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ORIGINAL ARTICLE

Biomarkers of neurotoxicity, oxidative stress, hepatotoxicity and lipid peroxidation in *Clarias gariepinus* exposed to melamine and polyvinyl chloride

Stanley Chidi Iheanacho[a,b#], Christiana Igberi[c], Akunna Amadi-Eke[d], Delight Chinonyerem[a], Angus Iheanacho[e] and Fred Avwemoya[f]

[a]Department of Fisheries and Aquaculture, Alex Ekwueme Federal University Ndufu Alike, Ikwo, Nigeria; [b]Department of Zoology and Environmental Biology, University of Nigeria, Nsukka, Nigeria; [c]Department of Agriculture, Alex Ekwueme Federal University Ndufu Alike, Ikwo, Nigeria; [d]Department of Fisheries and Aquaculture Technology, Federal University of Technology, Owerri, Nigeria; [e]Department of Chemistry and Industrial Chemistry, University of Nigeria, Nsukka, Nigeria; [f]Department of Fisheries, Nigerian Maritime University, Okerenkoko Warri, Nigeria

ABSTRACT

Purpose: Plastic particulates and chemicals are emerging environmental pollutants with significant impact on aquatic ecosystems. In this study, the effects of oral uptake of melamine, melamine formaldehyde, and polyvinyl chloride on serum biochemical profiles, antioxidant enzymes activities, lipid peroxidation levels and brain acetyl cholinesterase activities in *Clarias gariepinus* juveniles were investigated.

Methods: Fish specimens were fed diets spiked with melamine, melamine formaldehyde and poly vinyl chloride at 0.3% (3.0 g Kg^{-1}) dietary inclusion for 45 days. Toxicity effect of these plastic chemicals was estimated by assaying relevant biomarkers.

Results: After 45 days exposure, Serum glucose was significantly elevated, whereas plasma protein levels were substantially reduced in the exposed fish groups. Serum transaminases were significantly elevated in the exposed groups. Brain acetylcholinesterase and antioxidant enzyme activities declined significantly, while malondialdehyde levels were elevated in the exposed groups.

Conclusion: *C. gariepinus* is an important bioindicator to monitor the ecotoxicological impact of plastic chemicals such as melamine, and polyvinyl chloride.

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KEYWORDS
African catfish; melamine; polyvinyl chloride; toxicity; biomarkers

Introduction

Discarded plastic materials enter the aquatic environment as trash, industrial discharge, or litter through inland waterways, wastewater outflows, and transport by winds or tides (Ng *et al.* 2018). Plastic additive chemicals are usually added as antioxidants, pigments, plasticizers, fillers, or stabilizers (Chokwe and Okonkwo 2019). Melamine (a monomer and low molecular-weight compound), melamine formaldehyde (MEF) (a polymer) are used as coating materials and for dishware (ceramic plates, cups etc.) (Ng *et al.* 2018, Nuntapong *et al.* 2019), while polyvinyl chloride (PVC) is used for the production of water pipes, cables and leathers (Espinosa *et al.* 2018). One of the most important issues that remain controversial is whether these chemicals can cause toxic effects. ME has been detected in waste waters (266.67 mg L^{-1}; 1240 ng L^{-1}) (Qin *et al.* 2010), lake water, river water (347.0 mg L^{-1}) and sea water (186.0 mg L^{-1}) (Zhu and Kannan 2020). Considering its nitrogen-rich compounds which can mimic proteins, ME is unscrupulously incorporated into aquafeed and other protein-based edible products (infant milk, fish meal etc.) as adulterant to bloat their protein content (WHO 2009). ME resin has also been detected in aquaculture feed (> 400 mg L^{-1}) (USFDA 2007); also, in salmon fish (1200 mg L^{-1}) (Karunasagar 2009) and to a large extent, in processed aquatic meat supposedly meant for human consumption (WHO 2009). A higher deposition of ME up to 4437 mg Kg^{-1} has been detected in the kidney of chicken (Qin *et al.* 2010). PVC (high molecular-weight polymer) particles have also been found in water surface, sediments and column of Rivers, streams and Oceans (Mani *et al.* 2014, Isobe *et al.* 2017).

