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Malathion-induced Biochemical and Molecular Changes in the Brain of *Danio rerio* as Biomarkers of Oxidative Stress Damage

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ABSTRACT

Malathion is the most widely used organophosphorus pesticide in freshwater ecosystems around the world. In *Danio rerio* brain specimens, the effects of MAL exposure on oxidative stress stimulation and acetylcholinsterase, as well as gene expression and histology, were investigated. Antioxidant enzyme activities (superoxides dismutase and catalase) and AChE levels changed widely in brain. The mRNA levels of genes encoding antioxidant enzymes such as Cu/Zn–SOD, Mn–SOD, and COX-17 did not increase when zebrafish were exposed to varied levels of MAL for 5 and 25 days. CAT, GPx, CYP1A, and AChE transcription were all enhanced significantly following exposure to MAL levels ($P<0.05$). Variations in the brain's antioxidant enzyme did not match mRNA induction patterns. Furthermore, with higher exposure time and dose, pathological changes included more severe tissue harm. These alterations are common cellular responses for pesticides and are expected to be an important signal in ecotoxicology studies. Given our findings, the *Danio rerio* can be used as a model organism for the further research of pesticide effects on the CNS and the various mechanisms involved.

1. Introduction

Aquatic environmental pollution is a serious and growing issue. The number and quantity of industrial, commercial and agricultural substances released into the aquatic environment has increased, resulting in a variety of negative effects on aquatic creatures (Barky, et al., 2012). Malathion (MAL) is an insecticide that kills insects by blocking the enzyme acetylcholinsterase (AChE), which is responsible for the disruption of the neurotransmitter acetylcholine. Inactivation of AChE causes acetylcholine to accumulate at cholinergic synapses, resulting in synaptic blockage and signal transmission problems (Ali & Aldhamin, 2019). AChE activity was measured and biochemically identified in a variety of aquatic invertebrates (Ribeiro, et al., 2017). As a result, while there is agreement that discouragement is a marker of exposure to organophosphorus pesticide (Ops), there is disagreement regarding whether inhibiting has a detrimental consequence until it is coupled with physical toxicity. Feeding, escape, and reproductive behaviour, can all be disturbed by changes in AChE activity (Banaee, et al., 2011). Despite their neurotoxic effects, Ops insecticide have other qualities that can influence beings, such as cytotoxicity, genotoxicity, mutagenicity, and carcinogenicity (Ali & Aldhamin, 2019).

Scientists and environmental executives are conducting

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the sub-lethal impacts of pesticides on non-target aquatic creatures, which could be critical for the protection and conservation of such frail ecosystems of highly productive (Rickwood & Galloway, 2004). MAL is a popular Ops insecticide because of its low mammalian toxicity and high insect selectivity when compared to other OPs insecticides. Recent studies have employed antioxidant enzyme activity measurements in fish to assess oxidative damage in chemically influenced aquatic habitats (Abbas, et al., 2019). The enzymes of antioxidants are sensitive indicators of oxidative stresses (Guardiola, et al., 2017; Abdel-Tawwab, et al., 2018). Under normal circumstances, antioxidant enzymes cleanse and remove reactive oxygen species (ROS) and other oxidants of cells (Egea, et al., 2017; Ighodaro & Akinloye, 2018). Oxidative stress occurs when there is a discrepancy between the formation and elimination of reactive oxygen species (ROS), especially when the generation of ROS outnumbers the antioxidant mechanism (Ajima, et al., 2017; Iheanacho & Odo, 2020). ROS generation has been act a potential toxicity mechanism for aquatic species exposed to watery organic contaminants since it can cause oxidative damage (Lushchak, 2011).

As a result, various oxidation-related biomarkers are frequently utilized in aquatic environments to estimate pollution exposure and/or effects on native fish populations, including both enzymatic and molecular characteristics (Feng, et al., 2013). Then, a range of biochemical, molecular, and histopathological reactions can be used to exposed of quantify chemical and offer information regarding spatial and temporal variations in pollutant concentrations. It then applies to environmental quality and negative environmental repercussions. Lower levels of biological structure (biochemical and molecular responses) are more generic, sensitive, repeatable, and easy to distinguish in general (Au, 2004). As a result, the purpose of the study was to use a variety of biochemical, molecular, and histopathological parameters to establish the sub-lethal effects of MAL exposure in brain zebrafish. AChE levels in the brain were assessed, as well as antioxidant defense utilizing superoxides dismutase (SOD) and catalase (CAT). Because these enzymes metabolize a wide spectrum of organic toxicants in tissues of *Danio rerio*, they are commonly utilized as sensitive and accurate biomarkers for the poisonous behavior of major environmental contaminants (Song, et al., 2006).

2. Materials & Methods

Mixed-gender adults Sub-lethal testing was performed on *Danio rerio* (length 3.1-4.6 cm, weight 0.29-0.84 g). They are fed commercial fish food twice a day. In a 40-liter aquarium, fish samples were separated into four groups, each with 100 fish. Xingyinhe Chemical Engineering Co. provided MAL (diethyl [dimethoxy phosphino thioyl]butanediotae) with a purity of 95%. Using Finney's Probit Analysis (Finney, 1971), the 96-hour LC_{50} of MAL was (6.475mg l^{-1}) and group I was used as a control in tap water. For 5, 10, 15, 20, and 25 days, other groups were subjected to C1(0.432 mg MAL l^{-1}), C 2 (0.648 mg MAL l^{-1}) and C 3 (1.295 mg MAL l^{-1}) LC_{50} levels. The fish are collected from each tank at the end of each exposure period to be dissected. However, all institutional and national guidelines for the care and use of laboratory animals were followed.

