# Exploring the Interactive Effects of Perfluorooctane Sulfonate (PFOS) and Copper Oxide Nanoparticles in *Danio rerio* (H.)


**AHMED ATIULLAH**

**Registration No. 023112013011**

**DEPARTMENT OF ENVIRONMENTAL SCIENCES**

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**ISLAMABAD, PAKISTAN**

**2020-2022**

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A dissertation submitted in partial fulfillment of the requirement for the degree of

**Master of Philosophy**

**in**

**Environmental Sciences**


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I, **Ahmed Atiullah**, here by state that my M. Phil. Thesis titled **"Exploring the Interactive Effects of Perfluorooctane Sulfonate and Copper Oxide Nanoparticles in Adult Zebrafish *Danio rerio* (h.)"** is solely my research work with no significant contribution from any other person. Small contribution/help whatever taken has been duly acknowledged and complete thesis has been written by me.

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If my statement is found to be incorrect at any time, even after my graduation the university has the right to withdraw my M Phil degree.

**Ahmed Atiullah**

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Ahmed Atiullah

# LIST OF ABBERIVATIONS

| **Abbreviations** | **Stands for** |
|---|---|
| AChE | Acetylcholinesterase |
| AFFF | Aqueous Firefighting Foams |
| Ag | Silver |
| AG | Analytical Grade |
| AgNPs | Silver nanoparticles |
| AI | Acetylthiocholine iodide |
| Al | Aluminum |
| ANOVA | Analysis of Variance |
| Au | Gold |
| $C_{60}$ | Fullerene |
| $Ca^{+2}$ | Calcium ion |
| CAS | Chemical abstract number |
| CAT | Catalase |
| CT | Control Treatment |
| Cu | Copper |

| | |
|---|---|
| CuO | Copper oxide nanoparticles |
| CuO-NPs | Copper oxide nanoparticles |
| DEPPD | N, N-Diethyl-p-phenylenediamine sulphate salt |
| DMSO | Dimethyl sulfoxide |
| DNA | Deoxyribonucleic acid |
| EC | Electrical Conductivity |
| EDTA | Ethylenediaminetetraacetic acid |
| EDX | Energy dispersive x-ray spectroscopy |
| ENPs | Engineered nanoparticles |
| ε | Extinction Co-efficient |
| Fe | Iron |
| $FeSO_4$ | Iron sulphate |
| FTIR | Fourier-transform infrared spectroscopy |
| GPx | Glutathione Peroxidase |
| GSH | Glutathione |
| $H_2O_2$ | Hydrogen peroxide |
| HPLC | High performance liquid chromatography |
| IBR | Integrated biomarker response |

| | |
|---|---|
| ISO | International Organization for Standards |
| JCPDS | Joint Committee on Powder Diffraction Standards |
| JCPDS | Joint Committee on Powder Diffraction Standards |
| KBr | Potassium bromide |
| LMA | Low melting agrose |
| MDA | Malondialdehyde |
| Me-ENPs | Metal Engineered nanoparticles |
| Mg | Milligram |
| MPs | Microplastics |
| MWCNT | Multi-walled carbon nanotubes |
| NADPH | Nicotinamide Adenine Dinucleotide Phosphate Hydrogen |
| NaOH | Sodium Hydroxide |
| NBT | Nitrotetrazolium Blue chloride |
| NMA | Normal melting agarose |
| NPs | Nanoparticles |
| O | Oxygen |
| OD | Optical Density |
| OECD | Organization for economic co-operation and development |

| | |
|---|---|
| PAHs | Polycyclic aromatic hydrocarbons |
| PBS | Phosphate buffer saline |
| PBT | Persistent bioaccumulative toxic |
| PFAS | Per- and polyfluoroalkyl substances |
| PFCs | Perfluorinated compounds |
| PFOS | Perfluorooctane sulfonate |
| PFSA | Perfluoroalkyl sulfonamidoacetic acids |
| Pg/L | Picogram/liter |
| $pg/m^3$ | Picogram/meter cube |
| Ppb | Parts per billion |
| Ppm | Parts per million |
| PUF-PASs | polyurethane foam-based passive air samplers |
| ROS | Reactive oxygen species |
| S | Sulphur |
| SCGE | Single-cell gel electrophoresis |
| SD | Standard Deviation |
| SDS | Sodium Dodecyl Sulfate |
| SEM | Scanning electron microscopy |

| | |
|---|---|
| SOD | Superoxide dismutase |
| SPSS | Statistical Package for Social Sciences |
| SWCNT | Single walled carbon nanotubes |
| T1 | CuO-NPs treatment |
| T2 | PFOS treatment |
| T3 | Combined treatment (CuO-NPs+PFOS) |
| TBA | Thiobarbituric acid |
| TBARS | Thiobarbaturic Substances |
| $TiO_2$ | Titanium oxide nanoparticles |
| $TiO_2$-NPs | Titanium oxide nanoparticles |
| TSCA | Toxic Substances Control Act |
| US-EPA | United States Environmental Protection Agency |
| XRD | X-ray diffraction |
| Zn | Zinc |
| ZnO | Zinc oxide nanoparticles |
| ZnO-NPs | Zinc oxide nanoparticles |

# HIGHLIGHTS

- Singular and combined effects of CuO-NPs and PFOS in adult zebrafish were assessed in a 28-day experiment.
- ROS and MDA increased upon exposure to both contaminants, whereas inhibition of antioxidant enzymes (SOD & CAT) was observed.
- AChE activity in zebrafish brain was inhibited upon combined exposure CuO-NPs and PFOS.
- Comet assay revealed DNA damage with increased tail length and tail moment in all treatment groups.
- Histopathological alterations including segmental necrosis, degeneration and splitting of muscle fibers, necrosis, shortening of muscle fiber and atrophy were observed in skeletal muscle segments.
- Mixture of CuO-NPs and PFOS revealed greater damage in all biomarkers as compared to individual actions.

# ABSTRACT

**Background:** Perfluorooctane sulfonate has existed in the natural environment for decades, but emergence of novel pollutants, such as engineered nanoparticles, can alter toxicological risks associated to it upon interaction. To understand these interactive toxic effects, this work studies sublethal toxicity effects of perfluorooctane sulfonate (PFOS), copper oxide nanoparticles (CuO-NPs), and their mixture on adult zebrafish *Danio rerio*.

**Methodology:** Nanoparticles used in the study were characterized for size and morphology using SEM, whereas chemical composition, surface functional groups and crystallography information were determined using EDX, FTIR and XRD, respectively. The fish were subjected to four different treatments; control (DMSO 0.03%v/v); CuO-NPs (1mg/L); PFOS (300 µg/L); and CuO-NPs+PFOS (1mg/L+300 µg/L). Fish were exposed for 28 days in triplicates ($n=3$) and from each treatment, 5 fish were randomly sampled at 7, 14, 21 and 28 days and gills were obtained for evaluation of total protein levels, oxidative stress markers (ROS and MDA) and antioxidant enzymes (SOD and CAT). Further, AChE activity was measured in the brain and DNA damage was assessed in fish liver, whereas histopathology of skeletal muscle tissues was also examined.

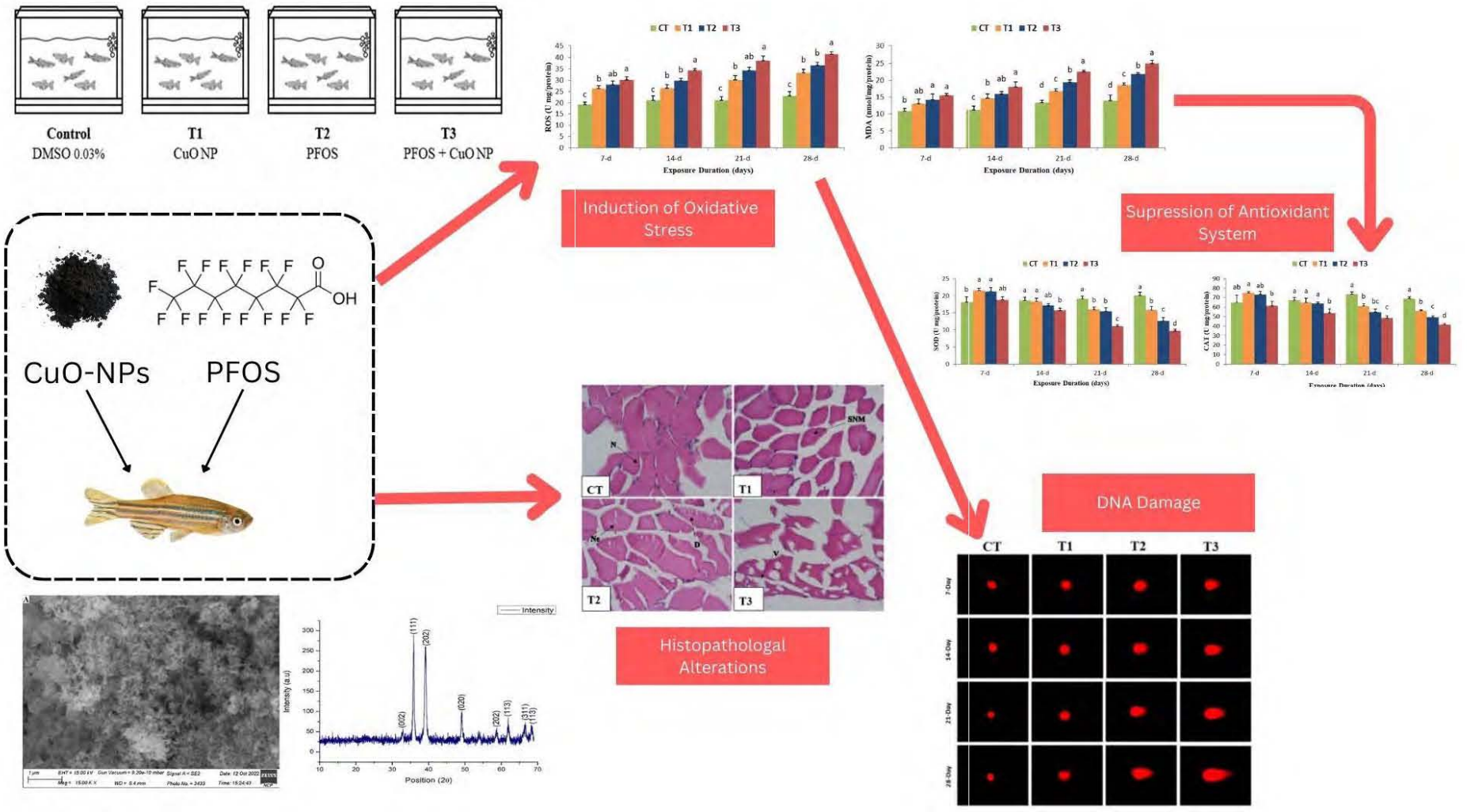
**Results:** Exposure to CuO-NPs and PFOS in individual and combined settings significantly ($p<0.05$) increased ROS and MDA content in zebrafish gills, while activities of SOD and CAT were significantly inhibited after 28 days. Induction of oxidative stress and inhibition of antioxidant enzymes was significantly higher in co-exposure treatment. Similarly, both contaminants significantly decreased AChE activity in zebrafish brain which was more pronounced under combined treatment. CuO-NPs and PFOS caused an increase in DNA tail length and tail moment revealing DNA damage, although the highest genotoxic action was observed in the co-exposure treatment. Histopathological alterations including segmental necrosis, degeneration and splitting of muscle fibers, necrosis, shortening of muscle fiber and atrophy were observed in all groups except control on all sampling intervals.

**Conclusion:** Overall, the findings suggest that CuO-NPs and PFOS interact synergistically to cause toxicity in adult zebrafish. Future studies should focus on interactive effects of PFOS and other PFCs with novel pollutants including nanoparticles and distribution of these contaminants across trophic levels under combined exposure.



Keywords: Oxidative stress; Nanotoxicity; AChE; DNA Damage; Co-exposure; PFAS; Histology

# GRAPHICAL ABSTRACT



# CHAPTER 1
# INTRODUCTION AND LITERATURE REVIEW

# 1 INTRODUCTION AND LITERATURE REVIEW

## 1.1 Per- and polyfluoroalkyl substances (PFASs)

The C-F linkages and terminal functional groups produce a class of synthetic persistent organic pollutants (POPs) known as per- and polyfluoroalkyl substances (PFAS). PFAS offer special qualities including great surface activity, corrosion resistance, thermal/chemical stability, and others (Li et al., 2022). Per- and polyfluoroalkyl substances (PFAS), also known as "forever chemicals" due to their inherent chemical stability, have been discovered to be pervasive environmental pollutants, found everywhere from metropolitan runoff to the extreme reaches of the planet's Arctic. Although PFAS have been produced for around 7 decades, public awareness of them is still relatively recent (Evich et at., 2022). More than 3000 PFAS are available on the market for deliberate purposes, according to Wang et al. (2017), including to produce surface repellents, metal plating, fluoropolymer manufacturing, semiconductors, andfirefighting foams (Radjenovic et al., 2020). Due to rising concerns about the potential dangers of PFAS, the Stockholm Convention designated PFOS and PFOA for global restriction and removalin 2009 and 2019, respectively (UNEP, 2019).

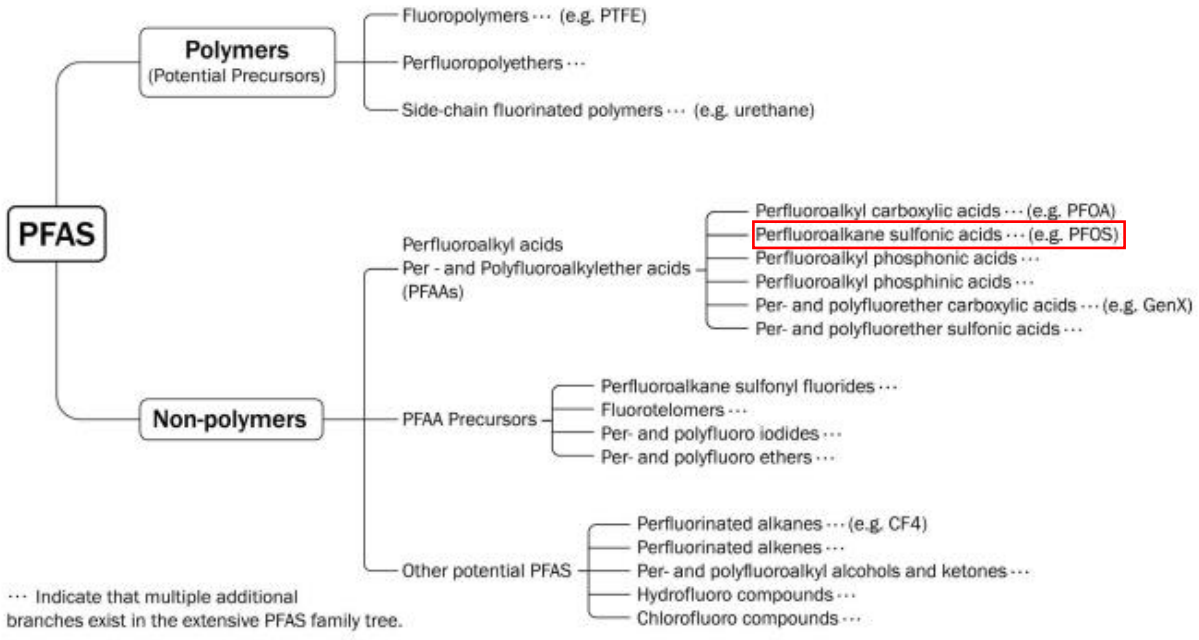


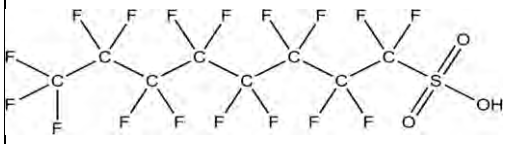

**Figure 1**: "Family Schematic diagram of PFAS" (Winchell et al., 2021).

PFAS are manufactured with the potential to be released into the environment (Li et al., 2019). Recent research has demonstrated the abundance of PFAS in environmental compartments, including water (Marchiandi et al., 2021; Zhang et al., 2021; Chen et al., 2021, 2020; Li et al., 2020; Joerss et al., 2019; Liu et al., 2019; Pan et al., 2018;), sediments (Ahmed et al., 2020; Ahrens et (Wang et al., 2021; Lin et al., 2020). Due to PFAS's relatively high water-solubility and moderate volatility, it is the aquatic environment that serves as its main reservoir as opposed to other POPs like polychlorinated dioxinsand polychlorinated biphenyls (Sharma et al., 2016). As a result, PFAS has the potential to damage surface water.

## 1.2 Perfluorooctane sulfonate (PFOS)

Perfluorooctane Sulfonate (PFOS) and its different salts are manmade organic substances having carbon chain and a special functional group, which can be a carboxylic group or sulfonate ($C_nF_{2n+1}$-R), that are members of the poly- and perfluoroalkyl substances (PFAS) category of chemicals (Ahrens 2011; Wang et al., 2017). In particular, PFOS is made up of a sulfonate functional group and an 8-carbon backbone. The perfluorooctane sulphonate anion,which is what is popularly referred to as PFOS, is not a chemical as per conventional definitions and hence no particular CAS has been assigned to it. It can be purchased commercially as salts, derivatives (PFOS- substances), and polymers (PFOS-polymers). Any or all chemicals that contain the PFOS moieties and may degrade in environmental compartments to produce PFOS are referred to as "PFOS related substances" (Environment Agency, 2004). Since the 1950s, PFOS and its salts have been utilized in a variety of industrial, commercial and user products, such as surface treatments for the resistance to soil and stains of textiles, paper, metals, and pesticides (Ahrens, 2011; Ahrens and Bundschuh, 2014). In 2002, PFOS was phased out except for few minor uses (Lindstrom et al., 2011).

**Table 1**: Chemical Characteristics of PFOS

| | |
|---|---|
| **Common name** | PFOS |
| **Chemical name (IUPAC)** | Perfluorooctane Sulphonates |
| **Chemical Class** | Perfluorinated Compounds |
| **Molecular Formula** | C8F17SO3 |
| **Molecular structure** |  |
| **CAS Number** | 2795-39-3 (Potassium Salt)<br>1763-23-1 (Acid)<br>29081-56-9 (Ammonium salt)<br>29457-72-5 (Lithium salt)<br>70225-39-5 (Diethanolamine salt)<br>56773-42-3 (Tetraethyl-ammonium salt)<br>251099-16-8 (Didecyldimethyl-ammoniumsalt) |
| **Molecular weight (g/mol) of Perfluorooctane Sulfonate Potassium salt** | 538 |
| **Molecular weight (g/mol) of Perfluorooctane Sulfonic acid** | 500 |

Perfluorooctane sulfonate is water-soluble due to the presence of the negatively charged sulfonate group, which increases its interaction with water molecules through electrostatic forces. The charged nature of PFOS allows it to form ion-dipole interactions with water, enhancing its solubility. As perfluorooctane sulfonate is extremely soluble in water, it is also very mobile in aqueous media. Additionally, it has extremely low vapor pressure, which raises the possibilityof its atmospheric presence. The moderate sorption capacity of PFOS toward solids (sediments/soil) at optimum conditions.

The strong persistent, bioaccumulative and toxic ( PBT) character of PFOS is similar to that of other PFCs. It endures in theenvironment for a long time and travels far without breaking. Eagles, mink, and other speciesat the top of the food chain were found to have greater PFOS levels, which is indicative of thesubstance's bioaccumulative properties (Giesy and Kannan, 2001).

### 1.2.1 Manufacture and environmental discharge

The 3M Company in the United States of America began producing PFOS in 1949 (Paul et al., 2009). Perfluorooctanesulfonyl fluoride (PFOSF), more commonly known as POSF, the raw material used in the electrochemical fluorination process to create PFOS, was previously primarily produced by the 3M Company, with small scale manufacturers in Asia and Europe (Paul et al., 2009; Lindstrom et al., 2011). The company 3M produced around 78% out of approximate 4650 tonnes of PFOSF produced globally in 2000 (OECD, 2002). Between 44000 and 96 000 tonnes of PFOSF have reportedly been produced overall until 2002 by the 3M Company and other Western businesses. Limited information is available on PFOSF production in Asia and other manufacturing regions, both historically and currently (Prevedourous et al., 2006; Paul et al., 2009).

The 3M Company decided to stop using and started looking suitable alternatives for PFOS primarily for a few minor applications among the variety of products after discussions with theUS Environmental Protection Agency (US-EPA) held in May 2000 (Lindstrom et al., 2011). Beginning around the same year, the US-EPA implemented a number of new use regulations to limit the manufacture and use of chemicals in the country that include PFOS andits precursors (Lindstrom et al., 2011). Additionally, PFOS, its salts, and its precursors were phased out in Canada (Environment and Climate Change Canada 2018). The StockholmConvention on

Persistent Organic Pollutants' Annex B, added in 2009, places ban on production and use of PFOS worldwide (Aherns, 2011; OECD, 2002). Potential sources of PFOS include precursor chemicals, and novel families of PFAS, all of which are still beinggenerated (Ahrens 2011). From 2002 onward, it was estimated that 1000 tonnes of PFOS were produced (Paul et al., 2009). However, producers in other nations, including Brazil and China, have increased their manufacturing to meet the demand not withstanding industrialized nations, such as US, phased out PFOS and its predecessors (Wang et al., 2013). Despite PFOS and its precursors being widely used in a range of consumer and industrial items around the world, there is and has only ever been minimal information on their sources, quantities, and emissions(Paul et al., 2009; Zhang et al., 2016; Ankley et al., 2020).

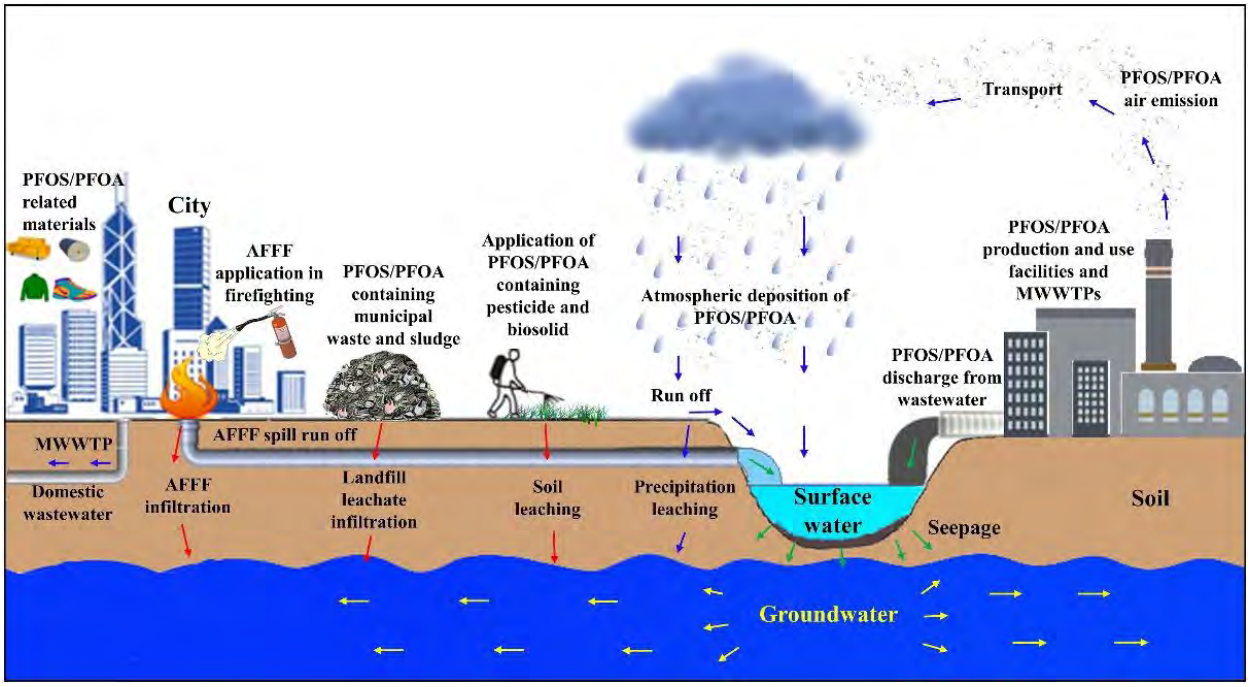



**Figure 2**: Different pathways of PFOS/PFAS into the environment. This figure is taken from the study of Lui et al. (2017).

PFAS that change and/or degrade into substances from the PFSA family of perfluoroalkyl substances, such as PFOS, are still manufactured (Ahrens 2011). It is known that PFOS is produced during the metabolic conversion of PFAS precursors like fluorotelomer sulfonates and perfluoroalkyl sulfonamidoacetic acids, as well as during the breakdown of volatile PFAS like

perfluoroalkyl sulfonamidoethanols (Lange, 2000; Boulanger et al., 2005; Rhoads et al., 2008; Benskin et al., 2009; Plumlee et al., 2009; Buck et al., 2011; Liu and Avendaño, 2013; Ahrens and Bundschuh, 2014; Wang et al., 2017). To completely comprehend the processes of transformation and their function as a source of PFOS for aquatic habitats, however, more research is required (Lau et al., 2007; Buck et al., 2011; Liu and Avendao, 2013; Wang et al., 2017). It is unclear and challenging to assess the how the precursors contribute to the existenceof perfluorooctane sulfonate in our environment. However, PFOS can be continuously produced from these precursors. Since PFOS manufacture has not taken place in the United States since 2002, the degradation of precursors may in particular constitute a large portion ofthe sources of perfluorooctane sulfonate in the aquatic environment (Buck et al., 2011; Liu andAvendao, 2013). But PFOS treated items like fabrics, paper, and some other articles are still brought into the US, where they are eventually discharged into the environment (Liu et al., 2014; Allred et al., 2015; Lang et al., 2016; Wang et al., 2014a, 2014b). According to the ToxicSubstances Control Act (TSCA), importing items treated with PFOS counts as production (US-Environmental Protection Agency, 2020).

### 1.2.2 PFOS concentration levels in the environment

Between 1980 and 2002,42,000 tonnes of PFOS were released into aquatic ecosystems, compared to 235 tonnes discharged into the atmosphere globally. These emissions are anticipated to act as a reservoir for PFOS in soil and aquatic environments (Paul et al., 2009; Rankin et al., 2016). PFAS are manmade substances with no known natural source, in contrast to other pollutants that are frequently found in aquatic habitats, such as metals. Therefore, thepresence of any PFAS chemical in the environment is a sign that it came from human activity (Ahrens et al., 2011). Comparatively to the other persistent bioaccumulative pollutants, quantitative analyses making, point and nonpoint source releases and quantification in the environment are scarce for PFOS (Ahrens and Bundschuh 2014; Zhang et al., 2016).

Studies have reported a variety of PFOS values in surface and groundwater. Median reported concentration in surface water as reported by studies is 3.2 ng/L (Saito et al., 2003; Taniyasu et al., 2003; Nakayama et al., 2007; So et al., 2007; Becker et al., 2008a; Becker et al., 2008b; Furdui et al., 2008; Ahrens et al., 2009a; Ahrens et al., 2009b; Jin et al., 2009; Quinete et al., 2009; Teng et al., 2009). The scientific literature is devoid of information on perfluorinated compounds

(PFC) concentrations in soils (Washington et al., 2008; Li et al., 2010; Yuan-Yuan et al., 2010; Wangand Shih, 2011); reported amounts are frequently below detection thresholds. Strynar et al. (2012) calculated an overall average soil content of 0.472 ng/gm for PFOS by examination of 10 soil samples each from of six nations (US, China, Greece, Norway, Mexico, and Japan). Additionally, 0.54 ng/gm, 69 ng/gm, and 11 ng/L, respectively, are the overall global reported median amounts of PFOS in sediments, sewage sludge, and wastewater treatment effluent (Zareitalabad et al., 2013).

In 2005, a worldwide study of perfluorinated acids in the oceans was conducted. Samples of seawater taken in various international research expeditions conducted between 2002 and 2004 from 19 places in the central to eastern Pacific Ocean, 5 in the South China and Sulu Seas, 12 in the north and mid-Atlantic Ocean, and 1 in the Labrador Sea (20). Further, 50 seawater samples from the coasts of different Asian nations (China, Korea, Japan) were examined. The recorded levels of PFOS concentrations ranged from lower values of 1.1-20 pg/L (surface water of the Central to Eastern Pacific Ocean) to average higher levels of 70- 2600 and 23-9680 pg/L in coastal areas of China and Hong Kong, respectively. Tokyo Bay recorded the highest amounts, ranging from 338 to 57,700 pg/L, whereas the Western Pacific,North, and Mid Atlantic had readings of less than 100 pg/L (Yamashita et al., 2005). Tokyo Bay may have greater quantities due to the six plants in Asia that produce and supply PFOS- related compounds, four of which are in Japan (Paul et al., 2009).

Few studies have used various experimental methodologies to measure and research the presence of PFOS in air, and there is a dearth of information on PFOS concentrations in the atmosphere and ambient air. Ionic perfluoroalkyl sulfonates (PFASs) were sampled with polyurethane foam-based passive air samplers (PUF-PASs) over 2-3 months in 3 locations: thenorth-west of England (15 sites), the UK-Norway transects (11 sites), and a European survey (23 sites). According to the study, PFOS levels in England ranged from 1.5 to 720 pg/sample/day. Only the UK samples from the UK-Norway transect campaign (2.7-7.7 pg persample per day) and the EU survey (1.9-69 pg/sample per day) were found to contain PFOS. In the US, ambient fine particulate matter (PM2.5) PFOS values varied from 0.18 to 14.1 pg/$m^3$, which is comparable to earlier studies of PM2.5 from Europe and Canada (0.02-3.5 pg/$m^3$) (Zhou et al., 2021). As of 2022, Pakistan has only conducted one study to measurethe presence of PFOS in any environmental matrix. In

Karachi, Pakistan, total suspended particle samples taken throughout the winter were analyzed for PFAS in this study. Having a median value of 1.70-0.79 pg/$m^3$, PFOS was measured and accounted for 17% of the total PFAS (Lin et al., 2022). To effectively combat flames involving hydrocarbons, aqueous film-forming foams (AFFF) products frequently contain PFOS and fluorotelomer sulfonic acids (FTSAs) as additives (D'Agostino and Mabury, 2017). Therefore, one of the potential sources may be employing AFFF related materials for firefighting exercises at adjacent military bases and airports. The average PFOS concentration in air samples (1.69 pg/$m^3$) was a bit lessthan that found in urban sites in China, including Nanjing (4.30 pg/$m^3$), Dalian (5.73 pg/$m^3$), and Changshu (8.37 pg/$m^3$) (Liu et al., 2017; Yu et al., 2018). Additionally, average concentrations in Japan's urban and rural areas were foundto be 5.3 and 0.6 pg/$m^3$, respectively (Sasaki et al., 2003).

Polar regions are excellent research locations because they are largely separated from other ecosystems and can be used to examine the environmental behavior of pollutants (Gao et al., 2020). Ionizable perfluoroalkyl compounds travel by ocean currents and even aerosols, whichare then deposited on land (Zhen et al., 2014; Casal et al., 2017b), whereas neutral perfluoroalkyls can travel over great distances due to their extensive half-life in the atmosphere and can thus travel to polar regions (Nash et al., 2010; Young and Mabury, 2010; Zhao et al., 2012; Stock et al., 2007) The Arctic and Antarctic regions have seen widespread detection of legacy PFASs, such as PFOS, in oceans, biota and sediments (Pedersen et al., 2015; Letcher et al., 2018; Cai et al., 2012; Llorca et al., 2012; Zhao et al., 2012). Antarctic sediments have been observed to contain PFOS up to 0.2 ng/gm (Gao et al., 2020). The concentrations of perfluorooctane sulfonate in Arctic snow ranged from 2.6 to 86 pg/L. There are three distinct parts to the PFOS time course in the Arctic. From 1996 to 1998, there was a rise ($p<0.001$), which was followed by a reduction ($p<0.001$) from 1998 to 2001. After 2001, there wasnodiscernible trend in the amounts of PFOS ($p = 0.094$). (Young et al., 2007).

### 1.2.3 Toxicological data for PFOS

PFOS are pervasive in the environment around the world and have been found in both terrestrial and aquatic living organisms, including people. These substances are persistent, bioaccumulative, and toxic, and exist detected at many ng/L values in the water environment of different counties. Perfluorooctane sulfonate has a prolonged half-life (more than 5 years), which makes it a

persistent contaminant in the natural environment due to overuse (Chowdhury et al., 2022). For instance, fish (0.49 mg/L), birds (2.50 mg/L), reptiles (0.17 mg/L) andmammals (3.07 mg/L) have been found to have higher perfluorooctane sulfonateconcentrations (Chen et al., 2018; Stylianou et al., 2019). Taniyasu et al. (2002) discovered theoccurrence of perfluorooctane sulfonate in fish samples taken from several locations in Japan,with values ranging from 2 to 834 ng/mL. Fish liver samples from Lake Minto, Quebec, and theGreat Whale River in Kuujuarapik, Canada, were both found to have PFOS, with concentrations ranging from 5.7-50 ng/gm (Martin et al., 2004). PFOS was detected in the livers of numerous species of fish that were examined from the Great Lakes and inland rivers of New York and Michigan (Sinclair et al., 2004).

Additionally, perfluorooctane sulfonate was found in fish eggs, however at amounts that were roughly twice as high as those found in the fish's livers. The adult female fish actively transmit PFOS to its eggs. Additionally, PFOS has the capacity to interfere with, breach, and accumulate across the blood-brain barrier (Yu et al., 2020). Researchers have discovered that PFOS can cross the placental barrier and cause developmental harm, including prenatal mortality and fetal growth retardation (Fuentess et al., 2005; Li et al., 2016). These findings were made in studies with pregnant mice. Taves and Shen (1974) were the first to discover organic fluoride in humans, and the organic substance was initially considered PFOA and potentially PFOS (Taves et al., 1976). The amount of PFOS found in non-occupationally exposed people ranged from 8.1 to 150.7 ng/L. (Wilhelm et al., 2009). PFOS was also found in human placentas and blood-brain barriers, as well as in breast milk (Lee et al., 2015; Wang et al., 2011).

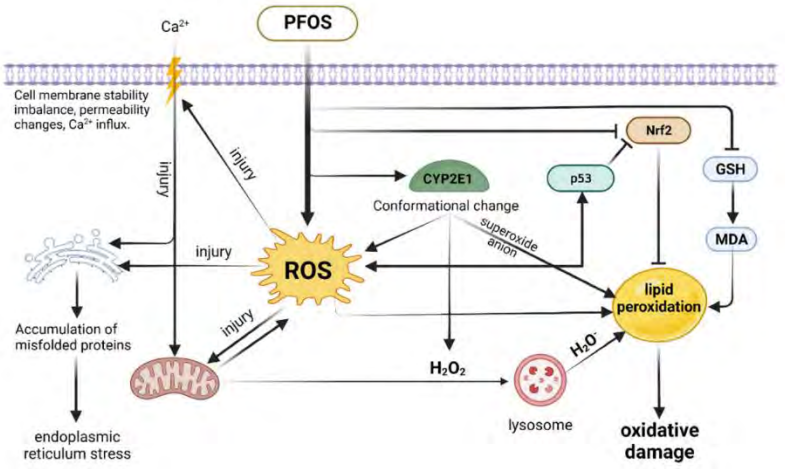



**Figure 3:** Mode of action of PFOS on liver. This figure has been obtained from the following study (Wang et al., 2022).

Potential toxic effects of PFOS have been researched upon in rats, fish, and monkeys (Seacat et al.,2002; Austin et al., 2003; Thibodeaux et al., 2003; Martin et al., 2003a; Martin et al., 2003b; and humans, Olsen et al., 1999; Olsen et al., 2003b; and Olsen et al., 2003c). According to toxicity studies, PFOS (0.75mg/kg/day) causes hypolipidemia, lowers serum cholesterol, raises liver triglycerideand cholesterol levels, and decreases food intake in monkeys (Butenhoff et al., 2002, Seacat et al., 2002). Further, PFOS caused hepatotoxicity, immunotoxicity, developmental toxicity, or neurotoxicity effects in land animals like rats or mice (Fuentes et al., 2007; Lau et al., 2003; Peden-Adams et al., 2008; Thibodeaux et al., 2003); and fish (Du et al (Ankley et al., 2005). The transport and metabolism of fatty acids, mitochondrial function, cell membrane integrity, liver superoxide dismutase activity and glutathione peroxidase, and reduction in levels of thyroid hormone are a few of the processes that these toxicities can influence (Lau et al., 2004; Lau et al., 2007). As an endocrine disruptor, PFOS has been shown to have an impact on rats'neuroendocrine systems (Austin et al., 2003). The 48-hr LC50 for *D. magna* and *D. pulicaria*, respectively, in those investigations was 130 mg/L and 169 mg/L, respectively. The LC50 for adult and embryonic zebrafish was reported by Sharpe et al. (2010) to be 22 mg/L and 3.05 mg/L, respectively, while the LC50 for zebrafish embryo was reported by Hagenaars et al. (2011) to be 58.47 mg/L (96 hrs).

Additionally, several research had suggested that PFOS might make other substances more harmful. Researchers have recommended using mixture toxicity models in the combined toxicity assessments (Altenburger et al., 2012). Early studies reported the toxicity of pollutants when combined. For instance, co-exposure to PFOS and cadmium increased the toxicity to zebrafish development and survival in the early life stages (Kim et al., 2011). Zebrafish survival rates were lowered by PFOS and BPA co-exposure (Keiter et al., 2012). To assess thecombined toxicity of pollutant mixtures, the co-exposure effects of the individual contaminants were considered (Sarigiannis and Hansen, 2012).


Algae


Earthworms


Zebrafish


Zooplankton


Albino mice

## 1.3 Nanotechnology

The manufacturing of a range of substances, including materials having at least one dimensionless than 100 nanometers (nm), is made possible by the advanced field of research known as Nanotechnology (Saleh et al., 2020). Richard Adolf Zsigmondy coined the phrase "nanometer"in 1914. (Santamaria et al., 2012). The length of a nanometer is better understood by imagining 5 silicon atoms or 10 hydrogen atoms, lined up, each of which is equal to one nanometer in length. If a material's size or one of its dimensions is between 1 and 100 nm, it is referred to as a nanomaterial (Baig et al., 2021).

When scientists discovered that a substance's size affects its physicochemical qualities,including chemical, mechanical, electrical, and optical properties, they began to understand thesignificance of nanomaterials. Due to their special qualities, nanoparticulate materials have garnered a lot of interest (Gleiter, 2000). Water treatment facilities, industrial processes, catalytic processes, petrochemical industries, structures and construction materials, diagnostics, and medicine delivery are just a few of the potential uses of nanoparticles (NPs).There are various ways to classify nanomaterials based on their properties, uses and synthesismethods, but in terms of chemistry and interactive behavior, we generally have three types; Inorganic, organic and hybrid nanomaterials, which are further classified into more specific groups (Figure 4).

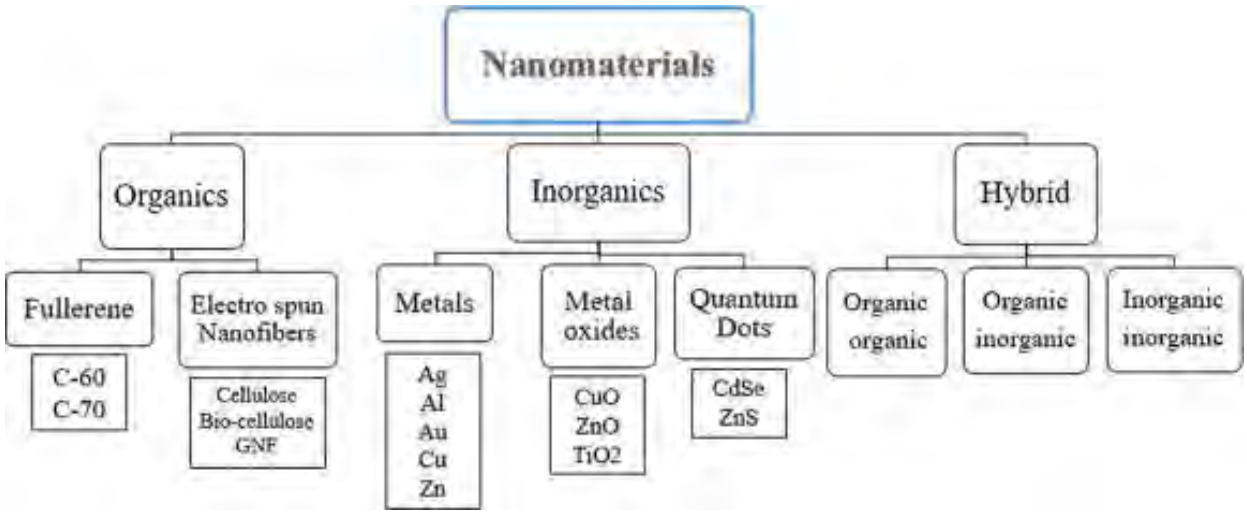



**Figure 4:** Types of Nanomaterials based on chemical characteristics.

## 1.4 Nanoparticles in the environment

It's a challenge to predict the appropriate the exact amounts of nanoparticles being released due to the fact that numerous entry points exist for manmade nanoparticles in the environment, including release directly into the environment, effluent, and sludge from wastewater treatment plants (Mueller et al., 2008; Gottschalk et al., 2010). Three emission scenarios are typically taken into consideration when NP is released into the environment throughout their life cycle: release from (i) the manufacture or raw materials and nano substances; (ii) application; and (iii) discharge after the disposal (waste handling) (Gottschalk et al., 2009; Gottschalk et al., 2013; Tolaymat et al., 2017). Additionally, changes to nanomaterials' surfacemoieties, agglomeration, dissolution, or other processes may have a significant impact on the manner and extent of environmental release (Maurer-Jones et al., 2013).

