Exploring the Interactive Toxicological Impacts of Sodium Arsenite and Copper Oxide Nanoparticles on Zebrafish *Danio rerio* (H.)

AQSA HUMA

Registration No. 02312111001

DEPARTMENT OF ENVIRONEMNTAL SCIENCES

FACULTY OF BIOLOGICAL SCIENCES

QUAID-I-AZAM UNIVERSITY

ISLAMABAD, PAKISTAN

2021-2023

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

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in

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APPROVAL CERTIFICATE

It is to certify that the research work presented in this thesis, entitled **"Exploring the Interactive Toxicological Impacts of Sodium Arsenite and Copper Oxide Nanoparticles on Zebrafish *Danio rerio* (H.)"** was conducted by **Ms. Aqsa Huma (Reg. No. 02312111001)** under the supervision of **Dr. Mazhar Iqbal Zafar**. No part of this thesis has been submitted else for any other degree. This thesis is submitted to the Department of Environmental Sciences, in the partial fulfillment of the requirements for the degree of Master of philosophy in the field of Environmental Science, Quaid-i-Azam University, Islamabad.

Ms. Aqsa Huma (M. Phil. Scholar) _____

Supervisor

Dr. Mazhar Iqbal Zafar _____

Associate Professor
Department of Environmental Sciences
Quaid-i-Azam University, Islamabad, Pakistan

External Examiner

Dr. Nazish Bostan _____

Assistant Professor
Department of Biosciences
COMSATS University, Islamabad

Chairperson

Dr. Abida Farooqi _____

Professor
Department of Environmental Sciences
Quaid-i-Azam University, Islamabad, Pakistan

Dated: 30-11-2023

AUTHOR'S DECLARATION

I, Aqsa Huma, hereby state that my MPhil thesis titled "**Exploring the Interactive Toxicological Impacts of Sodium Arsenite and Copper Oxide Nanoparticles on Zebrafish *Danio rerio* (H.)**" is solely my own work and has not been submitted in any institution prior to my admission in Quaid-i-Azam University.

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Ms. Aqsa Huma

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Ms. Aqsa Huma

DEDICATION

I dedicate this research work to my beloved parents and my hardworking sisters. It is because of their faith in me that I have been able to accomplish great things in my academic life. I am forever indebted to them for their endless encouragement and support throughout this journey.

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Aqsa Huma

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LIST OF ABBREVIATIONS

Abbreviations	**Stands for**
As	Arsenic
AsV	Arsenate
AsIII	Arsenite
AMPA	Aminomethyl Phosphoric Acid
ANOVA	Analysis of Variance
ATSDR	Agency for Toxic Substances and Disease Registry
ATP	Adenosine Triphosphate
CNT	Carbon Nano Tubes
DNA	Deoxyribonucleic Acid
DO	Dissolved Oxygen
EC	Electrical Conductivity
EDTA	Ethylenediaminetetraacetic Acid
EPSP	5-Enolpyruvylshikimate-3-Phosphate
EU	European Union
EDX	Energy Dispersive x-ray Spectroscopy
FAO	Food and Agriculture Organization
FT-IR	Fourier-Transform Infrared Spectroscopy
GST	Glutathione S-Transferases
GPX	Glutathione Peroxidase
GR	Glutathione Reductase
IBM	International Business Machines
LMA	Low Melting Agarose

MDA	Malondialdehyde
mM	Mili Molar
MPs	Microplastics
$NaAsO_2$	Sodium Arsenite
NBT	Nitrotetrazolium Blue Chloride
NBF	Neutral Buffer Formalin
Ne	Necrosis
NEQS	National Environmental Quality Standards
NMA	Normal Melting Agarose
NPs	Nanoparticles
NiO-NPs	Nickel Oxide Nanoparticles
OECD	Organization for Economic Co-Operation and Development
OD	Optical Density
O	Oxygen
PCRWR	Pakistan Council of Research in Water Resources
PPB	Parts Per Billion
PPM	Parts Per Million
PAK-EPA	Pakistan Environmental Protection Agency
PBS	Phosphate Buffer Saline
ROS	Reactive Oxygen Species
SCGE	Single-cell Gel Electrophoresis
SD	Standard Deviation
SDS	Sodium Dodecyl Sulfate

SEM	Scanning Electron Microscopy
SMF	Shortening of Muscle Fiber
SNM	Segmental Necrosis in Muscle Fiber
SOD	Superoxide Dismutase
SPSS	Statistical Package for Social Sciences
TDS	Total Dissolved Solids
TiO_2-NPs	Titanium Dioxide Nanoparticles
UNICEF	Nations International Children's Emergency Fund
US-EPA	United States Environmental Protection Agency
WHO	World Health Organization
XRD	X-ray Diffraction
ZnO-NPs	Zinc Oxide Nanoparticles

HIGHLIGHTS

- Individual and combined toxic effects of $NaAsO_2$ and CuO-NPs in adult zebrafish were evaluated in a 28-day experiment.
- ROS and MDA increased in the single and combined treatment groups, however antioxidant enzymes (SOD and CAT) were inhibited throughout the experiment.
- AChE activity in zebrafish brain was reduced as a result of single and joint toxicity test of $NaAsO_2$ and CuO-NPs.
- Comet assay indicated DNA damage with increased tail length and tail moment in all treatment groups.
- Histopathological alterations such as segmental necrosis, degeneration and splitting of muscle fibers, necrosis, and shortening of muscle fiber were examined in skeletal muscles in all treatment groups.
- Combined exposure of $NaAsO_2$ and CuO-NPs revealed significant damage in all biomarkers as compared to single exposure.

ABSTRACT

Background: Sodium arsenite is widely known for its use as herbicide in agriculture and has been found to be highly toxic to exposed organisms. Nanoparticles are emerging pollutants that can affect the toxicological risks associated with the already existing contaminants after interacting with them. Nanoparticles due to their variety of unique properties can impact the toxicity of sodium arsenite when jointly exposed. Therefore, the present study aims at evaluating the individual toxic effects of sodium arsenite ($NaAsO_2$), copper oxide nanoparticles (CuO-NPs) and their joint toxicity on zebrafish *Danio rerio*.

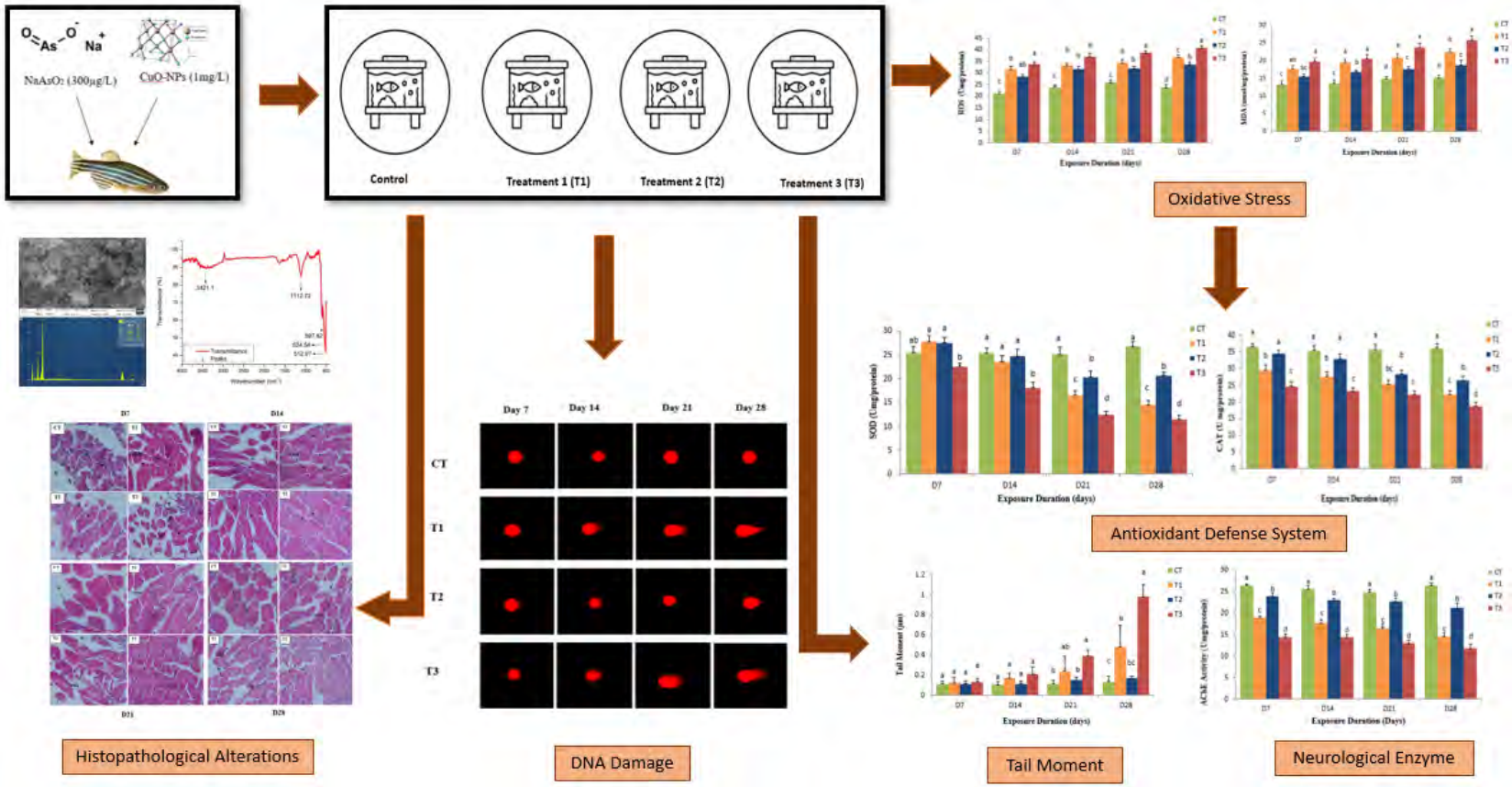
Methodology: Nanoparticles used in the study were characterized for size and morphology using SEM, however chemical composition, surface functional groups and crystallography information were analyzed using EDX, FTIR and XRD, respectively. The adult zebrafish were exposed to these contaminants in four different treatment groups; control group; $NaAsO_2$ (300µg/L); CuO-NPs (1mg/L); and $NaAsO_2$+CuO-NPs (300µg/L+1mg/L). The exposure period consisted of 28 days with samples taken at 7, 14, 21 and 28 days. The fish were exposed in triplicates ($n=3$) and the organs were obtained through dissection. Total protein content, oxidative stress (ROS and MDA) and antioxidant enzymes (SOD and CAT) were assessed in the gills, acetylcholinesterase (AChE) activity was measured in the brain and DNA damage was evaluated in the liver, histopathological alterations were assessed in skeletal muscles.

Result: Both contaminants significantly ($p<0.05$) increased ROS levels and MDA content, hence inducing oxidative stress in the exposed fish. Whereas the activities of SOD and CAT were inhibited significantly throughout the experiment. Furthermore, the exposure to both contaminants significantly ($p<0.05$) decreased AChE activity in the brain and synergistic effect was observed in the co-exposure treatment group. The DNA damage was caused by both $NaAsO_2$ and CuO-NPs inducing increase in tail length and movement. Furthermore, histopathological changes including segmental necrosis, degeneration and splitting of muscle fibers, necrosis and shortening of muscle fiber were observed in all treatment groups other than the control group at all sampling intervals.

Conclusion: The results of the present study indicate that the combined exposure of $NaAsO_2$ and CuO-NPs leads to a synergistic toxic effect in zebrafish *Danio rerio.*

Keywords: Zebrafish, Chronic exposure, Joint Toxicity, Sodium arsenite, Copper oxide nanoparticles, Histopathology, AChE activity, DNA damage, Oxidative stress, Antioxidant enzymes.

GRAPHICAL ABSTRACT



Chapter 1

Introduction and Literature Review

1. INTRODUCTION AND LITERATURE REVIEW

1.1 Environmental toxicity and its drivers

Since the onset of the human race, the quest for perfection and advancement in every aspect of human life has increased by leaps and bounds, therefore, it has seen no restriction whatsoever in history. Humans have carved out ways to satisfy their needs and desires to live a prosperous life. One of those eager ways has led to industrialization and the industrial revolution began in the 18th century (Szreter, 2004). The pollutants are released in the environment through multiple sources emerging both from anthropogenic and natural means. Due to rapid progression in industrial sector across the globe alongside urbanization, the levels of anthropogenic pollutants keep on ascending to variable extents in different regions of the world. Whereas chemicals from natural means seem to exist in nature and cause no meaningful harm upon exposures to organisms unless their quantities are altered either in response to human activities or as a result of natural disasters (Engwa et al., 2019). Industrialization is the main source of the existence of chemicals in the environmental followed by agricultural practices that mostly end up disrupting various life process and adversely impacting living organisms by altering their respective ecosystems (He et al., 2014). These chemicals become part of the water we drink, the food we eat and the air we breathe, hence, putting our lives at risk (Balistrieri and Mebane, 2014). Under such circumstances the term that encompasses such measures and studies them in detail is known as environmental pollution (EP). The main culprits are often categorized as heavy metals, pesticides, plastic, persistent organic pollutants (POP) and pharmaceuticals and personal care products (PPCPs), plastic products etc. are collectively known as pollutants (Coetzee et al., 2018; Rice et al., 2014; Boverhof and Zacharias, 2005). Hence, these pollutants have adverse effects on organisms that are encountering them. These effects might result in severe health implications for humans and animals and pose a threat to the well-being of plants (Wu et al., 2016).