Biochemical tests and enzyme extraction

After homogenizing brain tissues to a 1/10 (w/v) ratio with cold saline solution NaCl (0.86 percent), the supernatant was used for biochemical examination. At a wavelength of 412 nm, Ellman, et al., (1961) detected AChE activity. Iodide of acetylcholine and dithiobis of nitrobenzoic acid were utilized as substrates. The Can (2011) approach was used to assess the activities of SOD and CAT. Protein activity in units of U/mg has been used to describe basic activity. Bradford (1976) used bovine serum albumin as a standard to determine total protein concentration. The standard calibration curve was set at y=0.0051x-0.0013 (R^2=0.999).

Histopathological Analysis

Collecting and fixing brain tissue in paraformaldehyde, dehydrating with a graded ethanol series, clearing in xylene, and then embedding in paraffin was done immediately after 5 and 25 days of exposure to C1 and C3 mg l^{-1}. A paraffin block rotary microtome was used to generate 7 µ thick sections, which were subsequently stained with haematoxylin and eosin. Histopathological alterations were investigated using a Nikon Eclipse Bio microscope.

RNA Extraction and Real-time Quantitative RT-PCR

Total RNA was isolated from brain zebrafish (frozen in liquid N_2) according to the manufacturer's instructions using TRIzol®Reagent (Invitrogen). After each sample's RNA quality was assessed. The RNA was denatured for 5 minutes at 65° C. First-strand cDNA was synthesized using a Thermo Scientific first-strand cDNA synthesis kit. In addition to the β-actin control genes, PCR primers for target genes were developed, as well as the Primer Premier 5 software. Then sent to the Shanghai Sunny Biotechnology Co.,China for synthesis. Table 1 lists all of the primers used in real-time quantitative PCR.

Table 1
The primer pair sequences utilized in real-time quantitative PCR experiments

Gene	Primer sequence	Gene bank accession	Length
GPx	F:5′-GTGCTCGCTGTATCTCCT-3′ R: 5′-CGTCCAGAAACGCACCAC-3′	NM200222	463 bp
Mn–Sod	F:5′-ACGGACTATGTTAAGGCCATCT-3′ R: 5′-CCACTCGGTTGCTCTCTTTTCTCT-3′	AY195857	123bp
Cu/Zn-SOD	F:5′-TCAATCAAGAGGGTGAAAAGAAGCC-3′ R:5′-TGACCAAGTTAGCATTGCATCCTCG-3′	NM131294	262bp
AChE	F:5′-TTACTCTTGCCCACTGTCTACTC-3′ R:5′-CTTCACTCATCACTCTGTTGGGGTTC-3′	NM131846	331bp
COX-17	F:5′-GCGGCGCAGAAAAAGCCACT-3′ R: 5′-ACACAAGCAGTCACACACAT-3′	NM001004652	200bp
CYP1A	F:5′-CGAACATCCCAGACGGGCTAC-3′ R:5′-CCCTAATTACTGATGTGCTCCTCT-3′	AB078927	410bp
CAT	F:5′-ATGGCAACTGGGATCTTACA-3′ R: 5′-TGTATGGGACCAGACCTTGG-3′	AF170069	498 bp
β-actin	F :5′-AACTGGTGAAATTACTGGCCTTACT-3′	NM131031	452bp

R:5′-TGTAATTGTCAGCGGGCTAAGT-3′

An ABI 7900HT real-time PCR apparatus with SDS v2.3 Software was used to measure mRNA expression in duplicate (Applied Biosystems). GPx, AChE, COX-17, CYP1A, Mn–SOD, Cu/Zn–SOD, CAT, GPx and β-actin gene expression was measured using oligonucleotide primers and the SuperReal PreMix Plus Kit (SYBR Green (Tiangen)) with ROX as the reference dye, according to the manufacturer's instructions. Initial denaturation at 95°C for 15 min, 95°C for 10 sec for 40 cycles, 60°C for 20 sec, and 72°C for 30 sec were the cycling conditions. The β-actin mRNA ratio was calibrated to the control (control=1) values as the mRNA concentration increased. The $2^{-\Delta\Delta Ct}$ aproach was then used to measure the relative quantification of the amount of expression of the mRNA gene between treatment groups (Livak & Schmittgen, 2001).

Statistical analysis: Through a two-way Variance Analysis (ANOVA), the data collected are statistically analyzed. The differences between the control and exposed groups are accurately checked. The significance criteria was established at ($p<0.05$). GraphPad software was used to conduct the statistical analysis.

3. Results & Discussion

Effect of MAL on AChE and antioxidant enzymes

After 10 and 20 days of exposure to a lower MAL dose (C1mg l^{-1}), brain AChE decreased considerably ($p<0.01$) by 60.12 and 84.54 %, respectively, when compared to control. After 10, 15, 20, and 25 days of C2 and C3 mgl^{-1} treatment, MAL's AChE level decreased gradually (70.17, 60.97, 76.29, and 75 %) (Figure 1A). After 10, 15, 20 and 25days, the antioxidant enzymes CAT and SOD increased their dose- and time-dependent activity in brain tissues. As a result, antioxidant CAT activity increased significantly ($p < 0.01$) at all concentrations during the experiment (215.25, 387.88, 289.95, and 202.17) % (Figure 1C). In addition, when compared to the control, Figure 1B shows that the activity of SOD antioxidant enzymes increased to (237.09, 255.73, 254.25, and 188.62) %, respectively, at all concentrations.