It has been established that produced nanoparticles will inevitably be released into the aquatic environment as a result of increased production and use. According to several studies, metals supplied to organisms as engineered nanoparticles (ENPs) are ingested by them from food sources. Typically, ENPs are blended into sediments or added to food sources like algae. Predation of these organisms may result in the uptake and accumulation of Me-ENPs or, in cases of particle breakdown, at least the metal ions making dissolved from the NPs once they have been absorbed in the living organisms as dissolved metal or metal engineered nanoparticles taken up in the gut or absorbed across the epithelia. Because of the changes occurring once the particles get into the environment, particularly aquatic environments, it is challenging to describe the behavior and

Transport of intact nanoparticles along aquatic food webs. Although there are currently just a few studies on this subject, trophic transfer of nanoparticles has been reported in aquatic food chains, and this issue needs additional research. The trophic transmission of Me-ENPs in terrestrial habitats has also been studied in a few studies, with the tobacco hornworm receiving intact gold (Au) nanoparticles from tomato andtobacco plants. When transferred from low-level trophic level (i.e., accumulated in leaves), gold nanoparticles were accumulated in the hornworms; however, uptake was not observed when the nanoparticles were adsorbed (i.e., applied to leaf surfaces) on the leaves. These experiments showed how accumulation of metallic nanoparticles in the consumer organism can vary depending on how they are taken up by the prey along with potential

transportation of intact metal nanoparticles along the food web. Inhalation through the respiratory tract, ingestion or uptake in the gastrointestinal tract, dermal contact, and injection into the blood arethe most common ways that humans are exposed to nanoparticles (Wu and Tang 2018). Upon entering the human body, nanoparticles might pass through several biological barriers and canget to the most vulnerable targets, such as kidneys, lungs and the liver, causing damage to the DNA, and can ultimately lead to cell apoptosis/death (Ahamd et al., 2010; Bahadar et al., 2016; Shin et al., 2015; Tan et al., 2018).

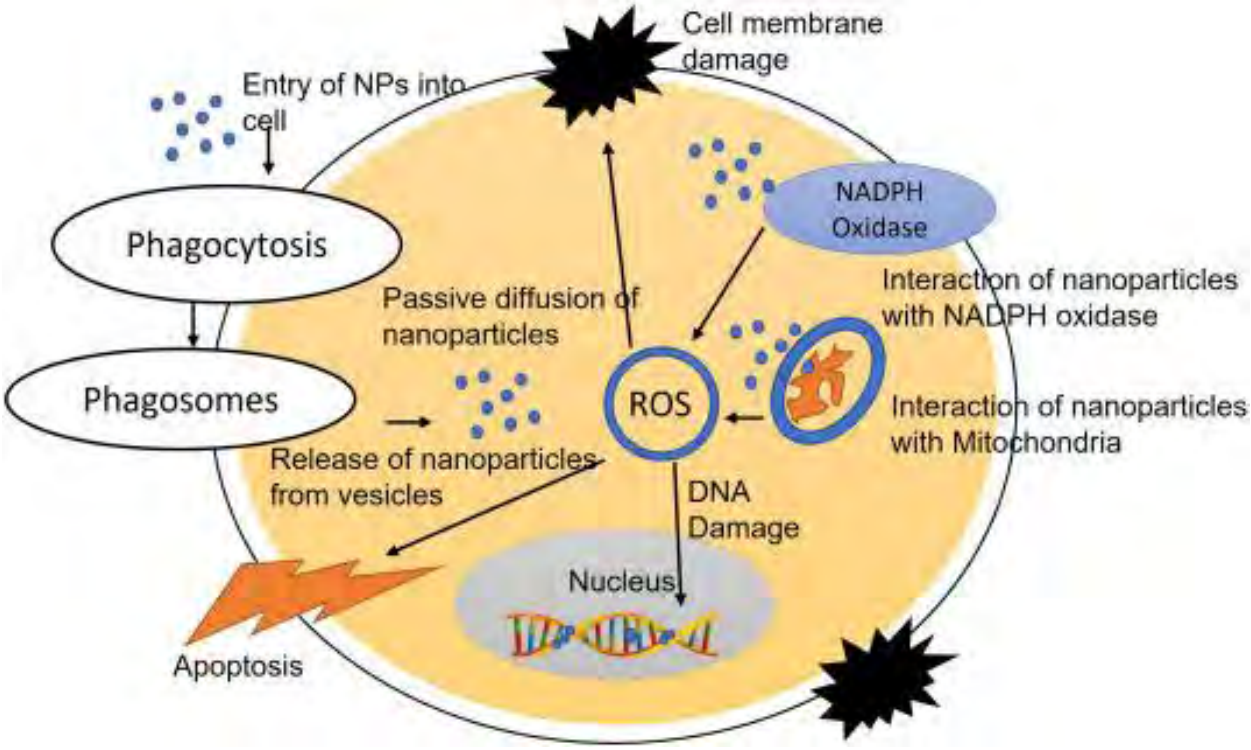



**Figure 5:** Nanoparticles can enter cells and cause cellular damage through various mechanisms. This image is taken from the study of PJ et al. (2021).

## 1.5 Copper oxide nanoparticles (CuO-NPs)

Copper oxide nanoparticles (CuO-NPs) are smart transition metal-oxide nanomaterials, and have a low bandgap of 2.0 eV, excellent electrochemical activity, a high specific-surface area, the appropriate redox potential, and exceptional solution strength (Khan et al., 2014; Giri et al., 2016). It is one of the most sought-after materials, second only to noble metal nanoparticles, because of its potential uses in a wide range of fields, including catalysis (Bhosale et al., 2014;

Momeni et al., 2018), electrochemistry (Pendashteh et al., 2013; Zampardi et al., 2018), sensors/biosensors(Alizadeh et al., 2014; Di Toc (Sivaraj et al., 2014). As nanotechnology was introduced in biomedical research, application of CuO nanoparticles in the field have developed dramatically. Owing to their large specific surface area and propensity to promote electron transfer processes at lower overpotential, they are used in non-enzymatic sensing of therapeutically relevant analytes (Rahimi-Nasrabadi et al., 2013; Shabnam et al., 2017). CuO nanoparticles have demonstrated their pharmacological activity, particularly in the treatment of cancer (Wang et al., 2013; Yang et al., 2017).

Their widespread use without any kind of oversight raises serious safety concerns. Asia alone will be responsible for between 8 and 10 percent of the CuO-NP contamination in the environment, with annual releases ranging from 22-200 metric tonnes (Keller and Lazareva 2013). It inevitably enters the aquatic system, damaging the aquatic habitat, just like any other contaminant (Mani et al., 2019). The availability of coppercontaining nanoparticles in the natural environment, however, is not well known. Maximum regional data for emissions to water bodies were estimated to be 3, 16, and 2 metric tonnes annually in Europe, Asia, and North America in 2010. (Keller and Lazareva, 2014). For majorTaiwanese rivers, the predicted environmental concentration of Cu NPs is 0.06 mg/L, with a 95% confidence interval of 0.01-0.92 mg/L (Chio et al., 2012).

CuO-NPs have a significant adverse effect on the ecosystem, with certain studies showing their trophic transmission through the food chain from organisms at the lower level to those at the top (Ates et al., 2015). The basic mechanism of copper oxide nanoparticle toxicity in the aquatic environment has been identified through studies on aquatic flora ( Zhao et al., 2017; Shi et al., 2011), crustaceans (Xiao et al., 2015; Wu et al., 2015), and teleost (Sun et al., 2016; Xu et al., 2017). It is currently unclear how they cause biological toxicity, with many reports presenting conflicting information. Increased bioavailability and toxicity are caused by the high surface area and reactivity (Bhatt and Tripathi 2011). Through Fenton's reaction, the high redox- potential of copper oxide nanoparticles produce free radicals that result in reactive oxygen species (ROS) (Chang et al., 2012). Their quick disintegration in water, made possible by theirsurface stability, is ingested by fish with the majority of buildup occurring in the muscle tissue.There have been reports of CuO nanoparticles causing toxicity in the kidney (Sarkar et al., 2011), lungs (Ahamed

et al., 2010), liver (Siddiqui et al., 2013), and brain (Wang et al., 2014).

Because ions of copper suppress the enzyme ZHE1 for hatching in zebrafish, exposing them at embryonic stage to CuO nanoparticles disrupts normal hatching (Lin et al., 2012). Another significant component that may have an impact on the material's toxicological reaction is the dissolution of CuO-NPs into Cu ions in aqueous suspension (Ahamed et al., 2015). The ionic form of copper is therefore more harmful than the NP form, as both copper oxide nanoparticlesand ions of copper caused reduction in hatching of embryos and increased deformity in zebrafish embryos (Vicario-Parés et al., 2014). Copper oxide nanoparticles have been therefore often reported to be less toxic to zebrafish than the ionic form (Lin et al., 2011, 2012; McNeil et al., 2014). Zebrafish embryos exposed to 0.05 milligram/L of copper nanoparticles experience toxicological effects that reduce the amount of functioning lateral neuroblasts andcause alteration of behavior. These toxic effects are greater when fish are exposed to CuSO4 than in fish subjected to Cu NPs (McNeil et al., 2014). However, the presence of free metal ions does not always, or only largely, account for the acute toxicity of the metal and metal oxide nanoparticles. Similar to this, the idea of the LC50 for nanoparticles is not standardizedbecause it varies depending on factors like size, synthesis method, behavior in test medium, and many others. According to Griffitt et al. (2008), the 48-hour LC50 for dissolved and nano-sized forms of copper in juvenile zebrafish is around 1.78 mg/L and 0.71 mg/L, respectively. The LC50 for CuO nanoparticles in adult zebrafish is 400 mg/L (Mani et al 2020). In addition, Griffitt et al. (2009) identified unique "nano" effects in adult zebrafish at the tissue (gill histology) and molecular (gill transcriptome) levels.

**Table 2:** Literature review of different studies of CuO-NPs evaluating the effects on Zebrafish.

| Contaminant | Exposure | Doses | Studied Parameters | Responses | References |
|---|---|---|---|---|---|
| CuO-NPs, 50–60 nm | Zebrafish Embryo, 96hpf | 0, 25, 12.5, 6.25, and 1 mg/L | Histology and Immunochemistry, SOD and Behavioral Analysis | CuO NP aqueous exposure at high doses (12.5 mg/L or above) activates xenobiotics-metabolizing enzymes, induces an inflammatory response, and shows developmental toxicity on the zebrafish liver and CNS. | Sun et al. (2016) |
| CuO-NPs, 20–95 nm | Zebrafish Embryos, 4 to 120 hpf | 0.1, 0.5, 5, and 50 mg/L | Genes related to cell migration of gastrulation, neural differentiation and cardiogenisis | The mortality in the 50-ppm CuO-NP exposed group was 60% at 24 hpf and increased to approximately 80% at 120 hpf. In the 5 ppm CuO-NP-exposed group, the mortalities increased to approximately 20% at 96 hpf and 40% at 120 hpf. CuO-NP exposure could induce abnormal phenotypes of a smaller head and smaller eyes, affect dorsoventral patterning, disturb cell migration of gastrulation, decrease the sizes of several structures in the neural system, and prevent looping of the heart tube during cardiogenesis | Xu et al. (2017) |
| CuO-NPs, Avg. 51 nm | Zebrafish Embryo, 96hpf | 0, 5, 10, 20, 40, 60, 80, 100 and 120 mg/L for LC50. 0, 40 and 60 mg/L for sublethal tests | Lethality, spinal cord malformation, end tail malformation, tail malformation, heart malformation, rachischisis, heart rate and hatching delay. Total protein content, AChE activity and Na+/K+-ATPase activity. Oxidative stress parameters | LC50 of CuO-NPs in zebrafish embryos was about 64 mg/L. CuO-NPs caused a 2% death rate at 5 ppm, and mortality increased up to 100% at 120 ppm at 48 hpf. Concentrations of 40 and 60 ppm were toxic to embryos | Ganesan et al. (2015) |
| CuO-NP, 6nm and Ionic Copper | Zebrafish Embryo and Fry, 96hpf | 0.1, 0.5, 2, 10, 50, and 200 µM Cu | Embryo mortality, Hatching success, Zebrafish locomotion and survival | Zebrafish fry mortality was only caused by Cu ions, whereas swimming activity was affected both by Cu ions and CuO-NPs. Overall, Cu ions were more toxic than CuO-NPs to both embryos and fry | Thit et al. (2017) |

| | | | | | |
|---|---|---|---|---|---|
| CuO-NPs, ≤50 nm. | Zebrafish Embryo, 96hpf | 0.5, 1, and 1.5 mg/L | Immunotoxicity. Other endpoints including mortality, hatching, and malformations were recorded | 1 and 1.5 mg/L CuO-NPs application caused significant decrease in the heartbeat rate at 48 and 72 hpf compared to the control groups. Mortality for 0.5, 1 and 5 mg/L was 13, 22 and 30 % at 96 hpf respectively. | Aksakal and Ciltas (2019) |
| CuO-NPs, Avg. 40.3 nm | Zebrafish Embryo, 96hpf | 0.2, 0.4, 0.6, 0.8, 1, 1.2, 1.4, 1.6, 1.8, and 2 mM | Cardiac performance assessment and molecular docking. Not very relevant | Copper oxide nanoparticles and carbofuran work synergistically to increase toxicity in the cardiac system of zebrafish showing irregular heartbeats | Saputra et al. (2021) |
| CuO-NPs, Avg. 30 nm | Zebrafish Embryo, 96hpf | 1, 5, and 10 mg/L | Mortality, hatching, heartbeat, and malformation | CuO NP toxicity increased with decrease in Ionic strength of exposure medium | Chao et al. (2021) |
| CuO-NPs, Avg 29.5 nm | Zebrafish Embryo, 120 hpf | 10 mg/L | Survival, hatching rates and malformation | Exposure to CuO-poly NPs or bulk CuO up to 10 mg Cu/L did not produce any significant decrease on embryo survival. A LC50 value of 3.083 mg/L was calculated for ionic copper which increased significantly embryo mortality at 5 and 10 mg/L. CuO-poly NPs showed higher toxicity than bulk CuO. Significantly decreased hatching rate was observed in embryos treated with CuO-poly NPs at 10 mg Cu/L | Vicario-Parés et al. (2014) |
| CuO-NPs, avg 29.5 nm and Ionic Copper | Adult Zebrafish, 3 days, 21-Days and 6 months | 10 µg/L Cu | Accumulation, Histology, Micronuclie Tests, Lysosomal Membrane Stability, and Transcriptomics | Significant copper accumulation was only detected in fish exposed to ionic copper. In all parameters, ionic copper was found to be more harmful than nano copper | Vicario-Parés et al. (2014) |
| CuO-NPs, <50 nm | Adult Zebrafish, 30 Days | 1 mg/L and 3 mg/L | Total protein content, functional markers, mRNA expression levels, histology and immunochemistry, oxidative stress, and AChE activity | Chronic exposure to CuO-NPs caused muscular toxicity which may lead to muscle degeneration in adult zebrafish | Mani et al. (2020) |
| CuO-NPs, Avg 18.2 | Zebrafish Embryo, 96 hpf | 1, 2, 5, 10, 20, 30, 40, 50, 60, 80 and 100 mg/L | Acute toxicity and hatch inhibition, activities of Na+/K+-ATPase and GSH | LC50 values for CuO-NPs were: 6.6 (4.5–8.5), 19.4 (11.6–27.2) and >100 mg L−1 at pH 5, pH 6 and pH 7. Calculated NOECs were 5 and 40 mg L−1 CuO-NPs at pH 6 and pH 7. LOECs for CuO-NPs were 1, 10 and 50 mg L−1 at pH 5, pH 6 and pH 7. At pH 7 calculated EC50 was 11.05 mg/L CuO-NPs | Boyle et al. (2020) |

## 1.6 Interactive toxicity of PFOS and nanoparticles

Numerous studies have found that PFOS is more readily absorbed and accumulated when nanoparticles, particularly metal oxide nanoparticles, are present. According to Qiang et al. (2016), the presence of nano-TiO2 may have an impact on the vertical distribution of PFOS in water and may promote its bioaccumulation in fish dwelling in various water strata. This was primarily caused by the creation of TiO2-PFOS complexes, which fish species ingested, increasing their exposure to PFOS. Fish quickly absorbed PFOS after it had been released from TiO2 and had entered their digestive systems, which increased the amount of PFOS they had accumulated. As a result, the presence of nano-TiO2 increased the danger of PFOS to the environment. Given that PFOS may be biomagnified and transmitted to humans who consumefish as one of their dietary sources, this caused significant alarm. When TiO2 is deposited in areal water system, it may transfer organic compounds to the sediment, raising the danger of exposure for benthic deposit-feeders, which consume a lot of particles. To comprehend the consequences of manmade nanomaterials on invertebrates in aquatic systems, more research is required. According to a different study, co-exposure to PFOS and ZnO-NPs can result in more severe oxidative stress and apoptosis than either substance alone. The interaction betweenPFOS and ZnO-NPs may be one of the key processes underlying their toxicity to zebrafish embryos (Du et al., 2017). ZnO nanoparticles and PFOS exposure together also caused thyroid dysfunction in developing zebrafish larvae (Du et al., 2016). According to Li et al. (2017), SWCNT may absorb PFOS from water, which lowers the bioconcentration in zebrafish tissueand increases it in skin. The effects of PFOS on ROS, SOD, CAT, and AChE activity were exacerbated by co-exposure. Co-exposure was the exposure circumstance that caused the highest stress, according to integrated biomarker response (IBR). This helps us better understand the complicated effects of single walled carbon nanotubes (SWCNT) on the toxicity of PFOS in aquatic environments. In a separate study, the effects of prolonged co-exposure to PFOS and nano-ZnO on growth in first-generation (F0) zebrafish as well as potential maternal transmission of these effects on the development of first-generation (F1) larvae were studied. Bioconcentration led to considerably lowergrowth as evaluated by body length and body weight, increased mortality, and reduced spawning in the F0 generation in zebrafish that were exposed to single- and co-exposure groupsfor

120 days. Less fertilization, fewer eggs hatching, more mortality, and more deformity were detected in the F1 generation after prolonged exposure. Transgenerational toxicity might be caused via the downregulation of genes and hormones. This study revealed that long-term exposure to PFOS and nano-ZnO negatively affects embryonic growth, reproduction in the F0 generation, and development (Du et al., 2018).

Heavy metallic cations like Cu (II), in addition to PFOS and nano-oxides, are frequently found in aquatic settings (Lu et al., 2016). Numerous investigations on the adsorption of heavy metals on nano-oxides have suggested that the surfaces of the divalent metal cations typically form inner-sphere surface complexes with the oxides (Hua et al., 2012). Heavy metallic cations have also been observed to affect the adsorption of ionizable organic pollutants (Hyun et al., 2015). The adsorption of PFOS at high concentrations onto carbon nanotubes (CNTs) was explored by Zhou et al. (2012), and they discovered that the addition of Cu ions greatly boosted the adsorption of PFOS (II). Due to its sulfonate group and hydrophobic fluorinated carbon chain, PFOS can be adsorbed on the surface of adsorbents through a variety of interactions, including hydrogen bonding, hydrophobic interaction, electrostatic interaction, and ligand exchange (PFOS molecules' sulfonate groups may serve as the paired groups for functionalities on adsorbents) (Qiang et al., 2015).

According to published research, copper-based nanoparticles (CuO-NPs) release enough copper ions in the right media to have an impact on biological systems. When compared to bulk CuO-NPs, CuO-NPs exhibit a 40– to 50–fold increase in toxicity. Cu and PFOS have been found to coexist in large quantities in China's aquatic environments; they were both found in the Yangtze and Dayan rivers. Additionally, Cu and PFOS were discovered in the waterways of other nations, such as the Tennessee River and the Danube River (Zheng et al., 2015). Compared to the effects of a single chemical, the combinations of heavy metals and organics can have diverse harmful effects on aquatic life. According to Feng et al. (2016), *L. hoffmeisteri* can become acutely toxic and experience oxidative stress after receiving short- term exposure to Cu (II) in the presence or absence of PFOS at various pH levels. These were supported by measurements of the 24hour LC50, weakened antioxidant defenses, and elevated levels of lipid peroxidation. Notably, the bioaccumulation and toxicity of Cu (II) in test species were dramatically impacted by the presence of PFOS. According to the IBR data, synergism was suggested for the combined effects.

Additionally, *L. hoffmeisteri* may experience more harm in conditions that are acidic. Therefore, pH should be taken into consideration when evaluating the toxicity of Cu (II) and PFOS in natural waters. Another study examined the single and combined toxicity of copper, perfluorooctanoic acid, and perfluorooctane sulfonate to *Carassius auratus*. The investigation's findings demonstrated that both individual andcombined exposure to PFOS, PFOA, and Cu led to an increase in lipid peroxidation and decreased antioxidant defense in the fish liver, which indicated an oxidative stress. Based onthe IBR data, a preliminary ranking of toxicity was made, and synergistic effects were postulated for any potential combined effects. Additionally, these treatments had a considerable impact on the homeostasis of trace elements in fish tissues, and it was also foundthat these test substances obviously bioaccumulated. However, more research is required to examine the joint-action toxicity and underlying mechanisms of PFCs and other pervasive contaminants on various tissues of aquatic species after chronic exposure due to the chemical complexity of the natural aquatic environment. Further research should look at the potential joint-action toxicity and processes on aquatic animals of Cu (II) and copper oxide nanoparticles mixed with other organics at different trophic levels in light of the chemical complexity in realistic waters.

**Table 3:** Literature review for studies reporting the interactive effects of different nanoparticles with PFOS focusing on zebrafish.

| Nanoparticle | PFOS | Experimental Animal | Nanoparticle | PFOS | Parameters Studied | Toxicological Impacts | References |
|---|---|---|---|---|---|---|---|
| ZnO 40nm | Heptadeca-fluorooctane sulfonic acid (PFOS) potassium salt | Zebrafish embryos | 1, 5, 10, 20, 50, 100, 200 mg/L | (1, 2, 4, 8, 16 mg/L) | Antioxidant enzymes activity | It has been proven that oxidative stress and apoptosis induced by the PFOS at different concentrations are enhanced in the presence of the ZnO-NPs after 96 h exposure | Du et al. (2016) |
| SWCNT 1–2 nm | Perfluorooctane sulfo nate potassium | Mature Zebrafish | 0, 5, 10, 15 mg/L | 200 µg/ L | Oxidative stress | Co-exposure enhanced the injury effect of PFOS on ROS, SOD, CAT and AChE activity | Li et al. (2017) |
| ZnO particle dispersion (CAS no. 1314-13-2, <100 nm) | PFOS (CAS no. 2795-39-3; ≥98% purity | Zebrafish embryos | (1.7, 3.4, 6.75 mg/L) nano-ZnO | (0.05, 0.1 or 0.2 mg/ L) | Parental Transfer | Exposure to PFOS and nano-ZnO in parents resulted in a lower fertilization rate, a lower hatch rate, higher mortality, and a higher malformation rate in F1 larvae | Du et al. (2018) |
| MWCNT, OD approximately 50 nm, and length 10–20 µm | PFOS (CAS no., 2795-39-3, ≥98% purity) | Embryo and Larvae Zebrafish | 10, 20, 50, 100, and 200 mg/L | 0.2, 0.4, 0.8, and 1.6 mg/ L | Oxidative stress | After zebrafish larvae were exposed to PFOS, the activities of SOD, CAT, and GSH-Px, as well as the levels of ROS and MDA, decreased in the presence of MWCNTs, suggesting that oxidative stress and lipid peroxidation were relieved by MWCNTs | Wang et al. (2017) |
| Anatase Titanium dioxide 20-30 nm, Rutile titanium dioxide | Potassium perfluorooctanesulfon ate (PFOS, 98%) | Adult Zebrafish | - | 600 ng/ L | Accumulation | It has been proven that oxidative stress and apoptosis induced by the PFOS at different concentrations are enhanced in the presence of the ZnO-NPs after 96 h exposure | Qiang et al. (2017) |
| ZnO particle dispersion | PFOS (CAS no. 2795-39-3; 98% purity) | Zebrafish Embryo | 50 mg/L | 0, 0.4, 0.8, and 1.6 mg/L | Histopathologi cal Analysis | Our results indicated that the joint effects of PFOS and ZnO– NPs on zebrafish were synergistic in acute and chronic mixture toxicity tests | Du et al. (2016) |

## 1.7 Zebrafish model for toxicity screening

A tropical freshwater fish in the minnow family (Cyprinidae), the zebrafish (*Danio rerio*) is indigenous to rivers in Pakistan and India. The main advantages of zebrafish in toxicological research are their tiny adult size (only 3–4 cm in length), which significantly reduces the amount of space and money needed for husbandry. The optimum reported temperature for zebrafish is 28.5 °C (Westerfield et al., 2000), but it prefers a pH range of 7-8 and a temperaturerange of 6.7-41.7 °C (Lawrence, 2007). Zebrafish also have a short generation interval, consistently reproduce year-round, and are simple to keep in small recirculating systems (4-5 months). The table below shows how zebrafish are taxonomical classification of zebrafish.

**Table 4**: Zebrafish taxonomic classification.

| | |
|---|---|
| **Kingdom** | Animalia |
| **Phylum** | Chordata |
| **Class** | Actinopterygii |
| **Order** | Cypriniformes |
| **Family** | Cyprinidae |
| **Subfamily** | Danioninae |
| **Genus** | *Danio* |
| **Species** | *Danio rerio* |

We may also locate a wealth of material on zebrafish trials, which are one of the most suggestedand frequently used animal models for toxicity investigations. The similarity of the circulatory, neurological, and digestive systems of zebrafish to those of mammals is another significant benefit of this model organism (Hsu et al., 2007). In addition, zebrafish and humans with a high level of genetic similarity share highly conserved signaling pathways (Beliaeva et al., 2010). Zebrafish are a suitable animal model for analytical investigations due to the high degree of similarity between their and human genomes (about 75% similarity) (Chakraborty etal., 2009). Zebrafish are now incredibly valuable for toxicity testing. As a result, the zebrafishis becoming a potent model organism for research into genetics, development, environmental toxicity,

pharmacology, DNA damage repair, cancer, and other illnesses (Dia et al., 2014).

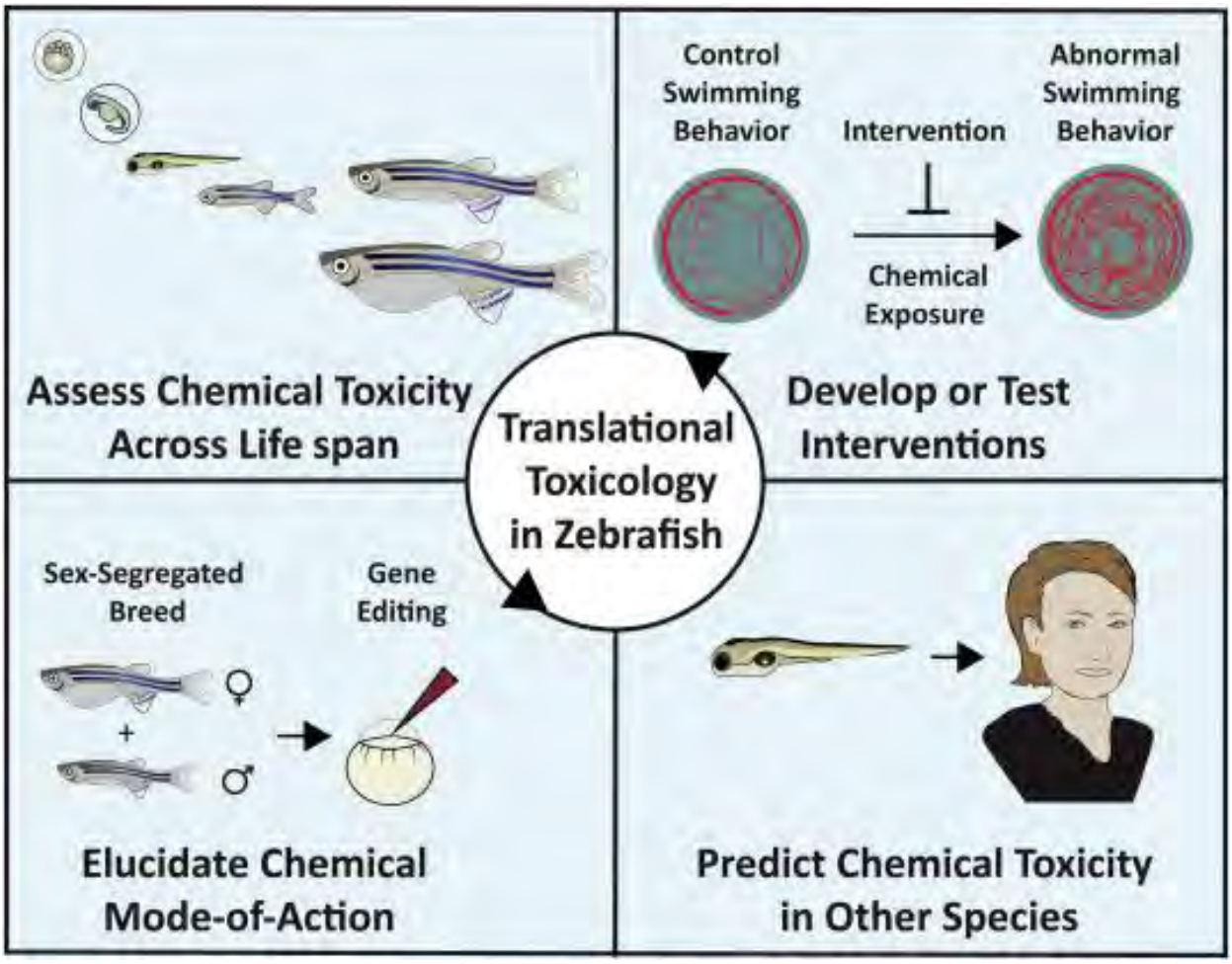



**Figure 6:** Zebrafish as a model species for standardized toxicity testing (Tal et al., 2020).

Use of *Danio rerio* as a recognized animal model for testing the toxicological effects of nanoparticles has increased significantly in recent years. Nanoparticle toxicity is measured using a variety of metrics, including success rate in hatching, developmental abnormalities ofdifferent organs, damage to the gills and skin, abnormality in behavior (movement), reproduction toxicity and lethality (Bruno et al., 2018). Similar to this, the zebrafish model has also been used to study persistent organic pollutants (POPs) and is nearly always used in mixture toxicity studies incorporating PFOS and nanoparticles (Almas, 2007; Du et al., 2018;Li et al., 2017; Qiang et al., 2016).

## 1.8 Toxicity biomarkers for the study

### 1.8.1 Acetylcholinesterase

The crucial neurotransmitter acetylcholinesterase (AChE) is involved in a number of functions for the central nervous system. This enzyme aids in the transmission of the neural message from one cholinergic neuron to the next by catalyzing the hydrolysis of acetylcholine in synaptic connections (Soreq and Seidman, 2001). AChE activity is frequently studied in brain and muscle tissue because the fish neuromuscular system is predominately cholinergic and because it is essential for optimal muscle behavior and function (Payne et al., 1996). Tetania, paralysis, and even death can occur as a result of AChE inhibition, which modifies brain behavior and overstimulates muscle fibers (Kirby et al., 2000). Fish brain AChE research hasreceived more attention than fish muscle AChE research (Ferrari et al., 2007).

### 1.8.2 Oxidative stress and antioxidant enzymes

Aquatic organisms may experience oxidative stress as a result of pollutants in the aquatic environment producing free radicals and reactive oxygen species (ROS). Chemicals in pollution can alter antioxidant defenses or directly cause oxidative damage to an organism byunbalancing ROS levels and antioxidant defense (Slaninova et al., 2009). The initial defense comprises of low-molecular-weight antioxidants including glutathione, vitamin E, and vitaminC, whereas the next line of defense consists of antioxidant enzymes (Valavanidis et al., 2006,Slaninova et al., 2009). SOD is typically regarded as the main enzyme that protects against reactive oxygen species and lipid peroxidation (Wang et al., 2011). SOD alters the toxic $O_2$ free radical into one that isless harmful, aiding in the catalysis of dismutation to create $H_2O_2$ (Halliwell and Gutteridge,2015). With the aid of the CAT enzyme, SOD generates $H_2O_2$ which is transformed into water and molecular oxygen. It is known that increased $O_2$ generation reduces activity of CAT(Kono and Fridovich 1982). For the elimination of $H_2O_2$, CAT and GPx enzyme collaborate.

The most popular analytical strategy in the field of free radical research is the oxidation of lipids since many species, particularly aquatic animals, have higher concentrations of lipids (Lushchak, 2011). There are a number of lipid peroxidation products that are frequently utilized, but the measurement of malondialdehyde levels probably takes first place. Thiobarbituric acid is used to measure it the most frequently (Lushchak and Semchyshyn, 2012). Due to the ability of thiobarbituric acid to react with a wide variety of substances, including various aldehydes, amino

acids, and carbohydrates, this approach is quite general. However, it is a widely used technique for evaluating lipid peroxidation in a variety of organisms (Lushchak, 2011).

### 1.8.3 DNA damage

Several chemicals in the polluted water have the potential to change biological processes, which can have an impact on certain populations and entire ecosystems. One of the key duties of environmental monitoring to reduce pollution is the evaluation of the genotoxic potential in surface water. To this purpose, studies on environmental biomonitoring have suggested using DNA damage, such as strand breaks, as a helpful indicator for evaluating the genotoxic effectsof environmental pollutants (Everaats et al., 1998; Felder et al., 1998; Theodorakis and Shugart,1998). Single cell gel electrophoresis (SCGE), also known as comet assay, is one of the methods used to evaluate the genotoxic potential of environmental toxicants. The method makes it possible to effectively see DNA damage or repair even in single cells (Olive et al., 1990). Any cell with a nucleus can be used in the experiment, and it only requires a small number of cells (a few hundred), is sensitive to a variety of mutagens, and uses a variety of tissues (Fairbairn et al., 1995). The comet assay exhibits strong dosage responses even at relatively low levels of naturally occurring genotoxicants and has an extraordinarily high sensitivity, detecting one break in 11010 Da (Gadik et al., 1992). (Mitchelmore and Chipman,1998; Rojas et al., 1999). In comparison to many other monitoring systems, the assay has a shorter processing time and costs less. And finally, no other standard environmental impact assessment method will estimate the cumulative genetic toxicity brought on by all the genotoxic contaminants to which organisms are exposed.

### 1.8.4 Histological alterations

Fish that have undergone pathological alterations have been exposed to environmental stresses.In order to evaluate the health status of fish under exposure to pollutants in both the field and laboratory, histopathology has been frequently employed as a biomarker. Because they may more accurately reflect the animal's true health status than other biomarker/diagnosis methods,Au et al. (2004) validated the histopathological changes in fish as a pivotal tool to detect the toxicological consequences of material (Al-Sawafi et al., 2017).

## 1.9 Research Gap

PFOS are known to be absorbed through a number of interactions. These include electrostatic

interaction, hydrogen bonding, hydrophobic interaction, and ligand exchange since it has a hydrophobic fluorinated carbon chain and a sulfonate group. PFOS and nanoparticles are widely detected in global environments, and very possibly coexist in the environment. Since nanoparticles aregood absorbents of PFOS, they can influence its toxicity. Many studies have reported mixturetoxicities of PFOS and nanoparticles, particularly metal oxide nanoparticles, and observed synergistic action. Copper ions and CuO bulk material has shown to increase absorptive capacity of PFOS on inorganic nanomaterials. Similarly, copper ions and PFOS exhibit synergistic toxicity in red worm *Limnodrilus hoffmeisteri* and Maize (Feng et al., 2016, Song et al., 2021). However, no study has till date explored mixture toxicity of PFOS and Copper oxide nanoparticles.

## 1.10 Problem statement

Both Copper oxide nanoparticles and Perfluorooctane sulfonate are widespread in the environment. They enter and are distributed in different environmental compartments through different sources, either point source or their precursors. Once in aquatic environments, they are taken up by aquatic organisms, and have toxicological consequences. However, once they interact, their mechanisms of uptake, accumulation and toxicity can differ as compared to singular action. Binary mixtures of PFOS with various metal oxide nanoparticles have shownto increase its toxicity, making it crucial to explore its interactive effects with CuO nanoparticles, given its chemical affinity towards copper containing compounds. Copper oxidenanoparticles can interact with perfluorooctane sulfonate within the exposure media or in vivo.This interaction can either be in the form of PFOS + Cu complexes as negatively charged PFOScan bind with Cu ions released from the nanoparticles via its sulfonic group; or it can adsorb on the surface of nanoparticles, in which case the nano-effect may be dominant rather than thechemical characteristics of copper. These complex interactions can influence the uptake, accumulation, depuration, and toxicities of copper oxide nanoparticles and PFOS.

## 1.11 Objectives

This study aims to understand the impact of Copper Oxide nanoparticles and perfluorooctane sulfonate on oxidative stress and antioxidant response in adult zebrafish. Neurotoxicity will be assessed in the form of Acetylcholine esterase inhibition. DNA damage will be studied to assess

genotoxicity and histological alterations will be studied in skeletal muscle. This will be the first study done on theco-exposure of PFOS with Copper Oxide Nanoparticles on zebrafish.

- To investigate the effects of CuO-NPs, PFOS and their co-exposure on total protein levels, oxidative stress markers ROS and MDA and antioxidant enzymes SOD and CAT in zebrafish gills.
- To measure neurological damage of CuO-NPs and PFOS via acetylcholinesterase activity in zebrafish brain.
- To assess DNA damage in response to CuO-NPs, PFOS and their co-exposure in zebrafish liver using Comet Assay.
- To examine histological alterations in zebrafish skeletal muscle segments following the chronic exposure of CuO-NPs and PFOS.

# Chapter 2

# Materials and Methods

# 2 MATERIALS AND METHODS

## 2.1 Chemical and Reagents

Copper oxide nanoparticles 40 nm diameter [CAS; 1317-38-0, Purity 99.5 %] were purchased from Macklin Biochemical Co. Ltd., China. Perfluorooctane sulfonic acid potassium salt [CAS; 2795-39-3, Purity 98%] was purchased from AK Scientific, USA. Dimethyl Sulfoxide (DMSO) HPLC grade was purchased from Honeywell Company (Morristown, NJ, USA). Acetylthiocholine iodide [CAS; 2260-50-6, purity: 99.0%] and Nitrotetrazolium blue chloride (NBT) [CAS Number; 298-83-9, purity; 98%] were purchasedfrom Sigma Aldrich® (Germany). Ethylenediamine tetra acetic acid (EDTA) [CAS: 60-00-4, purity; 99%] of Sigma Aldrich, Germany, Hydrogen peroxide [CAS: 7722-84-1] riboflavin [CAS:83-88-5, purity; 98%] of Merck (Darmstadt, Germany) were used. All other chemicals and reagents used in the study are of analytical grade (AG).

## 2.2 Characterization of nanoparticles

### 2.2.1 Scanning electron microscopy (SEM) and energy dispersive x-ray (EDX)

The particle size and morphology were characterized by Scanning Electron Microscopy (EV018 Carl Zeiss, Germany). The nanoparticles suspension of 10 mg/L was coated on a glass slide and subjected to gold sputtering after drying. Elemental composition was examined using EDX analysis.