It is high time to establish the possible linkages between the release of these chemicals and them becoming part of our environment as an existential threat. The field that mainly studies the toxic relationship of anthropogenic and natural pollutants with the biotic factors of the environment is “Environmental Toxicology”. The pollutants are studied individually

to determine their toxicological effects as well as the mixtures of chemicals are studied to get a better understanding of their toxic effects (Wu et al., 2016). It is essential in this regard to get a better understanding of the origin of these pollutants and from where they are emerging in the environment (Enfrin et al., 2019). This leads us to further determine their fate and consequences that they might have on living organisms upon exposure (Altenburger et al., 2013). The focus of environmental toxicology is to deeply study the effects of toxic agents that could be physical, chemical, and biological in nature exist in different environmental mediums such as air, water, and soil (Agathokleous and Calabrese, 2020). Therefore, it promises a multidisciplinary approach in this domain to comprehend the effects of toxicants on ecosystems and the population and communities they harbor (Asha et al., 2022).

1.2 Ecotoxicity and its effects on aquatic ecosystem

The recent advancement in industrial and agricultural sectors for the betterment of lifestyle on individual basis ranging from public places to household level has put the health of our ecosystem at the risk of irreversible damage (Sharma et al., 2019). These sectors are responsible for the occurrence and existence of a number of pollutants and contaminants in aquatic ecosystems (Hoang et al., 2020). Therefore, these pollutants are responsible for putting the life cycles of aquatic organisms in jeopardy (Mokarram et al., 2020). Agricultural runoff, industrial and household effluents are the main sources of the emergence of such pollutants (Saha and Paul, 2018). Micro-(Nano) plastics, heavy metals, pesticides, persistent organic pollutants, and endocrine disrupting chemicals (EDC) are notoriously famous for polluting the waterways and aquatic lifestyle. These pollutants have the ability to disrupt life processes by transforming into their highly toxic compounds such as heavy metals (Zhou et al., 2008). These compounds can further bioaccumulate in the organisms and magnify along the food chains, hence, making their ways to be consumed by humans at the end leading to various diseases such as immunotoxicity, behavioral abnormalities, altered metabolism and specific organ dysfunctions (Prüss-Ustün et al., 2011).

Toxic exposure of pollutants to aquatic organisms have been reported to exhibit severe abnormalities in organisms that include underdevelopment of internal organs such as heart,

liver, brain, eyes etc. and morphological malformations (Wang and Fowler, 2008). The severity these pollutants have on aquatic dwellers is dependent on their bioavailability and chemical forms (Adams et al., 2019). Therefore, various toxicity tests have been employed on living organisms such as zebrafish, brown mussel, earthworms, African catfish (Tortella et al., 2020). in order to expose them to a variety of pollutants to evaluate their toxicological effects. These models test organisms that are examined under the laboratory conditions to understand the interaction of living organisms and pollutants upon exposure. Such experiments are either done with a single chemical exposure or combined toxicity tests are carried out to analyze how certain mixtures of chemicals affect the well-being of aquatic species. For instance, Lin et al. (2019) examined the combined toxicity of Polychlorinated biphenyls (PCBs) and polystyrene nanoparticles on *Daphnia magna* and found out that the presence of polystyrene nanoplastics affects the toxic activity of polychlorinated biphenyls due to the adsorption of the latter on the nanoparticles. Further on it is essential to determine the toxic effects of pollutants on various life stages of organisms through acute or chronic exposure depending on the toxicity test. The toxicity tests of certain pollutants have been reported on embryonic stages, larval development, and adult organisms to have a better understanding of toxicity endpoints (He et al., 2014). Gandara-e-Silva et al. (2016) reported the toxicity of leachate of microplastics on larval development of brown mussels and concluded that indirect exposure of plastic debris is harmful to the aquatic organisms to a point that it can cause lethality in them.

1.3 Toxicological impacts of heavy metals

The exponential rise in the human population across the globe has caused an ever-growing surge in the dependence on energy as lifestyles have evolved in the recent years mainly due to industrial development (Armansyah and Jaman, 2023; Tchounwou et al., 2014). The global demand to acquire goods and modern-day technology has gone through the roof in the last few decades urging industrialists to come up with a variety of ways to make maximum economical gains (Muthusaravanan et al., 2019). This has led to their heavy reliance on heavy metals among other elements. Heavy metals are naturally occurring elements having high density of more than $5g/cm^3$ with atomic number greater than 20 and atomic weight five times higher than water (Briffa et al., 2020). They are naturally present

in the earth crust with varying compositions depending on the spatial distribution of these elements across the globe (Jaishankar et al., 2014). Normally, they are present in their trace quantities but when they exceed their threshold concentrations, they become toxic depending on the degree of their concentration in the respective environmental matrix (Bayata, 2020). Some of the heavy metals play an essential role in the biological well-being of the organisms but they exceed the required amount they cause toxicity in the same organisms whilst most of the heavy metals play no role and are just toxic (Garai et al., 2021). Moreover, their toxicity is heavily influenced by the pathways of exposures of these elements that determine their bioavailability to the target organisms across the ecosystems (Kim et al., 2015). It is essential to determine the fate and consequences of these heavy metals once they are released in the environment by conducting toxicological studies on organisms (Briffa et al., 2020).

The emergence of heavy metals from industries, agriculture and household sources have elevated the levels of their concentrations in the natural environment (Yang et al., 2018). Heavy metals are persistent in the environment and there is no natural mechanism for their biodegradation, hence, they remain in the environment unless removed through remediation techniques (Kapahi and Sachdeva, 2019). The living organisms are exposed to it once the persistent heavy contaminants become bioavailable to their targets in different ecosystems (Moiseenko and Gashkina, 2020). The contaminants are easily taken up by the roots to the rest of the plants when they enter soil and cause adversity upon their exposure (Wan et al., 2020). Similarly, when these contaminants enter water bodies, they put aquatic biota at risk by direct exposure after being dissolved in water (Joseph et al., 2019).

Heavy metals can cause damage to the nervous system of aquatic animals making up the food web (Kapoor and Singh, 2021). They can biomagnify along the food chain and remain persistent in the aquatic ecosystem (Nkwunonwo et al., 2020). The heavy metals that bioaccumulate and have toxic effect on organisms are Arsenic (As), Copper (Cu), Chromium (Cr), Cadmium (Cd), Lead (Pb), Mercury (Hg), and Zinc (Zn) (Sheikhzadeh and Hamidian, 2021). In rainbow trout (*Salmo gairdneri*) chromium affected the development of fish and embryo hatching when exposed to the concentration of 2 mg/L (van der Putte et al., 1982). Cadmium caused anemia in American eel fish at the concentration of 150 µg/L by reducing hemoglobin and erythrocyte counts (Gill and Epple,

1993). Furthermore, cadmium causes toxicity in fish by inhibiting the electron transfer chain in mitochondria and inducing reactive oxygen species (Wang et al., 2004). Copper has been reported to be neurotoxic to fish and affects the function of olfactory neurons in them (Johnson et al., 2007). Lead bioaccumulates in the organs of fish mainly it occurs in liver, spleen, kidney, and gills which affects locomotion and morphology of the fish (Hou et al., 2011; Cretì et al., 2009). Mercury enters the fish body mostly through skin and gills causing physiological, biochemical, and morphological alterations in the fish. For instance, methyl mercury can cross blood-brain barrier in the fish due to its lipophilic properties hence causing neurotoxicity by accumulating in the nervous system (Garai et al., 2021).

1.4 An overview of Arsenic (As)

Arsenic is a metalloid that is naturally present in the earth's crust and is also released in the aquatic environment from several anthropogenic activities. It is widely distributed in the environment and its compartments air, water, and land (Rehman et al., 2021; Han et al., 2019:). It hardly occurs in a free state and is mostly found in its combined form with sulfur, oxygen, and iron. It appears in the environment as trivalent arsenic (arsenite, AsIII) and pentavalent arsenic (arsenate, AsV). The trivalent arsenic is 60 times more toxic than pentavalent arsenate (Raju, 2022). AsV is the stable oxidation state of arsenic in oxygen-containing waters, but it can be reduced to AsIII under anoxic or reducing conditions (see Figure 1). The large amounts of arsenic from anthropogenic sources and trace quantities from natural sources collectively pose ecological toxicity to organisms coming in contact with the organisms and put their well-being at risk (Canivet et al., 2001).

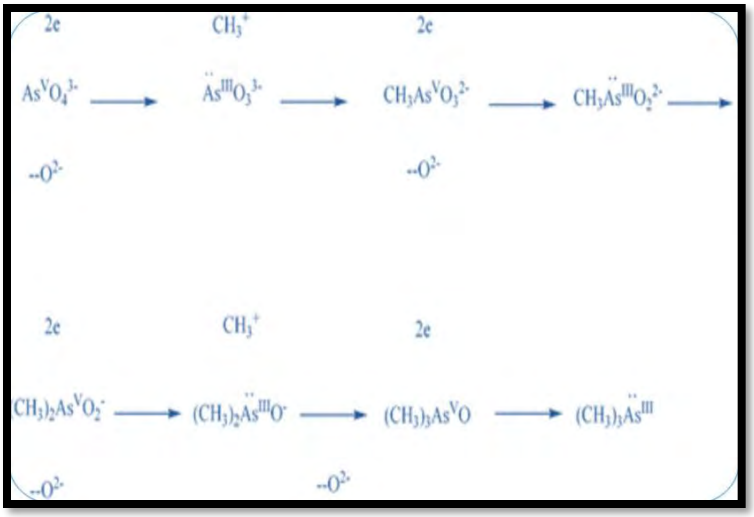


Figure 1: Mechanism of AsV reduction to AsIII (Kumari et al., 2017)

1.5 Presence of As in the environment

Naturally, As is found in huge quantities in sedimentary or igneous rocks alongside other elements and also as major constituent in more than 200 minerals for e.g., elemental arsenic, arsenide, arsenite and arsenate that are rarely found in the natural environment (Figure 2). These forms of arsenic are mostly released in the environment from anthropogenic sources as a result of industrial activities (Kumari et al., 2016). The low concentrations of Arsenic causes bioaccumulation in the vital organs of fish such as kidney and liver upon chronic exposure through food chain (Rahaman et al., 2021; Kumari et al., 2016). Arsenic alters histopathology in gills and tissues of liver in freshwater fish tilapia *Oreochromis mossambicus* (Ahmed et al., 2013). Sodium arsenate caused neurotoxicity in zebrafish at acute exposure of 6h with the concentration of 65 mg/L (Guidi et al., 2023). Arsenic is mainly exposed through consumption of As contaminated food and water by humans and animals (Ohno et al., 2007). Arain et al. (2008) examined the food irrigated by lake and canal water and estimated the daily intake of As concentration in the diet to be within the range of 9.7– 12.2 µg/kg body weight/day. As has been classified as a human carcinogen by WHO whereas low concentrations of As causes gastrointestinal issues, reduced production of red and white blood cells, affected heart rhythm, damaged blood vessels and sensation of “pins and needles” in hands and feet (Abernathy et al., 2003). Arsenic has been categorized as a number 1 toxic pollutant among top 20 priority pollutants

by the Agency of Toxic Substances and Disease Registry (ATSDR) and it reported arsenic to be responsible for impaired fetal development, low birth weight, fetal malformations ultimately causing fetal death during prenatal exposure (Murtaza et al., 2019). Also, It can cause blood vessel damage, lower IQ, reduced nerve function hence leading to mortality in children as a result of the exposure. Furthermore, it has been stated that arsenic is responsible for cardiovascular diseases and neurological disturbances (ATSDR, 2007).

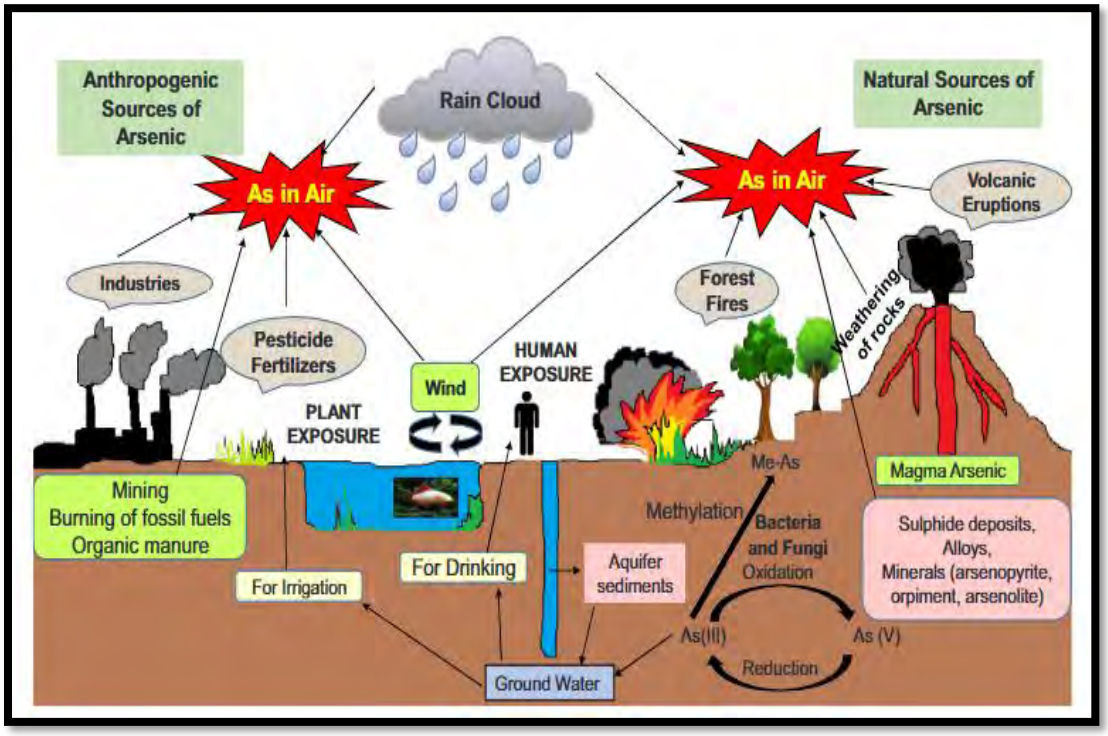


Figure 2: Interaction of naturally occurring and anthropogenic arsenic in the environmental matrices. This figure is taken from the study of Rehman et al. (2021).