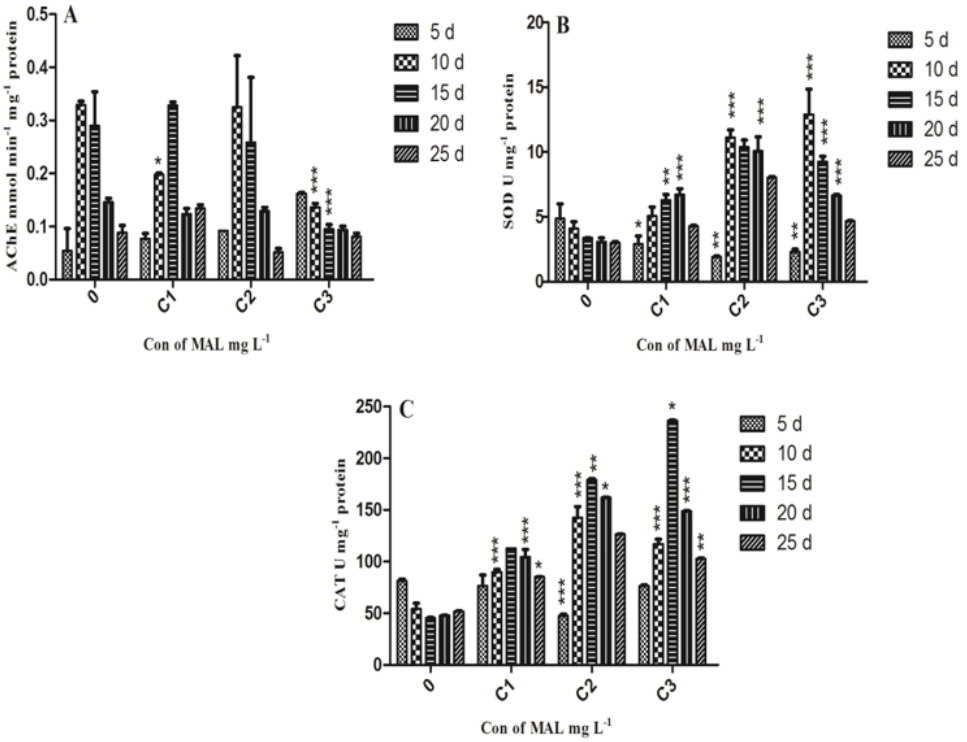

Fig. 1. AChE (A), SOD (B), and CAT (C) activities in the brain of zebrafish after 5, 10, 15, 20, and 25-day exposure to different levels of MAL. Values are shown as mean ± SD. When contrast to controls, the asterisk indicates a statistically significant difference; * at $p<0.05$, ** at $p<0.01$ and *** at $p<0.001$

Histological observation

The overall histological analysis revealed a low to medium incidence of chemical damage on zebrafish tissues after 5 and 25 days of exposure to MAL. In Figure 2, histopathological alterations in brain tissue are depicted. The control group found no histological changes in any of the tissues we investigated. The brain structure of MAL-treated fish, on the other hand, has changed, with the extent of the change increasing with the severity of exposure. The fish's brain showed histopathological changes that were more susceptible to C3 than C1. As shown in Figure 2, zebrafish brain tissue showed various degrees of granule cell loss, Nissl body reduction, Purkinje cell degeneration, and neuropil loss.

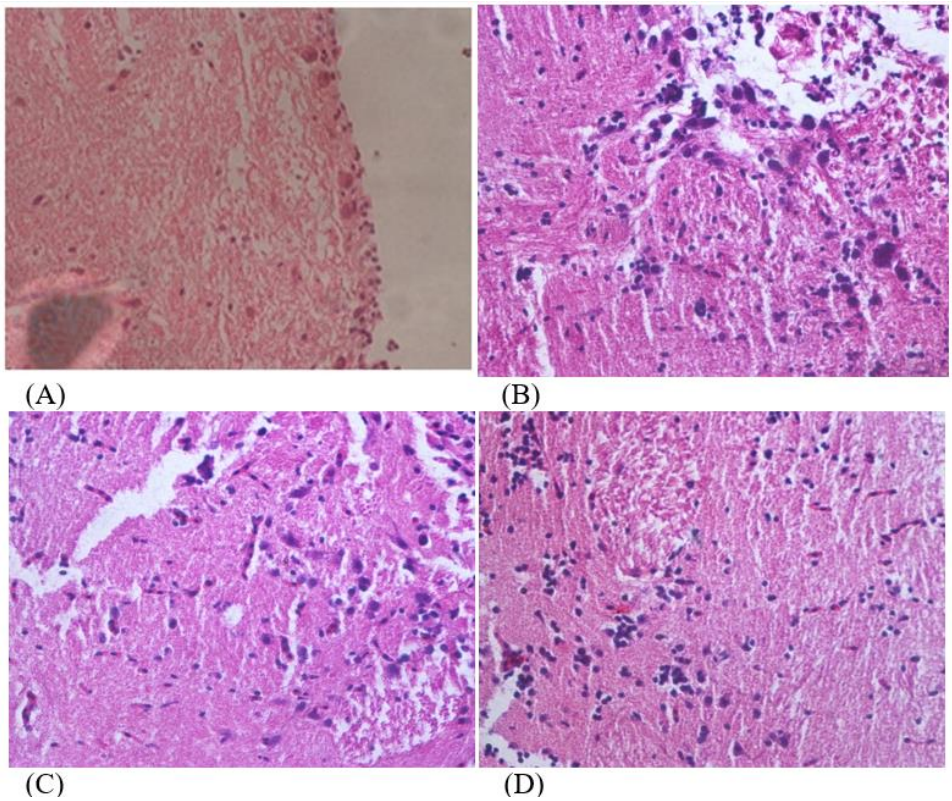

Fig. 2. In the brains of zebrafish, H&E staining revealed significant histopathological changes. (A) Control (B, C) Purkinje cell degeneration and severe cell loss after 5 days of MAL C1 and C3 exposure; (D) Purkinje cell degeneration and neuropil loss after 25 days of MAL C3 exposure

