

Combined toxicity of copper oxide nanoparticle and polystyrene nanoplastic in *Oreochromis niloticus*

Submitted in partial fulfillment of the requirement for
the award of a bachelor of Technology degree in
Biotechnology

By

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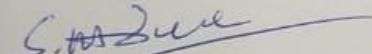
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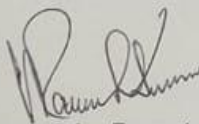
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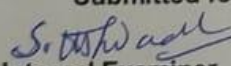

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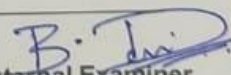
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I, ARJUN ADITIYA K R (40230003), hereby declare that the project work entitled **“COMBINED TOXICITY OF COPPER OXIDE NANOPARTICLE AND POLYSTYRENE NANOPLASTIC IN *Oreochromis niloticus*”** done by me under the guidance of **Dr. D. Inbakandan** (external guide) and **Dr. Usha Nandhini** (internal guide) at Centre for Ocean Research, Sathyabama Research Park, Sathyabama Institute of Science and Technology are submitted in the partial fulfillment for the requirements of a Bachelor of Technology degree in Biotechnology.

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Place: Chennai



SIGNATURE OF THE CANDIDATE

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ABSTRACT

The current research focuses on the combination toxicity of copper oxide nanoparticles (CuO NPs) and polystyrene nanoplastics (PS NPs) in *Oreochromis niloticus*. Fish were subjected to varied concentrations of CuO NPs and PS NPs, both alone and in combination, for 14 days. Toxicity was evaluated using biochemical and histopathological markers. The results show that CuO NPs and PS NPs were having greater impacts on oxidative stress indicators, organ damage, and overall fish health. The oxidative stress assessment such as ROS, LPO, and LDH has shown increased stress damage in each tissue (Gill, Muscle, Heart). MIX3 (PSNP+CuO) has shown greater stress damage compared to other concentration groups. Similarly, the results in the Antioxidant Biomarker response (SOD, CAT, GSH, GST) have elevated enzyme activity. Histopathology results showed cellular damage after exposure to PSNPs and CuO in Gill, Muscle, and Heart tissue. These findings highlight the necessity of knowing the interacting impacts of nanoparticle contaminants on aquatic life to effectively assess and control environmental risks.

KEYWORDS: Nanoplastics, Polystyrene, Copper, Toxicity, Oxidative stress, PSNPS, CuO.

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LIST OF ABBREVIATIONS

NPs	Nanoplastics
MPs	Microplastics
PS	Polystyrene
CuO	Copper Oxide
PSNP	Polystyrene Nanoplastics
ROS	Reactive Oxygen Species
MDA	Malondialdehyde
SOD	Superoxide dimutase
LDH	Lactate dehydrogenase
LPO	Lipid peroxidation
CAT	Catalase activity
GSH	Glutathione
GST	Glutathione S-transferase
TBARS	Thiobarbituric acid reactive substance
PBS	Phosphate Buffer Saline
NBF	Neutral Buffer Formalin
PCA	Principle Component Analysis
NADH	Nicotinamide Adenine Dinucleotide Hydrogen

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CHAPTER-1

INTRODUCTION

Fishes are one of the most abundant creatures in the aquatic environment (Roest Crolius & Weissenbach, 2005). Belonging to the cichlid family, Nile tilapia (*Oreochromis niloticus*) is widely recognised in Africa and is primarily valued for its quality flesh and nutritional benefits. Tilapias (all species) are the second most significant farmed fish after carp, and the most commonly cultivated (The 2022 edition of The State of World Fisheries and Aquaculture, 2022). It serves as a beneficial research model in toxicological investigations since it is straightforward to control and seen under experimental circumstances (Hamdy A. M. Soliman, 2017). This species has various characteristics that qualify it as an appropriate model for use as an indicator species. Its rapid growth rate, high tolerance to environmental stress, ease of reproduction, and significant market demand contribute to its suitability for this job (Abdel El-Sayed, 2006). Plastic materials have been extensively utilised due to their affordability, lightweight, durability, and adaptability (Nielsen et al., 2020). Polymers can either directly or indirectly increase in freshwater, harming the ecology (Yu et al., 2020). Every year, a large amount of plastic ends up in freshwater and marine settings. Freshwater lakes and rivers serve as crucial supplies for drinking water, industrial and agricultural consumption, and aquatic life habitats. Plastics are devoured by roughly 700 species in freshwater and marine habitats, including fish, crustaceans, molluscs, seabirds, and mammals (Ferreira et al., 2019). When plastics infiltrate freshwater habitats, they carry with them degrading compounds and other biological contaminants. These compounds can degrade freshwater quality, affecting the growth and development of aquatic vegetation and wildlife. Furthermore, they can influence the use of water resources for both livelihoods and daily activities (B. Zhang et al., 2021). The UN Environment Programme (UNEP) has highlighted the accumulation of plastic in aquatic ecosystems as an environmental crisis on pace with the severity of climate change. Roughly 800 million tons of plastic pollution in the

ocean are thought to come from land-based activities that involve fishing, various types of aquaculture, and coastal tourist attractions (Yee et al., 2021). Waste from plastics is often broken down into microplastics that have sizes between 1-6000 μm and nanoplastics sizes ought to be less than 1 μm . These minute particles are complex to eradicate from the aquatic environment by a combination of chemical, biological, and physical reactions (Triebkorn et al., 2019). PS is an aromatic hydrocarbon synthesised from the polymerisation of styrene monomers (Umamaheswari et al., 2021). Enormous PS manufacturing is carried out by catalytic dehydrogenation of ethylbenzene, which normally does not exceed 20 ppm in the plastics sector, which is far lower than the calculated value of chronic toxicity. Given its flexibility and benign qualities, Polystyrene is used to make Styrofoam and other polymer items such as home appliances, toys, automobile food packaging, components, healthcare equipment, and electronic gadgets for consumers (Rabie et al., 2008). Polystyrene particles have been utilised as representative particles in studies investigating the effects of numerous particle surfaces on biological variables. This is because they may easily be created in a variety of sizes (Loos et al., 2014). Styrene monomers have been shown to migrate into food products from PS containers, posing an imminent risk to the well-being of humans (World Health Organization. et al., 2008); (Pilevar et al., 2019). Microbeads constructed of tiny polystyrene (0.05 μm -0.1 μm) tend to be more deadly to aquatic invertebrates (Rist et al., 2017) ;(Jeong et al., 2016). It is commonly assumed that NPs/MPs enter the human body predominantly through the gastrointestinal (Van Cauwenberghe & Janssen, 2014) ;(Liebezeit and Liebezeit, 2013) and respiratory systems (Dris et al., 2016);(Prata, 2018). Nanoplastics in ambient air can cause respiratory exposure, whilst contaminants in food, drinking water, and air accumulation can promote digestive system exposure. Fish rely extensively on their gills for gas exchange, ion control, and waste removal. With a large surface area accessible to the external environment, gills are the initial target for waterborne contaminants (Mallatt, 1985) ;(Perry and Laurent, 1993). Tissue chunk and enzyme assays are commonly employed to detect the harmful effects of plastic particles (Oliveira et al., 2013) ;(von Moos et al., 2012). Apart

from PSNP's, Copper in the form of Cu^{2+} is a necessary micronutrient for living organisms, playing a key part in stimulating growth, aiding development, and promoting cellular functions (Cao et al., 2019). A disproportionate amount of Cu^{2+} in the environment poses a hazardous risk to aquatic species. Heavy metals' negative effects originate mostly from their capacity to diffuse, biomagnify throughout aquatic food chains, and accumulate in biological tissues, thereby contributing to their intrinsic toxicity (A. M. Soliman and M. M. Ragab, 2018). Copper's harmful effects on fish are presumed to be driven by the formation of ROS, or reactive oxygen species, via the Fenton and Haber-Weiss reactions (Maria and Bebianno, 2011). Copper has been shown to produce significant histopathological abnormalities in the gills of teleost fish at high doses (Jooyandeh et al., 2016). (Lease et al., 2003) discovered Gill histology can provide early indicators of fish health in the environment. The detrimental impacts of nanoparticles composed of metals and metal oxides are extensively studied in fish using histopathological, biochemical, and haematological indicators (Gül et al., 2004). These fish have an extensive antioxidant system that includes superoxide dismutase (SOD), catalase (CAT), glutathione S-transferase (GST), and glutathione peroxidase (GPx), which counteract the oxidative damage of reactive oxygen species (ROS) (Sayed & Soliman, 2018). Cellular immune processes, such as phagocytic capacity and reactive oxygen species (ROS) generation, can be effective indicators for assessing immunotoxic effects. During the respiratory burst, cells release harmful chemicals known as ROS, including superoxide anion (O_2^-), singlet oxygen (O_2), hydrogen peroxide (H_2O_2), and hydroxyl radicals (OH) (Haugland et al., 2012). Oxidative stress is characterised by increased reactive oxygen species (ROS), weakened anti-oxidant defence mechanisms, or the inability to repair oxidative damage (Dorval, 2003). Correlative research of nanoparticle and microscale materials is crucial because the fundamental characteristics of nanoparticles may be directly related to their potential toxicity. It is critical to characterise solutions containing these particles fully (Ribeiro et al., 2013). It is essential to emphasise that the link between heavy metals and nanoplastics may greatly affect the toxicity and accumulation of heavy metals, given their shared route of entry into the water (freshwater, lakes, rivers). Nevertheless, there needs to

be unanimity on the role of NPs in the exposure to these residing polluting substances. As a result, additional research is needed for a better understanding of the function of nanoplastics in the joint toxicity of intervening pollutants. This article seeks to give a comparative study between CuO nanoparticles and PSNP's nanoplastics at different concentrations and a combination of CuO NPs and PSNPs on oxidative stress response, DNA fragmentation, histopathology, LDH and bioaccumulation assessment in famous juvenile Nile tilapia (*Oreochromis niloticus*).

CHAPTER-2

LITERATURE SURVEY

(Zhou et al., 2021) Quantitative analysis of polystyrene and poly(methyl methacrylate) nanoplastics in tissues of aquatic animals

Micro- and nanoplastics can enter the body by drinking water, food, and breathing. This technique involves extracting nanoplastics from aquatic animal tissues using alkaline digestion and protein precipitation, followed by quantitative measurement using pyrolysis gas chromatography and mass spectrometry. The optimised technique has good reproducibility and sensitivity, with detection limits of 0.03 µg/g for PS nanoplastics and 0.09 µg/g for PMMA nanoplastics. This approach preserves the original shape and size of nanoplastics.

(Hamed et al., 2022) Investigation of the impact caused by different sizes of polyethylene plastics (nano, micro and macro) in common carp juveniles, *Cyprinus carpio* L., using multi-biomarkers.

Plastic pollution is a global hazard to aquatic life, yet the effects on many species are little documented and understood. This study investigates the effects of various sizes of polyethylene (PE) plastics on common carp and assesses their multi-biomarker responses. The histological structure and assessment of biochemical modifications, antioxidant enzymes, immunological responses, and variations in blood profiles of organisms after 15 days of exposure to a concentration of 100 mg/L of nano- (NPs), micro- (MPs), and macroplastics (MaPs). The fish's health was influenced only by the presence of PE particles. All biomarkers altered following exposure compared to the control group, with higher alterations being detected with the decreasing size of particles (NPs > MPs > MaPs) than in their absence.

(El-Din et al., 2011) Ameliorative effects of selenium and *Chlorella vulgaris* against polystyrene nanoplastics-induced hepatotoxicity in African catfish (*Clarias gariepinus*)

Polystyrene nanoplastic contamination is a global issue that has sparked concern among scientists due to potential health implications. This study examined the hepatotoxic effects of polystyrene nanoplastics (PS NPs) on catfish (*Clarias gariepinus*) by histopathology, biochemistry, and ultrastructural analysis. Biochemical assays revealed an increase in serum ALT and AST activity following exposure to NPs compared to the control group. Exposure to NPs and supplementation with *Chlorella* and selenium resulted in significantly lower blood enzyme activity compared to the NPs-intoxicated group. Histopathological study revealed hepatotoxic effects of NPs, including steatosis, coagulative necrosis, fibrosis, glycogen depletion, and toxic alterations in cellular ultrastructure. Supplementing with *chlorella* and selenium reduced the hepatotoxicity of NPs and improved liver tissue appearance and structure.

(Umamaheswari et al., 2021b) polystyrene microplastic induces apoptosis via ROS-mediated p53 signalling pathway in zebrafish.

Microplastic (MP) contamination is prevalent and poses a growing danger to aquatic biota. Recent research data indicate harmful effects at the cellular and organism levels, but the chemical mechanism remains unknown. This study investigates the molecular mechanisms that lead to apoptosis in the gills of zebrafish exposed to polystyrene microplastics (PS-MPs). PS-MPs at varying doses (10 and 100 $\mu\text{g L}^{-1}$) generated reactive oxygen species (ROS), influencing oxidative and immune defence mechanisms. Antioxidant gene cat; sod1, gpx1a and gstp1 showed substantial expression changes. PS MP's also drastically reduced neurotransmission in zebrafish. Furthermore, the PS-MPs exposure increased the expression of p53 gadd45ba, and casp3b, leading to apoptosis. PS-MP exposure can cause oxidative damage and cytological damage, including altered lamellar structures, capillary dilatation, and necrosis in gill histomaps. This study indicates that PS-MPs trigger dose- and time-dependent ROS-mediated apoptotic responses in zebrafish.

(Abarghouei et al., 2021) size-dependent effects of microplastic on uptake, immune system, related gene expression and histopathology of goldfish (*Carassius auratus*)

This study investigates the size-dependent effects of microplastics (MPs) on the uptake, immune system response, related gene expression, and histopathology in goldfish (*Carassius auratus*). Goldfish were exposed to microplastics of varying sizes, and their uptake rates were measured. Additionally, the impact of microplastic exposure on the immune system was assessed through immune response parameters and gene expression analysis. Histopathological examinations were conducted to evaluate any tissue damage caused by microplastic ingestion. The results suggest that the size of microplastics plays a crucial role in uptake rates and subsequent physiological effects in goldfish. Larger microplastics were found to have different impacts on immune system parameters, gene expression, and histopathological changes compared to smaller microplastics. Understanding these size-dependent effects is essential for assessing the ecological risks associated with microplastic pollution in aquatic environments and for developing effective mitigation strategies.

(Zhou et al., 2022a) Long-term exposure to polystyrene nanoplastics impairs the liver health of medaka.

The ubiquitous presence of nanoplastics in aquatic environments has raised concerns about their potential impacts on aquatic organisms. This study investigates the effects of long-term exposure to polystyrene nanoplastics on the liver health of medaka (*Oryzias latipes*), a commonly used model organism in aquatic toxicology. Medaka were exposed to environmentally relevant concentrations of polystyrene nanoplastics for an extended period, and liver health parameters were assessed using histological analysis, biochemical assays, and gene expression profiling. Results indicate that long-term exposure to polystyrene nanoplastics induces hepatotoxicity in medaka, characterized by hepatic inflammation, oxidative stress, altered lipid metabolism, and disruption of liver function markers. Gene expression analysis revealed dysregulation of genes involved in detoxification, inflammation, and lipid metabolism pathways. These findings underscore the potential adverse effects of nanoplastic pollution on aquatic organisms and highlight the importance of further research to elucidate the mechanisms underlying nanoplastic-induced hepatotoxicity. Addressing nanoplastic

pollution requires concerted efforts to mitigate its environmental impact and safeguard aquatic ecosystem health.

(Qiao et al., 2019a) Combined effects of polystyrene microplastics and natural organic matter on the accumulation and toxicity of copper in zebrafish

This study investigates the combined effects of polystyrene microplastics (PS-MPs) and natural organic matter (NOM) on the accumulation and toxicity of copper (Cu) in zebrafish (*Danio rerio*). Zebrafish were exposed to environmentally relevant concentrations of Cu in the presence of PS-MPs and NOM to simulate real-world conditions. The results indicate that the presence of PS-MPs and NOM influences the accumulation of Cu in zebrafish tissues, with potential implications for bioaccumulation and biomagnification in aquatic food webs. Furthermore, the combined exposure to Cu, PS-MPs, and NOM results in complex interactions that affect the toxicity and physiological responses of zebrafish. Understanding these interactions is critical for assessing the environmental risks associated with the co-occurrence of microplastics and metal contaminants in aquatic ecosystems.

(Ding et al., 2020) Toxicological effects of nano- and micro-polystyrene plastics on red tilapia: Are larger plastic particles more harmless?

This study investigates the toxicological effects of nano- and micro- polystyrene plastics on red tilapia (*Oreochromis spp.**) and examines whether larger plastic particles are relatively less harmful. Red tilapia were exposed to varying concentrations of nano- and micro-polystyrene plastics in controlled laboratory conditions. Assessment of physiological parameters, including growth rates, behavior, and biochemical markers, was conducted to evaluate the toxicological impact. Results suggest that both nano- and micro- polystyrene plastics induce adverse effects on red tilapia, including growth inhibition, altered behavior, and oxidative stress markers. Surprisingly, larger plastic particles did not exhibit significantly reduced toxicity compared to smaller particles. These findings underscore the importance of considering the harmful effects of plastic pollution on aquatic organisms, regardless of particle size, and highlight the urgent need for effective mitigation strategies to address plastic pollution in aquatic ecosystems.

(Thiagarajan et al., 2021) Interactive effects of micro/nanoplastics and nanomaterials/pharmaceuticals: Their ecotoxicological consequences in the aquatic systems

The proliferation of micro/nanoplastics and the widespread use of nanomaterials and pharmaceuticals have raised concerns about their interactive effects and ecotoxicological consequences in aquatic systems. This review examines the synergistic, antagonistic, and additive interactions between micro/nanoplastics and nanomaterials/pharmaceuticals, focusing on their impacts on aquatic organisms and ecosystems. The complex interplay between these contaminants can exacerbate ecological risks, leading to adverse effects on organismal health, reproduction, behaviour, and ecosystem functioning. Understanding the interactive effects of these pollutants is crucial for devising effective mitigation strategies and safeguarding the health and integrity of aquatic environments.