Toxicity of ME have been reported in different fish species, shrimp and other aquatic models which include growth inhibition, immunosuppression, behavioural anomalies, hepatotoxicity and oxidative stress (Phromkunthong *et al.* 2013, 2015, Mahardika *et al.* 2017, Nuntapong *et al.* 2019). In an *in vitro* experiment, 400 mg Kg^{-1} of ME was fed to fish specimens, of which up to 210 mg Kg^{-1} of ME residue was detected in edible tissues of fish even after 14 days depuration period, indicating its bioaccumulation tendency (Anderson *et al.* 2008).

Evidence of the toxicological effects of PVC in aquatic organisms have been documented based on *in vitro* and *in vivo* experiments (Jovanović 2017, Barboza *et al.* 2018,

CONTACT Stanley Chidi Iheanacho iheanacho.stanley@yahoo.com Department of Fisheries and Aquaculture, Alex Ekwueme Federal University Ndufu Alike, Ikwo, Nigeria
[#]Stanley Chidi Iheanacho is responsible for statisitical design and analysis. E-mail: iheanacho.stanley@yahoo.com (S. C. Iheanacho).

Foley *et al.* 2018, Iheanacho and Odo 2020a, 2020b). Neurotoxicity (Iheanacho and Odo 2020a), hepatotoxicity (Espinosa *et al.* 2018, Iheanacho and Odo 2020b), intestinal abrasion (Jovanović 2017), behavioural changes (Critchell and Hoogenboom 2018), alteration in lipid metabolism (Oliveira *et al.* 2013), and bioaccumulation potential in tissues (Ribeiro *et al.* 2017) are the biological consequences accompanied with PVC ingestion in fish.

Biomarkers are indispensable tools for detecting pathological conditions in animals (Adams *et al.* 2001, Ajima *et al.* 2017). Biomarkers are used to study different physiological responses to adverse environmental conditions (such as pollution) in animals (Hamed *et al.* 2020, Iheanacho and Odo 2020a). Oxidative stress scenario is prompted as a result of acute or chronic exposition to environmental or chemical induced stressors (Saravanan *et al.* 2012).

Clarias gariepinus is an important commercial species highly relished in Nigeria. The fish is an omnivorous species and widely cultivated due to its easy adaptability (aquaculture species), fast growth and nutritional quality (Ogueji *et al.* 2020). The hardy nature of *C. gariepinus* makes it suitable for ecotoxicological assays (Okoro *et al.* 2019).

The present study aims at evaluating the toxicity effects of ME, melamine formaldehyde (MEF) and PVC in *Clarias gariepinus*, using a battery of biomarkers of oxidative stress such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) (first line defense antioxidants), and lipid peroxidation, determined as malondialdehyde (MDA). As a neurotoxic biomarker, the acetylcholinesterase (AChE) activity was also measured. Being that the liver is the chief organ for detoxification in the physiological system, serum biochemical assay was conducted to check for liver damage and assessed using serum biomarkers such as serum enzymes [alkaline phosphatase (ALP), alanine amino transferase (ALT), aspartate amino transferase (AST)], serum protein and glucose as baseline for measuring hepatotoxicity in the bio model.

Clinical significance

- Toxicity effects of melamine/formaladehyde and polyvinyl chloride were evaluated via relevant biomarkers.
- Antioxidant enzymes activities were prominently altered in the biomodel following the chronic exposure to melamine/formaladehyde and polyvinyl chloride chemicals.
- Exposure to melamine/formaladehyde and polyvinyl chloride chemicals induced neurotoxic effect in bio-model.
- Hepatotoxicity, alterations of serum enzymes and metabolites were detected in melamine/formaldehyde and polyvinyl chloride exposed bio-model.
- Clarias gariepinus is an important bioindicator to measure the biological consequences of melamine/formaladehyde and polyvinyl chloride exposure.

Materials and methods

Fish and test chemical

Healthy African catfish juveniles *C. gariepinus* ($N = 180$; 15.1 ± 1.2 g) were obtained from the Department of Fisheries and Aquaculture farm, Alex Ekwueme Federal University Ndufu Alike, Nigeria. Fish species were transferred to the laboratory, acclimated in the test tanks for twelve (12) days, under 12 h light: 12 h darkness photo period in a semi-static culture system, and were fed twice daily (8:00 h, 17:00 h) with Coppens feed (45% crude protein) (Coppens International, Nettetal, Germany) at 3% body weight. Powdered purity grades of melamine (99.8%, CAS no. 108-78-1), melamine formaldehyde (99.3%, CAS no. 9003-08-1) and PVC (99.5%, CAS no 9002-86-2), were procured from Sigma-Aldrich (St. Louis, MO, USA).