MAL's effects on zebrafish brain mRNA expression

To investigate the effect of MAL on gene expression in the brain of zebrafish, researchers used a quantitative RT-PCR study to quantify mRNA quantities for several genes encoding proteins (Cu/Zn–Sod, Mn–Sod, COX-17, CAT, CYP1A, AChE and GPx). Cu/Zn-SOD gene expression was reduced in the brain after 5 and 25 days of MAL treatment in both concentrations evaluated (C1 and C3 mg l^{-1}) as shown in Figure. 3B. (approximately (39.85 and 54.93) %, respectively). In all treatment groups, Mn–Sod and COX-17 mRNA levels in the brain fell significantly ($p<0.05$) when compared to the control group (Figure 3C, and E). However, both high and low MAL exposure resulted in a steady increase in brain CAT mRNA expression ($p<0.01$), with a significant 4.8-fold increase at 5 days (MAL C1 mg l^{-1}), 7.1 and 7.4 fold increases in expression after 25 days at both concentrations studied (Figure. 3, D). We also noticed a significant 1.9-fold increase in brain CYP1A mRNA expression following 25 days of exposure to increased MAL concentrations (Figure 3F). However, after 5 days of exposure, mRNA levels of AChE in the brain of zebrafish increased somewhat by about 1.5-fold for both treatments, then reduced significantly in the 25-day treatment groups (MAL C1 and C3 mg l^{-1}), declining by around 44.6 percent compared to the control group (Figure 3A). After 25 days of exposure to C3 mg l^{-1} MAL, some of the genes, such as GPx, showed (8.3-fold) greater expression in zebrafish ($p<0.01$) (Figure 3G).

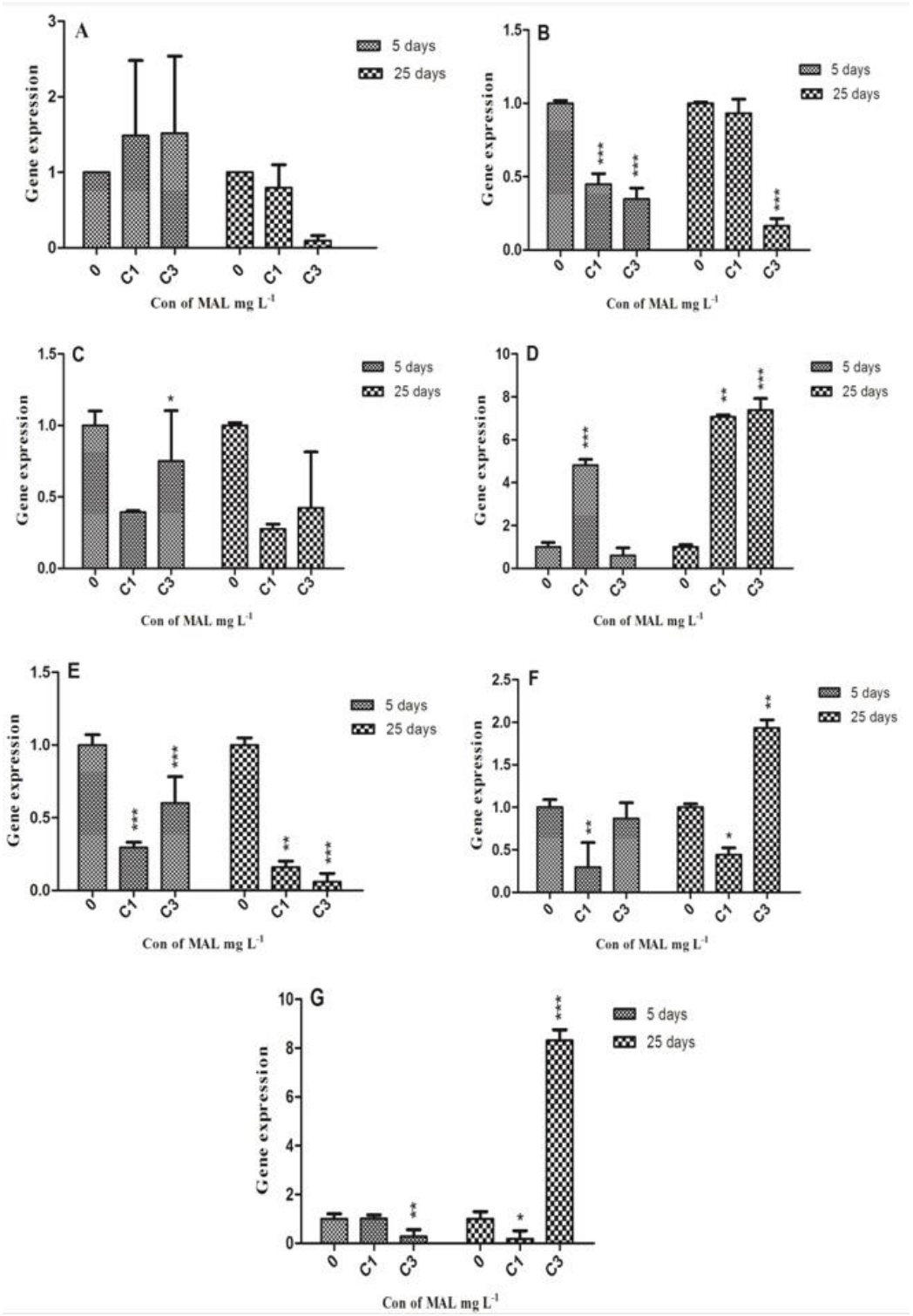

Fig. 3. AChE, Cu/Zn-Sod, Mn–Sod, CAT, COX-17, CYP1A, and GPx mRNA expression in the brains of zebrafish exposed to C1 and C3 mg L-1 MAL for 5, 25 days (A, B, C, D, E, F, G). Gene expression levels have been standardized to β-actin (a housekeeping gene), expressed as a control ratio (control = 1), and presented as ± SD. * at p0.05, ** at p0.01, and *** at p0.001 levels, the asterisk denotes a statistically significant difference from the controls