### 2.2.2 Fourier transform infrared spectroscopy (FTIR)

Functional groups in the CuO nanoparticles were analyzed using Fourier Transform Infra-Red (FTIR) spectroscopy (FT/IR-6600 type A Spectrometer, Jasco). The dry nanoparticle powder was added with KBr (Potassium bromide) and pressed under 5000–10,000 psi in the dye to form a transparent pellet. The transmittance spectrum was obtained and plotted in the wavenumber range of 4000 $cm^{-1}$ to 400 $cm^{-1}$ using OriginPro Version 8.5.

### 2.2.3 X-ray diffraction (XRD)

The particle crystalline structure was characterized using X-Ray Diffraction pattern. The scanning was done by 2.2 kW Cu anode radiation produced by the ceramic X-ray tube at a wavelength of 1.54 Å. The wavelength was recorded in the range of 5°-70°. The results were

cross-checked with the JCPDS (Joint Committee on Powder Diffraction Standards) database using PANanalytical X'Pert HighScore software version 2.2 to confirm its crystalline structure.

## 2.3 Preparation of stock and dose solutions of CuO-NPs and PFOS

Stock solutions of copper oxide nanoparticles and PFOS were prepared separatelyusing distilled water and analytical grade DMSO, respectively. For the control group, 4.5mL of pure analytical grade DMSO without PFOS was added to 15L of water to achieve 0.03% (v/v).

### 2.3.1 Copper oxide nanoparticles (CuO-NPs)

Stock suspension of CuO-NPs was made by adding 50mg of copper oxide nano powder (40 nm) in 1000mL of distilled water to achieve the final concentration 50 mg/L. To disperse the nanoparticles in the suspension, the suspension was sonicated with an ultrasonicator at 100W 40 KiloHertz for 60 minutes before exposure. A total of 300mL was taken from this solution and added to the tanks containing 15L of water to achieve a final concentration of 1mg/L.

### 2.3.2 Perfluorooctane sulfonate (PFOS)

The stock solution of Perfluorooctane Sulfonate was made in pure analytical grade DMSO solvent to enhance solubility in exposure medium. 50mg of PFOS was added to 50mL of DMSO to make a stock solution of 1mg/mL. From the stock solution, 4.5mL was added to each tank containing 15Lof water to achieve a final concentration of 300µg/L.

## 2.4 Experimental animals

Adult zebrafish (AB wild-type strain, 6 months old) were maintained at Chemical Stress Ecology & Aquatic Toxicology Lab, Quaid-i-Azam University, Islamabad. The fish were acclimatized for 30 days in dechlorinated tap water. 50 individuals were placed in every aquarium. Aeration was provided to maintain the dissolved oxygen at 7 ± 1 mg/L. Photoperiod was maintained at 14:10 h light and dark period, at 27 ± 1 C° temperature, and pH ranging from 7.4-8.1. Fish were fed daily at 5% body weight with Optimum Betta (commercial food for fish). Male and female fish were randomly distributed in the aquaria. The fish were observed regularly for behavior and health and signs of stress.

## 2.5 Experimental setup

A total of 240 healthy fish were selected of length (3-4cm) and weight (340-370 mg) for the

experiment andtransferred to 20-liter tanks (30×60×30cm) containing 15L exposure medium. The fish were subjected to three treatments (T1, T2 and T3) and a control group (CT) for 28-Days. Each tank contained 20 fish per aquarium, and all treatments were run in triplicate.

- Control (CT): DMSO (0.035 v/v)
- Treatment 1 (T1): 1mg/L of CuO-NPs. Change sequence
- Treatment 2 (T2): 300µg/L PFOS.
- Treatment 3 (T3): 300 µg/L PFOS and 1 mg/L CuO-NPs.

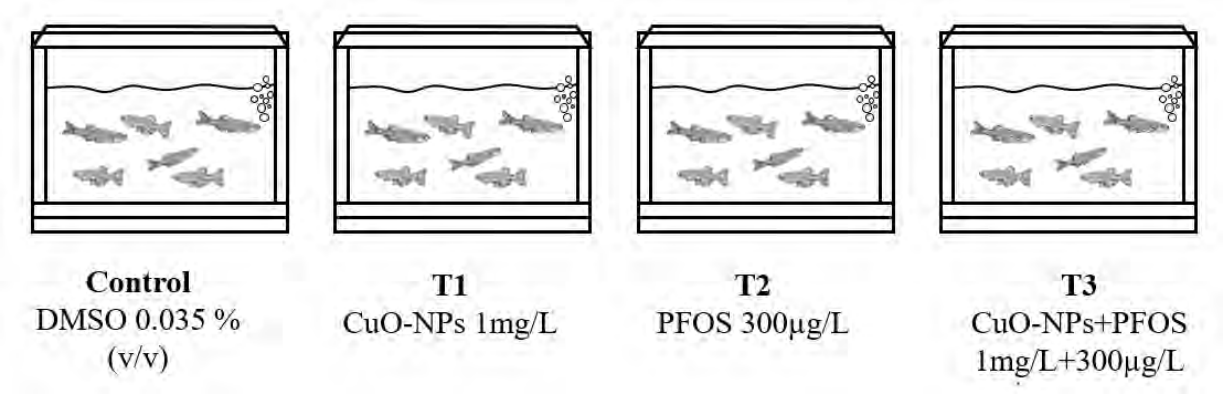



**Figure 7**: Schematic diagram of experimental set-up during the 28-day exposure.

The experiment was performed in dechlorinated tap water. Toensure good water quality and maintain the concentrations of the contaminants, water was changedevery alternate day. All glass aquaria were fitted with aerators to maintain dissolved oxygen within the required range. However, air stones were not used to avoid any brittle adsorption site to the contaminants. Water quality parameters were recorded daily. Samples were taken on day 7, 14, 21 and 28. At each sampling interval, 4 fish from each group were sampled for the measurement of different parameters. Fish were euthanized by immersion in ice cold water (4ºC) for 10 minutes or till they became inverted. Fish were then patted dry on a paper towel and sex was determined by observing coloration, belly shape, and anal fin size and were pinned on the dissection mat by inserting pins through the fleshy part of the tail and ventral part of the eye socket. Incision was made along the belly from the anus to the operculum, and skin was removed to reveal the organs. Fish brains, liversand gills were taken out and placed on PBS plates. Organs were carefully examined under the stereomicroscope and transferred to labeled eppendorfs containing Phosphate Buffer Saline (PBS) (pH7.4) and frozen immediately at -80º C. Skeletal muscles were dissected and washed in

PBS before being preserved in Neutral BufferFormalin (NBF) (pH 7) at 4º C.

## 2.6 Homogenate preparation

The SilentCrusher M Homogenizer from Heidolph Instruments was used to homogenize the brain, gills, and liver tissues individually in 50mM potassium phosphate buffer (pH 7.0) in 2mL eppendorf tubes. The homogenate was centrifuged at 10,000 rpm at 4º C for 10 minutes. For additional examination,the supernatant (cytosolic fraction) was removed and transferred to other labelled eppendorf tubes.

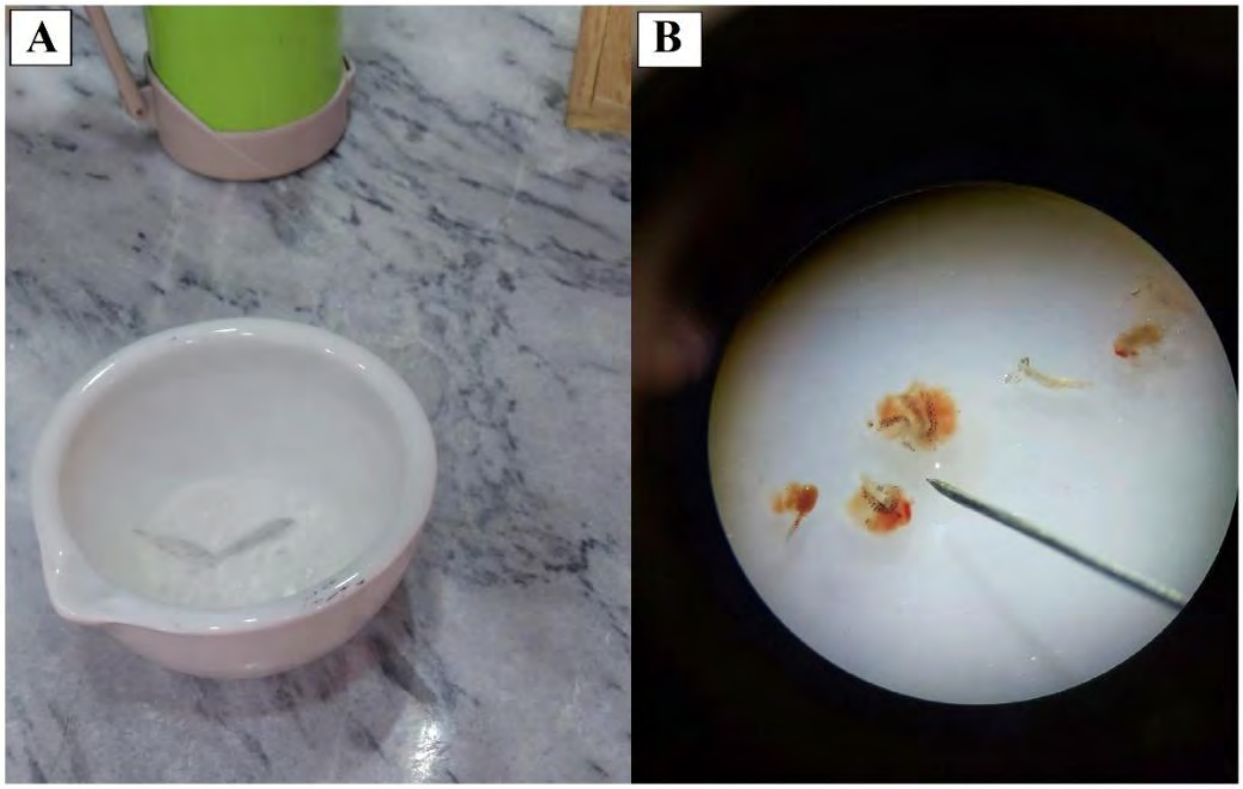



**Figure 8**: Fish snap frozen in liquid nitrogen (**A**) and gills obtained and observed under microscope (**B**).

## 2.7 Total protein estimation by Bradford method

Sigma Bradford Method was used to measure total protein content in zebrafish tissues, using bovine serum albumin (BSA) as standard. Bradford reagent was purchased from Sigma Aldrich®. Five dilutions of BSA (0, 10, 20, 40, 60, 80 and 100µg/mL) were prepared in Bradford Reagent. Absorbance of BSA dilutions was measured at 595 nm using UV-Vis Spectrophotometer

(HACH, USA, DR-5000) and standard curve was generated. Sample with BSA concentration 0µg/L was considered as blank. Level of protein was measured using BSA curve. Total protein is calculated using the following equation obtained from the standard curve:

- ➔ Y = 0.001x + 0.0055
- ➔ X = (Y – 0.0055) / 0.001

Whereas y is absorbance of the samples and x gives total protein estimation.

## 2.8 Oxidative stress parameters

### 2.8.1 Reactive oxygen species (ROS)

The ROS assay in the gill tissue of fish followed the methodology described by Hayashi et al. (2007).

To prepare a 0.1 M Sodium Acetate buffer, 4.1 g of sodium acetate was dissolved in 500 ml of distilled water, and the pH was adjusted to 4.8. Next, 10 mg of N, N-Diethyl-p-phenylenediamine sulphate salt (DEPPD) was dissolved in 100 ml of sodium acetate buffer. Additionally, a solution containing 50 mg of ferrous sulphate ($FeSO_4$) in 10 ml of sodium acetate buffer was prepared. The two solutions were mixed in a 1:25 ratio and incubated in the dark for 20 minutes at room temperature.

For the assay, a 20 µl sample was taken from the solution mixture, and 1.2 ml of buffer and 20 µl of liver tissue homogenate were added to a cuvette. The absorbance was then measured at 505 nm using a UV-Vis Spectrophotometer (HACH, USA, DR-5000). Three readings were taken for each sample at 15-second intervals. This process allows for the estimation of reactive oxygen species (ROS) in the liver tissue of the fish. Values were calculated using the following formula:

$$\textbf{Reactive Oxygen Species (ROS)} = \frac{(\textbf{Sample OD} \times \textbf{Cuvette Vol.} \times \textbf{10\^6})}{(\textbf{Protein} \times \textbf{Sample Vol.} \times \textbf{Δtime} \times \textbf{Constant 15600})}$$

Units = nanomoles / mL

Whereas:

Sample OD = Sample Absorbance (ΔSample - ΔBlank)

Cuvette Vol. = Volume of Cuvette

Protein = Total protein in sample (mg/mL)

Sample vol = Volume of Enzyme Supernatant

Δtime = Measurement Time

15600 = Extinction co-efficient ($M^{-1}$ $cm^{-1}$)

### 2.8.2 Malondialdehyde (MDA) content measurement

Thiobarbituric acid reactive substance assay (TBARS) was used to measure malondialdehyde content in gill tissues of zebrafish using the method described by (Zhang et al., 2013).

Reaction mixture contains:

- 200µL of enzyme supernatant
- 200µL of 8.1% Sodium Dodecyle Sulfate (SDS)
- 1500µL of 20% Acetic Acid (pH3.5)
- 100µL of 1% Thiobarbituric Acid
- And 1000µL distilled water

The resulting solution was then kept in a water-bath for one hour at 90°C. Afterwards, the solution was cooled and centrifuged at 3000 rpm for 15 minutes. Absorbance of resulting mixture was measured at 532nm using UV-Vis Spectrophotometer. In the assay, mixture without tissue homogenate was considered as blank. The MDA content was observed as the content of thiobarbituric acid reactive substances nanomoles per milligram of protein .

$$\textbf{Malondialdehyde (MDA)} = \frac{((\Delta\text{Sample} - \Delta\text{Blank}) \times \text{Cuvette Vol.} \times 10\text{^}6)}{(\text{Protein} \times \text{Sample Vol.} \times \Delta\text{time} \times \text{Constant}\ (15600))} =$$

Units = nanomoles / mg protein

Where:

ΔSample = Change in Sample Absorbance

ΔBlank = Change in Blank Sample Absorbance

Cuvette Vol. = Volume of Cuvette

Protein = Total protein in sample (mg/mL)

Sample vol = Volume of Enzyme Supernatant

Δtime = Measurement Time

15600 = Extinction co-efficient ($M^{-1}$ $cm^{-1}$)

## 2.9 Antioxidant parameters

### 2.9.1 Superoxide dismutase (SOD) activity

Protocol described by Shao et al. (2012) were followed to measure activity of superoxide dismutase activity

Reaction mixture was 3mL that contains:

- 50mM Phosphate Buffer (pH 7.8)
- 100µM Ethylenediaminetetraacetic acid (EDTA)
- 130mM Methionine
- 750µM Nitrobluetetrazolium chloride (NBT)
- 20µM Riboflavin
- And 50µL enzyme supernatant

The resulting mixture was irradiated for 20 minutes with 4000 1x fluorescent lamp, and absorbance of the mixture was measured at 560 nm using UV-Vis Spectrophotometer (T80+ UV/Visible spectrometer). One unit of superoxide dismutase activity was termed as the enzyme quantity necessary to cause inhibition of 50% of Nitrobluetetrazolium chloride photoreduction rate. Results obtained were expressed as U/mg of protein.

**Superoxide Dismutase (SOD)** $= \frac{((\mathbf{\Delta Sample - \Delta Blank}) \times \mathbf{Cuvette\ Vol.} \times \mathbf{10\hat{}6})}{(\mathbf{Protein} \times \mathbf{Sample\ Vol.} \times \mathbf{\Delta time} \times \mathbf{Constant}(6.22))} =$

Units = U/mg of protein

Whereas:

ΔSample = Change in Sample Absorbance

ΔBlank = Change in Blank Sample Absorbance

Cuvette Vol. = Volume of Cuvette

Protein = Total protein in sample (mg/mL)

Sample vol = Volume of Enzyme Supernatant

Δtime = Measurement Time

6.22 = Extinction co-efficient ($M^{-1}$ $cm^{-1}$)

### 2.9.2 Catalase (CAT) activity

Catalase activity was measured following the methods used by Muazzam et al.; (2019).

Reaction mixture contains:

- 500µL of 5.9mM $H_2O_2$
- 1mL of 50mM Potassium Phosphate Buffer
- 100µL of tissue homogenate

The resulting mixture was incubated for 15 minutes at 37°C. Absorbance was noted at 240nm. Activity of catalase was denoted in U/mg protein.

**Catalase (CAT)** $= \frac{((\mathbf{\Delta Sample - \Delta Blank}) \times \mathbf{Cuvette\ Vol.} \times \mathbf{10\hat{}6})}{(\mathbf{Protein} \times \mathbf{Sample\ Vol.} \times \mathbf{\Delta time} \times \mathbf{Constant\ 43.1})} =$

Units = U/mg of protein

Whereas:

ΔSample = Change in Sample Absorbance

ΔBlank = Change in Blank Sample Absorbance

Cuvette Vol. = Volume of Cuvette

Protein = Total protein in sample (mg/mL)

Sample vol = Volume of Enzyme Supernatant

Δtime = Measurement Time

43.1 = Extinction co-efficient ($M^{-1}$ $cm^{-1}$)

## 2.10 Determination of acetylcholinesterase (AChE) activity

AChE activity was measured according to the protocol by Ellman et al. (1961) with slight modifications.

The mixture contains:

- A 50µL of 0.5 mM DTNB
- 1% Sodium citrate
- 200µL 0.5 M Phosphate buffer (pH 8.0)
- 650µL $H_2O$
- 50µL enzyme supernatant
- 50µL 10 mM acetyl thiocholine iodide

The control cuvette did not contain acetyl thiocholine iodide. Enzyme activity was calculated by reading the changes in absorbance over 5 min at 412 nm.

Inhibition of AChE activity was measured using the following formula:

**Acetylcholinesterase activity (AChE)** $= \frac{(\mathbf{T}f - \mathbf{T}i)}{\mathbf{0.01}} =$

Units = U/min

Whereas:

$T_f$ = Final Absorbance

$T_i$ = Initial Absorbance

0.01 = Constant (Time Constant for 1 minute)

## 2.11 DNA damage by comet assay (SCGE)

DNA damage in zebrafish liver tissues was evaluated using Single-cell gel Electrophoresis. Method described by Ge et al. (2015) was used to perform Comet Assay.

### 2.11.1 Reagents preparations

Following reagents were prepared for performing Comet assay:

- Low Melting Agarose (LMA) was created by dissolving 250 mg of LMA in 50mL of 1% PBS. This solution was then maintained at a low temperature in the refrigerator andbrought to 37 °C just before use.
- Normal Melting Agarose: The solution was heated to dissolve 500 mg of normal melting agarose in 50mL of distilled water to prepare 1% NMA.
- Lysing solution: This solution was obtained by dissolving 1.46g of NaOH (2.5M), 37.2g of EDTA (100M), and 1.2g of Trizma base (100M) in distilled water and increasing the volume to 890mL. Following pH correction to 10.0 with HCl or NaOH, the final volume was increased to 1000mL. The final lysing solution was created by combining already prepared suspension with 10% DMSO and 1% Triton X. Before making slides, the final solution must be chilled for 30 minutes.
- Electrophoresis buffer: The electrophoresis buffer is composed of 0.5mL of EDTA (200mM) and 30mL of NaOH in 1000mL of distilled water. The pH was kept basic (>13).
- Neutralization buffer: A pH of 7.5 was achieved by dissolving 48.5g of 0.4 M tris in 1000mL of distilled water, and the mixture was stored at room temperature.
- Staining solution: 50mL of distilled water was used to dissolve 10mg of ethidium

bromide in the staining solution.

- PBS buffer: 990 milliliters of phosphate buffer saline were made, and their volume was increased to 1000 milliliters when the pH was set at 7.4.

### 2.11.2 Slides preparation

Slides were first cleaned with methanol and then burned to disinfect them. Onto frosted slides with a coating of NMA, 200 L of LMA and cell suspension were pipetted. As the first LMA layer solidifies, a second one (85L) is added. Slides were immersed in lysis solution and chilled for 120 minutes while being kept in the dark following the fixation of the second layer.

### 2.11.3 Electrophoresis

After two hours, the slides were removed from the lysis solution and kept in the horizontal gel apparatus. Slides were dipped into a recently made buffer solution. Power at 24 volts was provided for 30 minutes while waiting for the DNA to uncoil. After that, a neutralizing buffer was applied to the slides. The same steps were taken twice. 80 liters of 1X ethidium bromide were used for staining, and cover slips were placed over the plates.

### 2.11.4 Slides visualization

To evaluate DNA damage, a fluorescent microscope (Nikon, 40X) was used. The software CASP 1.2.3.b was useful in assessing the degree of DNA damage. DNA migration from head to tail was assessed in each sample, which contained between 50 and 100 cells. Comparing each cell's DNA transfer and the proportion of cells with a high DNA fragment migration ratio allowed for the mounting of results.

## 2.12 Skeletal muscle tissue histopathology

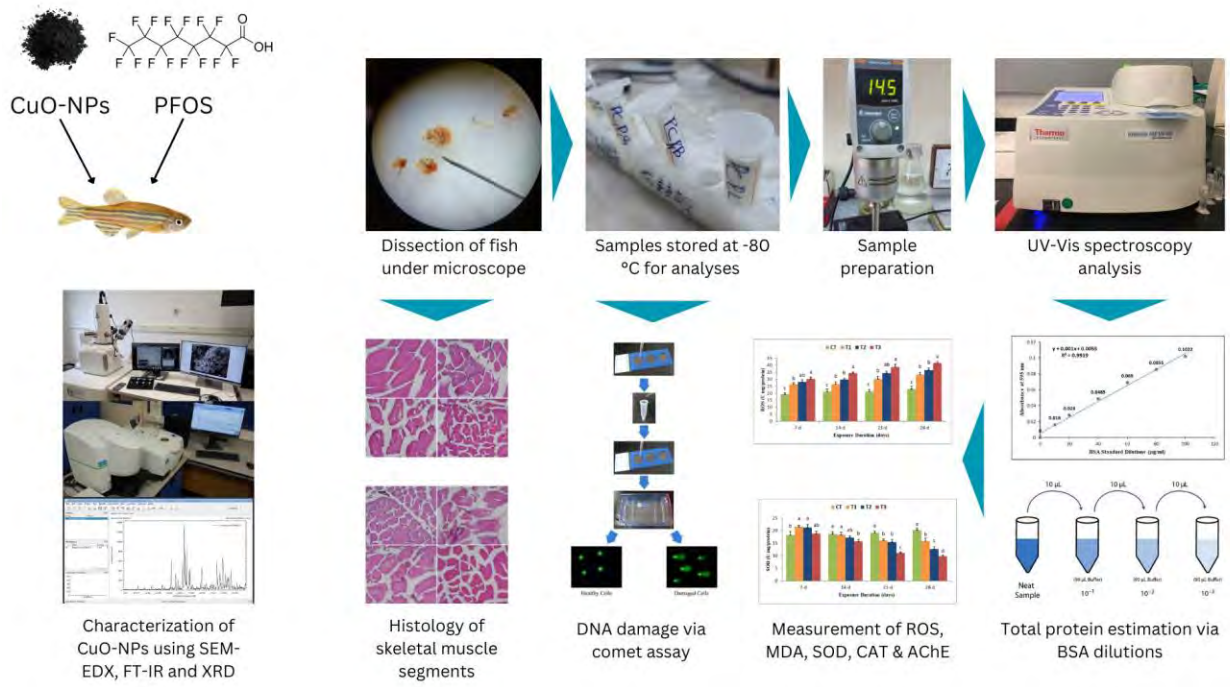
Skeletal muscle tissues were studied for histological changes. Fish were dissected, and skeletal muscle was taken in each sampling interval. They were transferred right away into 2mL eppendorf tubes with 1.5mL of neutral buffer formalin. Samples were kept at 4°C until additional analysis.

### 2.12.1 Hematoxylin & eosin (H&E) staining of skeletal muscles

Using the Hematoxylin & Eosin staining technique, the histopathological alterations in the muscle tissues following exposure to CuO-NPs, PFOS and co-exposure were evaluated. Fixation, dehydration,embedding, slide preparation, staining, and microscopy are some of the steps. The obtained tissueswere washed and rinsed with a saline solution containing 0.75% NaCl. Sections of

the tissues werecut into 4–6-micron thick pieces, embedded in paraffin wax, fixed in aqueous Bouins solution for 24 hours, processed in a graded series of alcohols, cleaned in xylene, stained with hematoxylin eosin, and dissolved in 70% alcohol (Hampton et al., 1985). An Olympus-CX41 light microscopewas used to analyze histopathological lesions on slides at a 1000X magnification, and a TUCSENdigital camera was used to take pictures of the liver and gill tissues (Model: ISH500).

**Figure 9**: Dissection of zebrafish under stereomicroscope to obtain organ samples to be transferred in pre-labeled eppendorf tubes.




Pictorial flowchart representing the summary of complete experimental methodology.

## 2.13 Statistical Analysis

Data was expressed as means and standard deviations (Means ± SD). Statistical analysis was performed on all experimental data via IBM SPSS Statistics, version 21.0. (SPSS Inc., USA). One-way analysis of variance (ANOVA) was done to determine the statistical differences between treatment groups. Means were separated using the Tukey's Post-Hoc comparison test. Differences were considered statically significant at $P<0.05$. Statistical analyses were performed using the SPSS statistical package (ver.22.0, SPSS Company, Chicago, IL, USA).

# Chapter 3

# Results

# 3 RESULTS

The present experiment was conducted for 28-Days to evaluate single and joint toxicity of copper oxide nanoparticles (CuO-NPs) and perfluorooctane sulfonate (PFOS) on adult zebrafish. The nanoparticles used in this study were characterized for surface morphology and elemental composition, spectral peaks, and x-ray diffraction. Water quality parameters were measured throughout the experiment. Fish samples were taken at days 7, 14, 21 and 28 and fish gills were analyzed for total protein, oxidative stress markers reactive oxygen species (ROS) and lipid peroxidation product malondialdehyde (MDA) and antioxidant enzymes superoxide dismutase (SOD) and catalase (CAT). Functional neuromarkar acetylcholinesterase activity (AChE) in the brain was also measured. Fish liver samples were observed for DNA damage and skeletal muscles were examined for histological alterations. Results are presented and interpreted in this section.

## 3.1 Characterization of CuO nanoparticles

### 3.1.1 Scanning electron microscopy (SEM) and energy dispersive X-ray (EDX)

The surface morphology of the synthesized CuO nanoparticles exhibit a rice-grain shaped structure as examined by SEM analysis. The nanoparticles are observed to be homogenous in nature and assembled into aggregates with slight irregularity (Fig.10**A**). The SEM image reveals nanoparticles to be in the size range of 50-100 nm. The Energy dispersive spectra of the sample confirmed the chemical composition of the synthesized material. The presence of strong peaks of copper (Cu) and oxygen (O), along with minor impurities observed suggest high purity of nanoparticles. Calculated weight percentages for copper and oxygen were found to be 71.4 and 20.9% respectively. Sharp and narrow peaks are evidence of the highly crystalline nature of the nanoparticles as shown in Figure 10**B**.

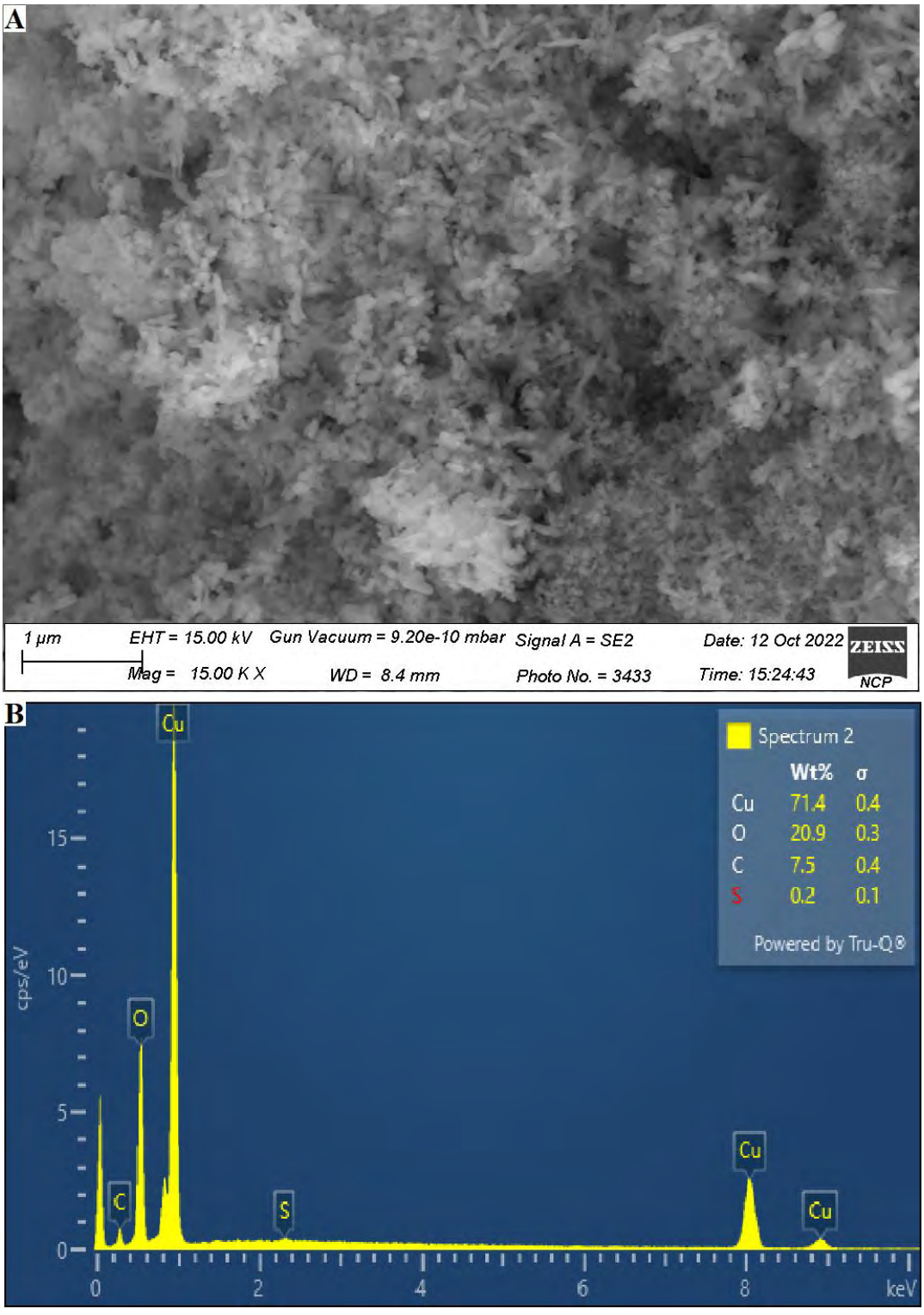



**Figure 10**: Scanning electron microscopy image at 15000x magnification (A) and EDX spectra of CuO nanoparticles (B) used in this study.

### 3.1.2 Fourier transform infrared spectroscopy (FTIR)

The FTIR spectral peak of CuO-NPs was recorded in the range of 4000–400 $cm^{-1}$. The broad spectral band at 3421.1 $cm^{-1}$ corresponds to OH stretching of the O-H hydroxy group. This is characteristic of absorbed water molecules on nanocrystalline structures due to their large surface area. The sharp peak at 1112.72 $cm^{-1}$ corresponds to C-N stretching vibrations of amine group (Fig.11). The intense peaks at 597.82, 524.54 and 512.97 $cm^{-1}$ are attributed to the stretching vibrations of Cu (II)-O bonds. There is sharp peak observed at 512 $cm^{-1}$ in the spectrum CuO nanoparticles which is the characteristics of Cu-O bond formation as indicated in Figure 11.

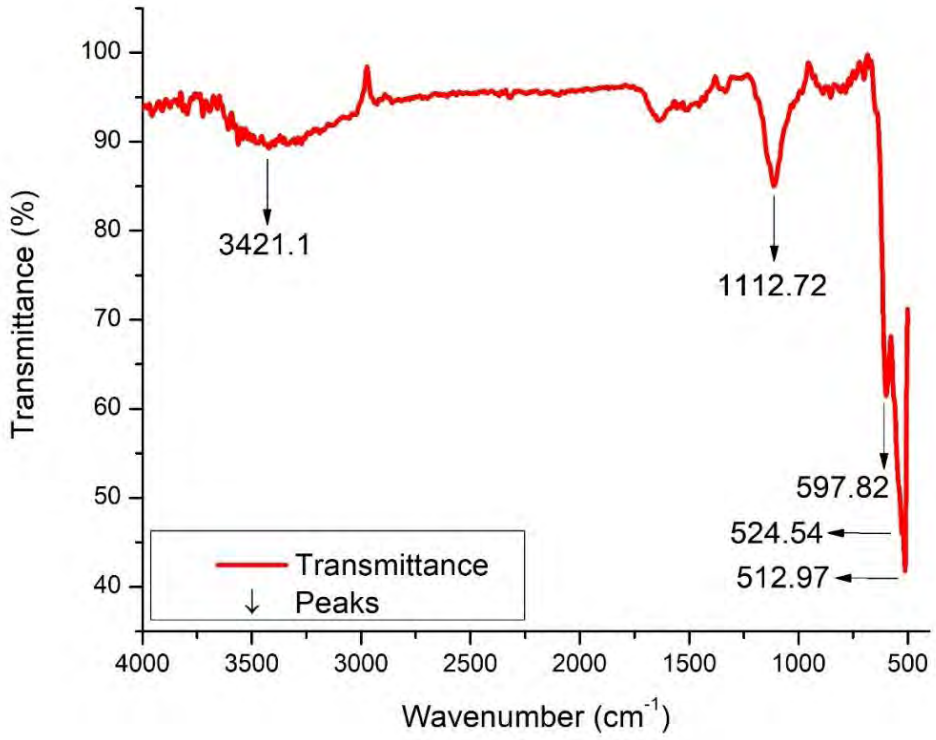



**Figure 11:** FTIR spectrum of CuO-NPs. Intense peaks 597.82, 524.54 and 512.97 $cm^{-1}$ confirm purity of the nanostructures.

### 3.1.3 X-ray diffraction (XRD)

The X-ray diffraction pattern revealed the crystalline nature of the CuO-NPs used in the study as shown in Figure 12. The XRD pattern showed intense peaks at 2θ = 35.5°, 38.6°, 48.8°, 53.5°, 58.2°, 61.5°, 66.3°, 68° positions corresponding to (002), (111), (202), (020), (202), (113), (311) and (113) planes of the monoclinic crystal system of CuO-NPs are in agreement with JCPDS card

no. 45-0937 (Ahamed et al., 2014), indicating Tenorite phase, which is a monoclinic system of copper oxide. Lack of impurity peaks suggests high quality of CuO-NPs.

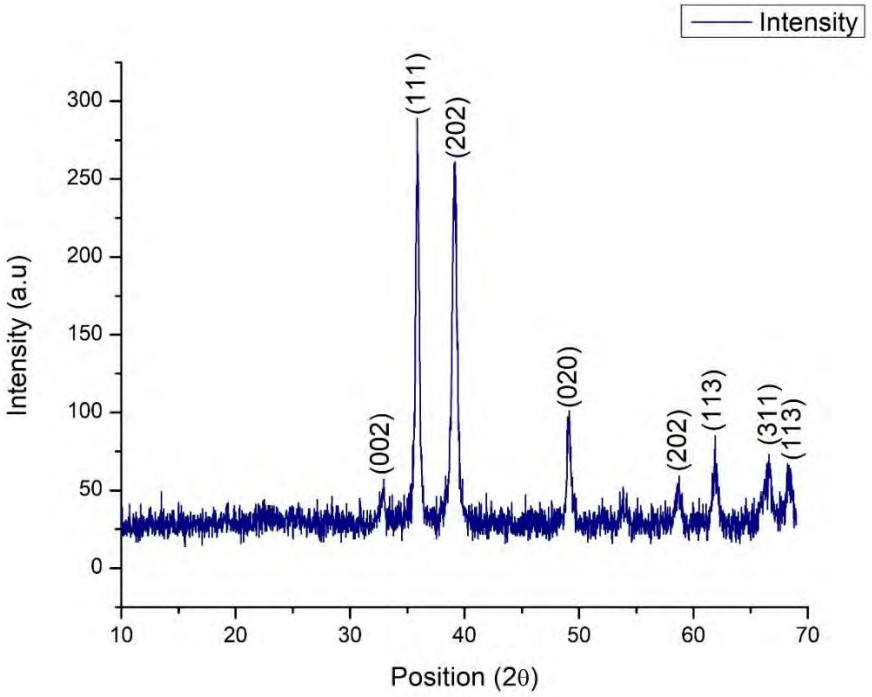



**Figure 12:** X-ray diffraction pattern of copper oxide nanoparticles used in this study.

## 3.2 Water quality parameters

Water quality parameters recorded during the experiment included pH, dissolved oxygen (mg/L), electrical conductivity (µS/cm), and temperature (C°) measured throughout the experiment. Fluctuations in pH (7-8.2), EC (6.7-60 (µs/cm) and Salinity (8-97ppm) were observed to be minor. Dissolved oxygen ranged from 5mg/L to 9mg/L. Temperature averaged at 26 ° C, ranging from 17.9 to 24.8° C. Water quality parameters in all treatment groups are presented in Table 5.

**Table 5**: Water quality *p*arameters in experimental treatments recorded during the experiment. The water quality remained within optimum range for zebrafish rearing conditions.

| Treatments | Temperature (C°) | | | EC (µS/cm) | | | DO (mg/L) | | | pH | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Min | Max | Mean | Min | Max | Mean | Min | Max | Mean | Min | Max | Mean |
| **CT** | 24.2 | 28.6 | 26.4 | 10 | 12 | 11 | 4.8 | 7.9 | 6.35 | 7.7 | 8.4 | 8.05 |
| **T1** | 24.1 | 28 | 26.05 | 6.7 | 56 | 31.35 | 5.5 | 8.5 | 7 | 7.9 | 8.2 | 8.025 |
| **T2** | 24.9 | 28.7 | 26.8 | 9.8 | 55 | 32.4 | 5.1 | 9 | 7.05 | 7.4 | 8.1 | 7.75 |
| **T3** | 24.2 | 28 | 26.1 | 9.5 | 60.1 | 34.8 | 5.3 | 8.5 | 6.9 | 7.1 | 7.4 | 7.25 |

**CT**: Control

**T1**: CuO-NPs (1mg/L)

**T2**: PFOS (300 µg/L)

**T3**: CuO-NPs+PFOS (1mg/L+300 µg/L)

## 3.3 Total protein estimation

Total protein (TP) levels are an important biochemical parameter used to determine the adverse effects of environmental pollutants in aquatic organisms (Kayhan et al., 2022). They are also precursors of enzymes, hormones, neurotransmitters, and cofactors (Qiu et al., 2019).

### 3.3.1 Calibration curve for total protein estimation using BSA.

Serial dilutions were made from Bovine Serum Albumin (BSA) solution to use as standards (Table 6). Absorbance was recorded at 595 nm and the values were used to plot a standard calibration curve (Fig.13) for calculating the total protein values in unknown samples.