Ethics statement

All experimental procedures were performed in compliance with the standards described by the institute of animal welfare act of Nigeria, in line with the National Environmental Standard Regulations Enforcement Agency Act of Nigeria on the protection of animals against cruelty.

Diet preparation

Four iso-nitrogenous and iso-lipidic diets were formulated for the experiment. The different feed stuffs and their inclusion levels were as follows: fish meal 47.7%, corn meal 15%, soybean meal 18%; fish oil 1.0%, wheat offal 10%, vitamin/mineral premixes 9%, starch (binder) 2%. Melamine, melamine formaldehyde, and polyvinyl chloride were incorporated into diets at 0.3% (3.0 g Kg^{-1}), while the feed for the control fish contained none of these substances. Although the quantity of plastic particulates in the wild is not known, however, an environmentally relevant concentration has been suggested to amount to 0.3% (3.33 g Kg^{-1}) of food consumed daily (Jovanović et al. 2018). Moreover, chronic concentration of ME exposure amounting to 3000 mg L^{-1}, have been reported in guppy fish (*Pecilia reticulata*); 500 mg L^{-1} in Japanese medeka fish (*Oryzias latipes*) and $>$ 2000 mg L^{-1}in *Salmon gairdneri* (OECD 1998). Invitro exposures to ME up to 10 g Kg^{-1} and 3.33 g Kg^{-1} have also been reported to induce toxic effects in Pacific white shrimp (*Litopenaeus vannamei*) (Nuntapong *et al.* 2019). Dietary inclusion levels of ME at 5–30 g Kg^{-1} have also been reported to induce toxic effects on the gills, liver and kidney of tilapia fish (*Oreochromis niloticus*) (Phromkunthong *et al.* 2013). Therefore, the selected dose (3.0 g Kg^{-1}) for this study reflects a possible extreme exposure scenario which was targeted at estimating the effects of these chemicals in *C. gariepinus*. The formulated diets were mechanically mixed and pelletized into 3 mm size using a locally fabricated machine. Pelleted diets were air dried at 25 ° C for 24 h and stored at 4 °C in a refrigerator until use. Approximate composition of the control diet is as follows; total protein 48.1%, crude fat 15.9%, crude fibre

5.2%, crude ash 10.3%, moisture 7.0% and nitrogen free extract 13.5%.

Experimental design and chemical exposure

Chemical exposure (dietary method) to fish was adopted from OECD (2012), Nuntapong *et al.* (2019), and Espinosa *et al.* (2019). A total of 180 juveniles *C. gariepinus* were randomly allotted to four treatment groups included the control one, in a completely randomized design. Each group contained 45 fish and was further randomized into three replicates of 15 fish per replicate. The fish were fed twice (morning and evening) daily at 3% body weight daily. The feeding trial lasted for 45 days. Water in the experimental tanks was renewed every three days (semi-static culture system) in order to maintain optimal water quality conditions. The physicochemical parameters of the experimental tank water were examined every 24 hours using a water quality test kit (ProLab^{TM}, Weston, FL, USA), and presented as mean ± SE; dissolved oxygen 5.6 ± 0.2 mg/L^{-1} temperature 28.2 ± 0.5 °C; pH 6.78–6.96; conductivity 39.9 ± 1.5 µs/cm and ammonia 0.02 ± 0.01 mg/L^{-1}.

Procedure for blood collection

Total of nine fish per group (three per replicate) were sampled at the of 45 days exposure trial for blood collection. The fish were randomly caught using hand net and anaesthetized with tricaine methane sulphonate (MS 222) at 50.0 mg/L^{-1} to minimize stress. The method for blood collection was done following the procedure of Bello *et al.* (2014). Sterilized 3 G needles and syringes (non-heparinized) of 2 mL were used to collect blood. At first, the ventral cavity of the fish was wiped with dry tissue paper to avoid contamination with mucus during blood collection. At a distance of 3–4 cm from the genital opening of each fish, the needle was inserted at a right angle to the vertebral column of the fish. Under gentle aspiration, 1.5 mL of blood was taken and the needle was gently withdrawn and transferred into plain tube for serum chemistry analysis.

