 and 96 h. The asterisk (*) on the bar shows statistical differences between different exposure times (24, 96 h) in the same groups according to the independent *t*-test (*$p < 0.05$). Different letters (a, b, c, d, e) on the bar show statistical differences among all groups in the same exposure time according to Duncan's multiple range test ($^{abc}p < 0.05$). Values represent mean ± SE.

end of 96 hours, it was determined that there was no statistically significant change in GSH levels ($p > 0.05$) (Figure 3).

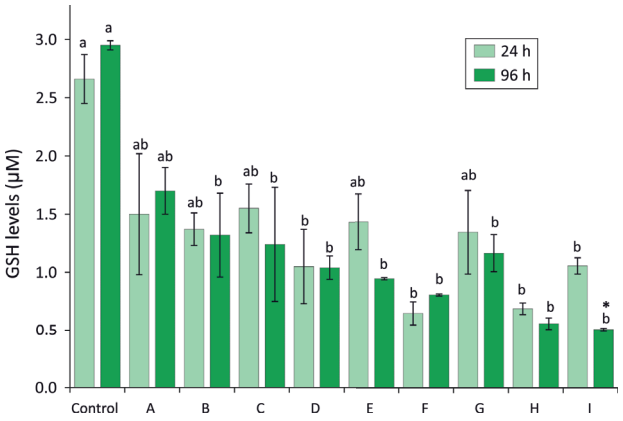

Figure 3

The Levels of GSH in *D. polymorpha* after single and combined DI and MA exposure during 24 and 96 h. The asterisk (*) on the bar shows statistical differences between different exposure times (24, 96 h) in the same groups according to the independent *t*-test (*$p < 0.05$). Different letters (a, b, c, d, e) on the bar show statistical differences among all groups in the same exposure time according to Duncan's multiple range test ($^{abc}p < 0.05$). Values represent mean ± SE.

3.2.4 TBARS Levels

The TBARS levels were increased in all exposure groups after 24 h and 96 h compared to the control group ($p < 0.05$). Compared to the single treatments, the levels of TBARS were increased in the combinated exposure after 96 h ($p < 0.05$). When the exposure times were compared, statistically significant changes were observed in groups B, D, E, G, H and I after 96 hours. ($p > 0.05$) (Figure 4).

4. Discussion

Pesticides are chemicals that are widely used, especially to increase food production efficiency and to control disease vectors. These chemicals are released into the environment from many sources and contaminate water, soil and foodstuffs. In this study, the toxic potential of DI and MA, currently used in agriculture, was investigated at field realistic exposure levels in *D. polymorpha*. The capacity of *D. polymorpha* to cope with the environmental pollution stress was clearly evidenced. The selected biomarkers including SOD, CAT, GSH and TBARS displayed significant changes in activities and levels at different times of exposure.

The acute toxicity value (LC50) in *G. pulex* of the DI pesticide was found to be 167 µg l^{-1} (Serdar 2019). Pandey et al. (2009) carried out a 96 hr static acute toxicity test to determine the LC50 value of DI on the freshwater airbreathing catfish *Heteropneustes fossilis* (Bloch). The LC50 values for DI for 24, 48, 72 and 96 h were 3.38, 3.23, 3.08 and 2.98 mg l^{-1}, respectively. MA is highly toxic to many organisms, although there is a wide variation from one species to another. The 96 h LC50 of MA was estimated for rohu in a semi static system and was found to be 5 µg l^{-1} (Ullah et al. 2016). There was a wide variation in the 48 to 96 h LC50 for 5 test Freshwater molluscs species. Thiara (two species) had figures of 31 to 37 µg l^{-1}; *Viviparus bengalensis* (96 hours) had 1510 to 2340 µg l^{-1}; *Pila globosa*; 10,000 µg l^{-1} to 15,490 µg l^{-1}; and *Biomphalaria havanensis*, 126,270 µg l^{-1} (Anzecc&Armcanz 2000). In this study, the average of the LC50 value of *D. polymorpha*, exposed to three repetitive DI pesticides, was found to be 120.14 ± 6.26 mg l^{-1}. The LC50 value of the MA was determined to be 52.06 ± 5.33 mg l^{-1}. The LC50 value of the MA and DI was determined to be 39.91 ± 2.47 mg l^{-1}.