**Table 6**: Absorbance for Bovine Serum Albumin (BSA) standards.

| BSA Standard Dilutions (µg/mL) | Absorbance at 595 nm |
|---|---|
| 0 | 0 |
| 10 | 0.016 |
| 20 | 0.028 |
| 40 | 0.0485 |
| 60 | 0.069 |
| 80 | 0.0855 |
| 100 | 0.1022 |

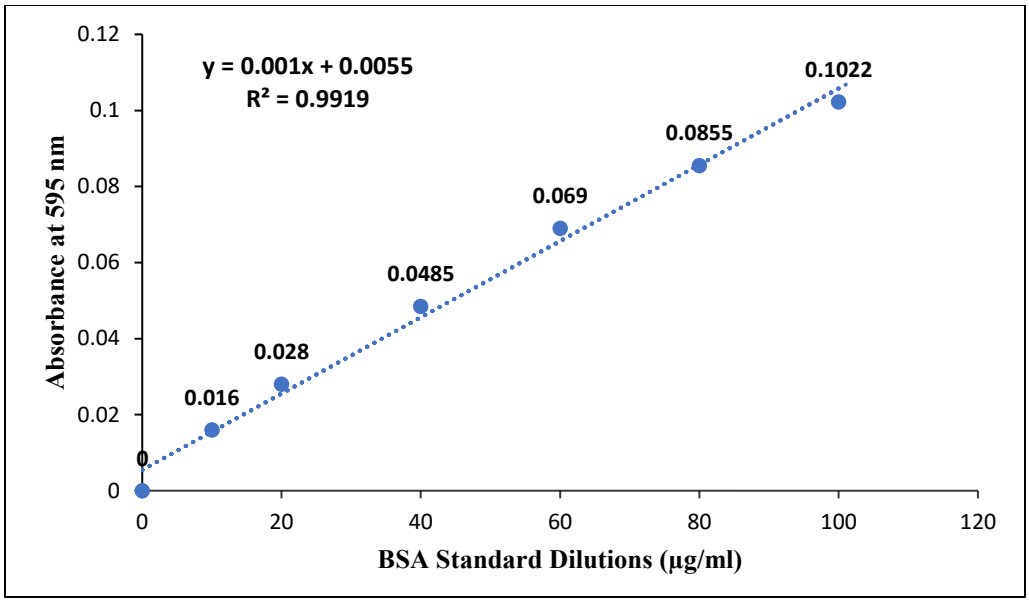



**Figure 13:** Standard Calibration Curve for Total Protein Estimation in *Danio rerio* gill samples at 595 nm.

## 3.4 Total protein content in zebrafish gills

Total protein content was estimated in zebrafish gills to evaluate the impact of copper oxide nanoparticles and perfluorooctane in individual and combined exposure. The results revealed statistically significant ($p<0.05$) decrease in groups exposed to CuO-NPs and PFOS and their mixtures (CuO NP + PFOS) as compared to the control (Figure 14). At 7-day sampling interval, CT (control group) showed highest total protein content, followed by T1 (CuO-NPs), and T2 (PFOS) which had further lower protein content compared to T1, and the lowest protein content was observed in T3 (CuO-NPs+PFOS). This trend persisted on sampling intervals at days 14, 21 and 28, with the decrease in total protein content in all groups being statistically significant in the manner CT>T1>T2>T3. The values for each interval are presented below in Figure 14.

**Table 7:** Total Protein content in *Danio rerio* gills following single and joint exposure to CuO-NPs and PFOS.

| Treatments | Exposure Days | | | |
|---|---|---|---|---|
| | **7-Day** | **14-Day** | **21-Day** | **28-Day** |
| **CT (Control)** | 15.58±0.74$^{a}$ | 16.72±0.50$^{a}$ | 16.38±0.56$^{a}$ | 16.24±0.98$^{a}$ |
| **T1 (CuO-NPs)** | 13.46±0.46$^{b}$ | 14.50±0.70$^{b}$ | 14.27±0.50$^{b}$ | 14.38±0.55$^{b}$ |
| **T2 (PFOS)** | 11.92±0.58$^{c}$ | 12.70±0.56$^{c}$ | 12.77±0.50$^{c}$ | 12.16±0.44$^{c}$ |
| **T3 (CuO-NPs+PFOS)** | 10.56±0.49$^{c}$ | 9.71±0.67$^{d}$ | 10.52±0.56$^{d}$ | 9.74±0.65$^{d}$ |

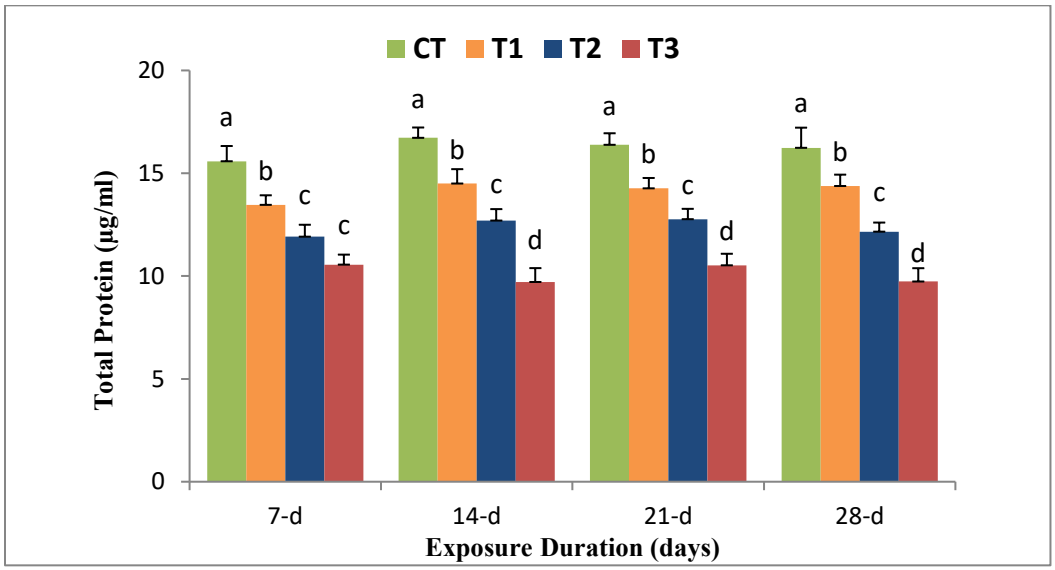



**Figure 14:** Total protein levels in *Danio rerio* gills following single and joint exposure to CuO-NPs and PFOS. Treatments are presented as following; **CT**: Control; **T1**: CuO-NPs; **T2**: PFOS, and **T3**: CuO-NPs+PFOS. Vertical bars represent mean ± SD of triplicates (n=3). Significant difference ($p<0.05$) in treatments is denoted by lowercase alphabets.

## 3.5 Oxidative stress parameters

A common pathway for contaminants to manifest toxicity and disease is via oxidative stress (Wu et al., 2019). Reactive Oxygen Species (ROS) play a vital role in evaluating oxidative stress resulting from pollutant exposure in fish. These ROS can induce cellular harm, affecting diverse physiological functions like metabolism and immunity in fish. Malondialdehyde (MDA), generated through ROS-triggered lipid peroxidation, emerges as a valuable biomarker for

assessing oxidative stress within fish tissues. Elevated MDA levels are indicative of heightened oxidative damage, offering valuable insights into the potential adverse impacts of environmental toxins on fish well-being and the broader aquatic ecosystem health.

### 3.5.1 Reactive oxygen species (ROS)

At the 7-day sampling interval, CT (control) had the lowest level of reactive oxygen species (ROS) among all treatment groups. The treatment group T1 (CuO-NPs) had a significantly higher level of ROS as compared to the control group, whereas T2 (PFOS) was not significantly different from T1 and T3 but was also significantly higher from the control group. The combined exposure in treatment T3 (CuO-NPs), was significantly different from CT and had the highest level of reactive oxygen species. During the second sampling interval at 14-day, there was a significant difference between treatment groups T1, T2 and T3 and the control group (CT), which was lower than the exposed groups. Treatment T1 showed an increase as compared to the control but was lower than T2. Treatment T3 had the greatest impact on ROS levels and was significantly higher than treatments T1 and T2 (Fig.15). At 21-Days, CT was still significantly lower than the treated groups. The CuO-NPs exposed group was higher than control. Treatment T2 was higher than T1 and lower than T3, but these differences were not statistically significant ($p<0.005$). Reactive oxygen species levels remained highest in the co-exposure group. The trend observed during the 28-day interval was similar in terms of CT being significantly different from T1, T2 and T3. The increase in ROS levels showed a clear pattern (CT<T1<T2<T3), with differences between all treatments being statistically significant ($p<0.05$). Reactive oxygen levels in all groups across all sampling intervals are presented below (Fig.15).

**Table 8:** Reactive Oxygen Species in *Danio rerio* gills following single and joint exposure to CuO-NPs and PFOS.

| Treatments | **Exposure Days** | | | |
|---|---|---|---|---|
| | **7-Day** | **14-Day** | **21-Day** | **28-Day** |
| **CT (Control)** | $19.13\pm1.09^{c}$ | $21.06\pm1.96^{c}$ | $21.10\pm1.62^{c}$ | $22.96\pm2.04^{c}$ |
| **T1 (CuO-NPs)** | $26.38\pm1.08^{b}$ | $26.56\pm1.44^{b}$ | $30.20\pm1.78^{b}$ | $33.17\pm1.58^{b}$ |
| **T2 (PFOS)** | $28.00\pm1.67^{ab}$ | $29.74\pm1.06^{b}$ | $34.21\pm1.49^{ab}$ | $36.58\pm1.37^{b}$ |
| **T3 (CuO-NPs+PFOS)** | $30.20\pm1.25^{a}$ | $34.24\pm0.95^{a}$ | $38.65\pm2.15^{a}$ | $41.62\pm1.05^{a}$ |

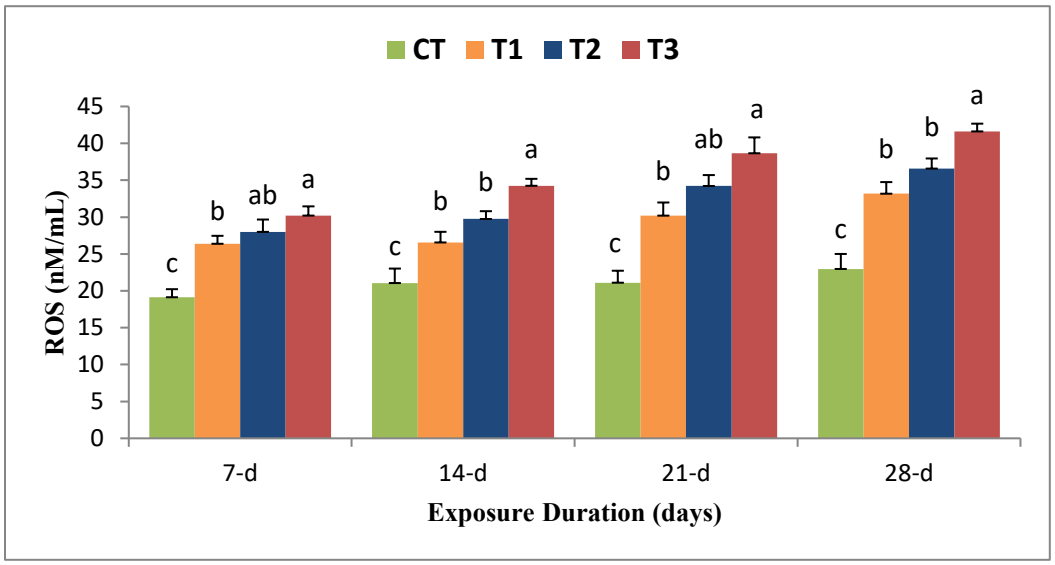



**Figure 15:** Reactive oxygen species in *Danio rerio* gills following single and joint exposure to CuO-NPs and PFOS. Treatments are presented as following; **CT**: Control, **T1**: CuO-NPs, **T2**: PFOS, and **T3**: CuO-NPs+PFOS. Vertical bars represent mean ± SD of triplicates (n=3). Significant difference ($p<0.05$) in treatments is denoted by lowercase alphabets.

### 3.5.2 Malondialdehyde (MDA) content

After 7 days of experiment, malondialdehyde (MDA) levels had slightly increased in exposed groups as compared to the control. The control treatment, CT, had the lowest values among all groups followed by T1, but the difference between the two was not statistically significant ($p<0.05$). Difference between treatments T2 and T3 was not significantly different, but both were significantly higher than the control. However, T2 and T3 were not significantly different from T1 at $p<0.05$. At the 14-day interval, all treatment groups were significantly higher than the control. Co-exposure (T3) had the highest levels of MDA content. Treatment T1 was significantly lower than T3, whereas T2 was not significantly different from T1 and T3. At 21 and 28-day, a similar increasing trend was observed. All treatments were statistically different. Values were lowest in CT, and showed an increase in treatments T1, T2 and T3 in the order CT<T1<T2<T3. Values for MDA content in all groups estimated as a measure of lipid peroxidation during the 28 days experiment are presented in Figure 16 below.

**Table 9:** Malondialdehyde levels in *Danio rerio* gills following single and joint exposure to CuO-NPs and PFOS.

| Treatments | Exposure Days | | | |
|---|---|---|---|---|
| | **7-Day** | **14-Day** | **21-Day** | **28-Day** |
| **CT (Control)** | 10.88±0.76$^{b}$ | 11.23±1.15$^{c}$ | 13.42±0.70$^{d}$ | 13.97±1.60$^{d}$ |
| **T1 (CuO-NPs)** | 13.06±1.38$^{ab}$ | 14.66±1.24$^{b}$ | 16.70±0.76$^{c}$ | 18.63±0.53$^{c}$ |
| **T2 (PFOS)** | 14.16±1.82$^{a}$ | 15.95±0.64$^{ab}$ | 19.36±0.75$^{b}$ | 21.82±0.50$^{b}$ |
| **T3 (CuO-NPs+PFOS)** | 15.47±0.62$^{a}$ | 18.02±1.51$^{a}$ | 22.48±0.52$^{a}$ | 24.92±1.00$^{a}$ |

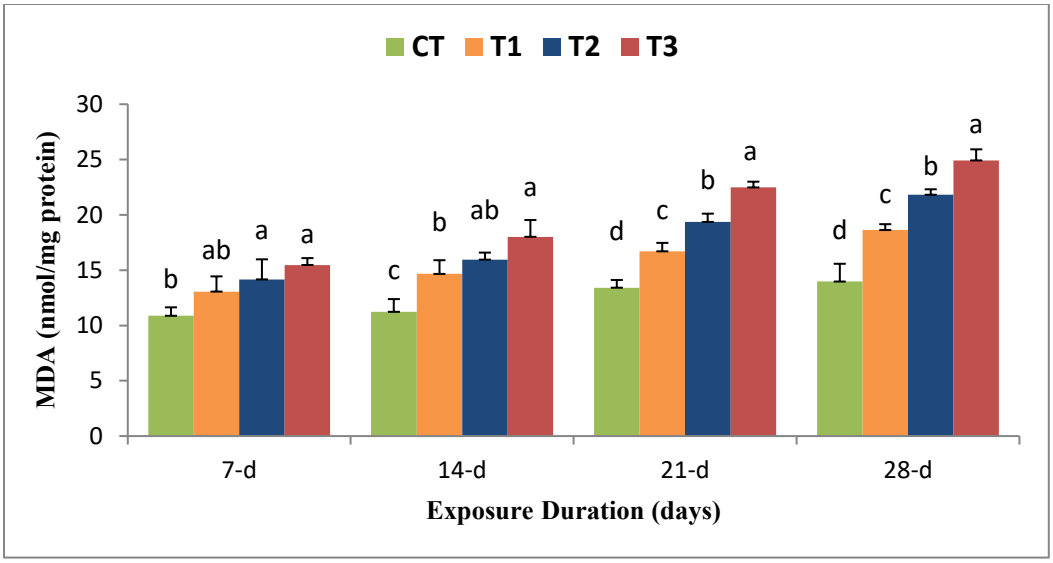



**Figure 16:** Malondialdehyde levels in *Danio rerio* gills following single and joint exposure to CuO-NPs and PFOS. Treatments are presented as following; **CT**: Control, **T1**: CuO-NPs, **T2**: PFOS, and **T3**: CuO-NPs+PFOS. Vertical bars represent mean ± SD of triplicates (n=3). Significant difference ($p<0.05$) in treatments is denoted by lowercase alphabets.

## 3.6 Antioxidant parameters

Antioxidant systems utilize different enzymes and molecules to counter oxidative stress caused by stressors. Superoxide dismutase (SOD) and Catalase (CAT) collaborate as the initial defense against the conversion of ROS production into harmless compounds. This process involves SOD converting superoxide radicals into hydrogen peroxide, which is subsequently broken down into water and molecular oxygen by CAT. Catalase, a group of enzymes primarily found in

peroxisomes (Modesto and Martinez, 2010), facilitates this breakdown. The induction of antioxidant enzymes can help organisms manage stress caused by hazardous exposure (Cossu et al., 1997). However, an excess of oxidants has the potential to deactivate enzymes engaged in the antioxidant defense mechanism (Modesto and Martinez, 2010).

### 3.6.1 Superoxide dismutase (SOD)

Activity of superoxide dismutase (SOD) showed a distinct pattern. The values were initially higher than the control during the first interval but decreased from 14-day onwards. At 7-day, treatments T1 (CuO-NPs) and T2 (PFOS) were significantly higher than CT (control group), whereas T3 (CuO-NPs+PFOS) was not significantly different ($p<0.05$) from all treatments as shown in Figure. During the 14-day interval, T3 had significantly lower enzyme activity than CT and T1. However, treatment T2 was not significantly different from the treatments CT, T1 and T3. At 21-day, enzyme activity remained lowest in T3, and was highest in CT at statistical significance $p<0.05$. Treatments T1 and T2 showed nonsignificant difference. At the end of experimental period (28-day), all groups were statistically different and showed a clear decreasing trend in the order CT>T1>T2>T3. Superoxide enzyme activity are presented in Figure 17 below.

**Table 10:** Superoxide dismutase activity in *Danio rerio* gills following single and joint exposure to CuO-NPs and PFOS.

| **Treatments** | **Exposure Days** | | | |
|---|---|---|---|---|
| | **7-Day** | **14-Day** | **21-Day** | **28-Day** |
| **CT (Control)** | $18.16\pm1.50^{b}$ | $18.59\pm0.99^{a}$ | $19.07\pm0.73^{a}$ | $20.07\pm0.92^{a}$ |
| **T1 (CuO-NPs)** | $21.50\pm0.67^{a}$ | $18.33\pm0.96^{a}$ | $16.02\pm0.64^{b}$ | $15.77\pm1.13^{b}$ |
| **T2 (PFOS)** | $21.18\pm1.20^{a}$ | $17.13\pm0.63^{ab}$ | $15.41\pm1.03^{b}$ | $12.59\pm0.95^{c}$ |
| **T3 (CuO-NPs+PFOS)** | $18.81\pm0.90^{ab}$ | $15.71\pm0.70^{b}$ | $11.09\pm0.43^{c}$ | $9.77\pm0.45^{d}$ |

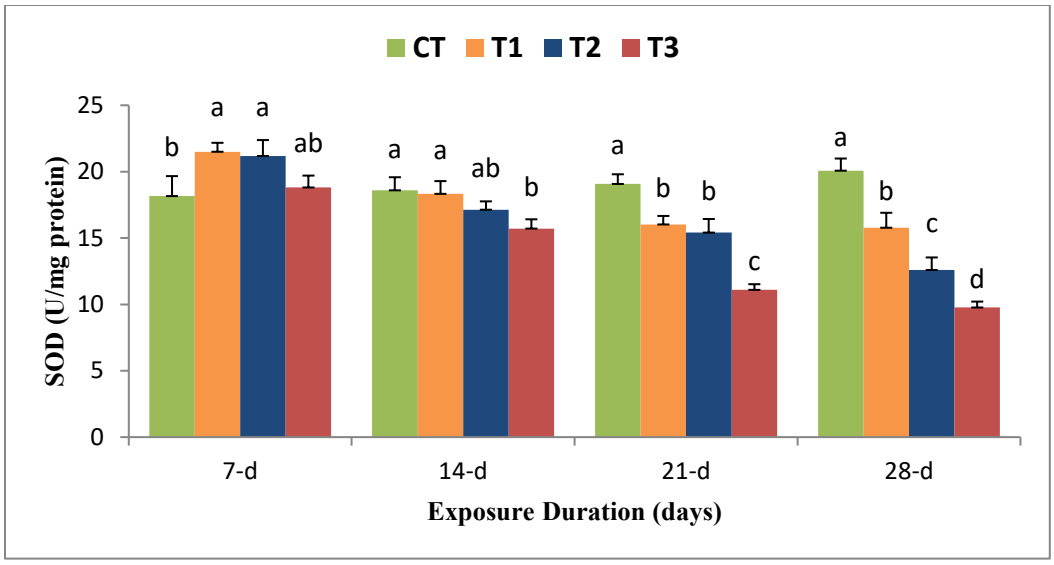



**Figure 17:** Superoxide dismutase activity in *Danio rerio* gills following single and joint exposure to CuO-NPs and PFOS. Treatments are presented as following; **CT**: Control, **T1**: CuO-NPs, **T2**: PFOS, and **T3**: CuO-NPs+PFOS. Vertical bars represent mean ± SD of triplicates (n=3). Significant difference ($p<0.05$) in treatments is denoted by lowercase alphabets.

### 3.6.2 Catalase (CAT) activity

There was no significant difference in catalase (CAT) activity between CT (control group) and the exposed groups T1 (CuO-NPS), T2, (PFOS) and T3(CuO-NPs+PFOS) after 7 days of exposure. However, CAT activity in T1 was significantly higher than that of T3. At the 14-day interval, there was no significant difference observed between CT, T1 and T2, whereas CAT activity in T3 was lowest at statistical significance ($p<0.05$). After 21 days of exposure, all exposed groups were significantly lower than that of CT (Fig.18). Treatment T1 significantly decreased as compared to control. The lowest value of enzyme activity remained in T3. Treatment T2 was significantly lower than control but was not significantly different than treatments T1 and T3. At the 28-day sampling, there was a significant decreasing trend observed in the order CT>T1>T2>T3. Enzyme activity across all sampling intervals is presented below Figure 18.

**Table 11:** Catalase activity in *Danio rerio* gills following single and joint exposure to CuO-NPs and PFOS.

| Treatments | Exposure Days | | | |
|---|---|---|---|---|
| | **7-Day** | **14-Day** | **21-Day** | **28-Day** |
| **CT (Control)** | 64.98±7.65$^{ab}$ | 67.15±2.94$^{a}$ | 73.49±2.47$^{a}$ | 68.88±2.28$^{a}$ |
| **T1 (CuO-NPs)** | 74.77±1.71$^{a}$ | 65.00±4.34$^{a}$ | 61.01±2.61$^{b}$ | 56.17±1.13$^{b}$ |
| **T2 (PFOS)** | 73.20±3.37$^{ab}$ | 63.69±1.61$^{a}$ | 54.89±2.96$^{bc}$ | 49.24±1.25$^{c}$ |
| **T3 (CuO-NPs+PFOS)** | 61.38±4.59$^{b}$ | 53.62±4.81$^{b}$ | 48.57±2.38$^{c}$ | 41.44±1.61$^{d}$ |

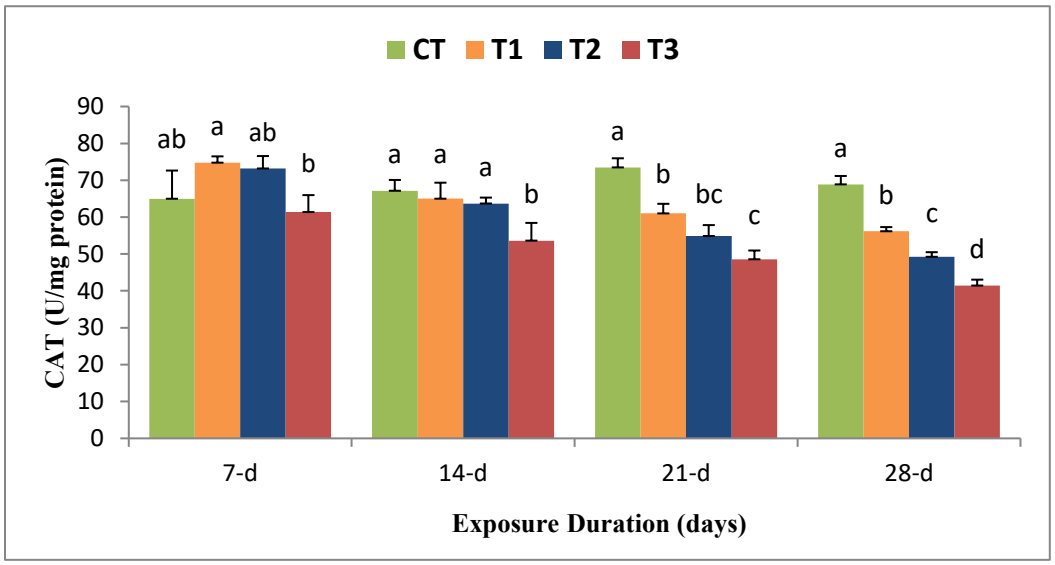



**Figure 18:** Catalase activity in *Danio rerio* gills following single and joint exposure to CuO-NPs and PFOS. Treatments are presented as following; **CT**: Control, **T1**: CuO-NPs, **T2**: PFOS, and **T3**: CuO-NPs+PFOS. Vertical bars represent mean ± SD of triplicates (n=3). Significant difference ($p<0.05$) in treatments is denoted by lowercase alphabets.

## 3.7 Acetylcholinesterase activity

Acetylcholinesterase (AChE) activity was measured in zebrafish brain at 7, 14, 21 and 28-day from all treatments. Inhibition of AChE was observed to be significantly lower in CT (control) as compared to T1 (CuO-NPs), T2 (PFOS) and T3 (CuO-NPs+PFOS) throughout the experimental duration ($p<0.05$). At 7 and 14-day interval, T3 had the highest inhibition of AChE,

whereas no significant difference was observed between T1 and T2 (Fig.19). At 21-day interval, AChE activity remained highest in CT and T2 with least inhibition of the enzyme activity, however, there was no significant difference between T1 and T2 ($p<0.05$), whereas difference between T1 and the lowest treatment, T3, was found to be nonsignificant. At 28-day, differences in groups followed similar trend as the 7 and 14-day intervals. The activity of AChE in all treatments across the experimental duration is presented below in Figure 19.

**Table 12:** Acetylcholinesterase activity in *Danio rerio* brain following single and joint exposure to CuO-NPs and PFOS.

| **Treatments** | **Exposure Days** | | | |
|---|---|---|---|---|
| | **7-Day** | **14-Day** | **21-Day** | **28-Day** |
| **CT (Control)** | $6.25\pm0.25^{a}$ | $6.10\pm0.17^{a}$ | $5.91\pm0.64^{a}$ | $5.97\pm0.33^{a}$ |
| **T1 (CuO-NPs)** | $5.37\pm0.15^{b}$ | $5.02\pm0.28^{b}$ | $4.63\pm0.25^{bc}$ | $4.50\pm0.20^{b}$ |
| **T2 (PFOS)** | $5.47\pm0.32^{b}$ | $5.16\pm0.38^{b}$ | $4.97\pm0.21^{ab}$ | $4.66\pm0.35^{b}$ |
| **T3 (CuO-NPs+PFOS)** | $4.75\pm0.15^{c}$ | $4.23\pm0.21^{c}$ | $3.80\pm0.35^{c}$ | $3.53\pm0.19^{c}$ |

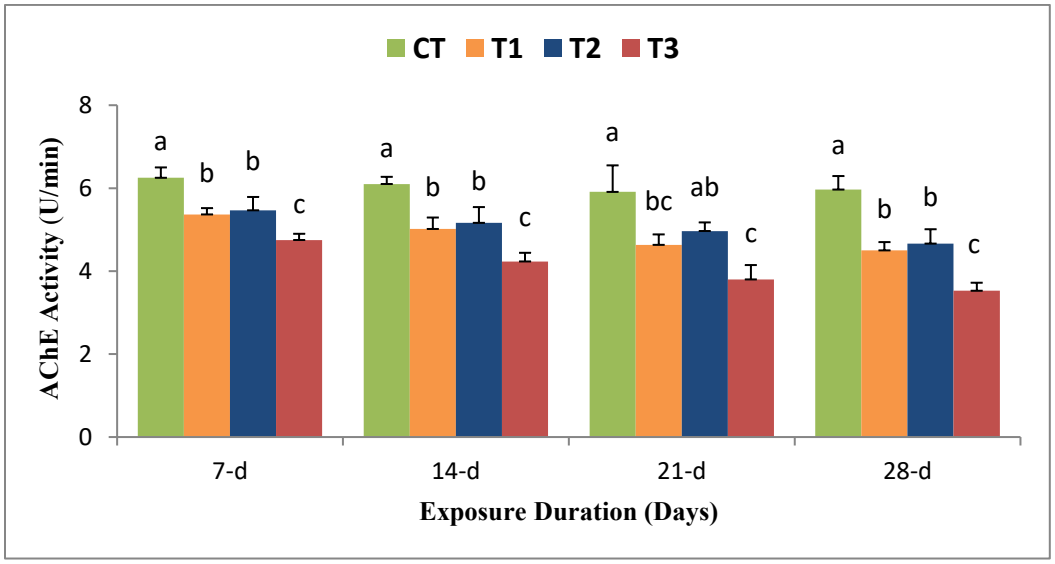



**Figure 19:** Acetylcholinesterase activity in *Danio rerio* brain following single and joint exposure to CuO-NPs and PFOS. Treatments are presented as following; **CT**: Control, **T1**: CuO-NPs, **T2**: PFOS, and **T3**: CuO-NPs+PFOS. Vertical bars represent mean ± SD of triplicates (n=3).

Significant difference ($p<0.05$) in treatments is denoted by lowercase alphabets.

## 3.8 DNA damage by comet assay

The extent of DNA damage brought on by singular and combined exposure to CuO-NPs and PFOS in zebrafish liver tissues was assessed using the comet assay. As summarized in Table 13, DNA gradually changed both before and after exposure. At all sampling intervals (i.e., day 7, 14, 21, and 28), head length gradually decreased in all treatments (T1, T2, and T3) whereas tail length increased as a result of damaged DNA migrating from the head to the tail. At all sampling points, DNA in the head remained between 86 and 88.5% in CT (control). From 7 to 28-day, DNA percentage in the tail increased in treatments T1 (CuO-NPs), T2 (PFOS) and T3 (CuO-NPs+PFOS). Compared to 7, 14 and 21-day, DNA in the tail was found to be the highest in T3 at 28-days. Throughout the experiment duration, DNA in the tail remained at 11.9 to 14.9% in CT. Compared to the CT, comet length also consistently increased at all sampling points with in T1, T2 and T3. Throughout the experiment, the percentage of DNA in the head followed the trend; T1>T2>T3, while the percentage of DNAin the tail followed the trend; T1>T2>T3 (Figure 21).

**Table 13:** Genotoxicity assessment in *Danio rerio* liver following single and joint exposure to CuO-NPs and PFOS. Values are presented as mean ± SD of triplicates (n=3).

| Treatment | Comet length (µm) | Head length (µm) | Tail length (µm) | % DNA in head | % DNA in tail | Tail moment (µm) |
|---|---|---|---|---|---|---|
| | | | **7-Day** | | | |
| **CT** | 38.6±2.1 | 33.2±2.5 | 5.4±0.9 | 86.01±3.8 | 13.98±3.8 | 0.10±0.03 |
| **T1** | 40.2±3.8 | 33.1±3.4 | 7.1±0.7 | 82.33±3.3 | 17.66±2.2 | 0.13±0.03 |
| **T2** | 41.8±4.2 | 34.7±3.8 | 7.2±1.5 | 83.01±3.1 | 16.98±1.9 | 0.12±0.04 |
| **T3** | 40.9±4.2 | 32.5±3.7 | 8.4±1.4 | 79.46±4.2 | 20.53±1.5 | 0.17±0.06 |
| **14-Day** | | | | | | |
| **CT** | 39.1±2.2 | 34.6±2.8 | 4.5±0.7 | 88.49±3.1 | 11.5±1.3 | 0.10±0.03 |
| **T1** | 41.5±3.4 | 35.1±2.6 | 6.4±1.0 | 84.57±3.2 | 15.42±1.6 | 0.11±0.03 |
| **T2** | 43.3±2.2 | 34.3±2.3 | 9.0±1.1 | 79.21±3.5 | 20.78±2.3 | 0.16±0.06 |
| **T3** | 43.9±3.1 | 34.1±2.8 | 9.8±1.7 | 77.67±2.5 | 22.32±4.1 | 0.21±0.05 |
| **21-Day** | | | | | | |
| **CT** | 37.2±3.1 | 32.1±2.3 | 5.1±1.7 | 86.29±3.2 | 13.7±2.4 | 0.12±0.03 |
| **T1** | 39.8±2.8 | 32.3±2.6 | 7.5±1.1 | 81.15±3.1 | 18.84±2.1 | 0.15±0.04 |
| **T2** | 42.1±3.8 | 31.1±3.4 | 11.01±0.9 | 73.87±3.2 | 26.12±2.5 | 0.22±0.06 |
| **T3** | 44.2±3.5 | 30.8±3.1 | 13.4±1.5 | 69.68±3.5 | 30.31±2.6 | 0.44±0.15 |
| **28-Day** | | | | | | |
| **CT** | 36.9±3.5 | 31.4±2.9 | 5.5±1.8 | 85.09±3.5 | 14.9±1.9 | 0.13±0.05 |
| **T1** | 40.2±3.2 | 32.1±2.8 | 8.1±1.7 | 79.85±3.7 | 20.15±1.2 | 0.17±0.02 |
| **T2** | 44.7±1.7 | 31.2±3.4 | 13.5±1.9 | 69.79±4.4 | 30.20±3.3 | 0.46±0.14 |
| **T3** | 46.1±3.6 | 29.3±1.3 | 16.8±1.8 | 63.55±3.1 | 36.44±3.2 | 1.01±0.23 |

CT: Control, T1: CuO-NPs (1mg/L), T2: PFOS (300 µg/L), T3: CuO-NPs+PFOS (1mg/L+300 µg/L)

## 3.9 Tail moment

Compared to CT (control), tail moment (TM) was found to be higher in all treatments T1 (CuO-NPs), T2 (PFOS) and T3 (CuO-NPs) (Fig). At 7 and 14-day intervals, no significant difference

in TM was found in CT and the treatments, however, after 21 days a significant increase was observed in T3 ($p<0.05$). Following 28 days of exposure, a significant increase in T2 and T3 was observed, whereas T1 was not statistically different from CT at $p<0.05$ (Fig.20). Increasing tail moment corresponded with chemical stress and increasing exposure duration. Compared to treatments T1 and T2, T3 had the highest levels of TM after 28 days of exposure. Combined exposure to CuO-NPs and PFOS was seen to exhibit DNA damage in zebrafish liver tissues, as summarized in Figure 20.

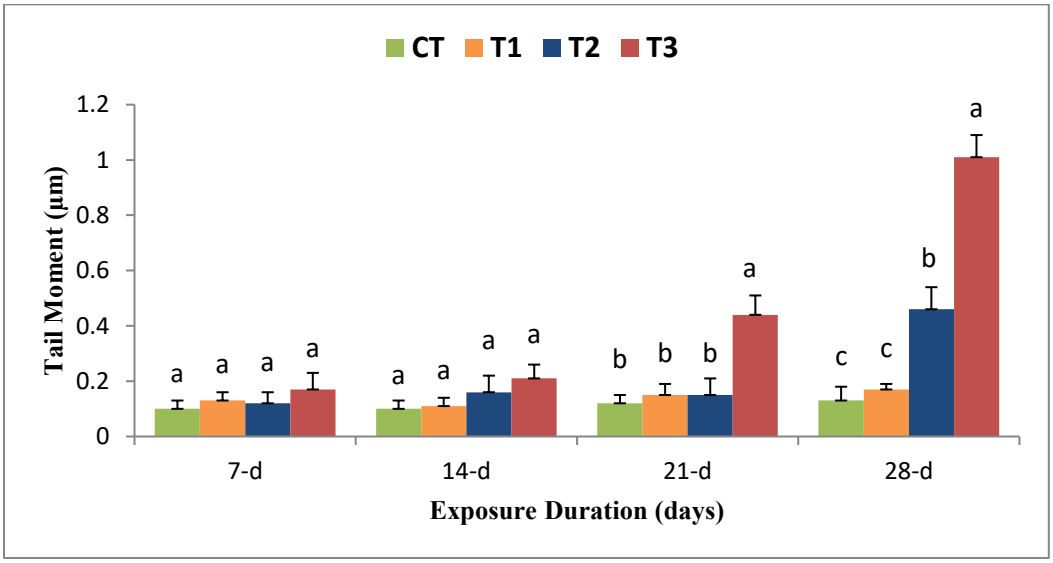



**Figure 20:** Tail moment in *Danio rerio* liver following single and joint exposure to CuO-NPs and PFOS. Treatments are presented as following; **CT**: Control, **T1**: CuO-NPs, **T2**: PFOS, and **T3**: CuO-NPs+PFOS. Vertical bars represent mean ± SD of triplicates (n=3). Significant difference ($p<0.05$) in treatments is denoted by lowercase alphabets.

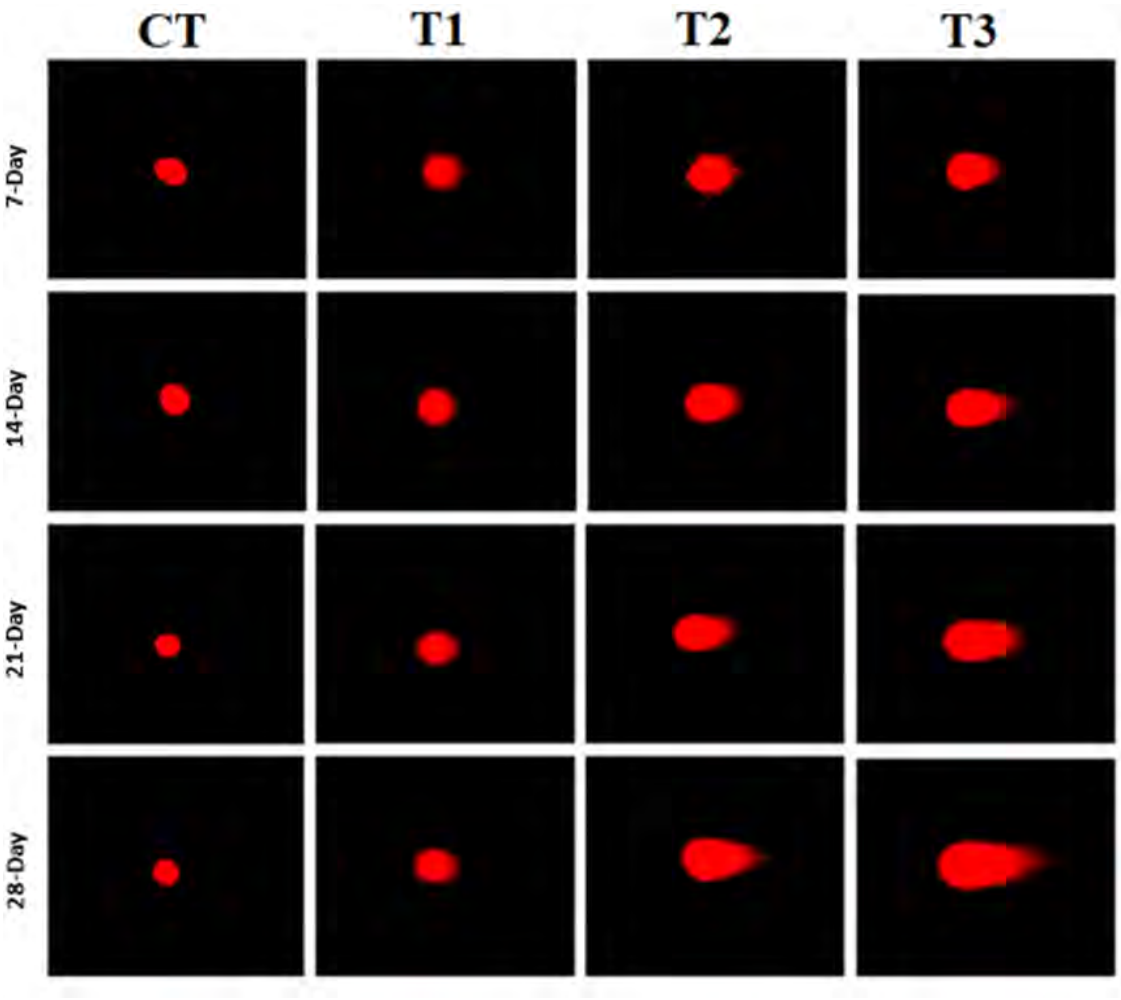



**Figure 21**: Fluorescence photomicrographs of DNA damage in Danio rerio liver following single and combined exposure to CuO-NPs and PFOS. Treatments are presented as following; CT: Control, T1: CuO-NPs, T2: PFOS, and T3: CuO-NPs+PFOS.