Serum biochemical analysis

The clotted blood in the plain tubes was transferred into clean dry centrifuge tubes with Hawsley minor bench centrifuge at 3000 r/min for 15 minutes (Hesser 1960). The supernatant (serum) formed after centrifugation was collected using a Pasteur pipette and transferred into a plain plastic test-tube for biochemical analysis. Total protein, glucose level, triglycerides and serum enzymes [alkaline phosphatase (ALP), alanine amino transferase (ALT), aspartate amino transferase (AST)] were determined by the use of a semi-automated biochemistry analyser (Sinothinker SK3002B, China). The use of reagents and procedures for the analysis were strictly adhered to, according to the manufacturer's instructions.

Tissue collection and preparation

After the blood collection, the same fish were further sacrificed for brain removal. The brain tissues were carefully collected using spatula to avoid damage. Brain samples ($n = 9$, with 3 fish per replicate) from each group were weighed and homogenized in a 0.25 mL (1:19 w/v) chilled sucrose. The homogenate was divided in two proportions; a portion was used for MDA assay, while the other portion was used for acetylcholinesterase and antioxidant enzymes assay.

Antioxidant enzymes assay

CAT activity was estimated according to the method of Beutler (1984). To 1.2 mL of 0.01 mmol/L phosphate buffer (pH 7.0), 0.5 mL tissue homogenate was added. The enzyme reaction started by the addition of 1.0 mL of 0.2 mmol/L hydrogen peroxide solution. The decrease in absorbance was measured at 250 nm for every 30 seconds up to 3 minutes. The enzyme blank was run simultaneously with 1.0 mL of distilled water instead of hydrogen peroxide. The enzyme activity was expressed as U mg protein^{-1}, where U = is the amount of enzyme that catalyses the reaction of 1 nmol of substrate per minute.

SOD activity in fish was estimated according to the method of Misra and Friedovich (1972). An aliquot of 0.1 mL of tissue homogenate was added to the tubes containing 0.75 mL ethanol and 0.15 mL chloroform under chilled condition and centrifuged. To 0.5 mL of supernatant, 0.5 mL of 0.6 mL EDTA solution and 1.0 mL of 0.1 mol/L carbonate-bicarbonate buffer pH 10.2) was added. The reaction was initiated by the addition of 0.5 mL of 1.8 mM epinephrine and the increase in absorbance at 30 second interval for 3 minutes was measured at 480 nm in a UV spectrophotometer (Shimadzu, Japan). One unit of superoxide dismutase activity was expressed as the amount of protein required for 50% of inhibition of epinephrine autoxidation/minute.

GPx activity in fish brain was measured according to Beutler (1984). Three grammes of tissue was homogenized in 2-ml Tris buffer. The reaction system contained 0.2-ml Tris buffer, 0.2-ml EDTA, 0.1-ml sodium azide and 0.5 ml of tissue aliquot. To this, mixture was added 0.2 ml of glutathione and 0.1 ml of hydrogen peroxide and shaken very well. The reaction mixture was incubated for 10 min at 30 °C. A control blank containing all the reagents except the test tissue homogenate was also set up. After 10 min, 0.5 ml of 10% TCA was added to and mixed before centrifuging the mixture. The supernatant was used to assay for GPx by reading the absorbance at 412 nm and value expressed as U mg protein^{-1}, where U = is the amount of enzyme that catalyses the reaction of 1 nmol of substrate per minute.

The estimation of thiobarbituric acid reactive substance evaluated as MDA content of the brain tissue was measured according to Sharma and Krishna Murti (1968). The results were expressed as nanomoles of MDA formed per hour per milligram of proteins (nmol TBARS mg prot^{-1}).

The AChE activity was determined according to Ellman *et al.* (1961). The homogenate was centrifuged at 3500 rpm