(Pang et al., 2021) Transcriptome sequencing and metabolite analysis reveal the toxic effects of nanoplastics on tilapia after exposure to polystyrene

This study investigates the toxic effects of nanoplastics on tilapia (*Oreochromis niloticus*) by employing transcriptome sequencing and metabolite analysis after exposure to polystyrene nanoparticles. Through transcriptome sequencing, alterations in gene expression profiles were observed, highlighting pathways potentially affected by nanoplastic exposure. Metabolite analysis further elucidated changes in biochemical pathways, shedding light on the physiological responses of tilapia to nanoplastic exposure. The findings underscore the detrimental impact of nanoplastics on aquatic organisms and provide valuable insights into the underlying molecular mechanisms involved in nanoplastic toxicity. Understanding these effects is crucial for developing strategies to mitigate nanoplastic pollution and safeguard aquatic ecosystems.

(Hamed et al., 2020) Antioxidants and molecular damage in Nile Tilapia (*Oreochromis niloticus*) after exposure to microplastics

This study investigates the impact of microplastic exposure on molecular damage and the role of antioxidants in Nile Tilapia (*Oreochromis niloticus*). Microplastics, ubiquitous pollutants in aquatic environments, pose a significant threat to marine organisms. Using Nile Tilapia as a model, we examined the oxidative stress response and molecular damage induced by microplastic exposure. Fish were exposed to environmentally relevant concentrations of microplastics, and markers of oxidative stress, including antioxidant enzyme activities and lipid peroxidation levels, were assessed. Our findings reveal a significant increase in oxidative stress markers and molecular damage in fish exposed to microplastics compared to control groups. Furthermore, we observed alterations in antioxidant defence mechanisms, suggesting a potential role of antioxidants in mitigating microplastic-induced oxidative damage. This study underscores the importance of understanding the mechanisms underlying microplastic toxicity and highlights the potential of antioxidants as protective agents against molecular damage in aquatic organisms.

(Zhang et al., 2019) Interactive effects of polystyrene microplastics and roxithromycin on bioaccumulation and biochemical status in the freshwater fish red tilapia (*Oreochromis niloticus*)

The interactive effects of polystyrene microplastics (PS-MPs) and roxithromycin (ROX) on bioaccumulation and biochemical status in the freshwater fish red tilapia (*Oreochromis niloticus*) were investigated. Red tilapia were exposed to PS-MPs, ROX, and their combination for a specified period, after which bioaccumulation levels of PS-MPs and ROX were measured in fish tissues. Additionally, biochemical parameters related to oxidative stress, antioxidant defence, and metabolic activity were assessed. Results indicated that exposure to PS-MPs and ROX individually led to bioaccumulation in fish tissues, with varying effects on biochemical markers. However, the combination of PS-MPs and ROX exhibited synergistic or antagonistic effects, altering bioaccumulation patterns and causing significant perturbations in biochemical status compared to single exposures. These findings highlight the complex interactions between microplastics and

pharmaceutical pollutants in freshwater ecosystems and underscore the importance of considering their combined effects on aquatic organisms.

(Y. Zhao et al., 2021) Metabolomic profiling reveals the intestinal toxicity of different lengths of microplastic fibers on zebrafish (*Danio rerio*)

Microplastic pollution in aquatic environments poses a significant threat to aquatic organisms, yet the mechanisms underlying its toxicity remain poorly understood. This study investigated the intestinal toxicity of microplastic fibers of varying lengths on zebrafish (*Danio rerio*) using metabolomic profiling. Zebrafish exposed to different lengths of microplastic fibers were analysed for alterations in their metabolomic profiles. Results revealed distinct metabolic changes associated with intestinal toxicity, including disruptions in energy metabolism, amino acid metabolism, and oxidative stress pathways. Furthermore, the length of microplastic fibers exerted differential effects on zebrafish metabolism, with longer fibers inducing more pronounced alterations. These findings provide insights into the intestinal toxicity mechanisms of microplastic fibers in zebrafish and highlight the importance of considering fiber length in assessing microplastic pollution impacts on aquatic ecosystems.

(Atamanalp et al., 2022) Interaction of Microplastic Presence and Oxidative Stress in Freshwater Fish: A Regional Scale Research, East Anatolia of Türkiye (Erzurum & Erzincan & Bingöl)

This study investigates the interaction between microplastic pollution and oxidative stress in freshwater fish within the East Anatolia region of Turkey, specifically focusing on Erzurum, Erzincan, and Bingöl provinces. Microplastics, ubiquitous pollutants in aquatic ecosystems, pose a significant threat to freshwater environments and aquatic organisms. Concurrently, oxidative stress, resulting from an imbalance between reactive oxygen species (ROS) and antioxidant defense mechanisms, can have detrimental effects on fish health and physiology. Through a regional-scale research approach, this study aims to assess the extent of microplastic contamination in freshwater bodies and evaluate its potential correlation with oxidative stress biomarkers in native fish species. By employing a combination of field

surveys, laboratory analyses, and statistical modeling techniques, we seek to elucidate the complex relationship between microplastic exposure and oxidative stress in freshwater fish populations. The findings of this research are expected to contribute valuable insights into the environmental implications of microplastic pollution and its impacts on aquatic ecosystems in the East Anatolia region of Turkey.

(Chen et al., 2021) Transgenerational neurotoxicity of polystyrene microplastics induced by oxidative stress in *Caenorhabditis elegans*

The widespread use and improper disposal of plastics have led to the accumulation of microplastics in various ecosystems, including marine environments. Despite growing concerns about their environmental impact, the potential neurotoxic effects of microplastics, particularly on subsequent generations, remain poorly understood. In this study, we investigated the transgenerational neurotoxicity of polystyrene microplastics (PS-MPs) induced by oxidative stress in *Caenorhabditis elegans* (*C. elegans*) as a model organism. Our results demonstrate that exposure to PS-MPs resulted in impaired locomotion behaviour and decreased lifespan in both exposed and subsequent generations of *C. elegans*. Furthermore, we observed elevated levels of oxidative stress markers, including reactive oxygen species (ROS) and lipid peroxidation, in exposed individuals and their progeny. These findings suggest that PS-MPs can induce transgenerational neurotoxic effects in *C. elegans* through mechanisms involving oxidative stress. Understanding the transgenerational effects of microplastics on neurotoxicity highlights the need for further research and underscores the importance of mitigating plastic pollution to safeguard environmental and human health.

(Raza et al., 2023a) Exploration of polyacrylamide microplastics and evaluation of their toxicity on multiple parameters of *Oreochromis niloticus* This study explores the presence and toxicity of polyacrylamide microplastics (PAM-MPs) on multiple parameters of *Oreochromis niloticus*, commonly known as Nile tilapia. Polyacrylamide microplastics, a type of microplastic pollutant, have gained attention due to their ubiquitous presence in aquatic environments and potential adverse effects on aquatic organisms. In this

research, water samples from freshwater bodies were analyzed to assess the abundance and distribution of PAM-MPs. Subsequently, Nile tilapia specimens were exposed to controlled concentrations of PAM-MPs, and various parameters including physiological, biochemical, and histological markers were evaluated to assess toxicity. The findings of this study provide valuable insights into the ecological implications of PAM-MPs contamination on aquatic ecosystems and contribute to the understanding of their impact on fish health and welfare.

(Zhang et al., 2022) Combined effects of polystyrene microplastics and copper on antioxidant capacity, immune response and intestinal microbiota of Nile tilapia (*Oreochromis niloticus*)

This study investigates the combined impact of polystyrene microplastics and copper on the antioxidant capacity, immune response, and intestinal microbiota of Nile tilapia (*Oreochromis niloticus*). Through controlled exposure experiments, we assessed the interactive effects of these pollutants on fish health. Results revealed significant alterations in antioxidant enzyme activities, immune parameters, and shifts in intestinal microbial composition. Specifically, the combined exposure led to compromised antioxidant defences suppressed immune function, and dysbiosis in the intestinal microbiota of Nile tilapia. These findings highlight the complex interactions between microplastics and metal pollutants, emphasizing the need for comprehensive assessment strategies to mitigate the adverse effects on aquatic organisms and ecosystems.

(Hong et al., 2023) Adverse effects of microplastics and nanoplastics on the reproductive system: A comprehensive review of fertility and potential harmful interactions

This comprehensive review examines the adverse effects of microplastics and nanoplastics on the reproductive system, focusing on fertility and potential harmful interactions. It synthesizes current research findings to elucidate the mechanisms through which these ubiquitous pollutants impact reproductive health. The review highlights the detrimental effects of microplastics and nanoplastics on gametogenesis, hormone regulation, and reproductive

organs, emphasizing their potential to disrupt fertility and reproductive function. Insights into the underlying pathways and interactions contribute to a deeper understanding of the reproductive toxicity of plastic pollution, informing strategies for mitigating its impact on human and environmental health.

(Shokr, 2015) Effect of Ammonia Stress on Blood Constitutes in Nile Tilapia
This study investigates the impact of ammonia stress on blood parameters in Nile tilapia (*Oreochromis niloticus*), a commonly farmed freshwater fish. Exposure to ammonia, a prevalent environmental pollutant in aquaculture systems, can significantly affect fish health. Blood serves as a vital indicator of physiological stress and overall health in aquatic organisms. Through experimental exposure to varying levels of ammonia, changes in key blood parameters including hematocrit, haemoglobin, and plasma biochemistry are analysed. Understanding the response of Nile tilapia to ammonia stress can inform aquaculture management practices, aiding in the development of strategies to mitigate the adverse effects of environmental pollutants on fish health and welfare.

(Guimarães et al., 2021) Toxic effects of naturally-aged microplastics on zebrafish juveniles: a more realistic approach to plastic pollution in freshwater ecosystems

This study investigates the toxic effects of naturally-aged microplastics on zebrafish juveniles, offering a more realistic perspective on plastic pollution in freshwater ecosystems. Through controlled experiments, the study examines the physiological and behavioural responses of zebrafish exposed to naturally weathered microplastics, simulating real-world conditions. Results indicate significant adverse effects on growth, development, and metabolic functions in zebrafish juveniles, highlighting the potential ecological implications of microplastic pollution in freshwater habitats. This research contributes to a deeper understanding of the environmental impact of microplastics and underscores the urgency of implementing effective mitigation strategies to preserve the health and integrity of freshwater ecosystems.

(Li et al., 2023a) Toxic effect of chronic exposure to polyethylene nano/microplastics on oxidative stress, neurotoxicity and gut microbiota of adult zebrafish (*Danio rerio*)

This study investigates the toxic effects of chronic exposure to polyethylene nano/microplastics on adult zebrafish (*Danio rerio*). Through comprehensive analysis, including assessment of oxidative stress markers, neurotoxicity indicators, and alterations in gut microbiota composition, the study aims to elucidate the potential adverse impacts of polyethylene pollution on aquatic organisms. Utilizing zebrafish as a model organism offers insights into the systemic effects of nanoplastic and microplastic exposure on physiological processes, providing valuable implications for environmental health and human well-being. Understanding the mechanisms underlying these toxic effects is crucial for developing effective strategies to mitigate plastic pollution and safeguard aquatic ecosystems.

(Falfushynska and Sokolova, 2023) Intermittent hypoxia differentially affects metabolic and oxidative stress responses in two species of cyprinid fish "Intermittent hypoxia, a common occurrence in aquatic environments, impacts metabolic and oxidative stress responses differently across species. This study investigates the effects of intermittent hypoxia on two cyprinid fish species. Results reveal species-specific variations in metabolic and oxidative stress responses, highlighting the importance of considering interspecies differences in hypoxia tolerance. Understanding these differential responses provides insights into the adaptive mechanisms of fish to fluctuating oxygen levels in their environment."

(Z. Li et al., 2021) Nanoplastic-Induced Genotoxicity and Intestinal Damage in Freshwater Benthic Clams (*Corbicula fluminea*): Comparison with Microplastics This study investigates the genotoxic and intestinal effects of nanoplastics on freshwater benthic clams (*Corbicula fluminea*), comparing them with microplastics. Results reveal significant nanoplastic-induced genotoxicity and intestinal damage, highlighting potential adverse impacts on aquatic organisms. Understanding the comparative effects of nanoplastics and

microplastics is crucial for assessing their environmental risks and informing strategies for mitigating plastic pollution's detrimental effects on freshwater ecosystems.

(Liu et al., 2020) Neurodevelopmental Toxicity of Polystyrene Nanoplastics in *Caenorhabditis elegans* and the Regulating Effect of Presenilin

This study investigates the neurodevelopmental toxicity of polystyrene nanoplastics in *Caenorhabditis elegans* and examines the regulating effect of Presenilin. Results suggest that exposure to polystyrene nanoplastics induces adverse effects on neurodevelopmental processes in *C. elegans*. Interestingly, Presenilin appears to mitigate these toxic effects, potentially through modulation of cellular pathways. These findings shed light on the neurotoxicity of nanoplastics and highlight the importance of understanding their impacts on neurological health. Further research on the mechanisms underlying Presenilin-mediated regulation may offer insights into potential therapeutic strategies for mitigating nanoplastic-induced neurodevelopmental toxicity.

(Ismail et al., 2021) Impacts of microplastics on reproductive performance of male tilapia (*Oreochromis niloticus*) pre-fed on *Amphora coffeaeformis*

This study investigates the effects of microplastic exposure on the reproductive performance of male tilapia (*Oreochromis niloticus*) pre-fed with *Amphora coffeaeformis*. Results indicate a significant reduction in sperm quality and quantity, as well as altered reproductive behaviours, suggesting detrimental impacts on male fertility. These findings underscore the need for further research on the reproductive toxicity of microplastics and emphasize the importance of mitigating plastic pollution to safeguard aquatic species' reproductive health.

(El Asely et al., 2020a) *Ziziphus mauritiana* supplementation of Nile tilapia (*Oreochromis niloticus*) diet for improvement of immune response to *Aeromonas hydrophila* infection

This study investigates the effects of *Ziziphus mauritiana* supplementation in Nile tilapia (*Oreochromis niloticus*) diets on immune response enhancement against *Aeromonas hydrophila* infection. Results suggest that dietary

supplementation with *Z. mauritiana* improves the immune response of Nile tilapia, potentially enhancing resistance to *A. hydrophila* infection. These findings highlight the potential of *Z. mauritiana* as a natural immunostimulant in aquaculture practices for improving disease resistance and overall health in Nile tilapia populations.

(Guerrera et al., 2021) Micro and Nano Plastics Distribution in Fish as Model Organisms: Histopathology, Blood Response and Bioaccumulation in Different Organs

This study investigates the distribution of micro and nano plastics in fish model organisms, examining histopathology, blood response, and bioaccumulation in various organs. Through histopathological analysis, blood response assessment, and organ-specific bioaccumulation studies, the research aims to elucidate the impact of plastic pollution on fish health. Understanding the distribution and physiological responses to micro and nano plastics in fish can provide valuable insights into the potential consequences of plastic pollution on aquatic ecosystems and human health.

(Abdel-Khalek et al., 2015) Comparative toxicity of copper oxide bulk and nano particles in Nile Tilapia; *Oreochromis niloticus*: Biochemical and oxidative stress

This study investigates the differential toxic effects of copper oxide bulk and nanoparticles on Nile Tilapia (*Oreochromis niloticus*). Biochemical and oxidative stress parameters are assessed to compare the impact of these two forms of copper oxide. Results reveal distinct responses in the fish, highlighting potential differences in toxicity mechanisms. Understanding the comparative toxicity of copper oxide bulk and nanoparticles provides valuable insights into their environmental implications and aids in the development of effective mitigation strategies.

(Soliman et al., 2021) Investigating the effects of copper sulfate and copper oxide nanoparticles in Nile tilapia (*Oreochromis niloticus*) using multiple biomarkers: the prophylactic role of *Spirulina*

This study examines the impacts of copper sulfate and copper oxide nanoparticles on Nile tilapia, assessing multiple biomarkers to gauge physiological responses. Additionally, the potential prophylactic effects of Spirulina supplementation are explored. Preliminary findings suggest varying degrees of stress and biochemical alterations in fish exposed to copper nanoparticles. However, Spirulina appears to mitigate some adverse effects, highlighting its potential as a protective agent. These findings shed light on the intricate interactions between nanoparticles, aquatic organisms, and potential ameliorative strategies in aquaculture.

CHAPTER-3

AIM AND OBJECTIVE

- **AIM**

To investigate the combined toxicity of copper oxide nanoparticles and polystyrene nanoplastics on *Oreochromis niloticus*, with a focus on understanding the oxidative stress-induced multivariate biomarkers activity.

- **OBJECTIVES**

1. Assess the individual chronic toxicity of copper oxide nanoparticles and polystyrene nanoplastics on *Oreochromis niloticus*.
2. Investigate the influence of copper oxide nanoparticles on bioaccumulation patterns of polystyrene nanoplastics in different tissues of *Oreochromis niloticus* using quantitative assay and qualitative fluorescent tracking, especially on gill.
3. Evaluate the impact of combined exposure on the oxidative stress levels in the tissues of *Oreochromis niloticus*, including the assessment of antioxidant enzyme activities.
4. Analyse the histopathological changes in the gills, liver, and other relevant organs of *Oreochromis niloticus* exposed to the combination of copper oxide nanoparticles and polystyrene nanoplastics.
5. Assess the genotoxic effects of combined exposure on *Oreochromis niloticus* by examining DNA damage.