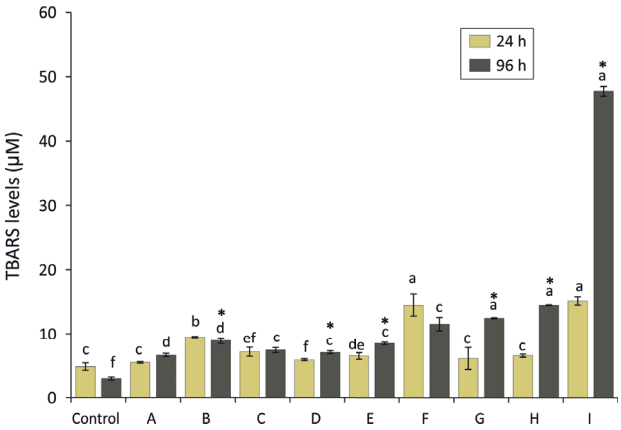

Figure 4

The Levels of TBARS in *D. polymorpha* after single and combined DI and MA exposure during 24 and 96 h. The asterisk (*) on the bar shows statistical differences between different exposure times (24, 96 h) in the same groups according to the independent *t*-test (*$p < 0.05$). Different letters (a, b, c, d, e) on the bar show statistical differences among all groups in the same exposure time according to Duncan's multiple range test ($^{abc}p < 0.05$). Values represent mean ± SE.

At low or non-high concentrations, ROS are considered a result of normal oxidative metabolism, but when they reach high levels, they cause many problems, including DNA damage, lipids, protein oxidation, and enzyme inactivation (Dal-pizzol et al. 2001). During the stress reactions caused by pollution, the antioxidant system increases its activities to scavenge harmful substances, reducing their toxicity and increasing their excretion. Pesticides may cause oxidative stress by increasing the formation of free radicals, resulting in changes in protective enzymatic and non-enzymatic antioxidants and lipid peroxidation (Abdollahi et al. 2004). Banaee et al. (2019b) investigated acute and subacute toxicity tests for chlorpyrifos and glyphosate performed on the crayfish *Astacus leptodactylus*. They found subacute concentrations of chlorpyrifos and glyphosate could lead to a collapse of cellular homeostasis and changes in the hemolymph biochemical parameters in the crayfish. Gunderson et al. (2018) indicate that the biomarkers are sensitive to the pollutants tested and that animals exposed in the wild are potentially compromised in their ability to detoxify environmental contaminants.

Lipid peroxidation is a degenerative process that affects polyunsaturated fatty acids in membrane phospholipids, resulting in toxic aldehydes that react with protein and non-protein substances causing diffuse changes in cell membranes. It is also suggested that organisms exposed to methamphetamine (MA) form covalent adducts between proteins and the carbonyl groups of malondialdehyde. Additionally, covalent adducts between proteins and the carbonyl groups of malondialdehyde are proposed to be generated in organisms exposed to MA (Chitra 2013). Lipid peroxidation and its end product, malondialdehyde (MDA), can occur when antioxidant defenses are not sufficient to neutralize excess ROS, presumably produced during the biotransformation process (Modesto & Martinez 2010). TBARs are used to measure the lipid peroxidation products in cells, tissues and bodily fluids (Frost et al. 2019). Previous studies have shown that DI intoxication causes oxidative stress by producing free radicals, resulting in cellular damage and lipid peroxidation (Singh et al. 2004). Wankhade (2012) demonstrated that sustained and prolonged exposure of mice to a non-lethal dose of MA induced lipid peroxidation.

Exposure to dimethoate has been reported to result in increased MDA in freshwater fish such as *Channa punctatus* (Ali et al. 2014), *Oncorhynchus mykiss* (Dogan & Can 2011), *Cyprinus carpiove* (Shadegan et al. 2018) and *Danio rerio* (Maharajan et al. 2018). Akhgari et al. (2003) found that MA exposure (100, 316, 1000, 1500 ppm) for 4 weeks resulted in increases in MDA levels in red blood cells (RBC) and the liver. Aksoy and Alper (2019) suggested that lipid peroxidation occurred in rats exposed to MA. Due to its lipophilic nature, MA was thought to penetrate the membrane and initiate lipid peroxidation. Similarly, in our study, TBARS levels were found to increase in all the exposure groups compared to the control after 24 and 96 h.

SOD is an important enzyme involved in removing oxyradicals and scavenges superoxide into hydrogen peroxide (H_2O_2) and oxygen (Su et al. 2014). The H_2O_2 is then converted to H_2O and O_2 by CAT, a type of enzyme containing Fe-protoporphyrin (Koivula et al. 2011). CAT activity in contaminated environments may be increased or suppressed depending on the type of contaminant (Sobjak et al. 2017). The reaction mechanism of SOD and CAT enzymes after exposure to pesticides differs according to the structure of the pesticides, the type of organism and the targeted part of the organism (Oruc&Usta 2007). In a study conducted by Serdar et al. (2021), *D. polymorpha* was exposed to non-lethal concentrations (1/20, 1/10 and 1/5 of the LC_{50}) of cyfluthrin for 24 and 96 hours. These researchers found that the enzyme activities of SOD were increased, but the CAT activity was inhibited compared to the control. In another study, Pala et al. (2019) found a statistically significant reduction in CAT activity and GSH levels when exposed to non-lethal concentrations of the glyphosate-based herbicide for 24 and 96 hours. Barski et al. (2012) showed that subchronic exposure of rats to DI produced a strong oxidative stress resulting in a significant increase in CAT and SOD activity in erythrocytes. In the study conducted by Serdar et al. (2021) on *D. polymorpha* exposed to Cyfluthrin, the SOD activity increased but CAT was inhibited compared to the control. Serdar et al. (2019) found that SOD activity has been decreased after DI exposure in *G. pulex*. However, John et al. (2001) found increased erythrocyte CAT and SOD activities in rats exposed to DI and/or MA compared to the control. They stated that SOD and CAT activities in erythrocytes increased in rats exposed to DI and/or MA, possibly to degrade superoxide anions ($O_2^{\cdot-}$) and decompose H_2O_2. Banaee et al. (2022) investigated chlorpyrifos-induced oxidative stress in *Cyprinus carpio,* They determined the activity of SOD, and total antioxidant levels, significantly decreased in the hepatocytes of *C. carpio* exposed to chlorpyrifos, while malondialdehyde (MDA) increased.