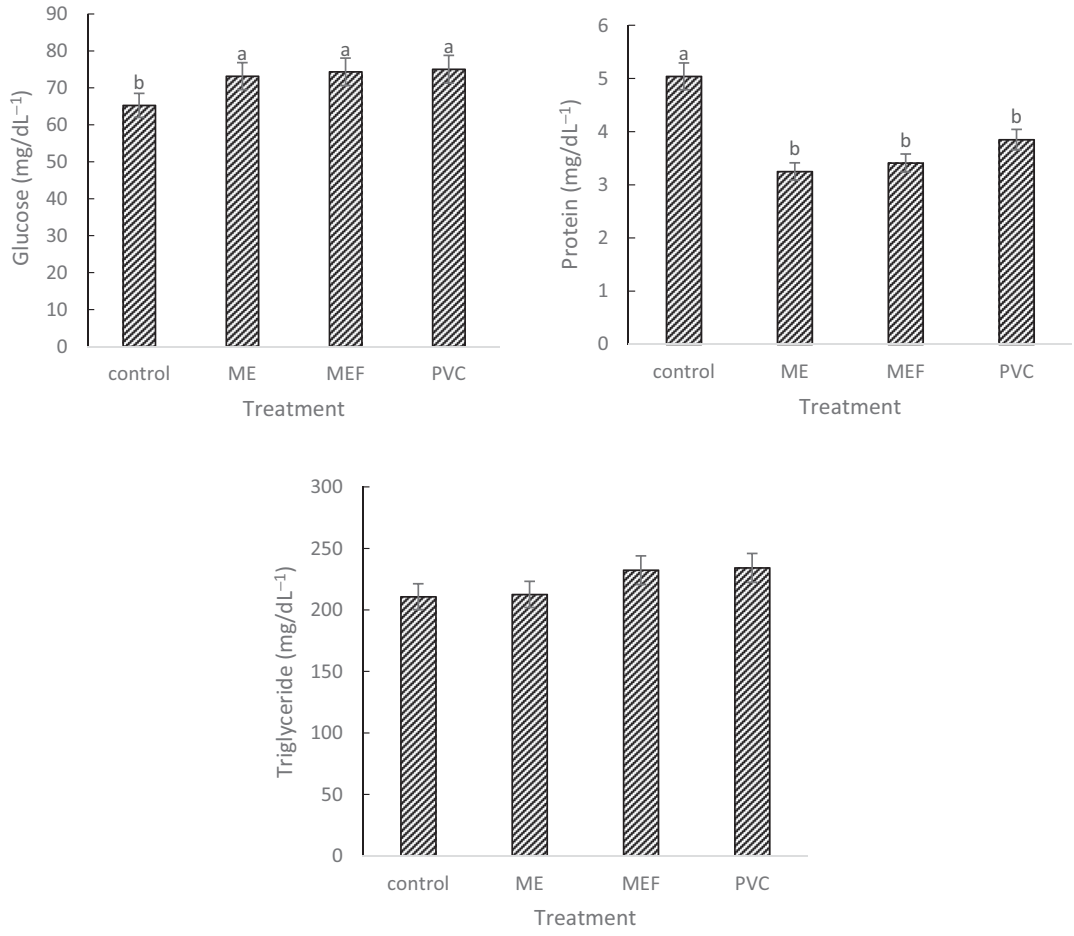

Figure 1. Biochemical profile (Glucose, protein and triglyceride) of *Clarias gariepinus* ($n = 9$) exposed to plastic additive chemicals (Melamine, Melamine formaldehyde and polyvinyl chloride). Bars with different alphabet letters denote significant difference ($p < 0.05$) based on ANOVA.

at 4 °C for 15 minutes and supernatant was removed. The AChE activity was measured 412 nm at time 5 minutes. The activity was expressed as U mg protein^{-1}, where U = is the amount of enzyme that catalyses the reaction of 1 nmol of substrate per minute.

Statistical analysis

Data generated are presented as mean ± standard error (SE), and were analysed using SPSS IBM version 22.0 computer program (SPSS, Chicago, IL, USA). Difference in the means were verified using one-way analysis of variance (ANOVA), while the levels of significance were declared at $p < 0.05$ using Ducan multiple range test (DMRT). Micro soft excel (version 16.0) was used to bar charts for proper interpretation of results.

Result

The effects of ME, MEF and PVC on the biochemical profile of *C. gariepinus* are presented in Figure 1. No mortality was observed in the exposed groups after the 45 days exposure trial. Glucose levels of the exposed fish groups increased significantly ($p < 0.05$) than the control, while total protein decreased substantially in the toxicant-exposed groups with respect to the control. However, changes in triglyceride levels among treatments were not significant ($p > 0.05$) when compared to the control. The effects of ME, MEF and PVC chemicals on the activities of serum enzymes in *C. gariepinus* are presented in Figure 2. In fact, AST and ALT levels from exposed fish increased significantly ($p < 0.05$) than control, while no significant change ($p > 0.05$) was observed for ALP activity between the control and the exposed groups.