1.6 National and International Existing levels of arsenic detected in water

The guideline for arsenic concentration in drinking water by the World Health Organization is 10 ppb (µg/L) (WHO, 2017). The permissible concentration of arsenic differs from country to country, for example it is as low as 7 ppb and 5 ppb in Australia and America, respectively, and as high as 50 ppb in countries like Pakistan, Argentina, Bangladesh, China, Chile etc. (Chakrabarti et al., 2019; Raju 2022; Rahman et al., 2009Ahmed et al., 2004). A geological survey by the United States of America to estimate the values of Arsenic found out the average concentration of Arsenic in groundwater to be

around 11g/L resulting in significant human exposure in drinking water (Fatoki and Badmus, 2022). In India, arsenic concentration was found to be more than 50 µg/L whereas the standard level for country is 50 µg/L exposing more than five million to arsenic toxicity (Sharma et al., 2018). Furthermore, a previous study reported arsenic concentration to be more than standard level of 50 µg/L of arsenic in groundwater in China (Rodriguez-Lado et al., 2013). In central China, the average concentration reported in drinking water was around 1.3 μg/L (Wang et al., 2022). Previously according to a study by Raessler (2018) in Bangladesh around 3 million tubewells out of 11 million had reported higher concentration of arsenic than WHO recommended guideline of arsenic i.e. 10 µg/L. Furthermore, arsenic concentration in groundwater of Nadia district, West Bengal ranged between 0.26 and 0.73 mg/L (Samal et al., 2021).

In Pakistan, the high levels of Arsenic were reported in the surface water of Nagarpakar, Sindh within the range of 360-683 µg/L whereas in the groundwater of Lahore, Punjab the levels were found to be in the increasing trend of 1-525 µg/L (Brahman et al., 2014; Sultana et al., 2013). According to National Environmental Quality Standards (NEQS) and Pakistan Environmental Protection Agency (Pak-EPA), the acceptable limit of arsenic is 50 µg/L in Pakistan (Shahid et al., 2022; NEQS, 2010; Pak-EPA, 2008). In 2016, a study reported the range of As concentration 3.0–50.0, and 13–106 µg/L in surface and ground water, respectively (Memon et al., 2016). According to the groundwater monitoring data by the Pakistan Council of Research in Water Resources (PCRWR) and United Nations International Children's Emergency Fund (UNICEF), concentration of As was found to be within10 to 200 µg/L in Punjab whereas, in Sindh higher concentration than 10µg/L exposing 16-36% people to As consumption (Shahab et al., 2018). Previously, a study by Shahid et al. (2017) reported arsenic concentration in groundwater of Vehari, Mailsi, and Burewala were 47.9 µg/L, 130 µg/L and 28 µg/L, respectively. Furthermore, a study conducted in various cities of Sindh and Punjab i.e. reported As concentration ranged between 0.2 and 501.1µg/L in groundwater hence exceeding WHO recommended guideline of Arsenic (10 µg/L) (Ali et al., 2019).

Table 1: The permissible drinking water limits for arsenic across the globe.

No.	Country	Permissible limit (ppb)	References
1	WHO	10	(Podgorski and Berg, 2020)
2	US-EPA	10	(Frisbie and Mitchell, 2022)
3	Pak-EPA (NEQS)	50	(Nawaz et al., 2023; Murtaza et al., 2019)
4	Australia	07	(Chakrabarti et al., 2019)
5	Bangladesh	50	(Hossain et al., 2023; Ahmed et al., 2004)
6	China	50	(Sun, 2004)
7	Chile	50	(Ahmad and Bhattacharya, 2019)
8	India	10	(Alsubih et al., 2021)
9	Nepal	50	(Gwachha et al., 2020)
10	New Zealand	10	(Robinson et al., 2003)
11	Pakistan	50	(Shahid et al., 2022)
12	Taiwan	10	(Ahmad and Bhattacharya, 2019)
13	United States of America	05	(Chakrabarti et al., 2019)

1.7 Sodium arsenite ($NaAsO_2$) and its characteristics

Typically, arsenic is found as arsenite (AsIII) and arsenate (AsV). Sodium arsenite ($NaAsO_2$), being a trivalent form of arsenic is an inorganic white-gray salt that is odorless, tasteless, and highly soluble in water. The chemical characteristics of $NaAsO_2$ are described in Figure 3. Sodium arsenite is used as a weed killer, rodenticide and is mostly used in the glass manufacturing processes. It is formed from the reaction of arsenic trioxide with caustic soda. Historically, arsenic based compounds were used as insecticides in the 18^{th} century as treatments against insects such as *Leptinotarsa decemlineata* (Colorado Potato Beetle) in potatoes, *Aclypea opaca* (Beet Carrion Beetle) in sugar beet and *Anthonomus grandis* (Boll Weevil) in cotton. Later on in the 19^{th} century, inorganic form of arsenic, $NaAsO_2$ was then used as herbicides. These practices were mainly carried out in agriculture and viticulture to prevent the crops from diseases in the 20^{th} century as

$NaAsO_2$ was also used as a fungicide against grapevine trunk diseases, anthracnose, phomopsis cane, and leaf spot. Furthermore, it was also used against the attacks by snails in mid-20th century (Songy et al., 2019).

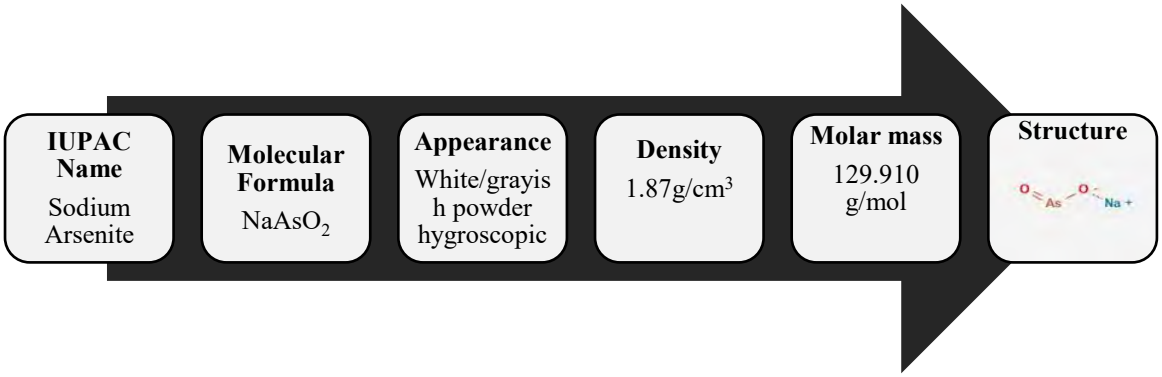


Figure 3: Chemical characteristics of sodium arsenite

Pertaining to its toxic effects and high risks, $NaAsO_2$ was banned in Europe in 2003 against its practice to control diseases caused by fungal pathogens in woods. It was found out that $NaAsO_2$ caused developed leaf senescence symptoms leading to decrease in chlorophyll content and increase in lipid peroxidation in grapevine plants treated with the compound. Furthermore, $NaAsO_2$ was found to have affected crops by penetrating into woody tissues, and moving from roots to shoots and berries and later on released in the soil (Li et al., 2015). It has also been found to trigger stress response in plants by adversely affecting plant growth and its productivity by altering ATP synthesis, photosynthesis, and nutrients levels (Abbas et al., 2018). Interaction of arsenite with sulfhydryl groups (-SH) of enzymes and the reactive oxygen species adversely affects plant metabolism by causing DNA damage (Finnegan and Chen, 2012). Sodium arsenite is an emerging pollutant in the aquatic environment from anthropogenic sources after mainly being used as herbicides, pesticides but also as dyes, antiseptics, soaps etc. (Hughes, 2002).

1.8 Fate of sodium arsenite in aquatic ecosystem

The inorganic form of Arsenic in trivalent oxidative state, $NaAsO_2$ is more toxic to the fish than arsenate as the former rapidly enters the fish and accumulates therein (Garai et al., 2021). Arsenite contamination in the water puts the life of aquatic organism at risk to great lengths due to certain toxicity mechanisms such as oxidative stress (Kumar and Banerjee,

2012; Ahmed et al., 2013). Since Arsenic is highly toxic to aquatic organisms at low concentrations, USEPA regulated a permissible limit for aquatic organisms to be 150 µg/L upon chronic exposure (USEPA, 1995; Sun et al., 2020). In aquatic ecosystem, both forms of arsenite (AsIII) and arsenate (AsV) are found to be present. Among the As species, AsV is majorly found in well-oxygenated water, but it can be eliminated easily through As removal techniques and technologies. Whereas AsIII is more toxic, soluble, and difficult to remove from the contaminated water. The conversion of AsIII to AsV in well-oxygenated water may take time depending on specific conditions. Therefore, the presence of AsIII is considered to be a potential threat to aquatic organisms (Dong et al., 2018).

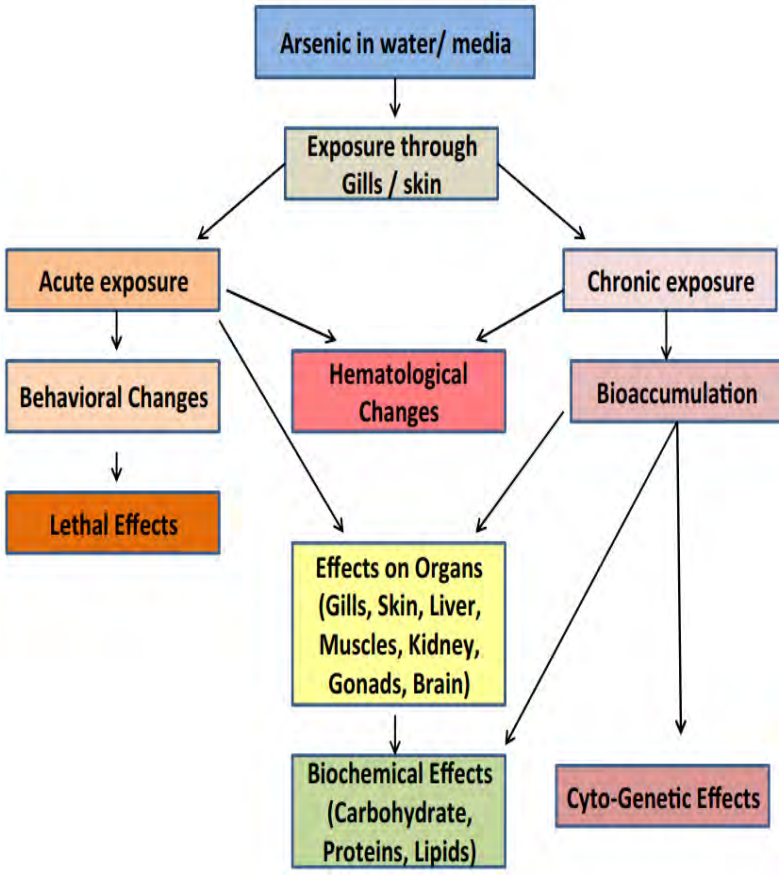


Figure 4: Chronic and acute toxicity of Arsenic in fish (Kumari et al., 2017)

Fish, the top predators in aquatic ecosystems, are the main target of As contamination than other aquatic organisms. Furthermore, low concentrations of AsIII can adversely impact fish (Sun et al., 2020). For instance, Datta et al. (2009) concluded that AsIII concentration at 100 µg/L is toxic to *Clarias batrachus* upon 30- days exposure by causing macrophage

apoptosis and adversely affecting immune system. Whereas as low concentration as 50 µg/L of AsIII affected antioxidant defense system in zebrafish (Sarkar et al., 2017). Sodium arsenite has been reported to cause several toxicity mechanisms in aquatic animals and also when the arsenite contaminated fish is consumed by humans, it causes toxicity in them as it travels along the food chain (Alarcón-Herrera and Gutiérrez, 2022). Sodium arsenite caused genotoxicity in a dose-dependent in adult and embryonic stages of *Danio rerio* within the low range of 0 -500 ppb concentration (Hallauer et al., 2016). Histological toxicity was observed in the gills of zebrafish alongside oxidative stress at environmentally relevant concentrations of sodium arsenite i.e., 0-150 µg/L (Sun et al., 2020). It is well reported that the main mechanism of sodium arsenite induced is oxidative stress as even at low concentrations, AsIII damages zebrafish antioxidant defense mechanisms (Dong et al., 2018). Severe morphological abnormalities were reported in *Labeo rohita* at the sodium arsenite concentrations of 2.5, 15 and 30 mg/L after 14 days of exposure (Rabbane et al., 2022).

Danio rerio

Labeo rohita

Gambusia affinis

Clarias batrachus

Figure 5: The aquatic organisms used as toxicity models to assess effects of $NaAsO_2$

Table 2: Toxicological studies of sodium arsenite conducted on aquatic organisms.