CHAPTER-4

Material and methods

Chronic Exposure Study of PS NPs and CuO NPs

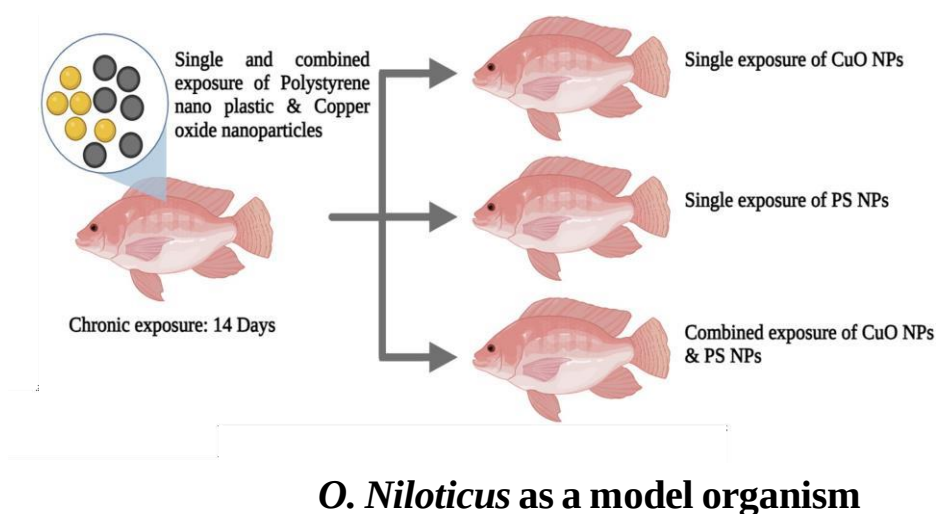


Fig4.1 Symmetric representation of the experimental design

1.1. Nano plastics and CuO

As a model for nano-plastics, the current study used pristine yellow-green fluorescent polystyrene polymers (PS NP; Sigma Aldrich, USA, Product No. L5155), with $\lambda_{ex} = 470$ nm and $\lambda_{em} = 505$ nm. One of the most widely produced materials in the world, polystyrene plastics are used in a wide range of items, such as food storage containers, wrapping, single-use cutlery, and commercial packaging (Muthukumar et al., 2024). Furthermore, because of their low rate of reuse, particles of this polymer are often found in aquatic environments (Kik et al., 2020). On the other hand, the current study used CuO-NPs purchased from Sigma-Aldrich. The stock solution of CuO-NPs was prepared according to the method of (Soliman, 2015).

1.2. Collection and acclimatization of Nile Tilapia

O. niloticus is one of the economically important edible fish across the globe. The animals were obtained from the sathyabama field facility and housed in a controlled laboratory environment with a temperature of 27.0 ± 1.5 °C, pH of 8.22 ± 0.05 , salinity of 33 ± 0.2 ppt, and dissolved oxygen (DO) concentrations ranging from 5.6 to 5.9 mg/L throughout a 12-hour light/dark cycle. This acclimatization phase lasted 10 days before the start of tests. The tanks were fitted with hatchery blowers to ensure aeration. Animals fed on commercial fish feed in the form of small pellets (guarantee levels: 45 % crude protein, 14 % ether extract, 5% crude fiber, 14 % mineral matter, and 87 % dry matter), twice a day (2% body weight). This species was selected as the experimental model due to its good adaptability to laboratory environments, resistance to both diseases and climatic variations, as well as because it had been previously used in different studies about environmental toxicology.

1.3. Experimental design and exposure

After the acclimatization phase, the animals were randomly assigned to the following experimental groups (n = 15 each): (i) the control group (C), consisting of animals devoid of PS NPs and CuO exposure; and several exposure groups, in which animals were exposed to varied amounts of pollutants, namely: (ii) PS NP-I group (10 µg/ l); (iii) PS NP-II group (50 µg/l); (iv) PS NP-III group (100 µg/l); (v) CuO -I group (0.25 mg/l); (vi) CuO -II group (5 mg/l); (vii) CuO -III group (10 mg/l); Mix-I: PS NP-I group (10 µg/ l)+ CuO -I group (0.25 mg/l); Mix-II: PS NP-II group (50 µg/ L)+ CuO -II group (5 mg/l) and Mix-III: PS NP-III group (100 µg/ L)+ CuO-III group (10 mg/l). Despite the lack of data about the environment of PS-NPs in freshwater ecosystems, these concentrations are close to the ones eventually found in the

surface water. This brings our experimental design closer to a more realistic condition. On the other hand, the concentration defined in the Mix III groups corresponded to a value of higher concentration comparatively. Animals were exposed to PS NPs and CuO for 14 days, either separately or in combination, and then subjected to further analyses. Water was renewed every two days, and PS NPs were delivered at the prescribed concentrations into the semi-static exposure system.

1.4. Quality control quality assessment

To reduce plastic contamination, aeration was aided with glass pipettes, and glass tanks were sealed to prevent airborne plastic contamination. To avoid further plastic contamination, no gloves or plastic materials/equipment were used during tissue dissection.

1.5. Sample collection

Following 14 days of exposure, three fishes were selected at random from both the control and treated tanks for biomarker analysis. The animal was anaesthetized in cold water, immersed in 75 μ l/liter of clove oil in a bucket for 3-5 minutes, and wiped with 70% ethanol, and dissection was done using sterile scissors, scalpel, forceps, and blades. Dissection was performed under a laminar bio hood which was sterilized by UV radiation for 10 mins and before dissection, the hood was also wiped with 70% ethanol. The gill, muscle and heart tissues were taken aseptically using sterile forceps and were thoroughly washed with sterile potassium phosphate buffer (50 mM; pH 7.4), was stored in NBF buffer. After 24hrs the tissue stored in the buffer was swapped to 70% chilled ethanol and kept at -80°C until further use. Blood was taken from the caudal vein with a sterile hypodermic syringe. The collected blood

was then stored in cryovials with a pinch of EDTA in it. The blood collected was used further in the comet assay.

1.6. Tissue extract preparation

The preserved tissues (gill, muscle and heart) were homogenized by crushing them with liquid nitrogen in a pre-chilled mortar and pestle. Homogenization was done using potassium phosphate buffer (50 mM, pH 7.4). Next, the homogenized mixture was centrifuged at 20,000×g for 15 minutes at 4°C. Total protein extraction from the tissues was accomplished, and the clear supernatant was separated into aliquots for analysis of several biomarkers.

1.7. Oxidative stress assessment

1.7.1. Assessment of intracellular Reactive oxygen species (ROS)

To assess the ROS production in exposed *O. niloticus*, a spectrophotometric-based fluorogenic 2,7-dichlorofluorescein diacetate (DCFH-DA) assay was performed using the previously described method with minor modification (T. Zhang et al., 2021). The principle of DCFH-DA is based upon the generation of a non-fluorescent 2,7-dichlorofluorescein during cellular esterase hydrolyses DCFH-DA (Gomes et al., 2005). The levels of reactive oxygen species (ROS) in Nile tilapia exposed to NPs and CuO (both alone and in combination) were measured in the gill, muscle, and heart tissues. Protein isolated from each tissue homogenate was used to calculate ROS levels. Equal volumes of tissue homogenate and DCFDA (0.01 mM) were mixed and incubated for 30 minutes in the dark. Thereafter, 200 µl of control and treated aliquot were taken into a black 96-microwell plate in dark conditions. Finally, changes in fluorescent intensity were measured by using a multimode reader

(BioTek, Synergy H1, USA) at an excitation of 490 nm and emission of 523 nm. The subsequent results were expressed as fluorescence units (abbreviated as AU).

1.7.2. Assessment of Thiobarbituric acid reactive substances (TBARS)

The thiobarbituric acid reactive substances (TBARS) assay is a widely used method for assessing lipid peroxidation, which indicates oxidative damage to lipids in biological materials. Lipid peroxidation occurs when reactive oxygen species (ROS) interact with polyunsaturated fatty acids in cell membranes, forming lipid hydroperoxides and producing several secondary products such as malondialdehyde (MDA) and other aldehydes. TBARS test was performed using the previously described method with minor modifications (Pothiwong et al., 2007). To assess, 100 μ L of tissue supernatants were mixed with 50 μ L of trichloroacetic acid (28% w/v in 0.25 N HCl), 50 μ L of thiobarbituric acid (1% in 0.25 N acetic acid), and 25 μ L of butylhydroxytoluene (5 mM). The mixture was then incubated for 15 minutes in a water bath set at 95°C. After incubation, the samples were centrifuged at 13,000 rpm for 10 minutes at room temperature. Thereafter, 200 μ L of supernatant from the control and treated aliquot were taken into a 96-microwell plate. Finally, absorbance was measured by using a multimode reader (BioTek, Synergy H1, USA) at 532 nm. MDA was used as the standard for the calibration curve and results were expressed in “nmol/ml”.

1.7.3. Assessment of Lactate Dehydrogenase (LDH) activity

The lactate dehydrogenase (LDH) assay is a widely used biochemical test that assesses the activity of the LDH enzyme. LDH is an enzyme that catalyzes the conversion of lactate and pyruvate during anaerobic and aerobic respiration. The

LDH assay typically includes converting lactate to pyruvate in the presence of nicotinamide adenine dinucleotide (NAD⁺) to produce reduced nicotinamide adenine dinucleotide (NADH). This indicates signs of cellular damage or injury when the cell membrane is disrupted or damaged. To assess the cellular membrane damage in exposed fishes, a spectrophotometric-based LDH assay was performed using the previously described method with minor modifications (Wróblewski and Ladue, 1955). Lactate dehydrogenase (LDH; L-lactate NAD⁺ oxidoreductase; EC.1.1.1.27) activity was determined using a reaction mixture containing 0.1M phosphate buffer (pH 7.5) and a 0.2 mM NADH solution in 0.1M phosphate buffer. The enzymatic reaction was commenced by adding the substrate, 0.2 mM sodium pyruvate, and the absorbance of the reaction mixture was measured at 340 nm by using a multimode reader (BioTek, Synergy H1, USA). This assay quantifies LDH activity by detecting the conversion of NADH to NAD⁺ as lactate is converted to pyruvate, providing insights into cellular metabolic processes and possible tissue damage.

1.8. Antioxidant biomarkers response

1.8.1. Estimation of Superoxide dismutase enzymatic (SOD) Activity

Superoxide dismutase (SOD) is a key antioxidant enzyme that aids in cellular defense against oxidative stress. SOD's enzymatic function entails catalyzing the dismutation of superoxide radical (O₂⁻) into molecular oxygen (O₂) and hydrogen peroxide (H₂O₂) (H. Zhao et al., 2021). To assess the SOD activity in exposed fishes, a spectrophotometric-based riboflavin-nitro blue tetrazolium (NBT) assay was performed using the previously described method with minor modifications (Giannopolitis and Ries, 1977). Briefly, the enzymatic activity of superoxide dismutase (SOD) was estimated in a 1.5 mL reaction mixture containing 50 mM potassium phosphate buffer at pH 7.6 (750 µl), 12 mM methionine (390 µl), 75 µM

nitroblue tetrazolium (NBT) (225 μ l), 2 μ M riboflavin (60 μ l), protein extract (50 μ l), and distilled water (25 μ l). The reaction mixture was incubated under white light for 30 minutes. This assay evaluates SOD activity by assessing the inhibition of NBT reduction, providing information on the protein extract's antioxidant ability and possible role in treating oxidative stress. Thereafter, 200 μ l of aliquot from the control and treated aliquot were taken into a 96-microwell plate. Finally, absorbance was measured by using a multimode reader (BioTek, Synergy H1, USA) at 560 nm. The results were expressed as a U/mg of protein.

1.8.2. Assessment of Catalase enzymatic (CAT) activity

Catalase is an essential antioxidant enzyme found in almost all organisms. It serves an important function in cellular defence against oxidative stress. To evaluate the catalase activity in exposed fishes, a spectrophotometric-based assay was performed using the previously described method with minor modifications (Aebi, 1974). Briefly, the reaction mixture (Volume 1.5 mL) contained 30 mM H_2O_2 (0.5 mL), 50 mM sodium phosphate buffer at pH 7 (0.5 mL), and a 1:10 dilution of protein extract from different experimental tissues. The drop in H_2O_2 was monitored for one minute, with measurements recorded at 10-second intervals with a multimode reader (BioTek, Synergy H1, USA) at 240 nm. The results were represented as Units/mg of protein, which provided insight into the enzyme's catalytic effectiveness in degrading hydrogen peroxide.

1.8.3. Assessment of Glutathione activity (GSH)

The glutathione content in the exposed mussels was measured using the previously described method with minor modifications (Watson, 1964). In a highly alkaline environment, glutathione (GSH) in the tissue combines with a combination of 0.067 M sodium nitroprusside and 1.5 M sodium cyanide, forming a red complex.

Thereafter, aliquots from the control and treated samples were taken into a 96-microwell plate. Finally, absorbance was measured by using a multimode reader (BioTek, Synergy H1, USA) at 520 nm. The results were expressed as a U/mg of protein.

1.8.4. Estimation of Glutathione S-transferases (GSTs) enzymatic activity

Glutathione S-transferases (GSTs) is a class of enzymes that play an important role in cellular detoxification by catalyzing the conjugation of glutathione (GSH). To evaluate the GST activity in exposed mussels, a spectrophotometric-based assay was performed using the previously described method with minor modifications (Griffith, 1980). Briefly, the reaction buffer was made consisting of 100 mM potassium phosphate buffer at pH 7.4, 1 mM reduced glutathione (GSH), 1 mM 1-chloro-2,4-dinitrobenzene (CDNB), and the supernatant in a total volume of 1 ml. Glutathione S-transferase (GST) activity was measured in units per milligram of tissue (U/mg. protein). Meanwhile, glutathione (GSH) levels were determined by measuring the absorbance change at 412 nm with a microplate reader (BioTek, Synergy H1, USA) and represented as units per milligram of protein using a standard curve as a reference.

1.9. Integrated biomarker response modelling analysis

The Integrated Biomarker Response Index (IBR) is a useful tool for understanding the biological effects of pollutants and simplifying the link between a battery of biomarkers and contamination levels (Damien et al., 2007). Essentially, the IBR combines data from various biomarkers into a single numerical index, allowing for a thorough assessment of the overall biological response to environmental stresses (Catteau et al., 2023). The IBR gives a comprehensive view of organismal health by integrating multiple biomarkers representing diverse biological pathways or

physiological processes, allowing it to efficiently explain the magnitude of pollutant-induced changes (Capolupo et al., 2016). This unified index simplifies the interpretation of complicated datasets, allowing researchers to more easily identify trends and patterns, thereby improving our understanding of pollutant exposure consequences on species and ecosystems (Sanchez et al., 2013). The Integrated Biomarker Response (IBR) allows the synthesis of many biomarker responses into a single numeric value. The concept of reference deviation, which compares a disturbed and an undisturbed state, is central to it. The IBR index was specifically intended to reduce reliance on biomarker arrangement and the possibility of biomarkers being produced or suppressed (Devin et al., 2014). The IBR index standardizes the assessment by including the concept of reference deviation, allowing for more objective comparisons across research and experimental setups. This approach improves the IBR index's robustness and dependability, ensuring its usefulness in accurately assessing the overall biological response to environmental stressors while avoiding confounding effects (Dagnino et al., 2007). In summary, the combined data of each biomarker (X_i) were compared to the data (X_0) of each biomarker from the control group and subjected to logarithmic transformation (Y_i) to reduce variance [$Y_i = \log (X_i/X_0)$]. Next, Y_i 's mean (μ) and standard deviation (σ) were calculated, and the data for each parameter was standardized using the equation:

$$Z_i = \left(\frac{Y_i - \mu}{\sigma} \right)$$

To construct a baseline cantered on controls and demonstrate parameter changes relative to this baseline, we used the mean of the standardized biomarker response (Z_i) and the mean of the unexposed biomarker (Z_0) to establish a biomarker deviation index (A):

$$A = Z_i - Z_0$$

Finally, to calculate the IBR index, the absolute value of A was obtained for each parameter under every experimental scenario, and these values were added:

$$IBRV2 = \sum |A_i|$$

The standardized dataset was used to determine the minimal value (referred to as "mini") for each biomarker. The score for each biomarker response (referred to as "Bi") was then computed as follows:

$$B_i = Y_i + |min_i|$$

Where, $B_i \geq 0$ and $|min_i|$ represents the absolute value. Ultimately, the computed scores from each biomarker were incorporated into the EIBR model;

$$EIBR = \frac{\sum_{i=1}^n B_i \times W_i}{\sum_{i=1}^n W_i}$$

The loading of each biomarker based on their level of biological organization, specifically physiological endpoints, is represented by W_i .

$$IBR = \frac{\sum_{i=1}^K S_i \times S_{i+1} \times \sin\left(\frac{2\pi}{K}\right)}{2}$$

1.10. Multivariate statistical analysis

1.10.1. Correlation network analysis

A Pearson correlation matrix is a tabular representation of the pairwise correlations between variables in a dataset, calculated using Pearson correlation coefficients. Each column in the matrix has a numerical value representing the strength and direction of the linear relationship between two variables (Mu et al., 2018). With values ranging from -1 to 1, where -1 represents a perfect negative correlation, 1 represents a perfect positive correlation, and 0 shows no correlation, the matrix

offers information on how closely variables are related (Benesty et al., 2009).

Typically, the matrix is symmetric along the diagonal, with each variable correlating with one itself. It indicates helps discover trends, dependencies, and potential multicollinearity issues in toxicological analysis.

1.10.2. Principal Component Analysis

Principal Component Analysis (PCA) is a linear mathematical technique that converts a set of correlated variables into a new set of uncorrelated variables known as principal components (PCs) (Jolliffe and Cadima, 2016). This adjustment is carried out so that the first few principal components capture the majority of the data's variance. Furthermore, PCA makes it easier to identify the original variables that contribute the most to the variance in the data using a technique known as contribution plot analysis. By concentrating on these essential components, researchers can reanalyse the data to see if certain combinations of critical variables account for variations between test pollutants (NPs and CuO) (Groth et al., 2013). For example, if NPs and CuO with higher toxicity consistently exhibit a discrete combination of physicochemical properties, they would appear as a separate cluster in the PCA plot, far apart from those with lesser toxicity.