The effect of ME, MEF and PVC on brain AChE activity in *C. gariepinus* after 45 days dietary exposure trial is presented in Figure 3. AChE activity of the exposed groups was significantly lower ($p < 0.05$) than the control. Antioxidant enzymes activities and MDA levels of *C. gariepinus* exposed to the chemicals are shown in Figure 4. Activities of SOD, GPx and CAT in the exposed groups, irrespective of the treatment significantly declined ($p < 0.05$) when compared to the control. MDA levels were significantly higher in the exposed groups when compared to control.

Discussion

The present study conspicuously demonstrated the effects of plastic chemicals (ME, MEF, PVC) on serum biochemistry, brain antioxidant enzymes activities (SOD, GPx, CAT), MDA and AChE levels of *C. gariepinus*. The adverse consequence of plastic chemicals on serum biochemistry was assessed via general biomarkers such as glucose, protein and serum

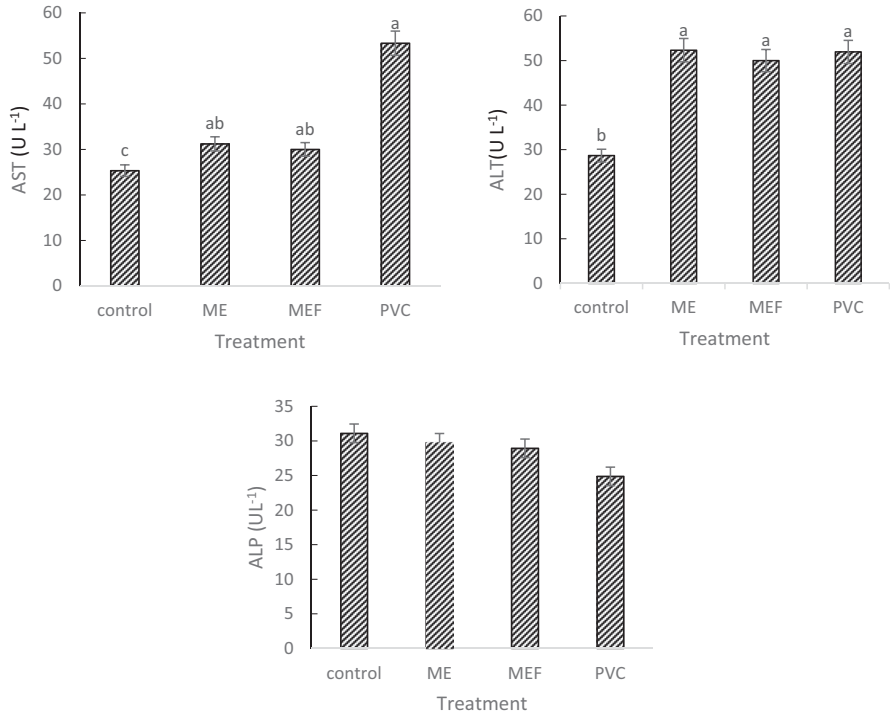

Figure 2. Serum enzymes activities (AST: aspartate aminotransferase; ALT: alanine amino transferase; ALP: alkaline phosphatase) of *Clarias gariepinus* ($n = 9$) exposed to plastic additive chemicals (ME: Melamine; MEF: Melamine formaldehyde; PVC: polyvinyl chloride). Bars with different alphabet letters denote significant difference ($p < 0.05$) based on ANOVA.

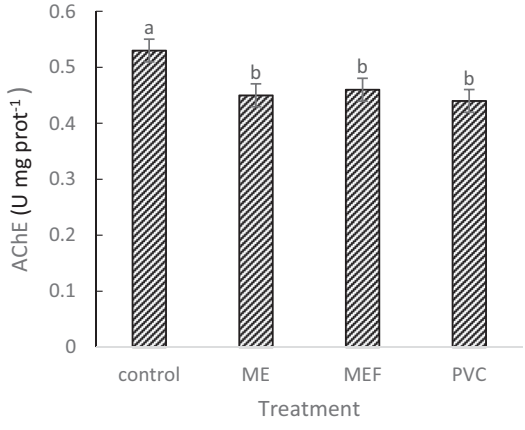

Figure 3. Brain acetylcholinesterase (AChE) activities of *Clarias gariepinus* ($n = 9$) exposed to plastic additive chemicals (ME: Melamine; MEF: Melamine formaldehyde; PVC: polyvinyl chloride). Bars with different alphabet letters denote significant difference ($p < 0.05$) based on ANOVA.