## 3.10 Histopathological changes in skeletal muscle of zebrafish

The skeletal muscles from CT (control) showed normal histoarchitecture with the nucleus lying at the periphery of the fibers. No significant alterations were observed in CT throughout the experimental duration. At 7-day, segmental necrosis of muscle was observed in T1 (CuO-NPs). Treatment T2 (PFOS) showed necrosis and degeneration of muscle fibers whereas T3 (CuO-NPs+PFOS) showed vacuolar degeneration of the muscle (see Fig. 22). As shown in Figure 23, splitting and degeneration of muscle fibers was observed in T1, while necrosis and shortening of fiber size was seen in T2 and T3, respectively after 14-day interval. During the 21-day interval, degeneration of muscle fibers continued in T1 whereas T2 showed segmental necrosis of muscle (Fig. 24). Skeletal muscle in T3 showed signs of degeneration along with atrophy. After 28 days of exposure, signs of segmental necrosis were observed in the combined exposure T3 while both T1 and T2 group showed degeneration. Splitting of muscle fibers was also seen in T2 group (Fig. 25).

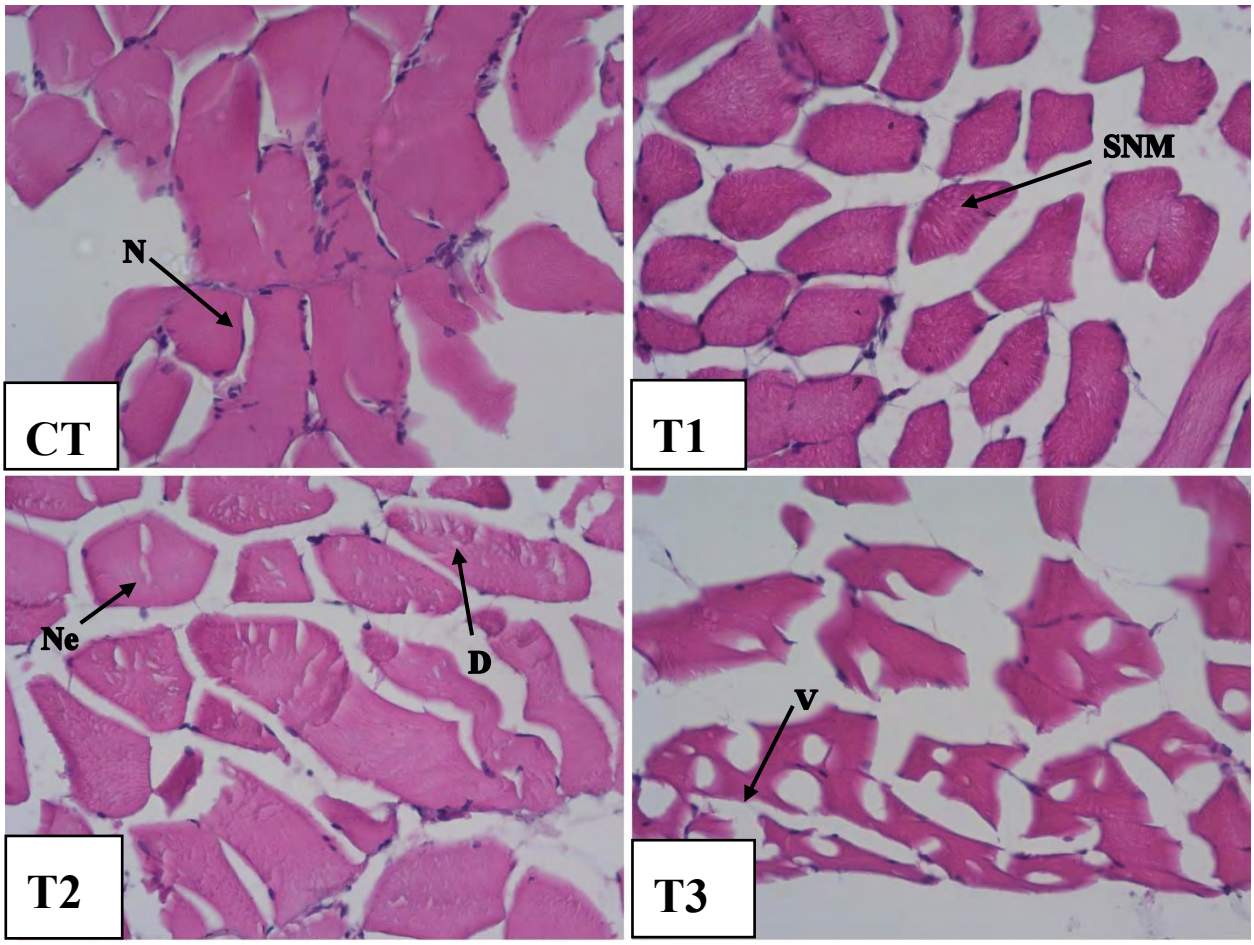



**Figure 22**: Photomicrographs of zebrafish skeletal muscle stained by H&E (40x magnification) after 7 days of exposure. CT (Control); T1 (CuO-NPs); T2 (PFOS); and T3 (CuO-NPs+PFOS). **N**: Nucleus located at periphery of muscle fibers, **SNM**: SegmentalNecrosis in Muscle Fiber, **Ne**: Necrosis, **D**: Degeneration in muscle bundles and **V**: Vacuolar degeneration.

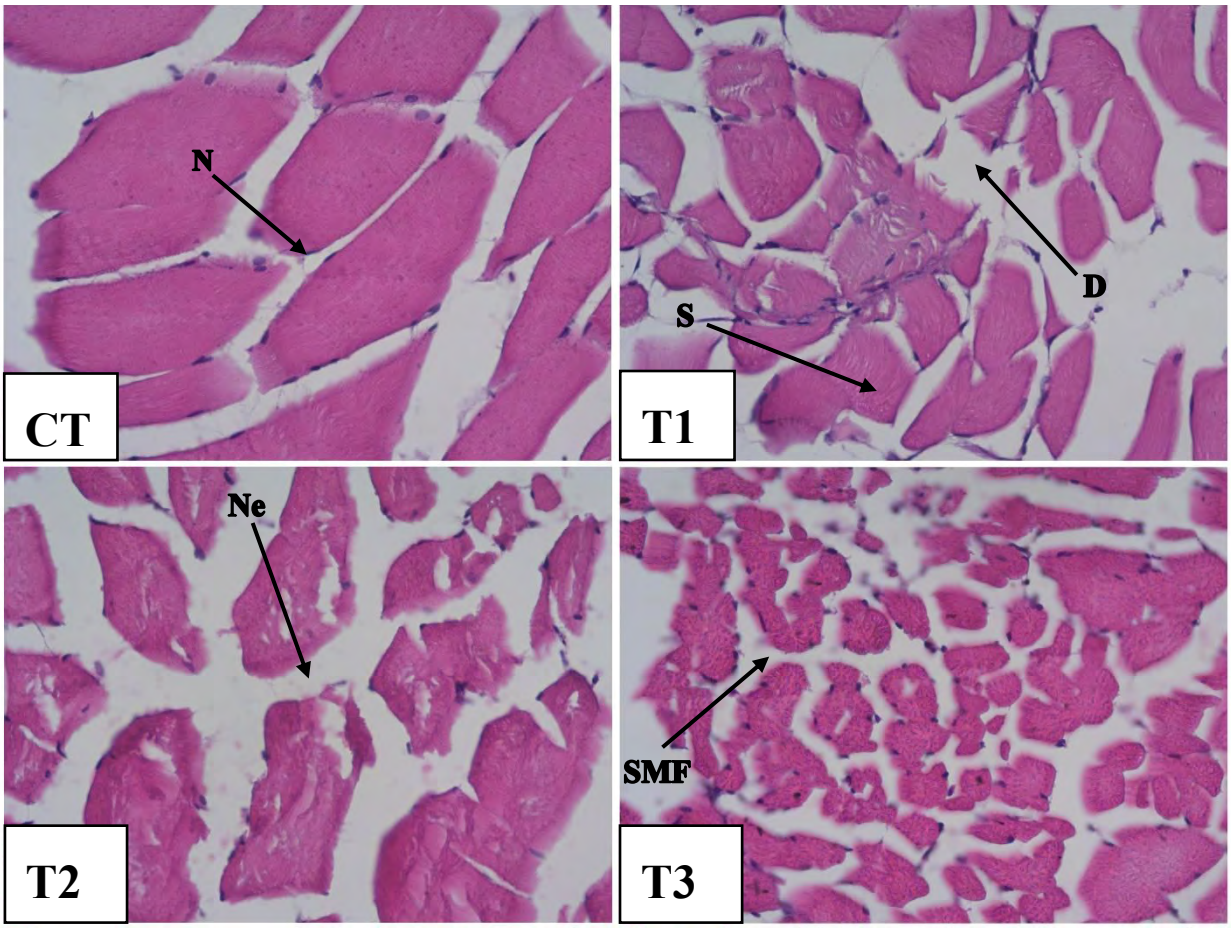



**Figure 23**: Photomicrographs of zebrafish skeletal muscle stained by H&E (40x magnification) after 14 days of exposure. CT (Control); T1 (CuO-NPs); T2 (PFOS); and T3 (CuO-NPs+PFOS). **N**: Nucleus located at periphery of muscle fibers, **S**: Splitting of muscle fibers, **Ne**: Necrosis, **D**: Degeneration in muscle bundles and **SMF**: Shortening of fiber size with overall reduction.

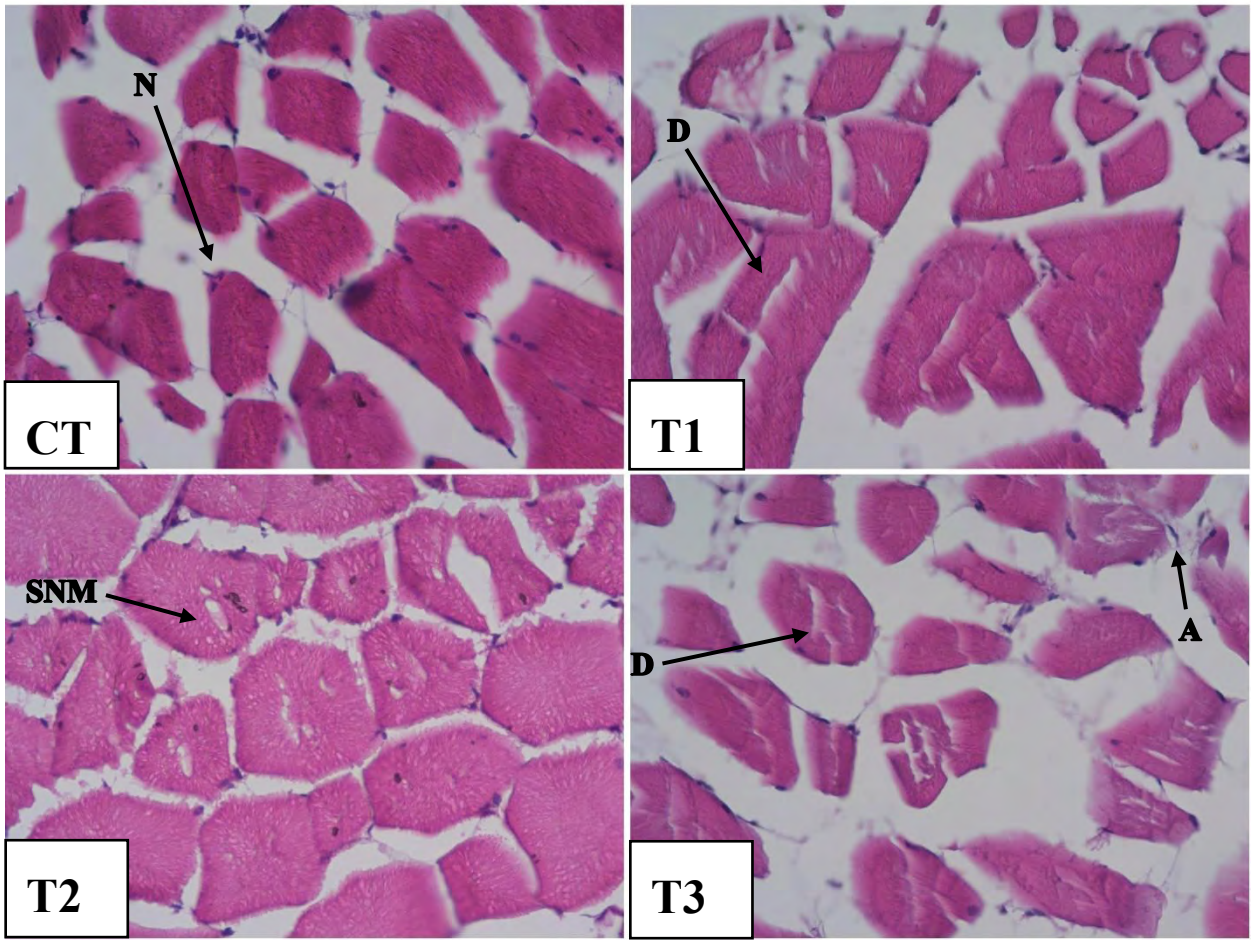



**Figure 24**: Photomicrographs of zebrafish skeletal muscle stained by H&E (40x magnification) after 21 days of exposure. CT (Control); T1 (CuO-NPs); T2 (PFOS); and T3 (CuO-NPs+PFOS). **N**: Nucleus located at periphery of muscle fibers, **D**: Degeneration in muscle bundles, **SNM:** Segmental necrosis of muscle and **A**: Atrophy.

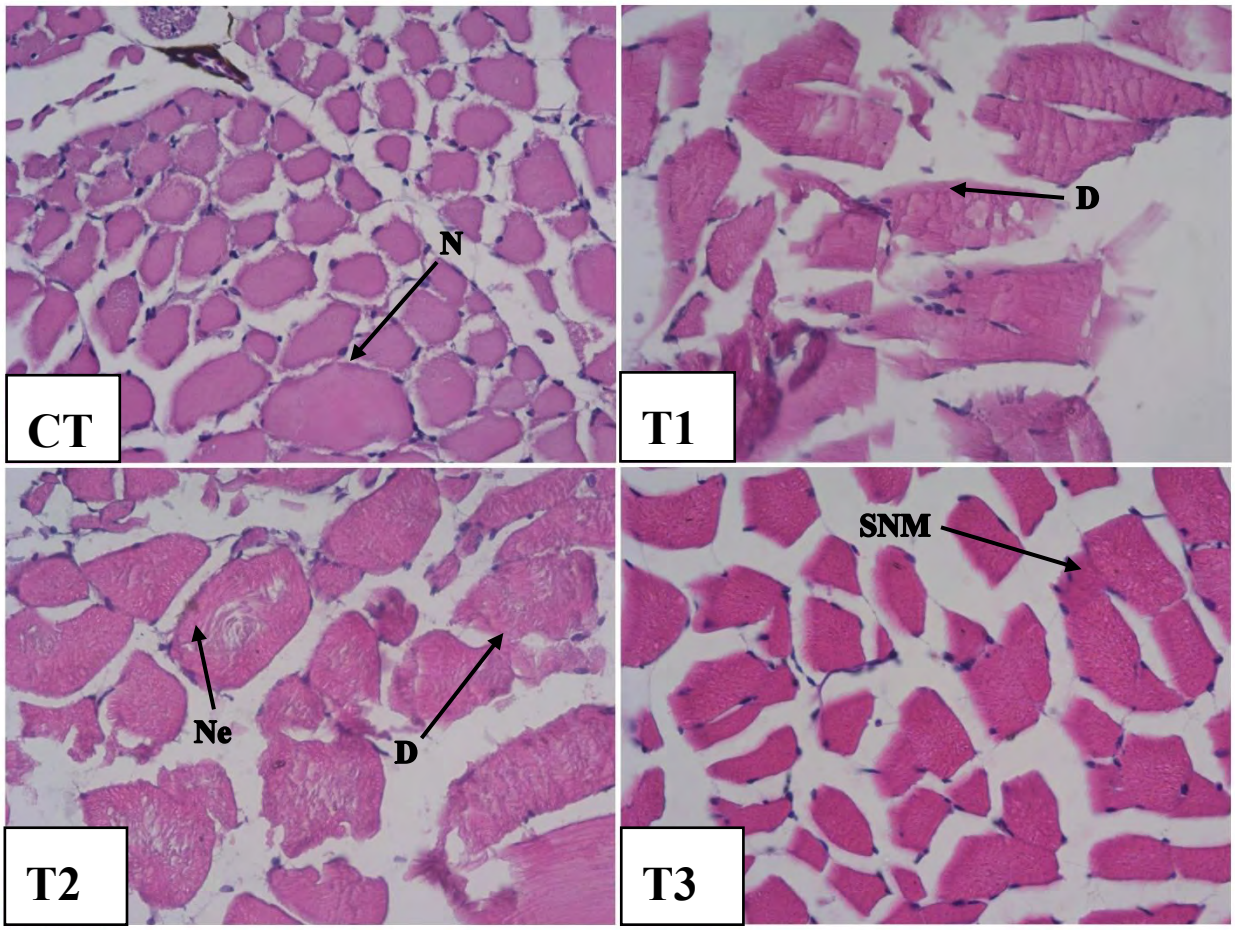



**Figure 25**: Photomicrographs of zebrafish skeletal muscle stained by H&E (40x magnification) after 28 days of exposure. CT (Control); T1 (CuO-NPs); T2 (PFOS) and T3 (CuO-NPs+PFOS). **N**: Nucleus located at periphery of muscle fibers, **D**: Degeneration in muscle bundles, **SNM**: Segmental necrosis of muscle and **Ne**: Necrosis.

# CHAPTER 4
# DISCUSSION

# 4 DISCUSSION

## 4.1 Characterization of copper oxide nanoparticles

Copper oxide nanoparticles (CuO-NPs) have been widely reported to be more toxic than their bulk counterparts (Chang et al., 2012). To understand and define toxic manifestation of CuO-NPs, it is crucial to understand the characterization of these nanoparticles, their routes of exposure and mechanism or pathways involved in their toxicity (Naz et al., 2019). Nanoparticles can cause toxicity through various mechanisms, but it is mostly a function of their physical and chemical characteristics such as size, shape, and chemical composition (Sukhanova et al., 2018).

The CuO-NPs used in this study were between 50-100nm in size and exhibited rice-grain like structures assembled into aggregates, as revealed by scanning electron microscope (SEM) in Figure 10. Similar morphology of CuO-NPs has been previously reported (Sukumar et al., 2020; Ananth et al., 2015). Aggregation and agglomeration tendency of CuO-NPs from the same manufacturer was observed by multiple authors (Ślosarczyk et al., 2023; Amirian et al., 2018). Lack of uniformity in size or shape can occur when there's no proper control on nanoparticle growth during synthesis (capping agent), whereas nanoparticles tend to aggregate to reduce Gibb's free energy in the absence of surfactants (Chowdhury et al., 2020; Wang et al., 2002). Elemental composition assessed by energy dispersive x-ray (EDX) exhibits prominent peaks of copper (Cu) and oxygen (O) with weight percentages 71.4 and 20.9% respectively as it clears from Figure 10. The presence of sharp and narrow peaks suggests high crystallinity of CuO-NPs (Sabeena et al., 2022; Sukumar et al., 2020).

**FTIR:** Fourier Transform Infrared (FTIR) Spectroscopy can detect the presence of any surface biomolecules or functional groups present on the nanoparticles (Badaway et al., 2021; Sukumar et al., 2020). Sharp peaks at 512.97, 52454 and 597.82 $cm^{-1}$ are characteristic of pure CuO nanostructure formation (Quirino et al., 2018; Hemelatha et al., 2017), which also suggests the presence of a monoclinic phase (Luna et al., 2015). This is also in agreement with x-ray diffraction (XRD) results, which indicate the formation of CuO in Tenorite phase with monoclinic crystal system, with characteristic peaks indexed according to JCPDS card No: 045-0937 (Zedan et al., 2028; Ahamed et al., 2014). No additional peaks were observed for commonly occurring impurities ($Cu_2O$ or Cu $(OH)_2$), suggesting high purity (Buledi et al., 2021).

## 4.2 Water quality parameters

Water quality parameters including temperature, electrical conductivity (EC), dissolved oxygen (DO), and pH were maintained throughout the experiment in accordance with OECD guidelines for testing of chemical (OECD, 2019). There was no significant difference in temperature, DO and pH in control and treated groups, and remained optimum for zebrafish. Water quality was maintained by changing exposure medium every alternative day to ensure adherence to OECD guidelines. Similar water quality conditions were also reported for zebrafish toxicity testing by Tang et al. (2019).

## 4.3 Total protein

Total protein (TP) content provides valuable insight into toxicological effects of pollutants on organisms, as proteins are essential for survival and growth of all living organisms (Guarda et al., 2003; Fu et al., 2020). Changes in total protein levels can occur due to a variety of processes, including inhibition of enzyme production, and high energy demand due to stressful conditions due to chemical exposure (Oruç et al., 2006; Morales et al., 2004). In the present study, total protein levels declined after singular and joint exposure to copper oxide nanoparticles (CuO-NPs) and perfluorooctane sulfonate (PFOS). Exposure to CuO-NPs for 7 days caused a decline in serum total protein levels in trout *O. niloticus* (Tuncsoy and Erdem, 2021). Similarly, thiazolidine was shown to reduce total protein levels in zebrafish *D. rerio* liver. Decrease in total protein may be due to cellular destruction or disruption in protein synthesis (Kayhan et al., 2022).

## 4.4 Oxidative stress

Contaminants in the environment cause toxicity in aquatic organisms by induction of oxidative damage (Stara et al., 2012 & 2013; Lushchak et al., 2005). For this reason, oxidative stress markers are studied to understand and evaluate environmental contamination and its impact on organisms (Slaninova et al., 2009). Chemical stress can cause excess production of reactive oxygen species (ROS), which cause cell damage via peroxidation of unsaturated fatty acids and through breakdown lipid hydroperoxide products (Fang and Zheng, 2002). One of the final breakdown products of lipid peroxidation (LPO) is malondialdehyde (MDA), which is evaluated as a biomarker to assess the severity of lipid peroxidation (Ge et al., 2015). Higher levels of ROS

have been linked to excess production of MDA content (Dong et al., 2013). Increased ROS and MDA content can cause DNA damage, mitochondrial respiratory chain dysfunction and cell death (Sun et al., 2018). In our study, exposure to copper oxide nanoparticles (CuO-NPs) and perfluorooctane sulfonate (PFOS) caused increasing production of ROS as compared to the control group. Exposure to CuO-NPs has been shown to cause oxidative stress via excess production of ROS and LPO in adult and embryonic zebrafish (Mani et al., 2020; Ganesan et al., 2015). Aziz et al. (2023 and 2022) reported increased production of thiobarbituric substances (TBARs), a marker for LPO, in bighead carp *H. nobilis* and rohu *L. rohita* following time-dependent exposure to CuO-NPs, which may be explained by overproduction of ROS by exposure to nanoparticles (Wang et al., 2012). CuO-NPs can penetrate cell membranes via diffusion and endocytosis and accumulate in cell organelles such as mitochondria, which is a major intracellular source of ROS (Wang et al., 2013a, b). Further, released copper (Cu) ions can exacerbate oxidative stress through one-electron oxidation-reduction (Haber-Weiss and Fenton's reaction) (Ganesan et al., 2016). Similarly, exposure to PFOS has been reported to cause oxidative stress (Feng et al. 2015; Lu et al. 2015; Shi and Zhou 2010). Increased ROS and MDA levels were observed in zebrafish embryos exposed to PFOS (Huang et al., 2021). Similarly, an increase in MDA content was observed in crustacean *G. insensibilis* following exposure to PFOS (Touaylia et al., 2019). Structural similarities of perfluorinated compounds (PFCs) to long-chain fatty acids have been associated with their capability to cause changes in mitochondrial permeability transition (MPT), resulting in cytotoxicity and oxidative stress (O'Biran et al., 2004). In the present study, combined exposure to CuO-NPs and PFOS resulted in significantly higher production of ROS and MDA as compared to individual exposure ($p<0.05$). Combined exposure to Cu and PFOS was reported to disrupt intracellular oxidant/antioxidant balance in *L. hoffmeisteri* under oxidative stress, which was higher than induvial exposure to both contaminants (Meng et al., 2016). Similarly, Du et al. (2017) reported synergistic toxic action of zinc oxide nanoparticles (ZnO-NPs) and PFOS on zebrafish, with significant induction in ROS accompanied by increase in MDA following co-exposure. Synergistic action may be caused by adsorption of PFOS via its sulfonic group on CuO-NPs or released Cu ions resulting in increased permeability and transmembrane transport of PFOS (Du et al., 2016; Meng et al., 2016).

## 4.5 Antioxidant response

In response to production of reactive oxygen species (ROS), aquatic organisms adopt an antioxidant defense system to scavenge and reduce oxidation (Pandey et al., 2003). Antioxidant enzymes superoxide dismutase (SOD) and catalase (CAT) are the primary defense system against oxidative damage (Fang and Zheng et al., 2002). SOD is a metalloenzyme which counters oxidative damage by converting harmful superoxide radicals ($O_2^-$) into hydrogen peroxide ($H_2O_2$) whereas CAT converts the $H_2O_2$ produced by SOD into oxygen ($O_2$) and water ($H_2O$), preventing the $H_2O_2$ from reacting with $O_2$ to form hydroxyl radical (·OH) (El-Garawani et al., 2021; Altun et al., 2017). When the SOD-CAT system is overpowered, an imbalance is created and risk of oxidative stress increases (Zheng et al., 2016). Both the enzymes have been extensively studied as markers for oxidative stress (Pinho et al. 2005; Gomi et al. 1998). In present study, exposure to copper oxide nanoparticles (CuO-NPs) and perfluorooctane sulfonate (PFOS) in single and combined settings influenced the antioxidant defense system. Exposure to both contaminants initially activated SOD and CAT activities at the 7-day interval, followed by a decline or inhibition of both enzymes after 14 days. Similar trends have been observed and explained by multiple authors. Initial exposure to thiamethoxam caused activation of SOD and CAT activities in zebrafish *D. rerio*, followed by a decrease, whereas prolonged exposure to endosulfan exhibited similar toxic effects (Yan et al., 2015; Shao et al., 2012). This pattern of early increase followed by a decrease in SOD and CAT enzyme activity can be explained by multiple phenomena. The initial increase is caused by the activation of zebrafish-defense system following induction of oxidative stress (Du et al., 2014). However, high production of ROS can overwhelm the antioxidant system and minimize the protective role of SOD and CAT in reducing oxidative stress by inhibition of enzyme activity (Dong et al., 2013). Increased ROS has been known to inactivate enzymes and production of superoxide ($O_2^-$) and peroxy radicals can inhibit CAT activity (Escobar et al., 1996; Tabataie & Floyd, 1994). Inhibition or inactivation of SOD and CAT by CuO-NPs and PFOS following an increase in oxidative stress has been previously reported by various studies. Exposure to increasing concentration of CuO-NPs caused increasing ROS and declining levels of SOD and CAT activity in *D. rerio* embryos and adults (Mani et al., 2020; Ganesan et al., 2015). Prolonged exposure to NPs has been shown to inhibit antioxidant resistance, whereas exposure to Cu ions can inhibit CAT activity in fish (Gomes et al., 2011; Atli

et al., 2006). An increase-decrease trend in enzymatic activities in Bighead Carp *H. nobilis* exposed to CuO-NPs was attributed to peroxidation and carbonization by ROS, as a function of metal toxicity (Aziz et al., 2023). Decrease in SOD and CAT activities was observed in gills of aquatic invertebrate *A. leptodactylus* after exposure to PFOS (Belek et al., 2022). Similarly, exposure to PFOS resulted in a concentration dependent decrease in SOD activity in crustacean *G. insensibilis* (Touaylia et al., 2019). No significant effect was observed on SOD and CAT activities in developing *D. rerio* following exposure to PFOS, whereas an increase in glutathione S-transferase (GST) and a decrease in glutathione GSH indicated that a conjugation reaction mediated PFOS induced oxidative stress, as action of antioxidant enzymes can be replaced by other antioxidants (Huang et al., 2021; Zheng et al., 2016). Lu et al. (2023) reported an increase in SOD and CAT activities in *D. rerio* after sub-acute exposure to PFOS, however, co-exposure of with cadmium (Cd) caused a decline in enzyme activities. In our study, combined exposure to CuO-NPs and PFOS had more deleterious effects than individual exposures. Comparable to our observations, combined exposure to Cu ions and PFOS had greater effect on SOD inhibition in *L. hoffmeisteri* than singular exposure, whereas interaction of ZnO-NPs and PFOS synergistically reduced CAT activity in *D. rerio* embryos (Du et al., 2017; Meng et al., 2016).

## 4.6 Acetylcholinesterase activity

Acetylcholinesterase (AChE) is an enzyme that hydrolyzes the neurotransmitter acetylcholine (ACh) present in the cholinergic synapses and is crucial for physiological functioning in organisms including locomotion, orientation, feeding and predator evasion (De Cuna et al., 2011). Changes in activity of AChE directly impact cholinergic neurotransmission, leading to neurological disorders (dos Santos Miron et al., 2005). Measurement of AChE activity is a good indicator of disturbances in neurological function (Li et al., 2017). Exposure to copper oxide nanoparticles (CuO-NPs) and perfluorooctane sulfonate (PFOS) led to inhibition of AChE activity upon exposure. The effect of CuO-NPs on AChE activity was found to be greater than that of PFOS, which are in consistency with previously documented results. A slight induction in AChE activity followed by a decrease with increasing concentration of CuO-NPs was observed in goldfish *C. auratus* (Jun et al., 2013). Exposure to CuO-NPs reduced AChE activity in embryonic and adult *D. rerio* (Mani et al., 2020; Ganesan et al., 2015). In juvenile carp *C. carpio*,

CuO-NPs had greater inhibitory action on cholinesterase (ChE) than bulk CuO, which may be explained by release of Cu ions in the body (Zhao et al., 2011). In agreement with our results, reduction in AChE activity occurred in *D. magna* exposed to PFOS and PFNA (Lu et al., 2015). Mahapatra et al. (2023) reported inhibition of AChE activity in *D. rerio* embryos in a dose-dependent manner and suggested that PFOS inhibits AChE in the same manner as other cholinesterase inhibitors. Contrary to these findings, exposure to PFOA and PFOS did not significantly alter AChE activity in common carp *C. carpio* (Kim et al., 2010). The same effect was observed in crustacean *G. insensibilis* following exposure to PFOS (Touaylia et al., 2019). However, microplastics (MPs) loaded with PFOS caused higher inhibition of AChE activity in clam *S. plana* compared to virgin MPs, resulting in greater neurotoxicity (Islam et al., 2021). The same phenomena were observed in our study, where co-exposure to CuO-NPs and PFOS resulted in significantly higher inhibition of AChE activity as compared to other treatments. Li et al. (2017) reported no significant alteration in AChE activity in zebrafish following individual exposure to PFOS, however, the author suggested that combined exposure with single walled carbon nanotubes (SWCNT) may have more enhanced effects.

## 4.7 DNA damage by Comet Assay

DNA damage has been established as a well-studied biomarker in ecotoxicological studies. Apart from direct action of toxicants, a close relationship between DNA damage and oxidative stress has been well-reported (Oliveria et al., 2009). Oxidative stress can induce DNA damage as reactive oxygen species (ROS) mediated free radicals attack cellular and sub-cellular components, whereas excessive malondialdehyde (MDA) can facilitate the entry of contaminants in cells by causing alteration in the cell membrane (Song et al., 2019; Gao et al., 2018). Single-cell gel electrophoresis (SCGE) or comet assay has been developed as a rapid and accurate method to analyze DNA damage (Jin et al., 2011). DNA damage is assessed by applying an electric field to exposed DNA, causing damaged DNA to migrate toward the anode which can be quantified (Muazzam et al., 2019). In present study, increased tail length and decreasing head length was observed in groups exposed to copper oxide nanoparticles (CuO-NPs) and perfluorooctane sulfonate (PFOS) with a significant increase in tail moment ($p<0.05$). Previously, genotoxic action of CuO-NPs on rohu *L. rohita* was reported, whereas a significant increase in

DNA tail length was observed in freshwater crayfish *P. clarkia* (Aziz et al., 2023; El-Atti et al., 2019). Furthermore, CuO-NPs were also shown to cause DNA damage in *C. gariepinus* and *H. nobilis* (Aziz et al., 2022; Ogunsuyi et al., 2019). CuO-NPs can enter cell nucleus via nuclear pores or punctures in the membrane getting into direct contact with the DNA, including release of Cu ions (Gupta et al., 2016; Huang et al., 2009). These Cu ions can create chemical bonds with the phosphate groups and nitrogenous bases present in the DNA, particularly cytosine (C) and guanine (G), by competition with hydrogen ions, which results in the breaking of links between nitrogenous bases and consequent unwinding of the DNA double-helical structure (Aziz et al., 2022). Exposure to PFOS resulted in a concentration-dependent increase in DNA tail length and tail DNA percentage in earthworm *E. fetida* coelomycetes (Xu et al., 2013). Similarly, PFOS induced breakage of DNA single strands in *C. carpio* (Kim et al., 2010). Hoff et al., 2003 hypothesized that PFOS may incur DNA damage by inducing cell necrosis associated membranous damage and/or by disrupting the balance between DNA damage and repair mechanisms, whereas it was proven that PFOS can influence DNA structure and attenuate DNA charge transport, causing damage to the DNA (Lu et al., 2012). In our study, the highest DNA damage was observed in fish exposed to CuO-NPs and PFOS in co-exposure, reflecting a synergistic action on genotoxicity. Interactive action of zinc oxide nanoparticles (ZnO-NPs) and PFOS reportedly caused greater DNA damage in *D. rerio* embryos than singular exposure (Du et al., 2014). In contrast, multiwalled carbon nanotubes (MWCNT) alleviated toxicity and DNA damage in peripheral blood cells caused by PFOS in *D. rerio* embryos, which may be due to antagonistic toxic action on oxidative stress induction (Changlu et al., 2015).

## 4.8 Histopathological Alterations in Muscle Tissues

Histopathological alterations are among the best indicators of toxicological manifestations of chemical substances. Au et al. (2004) argued that studying histopathological alterations in fish is an extremely important parameter to understand toxicological impacts as they reflect the health state of the animal under study better than other biomarkers or diagnosis methods. In zebrafish, muscles are secondary target as gills are the primary target of nanoparticles,not being in direct contact with the exposure medium (Wang et al., 2014). In our study, microscopicexamination of zebrafish skeletal muscle tissues showed significant alterations in histoarchitecture in fish

exposed to copper oxide nanoparticles (CuO-NPs) and perfluorooctane sulfonate (PFOS) in singular and combined settings. This is consistent with reported literature on exposure to both contaminants. Comparable with our observations, skeletal muscle of adult zebrafish *D. rerio* exposed to CuO-NPs showed fibrillary degeneration, mild perimysial inflammation, vascular degeneration, and atrophy with degeneration, while degeneration and increased extracellular space between muscle bundles was observed in rainbow trout *O. mykiss* (Mani et al., 2019; Al-Bairuty et al., 2013). Further, histological alterations were also observed in gills of African catfish *C. gariepinus* and Nile tilapia *O. niloticus* upon exposure to CuO-NPs including epithelial necrosis, desquamation, and reduced secondary gill lamellae (Abdel-Latif et al., 2021; Ogunsuyi et al., 2019), whereas CuO-NPs caused severe histological alterations in hepatopancreas of crayfish *P. clarkii* (El-Atti et al., 2019). CuO-NPs induced oxidative stress can cause apoptosis leading to muscle damage (Cole and Ross, 2001), whereas copper (Cu) ions can accumulate in muscle causing a decrease in muscle fiber size and depletion of muscle glycogen stores (Campbell et al., 2005; Handy, 1999; Vogel, 1959). In zebrafish larvae, PFOS has been reported to cause histological changes in swim bladder and gut section, as well as disintegration in somatic striated muscle cells, whereas disordered and loosened muscle arrays were observed in the tail section of larvae at 120 hours post fertilization (hpf) (Huang et al., 2021; Chen et al., 2014; Huang et al., 2010). Exposure to PFOS in adult *D. rerio* has been shown to induce histological alterations in thyroid, liver and cause granolumatous inflammation in different organs, and has also been reported to impair gonadal development in male and female zebrafish (Chen et al., 2016; Keiter et al., 2012). PFOS has been shown to alter expression of genes involved in muscle development, whereas oxidative stress induced apoptosis may also incur histopathological changes (Jantzen et al., 2016; Lui et a., 2007). Exposure to PFOS in combination with zinc oxide nanoparticles and cadmium caused greater histopathological anomalies in adult *D. rerio*, as compared to induvial exposure (Lu et al., 2023; Du et al., 2014). Similarly, mixture of CuO-NPs and $TiO_2$-NPs induced greater incidence of injury on *C. carpio* gills, kidney, intestine, and liver than exposure to both nanoparticles alone (Mansouri et al., 2016). This agrees with our findings, where interactive action of CuO-NPs and PFOS had more severe deleterious effects muscle histoarchitecture of adult *D. rerio* than induvial exposure to both contaminants.

# CHAPTER 5
# CONCLUSION AND RECOMMENDATIONS

# 5 CONCLUSION AND RECOMMENDATIONS

To the best of our knowledge, this is the first study on the combined exposure of perfluorooctane sulfonate (PFOS) and copper oxide nanoparticles (CuO-NPs) on zebrafish *Danio rerio*. This study exclusively provides insight into the interaction of nanoparticles with PFOS and their toxicological manifestations *in vivo*. The CuO-NPs used in the study were slightly aggregated and 50-100 nm in size, in tenorite phase with monoclinic crystal system, and were free from impurities. In the 28-day experiment, total protein levels, oxidative stress markers including ROS and MDA, activities of antioxidant enzymes SOD and CAT in gills, AChE in the brain, DNA damage in the liver and histopathological alterations in skeletal muscles of zebrafish were assessed. Singular and combined exposure caused significant elevation of ROS and MDA content as compared to the control, whereas activity of antioxidant enzymes SOD and CAT was initially activated but was suppressed after continued exposure. Both the contaminants led to inhibition of AChE activity and caused genotoxicity via DNA damage. Several histopathological alterations were observed in skeletal muscles in all treatment groups except control. The alterations and toxic action of contaminants increased with increased duration of the experiment. Interactive action of CuO-NPs and PFOS caused more adverse effects than individual action of both contaminants. Perfluorinated compounds have existed for almost 5 decades in the natural environments, but emergence of novel pollutants such as nanoparticles increases the ecotoxicological risks associated to them. Studies focusing on the influence of physical and chemical characteristics of nanoparticles in terms of their interaction with PFCs are needed to guide future risk assessment. Further studies on the interactive action of PFOS and other PFCs with emerging contaminants, particularly nanoparticles, and their distribution across trophic levels is crucial to better understand and assess the environmental risk posed by existing contaminants.

# BIBLIOGRAPHY

# BIBLIOGRAPHY


1. Abiraman, T., Ramanathan, E., Kavitha, G., Rengasamy, R., & Balasubramanian, S. (2017). Synthesis of chitosan capped copper oxide nanoleaves using high intensity (30 kHz) ultrasound sonication and their application in antifouling coatings. *Ultrasonics Sonochemistry*, 34, 781-791.
2. Action, V. D. (2020). Environmental Protection Agency 40 Cfr Part 721.
3. Ahamed, M., Akhtar, M. J., Alhadlaq, H. A., & Alrokayan, S. A. (2015). Assessment of the lung toxicity of copper oxide nanoparticles: current status. *Nanomedicine*, 10(15), 2365-2377.
4. Ahamed, M., Siddiqui, M. A., Akhtar, M. J., Ahmad, I., Pant, A. B., & Alhadlaq, H. A. (2010). Genotoxic potential of copper oxide nanoparticles in human lung epithelial cells. *Biochemical And Biophysical Research Communications,* 396(2), 578-583.
5. Ahmed, M. B., Johir, M. A. H., McLaughlan, R., Nguyen, L. N., Xu, B., & Nghiem, L. D. (2020). Per-and polyfluoroalkyl substances in soil and sediments: Occurrence, fate, remediation, and future outlook. *Science of the Total Environment*, 748, 141251.
6. Ahrens, L., & Bundschuh, M. (2014). Fate and effects of poly-and perfluoroalkyl substances in the aquatic environment: A Review. *Environmental Toxicology and Chemistry,* 33(9), 1921-1929.
7. Ahrens, L., Felizeter, S., & Ebinghaus, R. (2009). Spatial distribution of polyfluoroalkyl compounds in seawater of the German Bight. *Chemosphere*, 76(2), 179-184.
8. Ahrens, L., Felizeter, S., Sturm, R., Xie, Z., & Ebinghaus, R. (2009). Polyfluorinated compounds in wastewater treatment plant effluents and surface waters along the River Elbe, Germany. *Marine Pollution Bulletin,* 58(9), 1326-1333.
9. Ahrens, L., Gashaw, H., Sjöholm, M., Gebrehiwot, S. G., Getahun, A., Derbe, E., ... & Åkerblom, S. (2016). Poly-and perfluoroalkylated substances (PFASs) in water, sediment and fish muscle tissue from Lake Tana, Ethiopia, and implications for human exposure. *Chemosphere,* 165, 352-357.
10. Ahrens, L., Yeung, L. W., Taniyasu, S., Lam, P. K., & Yamashita, N. (2011). Partitioning of perfluorooctanoate (PFOA), perfluorooctane sulfonate (PFOS) and perfluorooctane sulfonamide (PFOSA) between water and sediment. *Chemosphere,* 85(5), 731-737.
11. Alizadeh, T., & Mirzagholipur, S. (2014). A Nafion-free non-enzymatic amperometric

glucose sensor based on copper oxide nanoparticles–graphene nanocomposite. *Sensors and Actuators B: Chemical,* 198, 438-447.