1.11. Qualitative assessment and fluorescent tracking of nano-plastic accumulation

The qualitative assessment and tracking of fluorescent nano-plastic accumulation in the gills of *O. niloticus* involves seeing and assessing the distribution and intensity of fluorescent signals emitted by the tagged nano-plastics using a fluorescence microscope. Following a 14-day exposure period, fishes were randomly sampled from each exposure concentration, including single exposures to nano-plastic and combined exposures to nano-plastic and Copper oxide. These fishes were subjected

to examination for nano-plastic accumulation at each concentration. In brief, gill samples were obtained from the fish and fixed in 4% paraformaldehyde at room temperature. Subsequently, all tissue samples were individually scrutinized and imaged using an Epifluorescence microscope (Carl Zeiss Axioscope 5, Germany) at a magnification of x40. The images obtained were processed utilizing the Zen 3.1 Lite built-in software (Carl Zeiss Axioscope 5, Germany) photomicrography system, employing an excitation wavelength of 470 nm. The distribution of nano-plastics within the gill tissues was discerned by tracking the fluorescently labelled polystyrene nanospheres. This approach provided insights into the accumulation and distribution patterns of nano-plastics within the gill tissues of the fishes across different exposure concentrations. Multiple samples from distinct exposure groups were examined and compared to follow the build-up of fluorescent nano-plastics over time or under different conditions. To enhance the qualitative visualization of nano-plastic accumulation, tissue areas covered by fluorescent signals were compared between exposure groups.

1.12. DNA damage assessment using single-cell gel electrophoresis (COMET)

The principle of DNA damage assessment using single-cell gel electrophoresis, often known as the comet assay, is based on the detection and quantification of DNA fragmentation in individual cells. To evaluate the genotoxicity and DNA damage in exposed fish, an alkaline single-cell gel electrophoresis assay was performed using the previously described method with minor modifications (Almeida et al., 2011; Singh et al., 1988). Briefly, Blood isolated from harvested samples was mixed with a coating of 0.65% low melting agarose (LMA) applied to pre-coated microscope slides. To ensure the sample's integrity, an extra layer of 0.65% LMA was applied.

The slides were then placed in a freshly made lysis buffer containing 2.5M NaCl, 100 mM Na₂ EDTA, 10 mM Tris HCl (pH 10), 1% Triton X-100, and 10% DMSO, and incubated for 15 minutes in darkness at 4°C. After that, the slides were washed with cooled distilled water. The slides were treated for 30 minutes in an electrophoresis apparatus containing electrophoresis buffer (300 mM NaOH, 1 mM Na₂ EDTA, pH > 13) before being electrophoresed for 30 minutes at 25 V/300 mA. Neutralization was then performed for 15 minutes with freshly made cold neutralizing buffer containing 0.4 M Tris HCl (pH 7.4). Following dehydration with 70% ethanol, the slides were stored in a moisture-free chamber. The slides were stained by incubating them with 80 µL of 20 µg/mL ethidium bromide for 10 minutes in the dark, then examined at x40 magnification using an epifluorescence microscope (Carl Zeiss Axioscope 5, Germany). The images obtained were processed utilizing the Zen 3.1 Lite built-in software (Carl Zeiss Axioscope 5, Germany) photomicrography system. DNA damage was measured as percentage tail DNA (%tail DNA), which represents the percentage of DNA that moved from the head to the tail in the comet-like form.

1.13. Histomorphology assessment

The control and exposed animal groups were chosen for dissection, which took place under a sterilized laminar bio hood that had been sanitized with ultraviolet light for 10 minutes. Before dissection, the hood was thoroughly cleaned with 70% ethanol. Fishes were dissected following the toxicity experiment, with their gills, muscle, and heart tissues immediately transferred to fixative solutions after being finely sectioned.

1.13.1. Methods of fixation, embedding, and decalcification

Fixation in 10% buffered formalin is typically followed by decalcification with either a 10% ethylenediaminetetraacetic acid (EDTA) solution in 0.1 M phosphate buffer (pH

7-8) for approximately 8-10 weeks, with weekly solution changes, or a 5% formic acid solution diluted in water for approximately 1 week. After decalcification, the tissues are embedded in paraffin. To speed up the decalcification process, particularly for bigger specimens, cut sequential portions about 5 mm apart. Midway through the decalcification process, joints in smaller specimens can be divided coronally with a sharp razor blade to speed decalcification, and the joint halves are returned to the decalcification solution. Although EDTA decalcification is preferable for maintaining proteoglycans and is highly recommended for in situ hybridization sections, formic acid decalcification is quicker. Decalcification is deemed complete when an aliquot of the decalcification solution no longer produces a calcium precipitate when ammonium oxalate is added. In summary, a 0.5 ml aliquot of the EDTA decalcification solution is mixed with 10 ml of citrate-phosphate buffer (0.2 M citric acid and 0.16 M dibasic potassium phosphate, pH adjusted to 3.2-3.6 using NaOH), then 2.5 ml of saturated ammonium oxalate (5% solution) is added and vortexed. The absence of a cloudy white precipitate over two consecutive days of testing, each lasting 20 minutes, indicates that decalcification is complete. Once at this stage, the tissue is thoroughly rinsed in buffer, with numerous changes of buffer solution. The entire joint is then immersed in paraffin and sectioned in either a coronal or sagittal position. For bigger joints, both sides are embedded in paraffin to aid in the creation of centre portions.

1.13.2. Methods for sectioning, staining, and microscopic observation

The species under study and the specific research goals influence the choice of sectioning orientation, known as the plane of sectioning, particularly in terms of which joint compartment is being examined. Maintaining constant slice thickness is critical for proper quantitative analyses, such as determining proteoglycan

concentration. Furthermore, having an adequate number of sections is critical to ensuring a complete investigation of all potentially affected areas. Tissue blocks were prepared with ethanol washing, dehydration, and paraffin embedding before being sectioned onto 4 μm thick slides. The sections were then fixed and stained with the standard hematoxylin and eosin (H&E) staining technique. The obtained slides were then examined under a Leica microscope to detect changes in cell morphology and tissue structure.

1.13.3. Data Curation and One way ANOVA analysis

The initial processing of experimental results involved employing MS Excel 2019 to compute averages, standard deviations, and standard errors. For data visualization and plotting purposes, Prism 8 and R version 4.3.0 and RStudio software were utilized. Additionally, One-way analysis of variance (ANOVA) was consistently employed for certain assays. Statistical significance was determined using Tukey's multiple comparisons test. For the entire statistical investigation, P value ($P < 0.05$) is considered statistically significant.

CHAPTER-5

RESULTS AND DISCUSSION

5.1 Oxidative stress assessment

5.1.1 ROS

In this work, PS-NPs (100µg/L) and CuO(10mg/L) significantly enhanced the production of ROS time- and dose-dependently (14 days) compared to the control group (Fig5.1 and Fig5.2). Correlated to other groups the combination of both PSNP+CuO has shown greater oxidative stress in each tissue (Fig5.3). Similarly, the exposure of the CuO groups shows lesser oxidative stress compared to other exposures (Fig5.1). Antioxidant biomarkers are widely used to track environmental stressor effects in aquatic organisms (Hook et al., 2014). Toxic substances cause reactive oxygen species (ROS) in fish, which enhances many defence systems to neutralise ROS effects (Sharbidre et al., 2011). In general, three distinct activities in the cell produce ROS when subjected to stress or operating normally. The mitochondrial respiratory chain produces ROS, whereas NADPH oxidases (NOX) release them. The latter is the creation of ROS from several enzymatic processes (Morgan et al., 2008). Microplastics cause ROS generation by mitochondrial injury or by boosting immune system responses while neutralising toxic foreign particles (Yang et al., 2020) Elevated ROS levels can cause lipid peroxidation, mitochondrial and lysosomal disruption, inflammation, neural malfunction, and the activation of antioxidant defences. All of them are major molecular and subcellular processes or targets associated with NP exposure, and they are expected to have repercussions at higher levels of biological organisation. In addition to the indirect effects of NPs on lysosomes and mitochondria via intracellular ROS, studies utilising fish and human models have revealed that NPs

(PS-NPs, 20 to 200 nm) can interact with or accumulate in these organelles (Brandts et al., 2020) ;(Johnston et al., 2010). When the marine copepod *Paracyclops nana* was exposed to PS (0.05, 0.5, and 6 μm , 20 $\mu\text{g mL}^{-1}$), it produced more ROS said (Jeong et al., 2017a). These above findings suggest that an increase in ROS levels causes damage to cell membranes, DNA etc and causes stress.

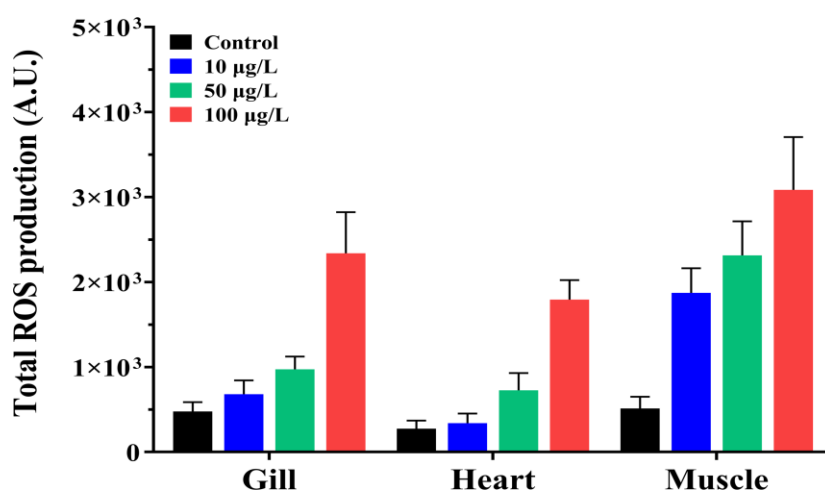


Fig5.1 Total ROS production assessment of different tissues (Gill, Heart, and Muscle) from *Oreochromis niloticus* after 14 days of chronic single exposure to PS-NPs

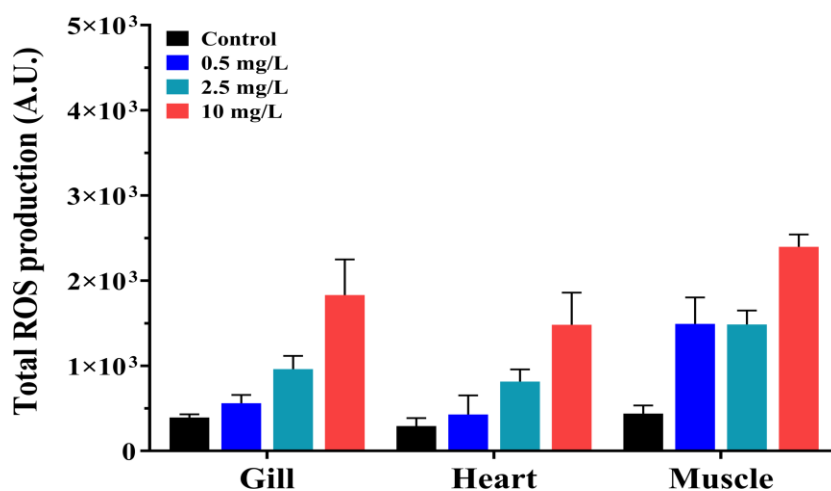


Fig5.2 Total ROS production assessment of different tissues (Gill, Heart, and Muscle) from *Oreochromis niloticus* after 14 days of chronic single exposure to CuO

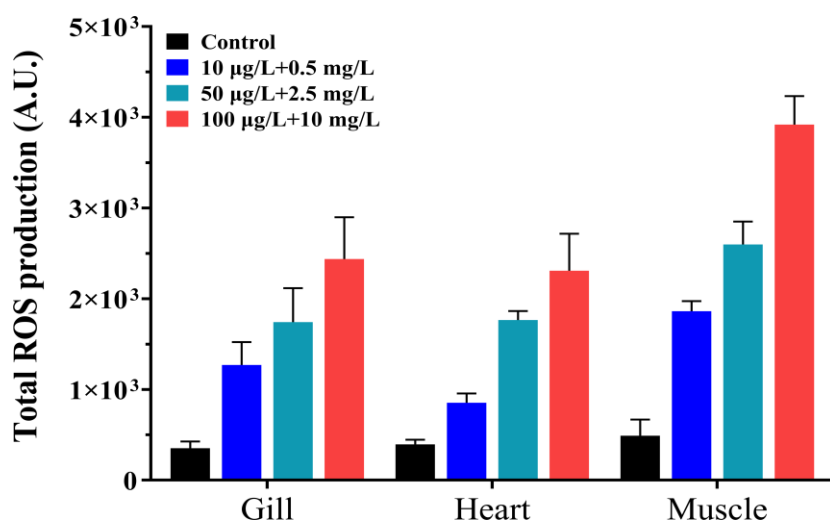


Fig5.3 Total ROS production assessment of different tissues (Gill, Heart, and Muscle) from *Oreochromis niloticus* after 14 days of chronic combined exposure to PS-NPs and CuO

5.1.2 Lipid peroxidation

Lipid peroxidation showed a significant increase ($P < 0.05$) after exposure to 0.5, 2.5, and 10 µg/L (Fig5.4) of nanoplastic for 14 days compared to the control group. Similarly, there has been a spike in the exposure of CuO nanoparticles with concentrations of 10, 50, and 100 mg/L (Fig5.5). In addition, the MDA level of the high combination of PSNP and CuO (100 µg/L+10 mg/L) group was significantly higher than that of the control group ($P < 0.05$) (Fig5.6). The MDA contents in muscle exposed to 10 mg/L NPs and 100 µg/L CuO were significantly increased at 14 days but thereafter, the MDA content in the NP and CuO treatment seemed to be followed by a decrease in other tissues. At the end of the exposure, the heart MDA content in every treatment was lower than the gill and muscle in every concentration. Elevated MDA levels, a by-product of lipid peroxidation, indicate oxidative damage (Ding et al.,

2018a). Previous studies demonstrated that exposure to MPs triggers ROS overproduction and oxidative stress, and this is consistent with the current findings (Eom et al., 2020) ;(Lee et al., 2021a). High concentrations of PSNPs resulted in lipid peroxidation and oxidative damage in Nile tilapia, indicating that inducing the antioxidant system was insufficient to prevent oxidative damage after 14 days of exposure to PSNPs, CuO, along a combination of the two. In previous studies, Exposure to PEMP elevated lipid peroxidation levels in young oceanic fish (*Pomatoschistus microps*) (Ferreira et al., 2016). Additionally, juvenile *D. labrax* treated with microplastics had greater LPO levels in their brains (Barboza et al., 2018c). (Lee et al., 2021b) found that treating the marine mysid (*Neomysis awatschensis*) with PS (5 x 10⁵ particles mL⁻¹, 1 and 10 µm) for 96 hours led to an increase in MDA levels. In this study, Polyacrylamide had a sub-lethal effect on the oxidative stress biomarker MDA in the gills of *O. niloticus* after 28 days of exposure. The results demonstrated a considerable rise in the activities of malondialdehyde (MDA) and lipid peroxide (LPO) in the gills of *O. niloticus*, whereas the activity of catalase was lowered in the high-dosage groups (Raza et al., 2023b). These above findings suggest that the fishes are exposed to pollutants and undergo oxidative stress.

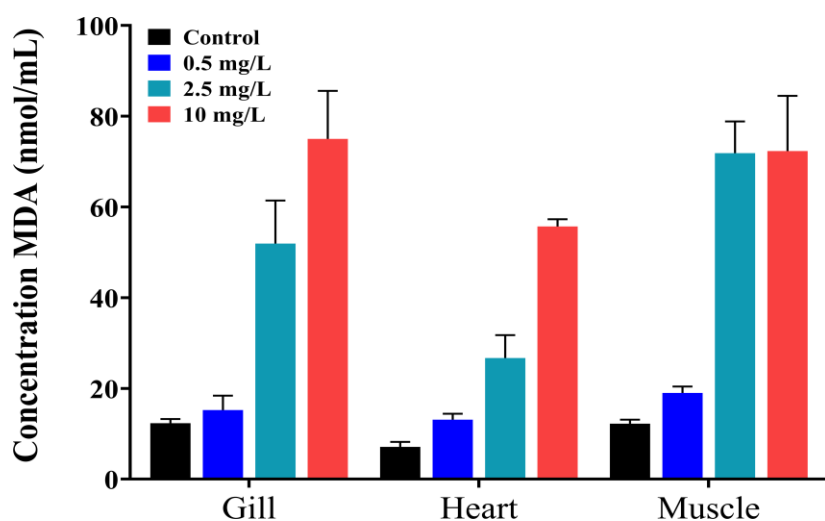


Fig5.4 Total Lipid peroxidation (LPO) assessment of different tissues (Gill, Heart, and Muscle) from *Oreochromis niloticus* after 14 days of single chronic exposure to PS-NPs

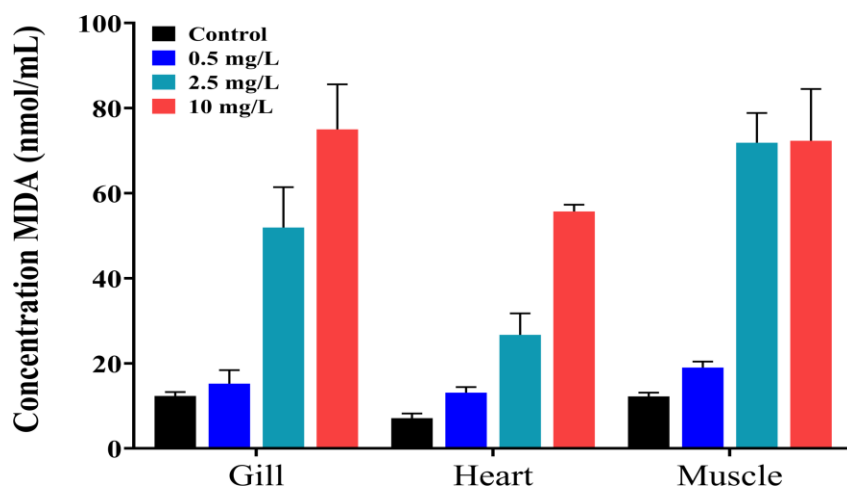


Fig5.5 Total Lipid peroxidation (LPO) assessment of different tissues (Gill, Heart, and Muscle) from *Oreochromis niloticus* after 14 days of single chronic exposure to CuO