The severity and extent of the stress, the species' susceptibility to exposure determine whether antioxidant activity increases or decreases under chemical pressure. However, increasing xenobiotic levels does not always result in increased antioxidant activity (Ballesteros, et al., 2009). Significant changes in the antioxidant system's functioning are a pathophysiological consequence of oxidative stress, which can lead to cell and tissue damage. The effects of pesticides on CAT and SOD activity in fish species were examined.

When European sea bass (*D. labrax*) were subjected to 100-500 mg kg^{-1} PVC microplastic, Espinosa, et al., (2019) found that their liver SOD activity did not decrease significantly. The activity of CAT and SOD was significantly increased in zebrafish brain tissue after exposure to atrazine (Al-Sawafi & Yan, 2013). According to Lu, et al., (2016), dietary uptake and accumulation of xenobiotics triggered oxidative stress in the gill and liver of the zebra fish *Danio rerio* through altering SOD and CAT levels. Exposure of plastic microparticles, on the other hand, resulted in a significant increase in SOD activity in the *Scrobicularia plana'* gills (Ribeiro, et al., 2017). In African Catfish (*Clarias gariepinus*), there is a considerable drop in brain CAT activity (Iheanacho & Odo, 2020). It is possible that it's due to an inflow of $O_{2^{-}}$ in the brain (Ribeiro, et al., 2017).

When compared to the control, Cd treatment in Nile tilapia (*Oreochromis niloticus L.*) fish resulted in an increase in CAT and SOD activities (Abbas, et al., 2019). SOD and CAT activity in the brain increased following MAL exposure at various periods and concentrations in this study. This shows that the rate of $O_2^{\bullet}$ generation and the enzyme's protective effect against pesticide-induced oxidative stress may have increased. The increase in MAL-exposed SOD activity will result in more H_2O_2 being produced. CAT activity, on the other hand, is responsible for detoxifying H_2O_2 in the water. Similarly, Abdel-Tawwab and Wafeek (2017), Mushtaq, et al., (2017), and Li, et al., (2018) found increased activity of oxidative system enzymes (CAT, SOD, and GST) in different fish species exposed to toxicants, indicating that the antioxidant system responds to metal stress and may shift toward a detoxification mechanism. Antioxidants found in fish have been found to be useful biomarkers of aquatic pollutant exposure (Regoli & Giuliani, 2014).

The AChE activity is another biochemical marker used to evaluate aquatic ecosystems that are fundamentally contaminated with pesticides. In organisms exposed to toxicants or bad environmental circumstances, AChE is a sensitive neurotoxicity biomarker (Ajima, et al., 2017). The level of AChE activity indicates the nerve system's physiological status (Ayşegül & Serdar, 2018). MAL exposure resulted in a decrease in brain AChE activity (Figure 1, A), which may have resulted in neurotoxicity in the exposed fish (Ali & Aldhamin, 2019). On the 45th day of exposure, the exposed fish's brain had decreased AChE activity (Ayşegül & Serdar, 2018). After malathion exposure to White albino rats (Ali & Aldhamin, 2019) and zebrafish brain and muscle of cadmium exposure (Al-Sawafi & Yan, 2013). As a result, in this investigation, we performed quantitative RT-PCR analysis to investigate the effects of exposure to sub-lethal MAL on AChE gene expression patterns. One of the neurotransmitter systems implicated in the pathophysiologic mechanism of mood disorders is the cholinergic system. It is a neurotransmitter that helps the brain conduct critical tasks, including memory and learning (da Luz Oliveira, et al., 2011).

Interestingly, following 5 days of exposure to MAL pesticide, AChE gene expression in the brain rose considerably in both doses examined. According to Kist, et al., (2012), MC-LR treatment significantly increased AChE mRNA levels in the brain of zebrafish. Various factors, such as cellular machinery and signal transduction pathways, control gene expression. As suggested by Vuaden, et al., (2012) the function of the enzyme is not directly tied to gene expression or protein levels. da Luz Oliveira, et al., (2011) noted that the increase in lithium-exposed enzyme activity was not directly connected with changes in the rate of expression in the brain of zebrafish. After that, we detected significant changes in GPx and CAT expression of brain (Figure 4, D, G), showing that oxidative stress caused by exposure of MAL increases genes transcription ($p<0.01$). Interestingly, there were no changes in the mRNA expression of Mn-SOD and Cu/Zn-SOD or COX-17 after 5 and 25 days of toxicant exposure in the current investigation (Figure. 4, B, C, E). In contrast to these transcriptional alterations, we detected a significant increase in brain SOD enzymatic activity (Figure 2, B). This shows that, at least in the case of SOD, certain antioxidant enzymes are first regulated by enzyme activation rather than transcription. 14 days of Pb treatment suppressed particular genes in the *Cyprinus carpio* liver, including the GPx , Mn-SOD and Cu-Zn SOD genes.