Species	Concentration	Experimental type/ Exposure duration	Studied Parameters	Results	References
Danio rerio (Embryos and Larvae)	100, 200, 300, 400, 500, 600 nM	Acute test (24 to 96 hours post fertilization (hpf)	• Determination of embryotoxicity • Evaluation of behavioral toxicity and malformation. Assessment of antioxidant enzymes. • Analysis of gene expression.	• Morphological abnormalities were observed at 300-600nM in embryos. • Touch response was severely affected at 500-600 nM in larvae, hence affecting behavioral patterns. • Abnormal turning behavior was observed at 400-600nM.	Piyushbhai et al. (2022)
Labeo rohita	2.5, 15, and 30 mg/L	Acute test (14 days)	• Morphological observations • Evaluated histopathology and gene expression.	• Severe morphological abnormalities were reported. • Highest mortality was reported at high conc of 30 mg/L whereas no mortality was found at 2.5 mg/L	Rabbane et al. (2022)
Gambusia affinis	0.75, 7.50 and 75 µg/L	Chronic test (30 days)	• Evaluated morphological and reproductive outcomes. •	• Altered fish morphology. • Adversely affected reproductive system.	Smith et al. (2022)
Danio rerio	0, 10, 50, 100, and 150 µg/L	Chronic test (28 days)	• Measurement of antioxidant response, oxidative damage. • Assessment of histological changes in the gills of zebrafish.	• Histological changes were induced in zebrafish gill. • Oxidative stress was observed. • CAT activity was increased by 46-87% while SOD decreased by 19%.	Sun et al. (2020)

Danio rerio	Embryos and larvae: 0, 50, 100, 300, and 500 µg/L Adult zebrafish: 50,100. 300 ppb	Chronic test (6 months for adults)	• Accumulation of As • Analysis neurological activity • Evaluation of oxidative stress	• No significant accumulation via exposure. • The highest levels were reported in liver, eye and skin. • Induced increased heart rate in dose-dependent manner, reduced locomotion at 100 and 300 ppb. • Oxidative stress was observed.	Hallauer et al. (2016)
Danio rerio	50 µg/L	Chronic test (90 days)	• Biochemical analysis	• Oxidative stress was induced in the brain of zebrafish at the low doses of arsenite affecting antioxidant enzymes activities.	Sarkar et al. (2014)
Clarias batrachus	1 mg/L	Chronic test (60 days)	• Bioaccumulation of sodium arsenite in different organs.	• Excessive deposition in tissue but no mortality. • The bioaccumulations increased with exposure period and were time dependent.	Kumar and Banerjee (2012)
Danio rerio	0, 10, 50, or 500 µg/L	Chronic test (7 and 21 days)	• Histopathology, RNA extraction and cDNA synthesis were evaluated.	• Genotoxicity was reported at low doses of sodium arsenite. • No histopathological changes were observed.	Canivet et al. (2001)

1.9 Key aspects of engineered nanoparticles

The world population stands around 7 billion and is expected to grow exponentially in the near future. Therefore, to meet the needs of the population it is essential to come up with innovative ways and ideas to counter the production costs whilst ensuring quality and a wide range of consumer products (Dunphy Guzmán et al., 2006). Recent decades have witnessed the production of nanoparticles from anthropogenic sources and their applications due to unique and multiple properties of nanotechnology (Lowry et al., 2012). The size of nanoscale materials ranges from 1-100 nm at least in one dimension hence resulting in higher surface area than macromaterials. Nanoparticles (NPs) have different types including metal based engineered nanoparticles for example, titanium oxide, copper oxide, iron oxide and also carbon-based nanotubes (Hristozov and Malsch, 2009). Owing to their novel characteristics (Figure 6) nanoparticles are being engineered into useful applications for a variety of fields such as medical, agriculture, information technology computers, automotive industry etc. (Helland et al., 2007). The diversity of nanosized materials can be put into use for the betterment of environment and counter existing pollution by reducing waste and our reliance on non-renewable resources (Rickerby and Morrison, 2007). For the last few years, the synthesis of nanoparticles is being employed for its strategic use in treating wastewater with carbon nanotubes, fullerenes, nanoploymers and is the subject of extensive research in this regard (Ali et al., 2021). The emergence of nanotechnology is expected to have a significantly positive impact on sustainable development (Sarkar et al., 2022). This innovation of nanoparticles implies the conservation of renewable resources through reduced energy consumption and efficient use of materials for large scale production in the industrial sector (Bour et al., 2015). Nanomaterials can be used for environmental remediation with an approach to propose commercially viable solutions for the treatment of wastewater, contaminated soil, and industrial waste due to widespread use of chemicals (Rafeeq et al., 2022).

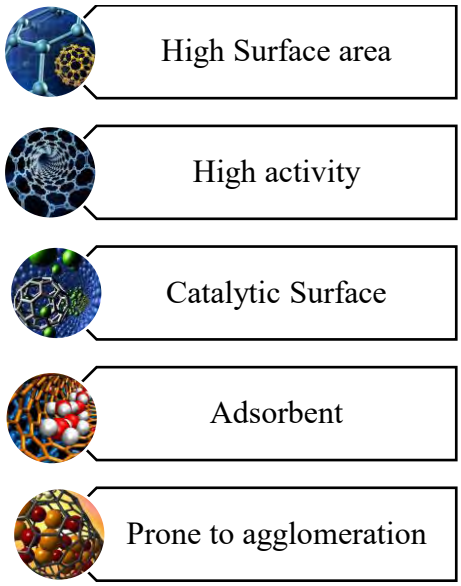


Figure 6: Key characteristics of nanoparticles NPs

Although manufactured nanoparticles have proven to be an economically viable solution to counter over-consumption of non-renewable resources, its potentially harmful impact on the environment cannot be ignored (Patil et al., 2022). NPs contaminate the environment through several pathways including discharge from the source during its synthesis, and after its usage. The functionalization nanomaterials through a specific coating for high activity can determine the fate and toxicity of NPs. It may also enter the environment from sewage plants, incinerators and landfills acting as the potential sources of NPs in soil and waterbodies (Dimkpa et al., 2013). The concern towards the impact of NPs on the environment due to its nanosized properties on plants, microbes, animals, human beings is being studied in detail to have a better understand of the mechanisms involved in its toxicity, for example, the increased production of reactive oxygen species (ROS) (Kiendrebeogo et al., 2022). A study found out that aluminum oxide nanoparticles potentially decreased ascorbate and glutathione proteins in soybean that in return increased oxidative stress by reducing ROS scavenging activity (Mustafa and Komatsu, 2016). In other ways the toxicity mechanisms involve the bioaccumulation and transfer of NPs along the food web because of the direct environmental exposures to NPs mainly due to consumption of contaminated food (Abbas et al., 2020; Tangaa et al., 2016). NPs have been documented to interact with the activity of microbes responsible for the decomposition and

degradation of natural organic matter and toxic pollutants (Ren et al., 2018). These nanoparticles become bioavailable to the microbes due to their dissolution and penetration of toxins through cell membrane of microbes, hence, altering with their chemical activities and inducing oxidative stress in them (Dinesh et al., 2012). According to recent studies, nanoparticles can be taken up by plants upon interaction with either the roots in the soil or parts that are above the ground (Rizwan et al., 2017). It has been reported that NPs interfere with early stages of plant growth and have an adverse effect of seed germination of food crops. For instance, seed germination was decreased by 10-20% in response to silver nanoparticles exposure as compared to the control experiment (El-Temsah and Joner, 2012).

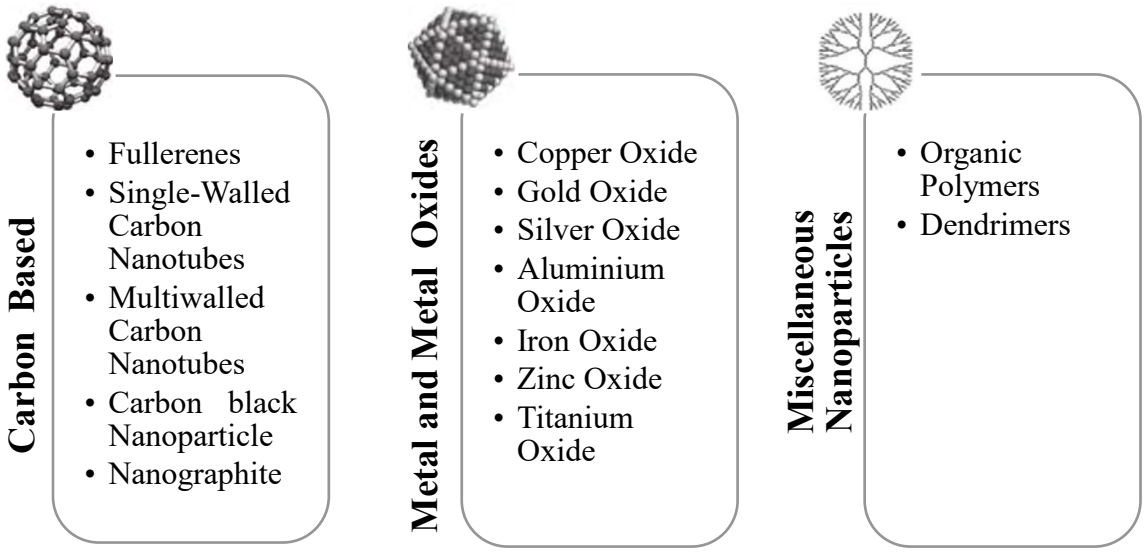


Figure 7: Classification and types of different types of nanoparticles

1.10 Threat of nanotoxicity to the aquatic ecosystem (Nanotoxicology)

The extensive use of nanoparticles across the globe has raised concerns about its potential environmental risks upon entering the aquatic ecosystems. They become part of waterbodies after being released through various point and non-point sources in considerable quantities (Vance et al., 2015). The discharge of wastewater effluents is a point source for nanomaterials mainly from treatment plants and industrial sectors into aquatic ecosystem (Zhang et al., 2019).

In order to comprehend and solve the mysteries behind the complex linkages between nanomaterials and their exposure to living groups on the receiving end, nanotoxicology has been introduced in the last few years as a research field to deal with the modern-day toxicology challenges (Walters et al., 2016). Nanotoxicology employs studies to understand the exposure routes of nanomaterials along the aquatic food chains and trophic transition of nanoparticles taking place in the process affecting living organisms (Zhang et al., 2018). Therefore, there is a heavy reliance found on research studies and findings on prominent ecological influences of nanoparticles on aquatic organisms. Nanotoxicity poses a serious threat to aquatic plants and animals involved in the food chain that links the exposure of NPs to humans as a potential final receptor (Vázquez Núñez and de la Rosa-Álvarez, 2018) (see Fig. 8).

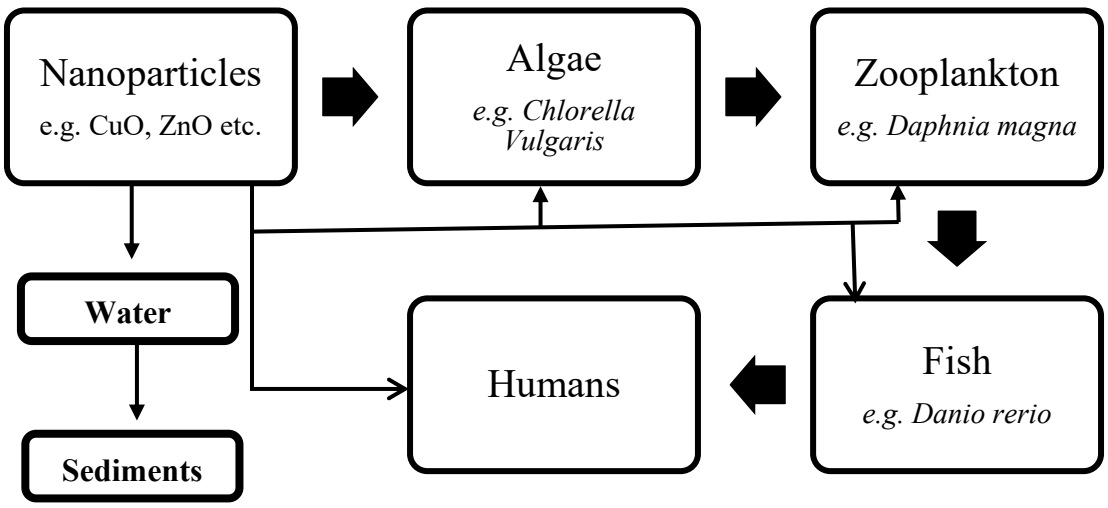


Figure 8: Schematic representation of nanoparticles pathway along the food chain. (This figure is developed from the following studies: Singh et al., 2021; Chen et al., 2012; Asghari et al., 2012; Kim et al., 2011)

Nanoparticles upon their release in the environment mostly end up in aquatic ecosystems, hence, altering and adversely influencing the aquatic balance of the species involved therein (Chae and An, 2017). NPs not only affect fish that is the main aquatic vertebrate ecologically responsible for aquatic functions, but also microbial communities, aquatic plants and phytoplankton are at risk (Casabianca et al., 2021). Titanium dioxide

nanoparticles (TiO_2-NPs) pose a threat to aquatic organisms at higher concentrations, proving that the aquatic systems have high tolerance for the respective metal (Sun et al., 2007). For instance, a study by Kim et al. (2011) showed that *Lemna paucicostata* plants exposed to high concentration of TiO_2-NPs i.e. 250 ppm had an adversely affected development compared to the low concentration of 1 ppm of silver nanoparticles. *Daphnia magna* being at the bottom of the aquatic food chain is an important species to understand the pathway of nanotoxicity across trophic levels (Banerjee and Roychoudhury, 2019). Silver nanoparticles have been reported to cause mortalities in *Daphnia magna* in a dose dependent manner by employing different types of silver nanoparticles to determine the lethal and toxic effect of composition of NPs on the crustacean species (Asghari et al., 2012). Furthermore, it was reported that the iron oxide nanoparticles caused the decrease in chlorophyll in *Chlorella vulgaris,* and this provided reasons for an adverse effect on CO_2 absorption and the net photosynthetic rate (Chen et al., 2012).