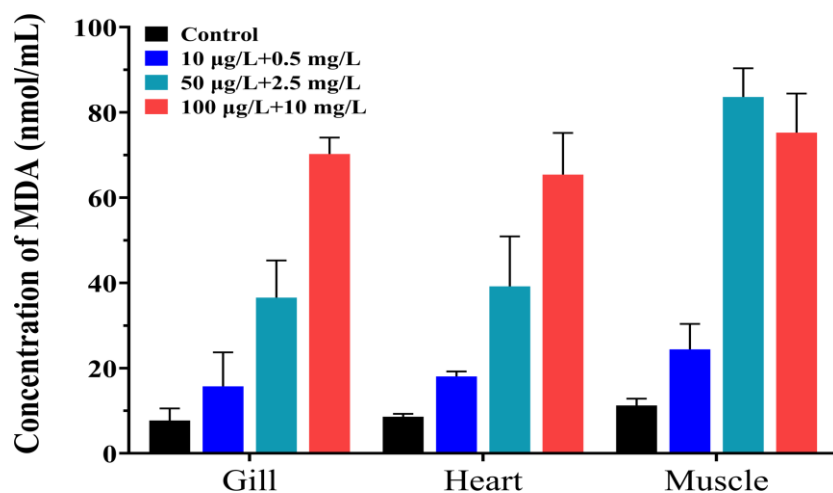


Fig5.6 Total Lipid peroxidation (LPO) assessment of different tissues (Gill, Heart, and Muscle) from *Oreochromis niloticus* after 14 days of combined chronic exposure to PS-NPs and CuO

5.1.3 Lactate dehydrogenase (LDH)

After 14 days of exposure to 0.5, 2.5, and 10 $\mu\text{g/L}$ of nanoplastic, lactate dehydrogenase levels were substantially higher ($P < 0.05$) than in the control group (Fig5.7). Similarly, there has been a spike in the exposure of CuO nanoparticles with concentrations of 10, 50, and 100 mg/L (Fig5.8). Unlike other antioxidant biomarkers, the gill tissue in exposure to a combination of PSNP+CuO has been effectively damaged (Fig5.9). According to the results, we can confirm that the elevated LDH values have caused the production of reactive oxygen species and subsequent cellular damage. LDH converts lactate to pyruvate, NAD^+ to NADH, and the other way around (V. Soleimany et al., 2016). Exposure to environmental pollutants can lead to increased LDH activity (Nunes et al., 2015). LDH is a measure of hypoxia and mitochondrial oxidative activity. Additionally, a rise in extracellular LDH may signal cell death or necrosis (Maes et al., 2015). Nanoplastics raised LDH levels, indicating a shift towards anaerobic energy generation in response to chemical stress. In previous studies, *C. carpio* and *S. aequifasciatus* treated with MPs coupled with Cd revealed increased LDH activity (Banaee et al., 2019) ;(Wen et al., 2018). Significant suppression of IDH was detected in the muscle of *D. labrax* upon short-term exposure to combinations of mercury and MP at a dosage of 0.69 mg/L , while the impact on LDH activity was significant (Barboza et al., 2018a). At the same time, Microplastic did not affect LDH activity in common carp (*Cyprinus carpio*) alone, but when combined with paraquat, activity increased significantly (Nematdoost Haghi and Banaee, 2017). These findings suggest that NPs have negative effects and can exacerbate the toxicity of other damaging environmental chemicals.

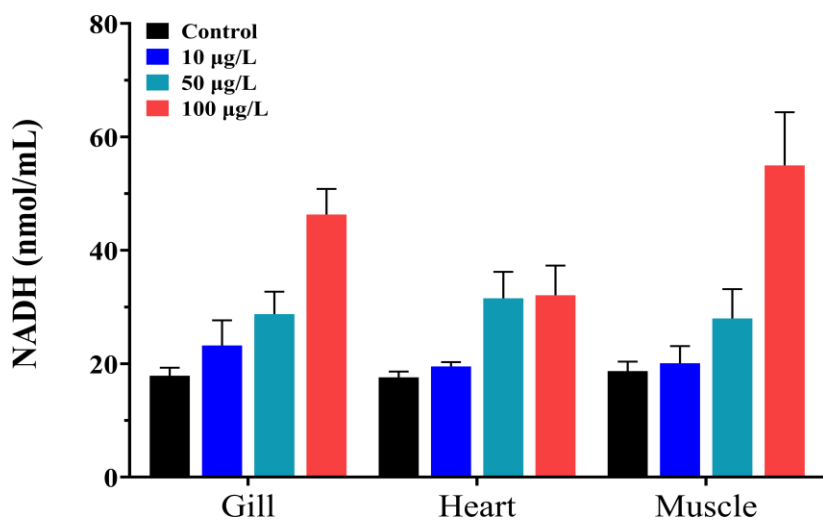


Fig5.7 Total membrane integrity (LDH) assessment of different tissues (Gill, Heart, and Muscle) from *Oreochromis niloticus* after 14 days of single chronic exposure to PS-NPs

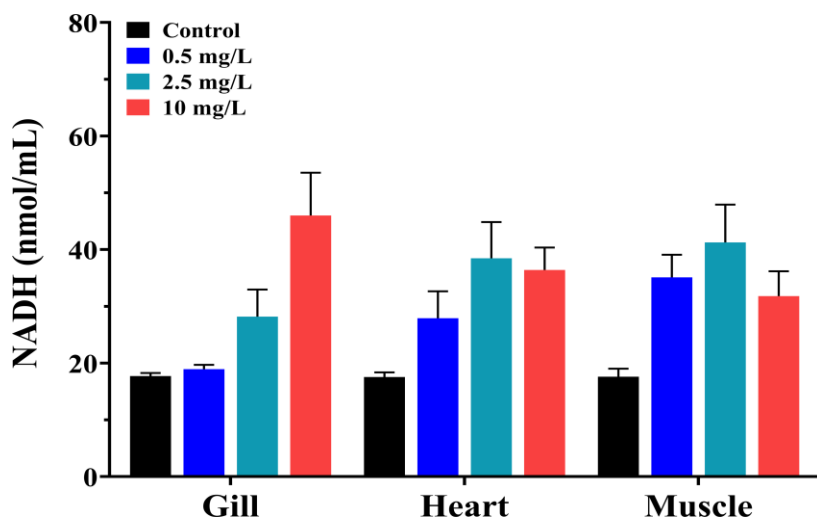


Fig5.8 Total membrane integrity (LDH) assessment of different tissues (Gill, Heart, and Muscle) from *Oreochromis niloticus* after 14 days of single chronic exposure to CuO

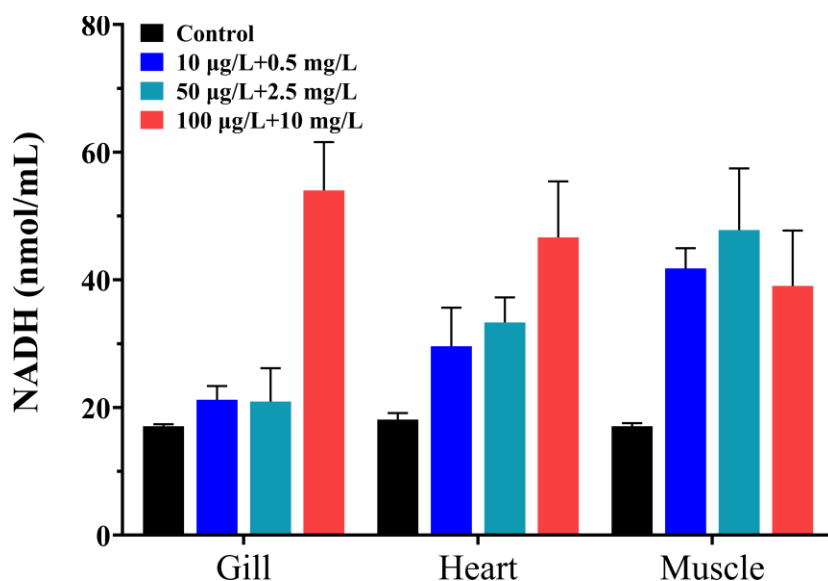


Fig5.9 Total membrane integrity (LDH) assessment of different tissues (Gill, Heart, and Muscle) from *Oreochromis niloticus* after 14 days of combined chronic exposure to PS-NPs and CuO

5.2 Antioxidant biomarkers response

5.2.1 Superoxide dismutase enzymatic (SOD) Activity

SOD levels elevated substantially ($P < 0.05$), following 14 days of exposure to nanoplastic at concentrations of $10\mu\text{g/L}$ (Fig5.10) compared to the control group. Similarly, in the exposure of CuO nanoparticles, CuNP3 has resulted in a drastic peak compared to the other concentrations of copper oxide in muscle tissue (Fig5.10c). However, Gill tissue in MIX 2 has dominated the stress peak compared to all other concentrations (Fig5.10a). SOD is an antioxidant defence mechanism found in a variety of aquatic animals (Alves de Almeida et al., 2007). SOD participates in the disproportionation process that transforms superoxide anions into H_2O_2 . (Bainy et al., 1996) discovered that (SOD) activity increased when Nile Tilapia were exposed to various contaminants. In previous studies, it was found that liver SOD activity increased in *Oreochromis mossambicus* following Cd exposure (Basha and Rani, 2003). Similarly, in this study the findings of this investigation demonstrate that compared to the control, an increase in the SOD enzyme activity was found in the liver of medaka treated with a modest concentration of (10 items/L) NPs, suggesting an active antioxidant defence system (Zhou et al., 2022b). Several additional species, including *Eriocheir sinensis*, *Danio rerio*, and

Macrobrachium nipponense, showed comparable findings (Li et al., 2020);(Lu et al., 2016a);(L. Li et al., 2021). Furthermore, SOD activity increased in red tilapia subjected to 0.1 μm PS-MPs (Ding et al., 2018b). From these above findings and demonstrations, we can conclude that exposure to pollutants such as NPs and metal oxides can increase SOD and weakens the antioxidant defense system.

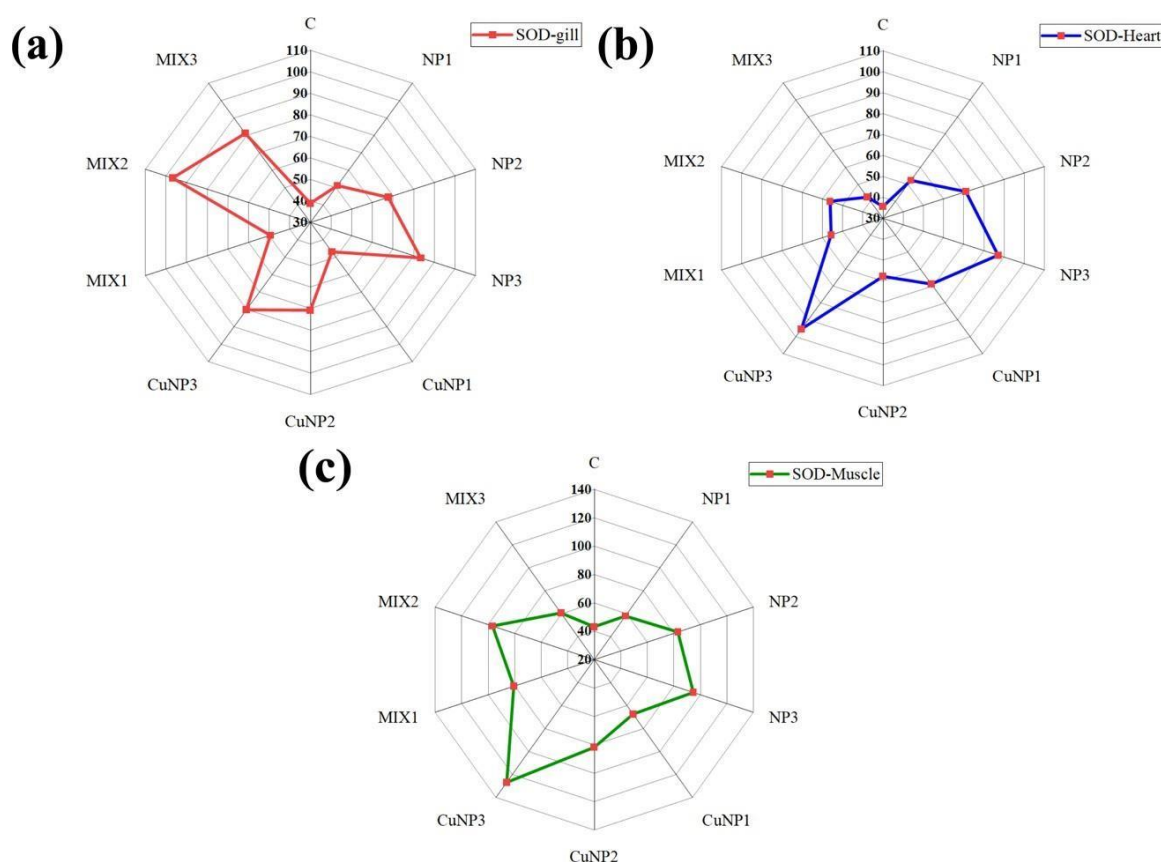


Fig5.10 Antioxidant (SOD) capacity of Nile tilapia after CuO and PS-NPs exposure test for 14 days. (a) SOD-Gill tissue, (b) SOD-Heart tissue, (c) SOD-Muscle.

5.2.2 Catalase enzymatic (CAT) activity

After 14 days of exposure to 0.5, 2.5, and 10 $\mu\text{g/L}$ of nanoplastic, CAT levels elevated ($P < 0.05$) in comparison to the other group (Fig5.11). Although, there has been an upsurge in exposure to CuO nanoparticles at concentrations of 100 mg/L in muscle tissue (Fig5.11c). Yet, MIX 3 in gill has dominated the stress peak compared to all other concentrations (Fig5.11a). CAT are important enzymes in the antioxidant defence system (Dorantes-Aranda et al., 2015). CAT suppresses the generation of harmful hydroxyl radicals by converting H_2O_2 to H_2O and oxygen. In previous studies, exposing zebrafish to 5- μm polystyrene microplastics boosted their catalase

Activity (Lu et al., 2016b). MPs exposure enhanced CAT activity. This can be explained by stimulating the cellular antioxidant system. CAT protects gill cells from oxidative damage by catalysing H₂O₂ and producing H₂O and O₂ after chronic exposure to MPs. Increased CAT activity led to increased H₂O₂ catalysis following exposure to MPs demonstrated by (Li et al., 2023b) *Pseudobagrus fulvidraco* had similar findings following exposure to PE-MPs, indicating that increased CAT activity aids in MP detoxification (Lee et al., 2023). In this research (Zhou et al., 2022c) indicated that, in contrast to the control group, there was a rise in CAT enzyme levels detected in the liver of medaka exposed to a low concentration (10 items/L) of NPs. This suggests an enhanced antioxidant defense mechanism at work. Furthermore, CAT activity was enhanced in the gut of zebrafish exposed to MPs (Qiao et al., 2019b). Similarly, irregular microplastics at a concentration of 50 mg/L promoted catalase (CAT) activity in sheepshead minnows (*Cyprinodon variegatus*). Based on the facts and demonstrations shown above, we may conclude that exposure to pollutants such as NPs and metal oxides may elevate CAT and minimize the antioxidant defence mechanism.

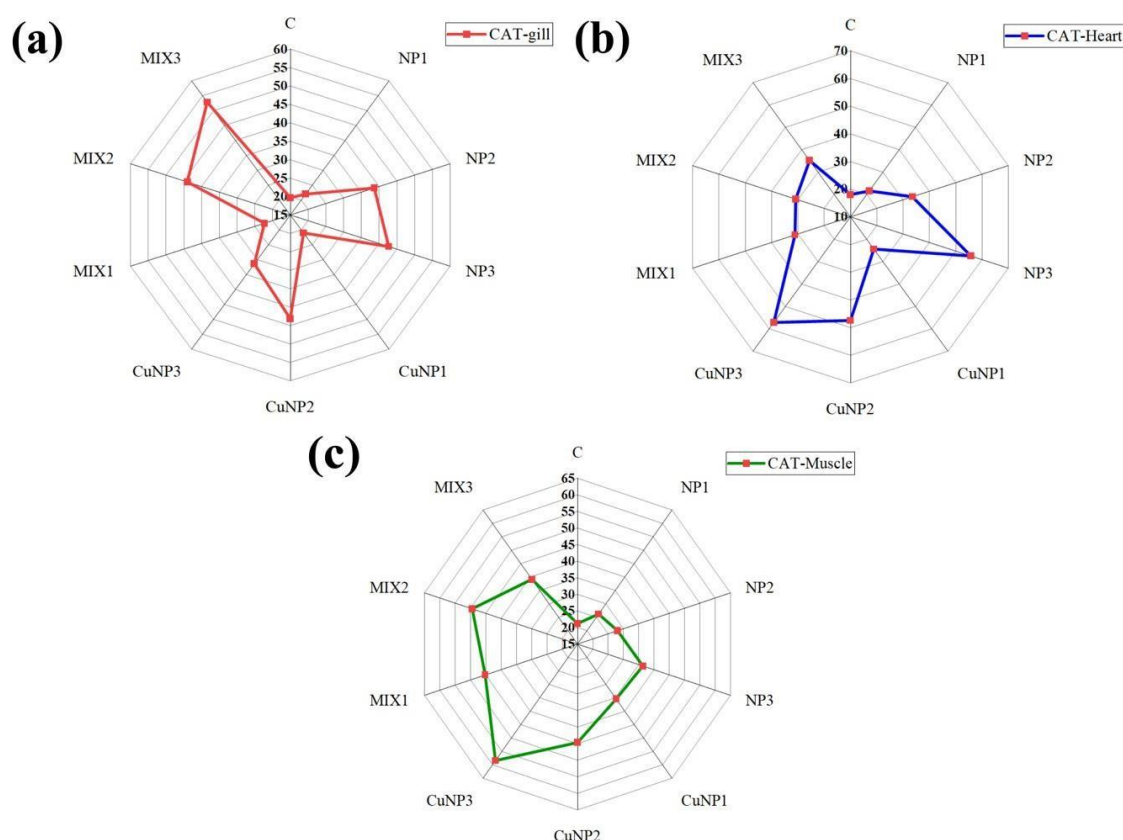


Fig5.11 Antioxidant (CAT) capacity of Nile tilapia after CuO and PS-NPs exposure test for 14 days. (a) CAT-Gill tissue, (b) CAT-Heart tissue, (c) CAT-Muscle.