**12.** Allred, B. M., Lang, J. R., Barlaz, M. A., & Field, J. A. (2015). Physical and biological release of poly-and perfluoroalkyl substances (PFASs) from municipal solid waste in anaerobic model landfill reactors. *Environmental Science and Technology,* 49(13), 7648-7656.

**13.** Almås, C. (2007). Effect studies of reallife mixtures of persistant organic pollutants (POPs) in a zebrafish model (Master's thesis).

**14.** Al-Sawafi, A. G. A., Wang, L., and Yan, Y. (2017). Cadmium accumulation and its histological effect on brain and skeletal muscle of zebrafish. *Journal of Heavy Metal Toxicity and Disease,* 2(1), 2.

**15.** Altenburger, R., Scholz, S., Schmitt-Jansen, M., Busch, W., and Escher, B. I. (2012). Mixture toxicity revisited from a toxicogenomic perspective. *Environmental Science and Technology,* 46(5), 2508-2522.

**16.** Ameri, B., Davarani, S. S. H., Roshani, R., Moazami, H. R., and Tadjarodi, A. (2017). A flexible mechanochemical route for the synthesis of copper oxide nanorods/nanoparticles/nanowires for supercapacitor applications: the effect of morphology on the charge storage ability. *Journal of Alloys and Compounds,* 695, 114-123.

**17.** Ankley, G. T., Cureton, P., Hoke, R. A., Houde, M., Kumar, A., Kurias, J., ... and Valsecchi, S. (2021). Assessing the ecological risks of per-and polyfluoroalkyl substances: Current state-of-the science and a proposed path forward. *Environmental Toxicology and Chemistry,* 40(3), 564-605.

**18.** Ankley, G. T., Kuehl, D. W., Kahl, M. D., Jensen, K. M., Linnum, A., Leino, R. L., and Villeneuve, D. A. (2005). Reproductive and developmental toxicity and bioconcentration of perfluorooctanesulfonate in a partial life-cycle test with the fathead minnow *(Pimephales promelas). Environmental Toxicology and Chemistry: An International Journal,* 24(9), 2316-2324.

**19.** Ates, M., Arslan, Z., Demir, V., Daniels, J., and Farah, I. O. (2015). Accumulation and toxicity of CuO and ZnO nanoparticles through waterborne and dietary exposure of goldfish (*Carassius auratus*). *Environmental Toxicology,* 30(1), 119-128.

**20.** Au, D. W. T. (2004). The application of histo-cytopathological biomarkers in marine pollution monitoring: A Review. *Marine Pollution Bulletin,* 48(9-10), 817-834.

**21.** Au, D. W. T. (2004). The application of histo-cytopathological biomarkers in marine pollution monitoring: A Review. *Marine Pollution Bulletin, 48*(9-10), 817-834.

**22.** Austin, M. E., Kasturi, B. S., Barber, M., Kannan, K., MohanKumar, P. S., and MohanKumar, S. M. (2003). Neuroendocrine effects of perfluorooctane sulfonate in rats. *Environmental Health Perspectives,* 111(12), 1485-1489.

**23.** Austin, M. E., Kasturi, B. S., Barber, M., Kannan, K., MohanKumar, P. S., and MohanKumar, S. M. (2003). Neuroendocrine effects of perfluorooctane sulfonate in rats. *Environmental Health Perspectives,* 111(12), 1485-1489.

**24.** Baig, N., Kammakakam, I., and Falath, W. (2021). Nanomaterials: A review of synthesis methods, properties, recent progress, and challenges. *Materials Advances,* 2(6), 1821-1871.

**25.** Barber, J. L., Berger, U., Chaemfa, C., Huber, S., Jahnke, A., Temme, C., and Jones, K. C. (2007). Analysis of per-and polyfluorinated alkyl substances in air samples from Northwest Europe. *Journal of Environmental Monitoring,* 9(6), 530-541.

**26.** Bar-Ilan, O., Chuang, C. C., Schwahn, D. J., Yang, S., Joshi, S., Pedersen, J. A., ... and Heideman, W. (2013). TiO2 nanoparticle exposure and illumination during zebrafish development: mortality at parts per billion concentrations. *Environmental Science and Technology*, *47*(9), 4726-4733.

**27.** Batley, G. E., Kirby, J. K., and McLaughlin, M. J. (2013). Fate and risks of nanomaterials in aquatic and terrestrial environments. *Accounts of Chemical Research,* 46(3), 854-862.

**28.** Becker, A. M., Gerstmann, S., and Frank, H. (2008). Perfluorooctane surfactants in waste waters, the major source of river pollution. *Chemosphere,* 72(1), 115-121.

**29.** Becker, A. M., Gerstmann, S., and Frank, H. (2008). Perfluorooctanoic acid and perfluorooctane sulfonate in the sediment of the Roter Man river, Bayreuth, Germany. *Environmental Pollution*, 156(3), 818-820.

**30.** Beliaeva, N. F., Kashirtseva, V. N., Medvedeva, N. V., IuIu, K., Ipatova, O. M., and Archakov, A. I. (2010). Zebrafish as a model organism for biomedical studies. *Biomeditsinskaia Khimiia,* 56(1), 120-131.

**31.** Bhatt, I., and Tripathi, B. N. (2011). Interaction of engineered nanoparticles with various

components of the environment and possible strategies for their risk assessment. *Chemosphere,* 82(3), 308-317.

**32.** Bhosale, M. A., and Bhanage, B. M. (2014). A facile one-step approach for the synthesis of uniform spherical Cu/Cu 2 O nano-and microparticles with high catalytic activity in the Buchwald–Hartwig amination reaction. *RSC Advances,* 4(29), 15122-15130.

**33.** Boudreau, T. M., Sibley, P. K., Mabury, S. A., Muir, D. G. C., and Solomon, K. R. (2003). Laboratory evaluation of the toxicity of perfluorooctane sulfonate (PFOS) on Selenastrum capricornutum, *Chlorella vulgaris*, *Lemna gibba*, *Daphnia magna*, and *Daphnia pulicaria*. *Archives of Environmental Contamination and Toxicology,* 44(3), 0307-0313.

**34.** Brundo, M. V., and Salvaggio, A. (2018). Zebrafish or *Danio rerio*: a new model in nanotoxicology study. *Recent Advances in Zebrafish Researches,* 10, 121-33.

**35.** Buck, R. C., Franklin, J., Berger, U., Conder, J. M., Cousins, I. T., De Voogt, P., ... and van Leeuwen, S. P. (2011). Perfluoroalkyl and polyfluoroalkyl substances in the environment: terminology, classification, and origins. *Integrated Environmental Assessment and Management,* 7(4), 513-541.

**36.** Buck, R. C., Franklin, J., Berger, U., Conder, J. M., Cousins, I. T., De Voogt, P., ... and van Leeuwen, S. P. (2011). Perfluoroalkyl and polyfluoroalkyl substances in the environment: terminology, classification, and origins. *Integrated Environmental Assessment and Management,* 7(4), 513-541.

**37.** Butenhoff, J., Costa, G., Elcombe, C., Farrar, D., Hansen, K., Iwai, H., ... and Thomford, P. (2002). Toxicity of ammonium perfluorooctanoate in male cynomolgus monkeys after oral dosing for 6 months. *Toxicological Sciences,* 69(1), 244-257.

**38.** Butt, C. M., Muir, D. C., Stirling, I., Kwan, M., and Mabury, S. A. (2007). Rapid response of Arctic ringed seals to changes in perfluoroalkyl production. *Environmental Science and Technology,* 41(1), 42-49.

**39.** Cai, M., Yang, H., Xie, Z., Zhao, Z., Wang, F., Lu, Z., ... and Ebinghaus, R. (2012). Per- and polyfluoroalkyl substances in snow, lake, surface runoff water and coastal seawater in Fildes Peninsula, King George Island, Antarctica. *Journal of Hazardous Materials,* 209, 335-342.

**40.** Casal, P., Zhang, Y., Martin, J. W., Pizarro, M., Jim. nez, B., and Dachs, J. (2017). Role of snow deposition of perfluoroalkylated substances at Coastal Livingston Island

(*Maritime Antarctica*). *Environmental Science and Technology,* 51(15), 8460-8470.

**41.** Chaemfa, C., Barber, J. L., Huber, S., Breivik, K., and Jones, K. C. (2010). Screening for PFOS and PFOA in European air using passive samplers. *Journal of Environmental Monitoring,* 12(5), 1100-1109.

**42.** Chakraborty, C., Hsu, C. H., Wen, Z. H., Lin, C. S., and Agoramoorthy, G. (2009). Zebrafish: a complete animal model for in vivo drug discovery and development. *Current Drug Metabolism,* 10(2), 116-124.

**43.** Chang, Y. N., Zhang, M., Xia, L., Zhang, J., and Xing, G. (2012). The toxic effects and mechanisms of CuO and ZnO nanoparticles. *Materials,* 5(12), 2850-2871.

**44.** Changlu, Z., Shutao, W., Jia, D., Weiqing, W., Yufeng, L., Hong, Y., and Rongjun, S. (2015). DNA Damage in Peripheral Blood Red Cells of Zebrafish Induced by Cotreatment of Perfluorooctane Sulfonate (PFOS) and Multiwall Carbon Nanotubes (MWCNTs). *Asian Journal of Ecotoxicology*, (3), 192-199.

**45.** Chen, C. E., Yang, Y. Y., Zhao, J. L., Liu, Y. S., Hu, L. X., Li, B. B., ... and Ying, G. G. (2021). Legacy and alternative per-and polyfluoroalkyl substances (PFASs) in the West River and North River, south China: Occurrence, fate, spatio-temporal variations and potential sources. *Chemosphere,* 283, 131301.

**46.** Chen, F., Wei, C., Chen, Q., Zhang, J., Wang, L., Zhou, Z., ... and Liang, Y. (2018). Internal concentrations of perfluorobutane sulfonate (PFBS) comparable to those of perfluorooctane sulfonate (PFOS) induce reproductive toxicity in Caenorhabditis elegans. *Ecotoxicology and Environmental Safety,* 158, 223-229.

**47.** Chen, H., Munoz, G., Duy, S. V., Zhang, L., Yao, Y., Zhao, Z., ... and Sauvé, S. (2020). Occurrence and distribution of per-and polyfluoroalkyl substances in Tianjin, China: The contribution of emerging and unknown analogues. *Environmental Science and Technology,* 54(22), 14254-14264.

**48.** Cheng, J., Vecitis, C. D., Park, H., Mader, B. T., and Hoffmann, M. R. (2010). Sonochemical degradation of perfluorooctane sulfonate (PFOS) and perfluorooctanoate (PFOA) in groundwater: kinetic effects of matrix inorganics. *Environmental Science and Technology,* 44(1), 445-450.

**49.** Chio, C. P., Chen, W. Y., Chou, W. C., Hsieh, N. H., Ling, M. P., and Liao, C. M. (2012). Assessing the potential risks to zebrafish posed by environmentally relevant copper and

silver nanoparticles. *Science of the Total Environment,* 420, 111-118.

**50.** Chowdhury, M. I., Sana, T., Panneerselvan, L., Sivaram, A. K., and Megharaj, M. (2022). Perfluorooctane sulfonate (PFOS) induces several behavioural defects in Caenorhabditis elegans that can also be transferred to the next generations. *Chemosphere,* 291, 132896.

**51.** D'Agostino, L. A., and Mabury, S. A. (2017). Certain perfluoroalkyl and polyfluoroalkyl substances associated with aqueous film forming foam are widespread in Canadian surface waters. *Environmental Science and Technology,* 51(23), 13603-13613.

**52.** Dai, Y. J., Jia, Y. F., Chen, N., Bian, W. P., Li, Q. K., Ma, Y. B., ... and Pei, D. S. (2014). Zebrafish as a model system to study toxicology. *Environmental Toxicology and Chemistry,* 33(1), 11-17.

**53.** Di Tocco, A., Robledo, S. N., Osuna, Y., Sandoval-Cortez, J., Granero, A. M., Vettorazzi, N. R., ... and Fernández, H. (2018). Development of an electrochemical biosensor for the determination of triglycerides in serum samples based on a lipase/magnetite-chitosan/copper oxide nanoparticles/multiwalled carbon nanotubes/pectin composite. *Talanta,* 190, 30-37.

**54.** Drottar, K. R., and Krueger, H. O. (2000). PFOS: A semi-static life-cycle toxicity test with the cladoceran *(Daphnia magna). Wildlife International Ltd., Project,* (454A-109).

**55.** Du, J., Cai, J., Wang, S., and You, H. (2017). Oxidative stress and apotosis to zebrafish *(Danio rerio)* embryos exposed to perfluorooctane sulfonate (PFOS) and ZnO nanoparticles. *International Journal of Occupational Medicine and Environmental Health,* 30(2), 213-229.

**56.** Du, J., Tang, J., Xu, S., Ge, J., Dong, Y., Li, H., and Jin, M. (2018). Parental transfer of perfluorooctane sulfonate and ZnO nanoparticles chronic co-exposure and inhibition of growth in F1 offspring. *Regulatory Toxicology and Pharmacology,* 98, 41-49.

**57.** Du, J., Wang, S., You, H., and Liu, Z. (2016). Effects of ZnO nanoparticles on perfluorooctane sulfonate induced thyroid-disrupting on zebrafish larvae. *Journal of Environmental Sciences,* 47, 153-164.

**58.** Du, J., Wang, S., You, H., Jiang, R., Zhuang, C., and Zhang, X. (2016). Developmental toxicity and DNA damage to zebrafish induced by perfluorooctane sulfonate in the presence of ZnO nanoparticles. *Environmental Toxicology*, 31(3), 360-371.

**59.** Du, Y., Shi, X., Liu, C., Yu, K., and Zhou, B. (2009). Chronic effects of water-borne

PFOS exposure on growth, survival and hepatotoxicity in zebrafish: a partial life-cycle test. *Chemosphere,* 74(5), 723-729.

**60.** Du, Y., Shi, X., Liu, C., Yu, K., and Zhou, B. (2009). Chronic effects of water-borne PFOS exposure on growth, survival and hepatotoxicity in zebrafish: a partial life-cycle test. *Chemosphere,* 74(5), 723-729.

**61.** Environment Agency (2004). Environmental Risk Evaluation Report: Perfluorooctanesulphonate (PFOS)

**62.** Everaarts, J. M., Den Besten, P. J., Hillebrand, M. T. J., Halbrook, R. S., and Shugart, L. R. (1998). DNA strand breaks, cytochrome P-450-dependent monooxygenase system activity and levels of chlorinated biphenyl congeners in the pyloric caeca of the seastar (*Asterias rubens*) from the North Sea. *Ecotoxicology,* 7(2), 69-79.

**63.** Evich, M. G., Davis, M. J., McCord, J. P., Acrey, B., Awkerman, J. A., Knappe, D. R., ... and Washington, J. W. (2022). Per-and polyfluoroalkyl substances in the environment. *Science,* 375(6580), eabg9065.

**64.** Fairbairn, D. W., Olive, P. L., and O'Neill, K. L. (1995). The comet assay: a comprehensive review. *Mutation Research/Reviews in Genetic Toxicology,* 339(1), 37-59.

**65.** Fang, C., Wu, X., Huang, Q., Liao, Y., Liu, L., Qiu, L., ... and Dong, S. (2012). PFOS elicits transcriptional responses of the ER, AHR and PPAR pathways in Oryzias melastigma in a stage-specific manner. *Aquatic Toxicology,* 106, 9-19.

**66.** Felder, D. P., D'surney, S. J., Rodgers, J. H., and Deardorff, T. L. (1998). A comprehensive environmental assessment of a receiving aquatic system near an unbleached kraft mill. *Ecotoxicology,* 7(6), 313-324.

**67.** Ferrari, A., Venturino, A., and de D'Angelo, A. M. P. (2007). Muscular and brain cholinesterase sensitivities to azinphos methyl and carbaryl in the juvenile rainbow trout Oncorhynchus mykiss. Comparative Biochemistry and Physiology Part C: *Toxicology and Pharmacology,* 146(3), 308-313.

**68.** Fiedler, H., Kallenborn, R., De Boer, J., and Sydnes, L. K. (2019). The Stockholm convention: a tool for the global regulation of persistent organic pollutants. *Chemistry International,* 41(2), 4-11.

**69.** Fuentes, S., Colomina, M. T., Rodriguez, J., Vicens, P., and Domingo, J. L. (2006).

Interactions in developmental toxicology: concurrent exposure to perfluorooctane sulfonate (PFOS) and stress in pregnant mice. *Toxicology Letters,* 164(1), 81-89.

**70.** Fuentes, S., Colomina, M. T., Vicens, P., Franco-Pons, N., and Domingo, J. L. (2007). Concurrent exposure to perfluorooctane sulfonate and restraint stress during pregnancy in mice: effects on postnatal development and behavior of the offspring. *Toxicological Sciences,* 98(2), 589-598.

**71.** Furdui, V. I., Crozier, P. W., Reiner, E. J., and Mabury, S. A. (2008). Trace level determination of perfluorinated compounds in water by direct injection. *Chemosphere,* 73(1), S24-S30.

**72.** Gedik, C. M., Ewen, S. W. B., and Collins, A. R. (1992). Single-cell gel electrophoresis applied to the analysis of UV-C damage and its repair in human cells. *International Journal of Radiation Biology,* 62(3), 313-320.

**73.** Giesy, J. P., and Kannan, K. (2001). Global distribution of perfluorooctane sulfonate in wildlife. *Environmental Science and Technology,* 35(7), 1339-1342.

**74.** Giri, S. D., and Sarkar, A. (2016). Electrochemical study of bulk and monolayer copper in alkaline solution. *Journal of the Electrochemical Society,* 163(3), H252.

**75.** Gleiter, H. (2000). Nanostructured materials: basic concepts and microstructure. *Acta Materialia,* 48(1), 1-29.

**76.** González, C., Salazar-García, S., Palestino, G., Martínez-Cuevas, P. P., Ramírez-Lee, M. A., Jurado-Manzano, B. B., ... and Ali, S. F. (2011). Effect of 45 nm silver nanoparticles (AgNPs) upon the smooth muscle of rat trachea: role of nitric oxide. *Toxicology Letters*, *207*(3), 306-313.

**77.** Gottschalk, F., Sonderer, T., Scholz, R. W., and Nowack, B. (2009). Modeled environmental concentrations of engineered nanomaterials (TiO2, ZnO, Ag, CNT, fullerenes) for different regions. *Environmental Science and Technology,* 43(24), 9216-9222.

**78.** Gottschalk, F., Sonderer, T., Scholz, R. W., and Nowack, B. (2010). Possibilities and limitations of modeling environmental exposure to engineered nanomaterials by probabilistic material flow analysis. *Environmental Toxicology and Chemistry,* 29(5), 1036-1048.

**79.** Gottschalk, F., Sun, T., and Nowack, B. (2013). Environmental concentrations of

engineered nanomaterials: review of modeling and analytical studies. *Environmental Pollution,* 181, 287-300.

**80.** Greaves, A. K., Letcher, R. J., Sonne, C., and Dietz, R. (2013). Brain region distribution and patterns of bioaccumulative perfluoroalkyl carboxylates and sulfonates in East Greenland polar bears *(Ursus maritimus). Environmental Toxicology and Chemistry,* 32(3), 713-722.

**81.** Griffitt, R. J., Hyndman, K., Denslow, N. D., and Barber, D. S. (2009). Comparison of molecular and histological changes in zebrafish gills exposed to metallic nanoparticles. *Toxicological Sciences,* 107(2), 404-415.

**82.** Griffitt, R. J., Luo, J., Gao, J., Bonzongo, J. C., and Barber, D. S. (2008). Effects of particle composition and species on toxicity of metallic nanomaterials in aquatic organisms. *Environmental Toxicology and Chemistry: An International Journal,* 27(9), 1972-1978.

**83.** Guy, W. S., Taves, D. R., and Brey Jr, W. S. (1976). Organic fluorocompounds in human plasma: Prevalence and characterization.

**84.** Hagenaars, A., Knapen, D., Meyer, I. J., Van der Ven, K., Hoff, P., and De Coen, W. (2008). Toxicity evaluation of perfluorooctane sulfonate (PFOS) in the liver of common carp *(Cyprinus carpio). Aquatic Toxicology, 88*(3), 155-163.

**85.** Hagenaars, A., Vergauwen, L., De Coen, W., and Knapen, D. (2011). Structure–activity relationship assessment of four perfluorinated chemicals using a prolonged zebrafish early life stage test. *Chemosphere,* 82(5), 764-772.

**86.** Halliwell, B., and Gutteridge, J. M. (2015). Free radicals in biology and medicine. Oxford university press, USA.

**87.** Hamid, H., Li, L. Y., and Grace, J. R. (2018). Review of the fate and transformation of per-and polyfluoroalkyl substances (PFASs) in landfills. *Environmental Pollution,* 235, 74-84.

**88.** Harrad, S., Wemken, N., Drage, D. S., Abdallah, M. A. E., and Coggins, A. M. (2019). Perfluoroalkyl substances in drinking water, indoor air and dust from Ireland: implications for human exposure. *Environmental Science and Technology,* 53(22), 13449-13457.

**89.** Hoff, P. T., Van Dongen, W., Esmans, E. L., Blust, R., and De Coen, W. M. (2003).

Evaluation of the toxicological effects of perfluorooctane sulfonic acid in the common carp *(Cyprinus carpio). Aquatic Toxicology,* 62(4), 349-359.

**90.** Hoff, P. T., Van Dongen, W., Esmans, E. L., Blust, R., and De Coen, W. M. (2003). Evaluation of the toxicological effects of perfluorooctane sulfonic acid in the common carp *(Cyprinus carpio). Aquatic Toxicology,* 62(4), 349-359.

**91.** Hsu, C. H., Wen, Z. H., Lin, C. S., and Chakraborty, C. (2007). The zebrafish model: use in studying cellular mechanisms for a spectrum of clinical disease entities. *Current Neurovascular Research,* 4(2), 111-120.

**92.** Hua, M., Zhang, S., Pan, B., Zhang, W., Lv, L., and Zhang, Q. (2012). Heavy metal removal from water/wastewater by nanosized metal oxides: a review. *Journal of Hazardous Materials,* 211, 317-331.

**93.** Huang, H., Huang, C., Wang, L., Ye, X., Bai, C., Simonich, M. T., ... and Dong, Q. (2010). Toxicity, uptake kinetics and behavior assessment in zebrafish embryos following exposure to perfluorooctanesulphonicacid (PFOS). *Aquatic Toxicology*, *98*(2), 139-147.

**94.** Huset, C. A., Chiaia, A. C., Barofsky, D. F., Jonkers, N., Kohler, H. P. E., Ort, C., ... and Field, J. A. (2008). Occurrence and mass flows of fluorochemicals in the Glatt Valley watershed, Switzerland. *Environmental Science and Technology,* 42(17), 6369-6377.

**95.** Hyun, S., and Lee, L. S. (2005). Quantifying the contribution of different sorption mechanisms for 2, 4-dichlorophenoxyacetic acid sorption by several variable-charge soils. *Environmental Science and Technology,* 39(8), 2522-2528.

**96.** Ishii, K. J., and Tang, C. K. (2013). Biological DNA Sensor: The Impact of Nucleic Acids on Diseases and Vaccinology, 1–352. https://doi.org/10.1016/C2012-0-01094-0

**97.** Ispas, C., Andreescu, D., Patel, A., Goia, D. V., Andreescu, S., and Wallace, K. N. (2009). Toxicity and developmental defects of different sizes and shape nickel nanoparticles in zebrafish. *Environmental Science and Technology*, *43*(16), 6349-6356.

**98.** Jeong, T. Y., Yuk, M. S., Jeon, J., and Kim, S. D. (2016). Multigenerational effect of perfluorooctane sulfonate (PFOS) on the individual fitness and population growth of Daphnia magna. *Science of the Total Environment,* 569, 1553-1560.

**99.** Ji, K., Kim, Y., Oh, S., Ahn, B., Jo, H., and Choi, K. (2008). Toxicity of perfluorooctane sulfonic acid and perfluorooctanoic acid on freshwater macroinvertebrates *(Daphnia magna* and *Moina macrocopa)* and fish *(Oryzias latipes). Environmental Toxicology and*

*Chemistry: An International Journal,* 27(10), 2159-2168.

**100.** Jin, Y. H., Liu, W., Sato, I., Nakayama, S. F., Sasaki, K., Saito, N., and Tsuda, S. (2009). PFOS and PFOA in environmental and tap water in China. *Chemosphere,* 77(5), 605-611.

**101.** Joerss, H., Apel, C., and Ebinghaus, R. (2019). Emerging per-and polyfluoroalkyl substances (PFASs) in surface water and sediment of the North and Baltic Seas. *Science of the Total Environment,* 686, 360-369.

**102.** Johansson, N., Eriksson, P., and Viberg, H. (2009). Neonatal exposure to PFOS and PFOA in mice results in changes in proteins which are important for neuronal growth and synaptogenesis in the developing brain. *Toxicological Sciences,* 108(2), 412-418.

**103.** Kannan, K. (2011). Perfluoroalkyl and polyfluoroalkyl substances: current and future perspectives. *Environmental Chemistry,* 8(4), 333-338.

**104.** Keil, D. E., Mehlmann, T., Butterworth, L., and Peden-Adams, M. M. (2008). Gestational exposure to perfluorooctane sulfonate suppresses immune function in B6C3F1 mice. *Toxicological Sciences,* 103(1), 77-85.

**105.** Keiter, S., Baumann, L., Färber, H., Holbech, H., Skutlarek, D., Engwall, M., and Braunbeck, T. (2012). Long-term effects of a binary mixture of perfluorooctane sulfonate (PFOS) and bisphenol A (BPA) in zebrafish (*Danio rerio*). *Aquatic Toxicology,* 118, 116-129.

**106.** Keller, A. A., and Lazareva, A. (2014). Predicted releases of engineered nanomaterials: from global to regional to local. *Environmental Science and Technology Letters,* 1(1), 65-70.

**107.** Khan, R., Ahmad, R., Rai, P., Jang, L. W., Yun, J. H., Yu, Y. T., ... and Lee, I. H. (2014). Glucose-assisted synthesis of Cu2O shuriken-like nanostructures and their application as nonenzymatic glucose biosensors. *Sensors and Actuators B: Chemical,* 203, 471-476.

**108.** Kim, S., Ji, K., Lee, S., Lee, J., Kim, J., Kim, S., ... and Choi, K. (2011). Perfluorooctane sulfonic acid exposure increases cadmium toxicity in early life stage of zebrafish, *Danio rerio. Environmental Toxicology and Chemistry,* 30(4), 870-877.

**109.** Kirby, M. F., Morris, S., Hurst, M., Kirby, S. J., Neall, P., Tylor, T., and Fagg, A. (2000). The use of cholinesterase activity in flounder *(Platichthys flesus)* muscle tissue as

a biomarker of neurotoxic contamination in UK estuaries. *Marine Pollution Bulletin,* 40(9), 780-791.

**110.** Klánová, J., Matykiewiczová, N., Máčka, Z., Prošek, P., Láska, K., and Klán, P. (2008). Persistent organic pollutants in soils and sediments from James Ross Island, Antarctica. *Environmental Pollution,* 152(2), 416-423.

**111.** Kono, Y., and Fridovich, I. (1982). Superoxide radical inhibits catalase. *Journal of Biological Chemistry,* 257(10), 5751-5754.

**112.** Korani, M., Rezayat, S. M., and Bidgoli, S. A. (2013). Sub-chronic dermal toxicity of silver nanoparticles in guinea pig: special emphasis to heart, bone and kidney toxicities. *Iranian Journal of Pharmaceutical Research: IJPR*, *12*(3), 511.

**113.** Lam, N. H., Cho, C. R., Kannan, K., and Cho, H. S. (2017). A nationwide survey of perfluorinated alkyl substances in waters, sediment and biota collected from aquatic environment in Vietnam: Distributions and bioconcentration profiles. *Journal of Hazardous Materials,* 323, 116-127.

**114.** Lang, J. R., Allred, B. M., Peaslee, G. F., Field, J. A., and Barlaz, M. A. (2016). Release of per-and polyfluoroalkyl substances (PFASs) from carpet and clothing in model anaerobic landfill reactors. *Environmental Science and Technology,* 50(10), 5024-5032.

**115.** Lau, C., Anitole, K., Hodes, C., Lai, D., Pfahles-Hutchens, A., and Seed, J. (2007). Perfluoroalkyl acids: a review of monitoring and toxicological findings. *Toxicological Sciences,* 99(2), 366-394.

**116.** Lau, C., Anitole, K., Hodes, C., Lai, D., Pfahles-Hutchens, A., and Seed, J. (2007). Perfluoroalkyl acids: a review of monitoring and toxicological findings. Toxicological sciences, 99(2), 366-394. Lau, C., Butenhoff, J. L., and Rogers, J. M. (2004). The developmental toxicity of perfluoroalkyl acids and their derivatives. *Toxicology and Applied Pharmacology,* 198(2), 231-241.

**117.** Lau, C., Butenhoff, J. L., and Rogers, J. M. (2004). The developmental toxicity of perfluoroalkyl acids and their derivatives. *Toxicology and Applied Pharmacology,* 198(2), 231-241.

**118.** Lau, C., Thibodeaux, J. R., Hanson, R. G., Rogers, J. M., Grey, B. E., Stanton, M. E., ... and Stevenson, L. A. (2003). Exposure to perfluorooctane sulfonate during pregnancy in rat and mouse. II: postnatal evaluation. *Toxicological Sciences,* 74(2), 382-

392.

**119.** Lawrence, C. (2007). The husbandry of zebrafish (*Danio rerio*): A Review. *Aquaculture,* 269(1-4), 1-20.

**120.** Lee, C. K., Kang, S. G., Lee, J. T., Lee, S. W., Kim, J. H., Kim, D. H., ... and Park, Y. B. (2015). Effects of perfluorooctane sulfuric acid on placental PRL-family hormone production and fetal growth retardation in mice. *Molecular and Cellular Endocrinology,* 401, 165-172.

**121.** Letcher, R. J., Morris, A. D., Dyck, M., Sverko, E., Reiner, E. J., Blair, D. A. D., ... and Shen, L. (2018). Legacy and new halogenated persistent organic pollutants in polar bears from a contamination hotspot in the Arctic, Hudson Bay Canada. *Science of the Total Environment,* 610, 121-136.

**122.** Li, F., Zhang, C., Qu, Y., Chen, J., Chen, L., Liu, Y., and Zhou, Q. (2010). Quantitative characterization of short-and long-chain perfluorinated acids in solid matrices in Shanghai, China. *Science of the Total Environment,* 408(3), 617-623.

**123.** Li, J., Gao, Y., Xu, N., Li, B., An, R., Sun, W., ... and Ni, J. (2020). Perfluoroalkyl substances in the Yangtze River: Changing exposure and its implications after operation of the Three Gorges Dam. *Water Research,* 182, 115933.

**124.** Li, X., Ye, L., Ge, Y., Yuan, K., Zhang, Y., Liang, Y., ... and Ge, R. S. (2016). In utero perfluorooctane sulfonate exposure causes low body weights of fetal rats: A Mechanism Study. *Placenta,* 39, 125-133.

**125.** Li, Y., Li, J., Zhang, L., Huang, Z., Liu, Y., Wu, N., ... and Niu, Z. (2019). Perfluoroalkyl acids in drinking water of China in 2017: distribution characteristics, influencing factors and potential risks. *Environment International,* 123, 87-95.

**126.** Li, Y., Men, B., He, Y., Xu, H., Liu, M., and Wang, D. (2017). Effect of single-wall carbon nanotubes on bioconcentration and toxicity of perfluorooctane sulfonate in zebrafish (*Danio rerio*). *Science of the Total Environment,* 607, 509-518.

**127.** Li, Y., Niu, Z., and Zhang, Y. (2022). Occurrence of legacy and emerging poly-and perfluoroalkyl substances in water: A case study in Tianjin (China). *Chemosphere,* 287, 132409.

**128.** Liang, L., Pan, Y., Bin, L., Liu, Y., Huang, W., Li, R., and Lai, K. P. (2021). Immunotoxicity mechanisms of perfluorinated compounds PFOA and PFOS.

*Chemosphere,* 132892.

**129.** Lin, H., Taniyasu, S., Yamashita, N., Khan, M. K., Masood, S. S., Saied, S., and Khwaja, H. A. (2022). Per-and polyfluoroalkyl substances in the atmospheric total suspended particles in Karachi, Pakistan: Profiles, potential sources, and daily intake estimates. *Chemosphere,* 288, 132432.

**130.** Lin, H., Taniyasu, S., Yamazaki, E., Wei, S., Wang, X., Gai, N., ... and Yamashita, N. (2020). Per-and polyfluoroalkyl substances in the air particles of Asia: levels, seasonality, and size-dependent distribution. *Environmental Science and Technology,* 54(22), 14182-14191.

**131.** Lin, H., Taniyasu, S., Yamazaki, E., Wei, S., Wang, X., Gai, N., ... and Yamashita, N. (2020). Per-and polyfluoroalkyl substances in the air particles of Asia: levels, seasonality, and size-dependent distribution. *Environmental Science and Technology,* 54(22), 14182-14191.

**132.** Lin, S., Zhao, Y., Ji, Z., Ear, J., Chang, C. H., Zhang, H., ... and Nel, A. E. (2013). Zebrafish high-throughput screening to study the impact of dissolvable metal oxide nanoparticles on the hatching enzyme, ZHE1. *Small,* 9(9-10), 1776-1785.

**133.** Lin, S., Zhao, Y., Ji, Z., Ear, J., Chang, C. H., Zhang, H., ... and Nel, A. E. (2013). Zebrafish high-throughput screening to study the impact of dissolvable metal oxide nanoparticles on the hatching enzyme, ZHE1. *Small,* 9(9-10), 1776-1785.

**134.** Lindstrom, A. B., Strynar, M. J., and Libelo, E. L. (2011). Polyfluorinated compounds: past, present, and future. *Environmental Science and Technology,* 45(19), 7954-7961.

**135.** Liu, C., Yu, K., Shi, X., Wang, J., Lam, P. K., Wu, R. S., and Zhou, B. (2007). Induction of oxidative stress and apoptosis by PFOS and PFOA in primary cultured hepatocytes of freshwater tilapia (*Oreochromis niloticus*). *Aquatic Toxicology,* 82(2), 135-143.

**136.** Liu, J., and Avendaño, S. M. (2013). Microbial degradation of polyfluoroalkyl chemicals in the environment: A Review. *Environment International,* 61, 98-114.

**137.** Liu, J., and Avendaño, S. M. (2013). Microbial degradation of polyfluoroalkyl chemicals in the environment: A Review. *Environment International,* 61, 98-114.

**138.** Liu, W., Qin, H., Li, J., Zhang, Q., Zhang, H., Wang, Z., and He, X. (2017).

Atmospheric chlorinated polyfluorinated ether sulfonate and ionic perfluoroalkyl acids in 2006 to 2014 in Dalian, China. *Environmental Toxicology and Chemistry,* 36(10), 2581-2586.

**139.** Liu, X., Guo, Z., Krebs, K. A., Pope, R. H., and Roache, N. F. (2014). Concentrations and trends of perfluorinated chemicals in potential indoor sources from 2007 through 2011 in the US. *Chemosphere,* 98, 51-57.

**140.** Liu, Y., Zhang, Y., Li, J., Wu, N., Li, W., and Niu, Z. (2019). Distribution, partitioning behavior and positive matrix factorization-based source analysis of legacy and emerging polyfluorinated alkyl substances in the dissolved phase, surface sediment and suspended particulate matter around coastal areas of Bohai Bay, China. *Environmental Pollution,* 246, 34-44.

**141.** Llorca, M., Farré, M., Tavano, M. S., Alonso, B., Koremblit, G., and Barceló, D. (2012). Fate of a broad spectrum of perfluorinated compounds in soils and biota from Tierra del Fuego and Antarctica. *Environmental Pollution,* 163, 158-166.

**142.** Lu, X., Deng, S., Wang, B., Huang, J., Wang, Y., and Yu, G. (2016). Adsorption behavior and mechanism of perfluorooctane sulfonate on nanosized inorganic oxides. *Journal of Colloid and Interface Science,* 474, 199-205.

**143.** Lushchak, V. I. (2011). Environmentally induced oxidative stress in aquatic animals. *Aquatic Toxicology,* 101(1), 13-30.

**144.** Mani, R., Balasubramanian, S., Raghunath, A., and Perumal, E. (2020). Chronic exposure to copper oxide nanoparticles causes muscle toxicity in adult zebrafish. *Environmental Science and Pollution Research,* 27(22), 27358-27369.

**145.** Marchiandi, J., Szabo, D., Dagnino, S., Green, M. P., and Clarke, B. O. (2021). Occurrence and fate of legacy and novel per-and polyfluoroalkyl substances (PFASs) in freshwater after an industrial fire of unknown chemical stockpiles. *Environmental Pollution,* 278, 116839.

**146.** Martin, J. W., Mabury, S. A., Solomon, K. R., and Muir, D. C. (2003). Dietary accumulation of perfluorinated acids in juvenile rainbow trout (*Oncorhynchus mykiss*). *Environmental Toxicology and Chemistry: An International Journal,* 22(1), 189-195.

**147.** Martin, J. W., Mabury, S. A., Solomon, K. R., and Muir, D. C. (2003). Bioconcentration and tissue distribution of perfluorinated acids in rainbow trout

(*Oncorhynchus mykiss*). *Environmental Toxicology and Chemistry: An International Journal,* 22(1), 196-204.

**148.** Martin, J. W., Smithwick, M. M., Braune, B. M., Hoekstra, P. F., Muir, D. C., and Mabury, S. A. (2004). Identification of long-chain perfluorinated acids in biota from the Canadian Arctic. *Environmental Science and Technology,* 38(2), 373-380.

**149.** Maurer-Jones, M. A., Gunsolus, I. L., Murphy, C. J., and Haynes, C. L. (2013). Toxicity of engineered nanoparticles in the environment. *Analytical Chemistry,* 85(6), 3036-3049.

**150.** McKelvey-Martin, V. J., Green, M. H. L., Schmezer, P., Pool-Zobel, B. L., De Meo, M. P., and Collins, A. (1993). The single cell gel electrophoresis assay (comet assay): A European Review. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis,* 288(1), 47-63.

**151.** McNeil, P. L., Boyle, D., Henry, T. B., Handy, R. D., and Sloman, K. A. (2014). Effects of metal nanoparticles on the lateral line system and behaviour in early life stages of zebrafish (*Danio rerio*). *Aquatic Toxicology,* 152, 318-323.

**152.** Meghana, S., Kabra, P., Chakraborty, S., and Padmavathy, N. (2015). Understanding the pathway of antibacterial activity of copper oxide nanoparticles. *RSC Advances,* 5(16), 12293-12299.

**153.** Meng, L., Yang, S., Feng, M., Qu, R., Li, Y., Liu, J., ... and Sun, C. (2016). Toxicity and bioaccumulation of copper in *Limnodrilus hoffmeisteri* under different pH values: Impacts of perfluorooctane sulfonate. *Journal of Hazardous Materials,* 305, 219-228.

**154.** Mitchelmore, C. L., and Chipman, J. K. (1998). DNA strand breakage in aquatic organisms and the potential value of the comet assay in environmental monitoring. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis,* 399(2), 135-147.