The cytochrome P450s (CYP1A), a broad family of super proteins, is active metabolizers of pollutants and plays an important role in drug metabolism (Velki, et al., 2019). In response to pollutants, there was significantly more CYP1A-immunopositive protein in the liver of *Cyprinus carpio L.* after exposure to Pb in zebrafish (*Danio rerio*) embryos exposed to endosulfan (Moon, et al., 2016), and in the liver of common carp (*C. carpio L.*) exposed to chlorpyrifos (Xing, et al., 2014). In contrast, Abbas, et al., (2019) claimed that increased CYP gene expression causes an increase in ROS, resulting in oxidative stress and tissue damage. When compared to control expression, CYP1A expression in MAL-exposed fish was over-regulated (Figure 3). Nonetheless, they can obstruct CYP450 synthesis by increasing the rates of active substances that, since they are not metabolized, have the potential to induce a negative reaction. Changes in the expression of CYP1A can lead to the formation of a variety of chemical compounds, making it a useful biomarker for detecting organic pollutants. The brain expression of CYP1A was commonly used to research responses to toxic chemicals.

CYP1A continues to be used as a common biomarker within the subfamily due to its distinctive activation or suppression in the brain following exposure to contaminants (Jönsson, et al., 2007). As indicated by a series of brain tissue lesions caused by Purkinje cell degeneration and neuropil loss, which may subsequently compromise normal physiological activity, MAL produces significant injury to the fish brain. These changes become more apparent as MAL dosages in the brain increase, showing that these tissues are sensitive to MAL poisoning. In addition, histopathology can be employed as a biomonitoring technique for health in toxicity research. Environmental stressors are detected by histopathological alterations, which are biomarkers that identify changes in biochemistry and physiology. Histopathology, cellular and tissue damage or abnormalities can be used to determine the level of exposure, especially for sublethal and chronic effects (Sayeed, et al., 2003). Similarly, Xing, et al., (2012) demonstrated that pathological alterations included increasing substantial tissue harm with increasing exposure dose for common carp brain and kidney tissue after a 40-day ATR / CPF exposure mixture. With greater Cd exposure, zebrafish neural cells showed varying degrees of granule cell loss, Purkinje cell degeneration, gliosis aggregation, and multiple areas of necrosis. After exposure to atrazine, Chirruh snow trout (*Schizothorax esocinus*) developed modest necrosis and blood congestion, mild pyknosis, and necrosis (Akhtar, et al., 2019).

4. Conclusion

To assess the aquatic ecosystem and health state of *Danio rerio*, we employed stress-responsive genes encoding Mn-SOD, CAT, GPx, Cu / Zn-SOD, COX-17, CYP1A and AChE as molecular indicators. In addition, after 25 days of MAL use, we detected a significant decrease in Mn-SOD, COX-17 and Cu/Zn-SOD mRNA expression in the zebrafish brain. More research is needed to determine the utility of these genes' mRNA quantification as indicators of pesticide-induced oxidative stress in fish, particularly in long-term exposure tests. According to our findings, the histological parameters in the brains of MAL-exposed zebrafish were also significantly altered. As a result, the information presented in this study may be useful in understanding the processes of oxidative stress produced by MAL in fish.

Laboratory Animals

All institutional and national guidelines for the care and use of laboratory animals were followed.

Competing Interests

The authors had no competing interests.

References

Abbas, T. W., MN Authman, M., A Darwish, D., M Kenawy, A., MK Abumourad, I., & B Ibrahim, T. (2019). Cadmium toxicity-induced oxidative stress and genotoxic effects on Nile tilapia (Oreochromis niloticus L.) fish: The protective role of fenugreek (Trigonella foenum-graecum) seeds. *Egyptian Journal of Aquatic Biology and Fisheries*, *23*(5 (Special Issue)), 193-215. **https://dx.doi.org/10.21608/ejabf.2019.64715**

Abdel-Tawwab, M., & Wafeek, M. (2017). Fluctuations in water temperature affected waterborne cadmium toxicity: hematology, anaerobic glucose pathway, and oxidative stress status of Nile tilapia, Oreochromis niloticus (L.). *Aquaculture*, *477*, 106-111. **https://doi.org/10.1016/j.aquaculture.2017.05.007**

Abdel-Tawwab, M., Adeshina, I., Jenyo-Oni, A., Ajani, E. K., & Emikpe, B. O. (2018). Growth, physiological, antioxidants, and immune response of African catfish, Clarias gariepinus (B.), to dietary clove basil, Ocimum gratissimum, leaf extract and its susceptibility to Listeria monocytogenes infection. *Fish & shellfish immunology*, *78*, 346-354. **https://doi.org/10.1016/j.fsi.2018.04.057**

Ajima, M. N., Pandey, P. K., Kumar, K., & Poojary, N. (2017). Neurotoxic effects, molecular responses and oxidative stress biomarkers in Nile tilapia, Oreochromis niloticus (Linnaeus, 1758) exposed to verapamil. *Comparative biochemistry and physiology part C: Toxicology & pharmacology*, *196*, 44-52. **https://doi.org/10.1016/j.cbpc.2017.03.009**

Akhtar, N., Khan, M. F., & Tabassum, S. (2019). Sub lethal effects of atrazine on hematology, histopathology and biochemistry of chirruh snowtrout (Schizothorax esocinus). *JAPS, Journal of Animal and Plant Sciences*, *29*(5), 1447-1454.

Ali, D. T., & Aldhamin, A. S. (2019). Changes in acetylcholinesterase and DNA damage due to the use of Malathion on white albino rat. *Biochemical and Cellular Archives*, *19*(1), 1915-1918.

Al-Sawafi, A. G. A., & Yan, Y. (2013). Alterations of acetylcholinesterase activity and antioxidant capacity of zebrafish brain and muscle exposed to sublethal level of cadmium. *International Journal of Environmental Science and Development*, *4*(3), 327.