1.11 Toxic Effects of nanoparticles on fish

Fish have been the prime choice to examine toxic effects of nanoparticles on aquatic vertebrates in order to broaden the spectrum of ecotoxicology in aquatic ecosystems (Banerjee and Roychoudhury, 2019). Nanoparticles have been reported to induce oxidative stress in fish, accumulation in vital organs and in extreme cases death (Singh et al., 2021). A study on *Danio rerio* concluded that copper oxide nanoparticle caused damage to gills and posed acute lethal toxicity to the fish against environmentally realistic concentration i.e. the LC50 was found to be at 1.5 mg/L for 48h (Griffitt et al., 2007). Silver nanoparticles (Ag-NPs) decreased the efficiency of antioxidant defense mechanism in embryos of the African catfish to the concentration of 25 ng/L and its toxicity kept on fluctuating with higher doses succeeding this manner ultimately causing severe oxidative stress hence setting the basis for Ag-NPs toxicity in the developmental stages of *Clarias gariepinus* (Sayed and Soliman, 2017). Andrade et al. (2019) investigated the effect of temperature on carbon nanotubes (CNTs) toxicity on brown mussels and found the over production of reactive oxygen species and ineffective antioxidant levels in response to CNTs toxicity combined with high temperature whereas no synergistic effects were observed. Titanium dioxide nanoparticles (TiO_2-NPs) affected the growth rate of the goldfish at 10 and 100 mg/L concentrations upon acute exposure by decreasing its body weight. It was found out

that 1.8% of the body weight increased at 10 mg/L compared to the control group which reported 8.1% increase in bodyweight whereas at high concentration of 100 mg/L 19.7% decrease in bodyweight was measured. The same study reported accumulations in gills and intestines of the fish whilst no TiO_2-NPs accumulation was detected on the muscle and brain (Ates et al., 2013). It was observed that polystyrene nanoparticles induced genotoxicity and oxidative stress when combined with copper oxide and zinc oxide nanoparticles (ZnO-NPs). However, genotoxicity reduced upon co-exposure of polystyrene nanoparticles and polycyclic aromatic hydrocarbons (Singh et al., 2021).

1.12 Copper oxide nanoparticles (CuO-NPs)

The present study focuses on the toxicity of copper oxide nanoparticles (CuO-NPs) that are widely known for their use as industrial catalysts and to enhance mechanical strength by mixing with other materials (Siddiqi and Husen, 2020). CuO-NPs are formed from copper and oxygen that are block d and block p elements in periodic table, respectively. It is also known as cupric oxide, a black transition metal oxide that has a monoclinic crystal structure with a bandgap of 1.2-1.9 eV as shown in Figure 9) (Narayan et al., 2018; Singh et al., 2021). It has several properties such as high thermal conductivity, high stability, and photovoltaic abilities. They are extensively used in several applications such as semi-conductors, gas sensors, anti-microbial fluids, and in machines as heat transfer fluids (Carmona et al., 2015). The practice of engineered copper oxide nanoparticles has advanced exponentially in electronics, biomedicines, and environmental remediation due to their unique set of properties (Khatoon et al., 2018). CuO-NPs have effective electrochemical, anti-fungal, and anti-microbial properties that may lead to their use in plastics, coatings, graphite surfaces and textiles etc. (Bondarenko et al., 2013). The usage of CuO-NPs has urged the need of its toxicity assessment to evaluate how it is impacting organisms in different ecosystems after their unchecked release across different compartments of environment. The CuO-NPs have adverse impact on aquatic organisms upon emergence from industrial plants, textile units, health sector, electrical appliances, and automobiles as its potential sources. Like any other nanoparticle, it is essential to learn the pathway of CuO-NPs to determine its exposure routes, fate and consequences in aquatic ecosystem (Hu et al., 2014).

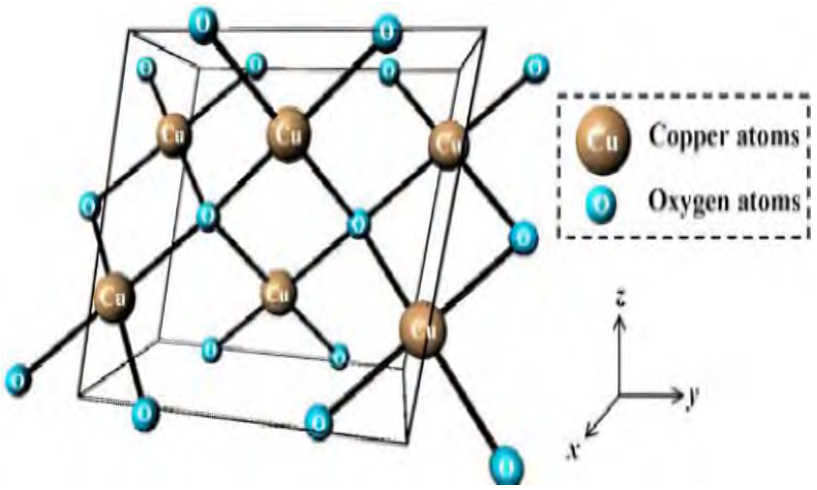


Figure 9: Monoclinic structure of copper oxide (Singh et al., 2021)

1.13 Potential toxicity of CuO-NPs

To establish the possible influence of CuO-NPs size, shape and charge on the mechanisms that determine its toxicity in organisms exposed to them, it is essential to characterize nanoparticles prior to their study. These mechanisms mainly include cellular uptake, interaction with targeted organisms and intracellular stability upon internalization within cells and tissues (Naz et al., 2020). A comparative study showed that small nanoparticles are more toxic than large nanoparticles. Due to high surface area of copper nanoparticles (25 nm) compared to copper microparticle (14-25 µm) were found to adversely disrupt cellular structures by crossing cell membranes and translocation between cells in rats (Lee et al., 2016). Humans are extremely prone to CuO-NPs due to their use in pharmaceutical industry and are exposed to it through skin and inhalation. Therefore, a study was conducted to examine the cytotoxic effect of CuO-NPs on human blood lymphocytes and concluded that cell viability decreased in a dose-dependent manner with inhibition concentration of 382 µM. Also, CuO-NPs effectively caused oxidative stress in the lymphocytes (Assadian et al., 2017).

Furthermore, copper oxide nanoparticles cause oxidative stress, DNA damage, inflammation of organs, affects growth performance, and in extreme case cell death that might lead to lethality in aquatic organisms. Genotoxicity, cytotoxicity, immunotoxicity and teratogenicity are one of the many adverse effects of copper oxide nanoparticles on

different life stages of organisms in different ecosystems (Ganesan et al., 2015; Li et al., 2016). These toxic effects are heavily influenced and determined by the concentration of nanoparticles and the duration depending on acute and chronic exposures of NPs in aquatic systems (Naz et al., 2020). A study concluded that the aquatic species *Ceriodaphnia silvestrii* and *Hyphessobrycon eques* were sensitive to CuO nanoparticles showing production of ROS in both organisms. The 48-h EC_{50} for *Ceriodaphnia silvestrii* was found to be at 12.6 µg/L and for *Hyphessobrycon eques* the 96 h LC_{50} was observed to be at 211.4µg/L of CuO nanoparticles (Mansano et al., 2018). In another study, the growth of *Cyprinus carpio* fish was inhibited by CuO nanoparticles at the concentration of 100 mg/L upon chronic exposure of 30 days (Zhao et al., 2011). CuO-NPs induced oxidative stress in liver and gills of *Carassius auratus* post 21-day exposure at the concentration of 1 and 10 mg/L (Ates et al., 2014). In a comparative study, copper oxide, zinc oxide and nickel oxide nanoparticles (NiO-NPs) were investigated against their toxic effects on *Danio rerio,* and it concluded CuO-NPs to be more lethal than other metal oxide nanoparticles in the study. The LC50 at 96h was 2.25 mg/L for CuO-NPs whereas for ZnO-NPs and NiO-NPs were 48.2 mg/L and 175 mg/L, respectively (Hou et al., 2018). CuO nanoparticles with diameter in the range of 20-95 nm caused 60% mortality of zebrafish embryos at the concentration of 50 ppm while at 5ppm the mortalities increased by 20% and 40% at 96hpf and 120 hpf, respectively (Xu et al., 2017). In another study, CuO-NPs delayed hatching in zebrafish embryos by inducing oxidative stress and suppressing embryonic motility (Zhang et al., 2018).

Table 3: Toxicological studies of CuO-NPs conducted and its effects on aquatic organisms.

Species	Concentrations	Experimental Type/ Exposure duration	Studied Parameters	Results	References
Danio rerio	0, 50, 100, and 200 µg/L	Acute test (24h)	• Assessment of metabolic rate, specific ammonia excretion, and swimming ability.	• Fish metabolism was adversely affected. • Decrease in specific ammonia excretion was observed by 34% and 83% and in swimming capacity by 34% and 55% at concentrations of 100 and 200 µg/L, respectively.	de Oliveira Eiras et al. (2022)
Danio rerio (Embryos)	1, 5, and 10 mg/L	Acute test (96hpf)	• Mortality, hatching, heartbeat, and malformation	• CuO-NPs toxicity increased with decrease in ionic strength of exposure medium.	Chao et al. (2021)
Danio rerio	1 and 3 mg/L	Chronic test (30 days)	• Total protein content, functional markers, mRNA expression levels, histology and immunochemistry, oxidative stress, and AChE activity.	• Muscle toxicity, oxidative stress, SOD and CAT levels decreased as the concentration increased.	Mani et al. (2019)
Danio rerio (Embryos and Larvae)	CuO-NPs (50, 25, 12.5, 6.25, or 1 mg/L	Acute test (4 hpf)	• Measurement of SOD activity. • Examination of histopathology	• The embryos were not affected at low conc. But at 12.5 mg/L or higher doses led to phenotypic malformations. • Locomotor ability was affected.	Li et al. (2016)

			• Immunohistochemistry and behavioral analysis.	• Analysis showed underdeveloped liver and delayed retinal growth. • SOD activity was reduced hence affecting antioxidant defense system.	
Danio rerio (Embryos)	10 mg/L	Acute test (120 hpf)	• Survival, hatching rates and malformation.	• Exposure to CuO- NPs or bulk CuO up to 10 mg Cu/L did not produce any significant decrease on embryo survival. An LC50 value of 3.083 mg/L was calculated for ionic copper which significantly increased 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- NPs at 10 mg/L.	Vicario-Parés et al. (2014)
Cyprinus carpio	100 mg/L	Chronic test (30 days)	• Cholinesterase activity assay Accumulation, tissue distribution and excretion of nanoparticles.	• Accumulation was found in intestines, gills, liver, brain, muscle and scale. Neurotoxicity as cholinesterase activity was inhibited.	Zhao et al. (2011)
Danio rerio	0.25, 1.5 mg/L	Acute test (48h)	• Biochemical, and histology analysis.	• Acute toxic effects in the fish were observed that included histopathology was induced primarily in the gills.	Griffitt et al. (2007)

1.14 Interactive Toxicity of Arsenic and Nanoparticles with Other Pollutants

Because of increasing population and rapid industrialization, several chemicals are released into the environment in different temporal and spatial settings (Tazitdinova et al., 2018). These chemicals interact with each other in different environmental compartments and collectively pose a threat to the relevant biota in ecosystems (Granero and Domingo, 2002). This cocktail of contaminants puts the lives of the exposed organisms at risk and threatens their life systems (Tongesayi et al., 2013). However, there is a lack of sufficient information on this matter to better understand and evaluate the composition of these environmental mixtures and interaction with each other. Previously, it has been reported that co-exposure of copper ions and TiO_2 nanoparticles enhances the uptake and accumulation of copper in *Daphnia magna* (Liu et al., 2015). Hou et al. (2018) investigated the joint toxicity of copper oxide, zinc oxide, and nickel oxide nanoparticles in zebrafish through microarray analysis and comet assay. It was found out that CuO-NPs were more toxic than zinc oxide nanoparticles and nickel oxide nanoparticles with increased DNA damage and oxidative stress. Furthermore, copper oxide nanoparticles were used to evaluate its interactive toxicity with zinc oxide nanoparticles and titanium dioxide nanoparticles in developing zebrafish embryos upon exposure for 120 hours. It was found out that that CuO-NPs were the most toxic nanoparticles by delaying hatching and increasing malformations (Vicario-Parés et al., 2014). Freitas et al. (2018) reported neurotoxicity in clams upon co-exposure of carbon nanoparticles and arsenic. The combined exposure of aluminum and zinc oxide nanoparticles had synergistic toxic effect on *Carassius auratus* (Benavides et al., 2016). Another study reported the synergetic role of TiO_2 nanoparticles in the accumulation of arsenate in *Cyprimus carpio* (Sun et al., 2006).