**155.** Möller, A., Ahrens, L., Surm, R., Westerveld, J., van der Wielen, F., Ebinghaus, R., and de Voogt, P. (2010). Distribution and sources of polyfluoroalkyl substances (PFAS) in the River Rhine watershed. *Environmental Pollution,* 158(10), 3243-3250.

**156.** Momeni, S., and Sedaghati, F. (2018). CuO/Cu2O nanoparticles: A simple and green synthesis, characterization and their electrocatalytic performance toward

formaldehyde oxidation. *Microchemical Journal,* 143, 64-71.

**157.** Moody, C. A., Hebert, G. N., Strauss, S. H., and Field, J. A. (2003). Occurrence and persistence of perfluorooctane sulfonate and other perfluorinated surfactants in groundwater at a fire-training area at Wurtsmith Air Force Base, Michigan, USA. *Journal of Environmental Monitoring,* 5(2), 341-345.

**158.** Muazzam, B., Munawar, K., Khan, I. A., Jahan, S., Iqbal, M., Asi, M. R., ... and Zafar, M. I. (2019). Stress response and toxicity studies on zebrafish exposed to endosulfan and imidacloprid present in water. *Journal of Water Supply: Research and Technology-Aqua*, *68*(8), 718-730.

**159.** Mueller, N. C., and Nowack, B. (2008). Exposure modeling of engineered nanoparticles in the environment. *Environmental Science and Technology,* 42(12), 4447-4453.

**160.** Nakayama, S., Strynar, M. J., Helfant, L., Egeghy, P., Ye, X., and Lindstrom, A. B. (2007). Perfluorinated compounds in the Cape Fear drainage basin in North Carolina. *Environmental Science and Technology,* 41(15), 5271-5276.

**161.** Nash, S. B., Rintoul, S. R., Kawaguchi, S., Staniland, I., van den Hoff, J., Tierney, M., and Bossi, R. (2010). Perfluorinated compounds in the Antarctic region: ocean circulation provides prolonged protection from distant sources. *Environmental Pollution,* 158(9), 2985-2991.

**162.** Nwani, C. D., Lakra, W. S., Nagpure, N. S., Kumar, R., Kushwaha, B., and Srivastava, S. K. (2010). Toxicity of the herbicide atrazine: effects on lipid peroxidation and activities of antioxidant enzymes in the freshwater fish Channa punctatus (*Bloch*). *International Journal of Environmental Research and Public Health,* 7(8), 3298-3312.

**163.** Olive, P. L., Banáth, J. P., and Durand, R. E. (1990). Heterogeneity in radiation-induced DNA damage and repair in tumor and normal cells measured using the" comet" assay. *Radiation Research,* 122(1), 86-94.

**164.** Olsen, G. W., Burris, J. M., Burlew, M. M., and Mandel, J. H. (2003). Epidemiologic assessment of worker serum perfluorooctanesulfonate (PFOS) and perfluorooctanoate (PFOA) concentrations and medical surveillance examinations. *Journal of Occupational and Environmental Medicine,* 260-270.

**165.** Olsen, G. W., Burris, J. M., Mandel, J. H., and Zobel, L. R. (1999). Serum

perfluorooctane sulfonate and hepatic and lipid clinical chemistry tests in fluorochemical production employees. *Journal of Occupational and Environmental Medicine,* 799-806.

**166.** Olsen, G. W., Logan, P. W., Hansen, K. J., Simpson, C. A., Burris, J. M., Burlew, M. M., ... and Mandel, J. H. (2003). An occupational exposure assessment of a perfluorooctanesulfonyl fluoride production site: biomonitoring. *AIHA Journal,* 64(5), 651-659.

**167.** Organization for Economic Cooperation and Development. (2002). Hazard assessment of perfluorooctane sulfonate (PFOS) and its salts. November 21, ENV/JM/RD 17/Final.

**168.** Pan, Y. Y., Shi, Y. L., and Cai, Y. (2010). Determination of perfluorinated compounds in soil, sediment and sludge using HPLC MS/MS. *Environmental Chemistry,* 3, 519-523.

**169.** Pan, Y., Zhang, H., Cui, Q., Sheng, N., Yeung, L. W., Sun, Y., ... and Dai, J. (2018). Worldwide distribution of novel perfluoroether carboxylic and sulfonic acids in surface water. *Environmental Science and Technology,* 52(14), 7621-7629.

**170.** Pandey, S., Parvez, S., Sayeed, I., Haque, R., Bin-Hafeez, B., and Raisuddin, S. (2003). Biomarkers of oxidative stress: a comparative study of river Yamuna fish Wallago attu (*Bl. and Schn.*). *Science of the Total Environment,* 309(1-3), 105-115.

**171.** Paul, A. G., Jones, K. C., and Sweetman, A. J. (2009). A first global production, emission, and environmental inventory for perfluorooctane sulfonate. *Environmental Science and Technology,* 43(2), 386-392.

**172.** Payne, J. F., Mathieu, A., Melvin, W., and Fancey, L. L. (1996). Acetylcholinesterase, an old biomarker with a new future? Field trials in association with two urban rivers and a paper mill in Newfoundland. *Marine Pollution Bulletin,* 32(2), 225-231.

**173.** Peden-Adams, M. M., Keller, J. M., EuDaly, J. G., Berger, J., Gilkeson, G. S., and Keil, D. E. (2008). Suppression of humoral immunity in mice following exposure to perfluorooctane sulfonate. *Toxicological Sciences,* 104(1), 144-154.

**174.** Pedersen, K. E., Basu, N., Letcher, R., Greaves, A. K., Sonne, C., Dietz, R., and Styrishave, B. (2015). Brain region-specific perfluoroalkylated sulfonate (PFSA) and carboxylic acid (PFCA) accumulation and neurochemical biomarker responses in east

Greenland polar bears *(Ursus maritimus). Environmental Research,* 138, 22-31.

**175.** Pendashteh, A., Mousavi, M. F., and Rahmanifar, M. S. (2013). Fabrication of anchored copper oxide nanoparticles on graphene oxide nanosheets via an electrostatic coprecipitation and its application as supercapacitor. *Electrochimica Acta,* 88, 347-357.

**176.** Pokropivny, V. V., and Skorokhod, V. V. (2007). Classification of nanostructures by dimensionality and concept of surface forms engineering in nanomaterial science. *Materials Science and Engineering:* C, 27(5-8), 990-993.

**177.** Prasad, K. P., Dhawale, D. S., Joseph, S., Anand, C., Wahab, M. A., Mano, A., ... and Vinu, A. (2013). Post-synthetic functionalization of mesoporous carbon electrodes with copper oxide nanoparticles for supercapacitor application. *Microporous and Mesoporous Materials,* 172, 77-86.

**178.** Prevedouros, K., Cousins, I. T., Buck, R. C., and Korzeniowski, S. H. (2006). Sources, fate and transport of perfluorocarboxylates. *Environmental Science and Technology,* 40(1), 32-44.

**179.** Qiang, L., Pan, X., Zhu, L., Fang, S., and Tian, S. (2016). Effects of nano-TiO2 on perfluorooctanesulfonate bioaccumulation in fishes living in different water layers: implications for enhanced risk of perfluorooctanesulfonate. *Nanotoxicology,* 10(4), 471-479.

**180.** Qiang, L., Shi, X., Pan, X., Zhu, L., Chen, M., and Han, Y. (2015). Facilitated bioaccumulation of perfluorooctanesulfonate in zebrafish by nano-TiO2 in two crystalline phases. *Environmental Pollution,* 206, 644-651.

**181.** Quinete, N., Wu, Q., Zhang, T., Yun, S. H., Moreira, I., and Kannan, K. (2009). Specific profiles of perfluorinated compounds in surface and drinking waters and accumulation in mussels, fish, and dolphins from southeastern Brazil. *Chemosphere,* 77(6), 863-869.

**182.** Radjenovic, J., Duinslaeger, N., Avval, S. S., and Chaplin, B. P. (2020). Facing the challenge of poly-and perfluoroalkyl substances in water: is electrochemical oxidation the answer?. *Environmental Science and Technology,* 54(23), 14815-14829.

**183.** Rahimi-Nasrabadi, M., Pourmortazavi, S. M., Davoudi-Dehaghani, A. A., Hajimirsadeghi, S. S., and Zahedi, M. M. (2013). Synthesis and characterization of copper oxalate and copper oxide nanoparticles by statistically optimized controlled precipitation

and calcination of precursor. *CrystEngComm*, 15(20), 4077-4086.

**184.** Rankin, K., Mabury, S. A., Jenkins, T. M., and Washington, J. W. (2016). A North American and global survey of perfluoroalkyl substances in surface soils: Distribution patterns and mode of occurrence. *Chemosphere,* 161, 333-341.

**185.** Rankin, K., Mabury, S. A., Jenkins, T. M., and Washington, J. W. (2016). A North American and global survey of perfluoroalkyl substances in surface soils: Distribution patterns and mode of occurrence. *Chemosphere,* 161, 333-341

**186.** Roduner, E. (2006). Size matters: why nanomaterials are different. *Chemical Society Reviews,* 35(7), 583-592.

**187.** Saito, N., Sasaki, K., Nakatome, K., Harada, K., Yoshinaga, T., and Koizumi, A. (2003). Perfluorooctane sulfonate concentrations in surface water in Japan. *Archives of Environmental Contamination and Toxicology,* 45(2), 149-158.

**188.** Saleh, T. A. (2020). Nanomaterials: Classification, properties, and environmental toxicities. *Environmental Technology and Innovation,* 20, 101067.

**189.** Sarigiannis, D. A., and Hansen, U. (2012). Considering the cumulative risk of mixtures of chemicals–a challenge for policy makers. *Environmental Health,* 11(1), 1-12.

**190.** Sarkar, A., Das, J., Manna, P., and Sil, P. C. (2011). Nano-copper induces oxidative stress and apoptosis in kidney via both extrinsic and intrinsic pathways. *Toxicology,* 290(2-3), 208-217.

**191.** Sasaki, K., Harada, K., Saito, N., Tsutsui, T., Nakanishi, S., Tsuzuki, H., and Koizumi, A. (2003). Impact of airborne perfluorooctane sulfonate on the human body burden and the ecological system. *Bulletin of Environmental Contamination and Toxicology,* 71(2), 408-413.

**192.** Seacat, A. M., Thomford, P. J., Hansen, K. J., Clemen, L. A., Eldridge, S. R., Elcombe, C. R., and Butenhoff, J. L. (2003). Sub-chronic dietary toxicity of potassium perfluorooctanesulfonate in rats. *Toxicology,* 183(1-3), 117-131.

**193.** Seacat, A. M., Thomford, P. J., Hansen, K. J., Olsen, G. W., Case, M. T., and Butenhoff, J. L. (2002). Subchronic toxicity studies on perfluorooctanesulfonate potassium salt in cynomolgus monkeys. *Toxicological Sciences,* 68(1), 249-264.

**194.** Seacat, A. M., Thomford, P. J., Hansen, K. J., Olsen, G. W., Case, M. T., and Butenhoff, J. L. (2002). Subchronic toxicity studies on perfluorooctanesulfonate

potassium salt in cynomolgus monkeys. *Toxicological Sciences,* 68(1), 249-264.

**195.** Segner, H. (2011). Reproductive and developmental toxicity in fishes. In Reproductive and developmental toxicology (pp. 1145-1166). Academic Press.

**196.** Semchyshyn, H. M., and Lushchak, V. I. (2012). Interplay between oxidative and carbonyl stresses: molecular mechanisms, biological effects and therapeutic strategies of protection. *Oxidative Stress—Molecular Mechanisms and Biological Effects,* 25, 15-46.

**197.** Shabnam, L., Faisal, S. N., Roy, A. K., Haque, E., Minett, A. I., and Gomes, V. G. (2017). Doped graphene/Cu nanocomposite: a high sensitivity non-enzymatic glucose sensor for food. *Food chemistry,* 221, 751-759.

**198.** Sharma, B. M., Bharat, G. K., Tayal, S., Larssen, T., Bečanová, J., Karásková, P., ... and Nizzetto, L. (2016). Perfluoroalkyl substances (PFAS) in river and ground/drinking water of the Ganges River basin: emissions and implications for human exposure. *Environmental Pollution,* 208, 704-713.

**199.** Sharpe, R. L., Benskin, J. P., Laarman, A. H., MacLeod, S. L., Martin, J. W., Wong, C. S., and Goss, G. G. (2010). Perfluorooctane sulfonate toxicity, isomer-specific accumulation, and maternal transfer in zebrafish *(Danio rerio)* and rainbow trout *(Oncorhynchus mykiss). Environmental Toxicology and Chemistry,* 29(9), 1957-1966.

**200.** Shaw, I., and Chadwick, J. (1998). Principles of Environmental Toxicology (1st ed.). CRC Press. https://doi.org/10.1201/9781315273785

**201.** Shen, Y-W., and Donald R. Taves. "Fluoride concentrations in the human placenta and maternal and cord blood." *American Journal of Obstetrics and Gynecology* 119, no. 2 (1974): 205-207.

**202.** Shi, J., Abid, A. D., Kennedy, I. M., Hristova, K. R., and Silk, W. K. (2011). To duckweeds *(Landoltia punctata),* nanoparticulate copper oxide is more inhibitory than the soluble copper in the bulk solution. *Environmental Pollution,* 159(5), 1277-1282.

**203.** Shi, X., and Zhou, B. (2010). The role of Nrf2 and MAPK pathways in PFOS-induced oxidative stress in zebrafish embryos. *Toxicological Sciences,* 115(2), 391-400.

**204.** Shi, X., Du, Y., Lam, P. K., Wu, R. S., and Zhou, B. (2008). Developmental toxicity and alteration of gene expression in zebrafish embryos exposed to PFOS. *Toxicology and Applied Pharmacology,* 230(1), 23-32.

**205.** Shi, X., Du, Y., Lam, P. K., Wu, R. S., and Zhou, B. (2008). Developmental

toxicity and alteration of gene expression in zebrafish embryos exposed to PFOS. *Toxicology and Applied Pharmacology*, *230*(1), 23-32.

**206.** Siddiqui, M. A., Alhadlaq, H. A., Ahmad, J., Al-Khedhairy, A. A., Musarrat, J., and Ahamed, M. (2013). Copper oxide nanoparticles induced mitochondria mediated apoptosis in human hepatocarcinoma cells. *PloS One,* 8(8), e69534.

**207.** Sinclair, E., and Kannan, K. (2006). Mass loading and fate of perfluoroalkyl surfactants in wastewater treatment plants. *Environmental Science and Technology,* 40(5), 1408-1414.

**208.** Sinclair, E., Kannan, K., Taniyasu, S., and Yamashita, N. (2004). Perfluorooctanoic acid and perfluorooctane sulfonate in Michigan and New York waters.

**209.** Sivaraj, R., Rahman, P. K., Rajiv, P., Salam, H. A., and Venckatesh, R. (2014). Biogenic copper oxide nanoparticles synthesis using Tabernaemontana divaricate leaf extract and its antibacterial activity against urinary tract pathogen. *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy,* 133, 178-181.

**210.** Slaninova, A., Smutna, M., Modra, H., and Svobodova, Z. (2009). REVIEWS Oxidative stress in fish induced by pesticides. *Neuroendocrinology Letters,* 30(1), 2.

**211.** So, M. K., Miyake, Y., Yeung, W. Y., Ho, Y. M., Taniyasu, S., Rostkowski, P., ... and Lam, P. K. S. (2007). Perfluorinated compounds in the Pearl river and Yangtze river of China. *Chemosphere,* 68(11), 2085-2095.

**212.** Stock, N. L., Furdui, V. I., Muir, D. C., and Mabury, S. A. (2007). Perfluoroalkyl contaminants in the Canadian Arctic: evidence of atmospheric transport and local contamination. *Environmental Science and Technology,* 41(10), 3529-3536.

**213.** Strynar, M. J., Lindstrom, A. B., Nakayama, S. F., Egeghy, P. P., and Helfant, L. J. (2012). Pilot scale application of a method for the analysis of perfluorinated compounds in surface soils. *Chemosphere,* 86(3), 252-257.

**214.** Stylianou, M., Björnsdotter, M. K., Olsson, P. E., Jogsten, I. E., and Jass, J. (2019). Distinct transcriptional response of Caenorhabditis elegans to different exposure routes of perfluorooctane sulfonic acid. *Environmental Research,* 168, 406-413.

**215.** Sun, H., Gerecke, A. C., Giger, W., and Alder, A. C. (2011). Long-chain perfluorinated chemicals in digested sewage sludges in Switzerland. *Environmental Pollution,* 159(2), 654-662.

**216.** Sun, Y., Zhang, G., He, Z., Wang, Y., Cui, J., and Li, Y. (2016). Effects of copper oxide nanoparticles on developing zebrafish embryos and larvae. *International Journal of Nanomedicine,* 11, 905.

**217.** Taniguchi, N. (1974). On the basic concept of nano-technology proceedings of the international conference on production engineering tokyo part ii japan society of precision engineering. Pabbati et al.

**218.** Taniyasu, S., Kannan, K., Horii, Y., Hanari, N., and Yamashita, N. (2003). A survey of perfluorooctane sulfonate and related perfluorinated organic compounds in water, fish, birds, and humans from Japan. *Environmental Science and Technology,* 37(12), 2634-2639.

**219.** Taniyasu, S., Kannan, K., Horii, Y., Hanari, N., and Yamashita, N. (2003). A survey of perfluorooctane sulfonate and related perfluorinated organic compounds in water, fish, birds, and humans from Japan. *Environmental Science and Technology,* 37(12), 2634-2639.

**220.** Taves, D. R. (1968). Evidence that there are two forms of fluoride in human serum. *Nature,* 217(5133), 1050-1051.

**221.** Teng, J., Tang, S., and Ou, S. (2009). Determination of perfluorooctanesulfonate and perfluorooctanoate in water samples by SPE-HPLC/electrospray ion trap mass spectrometry. *Microchemical Journal,* 93(1), 55-59.

**222.** Theodorakis, C. W., and Shugart, L. R. (1998). Genetic ecotoxicology III: the relationship between DNA strand breaks and genotype in mosquito fish exposed to radiation. *Ecotoxicology,* 7(4), 227-235.

**223.** Thibodeaux, J. R., Hanson, R. G., Rogers, J. M., Grey, B. E., Barbee, B. D., Richards, J. H., ... and Lau, C. (2003). Exposure to perfluorooctane sulfonate during pregnancy in rat and mouse. I: maternal and prenatal evaluations. *Toxicological Sciences,* 74(2), 369-381.

**224.** Thibodeaux, J. R., Hanson, R. G., Rogers, J. M., Grey, B. E., Barbee, B. D., Richards, J. H., ... and Lau, C. (2003). Exposure to perfluorooctane sulfonate during pregnancy in rat and mouse. I: maternal and prenatal evaluations. *Toxicological Sciences,* 74(2), 369-381.

**225.** Tolaymat, T., El Badawy, A., Genaidy, A., Abdelraheem, W., and Sequeira, R.

(2017). Analysis of metallic and metal oxide nanomaterial environmental emissions. *Journal of Cleaner Production,* 143, 401-412.

**226.** Valavanidis, A., Vlahogianni, T., Dassenakis, M., and Scoullos, M. (2006). Molecular biomarkers of oxidative stress in aquatic organisms in relation to toxic environmental pollutants. *Ecotoxicology and Environmental Safety,* 64(2), 178-189.

**227.** Valko, M., Leibfritz, D., Moncol, J., Cronin, M. T., Mazur, M., and Telser, J. (2007). Free radicals and antioxidants in normal physiological functions and human disease. *The International Journal of Biochemistry and Cell Biology,* 39(1), 44-84.

**228.** Vicario-Parés, U., Castañaga, L., Lacave, J. M., Oron, M., Reip, P., Berhanu, D., ... and Orbea, A. (2014). Comparative toxicity of metal oxide nanoparticles (CuO-NPs, ZnO and TiO2) to developing zebrafish embryos. *Journal of Nanoparticle Research,* 16(8), 1-16.

***229.*** Wang, B., Yao, Y., Wang, Y., Chen, H., and Sun, H. (2021). Per-and polyfluoroalkyl substances in outdoor and indoor dust from mainland China: Contributions of unknown precursors and implications for human exposure. *Environmental Science and Technology.*

**230.** Wang, F., and Shih, K. (2011). Adsorption of perfluorooctanesulfonate (PFOS) and perfluorooctanoate (PFOA) on alumina: Influence of solution pH and cations. *Water Research,* 45(9), 2925-2930.

**231.** Wang, H., Zhong, X., Shi, W. Y., and Guo, B. (2011). Study of malondialdehyde (MDA) content, superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) activities in chickens infected with avian infectious bronchitis virus. *African Journal of Biotechnology,* 10(45), 9213-9217.

**232.** Wang, T., Long, X., Cheng, Y., Liu, Z., and Yan, S. (2014). The potential toxicity of copper nanoparticles and copper sulphate on juvenile *Epinephelus coioides*. *Aquatic Toxicology,* 152, 96-104.

**233.** Wang, T., Long, X., Cheng, Y., Liu, Z., and Yan, S. (2014). The potential toxicity of copper nanoparticles and copper sulphate on juvenile *Epinephelus coioides*. *Aquatic Toxicology*, *152*, 96-104.

**234.** Wang, X., Li, B., Zhao, W. D., Liu, Y. J., Shang, D. S., Fang, W. G., and Chen, Y. H. (2011). Perfluorooctane sulfonate triggers tight junction "opening" in brain

endothelial cells via phosphatidylinositol 3-kinase. *Biochemical and Biophysical Research Communications,* 410(2), 258-263.

**235.** Wang, Y., Yang, F., Zhang, H. X., Zi, X. Y., Pan, X. H., Chen, F., ... and Hu, Y. P. (2013). Cuprous oxide nanoparticles inhibit the growth and metastasis of melanoma by targeting mitochondria. *Cell Death and Disease,* 4(8), e783-e783.

**236.** Wang, Z., Cousins, I. T., Scheringer, M., and Hungerbühler, K. (2013). Fluorinated alternatives to long-chain perfluoroalkyl carboxylic acids (PFCAs), perfluoroalkane sulfonic acids (PFSAs) and their potential precursors. *Environment International,* 60, 242-248.

**237.** Wang, Z., DeWitt, J. C., Higgins, C. P., and Cousins, I. T. (2017). A never-ending story of per-and polyfluoroalkyl substances (PFASs)?.

**238.** Wang, Z., DeWitt, J. C., Higgins, C. P., and Cousins, I. T. (2017). A never-ending story of per-and polyfluoroalkyl substances (PFASs)?.

**239.** Wang, Z., Xie, Z., Mi, W., Möller, A., Wolschke, H., and Ebinghaus, R. (2015). Neutral poly/per-fluoroalkyl substances in air from the Atlantic to the Southern Ocean and in Antarctic snow. *Environmental Science and Technology,* 49(13), 7770-7775.

**240.** Wang, Z., Xie, Z., Möller, A., Mi, W., Wolschke, H., and Ebinghaus, R. (2014). Atmospheric concentrations and gas/particle partitioning of neutral poly-and perfluoroalkyl substances in northern German coast. *Atmospheric Environment,* 95, 207-213.

**241.** Washington, J. W., Henderson, W. M., Ellington, J. J., Jenkins, T. M., and Evans, J. J. (2008). Analysis of perfluorinated carboxylic acids in soils II: optimization of chromatography and extraction. *Journal of Chromatography A,* 1181(1-2), 21-32.

**242.** Westerfield, M. (2000). The zebrafish book: a guide for the laboratory use of zebrafish. http://zfin. org/zf_info/zfbook/zfbk. htmL.

**243.** Wilhelm, M., Angerer, J., Fromme, H., and Hölzer, J. (2009). Contribution to the evaluation of reference values for PFOA and PFOS in plasma of children and adults from Germany. *International Journal of Hygiene and Environmental Health,* 212(1), 56-60.

**244.** Wu, B., Torres-Duarte, C., Cole, B. J., and Cherr, G. N. (2015). Copper oxide and zinc oxide nanomaterials act as inhibitors of multidrug resistance transport in sea urchin embryos: their role as chemosensitizers. *Environmental Science and Technology,* 49(9),

5760-5770.

**245.** Xia, J. G., Nie, L. J., Mi, X. M., Wang, W. Z., Ma, Y. J., Cao, Z. D., and Fu, S. J. (2015). Behavior, metabolism and swimming physiology in juvenile Spinibarbus sinensis exposed to PFOS under different temperatures. *Fish Physiology and Biochemistry,* 41(5), 1293-1304.

**246.** Xiao, Y., Vijver, M. G., Chen, G., and Peijnenburg, W. J. (2015). Toxicity and accumulation of Cu and ZnO nanoparticles in *Daphnia magna*. *Environmental Science and Technology,* 49(7), 4657-4664.

**247.** Xu, J., Zhang, Q., Li, X., Zhan, S., Wang, L., and Chen, D. (2017). The effects of copper oxide nanoparticles on dorsoventral patterning, convergent extension, and neural and cardiac development of zebrafish. *Aquatic Toxicology,* 188, 130-137.

**248.** Yamashita, N., Kannan, K., Taniyasu, S., Horii, Y., Petrick, G., and Gamo, T. (2005). A global survey of perfluorinated acids in oceans. *Marine Pollution Bulletin,* 51(8-12), 658-668.

**249.** Yang, Q., Wang, Y. E., Yang, Q., Gao, Y., Duan, X., Fu, Q., ... and Sun, Y. (2017). Cuprous oxide nanoparticles trigger ER stress-induced apoptosis by regulating copper trafficking and overcoming resistance to sunitinib therapy in renal cancer. *Biomaterials,* 146, 72-85.

**250.** Young, C. J., and Mabury, S. A. (2010). Atmospheric perfluorinated acid precursors: chemistry, occurrence, and impacts. *Reviews of Environmental Contamination and Toxicology Volume* 208, 1-109.

**251.** Young, C. J., Furdui, V. I., Franklin, J., Koerner, R. M., Muir, D. C., and Mabury, S. A. (2007). Perfluorinated acids in arctic snow: new evidence for atmospheric formation. *Environmental Science and Technology,* 41(10), 3455-3461.

**252.** Yu, J., Hu, J., Tanaka, S., and Fujii, S. (2009). Perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA) in sewage treatment plants. *Water Research,* 43(9), 2399-2408.

**253.** Yu, N., Guo, H., Yang, J., Jin, L., Wang, X., Shi, W., ... and Wei, S. (2018). Non-target and suspect screening of per-and polyfluoroalkyl substances in airborne particulate matter in China. *Environmental Science and Technology,* 52(15), 8205-8214.

**254.** Yu, Y., Wang, C., Zhang, X., Zhu, J., Wang, L., Ji, M., ... and Wang, S. L. (2020).

Perfluorooctane sulfonate disrupts the blood brain barrier through the crosstalk between endothelial cells and astrocytes in mice. *Environmental Pollution,* 256, 113429.

**255.** Zampardi, G., Thöming, J., Naatz, H., Amin, H. M., Pokhrel, S., Mädler, L., and Compton, R. G. (2018). Electrochemical behavior of single CuO nanoparticles: implications for the assessment of their environmental fate. *Small,* 14(32), 1801765.

**256.** Zareitalabad, P., Siemens, J., Hamer, M., and Amelung, W. (2013). Perfluorooctanoic acid (PFOA) and perfluorooctanesulfonic acid (PFOS) in surface waters, sediments, soils and wastewater–A review on concentrations and distribution coefficients. *Chemosphere,* 91(6), 725-732.

**257.** Zhang, D., Li, X., Wang, M., and Xie, W. (2021). Occurrence and distribution of poly-and perfluoroalkyl substances (PFASs) in a surface flow constructed wetland. *Ecological Engineering,* 169, 106291.

**258.** Zhang, L., Li, Y. Y., Chen, T., Xia, W., Zhou, Y., Wan, Y. J., ... and Xu, S. Q. (2011). Abnormal development of motor neurons in perfluorooctane sulphonate exposed zebrafish embryos. *Ecotoxicology,* 20(4), 643-652.

**259.** Zhang, L., Niu, J., Wang, Y., Shi, J., and Huang, Q. (2014). Chronic effects of PFOA and PFOS on sexual reproduction of freshwater rotifer Brachionus calyciflorus. *Chemosphere,* 114, 114-120.

**260.** Zhang, M., An, C., Gao, Y., Leak, R. K., Chen, J., and Zhang, F. (2013). Emerging roles of Nrf2 and phase II antioxidant enzymes in neuroprotection. *Progress in Neurobiology,* 100, 30-47.

**261.** Zhang, X., Lohmann, R., Dassuncao, C., Hu, X. C., Weber, A. K., Vecitis, C. D., and Sunderland, E. M. (2016). Source attribution of poly-and perfluoroalkyl substances (PFASs) in surface waters from Rhode Island and the New York Metropolitan Area. *Environmental Science and Technology Letters,* 3(9), 316-321.

**262.** Zhao, C., Lv, J., Xu, X., Zhang, G., Yang, Y., and Yang, F. (2017). Highly antifouling and antibacterial performance of poly (vinylidene fluoride) ultrafiltration membranes blending with copper oxide and graphene oxide nanofillers for effective wastewater treatment. *Journal of Colloid and Interface Science,* 505, 341-351.

**263.** Zhao, J., Liu, Y., Pan, B., Gao, G., Liu, Y., Liu, S., ... and Peijnenburg, W. J. (2017). Tannic acid promotes ion release of copper oxide nanoparticles: Impacts from

solution pH change and complexation reactions. *Water Research,* 127, 59-67.

**264.** Zhao, X., Wang, S., Wu, Y., You, H., and Lv, L. (2013). Acute ZnO nanoparticles exposure induces developmental toxicity, oxidative stress, and DNA damage in embryo-larval zebrafish. *Aquatic Toxicology*, *136*, 49-59.

**265.** Zhao, Z., Cheng, X., Hua, X., Jiang, B., Tian, C., Tang, J., ... and Zhang, G. (2020). Emerging and legacy per-and polyfluoroalkyl substances in water, sediment, and air of the Bohai Sea and its surrounding rivers. *Environmental Pollution,* 263, 114391.

**266.** Zhao, Z., Xie, Z., Möller, A., Sturm, R., Tang, J., Zhang, G., and Ebinghaus, R. (2012). Distribution and long-range transport of polyfluoroalkyl substances in the Arctic, Atlantic Ocean and Antarctic coast. *Environmental pollution,* 170, 71-77.

**267.** Zhao, Z., Xie, Z., Möller, A., Sturm, R., Tang, J., Zhang, G., and Ebinghaus, R. (2012). Distribution and long-range transport of polyfluoroalkyl substances in the Arctic, Atlantic Ocean and Antarctic coast. *Environmental pollution,* 170, 71-77.

**268.** Zheng, H., Hu, G., Xu, Z., Li, H., Zhang, L., Zheng, J., ... and He, D. (2015). Characterization and distribution of heavy metals, polybrominated diphenyl ethers and perfluoroalkyl substances in surface sediment from the Dayan River, South China. *Bulletin of Environmental Contamination and Toxicology,* 94(4), 503-510.

**269.** Zhou, J., Baumann, K., Mead, R. N., Skrabal, S. A., Kieber, R. J., Avery, G. B., ... and Turpin, B. J. (2021). PFOS dominates PFAS composition in ambient fine particulate matter (PM 2.5) collected across North Carolina nearly 20 years after the end of its US production. *Environmental Science: Processes and Impacts,* 23(4), 580-587.

**270.** Zhou, Y., Wen, B., Pei, Z., Chen, G., Lv, J., Fang, J., ... and Zhang, S. (2012). Coadsorption of copper and perfluorooctane sulfonate onto multi-walled carbon nanotubes. *Chemical Engineering Journal,* 203, 148-157.

**271.** Winchell, Lloyd J., John J. Ross, Martha JM Wells, Xavier Fonoll, John W. Norton Jr, and Katherine Y. Bell. "Per-and polyfluoroalkyl substances thermal destruction at water resource recovery facilities: A state of the science review." *Water Environment Research* 93, no. 6 (2021): 826-843.

**272.** Liu, Zhaoyang, Yonglong Lu, Pei Wang, Tieyu Wang, Shijie Liu, Andrew C. Johnson, Andrew J. Sweetman, and Yvette Baninla. "Pollution pathways and release estimation of perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA) in

central and eastern China." *Science of the Total Environment* 580 (2017): 1247-1256.

**273.** PJ, J. C., Saigeetha, S., Samrot, A. V., Ponniah, P., and Chakravarthi, S. (2021). Overview on toxicity of nanoparticles, it's mechanism, models used in toxicity studies and disposal methods–A review. *Biocatalysis and Agricultural Biotechnology,* 36, 102117.

**274.** Chang, Y. N., Zhang, M., Xia, L., Zhang, J., and Xing, G. (2012). The toxic effects and mechanisms of CuO and ZnO nanoparticles. *Materials,* 5(12), 2850-2871.

**275.** Sukhanova, A., Bozrova, S., Sokolov, P., Berestovoy, M., Karaulov, A., and Nabiev, I. (2018). Dependence of nanoparticle toxicity on their physical and chemical properties. *Nanoscale Research Letters,* 13, 1-21.

**276.** Naz, S., Gul, A., and Zia, M. (2020). Toxicity of copper oxide nanoparticles: a review study. *IET Nanobiotechnology,* 14(1), 1-13.

**277.** Chowdhury, R., Khan, A., and Rashid, M. H. (2020). Green synthesis of CuO nanoparticles using Lantana camara flower extract and their potential catalytic activity towards the aza-Michael reaction. *RSC Advances,* 10(24), 14374-14385.

**278.** Wang, H., Xu, J. Z., Zhu, J. J., and Chen, H. Y. (2002). Preparation of CuO nanoparticles by microwave irradiation. *Journal of Crystal Growth,* 244(1), 88-94.

**279.** Ananth, A., Dharaneedharan, S., Heo, M. S., and Mok, Y. S. (2015). Copper oxide nanomaterials: Synthesis, characterization and structure-specific antibacterial performance. *Chemical Engineering Journal,* 262, 179-188.

**280.** Sukumar, S., Rudrasenan, A., and Padmanabhan Nambiar, D. (2020). Green-synthesized rice-shaped copper oxide nanoparticles using Caesalpinia bonducella seed extract and their applications. *ACS Omega,* 5(2), 1040-1051.

**281.** Ślosarczyk, A., Klapiszewska, I., Parus, A., Balicki, S., Kornaus, K., Gapiński, B., ... and Klapiszewski, Ł. (2023). Antimicrobial action and chemical and physical properties of CuO-doped engineered cementitious composites. *Scientific Reports,* 13(1), 10404.

**282.** Chowdhury, R., Khan, A., and Rashid, M. H. (2020). Green synthesis of CuO nanoparticles using Lantana camara flower extract and their potential catalytic activity towards the aza-Michael reaction. *RSC Advances,* 10(24), 14374-14385.

**283.** Sukumar, S., Rudrasenan, A., and Padmanabhan Nambiar, D. (2020). Green-synthesized rice-shaped copper oxide nanoparticles using *Caesalpinia bonducella* seed

extract and their applications. *ACS Omega,* 5(2), 1040-1051.

**284.** Sabeena, G., Rajaduraipandian, S., Pushpalakshmi, E., Alhadlaq, H. A., Mohan, R., Annadurai, G., and Ahamed, M. (2022). Green and chemical synthesis of CuO nanoparticles: A comparative study for several in vitro bioactivities and in vivo toxicity in zebrafish embryos. *Journal of King Saud University-Science,* 34(5), 102092.

**285.** Luna, I. Z., and Bangladesh Atomic Energy Commission. (2015). Preparation and characterization of copper oxide nanoparticles synthesized via chemical precipitation method. *Open Access Library Journal,* 2(03), 1.

**286.** Quirino, M. R., Lucena, G. L., Medeiros, J. A., Santos, I. M. G. D., and Oliveira, M. J. C. D. (2018). CuO rapid synthesis with different morphologies by the microwave hydrothermal method. *Materials Research,* 21.

**287.** Badawy, A. A., Abdelfattah, N. A., Salem, S. S., Awad, M. F., and Fouda, A. (2021). Efficacy assessment of biosynthesized copper oxide nanoparticles (CuO-NPs) on stored grain insects and their impacts on morphological and physiological traits of wheat *(Triticum aestivum L.)* plant. *Biology,* 10(3), 233.

**288.** Hemalatha, S., and Makeswari, M. (2017). Green synthesis, characterization and antibacterial studies of CuO nanoparticles from Eichhornia crassipes. *Rasayan Journal of Chemistry,* 10(3), 838-843.

**289.** Zedan, A. F., Mohamed, A. T., El-Shall, M. S., AlQaradawi, S. Y., and AlJaber, A. S. (2018). Tailoring the reducibility and catalytic activity of CuO nanoparticles for low temperature CO oxidation. *RSC Advances,* 8(35), 19499-19511.

**290.** Buledi, J. A., Ameen, S., Memon, S. A., Fatima, A., Solangi, A. R., Mallah, A., ... and Gupta, V. K. (2021). An improved non-enzymatic electrochemical sensor amplified with CuO nanostructures for sensitive determination of uric acid. *Open Chemistry,* 19(1), 481-491.

**291.** Fu, J., Tan, Y. X. R., Gong, Z., and Bae, S. (2020). The toxic effect of triclosan and methyl-triclosan on biological pathways revealed by metabolomics and gene expression in zebrafish embryos. *Ecotoxicology and Environmental Safety,* 189, 110039.

**292.** Guarda, V. L. M., Pereira, M. A., De Simone, C. A., Albuquerque, J. F. C., Galdino, S. L., Chantegrel, J., ... and Luu-Duc, C. (2003). Synthesis and structural study of arylidene thiazolidine and benzothiazine compounds. *Sulfur Letters,* 26(1), 17-27.

**293.** Guarda, V. L. M., Pereira, M. A., De Simone, C. A., Albuquerque, J. F. C., Galdino, S. L., Chantegrel, J., ... and Luu-Duc, C. (2003). Synthesis and structural study of arylidene thiazolidine and benzothiazine compounds. *Sulfur Letters,* 26(1), 17-27.

**294.** Morales, A. E., Pérez-Jiménez, A., Hidalgo, M. C., Abellán, E., and Cardenete, G. (2004). Oxidative stress and antioxidant defenses after prolonged starvation in Dentex dentex liver. *Comparative Biochemistry and Physiology Part C: Toxicology and Pharmacology,* 139(1-3), 153-161.

**295.** Tunçsoy, M., and Erdem, C. (2021). Comparison to toxic effects of copper oxide nanoparticles and copper sulphate on some serum parameters and enzyme activities of Oreochromis niloticus. *Journal of Anatolian Environmental and Animal Sciences,* 6(4), 514-521.

**296.** Kayhan, F. E., Esmer Duruel, H. E., Tartar Kizilkaya, Ş., Kaymak, G., Akbulut, C., Fakültesi, F. E., ... and Yön Ertuğ, N. D. (2022). Effects of thiazolidine on AChE enzyme activity and total protein level in zebrafish *(Danio rerio)* gill and liver tissue.

**297.** Aziz, S., and Abdullah, S. (2023). Evaluation of Toxicity Induced by Engineered CuO Nanoparticles in Freshwater Fish, *Labeo rohita*. *Turkish Journal of Fisheries and Aquatic Sciences,* 23(8).

**298.** Aziz, S., Abdullah, S., Anwar, H., and Latif, F. (2022). DNA Damage and Oxidative Stress in Economically Important Fish, Bighead Carp (*Hypophthalmichthys nobilis*) Exposed to Engineered Copper Oxide Nanoparticles. *Pakistan Veterinary Journal,* 42(1).

**299.** Dong, M., Zhu, L., Zhu, S., Wang, J., Wang, J., Xie, H., and Du, Z. (2013). Toxic effects of 1-decyl-3-methylimidazolium bromide ionic liquid on the antioxidant enzyme system and DNA in zebrafish *(Danio rerio)* livers. *Chemosphere,* 91(8), 1107-1112.

**300.** Fang, Y. Z., and Zheng, R. L. (2002). Theory and application of free radical biology. Beijing, Sciencex, 56-78.

**301.** Ganesan, S., Anaimalai Thirumurthi, N., Raghunath, A., Vijayakumar, S., and Perumal, E. (2016). Acute and sub-lethal exposure to copper oxide nanoparticles causes oxidative stress and teratogenicity in zebrafish embryos. *Journal of Applied Toxicology,* 36(4), 554-567.