Al-Sawafi, A. G. A., & Yan, Y. (2013). Bioconcentration and antioxidant status responses in zebrafish (Danio rerio) under atrazine exposure. *International Journal of Chemical Engineering and Applications*, *4*(4), 204.

Au, D. W. T. (2004). The application of histo-cytopathological biomarkers in marine pollution monitoring: a review. *Marine Pollution Bulletin*, *48*(9-10), 817-834. **https://doi.org/10.1016/j.marpolbul.2004.02.032**

Ayşegül, P. A. L. A., & Serdar, O. (2018). Seasonal variation of acetylcholinesterase activity as a biomarker in brain tissue of Capoeta umbla in Pülümür Stream. *Journal of Limnology and Freshwater Fisheries Research*, *4*(2), 98-102. **https://doi.org/10.17216/limnofish.407339**

Ballesteros, M. L., Wunderlin, D. A., & Bistoni, M. A. (2009). Oxidative stress responses in different organs of Jenynsia multidentata exposed to endosulfan. *Ecotoxicology and Environmental Safety*, *72*(1), 199-205. **https://doi.org/10.1016/j.ecoenv.2008.01.008**

Banaee, M., Sureda, A., Mirvaghefi, A. R., & Ahmadi, K. (2011). Effects of diazinon on biochemical parameters of blood in rainbow trout (Oncorhynchus mykiss). *Pesticide Biochemistry and Physiology*, *99*(1), 1-6. **https://doi.org/10.1016/j.pestbp.2010.09.001**

Barky, F. A., Abdelsalam, H. A., Mahmoud, M. B., & Hamdi, S. A. (2012). Influence of Atrazine and Roundup pesticides on biochemical and molecular aspects of Biomphalaria alexandrina snails. *Pesticide biochemistry and physiology*, *104*(1), 9-18. **https://doi.org/10.1016/j.pestbp.2012.05.012**

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, *72*(1-2), 248-254. **https://doi.org/10.1016/0003-2697(76)90527-3**

Can, Z. (2011). Toxic effects of Butyl-Benzyl phthalate on Zebrafish and cloning, Expression of Acetylcholinesterase Gene. *A thesis of MS Degree in Biochemical and Molecular Biology*.

da Luz Oliveira, R., Seibt, K. J., Rico, E. P., Bogo, M. R., & Bonan, C. D. (2011). Inhibitory effect of lithium on nucleotide hydrolysis and acetylcholinesterase activity in zebrafish (Danio rerio) brain. *Neurotoxicology and teratology*, *33*(6), 651-657. **https://doi.org/10.1016/j.ntt.2011.05.005**

Egea, J., Fabregat, I., Frapart, Y. M., Ghezzi, P., Görlach, A., Kietzmann, T., ... & Di Lisa, F. (2017). European contribution to the study of ROS: A summary of the findings and prospects for the future from the COST action BM1203 (EU-ROS). *Redox biology*, *13*, 94-162. **https://doi.org/10.1016/j.redox.2017.05.007**

Ellman, G. L., Courtney, K. D., Andres Jr, V., & Featherstone, R. M. (1961). A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochemical pharmacology*, *7*(2), 88-95. **https://doi.org/10.1016/0006-2952(61)90145-9**

Espinosa, C. R., Esteban, A. A., & Cuesta, A. (2019). Dietary administration of polyvinylchloride and polyethene microplastics produces histological damage, oxidative stress and immunoregulation in European sea bass (Dicentrarchus labrax L.). *Fish & Shellfish Immunology*, *95*, 574-583. **https://doi.org/10.1016/j.fsi.2019.10.072**

Feng, M., Qu, R., Wang, C., Wang, L., & Wang, Z. (2013). Comparative antioxidant status in freshwater fish Carassius auratus exposed to six current-use brominated flame retardants: a combined experimental and theoretical study. *Aquatic toxicology*, *140*, 314-323. **https://doi.org/10.1016/j.aquatox.2013.07.001**

Finney, D. J. (1971). *Probit analysis: 3d ed.* Cambridge University Press.

Guardiola, F. A., Bahi, A., Messina, C. M., Mahdhi, A., Santulli, A., Arena, R., ... & Esteban, M. A. (2017). Quality and antioxidant response of gilthead seabream (Sparus aurata L.) to dietary supplements of fenugreek (Trigonella foenum graecum) alone or combined with probiotic strains. *Fish & Shellfish Immunology*, *63*, 277-284. **https://doi.org/10.1016/j.fsi.2017.02.029**

Ighodaro, O. M., & Akinloye, O. A. (2018). First line defence antioxidants-superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX): Their fundamental role in the entire antioxidant defence grid. *Alexandria journal of medicine*, *54*(4), 287-293. **https://doi.org/10.1016/j.ajme.2017.09.001**

Iheanacho, S. C., & Odo, G. E. (2020). Dietary exposure to polyvinyl chloride microparticles induced oxidative stress and hepatic damage in Clarias gariepinus (Burchell, 1822). *Environmental Science and Pollution Research*, *27*(17), 21159-21173. **https://doi.org/10.1007/s11356-020-08611-9**

Iheanacho, S. C., & Odo, G. E. (2020). Neurotoxicity, oxidative stress biomarkers and haematological responses in African catfish (Clarias gariepinus) exposed to polyvinyl chloride microparticles. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, *232*, 108741. **https://doi.org/10.1016/j.cbpc.2020.108741**