The increase in the activity of these enzymes likely occurred as a response to increased ROS formation due to organophosphate toxicity. Shadegan et al. (2018) found that a significant increase in MDA and CAT activity occurred in fish liver and kidneys exposed

to dimethoate alone or in combination with Bacilar. Demirci et al. (2018) investigated the toxic effects of the combined exposure of the herbicide atrazine and the insecticides endosulfan, indoxacarb and thiamethoxam using oxidative stress biomarkers found in *Gammarus kischineffensis*. They determined higher CAT and SOD activities when atrazine was combined with either endosulfan or indoxacarb compared with atrazine alone. In the present study, an increase was detected in DI+ MA exposure groups after 96 h compared to the control group ($p < 0.05$). CAT activity increased in all the DI exposure groups after 24 h compared to the control ($p < 0.05$). Compared to the single exposure, the CAT activity was reduced in the combined exposure at 24 h ($p < 0.05$). The increase in activity of the CAT enzyme could be due to the engagement of CAT enzyme to convert hydrogen peroxide into water molecules, whereas the decrease in activity detected could be due to the saturation of hydrogen peroxide leading the deactivation or suppression of CAT enzymes for a duration of time (Pawar 2020). Aksoy and Alper (2019) found that SOD and CAT concentrations in rats exposed to MA decreased significantly. In the present study, It was observed that SOD activity decreased in all groups after 24 h compared to the control group ($p < 0.05$). The constant level of increase in the ROS generation and at the same time inadequate supply of SOD to detoxify these ROS', results into a decrease of the level of SOD enzymes in organisms (Pawar 2020).

GSH, which is an important tripeptide in the detoxification system, scavenges organic and metallic xenobiotics with its reduced form (Vasseur and Leguille 2004). It also plays an important role as a reducing agent in preventing the damaging effect of ROS (Gismondi et al. 2012). An increase in GSH levels was observed for diazinon and diuron pesticides in the study conducted by (Velki et al. 2019). They also suggested that the increased GSH levels indicate the occurrence of oxidative stress. Aksoy & Alper (2019) investigated the effects of royal jelly on toxicity and biochemical changes in rats exposed to MA. They determined that the GSH concentrations increased in the brain and decreased in the erythrocyte, liver and kidneys in the group exposed to MA compared to the control groups. They suggested that MA administration increased toxicity in the erythrocyte, liver and kidney tissues and that GSH was utilized as an antioxidant defence agent. Taherdehi et al. (2019) investigated the effects of MA on the GSH level in the testis of male rats. They showed that MA decreased the GSH level compared with the control group. Serdar et al. (2019) determined the response of some biochemical biomarkers in *G. pulex* exposed to DI pesticide. They found that GSH levels in *G. pulex* exposed to the DI pesticide were reduced at both 24 and 96 hours compared to the control group. Smilarly, in the present study GSH levels were decreased in all the exposure groups after 24 h and 96 h compared to the control. The decrease in the GSH levels may be due to the fact that it is the substrate of GST, which takes part in the detoxification reaction (Serdar et al. 2019). This decrease in GSH levels with exposure to pesticides may be related to the increased use of GSH converted to oxidized glutathione and insufficient GSH production.

5. Conclusion

The present study investigated the toxic potential of insecticides DI and MA in *D. polymorpha* at field-realistic exposure levels. The results show that the toxicity of the combination of DI and MA was dependent on their concentrations. The exposed higher concentrations of DI and MA induced significant changes in some biochemical parameters. Sub-lethal concentrations of DI and/or MA caused oxidative damages in *D. polymorpha*.

Exposure times also had an effect on some biomarkers. Therefore, different combinations of pesticides can cause changes in their toxicity. To determine the combined effect of pesticides, it is necessary to evaluate the responses of the right biomarkers, and this is quite difficult. According to the results of our study, it was concluded that the multiple biomarkers we used are suitable for determining the single and combined toxicity of DI and MA.

Ethical approval statement

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

Conflict of interest

All authors declare that they have no confict of interest.

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