**302.** Ge, W., Yan, S., Wang, J., Zhu, L., Chen, A., and Wang, J. (2015). Oxidative

stress and DNA damage induced by imidacloprid in zebrafish *(Danio rerio). Journal of Agricultural and Food Chemistry,* 63(6), 1856-1862.

**303.** Lushchak, V. I., Bagnyukova, T. V., Husak, V. V., Luzhna, L. I., Lushchak, V., and Storey, K. B. (2005). Hyperoxia results in transient oxidative stress and an adaptive response by antioxidant enzymes in goldfish tissues. *The International Journal of Biochemistry and Cell Biology,* 37(8), 1670-1680.

**304.** Mani, R., Balasubramanian, S., Raghunath, A., and Perumal, E. (2020). Chronic exposure to copper oxide nanoparticles causes muscle toxicity in adult zebrafish. *Environmental Science and Pollution Research,* 27, 27358-27369.

**305.** Shi, X., and Zhou, B. (2010). The role of Nrf2 and MAPK pathways in PFOS-induced oxidative stress in zebrafish embryos. *Toxicological Sciences,* 115(2), 391-400.

**306.** Slaninova, A., Smutna, M., Modra, H., and Svobodova, Z. (2009). Reviews Oxidative stress in fish induced by pesticides. *Neuroendocrinology Letters,* 30(1), 2.

**307.** Stara, A., Machova, J., and Velisek, J. (2012). Effect of chronic exposure to prometryne on oxidative stress and antioxidant response in early life stages of common carp *(Cyprinus carpio L.). Neuroendocrinol Lett,* 33(Suppl 3), 130-135.

**308.** Stara, A., Steinbach, C., Wlasow, T., Gomulka, P., Ziemok, E., Machova, J., and Velisek, J. (2013). Effect of zeta-cypermethrin on common carp *(Cyprinus carpio L.). Neuroendocrinol Lett,* 34(Suppl 2), 37-42.

**309.** Sun, P., Nie, X., Chen, X., Yin, L., Luo, J., Sun, L., ... and Jiang, S. (2018). Nrf2 signaling elicits a neuroprotective role against PFOS-mediated oxidative damage and apoptosis. *Neurochemical Research,* 43, 2446-2459.

**310.** Wang, Y., Yang, F., Zhang, H. X., Zi, X. Y., Pan, X. H., Chen, F., ... and Hu, Y. P. (2013). Cuprous oxide nanoparticles inhibit the growth and metastasis of melanoma by targeting mitochondria. *Cell Death and Disease,* 4(8), e783-e783.

**311.** Wang, Z., Li, N., Zhao, J., White, J. C., Qu, P., and Xing, B. (2012). CuO nanoparticle interaction with human epithelial cells: cellular uptake, location, export, and genotoxicity. *Chemical Research in Toxicology,* 25(7), 1512-1521.

**312.** Wang, Z., Von Dem Bussche, A., Kabadi, P. K., Kane, A. B., and Hurt, R. H. (2013). Biological and environmental transformations of copper-based nanomaterials. *ACS Nano,* 7(10), 8715-8727.

**313.** Feng, M., He, Q., Meng, L., Zhang, X., Sun, P., and Wang, Z. (2015). Evaluation of single and joint toxicity of perfluorooctane sulfonate, perfluorooctanoic acid, and copper to Carassius auratus using oxidative stress biomarkers. *Aquatic Toxicology,* 161, 108-116.

**314.** Lu, G. H., Liu, J. C., Sun, L. S., and Yuan, L. J. (2015). Toxicity of perfluorononanoic acid and perfluorooctane sulfonate to *Daphnia magna. Water Science and Engineering,* 8(1), 40-48.

**315.** Touaylia, S., Khazri, A., Mezni, A., and Bejaoui, M. (2019). Effects of emerging persistent organic pollutant perfluorooctane sulfonate (PFOS) on the Crustacean Gammarus insensibilis. *Human and Ecological Risk Assessment: An International Journal,* 25(8), 2133-2141.

**316.** Huang, J., Wang, Q., Liu, S., Zhang, M., Liu, Y., Sun, L., ... and Tu, W. (2021). Crosstalk between histological alterations, oxidative stress and immune aberrations of the emerging PFOS alternative OBS in developing zebrafish. *Science of the Total Environment,* 774, 145443.

**317.** O'Brien, T. M., and Wallace, K. B. (2004). Mitochondrial permeability transition as the critical target of N-acetyl perfluorooctane sulfonamide toxicity in vitro. *Toxicological Sciences,* 82(1), 333-340.

**318.** Meng, L., Yang, S., Feng, M., Qu, R., Li, Y., Liu, J., ... and Sun, C. (2016). Toxicity and bioaccumulation of copper in *Limnodrilus hoffmeisteri* under different pH values: Impacts of perfluorooctane sulfonate. *Journal of Hazardous Materials,* 305, 219-228.

**319.** Du, J., Cai, J., Wang, S., and You, H. (2017). Oxidative stress and apotosis to zebrafish *(Danio rerio)* embryos exposed to perfluorooctane sulfonate (PFOS) and ZnO nanoparticles. *International Journal of Occupational Medicine and Environmental Health,* 30(2), 213-229.

**320.** Du, J., Wang, S., You, H., Jiang, R., Zhuang, C., and Zhang, X. (2016). Developmental toxicity and DNA damage to zebrafish induced by perfluorooctane sulfonate in the presence of ZnO nanoparticles. *Environmental Toxicology,* 31(3), 360-371.

**321.** Pandey, S., Parvez, S., Sayeed, I., Haque, R., Bin-Hafeez, B., and Raisuddin, S.

(2003). Biomarkers of oxidative stress: a comparative study of river Yamuna fish *Wallago attu* (Bl. and Schn.). *Science of the Total Environment,* 309(1-3), 105-115.

**322.** Fang, Y. Z., and Zheng, R. L. (2002). Theory and application of free radical biology. Beijing, Sciencex, 56-78.

**323.** Zheng, J. L., Yuan, S. S., Wu, C. W., and Li, W. Y. (2016). Chronic waterborne zinc and cadmium exposures induced different responses towards oxidative stress in the liver of zebrafish. *Aquatic Toxicology,* 177, 261-268.

**324.** Altun, S., Özdemir, S., and Arslan, H. (2017). Histopathological effects, responses of oxidative stress, inflammation, apoptosis biomarkers and alteration of gene expressions related to apoptosis, oxidative stress, and reproductive system in chlorpyrifos-exposed common carp *(Cyprinus carpio L.). Environmental Pollution,* 230, 432-443.

**325.** El-Garawani, I. M., Khallaf, E. A., Elgendy, R. G., Mersal, G. A., and El-Seedi, H. R. (2021). Imidacloprid Induces Oxidative Stress and Genotoxicity in *Nile Tilapia*: The Role of Ascorbic Acid Combined Exposure.

**326.** Pinho, G. L. L., Da Rosa, C. M., Maciel, F. E., Bianchini, A., Yunes, J. S., Proença, L. A. D. O., and Monserrat, J. M. (2005). Antioxidant responses and oxidative stress after microcystin exposure in the hepatopancreas of an estuarine crab species. *Ecotoxicology and Environmental Safety,* 61(3), 353-360.

**327.** Gomi, F., and Matsuo, M. (1998). Effects of aging and food restriction on the antioxidant enzyme activity of rat livers. *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences,* 53(3), B161-B167.

**328.** Shao, B., Zhu, L., Dong, M., Wang, J., Wang, J., Xie, H., ... and Zhu, S. (2012). DNA damage and oxidative stress induced by endosulfan exposure in zebrafish *(Danio rerio). Ecotoxicology,* 21, 1533-1540.

**329.** Yan, S. H., Wang, J. H., Zhu, L. S., Chen, A. M., and Wang, J. (2016). Thiamethoxam induces oxidative stress and antioxidant response in zebrafish *(Danio rerio)* livers. *Environmental Toxicology,* 31(12), 2006-2015.

**330.** Du, Z., Zhu, L., Dong, M., Wang, J., Wang, J., Xie, H., ... and Guo, Y. (2014). Oxidative stress and genotoxicity of the ionic liquid 1-octyl-3-methylimidazolium bromide in zebrafish *(Danio rerio). Archives of Environmental Contamination and Toxicology,* 67, 261-269.

**331.** Dong, M., Zhu, L., Zhu, S., Wang, J., Wang, J., Xie, H., and Du, Z. (2013). Toxic effects of 1-decyl-3-methylimidazolium bromide ionic liquid on the antioxidant enzyme system and DNA in zebrafish *(Danio rerio)* livers. *Chemosphere,* 91(8), 1107-1112.

**332.** Escobar, J. A., Rubio, M. A., and Lissi, E. A. (1996). SOD and catalase inactivation by singlet oxygen and peroxyl radicals. Free *Radical Biology and Medicine,* 20(3), 285-290.

**333.** Tabatabaie, T., and Floyd, R. A. (1994). Susceptibility of glutathione peroxidase and glutathione reductase to oxidative damage and the protective effect of spin trapping agents. *Archives of Biochemistry and Biophysics,* 314(1), 112-119.

**334.** Atli, G., Alptekin, Ö., Tükel, S., and Canli, M. (2006). Response of catalase activity to Ag+, Cd2+, Cr6+, Cu2+ and Zn2+ in five tissues of freshwater fish *Oreochromis niloticus. Comparative Biochemistry and Physiology Part C: Toxicology and Pharmacology,* 143(2), 218-224.

**335.** Gomes, T., Pinheiro, J. P., Cancio, I., Pereira, C. G., Cardoso, C., and Bebianno, M. J. (2011). Effects of copper nanoparticles exposure in the mussel *Mytilus galloprovincialis*. *Environmental Science and Technology,* 45(21), 9356-9362.

**336.** Belek, N., Erkmen, B., Dinçel, A. S., and Gunal, A. C. (2022). Does persistent organic pollutant PFOS (perfluorooctane sulfonate) negative impacts on the aquatic invertebrate organism, *Astacus leptodactylus* [Eschscholtz, 1823]. *Ecotoxicology,* 31(8), 1217-1230.

**337.** Zhang, Q. F., Li, Y. W., Liu, Z. H., and Chen, Q. L. (2016). Exposure to mercuric chloride induces developmental damage, oxidative stress and immunotoxicity in zebrafish embryos-larvae. *Aquatic Toxicology,* 181, 76-85.

**338.** Da Cuna, R. H., Vazquez, G. R., Piol, M. N., Guerrero, N. V., Maggese, M. C., and Nostro, F. L. L. (2011). Assessment of the acute toxicity of the organochlorine pesticide endosulfan in *Cichlasoma dimerus* (Teleostei, Perciformes). *Ecotoxicology and Environmental Safety,* 74(4), 1065-1073.

**339.** dos Santos Miron, D., Crestani, M., Shettinger, M. R., Morsch, V. M., Baldisserotto, B., Tierno, M. A., ... and Vieira, V. L. P. (2005). Effects of the herbicides clomazone, quinclorac, and metsulfuron methyl on acetylcholinesterase activity in the silver catfish *(Rhamdia quelen)* (Heptapteridae). *Ecotoxicology and Environmental*

*Safety,* 61(3), 398-403.

**340.** Zhao, J., Wang, Z., Liu, X., Xie, X., Zhang, K., and Xing, B. (2011). Distribution of CuO nanoparticles in juvenile carp *(Cyprinus carpio)* and their potential toxicity. *Journal of Hazardous Materials,* 197, 304-310.

**341.** Mahapatra, A., Gupta, P., Suman, A., Ray, S. S., Malafaia, G., and Singh, R. K. (2023). Unraveling the mechanisms of perfluorooctanesulfonic acid-induced dopaminergic neurotoxicity and microglial activation in developing zebrafish. *Science of the Total Environment,* 887, 164030.

**342.** Islam, N., da Fonseca, T. G., Vilke, J., Gonçalves, J. M., Pedro, P., Keiter, S., ... and Bebianno, M. J. (2021). Perfluorooctane sulfonic acid (PFOS) adsorbed to polyethylene microplastics: accumulation and ecotoxicological effects in the clam *Scrobicularia plana. Marine Environmental Research,* 164, 105249.

**343.** Oliveira, R., Domingues, I., Koppe Grisolia, C., and Soares, A. M. (2009). Effects of triclosan on zebrafish early-life stages and adults. *Environmental Science and Pollution Research,* 16, 679-688.

**344.** Gao, M., Yang, Y., Lv, M., Song, W., and Song, Z. (2018). Oxidative stress and DNA damage in zebrafish liver due to hydroxyapatite nanoparticles-loaded cadmium. *Chemosphere,* 202, 498-505.

**345.** Song, P., Gao, J., Li, X., Zhang, C., Zhu, L., Wang, J., and Wang, J. (2019). Phthalate induced oxidative stress and DNA damage in earthworms *(Eisenia fetida). Environment International,* 129, 10-17.

**346.** Jin, Y., Zheng, S., Pu, Y., Shu, L., Sun, L., Liu, W., and Fu, Z. (2011). Cypermethrin has the potential to induce hepatic oxidative stress, DNA damage and apoptosis in adult zebrafish *(Danio rerio). Chemosphere,* 82(3), 398-404.

**347.** El-Atti, A., Desouky, M., Mohamadien, A., and Said, R. (2019). Impact of Copper Oxide Nanoparticles on freshwater crayfish, *Procambarus clarkia*. A combined histopathological, Biochemical and genotoxicological study. *Journal of Egyptian Academic Society for Environmental Development. D, Environmental Studies,* 20(1), 1-18.

**348.** Ogunsuyi, O. I., Fadoju, O. M., Akanni, O. O., Alabi, O. A., Alimba, C. G., Cambier, S., ... and Bakare, A. A. (2019). Genetic and systemic toxicity induced by silver

and copper oxide nanoparticles, and their mixture in *Clarias gariepinus* (Burchell, 1822). *Environmental Science and Pollution Research,* 26, 27470-27481.

**349.** Gupta, Y. R., Sellegounder, D., Kannan, M., Deepa, S., Senthilkumaran, B., and Basavaraju, Y. (2016). Effect of copper nanoparticles exposure in the physiology of the common carp *(Cyprinus carpio)*: *Biochemical, histological and proteomic approaches. Aquaculture and Fisheries,* 1, 15-23.

**350.** Xu, D., Li, C., Wen, Y., and Liu, W. (2013). Antioxidant defense system responses and DNA damage of earthworms exposed to perfluorooctane sulfonate (PFOS). *Environmental Pollution,* 174, 121-127.

**351.** Xu, D., Li, C., Wen, Y., and Liu, W. (2013). Antioxidant defense system responses and DNA damage of earthworms exposed to perfluorooctane sulfonate (PFOS). *Environmental Pollution,* 174, 121-127.

**352.** Hoff, P. T., Van Dongen, W., Esmans, E. L., Blust, R., and De Coen, W. M. (2003). Evaluation of the toxicological effects of perfluorooctane sulfonic acid in the common carp *(Cyprinus carpio). Aquatic Toxicology,* 62(4), 349-359.

**353.** Lu, L., Xu, L., Kang, T., and Cheng, S. (2012). Investigation of DNA damage treated with perfluorooctane sulfonate (PFOS) on ZrO2/DDAB active nano-order film. *Biosensors and Bioelectronics,* 35(1), 180-185.

**354.** Al-Bairuty, G. A., Shaw, B. J., Handy, R. D., and Henry, T. B. (2013). Histopathological effects of waterborne copper nanoparticles and copper sulphate on the organs of rainbow trout *(Oncorhynchus mykiss). Aquatic Toxicology,* 126, 104-115.

**355.** Abdel-Latif, H. M., Dawood, M. A., Mahmoud, S. F., Shukry, M., Noreldin, A. E., Ghetas, H. A., and Khallaf, M. A. (2021). Copper oxide nanoparticles alter serum biochemical indices, induce histopathological alterations, and modulate transcription of cytokines, HSP70, and oxidative stress genes in *Oreochromis niloticus*. *Animals,* 11(3), 652.

**356.** Cole, L. K., and Ross, L. S. (2001). Apoptosis in the developing zebrafish embryo. *Developmental Biology,* 240(1), 123-142.

**357.** Vogel, F. S. (1959). The deposition of exogenous copper under experimental conditions with observations on its neurotoxic and nephrotoxic properties in relation to Wilson's disease. *The Journal of Experimental Medicine,* 110(5), 801-810.

**358.** Campbell, H. A., Handy, R. D., and Sims, D. W. (2005). Shifts in a fish's resource holding power during a contact paired interaction: the influence of a copper-contaminated diet in rainbow trout. *Physiological and Biochemical Zoology,* 78(5), 706-714.

**359.** Handy, R. D., Sims, D. W., Giles, A., Campbell, H. A., and Musonda, M. M. (1999). Metabolic trade-off between locomotion and detoxification for maintenance of blood chemistry and growth parameters by rainbow trout *(Oncorhynchus mykiss)* during chronic dietary exposure to copper. *Aquatic Toxicology,* 47(1), 23-41.

**360.** Jantzen, C. E., Annunziato, K. A., Bugel, S. M., and Cooper, K. R. (2016). PFOS, PFNA, and PFOA sub-lethal exposure to embryonic zebrafish have different toxicity profiles in terms of morphometrics, behavior and gene expression. *Aquatic Toxicology,* 175, 160-170.

**361.** Liu, C., Yu, K., Shi, X., Wang, J., Lam, P. K., Wu, R. S., and Zhou, B. (2007). Induction of oxidative stress and apoptosis by PFOS and PFOA in primary cultured hepatocytes of freshwater tilapia *(Oreochromis niloticus). Aquatic Toxicology,* 82(2), 135-143.

**362.** Huang, H., Huang, C., Wang, L., Ye, X., Bai, C., Simonich, M. T., ... and Dong, Q. (2010). Toxicity, uptake kinetics and behavior assessment in zebrafish embryos following exposure to perfluorooctane sulphonic acid (PFOS). *Aquatic Toxicology,* 98(2), 139-147.

**363.** Chen, J., Wang, X., Ge, X., Wang, D., Wang, T., Zhang, L., ... and Dong, Q. (2016). Chronic perfluorooctane sulphonic acid (PFOS) exposure produces estrogenic effects in zebrafish. *Environmental Pollution,* 218, 702-708.

**364.** Keiter, S., Baumann, L., Färber, H., Holbech, H., Skutlarek, D., Engwall, M., and Braunbeck, T. (2012). Long-term effects of a binary mixture of perfluorooctane sulfonate (PFOS) and bisphenol A (BPA) in zebrafish *(Danio rerio). Aquatic Toxicology,* 118, 116-129.

**365.** Chen, J., Tanguay, R. L., Tal, T. L., Gai, Z., Ma, X., Bai, C., ... and Dong, Q. (2014). Early life perfluorooctane sulphonic acid (PFOS) exposure impairs zebrafish organogenesis. *Aquatic Toxicology,* 150, 124-132.

# ANNEXURES

# ANNEXURES

## Annexure 1

**Preparation of Exposure Solution for PFOS and CuO Nanoparticles**

- PFOS stock solution = 1mg/mL in DMSO
- CuO-NPs stock solution = 50 mg/L in distilled water

1. **PFOS Stock Solution: 1 mg/ mL**
   **Required Exposure Concentration: 300 µg/L in 17.5 Liters**

**Use Equation: $C_1V_1 = C_2V_2$**

$$\frac{1mg}{1ml} \times V1 = \frac{0.3mg}{1000ml} \times 17500ml$$

$$\frac{\cancel{1mg}}{\cancel{1ml}} \times V1 = \frac{0.3\cancel{mg}}{1000\cancel{ml}} \times 17500ml$$

$$V1 = \frac{0.3}{\cancel{1000}} \times 1\cancel{7500}ml => 0.3 \times 17.5$$

$$\boldsymbol{V1 = 5.25ml}$$

From PFOS stock solution (1mg/1ml), We will take 5.25 ml and add into 17494.75ml water to achieve our final required concentration of 300 µg/L.

2. **CuO-NPs Stock Solution: 50 mg/ L**
   **Required Exposure Concentration: 1mg/L in 17.5 Liters**

**$C_1V_1 = C_2V_2$**

$$\frac{50mg}{1000ml} \times V1 = \frac{1mg}{1000ml} \times 17500ml$$

$$\frac{50\cancel{mg}}{\cancel{1000ml}} \times V1 = \frac{1\cancel{mg}}{\cancel{1000ml}} \times 17500ml$$

$$V1 = \frac{1\cancel{7500}ml}{\cancel{50}} = 350ml$$

$$\boldsymbol{V1 = 350ml}$$

From CuO-NPs stock solution (50 mg/L), we will take 350 ml and add into 17150 ml water to achieve final required concentration of 1mg/L.

**3. Mixture Exposure Solution CuO-NPs+PFOS (1mg/L + 300µg/L) in 22.5 Liters**

- **For CuO-NPs Concentration in mixture treatment**

**$C_1V_1 = C_2V_2$**

$$\frac{50mg}{1000ml} \times V1 = \frac{1mg}{1000ml} \times 22500ml$$

$$\frac{50\cancel{mg}}{1000\cancel{ml}} \times V1 = \frac{1\cancel{mg}}{1000\cancel{ml}} \times 22500ml$$

$$V1 = \frac{\cancel{22500}ml}{\cancel{50}} = 450ml$$

$$\boldsymbol{V1 = 450ml}$$

From CuO-NPs stock solution (50mg/L), we will take 450 ml and add 22050 ml water to acheive final required concentration of 1mg/L.

- **For Perfluorooctane Sulfonate Concentration**

**$C_1V_1 = C_2V_2$**

$$\frac{1mg}{1ml} \times V1 = \frac{0.3mg}{1000ml} \times 22500ml$$

$$\frac{\cancel{1mg}}{\cancel{1ml}} \times V1 = \frac{0.3\cancel{mg}}{1000\cancel{ml}} \times 22500ml$$

$$V1 = \frac{0.3}{\cancel{1000}} \times \cancel{22500}ml \Rightarrow 0.3 \times 22.5$$

$$\boldsymbol{V1 = 6.75ml}$$

From PFOS stock solution (1mg/1ml), we will take 6.75 ml and add into 22493.25ml water to achieve final concentration of 300 µg/L.

## Annexure 2

**Formulae for calculations of Total Protein, Oxidative Stress, and Antioxidant Enzyme activities by Mr. Muhib**

➔ **Formula for Total Protein Estimation using Calibration Curve**:

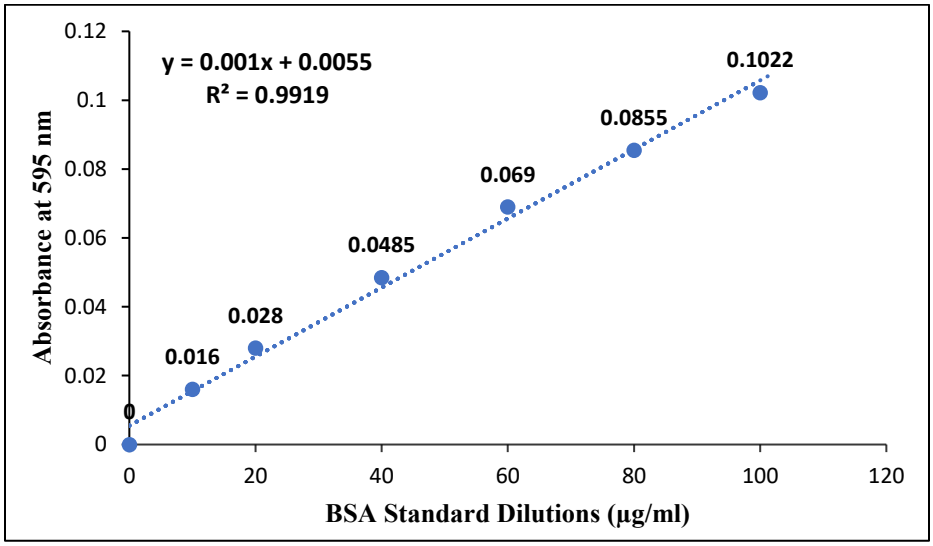



Figure: Standard curve of absorbance of Bovine serum Albumin (BSA) dilutions for determination of total protein in unknown sample.

Slope equation: $Y = 0.001X + 0.0055$

$X = (Y - 0.0055) / 0.001$

Whereas:

Y: Absorbance of unknown sample

X: Total protein in unknown sample (µg/ml)

**********

**Formula for Calculation of MDA**:

$$\text{Malondialdehyde (MDA)} = \frac{((\Delta\text{Sample} - \Delta\text{Blank}) \times \text{Cuvette Vol.} \times 10\text{^}6)}{(\text{Protein} \times \text{Sample Vol.} \times \Delta\text{time} \times \text{Constant } 15600)} = \text{U/mg protein}$$

Whereas:

ΔSample = Change in Sample Absorbance

ΔBlank = Change in Blank Sample Absorbance

Cuvette Vol. = Volume of Cuvette

Protein = Total protein in sample (mg/mL)

Sample vol = Volume of Enzyme Supernatant

Δtime = Measurement Time

15600 = Extinction co-efficient ($M^{-1}$ $cm^{-1}$)

**********

**Formula for Calculation of SOD**:

$$\text{Superoxide Dismutase (SOD)} = \frac{((\Delta\text{Sample} - \Delta\text{Blank}) \times \text{Cuvette Vol.} \times 10\text{^}6)}{(\text{Protein} \times \text{Sample Vol.} \times \Delta\text{time} \times (\text{Constant } 6.22))} = \text{U/mg of protein}$$

Whereas:

ΔSample = Change in Sample Absorbance

ΔBlank = Change in Blank Sample Absorbance

Cuvette Vol. = Volume of Cuvette

Protein = Total protein in sample (mg/mL)

Sample vol = Volume of Enzyme Supernatant

Δtime = Measurement Time

6.22 = Extinction co-efficient ($M^{-1}$ $cm^{-1}$)

**********

**Formula for Calculation of CAT**:

$$\text{Catalase (CAT)} = \frac{((\Delta\text{Sample} - \Delta\text{Blank}) \times \text{Cuvette Vol.} \times 10\text{^}6)}{(\text{Protein} \times \text{Sample Vol.} \times \Delta\text{time} \times (\text{Constant } 43.1))} = \text{U/mg of protein}$$

Whereas:

ΔSample = Change in Sample Absorbance

ΔBlank = Change in Blank Sample Absorbance

Cuvette Vol. = Volume of Cuvette

Protein = Total protein in sample (mg/mL)

Sample vol = Volume of Enzyme Supernatant

Δtime = Measurement Time

43.1 = Extinction co-efficient ($M^{-1}$ $cm^{-1}$)

## Annexure 3

**Formulae for calculations of AChE, Oxidative Stress, and Antioxidant Enzyme activities by Dr. Majid**

**Acetylcholine Esterase activity (AChE)** $= \frac{\boldsymbol{Tf - Ti}}{\mathbf{0.01}}$ = U/min

Units = U/min

Whereas:

Tf = Final Absorbance

Ti = Initial Absorbance

0.01 = Time Constant (for 1 minute)

---

**Catalase (CAT)** $= \frac{\boldsymbol{Tf - Ti}}{\mathbf{0.01}}$ = U/min

Units = U/min

Whereas:

Tf = Final Absorbance

Ti = Initial Absorbance

0.01 = Time Constant (for 1 minute)

**Reactive Oxygen Species (ROS)** $= \frac{(\mathbf{Sample\ O.D \times Cuvette\ Vol \times Protein})}{(\mathbf{Constant \times Sample\ Vol. \times Time})}$ = nM/mL

**Malondialdehyde (MDA)** $= \frac{(\mathbf{Sample\ O.D \times Cuvette\ Vol \times Protein})}{(\mathbf{Constant \times Sample\ Vol. \times Time})}$ = nM/mL

**Superoxide Dismutase (SOD)** $= \frac{(\mathbf{\Delta Sample - \Delta Blank}) \times \mathbf{Cuvette\ Vol \times Protein})}{(\mathbf{Constant \times Sample\ Vol. \times Time})}$ = U/min

# Annexure 4

- **Calculation for Antioxidants Enzymes Formula Sheet**

Calculate protein content from linear regression equation:

Y= 0.005X + 0.030

Y= absorbance (OD)

X has to find = Protein (Pr)

For all enzymes:

Enzyme activity = $\frac{OD}{t\ (min)} \times \frac{1}{\boldsymbol{\varepsilon}} \times \frac{Total\ vol(ml)}{Volume\ of\ enzyme\ extract(ml)} \times \frac{Total\ enzyme\ extract}{Weight\ of\ tissue\ (mg)} \times \frac{1}{Protein}$

Enzyme activity = µmol/mg/min

Here,

OD = $A_S$ - $A_b$

OD = Optical Density

As = Absorbance of the Sample

Ab = Absorbance of the Blank

ε = extinction coefficient

ε = CAT = 39.4 $Mm^{-1}$ $cm^{-1}$

ε = POD = 26.6 $Mm^{-1}$ $cm^{-1}$

t = incubation time

Total volume = Mixture of substrate, enzyme and buffer

In case of SOD,

$\frac{OD}{t}$ = Y → = $\frac{Ac-At}{Ac} \times 100$ = x%

= $\frac{x}{50}$ = Y ($Umin^{-1}$ $mg^{-1}$ FW).

- ✓ Ac = Absorbance of Control
- ✓ At = Absorbance of the Sample at respective time interval
- ❖ Second Method

Specific activity of enzyme = $\frac{A}{\varepsilon \times l \times t} \times \frac{Total\ volume\ (ml)}{Volume\ of\ enzyme\ extract\ (ml)} \times \frac{1}{Protein}$

A = absorbance (OD)
ε = Extinction coefficient
l = path length (1cm)
t = Incubation period/time of hydrolysis
Pr = Protein calculated from linear regression equation. x=?

**Example**:

980 µl = substrate

20 µl = enzyme extract

3 ml = buffer

Total volume = mixture of above all (µml)

5min = t (Incubation time)

e.g. A = 0.35

$\frac{\Delta A}{t} = \frac{0.35}{5}$

$\frac{\Delta A}{t}$ = 0.07 $min^{-1}$

$\frac{\Delta A}{t \times l \times \varepsilon}$ = 0.07 $min^{-1}$ $(\frac{1}{4600\ Lmol{-}1\ cm{-}1 \times 1cm})$

**OR**

= 1.52 µmol $L^{-1}$ $min^{-1}$

It can also be expressed as:

$= \frac{15.2\ \mu mol}{1000\ ml}$ $min^{-1}$

$\frac{\boldsymbol{A}}{\boldsymbol{t \times l \times \varepsilon}}$× total volume $= \frac{\boldsymbol{15.2\ \mu mol\ min{-}1}}{\boldsymbol{1000\ ml}} \times$ 4 ml

= 0.0609 µmol $min^{-1}$

$\frac{\boldsymbol{A}}{\boldsymbol{t \times l \times \varepsilon}} \times \frac{\textbf{total volume}}{\boldsymbol{Vol.of\ Enzyne\ extract}} = \frac{\boldsymbol{0.0609\ \mu mol\ min{-}1}}{\boldsymbol{20\ \mu L}}$

Convert 20µl to ml = $\frac{20}{1000}$ = 0.02 ml

$= \frac{0.006090\ \mu mol\ mim{-}1}{0.02ml}$

= 3.04 µmol $min^{-1}$ $ml^{-1}$

- Protein = 10 mg $ml^{-1}$

$\frac{\boldsymbol{A}}{\boldsymbol{t \times l \times \varepsilon}} \times \frac{\textbf{total volume}}{\boldsymbol{Vol.of\ Enzyne\ extract}} \times \frac{\boldsymbol{1}}{\boldsymbol{Pr}} = \frac{\boldsymbol{3.04\ \mu mol\ min{-}1\ ml{-}1}}{\boldsymbol{10 mg\ ml{-}1}}$

A = 0.304 µmol $min^{-1}$ $mg^{-1}$ Pr.

Conversions

U = µmol $min^{-1}$

U/mg = µml $min^{-1}$ $mg^{-1}$

U/ml = µmol $min^{-1}$ $ml^{-1}$

## Annexure 5

Activity = (ΔE x Vf) / (Δt x € x Vs x d)

Whereas:

ΔE → is change in absorbance,

Vf → final reaction volume

Vs → volume of enzyme extract used.

Δt → is time of hydrolysis.

€ → is extinction coefficient.

d → is diameter of cuvette used.

**The final answer is divided by mg or g protein of the tissue**.

➔ You can calculate enzyme activity of enzyme by using this:

Enzyme activity = change in OD / time taken (min) × 1 / extinction coefficient of enzyme × total reaction volume / volume of enzyme extract taken x total volume of enzyme extract / Fresh weight of tissue (g) × total protein × 1000 nano moles of enzyme present per gram of sample tissue. For catalase extinction coefficient is 39.4 $mM^{-1}$ $cm^{-1}$ and for peroxidases 26.6 $mM^{-1}$ $cm^{-1}$.

In case of SOD % inhibition = control OD - treatment OD/ control × 100 = X% inhibition.

50% inhibition is equal to 1 unit of enzyme, then X% is equal to 1/50 x X= Y unit.

➔ Calculate activity by inserting value of Y in above formula of activity in place of change in OD w.r.t. time. Rest of formula will be same.

- ✓ SOD → µmol / min / mg protein (µmol $min^{-1}$ $mg^{-1}$ protein)
- ✓ CAT → µmol / min / mg protein (µmol $min^{-1}$ $mg^{-1}$ protein)
- ✓ POD → nmol / min / mg protein (nmol $min^{-1}$ $mg^{-1}$ protein)
- ✓ GR → nmol / min / mg protein (nmol $min^{-1}$ $mg^{-1}$ protein)

# Exploring the Interactive Effects of Perfluorooctane Sulfonate (PFOS) and Copper Oxide Nanoparticles in Danio rerio (H.)

**Authors**
Ahmed Atiullah; Mazhar Iqbal Zafar

**Affiliations**
Quaid-i-Azam University, Islamabad

## 1 Introduction

- Per- and polyfluoroalkyl substances (PFAS), also known as "forever chemicals", are pervasive in the environment, from municipalities to the Arctic!
- Perfluorooctane sulfonate (PFOS) is the most infamous among PFAS, owing to its strong persistent, bioaccumulative and toxic (PBT) character.
- PFOS has existed in the natural environment for decades, but interaction with novel engineered nanoparticles can alter toxicological risks associated to it.
- Copper oxide nanoparticles (CuO-NPs) are manufactured for a wide range of applications, resulting in unmonitored release into the environment.
- Interaction of PFOS with existing nanoparticles (TiO2, ZnO and SWCNT) has been reported to enhance its toxicity.
- PFOS has also been reported to cause synergistic toxic effects upon interaction with Cu Ions.
- This study exclusively explores the interaction of CuO-NPs with PFOS and their toxicological manifestations in toxicity model zebrafish *D. rerio*.

## 2 Objectives

- Study the effects of CuO-NPs and PFOS on oxidative stress and antioxidant response in zebrafish gills.
- Measure neurological damage via monitoring AChE activity in brain tissues.
- Assess DNA damage in fish liver and study histopathology in skeletal muscle.

## 3 Methodology


## 4 Results

- ROS and MDA significantly ($p<0.05$)increased upon exposure to both contaminants, whereas inhibition of antioxidant enzymes (SOD & CAT) was observed.
- AChE activity in zebrafish brain was significantly inhibited upon combined exposure CuO-NPs and PFOS.
- Comet assay revealed DNA damage with increased tail length and tail moment in all treatment groups.
- Histopathological alterations including segmental necrosis, degeneration.
- n and splitting of muscle fibers, necrosis, shortening of muscle fiber and atrophy were observed in skeletal muscle.
- Mixture of CuO-NPs and PFOS revealed greater damage in all biomarkers as compared to their individual actions.

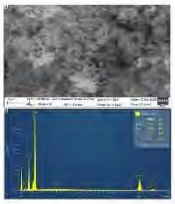


Figure 1: SEM-EDX of CuO-NPs

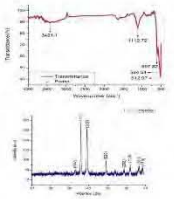


Figure 2: FTIR & XRD of CuO-NPs

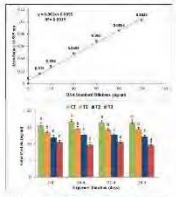


Figure 3: TP Estimation standard curve; TP levels during experiment

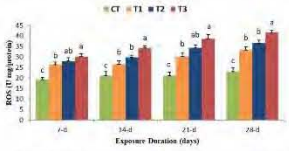



Figure 4: ROS levels in zebrafish gills during experiment

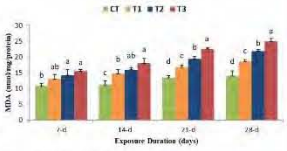



Figure 5: MDA content in zebrafish gills during experiment

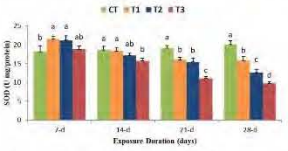



Figure 6: SOD activity in zebrafish gills during experiment

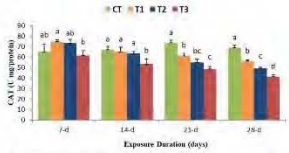



Figure 7: CAT activity in zebrafish gills during experiment

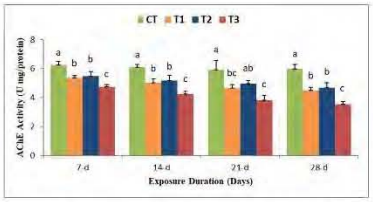



Figure 8: AChE activity in zebrafish brain during experiment

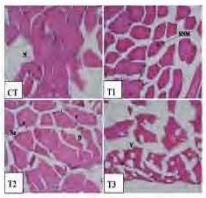



Figure 9: Histological alterations in muscle after 7 days

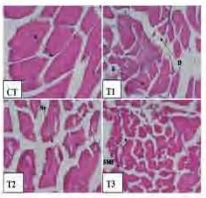



Figure 10: Histological alterations in muscle after 14 days

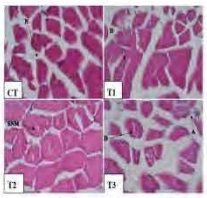



Figure 11: Histological alterations in muscle after 21 days

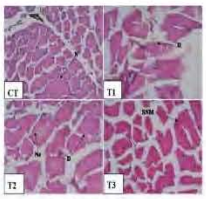



Figure 12: Histological alterations in muscle after 28 days

## 6 Conclusion

- This is the first study on the combined effects of PFOS and CuO-NPs on zebrafish *Danio rerio*.
- The CuO-NPs used in the study were slightly aggregated and 50-100 nm in size, in Tenorite phase with monoclinic crystal system, and were free from impurities.
- CuO-NPs and PFOS caused synergistic toxicity via induction of ROS, MDA, and reduced SOD and CAT activities.
- Combined exposure resulted in greater DNA damage and caused histological alterations in muscle segments.
- Further studies on the interactive action of PFOS and other PFCs with emerging contaminants, particularly nanoparticles, and their distribution across trophic levels.

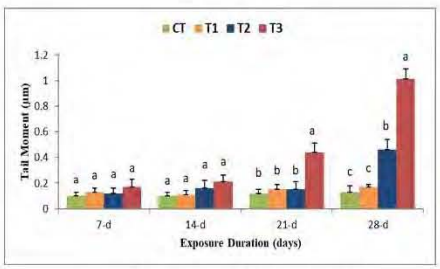



Figure 13: Tail moment in zebrafish liver during experiment

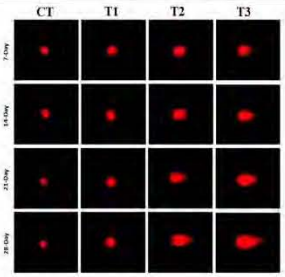



Figure 14: DNA damage in zebrafish liver during experiment

## 7 Acknowledgement

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**Related Literature**


- Qiang, L., Shi, X., Pan, X., Zhu, L., Chen, M., & Han, Y. (2015). Facilitated bioaccumulation of perfluorooctanesulfonate in zebrafish by nano-TiO2 in two crystalline phases. *Environmental Pollution*, 206, 644-651.
- Du, J., Wang, S., You, H., Jiang, R., Zhuang, C., & Zhang, X. (2016). Developmental toxicity and DNA damage to zebrafish induced by perfluorooctane sulfonate in the presence of ZnO nanoparticles. *Environmental toxicology*, 31(3), 360-371.
- Li, Y., Men, B., He, Y., Xu, H., Liu, M., & Wang, D. (2017). Effect of single-wall carbon nanotubes on bioconcentration and toxicity of perfluorooctane sulfonate in zebrafish (*Danio rerio*). *Science of the Total Environment*, 607, 509-518.