A study on combined exposure of copper and arsenite was concluded to be synergistic in brain tissues of *Gallus gallus* as it induced oxidative stress and autophagy (Sun et al., 2018). Similar results were reported by Liu et al. (2020) that copper elevated the toxicity of arsenic upon co-exposure by causing oxidative stress and autophagy in chicken brain. Whereas Wang et al. (2021) upon examining the combined toxicity of arsenite and Zinc (Zn) concluded that Zn effectively reduces the accumulation of arsenite and alleviated oxidative stress by improving antioxidant defense in common carp. On the contrary, nanoplastics, an emerging pollutant, promoted the adverse effects of arsenic by

elevating its accumulation in zebrafish brain hence increasing oxidative stress and causing histological alterations (Zhang et al., 2023).

1.15 Zebrafish (*Danio rerio*): A Toxicological Model Organism

1.15.1 Characteristics of Zebrafish

Zebrafish (*Danio rerio*) is considered as a model organism in research. The size of an adult zebrafish is typically found between 3 and 5 cm. It has a streamlined body form with dorsal, anal, and caudal fin. The color of the body can vary such as wild-type zebrafish having blue-gray bodies with black stripes. Figure 7 shows the detailed anatomy and morphology of zebrafish (Gupta and Mullins, 2010; Liu et al., 2016).

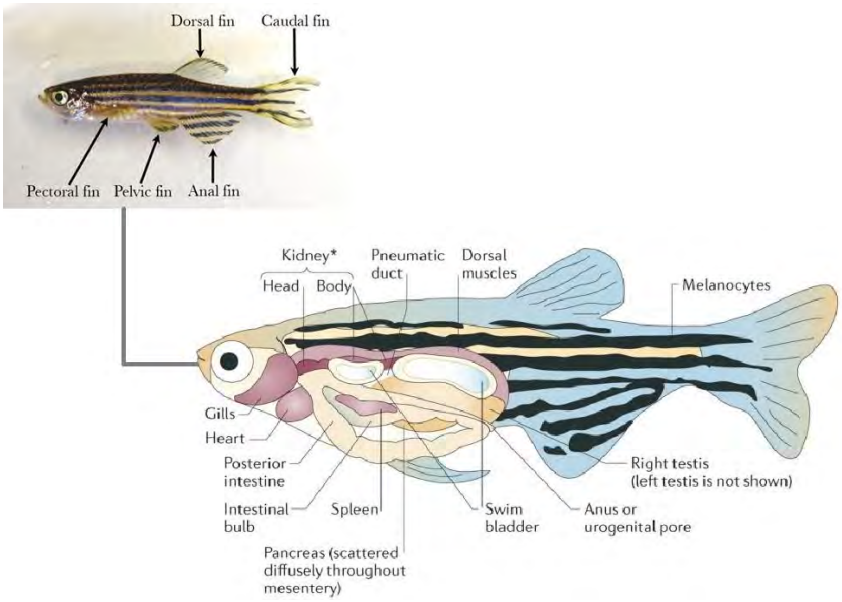


Figure 10: Morphology and anatomy of zebrafish

The average life span for zebrafish is 2-3 years, although under ideal conditions they can live up to 5 years. Being oviparous, zebrafish reproduce by laying eggs. The female releases the eggs, and the male fertilizes them externally, a process known as external fertilization. (Parichy et al., 2009). Adult zebrafish show a range of behaviors, such as feeding, swimming, interacting with their environment, and mating. They are nocturnal and exhibit shoaling behavior, preferring to be in groups. The fins, heart, and central nervous system of zebrafish are proficient in regeneration. They serve as an excellent model for studying tissue regeneration and repair since they are capable of regenerating

lost tissue and organs (Poss, 2002). Taxonomic classification of Zebrafish is provided in Table 3.

Table 4: Scientific taxonomy of zebrafish (*Danio rerio*)

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

1.15.2 Zebrafish as an Ideal Toxicity Indicator

Zebrafish (*D. rerio*) is a tropical freshwater vertebrate animal popularly used as a toxicity model in ecotoxicology (He et al., 2014). Its use is mainly employed to understand the toxic mechanisms induced by contaminants upon exposure in humans and animals leading to certain diseases. Genomic studies confirm high genetic homology to humans hence sharing a common set of genes i.e. around 75% similarity (Catchen et al., 2011; Woods, 2005). The fish has certain unique characteristics that include its small size (3-4cm), high sociability, short reproductive cycle, transparent embryo, and rapid embryo development (Zakaria et al., 2022).

Zebrafish can be used to monitor and evaluate environmental pollution due to heavy metals, organic pollutants, nanoparticles etc. (Dai et al., 2013). Another advantage that is notable in this regard is its cost-effectiveness and also, it's readily available in the market. Therefore, the zebrafish is becoming an effective toxicity modal in environmental toxicology, genetics, pharmacology and developmental studies to evaluate the effect of drugs and pollutants (Ray et al., 2017; Sarkar et al., 2014).

Figure 11: Toxicity endpoints of zebrafish (Bhagat et al., 2020)

Biomarkers and endpoints in zebrafish for heavy metals include Acetylcholinesterase activity, oxidative stress, SOD and CAT activity, bioaccumulation in vital organs and genotoxicity (Senger et al., 2010) (Fig.10). It was reported that copper and cadmium were highly toxic to zebrafish embryos by inhibiting hatching rate and morphological malformations (Santos et al., 2021). It was found out that fullerenes caused malformations, edema and mortality in embryos at the concentration of 200 µg/L (Usenko et al., 2007). Polystyrene nanoparticles caused accumulation in the intestine of adult zebrafish after 21 days of exposure (Gu et al., 2020). The combined toxicity of microplastics and cadmium showed that microplastics reduced the lethal effects of cadmium on zebrafish embryos whereas synergistic effects were observed on the development (Zhang et al., 2020).

1.15.4 Distribution of Zebrafish in Pakistan

Zebrafish (*Danio rerio*) is a freshwater fish that is native to South Asia and is a tropical and subtropical fish. Its location ranges in Pakistan, Bangladesh, Nepal, Bangladesh, and Bhutan. It can be found in a variety of freshwater bodies in Pakistan, including canals, streams, ponds, ditches, and rice paddies. Zebrafish are found in Mehran province. This region includes 50% of Kashmir and Pakistan. Also, Peshawar's hills, valleys, and Indus Plain are included in this region. The climate in this area can range from marine to subtropical, with chilly winters and hot summers with moderate temperatures (Mirza et al., 1994).

1.16 Toxicity biomarkers

Toxicity biomarkers are biological parameters of organisms that can be measured in response to exposure or the impacts of environmental pollutants. They indicate the level of stress and toxicity in the affected organisms upon exposure to environmental pollutants (Azize et al., 2017). The following toxicity biomarkers have been determined in this study to assess toxicity in zebrafish:

1.16.1 Oxidative stress

It is of great importance to assess aquatic toxicology by analyzing oxidative stress in a species that is exposed to various pollutants. Naturally, there is a balance between reactive oxygen species (ROS) and the antioxidant systems to protect the cells from ROS (Slaninova et al., 2009). ROS are generated by various mechanisms as a response to a stress hence disrupting the biochemical balance (Figure. 12 and 13) (Davies et al., 1994). Numerous pollutants such as pesticides, heavy metals etc. can induce oxidative stress through biochemical mechanisms that may include impairment of membrane-bound electron transport and inactivation of antioxidants. The resultant action of ROS production is lipid peroxidation that is determined by measuring Malondialdehyde (MDA) which is mainly a product of lipid peroxidation consisting of polysaturated fatty acids (Marnett, 1999). The combined study of arsenic and nanoplastics on zebrafish was found to significantly increase the MDA content at environmentally relevant concentrations of 200µg/L and 1mg/L, respectively. Furthermore, ROS levels also increased significantly in the same study at same concentrations (Zhang et al., 2023).

1.16.2 Antioxidant defense systems

When a body of an organism produces ROS in response to a xenobiotic, it reacts by lining up a defense system to ROS, low-molecular weight compounds that are involved in scavenging and eliminating ROS out of the living system (Chowdhury and Saikia, 2022).

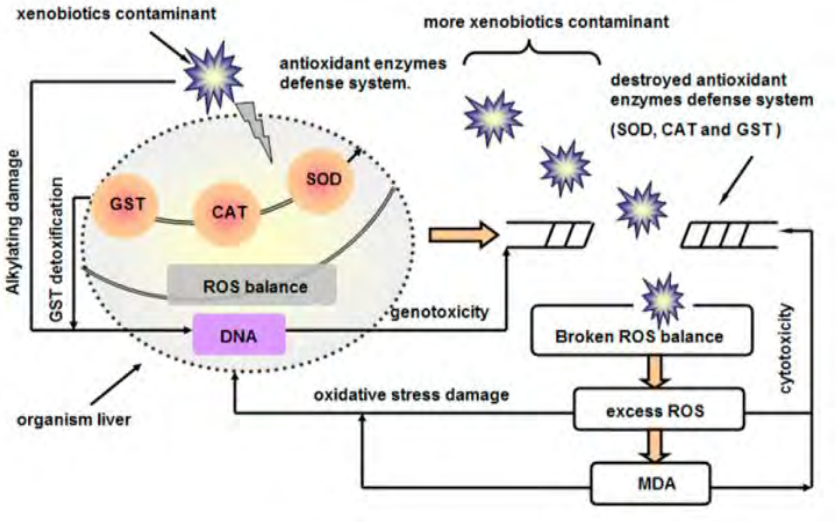


Figure 12: Schematic diagram of relationship between SOD, CAT, GST activities, ROS level, MDA content, and DNA damage in organs of zebrafish. This figure is obtained from the study by Ge et al. (2015).

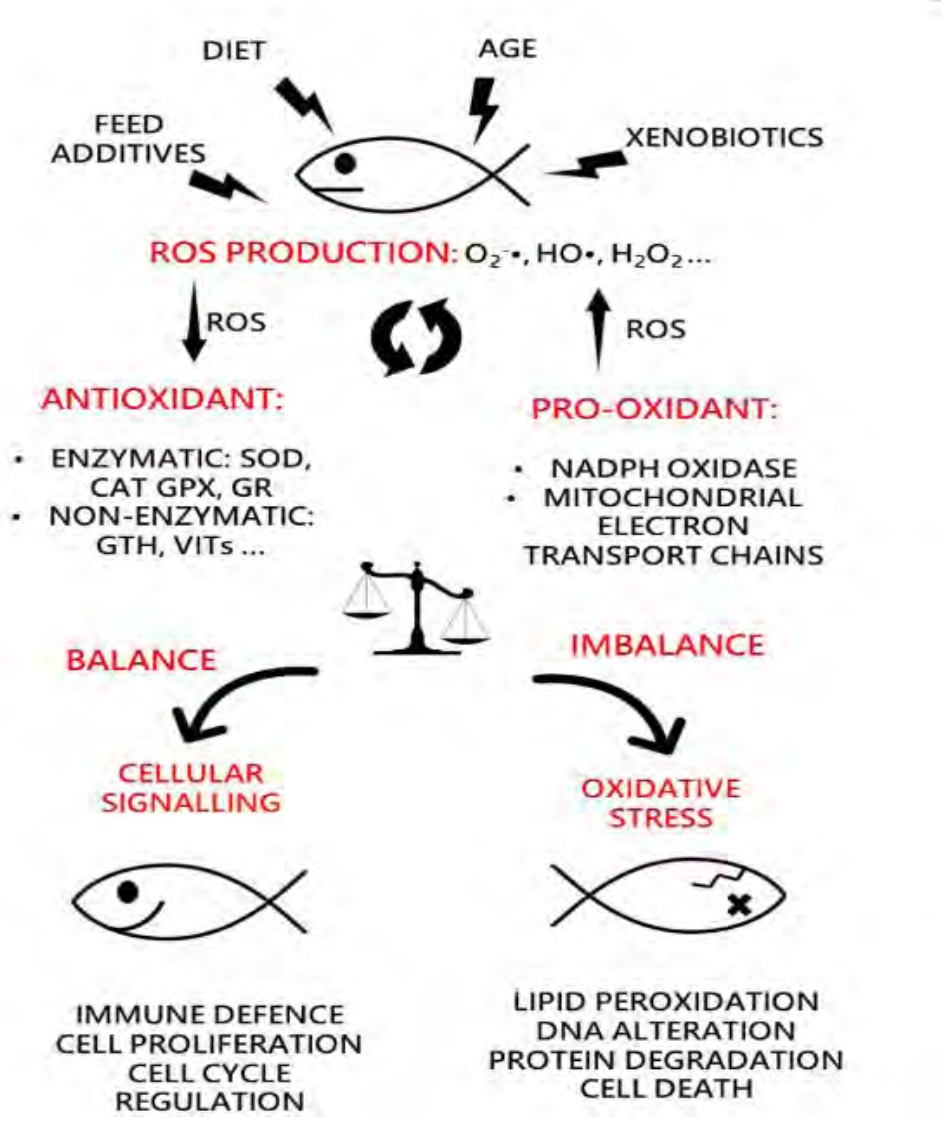


Figure 13: Antioxidant defense system in aquatic organisms (Hoseinifar et al., 2020)

The most important enzymes responsible for the detoxification of ROS are primarily superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidases (GPXs) (Slaninova et al., 2009). SOD is involved in altering O_2 free radical into a less toxic form and eliminating superoxide that further facilitates the dismutation of two molecules of O_2^{-} into oxygen and hydrogen peroxide (H_2O_2). Without SOD, these free radicals aid the formation of hydroxyl radicals. However, with the production of CAT, SOD creates H_2O_2 that eventually results in water and molecular oxygen. H_2O_2 is eliminated by CAT and GPx and with O_2 increasing, the activity of CAT decreases (Hoseinifar et al., 2020). In a study by Purushothaman et al. 2014, it was reported that acute exposure to Titanium dioxide

nanoparticles at 5 mg/L concentration for 24 and 48hr increased reactive oxygen species and lipid peroxidation products in zebrafish gill tissues. Therefore, causing tissue damage as the low levels of SOD and CAT were unable to defend the system against ROS production.