Jönsson, M. E., Orrego, R., Woodin, B. R., Goldstone, J. V., & Stegeman, J. J. (2007). Basal and 3, 3′, 4, 4′, 5-pentachlorobiphenyl-induced expression of cytochrome P450 1A, 1B and 1C genes in zebrafish. *Toxicology and Applied Pharmacology*, *221*(1), 29-41. **https://doi.org/10.1016/j.taap.2007.02.017**

Kist, L. W., Rosemberg, D. B., Pereira, T. C. B., De Azevedo, M. B., Richetti, S. K., de Castro Leão, J., ... & Bogo, M. R. (2012). Microcystin-LR acute exposure increases AChE activity via transcriptional ache activation in zebrafish (Danio rerio) brain. *Comparative Biochemistry and Physiology Part c: Toxicology & Pharmacology*, *155*(2), 247-252. **https://doi.org/10.1016/j.cbpc.2011.09.002**

Li, J., Yan, Y., & Xie, X. (2018). Tissue-specific antioxidative responses and cadmium accumulation in Silurus meridionalis under chronic waterborne cadmium exposure. *Bulletin of environmental contamination and toxicology*, *100*, 485-491. **https://doi.org/10.1007/s00128-018-2294-8**

Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2− ΔΔCT method. *Methods*, *25*(4), 402-408. **https://doi.org/10.1006/meth.2001.1262**

Lu, Y., Zhang, Y., Deng, Y., Jiang, W., Zhao, Y., Geng, J., ... & Ren, H. (2016). Uptake and accumulation of polystyrene microplastics in zebrafish (Danio rerio) and toxic effects in liver. *Environmental science & technology*, *50*(7), 4054-4060. **https://doi.org/10.1021/acs.est.6b00183**

Lushchak, V. I. (2011). Environmentally induced oxidative stress in aquatic animals. *Aquatic Toxicology*, *101*(1), 13-30. **https://doi.org/10.1016/j.aquatox.2010.10.006**

Moon, Y. S., Jeon, H. J., Nam, T. H., Choi, S. D., Park, B. J., Ok, Y. S., & Lee, S. E. (2016). Acute toxicity and gene responses induced by endosulfan in zebrafish (Danio rerio) embryos. *Chemical Speciation & Bioavailability*, *28*(1-4), 103-109. **https://doi.org/10.1080/09542299.2016.1198681**

Mushtaq, T., Javed, M., & Abbas, S. (2017). Peroxidase activity in liver and kidney of Labeo rohita exposed to zinc chloride. *Pakistan Journal of Zoology*, *49*(6). **http://dx.doi.org/10.17582/journal.pjz/2017.49.6.sc5**

Regoli, F., & Giuliani, M. E. (2014). Oxidative pathways of chemical toxicity and oxidative stress biomarkers in marine organisms. *Marine environmental research*, *93*, 106-117. **https://doi.org/10.1016/j.marenvres.2013.07.006**

Ribeiro, F., Garcia, A. R., Pereira, B. P., Fonseca, M., Mestre, N. C., Fonseca, T. G., ... & Bebianno, M. J. (2017). Microplastics effects in Scrobicularia plana. *Marine Pollution Bulletin*, *122*(1-2), 379-391. **https://doi.org/10.1016/j.marpolbul.2017.06.078**

Rickwood, C. J., & Galloway, T. S. (2004). Acetylcholinesterase inhibition as a biomarker of adverse effect: a study of Mytilus edulis exposed to the priority pollutant chlorfenvinphos. *Aquatic Toxicology*, *67*(1), 45-56. **https://doi.org/10.1016/j.aquatox.2003.11.004**

Sayeed, I., Parvez, S., Pandey, S., Bin-Hafeez, B., Haque, R., & Raisuddin, S. (2003). Oxidative stress biomarkers of exposure to deltamethrin in freshwater fish, Channa punctatus Bloch. *Ecotoxicology and Environmental Safety*, *56*(2), 295-301. **https://doi.org/10.1016/S0147-6513(03)00009-5**

Song, S. B., Xu, Y., & Zhou, B. S. (2006). Effects of hexachlorobenzene on antioxidant status of liver and brain of common carp (Cyprinus carpio). *Chemosphere*, *65*(4), 699-706. **https://doi.org/10.1016/j.chemosphere.2006.01.033**

Velki, M., Lackmann, C., Barranco, A., Ereno Artabe, A., Rainieri, S., Hollert, H., & Seiler, T. B. (2019). Pesticides diazinon and diuron increase glutathione levels and affect

multixenobiotic resistance activity and biomarker responses in zebrafish (Danio rerio) embryos and larvae. *Environmental Sciences Europe*, *31*, 1-18. **https://doi.org/10.1186/s12302-019-0186-0**

Vuaden, F. C., Savio, L. E. B., Piato, A. L., Pereira, T. C., Vianna, M. R., Bogo, M. R., ... & Wyse, A. T. (2012). Long-term methionine exposure induces memory impairment on inhibitory avoidance task and alters acetylcholinesterase activity and expression in zebrafish (Danio rerio). *Neurochemical research*, *37*, 1545-1553. **https://doi.org/10.1007/s11064-012-0749-6**

Xing, H., Li, S., Wang, Z., Gao, X., Xu, S., & Wang, X. (2012). Histopathological changes and antioxidant response in brain and kidney of common carp exposed to atrazine and chlorpyrifos. *Chemosphere*, *88*(4), 377-383. **https://doi.org/10.1016/j.chemosphere.2012.02.049**

Xing, H., Zhang, Z., Yao, H., Liu, T., Wang, L., Xu, S., & Li, S. (2014). Effects of atrazine and chlorpyrifos on cytochrome P450 in common carp liver. *Chemosphere*, *104*, 244-250. **https://doi.org/10.1016/j.chemosphere.2014.01.002**