5.2.3 Glutathione activity (GSH)

After 14 days of exposure to nanoplastic and copper oxide pollutants, muscle and gill MIX2 showed a greater spike than any other concentration in other groups (Fig5.12c and Fig5.12a). Similarly, MIX3 has the highest peak in heart tissue (Fig5.12b). GSH, the most common non-protein thiol in cells, serves as a non-enzymatic antioxidant and plays a crucial role in redox processes, amino acid transport, and contaminant removal (Burgos-Aceves et al., 2018). In previous studies, the GSH level in G3 was substantially greater than in the other groups (El Asely et al., 2020b). This enhanced reaction may be associated with ROS-induced oxidative stress, in which the organism produces GST for detoxification. Exposure to Cu led to a decrease in GSH content in *P. lineatus* due to its ability to inhibit the enzyme glutathione reductase (GR), which reduces oxidised glutathione into reduced glutathione, and use GSH as a substrate for glutathione-S-transferase in the reaction against ROS (Gravato et al., 2006). Similarly, Animals exposed to the combination saw a drop in GSH due to the presence of Cu, but those exposed to the pure MP showed no significant decline (Roda et al., 2020). The marine mysid (*Neomysis awatschensis*) was subjected to treatment with PS (5×10^5 particles mL⁻¹, 1 and 10 μ m) for 96 hours, resulting in an upsurge in antioxidant enzymes GSH activity in organisms exposed to both 7 and 30 days after hatching (Lee et al., 2021c). Based on the facts and demonstrations mentioned above, we can deduce that exposure to contaminants such as NPs and metal oxides, caused activation of the detoxification system could decrease GSH.

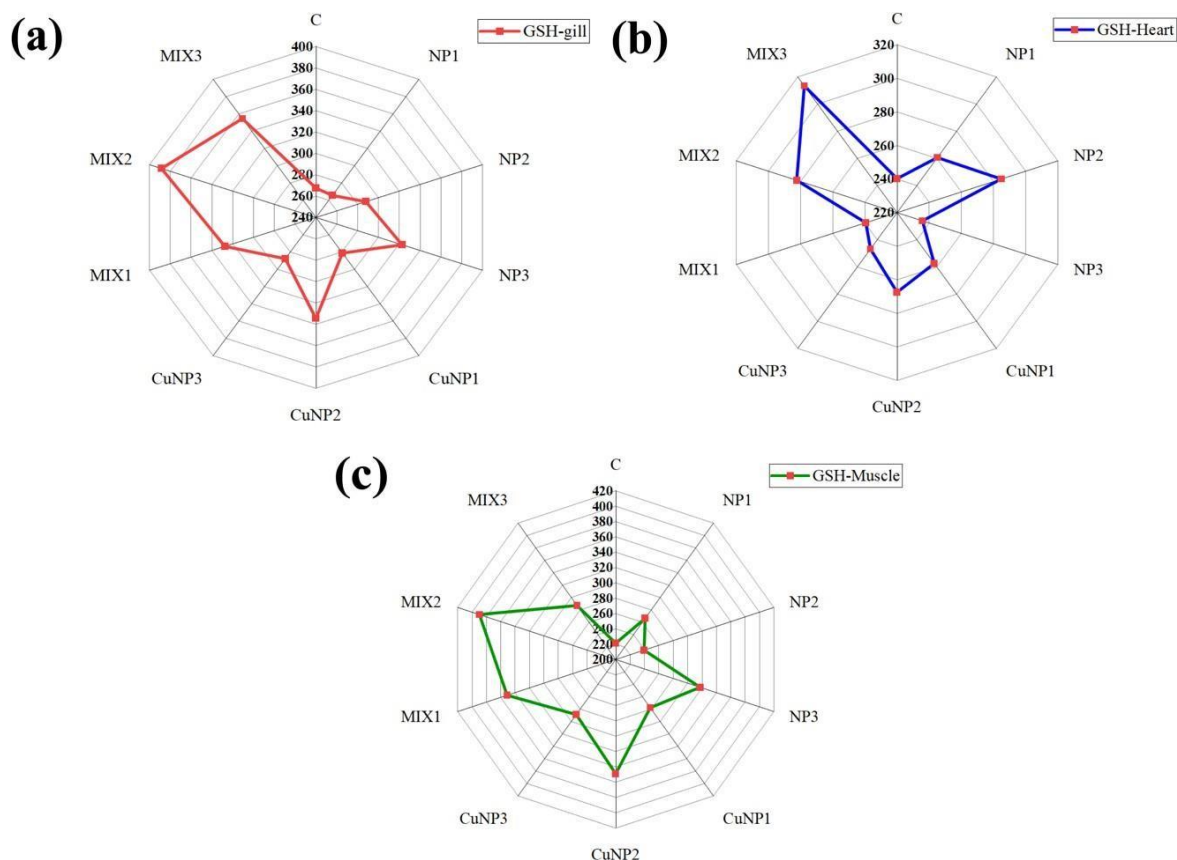


Fig5.12 Antioxidant (GSH) capacity of Nile tilapia after CuO and PS-NPs exposure test for 14 days. (a) GSH-Gill tissue, (b) GSH-Heart tissue, (c) GSH-Muscle.

5.2.4 Glutathione S-transferases (GSTs) enzymatic activity

After 14 days of exposure to nanoplastic and copper oxide pollution, MIX1 concentration exhibited a smaller rise in every tissue. In comparison with control groups other groups have inhibited the activity of the enzyme which indicated the activation of the detoxifying system (Fig5.13). Glutathione S-transferase (GST) is an antioxidant enzyme that responds to oxidative stress. Detecting GST activity can help determine oxidative stress levels. In the presence of NPs, inhibiting CAT or Glutathione Peroxidase (GPx) might enhance the activity of GST, a possible defence strategy for the organism (Santos et al., 2022). Similarly, Exposure to PS-MPs results in augmented GST activity in *Paracyclopsina nana* cells (Jeong et al., 2017b). Nevertheless, this study found that GST activity increased significantly under NP stress (Li et al., 2023c). Increased GST activity suggests that MP/NPs cause oxidative stress and activate the GST detoxifying system. Earlier studies have linked higher GST to activation of the detoxification mechanism, which encourages the

current conclusions (Barboza et al., 2018b) ;(Sinha et al., 2020). (Qiao et al., 2019c) evidenced that Zebrafish treated with PS (50 and 500 $\mu\text{g L}^{-1}$ for 21 days) showed inflammation, OS, elevated GSH levels, intestinal permeability, and dysbiosis. Likewise, A substantial increase in GST enzymatic activity was described in the liver of striped red mullets (Alomar et al., 2017). Based on the facts and demonstrations mentioned above, we may conclude that exposure to contaminants such as NPs and metal oxides, which cause activation of the detoxifying system, may enhance GST.

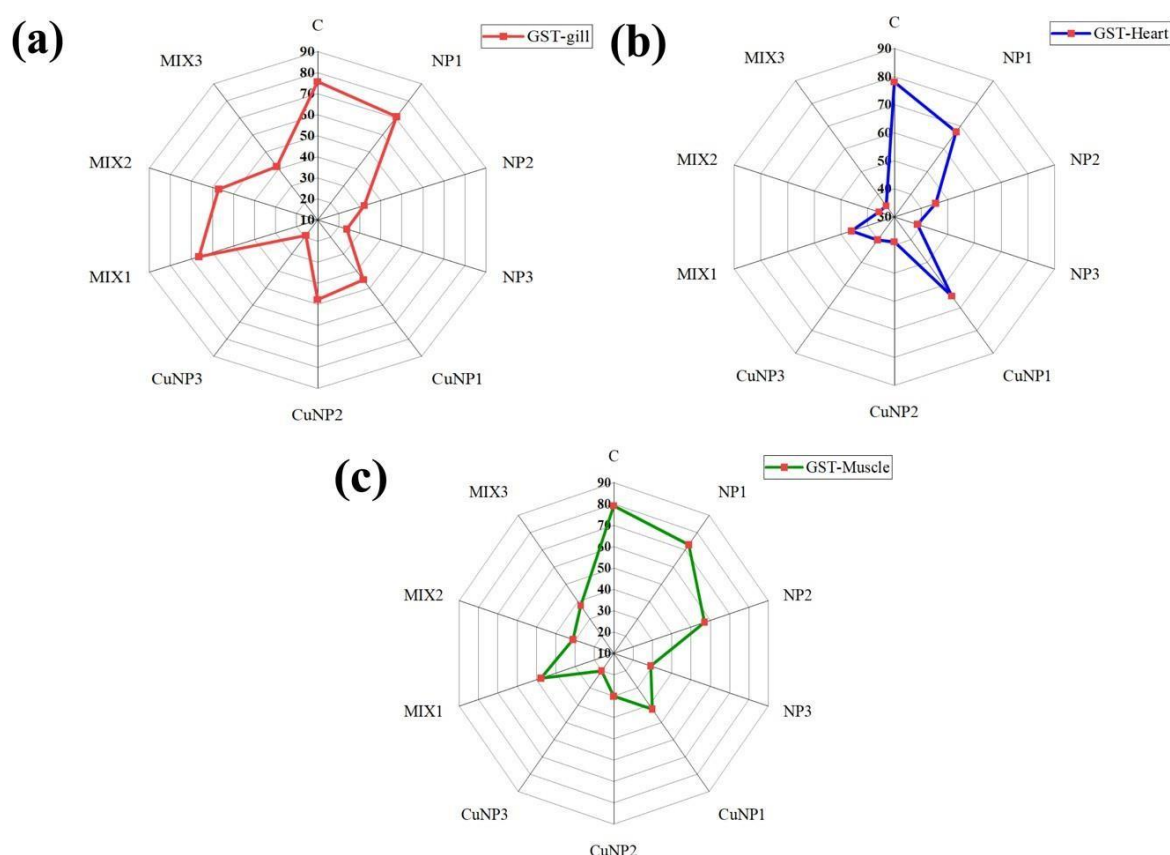


Fig5.13 Antioxidant (GST) capacity of Nile tilapia after CuO and PS-NPs exposure test for 14 days. (a) GST-Gill tissue, (b) GST-Heart tissue, (c) GST-Muscle.

5.3 Integrated biomarker response modelling analysis (IBR)

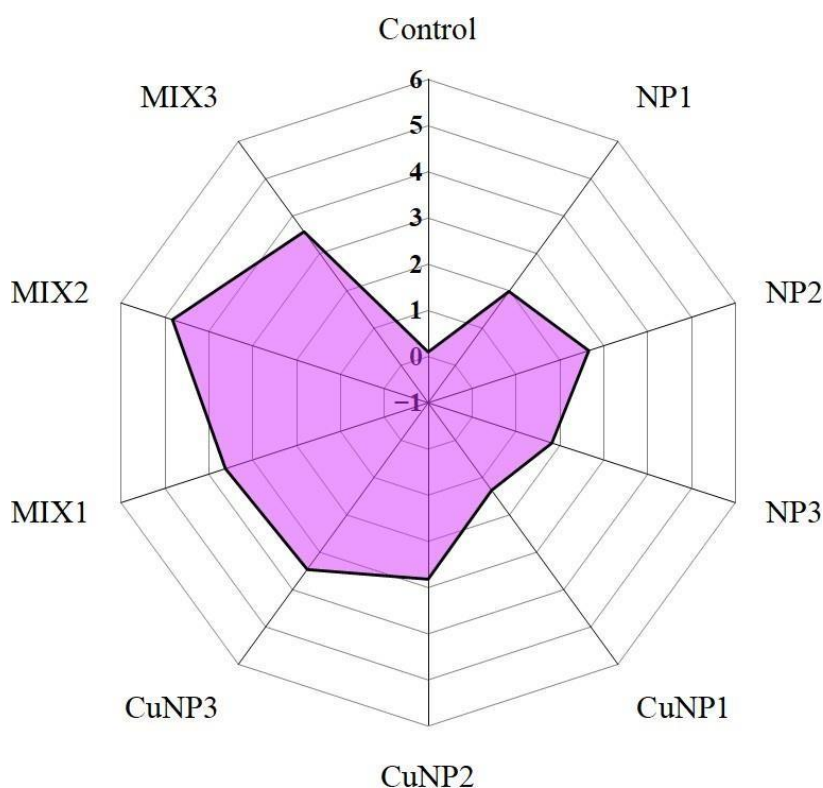


Fig5.14 IBR of chronic single and combined exposure to polystyrene nanoplastics and CuNP on the oxidative stress biomarkers and antioxidant enzymes

Integrated Biomarker Response (IBR) serves as a significant tool in toxicity experiments within the realm of multiple biomarkers and experimental conditions, offering a holistic perspective on the response of organisms exposed to various conditions. In these experiments, a suite of biomarkers is measured concurrently to capture the multifaceted responses to stress. Following exposure to stressors, such as PS-NP and CuNP, biomarker data are normalised and integrated using mathematical or statistical approaches to derive a comprehensive index of biological response. It indicated the cumulative effects of pollutants, distinguished between different levels of conditions, and evaluated the relative toxicity of various treatments and biomarkers. In this study, the Integrated Biomarker Response (IBR) was calculated from each experimental tissue at each experimental condition of single and combined exposure of polystyrene nanoplastics and CuNP. The IBR of chronic single and combined exposure to polystyrene nanoplastics and CuNP on the

oxidative stress biomarkers and antioxidant enzymes of the candidate animal was illustrated in Figure 5.14. IBR pattern was treatment-dependent and tissue-dependent. A combined IBR score revealed experimental group-dependent responses. In NP3, CuNP3, MIX2, and MIX3 highest IBR score revealed the highest toxicity responses compared to other experimental groups.

5.4 Multivariate statistical analysis

5.4.1 Correlation Network Analysis

Pearson correlation matrix plays a crucial role in elucidating relationships between various experimental factors and toxicity outcomes. This matrix provides a comprehensive overview of the linear associations between pairs of variables, such as dosage, exposure duration, biological parameters, and toxicity responses. A high positive correlation between certain variables may suggest a synergistic effect, whereas a negative correlation may indicate a mitigating influence. Additionally, variables showing low or no correlation may require further investigation to understand their independent contributions. The correlation matrix analysis and correlation network plot were carried out to assess the correlation efficiency among different response biomarkers and experimental tissues. As represented in Figure 5.15, there are strong correlations among the indexes.

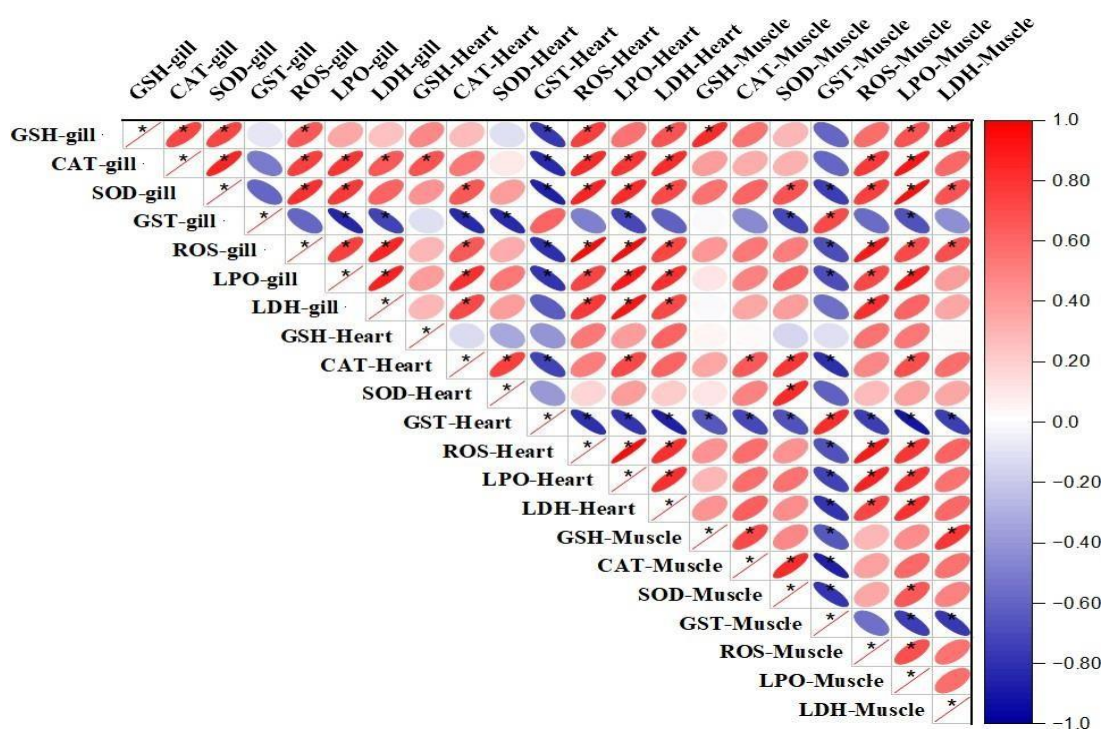


Fig5.15 Correlation matrix analysis and correlation network plot were carried out to assess the correlation efficiency among different response biomarkers and experimental tissues