1.16.3 Acetylcholinesterase inhibition

Acetylcholinesterase (AChE) is a neurological enzyme present in both vertebrates and invertebrates. It is mostly found at neuromuscular junctions and is also reportedly present in erythrocytes, liver, and muscles (Nayak and Patnaik, 2021). It is involved in the functions related to the central nervous systems and is essential in ceasing the neurotransmitter's function by catalyzing hydrolysis of acetylcholine. AChE activity and structure are reportedly different in different aquatic organisms such as crustaceans, mollusks, and fish (Bertrand et al., 2000).

Therefore, it can be used as an important biochemical biomarker in the brain for toxicological studies alongside antioxidant enzymes to evaluate neurotoxicity (Sharma et al., 2017). Numerous pollutants such as pesticides, herbicides, heavy metals, industrial chemicals, textile effluents etc. can inhibit AChE activity and alter its mode of action by disrupting neurotransmission hence affecting behavior and survival of the organism (Thi Tu et al., 2009). AChE activity is preferably examined in brain and muscles of the organism under test as it is a cholinergic agent and is responsible for muscle behavior and function (Stefânia Konrad Richetti et al., 2011).

1.16.4 DNA damage

Pollutants have the potential to alter biological processes of exposed population in aquatic ecosystems. Therefore, it is essential to examine genotoxicity of the contaminants on the targeted organisms. Evaluation of the genotoxic effects of environmental pollutants on the fish can be done systematically studying DNA damage (Pei and Strauss, 2013). In this regard, comet assay is a single cell gel electrophoresis that has a strong dose-response pattern even at relatively low concentrations. The assay has a shorter duration and is cost effective. It efficiently evaluates the acute and chronic effects of genotoxin on exposed aquatic organisms (Du et al., 2012).

1.16.5 Histopathological alterations

As a biomarker histopathology studies, the effect of chemical stressors existing in the environment on the muscles of the fish to examine the possible damages caused to the organisms upon chronic or acute exposure It is frequently employed to observe the health status of the exposed fish. (Brandts et al., 2022). Histopathological findings may include cellular alterations such as necrosis, atrophy, apoptosis, inflammatory response, fibrosis etc. (Azize et al., 2017; Menke et al., 2011).

1.17 Research Gap

Numerous research studies have reported singular toxicological effects of sodium arsenite and CuO nanoplastics on zebrafish as a toxicity modal organism. However, there is a dearth of literature on the combined exposure of the above-mentioned pollutants sodium arsenite and copper oxide nanoparticles. Sodium arsenite has been reported to have caused combined toxicity with other pollutants such as heavy metals and organic pollutants upon exposure to living organisms. On the other hand, Copper oxide nanoparticles have a high surface area to absorb such pollutants therefore it is essential to understand its interactive potential towards sodium arsenite. Several research studies have reported the emergence of sodium arsenite and CuO nanoparticles in the aquatic environment hence it is important to examine their combined exposure on aquatic organisms, but little is known about their ecological toxicity. It is essentially important to understand the interactive toxic effects of CuO-NPs and sodium arsenite on aquatic organisms due to the current lack of information in this regard. The present literature is not sufficient to bridge the gap concerning the interaction of sodium arsenite and copper oxide nanoparticles in order to determine the extent of their adversity once combined in different environmental matrices. Therefore, research should be carried out concerning joint toxicity of sodium arsenite and CuO-NPs to solve the mystery of the mechanisms upon interaction with each other in the environment and upon exposure to affected organisms.

1.18 Problem Statement

Presently, Arsenic (As) is considered a highly toxic element that is released into the aquatic environment through anthropogenic sources after its extensive use in agriculture as

pesticides, burning of fossil fuels, and mining activities. In Pakistan, As contamination is reported in groundwater that is used for drinking purposes, hence putting the lives of people at risk. Its trivalent form (arsenite) is observed to be more toxic than its pentavalent form (arsenate). However, sodium arsenite is an inorganic herbicide that emerges into the environment after its use mostly through agricultural runoff. CuO nanoparticles is an emerging pollutant that can increase the toxicity of other harmful pollutants such as arsenic once coming into contact with them. It ends up interfering with the well-being of the fish and inhibiting their growth upon entering the aquatic ecosystem. Several studies have reported combined toxicity of various chemical mixtures to understand and evaluate the effects of these mixtures in aquatic ecosystems. Zebrafish is an emerging toxicity modal mainly used to understand the toxic effects of chemical stressors in the environment not only on aquatic life but also humans. As both zebrafish and humans share the same set of genes, it is used to carry out toxicological studies. Therefore, the toxicity studies on zebrafish can guide our way in understanding the complex relationship of chemicals and their effects on humans and other aquatic life.

1.19 Objectives

The aim of the present study was to understand the interactive toxicological impact of sodium arsenite and copper oxide nanoparticles on adult zebrafish through chronic toxicity tests. The objectives of this study include:

- To evaluate the oxidative stress by measuring total protein levels, reactive oxygen species (ROS) and malondialdehyde (MDA) levels and activity of antioxidant enzymes superoxide dismutase (SOD) and catalase (CAT) in the gills of zebrafish.
- To examine the combined toxicity of $NaAsO_2$ and CuO-NPs on the activity of acetylcholinesterase (AChE) in brain.
- To analyze the impact of combined toxicity on DNA damage in liver tissue of zebrafish through comet assay.
- To examine muscle damage by histopathological alterations in muscle tissue of zebrafish.

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. Sodium Arsenite [CAS;7784-46-5; Purity 99%] was purchased from Fluka Chemika. Dimethyl Sulfoxide (DMSO) 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 purchased from 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 solvents used were of analytical grade (AG).

2.2 Characterization of Copper Oxide Nanoparticles

2.2.1 SEM/EDX

The characterization of particle size and morphology was done by Scanning Electron Microscopy (SEM) (EV018 Carl Zeiss, Germany). The suspension of nanoparticles of 10 mg/L was coated on a glass slide and sputtered with gold after drying. The sample was then observed under a high magnification scanning electron microscope prior to EDX analysis.

2.2.2 Fourier transform infrared spectroscopy (FTIR)

The functional groups of CuO-NPs were analyzed using Fourier Transform Infra-Red (FTIR) spectroscopy (FT/IR-6600typeA Spectrometer, Jasco). The mixture of dry nanoparticle powder and Potassium bromide (KBr) was pressed under 5000–10,000 psi in the dye in order to create a transparent pellet. The transmittance spectrum was attained and plotted within the wavenumber range of 4000 cm^{-1} to 400 cm^{-1} using OriginPro 8.5.

2.2.3 X-ray diffraction (XRD)

The crystalline structure of nanoparticles was characterized by 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 Joint Committee on Powder Diffraction Standards (JCPDS) database by using X'Pert HighScore software to confirm its crystalline structure.

2.3 Preparation of stock solution

The stock solutions of Copper Oxide Nanoparticles and Sodium Arsenite were prepared in distilled water.

2.3.1 Sodium arsenite

To achieve the final concentration of 300µg/L, the 100ppm stock solution of sodium arsenite was prepared in distilled water. Finally, 45 ml was added to the tanks with 15-Lwater to attain the final concentration of the exposure medium. The solution of the required concentration was prepared through the following formula:

$C_1V_1=C_2V_2$

2.3.2 Copper oxide nanoparticles

The stock suspension was made by adding 50mg of CuO-NPs (40 nm) in 1000 ml of distilled water to attain the final concentration of 50ppm. Next the suspension was sonicated with an ultrasonicator at 100W 40 KiloHertz for 60 minutes to disperse the nanoparticles before exposing the zebrafish. 300ml of this solution was added to the aquaria for the final concentration of 1mg/L. The solution of the required concentrations was prepared through the following formula.

$C_1V_1=C_2V_2$

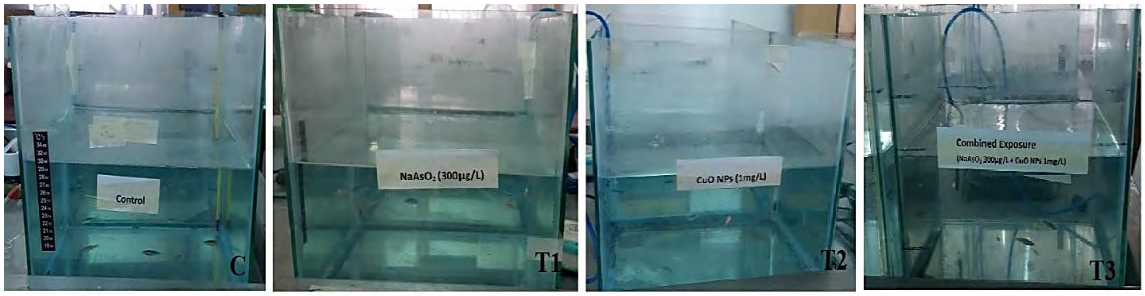
2.4 Experimental model animals

Adult zebrafish (AB wild-type strain, 6 months old) were maintained at Environmental Toxicology and Chemical Stress Ecology Lab, Quaid-i-Azam University, Islamabad. The fish were purchased from a local vendor in Rawalpindi. The fish were acclimatized for 14 days in dechlorinated water. Each aquarium contained 50 individuals with aeration for dissolved oxygen maintained at 7 ± 1 mg/L. The photoperiod of 14:10 h light and dark was maintained at 27± 1 C° temperature, and pH within the range of 7.4-8.1. Optimum Betta was used to feed the fish daily at 5% body weight. The fish were not placed in the aquaria on the basis of gender but randomly.

2.5 Experimental design

For the experiment, 250 healthy fish were selected for the experiment of length (3-4 cm) and weight (350mg ±20) and placed in 20L tanks containing 15L of exposure medium. Three treatment groups were assigned in total (T1, T2 and T3). One of the tanks was kept as control group (C) while all the treatment groups were run in replicates. To maintain dissolved oxygen aerators were put in the tanks. The experiment was carried out in dechlorinated water whereas water quality parameters were checked and recorded on a daily basis.

The experiment examined the sub-lethal toxicity effects of single and co-exposure of $NaAsO_2$ and CuO-NPs on the zebrafish. Treatment 1 (T1) was $NaAsO_2$ with the concentration of 300µg/L, Treatment 2 (T2) was assigned to CuO-NPs group exposed to 1mg/L of CuO-NPs and Treatment 3 (T3) was the co-exposure group of $NaAsO_2$ and CuO-NPs with the concentration of 300µg/L and 1mg/L, respectively. The fish were exposed for 28 days while the samples were taken on 7, 14, 21, and 28 days of exposure. The water in the treatment tanks was changed every alternate day to ensure good quality of the water and maintain concentrations of the contaminants. To evaluate the sublethal effects, the test concentration for sodium arsenite of 300µg/L was based on a study by Hallauer et al., 2016 that explored the toxic effects of chronic exposure of arsenic in zebrafish at the same concentration. Whereas for CuO-NPs Mani et al., 2019 reported sub-lethal effects at the same concentration used in the current study i.e. 1mg/L in adult zebrafish. The criteria for the test concentrations used in singular and joint exposure of sodium arsenite and CuO-NPs in this study was based on environmental concentrations (Ali et al., 2019; Shahid et al., 2017; Sultana et al., 2013) and the results of the previous studies (Zhang et al., 2023; Sun et al., 2020). The entire experimental trial was subject to approval by Bioethics Committee, Quaid-i-Azam University.



Control Treatment (CT)	**Treatment 1 (T1)** (**(**$NaAsO_2$ 300 µg/L)	**Treatment 2 (T2)** (CuO-NPs 1mg/L)	**Treatment 3 (T3)** (**(**$NaAsO_2$ 300 µg/L + CuO-NPs 1mg/L)

Figure 14: Treatment plan used in the experimental setup during the 28-day exposure.

At each sampling interval, four fish were taken from each treatment group and organs were preserved as one sample. Prior to the dissection of the fish, they were euthanized in ice-cold water (Figure11). The organs that were obtained upon dissection included brains, livers, and gills (Figure12) on ice plate and further on transferred to eppendorfs with Phosphate Buffer Saline with pH 7.4 and immediately frozen at -80ºC. The muscles were cut and preserved in Neutral Buffer Formalin (NBF) at 4 ºC.