enzymes. Higher serum glucose levels in exposed fish may suggest an increased demand cum utilization of glucose in order to cushion the impact of these toxicants which eventually culminated to stress (Saravanan *et al.* 2012). Min and Kane (2008) stated that stress condition is usually a resultant effect of elevated levels of corticosteroids which is often associated with hyperglycaemia. Iheanacho and Odo (2020b) reported a significant increase in glucose level of PVC exposed fish. Previous study revealed that PVC-MP exposure exerted prominent effect (increase) on triglyceride content of treated fish (*C. gariepinus*) specifically on the 45th day exposure as against 15th and 30th day exposure, however, comparative assessment based on the different exposure durations as well as treatment and duration interactions revealed that the toxicant had no substantial effect on the triglyceride contents of the exposed fish (Iheanacho and Odo 2020b).

Serum total proteins are usually synthesized in the liver and are known to be important marker of liver impairment (Iheanacho *et al.* 2019). Significant protein reduction observed in the chemical exposed groups may suggest an increased protein breakdown as a functional response to deal with extra energy requirements needed to cope with the stress caused by the damaging effects of the toxicants. Lavanya *et al.* (2011) stated that reduction in protein level is usually an indication of liver damage induced by toxicants. In addition, the liver status of the exposed fish was also assessed via the activities of some intracellular enzymes (ALT, AST and ALP). AST and ALT are involved in the metabolism of amino acids and are important indicators of tissue damage in organs such as liver and kidney (Saravanan *et al.* 2012). Elevation in transaminase activities could indicate the utilization of amino acids for oxidation or glycogenesis (elevating glycaemia as observed in the present study). Exposure to melamine caused ALT and AST elevations in *Clarias batrachus* (Pirarat *et al.* 2012). Similar findings have been reported in *Sparus aurata* fed 0.5 g Kg^{-1} PVC after 30 days exposure trial (Espinosa *et al.* 2017).

Assessment of the impact of plastic chemicals (ME, MEF and PVC) on the nervous system of the exposed fish was measured via AChE activity. AChE activity is an essential biomarker, whose activity indicates the physiological condition of the nervous system (Pala and Serdar 2018). The present study revealed significant inhibition of brain AChE activity in the exposed groups. This result could be attributed to an elevated acetylcholine accumulation in the brain of the