5.5 Principal Component Analysis

Principal component analysis (PCA) was carried out with the results obtained to understand the effects of active bromide concentrations and experimental durations on the Oxidative stress biomarkers viz., ROS, LPO, LDH, SOD, CAT, GST, and GSH of different mussel organs (Gill, mantle, and digestive gland) individually. The correlations between the parameters are studied based on the directions of the vector i.e., if the vectors are close and forming a small angle then they represent positive correlation, if they meet at 90° , they are not likely to correlate with each other, and when they diverge and meet at 180° , they are negatively correlated. The representation of the principal component analysis is illustrated as a biplot in Figure 5.16. Experimental biomarkers like ROS, LPO, LDH, SOD, CAT, GST, and GSH exhibited a strong correlation. From the analysis of gill tissue biomarkers and experimental duration (Figure 5.16a), it is observed that the components, PC1 and PC2 collectively sum up to 89.15 % (PC1 71.42 % and PC2 17.73 %) of data variability. The experimental biomarkers like ROS, SOD, CAT, and GSH all fall in

component 2 of the scatter plot. From the scatter plot it is clear that with the increase in these parameters in component 2, the toxicity increases. The MIX2, CuNP2, and MIX3 all fall in the segment of component 2 indicating also maximum toxicity. The LPO and LDH are the only parameter that falls in component 1 along with the NP3, NP2, and CuNP3. This suggests that these treatment groups can be directly correlated with LPO and LDH activity. The other experimental conditions like Control, NP1, and CuNP1 were clubbed and all fell together in the other domain devoid of any parameters. This suggests the above-mentioned groups expressed lower toxicity as they all fall in the scatter plot opposite to the seven main biomarkers. It suggests that they comprise a separate cluster that is in negative correlation with the others thereby indicating the least toxicity compared to other experimental groups. Accordingly, from the analysis of heart tissue, biomarkers, and experimental duration (Figure 5.16b), it is represented that the components, PC1 and PC2 collectively sum up to 88.6% (PC1 63.32 % and PC2 25.28 %) of data variability. In addition, the experimental biomarkers like SOD and CAT, and both fall in component 2 of the scatter plot. From the scatter plot it is clear that with the increase in these parameters in component 2, the toxicity increases. The experimental condition of NP3, CuNP3, and CuNP2 all falls in the segment of component 2 indicating also maximum toxicity. Consequently, The GSH, ROS, LDH, and LPO activity are the other parameters that fall in component 1 along with MIX2, MIX3, and NP2. This suggests that these treatment groups can be directly correlated with GSH, ROS, LDH, and LPO activity. The other experimental conditions like control, NP1, fall together in the other domain devoid of any parameters. This suggests those experimental conditions expressed lower toxicity as they all fall in the scatter plot opposite to the five main biomarkers. It suggests that they comprise a separate cluster that is in negative correlation with the others thereby indicating the normal response and least toxicity compared to other experimental groups. In addition, from the analysis of Muscle tissue biomarkers and experimental duration (Figure 5.16c), it is observed that the components, PC1 and PC2 collectively sum up to 80.08 % (PC1 66.9 % and PC2 13.18 %) of data variability. The experimental biomarkers like LDH, LPO, and ROS all fall in component 2 of the scatter plot. The experimental conditions of MIX3, and NP3 both fall in the segment of component 2 indicating also maximum toxicity. From the scatter plot it is clear that with the increase of activity in these parameters in component 2, the toxicity increases. Accordingly, the CAT,

GSH, and SOD activity are the other parameters that fall in component 1 along with MIX1, CuNP2, CuNP3, and MIX2. This suggests that these treatment groups can be directly correlated with MIX1, CuNP2, CuNP3, and MIX2. Similarly, the other experimental durations like CuNP1 along with control both fall together in the other domain devoid of any parameters. This suggests those durations and concentrations expressed lower toxicity as they all fall in the scatter plot opposite to the five main biomarkers. Thus, PCA can provide information on the correlation between individual and binary mixture toxicity using chemometric methodology.

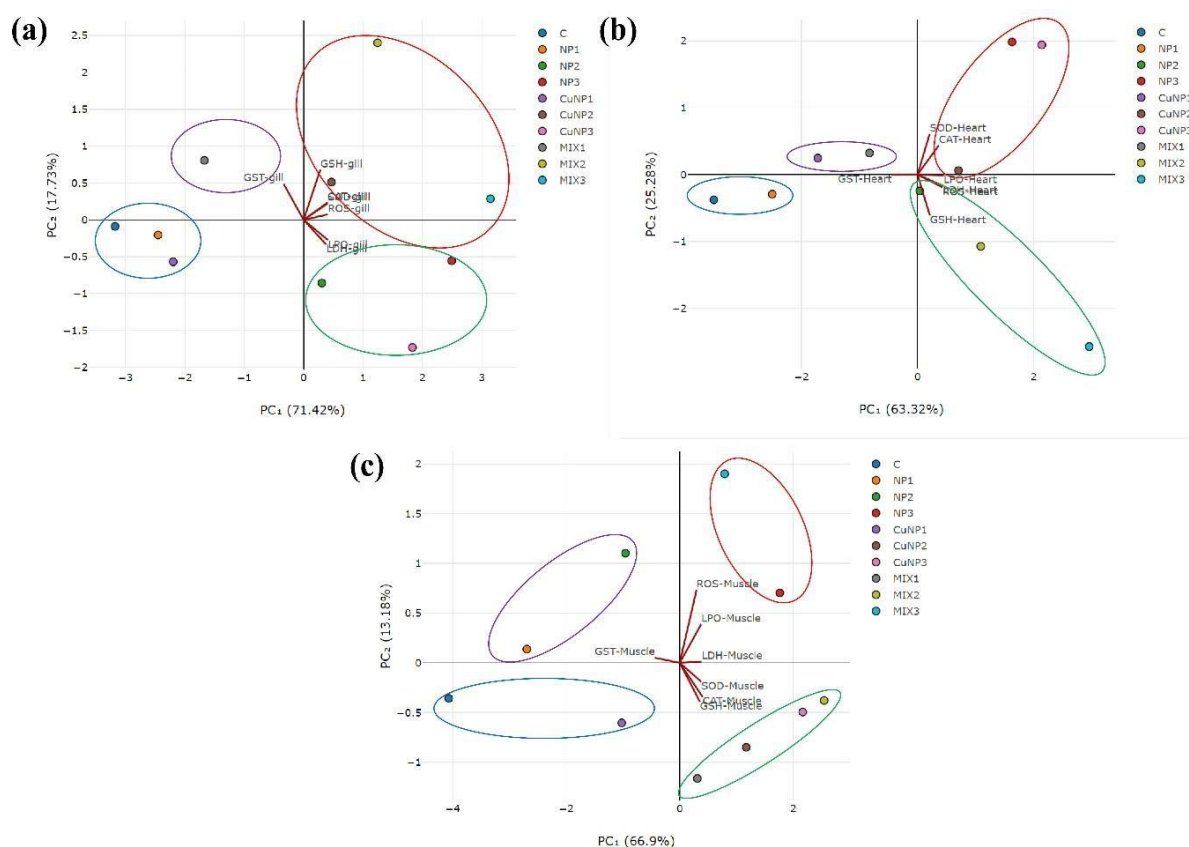


Fig5.16 Principal component analysis **a)** analysis of gill tissue biomarkers and experimental duration **b)** analysis of heart tissue, biomarkers, and experimental duration **c)** analysis of Muscle tissue biomarkers and experimental duration.

5.6 Qualitative assessment and fluorescent tracking of nano-plastic accumulation

In this present study, after 96 hours of exposure, the accumulation of a combination of PSNP's and CuO nanoparticles in the gill increased with the increase of PSNP's and CuO concentration in the water. The accumulation of PSNP's and CuO in the gill was higher in comparison to the other exposure groups. Fluorescence images of the gill are taken after the exposure of a combination of PSNPs and CuO (Fig5.17). One possible explanation is that the particle size of the NPs utilised in this investigation was tiny, resulting in restricted ingestion. Smaller MPs are more likely to remain in organisms than bigger particle sizes demonstrated by (Rist et al., 2017b). This explanation will apply to nanoplastics too. PS-NPs are difficult for organisms to metabolise and destroy due to their large molecular weight and hydrophobic nature. (Yang et al., 2015) discovered Polystyrene biodegradation rates in diverse microbial consortia ranged from 0.01% to less than 3% during four months. (Bussolaro et al., 2019) discovered that polystyrene nanoparticles affected the viability of rainbow trout gill epithelial cells in a concentration-dependent way. On the other hand, (Hu and Palić, 2020) conducted an in vivo investigation in which adult Japanese medaka (*O. latipes*) were exposed to polyester (PES) and polypropylene (PP) MP fibres. Scanning electron microscopy (SEM) revealed increased mucus in the gills, epithelial denudation on arches, and initial lamellar fusion. Polystyrene nanoparticles (PS NPs) were observed to accumulate in the larval or adult gut of Medaka (*O. melastigma*), with higher mortality and a drop in average lengths and weights (Cong et al., 2019). These above findings suggest that the bioaccumulation of the NPs causes damage to the internal organs of the fishes.

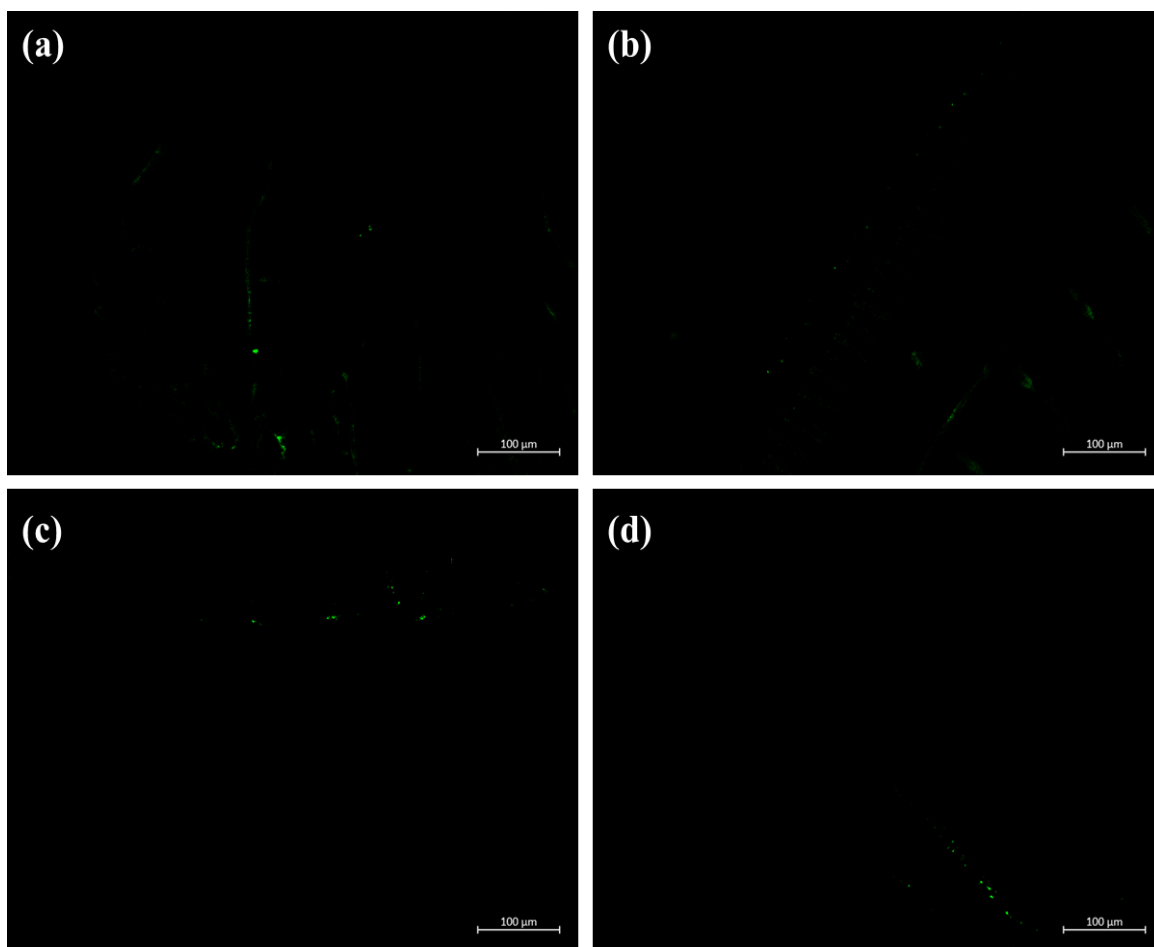


Fig5.17 Accumulation of a combination of PSNP's and CuO nanoparticles in Gill tissue.

5.7 DNA damage assessment using single-cell gel electrophoresis (COMET)

This study's findings show that administering NPs and CuO resulted in an enormous surge in DNA damage in the blood of *O.niloticus* compared to the control(Fig5.18a). Furthermore, this research detected an increase in damage markers, such as tail DNA percentage and tail length shown in (Fig5.18) (Fig5.19). All DNA damage metrics rose considerably in the NPs+CuO combination as compared to the control and other treatment groups.

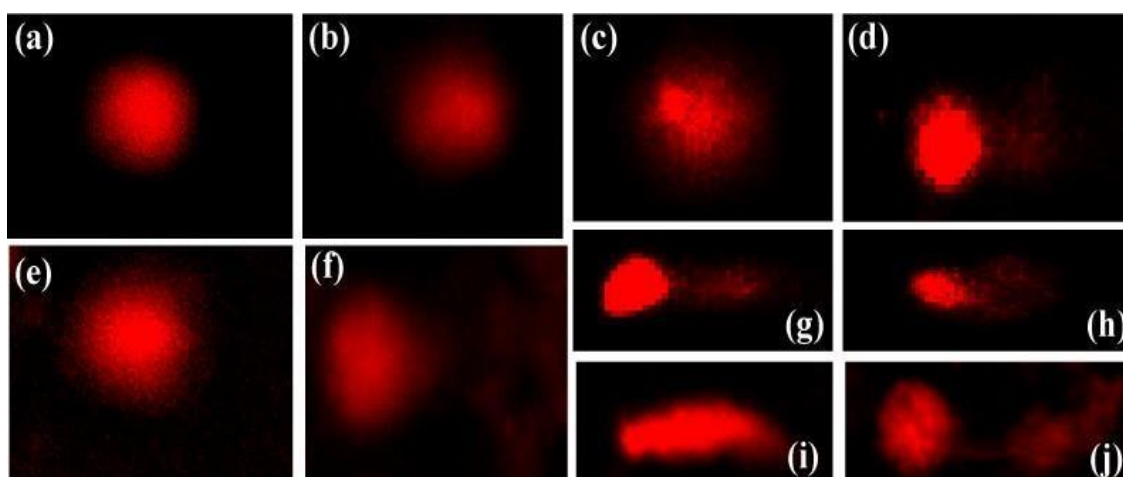


Fig5.18 COMET Assay (a) control; (b) CuO-I(0.25 mg/l); (c) CuO-II(5 mg/l); (d) CuO-III(10 mg/l); (e) PS NP-I (10 µg/l); (f) PS NP-II (50 µg/l); (g) PS NP-III (100 µg/l); (h) Mix-I(10 µg/l+0.25 mg/l); (i) Mix-II(50 µg/l+5 mg/l); (j) Mix-III(100 µg/l+10 mg/l).

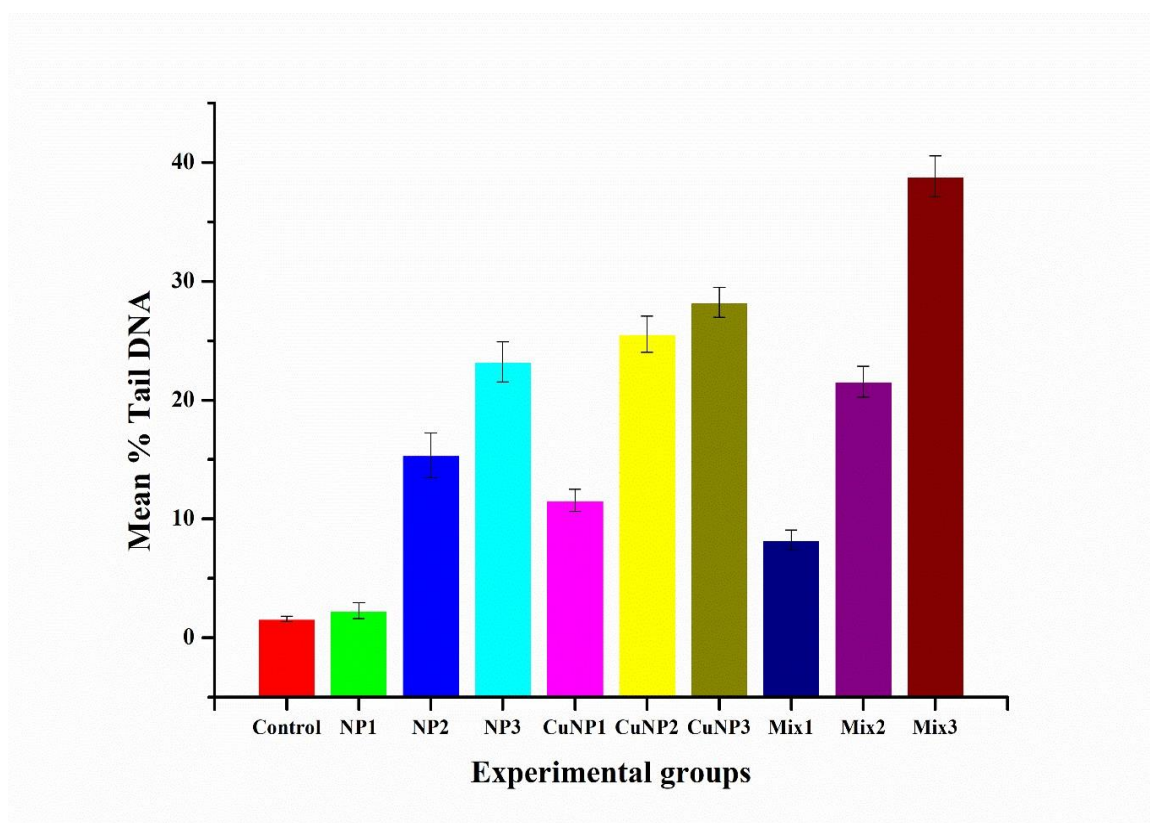


Fig5.19 Graphical representation of tail DNA percentage in the blood of *O. niloticus*

Intake of nanoplastics by aquatic species induces genotoxicity by producing ROS in their bodies (Sun et al., 2020). The DNA damage (comet-shaped cells) was assessed using the comet test. The comet test uses alkaline elution to determine DNA damage in single and double standards (Gobi et al., 2018); (Lee and Steinert, 2003). In our research, the ROS amount drastically increased in 14 days exposure of combination of PSNP+CuO, only PSNP and only CuO nanoparticles exposure groups (group1- 100µg/L+ 10 mg/L, group2- 100µg/L, group3- 10mg/L) respectively of *Oreochromis niloticus*. Results showed that the ingestion of polystyrene nanoplastics and copper oxide has increased the DNA damage compared to the control group. On the other side, our data suggest that both PS- NPs and the mix of pollutants (alone or in association) were able to induce damage to the erythrocyte DNA of fish. In the control group, the DNA was condensed (visualised in a round shape). Nevertheless, in a combination of PSNP+CuO exposure, the DNA cells are stretched out and look comet shaped. The findings indicated that 14 days of exposure to polystyrene nanoplastics and copper oxide nanoparticles causes DNA damage in *O. niloticus*. It may cause apoptosis or cell death in liver tissues. Similarly, in previous studies consuming polystyrene microplastics in freshwater fish, *Danio rerio* and *Perca fluviatilis* induce DNA damage in the liver and gill tissue (Kaloyianni et al., 2021).

5.8 Histopathology

Despite both single and combined exposure circumstances, treated fish's hearts exhibited significant changes as compared to control fish, demonstrating that cardiac tissue is not resistant to this exposure (Fig5.21). The heart of an untreated *O. niloticus* was made up of a mass of well-organised cardiomyocytes. Furthermore, no signs of necrosis could be identified. Moreover, In muscle tissue

PS-NPs enhanced muscle fibre voids and lysis compared to the other exposure dosages (Fig5.22). Similarly, in gill tissue PS-NPs developed atrophy and deformity in gill filaments (Fig5.20). Nanoplastic and Copper Oxide are the common pollutants to which the fishes are exposed or ingested. Ingestion of these pollutants can lead to accumulation and tissue harm (He et al., 2022). Intramuscular injection of PS-NPs and CuO damaged the tissue architecture of *O.niloticus*, resulting in muscle fibre dissolution, damaged cardiomyocytes, and gill filament atrophy. PS-NPs and other metal oxide pollutants have been linked to histopathological damage in several aquatic animals.

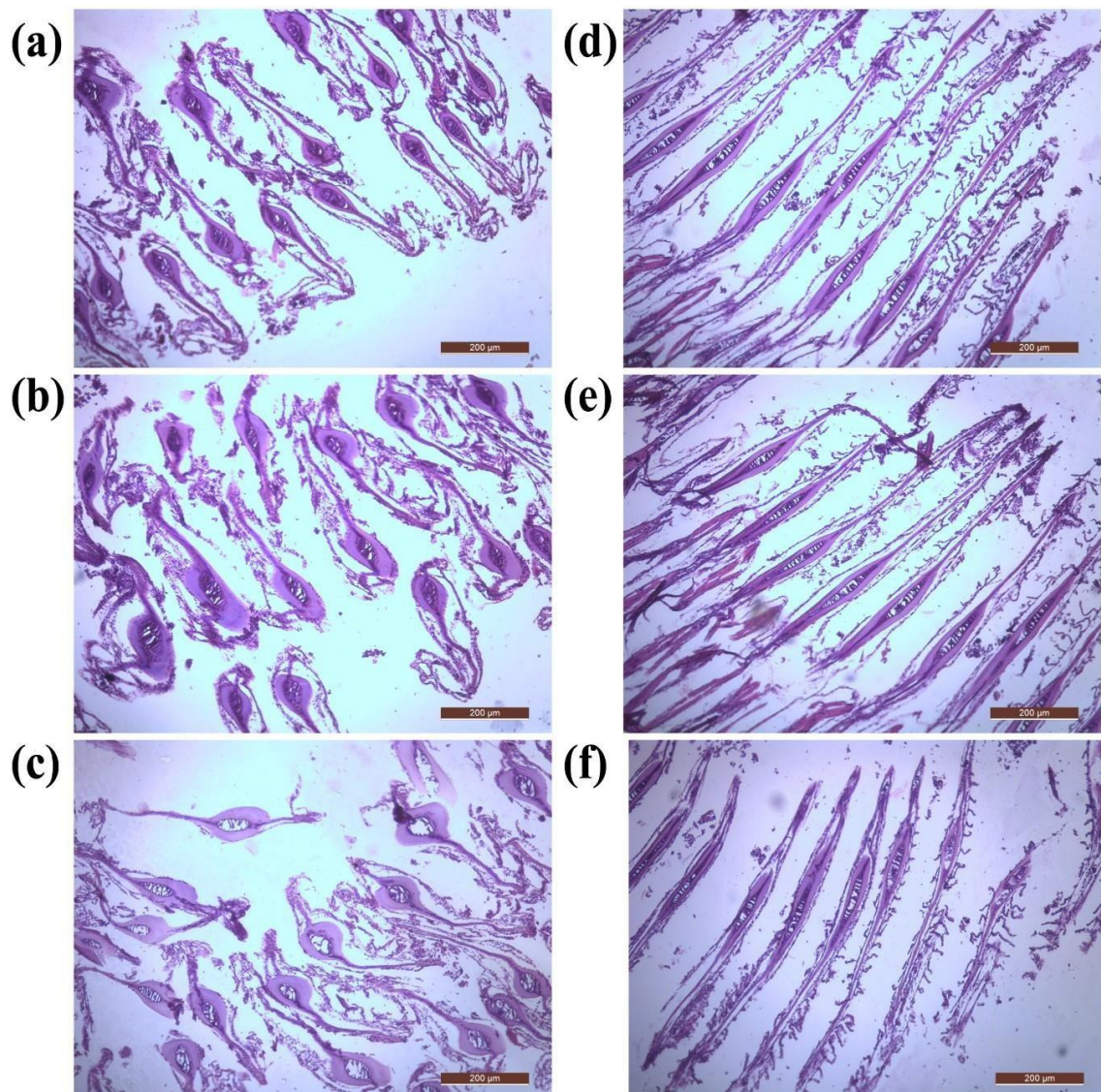


Fig5.20 Histopathological changes in Gill of Nile tilapia in Six groups. (a) PS NP-I (10 $\mu\text{g/l}$), (b) PS NP-II (50 $\mu\text{g/l}$), (c) PS NP-III (100 $\mu\text{g/l}$), (d) Mix-I(10 $\mu\text{g/l}$ +0.25 mg/l), (e) Mix-II(50 $\mu\text{g/l}$ +5 mg/l), (f) Mix-III(100 $\mu\text{g/l}$ +10 mg/l).

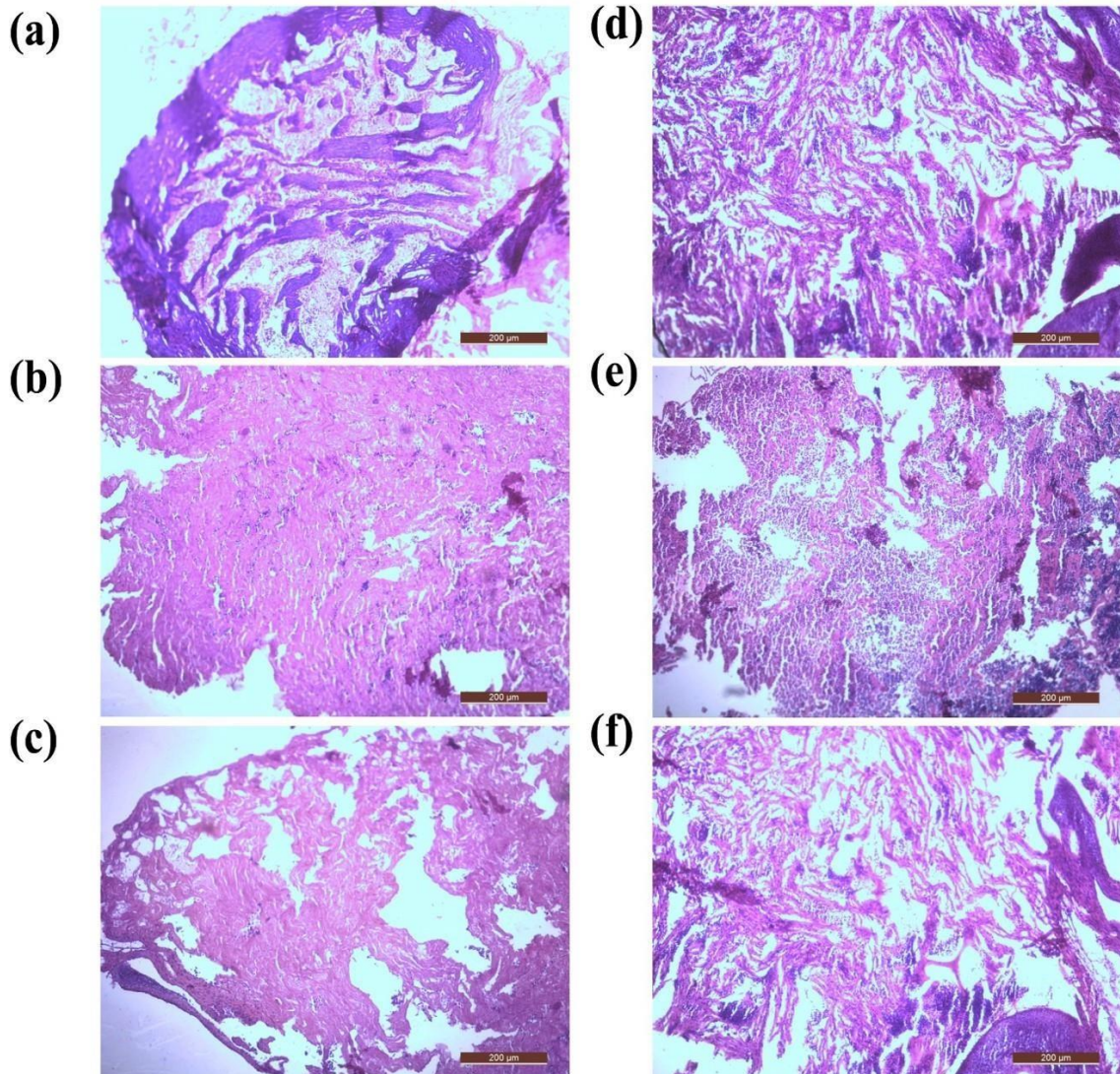


Fig5.21 Histopathological changes in Heart of Nile tilapia in Six groups. (a) PS NP-I (10 $\mu\text{g/l}$), (b) PS NP-II (50 $\mu\text{g/l}$), (c) PS NP-III (100 $\mu\text{g/l}$), (d) Mix-I(10 $\mu\text{g/l}$ +0.25 mg/l), (e) Mix-II(50 $\mu\text{g/l}$ +5 mg/l), (f) Mix-III(100 $\mu\text{g/l}$ +10 mg/l).

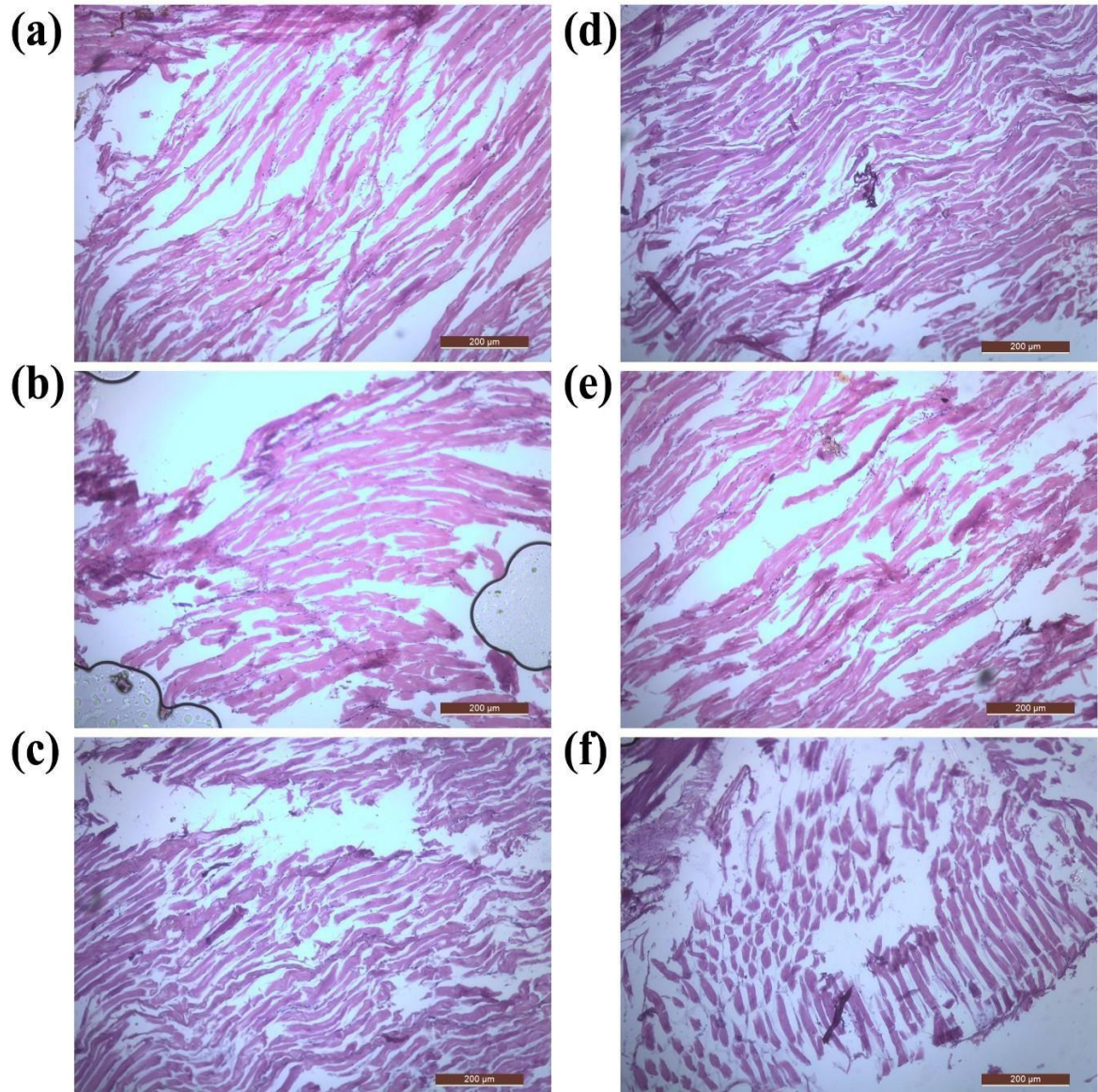


Fig5.22 Histopathological changes in Muscle of Nile tilapia in Six groups. (a) PS NP-I (10 µg/ l), (b) PS NP-II (50 µg/ l), (c) PS NP-III (100 µg/l), (d) Mix-I(10 µg/ l+0.25 mg/l), (e) Mix-II(50 µg/ l+5 mg/l), (f) Mix-III(100 µg/l+10 mg/l).

CHAPTER-6

SUMMARY&CONCLUSION

Finally, our investigation on the combined toxicity of copper oxide nanoparticles (CuO NPs) and polystyrene nanoplastics (PS NPs) in *Oreochromis niloticus*, Nile tilapia, indicates significant negative impacts on a variety of biochemical markers. Over time, the presence of both CuO NPs and PS NPs resulted in synergistic toxicity, worsening the specific effects of each nanoparticle type. We discovered that the combined exposure caused significant changes in oxidative stress indicators, including higher catalase (CAT) levels and higher lipid peroxidation (LPO) levels, indicating greater oxidative damage. Furthermore, histological investigations indicated severe tissue damage, notably in the heart, muscle and gills, indicating impairment of critical physiological activities. Overall, our findings emphasise the need to conduct extensive studies of the combination of nanoparticle and metal oxide interactions and their ecological implications. Mitigation solutions and regulatory measures are needed to reduce the environmental discharge of nanoplastics and metal nanoparticles, protecting aquatic ecosystems and the health of aquatic species such as *Oreochromis niloticus*. More study is needed to understand the processes causing nanoplastic toxicity and create effective ways to reduce their Negative impacts in aquatic settings.

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Test Report Form

ACADEMIC

NOTE Admission to undergraduate and post graduate courses should be based on the ACADEMIC Reading and Writing Modules.
GENERAL TRAINING Reading and Writing Modules are **not** designed to test the full range of language skills required for academic purposes.
It is recommended that the candidate's language ability as indicated in this Test Report Form be re-assessed **after two years** from the date of the test.

Centre Number Date Candidate Number

Candidate Details

Family Name

First Name

Candidate ID



Date of Birth Sex (M/F) Scheme Code

Country or Region of Origin

Country of Nationality

First Language

Test Results

Listening Reading Writing Speaking Overall Band Score CEFR Level

Administrator Comments

Centre stamp



Validation stamp



Administrator's Signature

Date

Test Report Form Number



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INSTITUTE OF SCIENCE AND TECHNOLOGY

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Name of the student : Arjun Aditiya K R

Register Number 40230003

Title of the Thesis : Combined Toxicity of Copper Oxide and Polystyrene Nano plastics in *Oreochromis Niloticus*

Internal Guide Name : Dr. S. Usha Nandhini

External Guide Name : Dr. D. Inbakandan

PO / PSO / PEO / SDG	Justification
PO4	To investigate the combined toxicity of copper oxide nanoparticles and polystyrene nano plastics on <i>Oreochromis Niloticus</i> , with the focus on understanding the oxidative stress - induced multivariate biomarkers activity
PEO2	To perform various biomarkers and analytical tests and identify the toxicity of the studied compound on the organism
SDG14	Work done helps in studying the marine organism and its behaviour due to the toxicants.