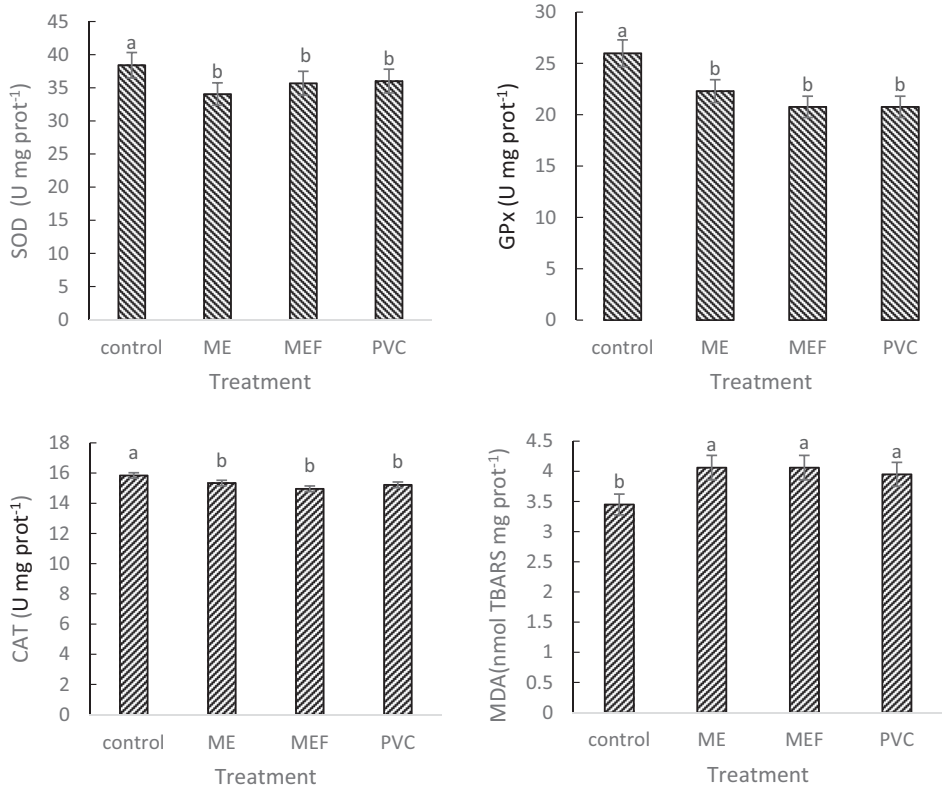

Figure 4. Antioxidant enzymes activity (SOD: superoxide dismutase; GPx: glutathione peroxidase; CAT: catalase) and malonaldehyde levels (MDA) of *Clarias gariepinus* ($n = 9$) exposed to plastic additive chemicals (ME: Melamine; MEF: Melamine formaldehyde; PVC: polyvinyl chloride). Bars with different alphabet letters denote significant difference ($p < 0.05$) based on ANOVA.

exposed groups, hence suggests neurotoxicity. Neurotoxicity and oxidative stress have been reported in polystyrene exposed fish *Scrobicularia plana* (Ribeiro *et al.* 2017). A similar finding has also been reported in pyrene treated fish (*Pomatoschistus microps*) (Oliveira *et al.* 2013). Iheanacho and Odo (2020a) stated that PVC induced neurotoxicity in *C. gariepinus* after 45 days dietary exposure trial.

Brain antioxidant enzymes activities (SOD, GPx, CAT) were measured in the present study to ascertain the oxidative physiological condition of the exposed groups. Antioxidant enzymes are important biomarkers of oxidative stress, whose functions are mainly to eliminate ROS and other pro-oxidants from cells (Egea *et al.* 2017). Imbalance between the production and elimination of ROS brings about oxidative stress especially when the generation of these ROS surpasses its elimination from the cell (Ajima *et al.* 2017). SOD, CAT and GPx are the first line defense antioxidants and are known to play protective role against free radical attack in the antioxidant system which include suppressing or thwarting the formation of free radicals or ROS in cells (Ighodaro and Akinloye 2018). These enzymes are proactive in the dismutation of superoxide radical, and further catalyse the degradation of hydrogen peroxide to water and oxygen molecule (Ribeiro *et al.* 2017, Ighodaro and Akinloye 2018). The findings of the present study revealed significant inhibitions of SOD, GPx and CAT in the exposed groups, hence suggest substantial production or generation of ROS caused by the noxious actions of these chemicals which resulted to oxidative stress induced toxicity. Nuntapong *et al.* (2019) reported significant inhibitions of SOD, CAT and GPx in ME exposed Pacific white shrimp. Espinosa *et al.* (2018) stated that dietary ingested PVC encouraged oxidative stress in European sea bass, by impairing cellular innate immune activities. Inhibited SOD, CAT and GPx activities were observed to be triggered by dietary ingested PVC in *C. gariepinus* (Iheanacho and Odo 2020a). MDA level is a useful biomarker of lipid peroxidation in cells (Ajima *et al.* 2017), of which its elevation denotes oxidative dysfunction and damage. Significant elevation of MDA levels observed in the exposed groups is in agreement with their lower antioxidant enzyme activities. It is known that brain is particularly prone to suffer oxidative stress due to its comparatively low antioxidant defense system, high rate of oxidative metabolism, presence of auto-oxidizable neuro-transmitters and cytochrome P450s and high levels of unsaturated fatty acids (Halliwell and Gutteridge 2007). Nuntapong *et al.* (2019) reported significant increase of MDA levels in ME and cyanuric acid exposed shrimp. Elevated MDA levels have been reported in PVC exposed fish *C. gariepinus* (Iheanacho and Odo 2020a).

Conclusions

The present study revealed that ME, MEF and PVC induced oxidative stress and neurotoxicity in the exposed fish. Additionally, the exposed fish suffered hepatic damage, following the elevated activities of serum transaminases (AST and ALT) and hypoproteinemia in exposed fish. The findings of this study are indispensable for monitoring and assessing the impact of plastic based environmental pollution and its associated health risk/defects on aquatic models and humans

at large. Illicit practices of incorporating ME resin into dairy products and aquafeed portends serious health risk as revealed from the present study, hence the need to strictly monitor food production chains and systems by relevant agencies is strongly encouraged. Additional studies are needed to appraise the impact of plastic chemical resins on aquatic biota and their mechanisms of action within the physiological system.

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Disclosure statement

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ORCID

Stanley Chidi Iheanacho https://orcid.org/0000-0001-8233-2604

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