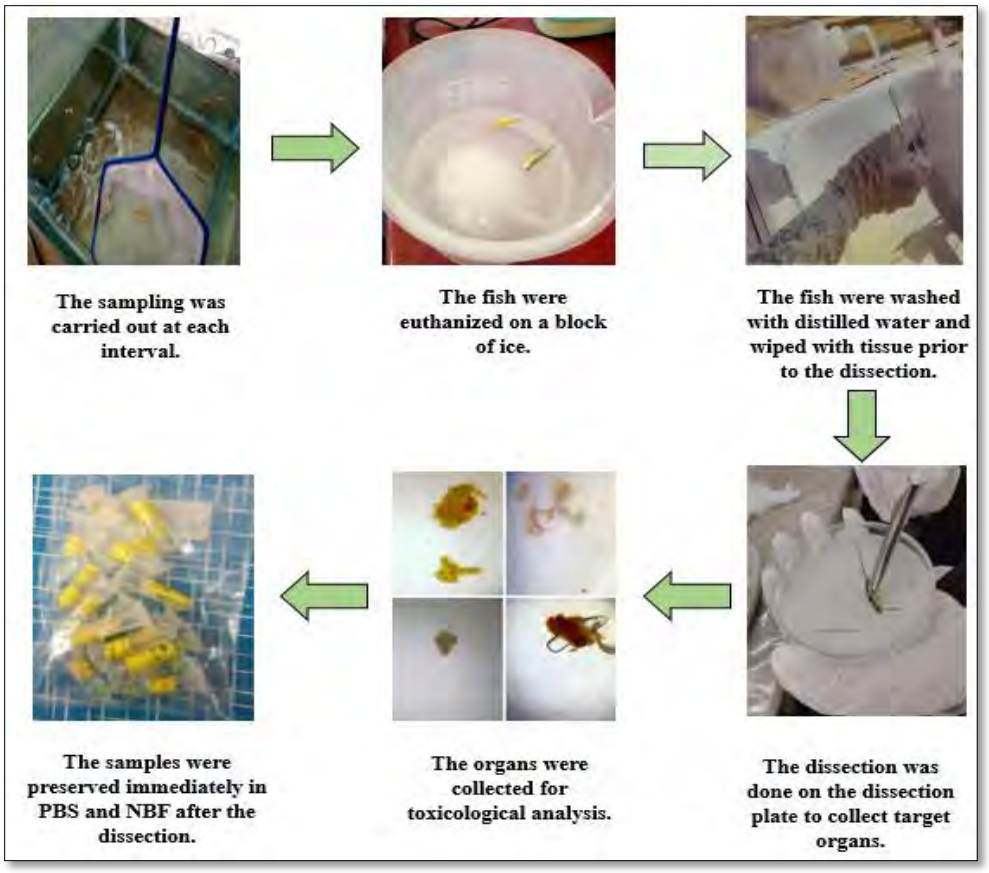


Figure 15: Schematic representation for the dissection of the fish at each sampling interval throughout the experiment.

Figure 16: Dissection process of zebrafish at each sampling interval during the experiment.

2.6 Phosphate buffer saline (PBS) preparation

Phosphate buffer saline solution (PBS) was prepared from PBS tablets (CAT NO. 2810305) which was purchased from Bio World. The tablets were dissolved in 1000 mL distilled water to make 1 Liter of PBS (1 tablet/100mL).

2.7 Neutral buffer formalin (NBF) preparation

The preferred fixative for histopathology was 10% natural buffered formalin (NBF). The NBF contained 37-40% formaldehyde in an aqueous solution. It is an un-buffered solution and 10% from this solution was mixed with 90% distilled water to create a histological fixative. The pH of formalin was maintained at 3-4 and the following chemicals were used to prepare the formalin solution:

- 100 ml formalin (37-40% stock solution)
- 900ml distilled water
- 4g Monobasic Salt (NaH_2PO_4)
- 6g Dibasic Anhydrous Salt (Na_2HPO_4)

These salts were mixed and stirred for ten minutes using a magnetic stirrer and the buffer formalin solution was stored in the refrigerator for usage.

2.8 Homogenate preparation

The tissues of brain, gills, and liver were homogenized using a Bertin Technologies Precellys Evolution Homogenizer on individual basis in 50mM potassium phosphate buffer with pH 7.0 in 2ml eppendorfs. The homogenizer was centrifuged at 10,000 rpm for 10 minutes. The supernatant was removed and transferred to other eppendorfs for additional examination.

Figure 17: The Homogenizer and temperature-controlled centrifuge used for homogenization of the samples.

2.9 Estimation of total protein by Bradford Method

The total protein content of zebrafish was assessed using Bovine Serum Albumin (BSA) as standard and through the Sigma Bradford Method. The Bradford was purchased from Sigma Aldrich. Five dilutions of BSA (10, 20, 40, 60, 80 and 100µg/L) were prepared in the reagent. The absorbance of BSA was measured at 595nm on UV-Vis Spectrophotometer and standard curve was generated. The level of protein was determined by using BSA curve. The following equation was used to calculate the total protein content:

$$Y=0.008X + 0.0097$$

$$X= Y - 0.0097/0.008$$

Where, Y stands for absorbance of the samples and X is the total protein estimation.

2.10 Measurement of reactive oxygen species (ROS)

The methodology employed for evaluating ROS in gills tissue of zebrafish was based on the protocol described by Hayashi et al. (2007). To prepare 0.1 molar sodium acetate buffer, 4.1g of sodium acetate was added and dissolved in 500ml of distilled water. The pH was maintained at 4.8 by adding 10 mg of N-Diethyl-p-phenylenediamine sulphate salt (DEPPD) in 100 ml of sodium acetate buffer and 50mg of Ferrous Sulphate ($FeSO_4$) was

added in 10 ml of sodium acetate buffer. The solutions were then mixed in a 1:25 ratio and incubated in the dark for 20 minutes at room temperature. The reaction mixture in the cuvette contained the following:

- 20 µL of solution mixture
- 1.2 mL of sodium acetate buffer
- 20 µL of enzyme supernatant

The absorbance was measured at 505nm using UV-Vis Spectroscopy. Each sample received three readings every 15 seconds and calculated by using the following formula:

$$\textbf{ROS} = \frac{(\boldsymbol{Sample\ OD \times Cuvette\ Vol. \times 10\text{^}6})}{\boldsymbol{Protein \times Sample\ Vol. \times \Delta Time \times Constant\ 15600}}$$

Units = nanomoles/mg protein

Where:

- Sample OD = Sample Absorbance
- Cuvette Volume = Volume of Cuvette
- Protein = Total Protein in Sample (mg/mL)
- Sample Volume = Volume of Enzyme Supernatant
- Time = Measurement Time
- 15600 =Extinction Co-efficient (M^{-1} cm^{-1})

2.11 Measurement of Malondialdehyde (MDA) Content

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

Reaction mixture contained:

- 200µL of enzyme supernatant
- 200µL of 8.1% Sodium Dodecyl 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 water bath for one hour at 90°C. After that, 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.

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

Units = nanomoles/mg protein

Where:

- ΔSample = Change in Sample Absorbance
- ΔBlank = Change in Blank Sample Absorbance
- Cuvette Volume = 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})

2.12 Estimation of 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 contained:

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

The resulting mixture was irradiated with 4000 1x fluorescent lamp for 20 minutes, 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 (NBT) photoreduction rate. The results obtained were expressed as U/mg of protein.

$$\textbf{SOD} = \frac{((\Delta\text{Sample} - \Delta\text{Blank})\times \textit{Cuvette Vol.}\times 10\text{^}6)}{(\text{Protein} \times \text{Sample Vol.}\times \Delta\text{time} \times \text{Constant}(6.22))}$$

Units = U/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
- **Δ** time = Measurement Time
- 6.22 =Extinction Co-efficient (M^{-1} cm^{-1})

2.13 Estimation of 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 at 37°C for 15 minutes. Absorbance was noted at 240nm. Activity of catalase was denoted in U/mg protein.

$$\textbf{CAT} = \frac{((\Delta\text{Sample} - \Delta\text{Blank})\times \text{Cuvette Vol.}\times 10\text{^}6)}{(\text{Protein} \times \text{Sample Vol.}\times \Delta\text{time} \times \text{Constant } 43.1)}$$

Units = U/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
- **Δ**time = Measurement Time
- 43.1 =Extinction Co-efficient (M^{-1} cm^{-1})

2.14 Inhibition of Acetylcholine Esterase (AChE)

The acetylcholine esterase inhibition in zebrafish was determined by using a test for sub-lethal toxicity. For the purpose of determining AChE inhibition, the brain tissues of exposed fish were dissected on an ice-cold plate and then immediately transferred to 2 mL Eppendorf tubes having 1.5 mL buffer formalin, and stored below -20 °C.

2.14.1 Determination of AChE Activity

The AChE activity was measured by following a modified study of Ellman et al. (1961).

The mixture contains:

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

Figure 18: Solution preparation and UV-spectroscopy for determination of AChE activity

Acetylthiocholine iodide was not present in the control cuvette. Enzyme activity was determined by reading the changes in absorbance over course of 5 minutes with readings taken at every minute at 412 nm on UV Spectrometer. The activity was measured using the following formula:

AChE Activity = $\frac{(\boldsymbol{Tf - Ti})}{\mathbf{0.01}}$

Units = U/min

Where:

- T*f* = Final Absorbance
- T*i* = Initial Absorbance
- 0.01= Constant (Time Constant for 1 minute)

2.15 Analysis of DNA Damage by Comet Assay (SCGE)

DNA damage in zebrafish liver tissues was evaluated using Single-cell gel Electrophoresis. In order to perform Comet Assay, the method described by Ge et al.; (2015) was employed.

2.15.1 Reagents Preparations

Following reagents were prepared to perform Comet Assay:

- Low Melting Agarose (LMA) was prepared by dissolving 250 mg of LMA in 50 ml of 1% PBS. This solution was then maintained at a low temperature in the refrigerator and brought to 37 °C just prior to use.
- Normal Melting Agarose: In order to dissolve 500 mg of normal melting agarose in 50 ml of distilled water the solution was heated 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. The pH was adjusted at 10.0 with HCl or NaOH and the final volume was increased to 1000 ml. The final lysing solution was prepared by combining already prepared suspension with 10% DMSO and 1% Triton X. The final solution must be chilled for 30 minutes, prior to making slides.

- Electrophoresis buffer: The electrophoresis buffer contains 0.5 ml of EDTA (200 mM) and 30 ml of NaOH in 1000 ml of distilled water. The pH was kept basic (>13).
- Neutralization buffer: A pH of 7.5 was attained 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 ml of phosphate buffer saline was prepared, and the volume was raised to 1000 ml when the pH was set at 7.

2.15.2 Slides Preparation

The slides were cleaned with methanol prior to use and then burned in order to disinfect them. The slides were then coated with NMA, 200 µL of LMA and cell suspension were pipetted. After the first layer of LMA solidified, the second one (85 µL) was added. Slides were then immersed in lysis solution and chilled for 120 minutes while being kept in the dark following the fixation of the second layer.

2.15.3 Electrophoresis

After two hours, the slides were removed from the lysis solution and placed in the horizontal gel apparatus. Slides were dipped into a previously made buffer solution. Power at 24 volts was provided for 30 minutes whilst 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 was used for staining, and cover slips were put over the plates.

2.15.4 Slides Visualization

To analyze 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.

2.16 Evaluation of Tissue Histopathology

The skeletal muscle tissues were examined for histological alterations. Fish were dissected and skeletal muscle was cut out in each sampling interval. They were immediately transferred into 2mL Eppendorf tubes with 1.5 mL of NBF. Samples were kept at 4°C until further analysis was done.

2.16.1 Hematoxylin and Eosin (H and E) Staining of Skeletal Muscle

Using the Hematoxylin and Eosin staining technique, the histopathological alterations in the muscle tissues following exposure to CuO, Sodium Arsenite and co-exposure were analyzed. Fixation, dehydration, embedding, slide preparation, staining, and microscopy are some of the steps. The obtained tissues were washed and rinsed with a saline solution containing 0.75% NaCl. Sections of the tissues were cut 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 microscope was used to analyse histopathological lesions on slides at a 1000X magnification, and a TUCSEN digital camera was used to take pictures of the liver and gill tissues (Model: ISH500).

Figure 19:Examination of histopathological alteration on light microscope (Olympus-CX41)

2.17 Statistical Analysis

The studies were run in triplicate for each tested concentration (treatment), and the calculations considered the means and standard deviations. Statistical analysis was performed on all experimental data via IBM SPSS Statistics, version 21.0. (SPSS Inc., USA). Three replicates mean (n=3) and standard deviation are used to express the data (SD). One-way analysis of variance (ANOVA) was done to determine the statistical differences between treatment and control groups. Means were separated using the Tukey's Post-Hoc comparison test. The p-value of 0.05 being considered significant.

Chapter 3

Results

3. RESULTS

The current study was carried out for a period of 28-days, during which we investigated the individual and joint toxicity of sodium arsenite ($NaAsO_2$) and copper oxide nanoparticles (CuO-NPs) on adult zebrafish. The nanoparticles used in this study were characterized to assess the surface morphology, elemental composition, spectral peaks, and x-ray diffraction. Furthermore, water quality parameters were measured continuously throughout the experiment. The fish samples were collected at specific intervals (7, 14, 21, and 28 days) and analyzed to determine total protein, oxidative stress markers including reactive oxygen species (ROS) and malondialdehyde content (MDA) and antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT) in the gills. Moreover, functional neuromarker acetylcholinesterase (AChE) was assessed in the brain and DNA damage was evaluated in the liver. Histopathological alterations were examined in the skeletal muscles of the fish. The results of the present study are presented and explained in this section.

3.1 Characterization of Copper Oxide Nanoparticles (CuO-NPs)

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

The surface morphology of the CuO-NPs was observed to be a rice-grained shaped structure as examined by scanning electron microscopy (SEM). The size of the nanoparticles ranged between 50 to 100nm as revealed by SEM analysis. The purity and chemical composition of the synthesized materials was confirmed by the energy dispersive spectra of the sample. Despite minor impurities, the strong peaks of Copper (Cu) and Oxygen (O) indicate high purity of nanoparticles. Furthermore, sharp, and narrow peaks showed the highly crystalline nature of the CuO-NPs. The CuO-NPs were found to be homogenous in nature and showed aggregation with slight irregularity. The calculated weight percentage for copper was 71.4% whereas for oxygen it was found to be 20.9%. The SEM image of CuO-NPs has been shown in Figure 19.

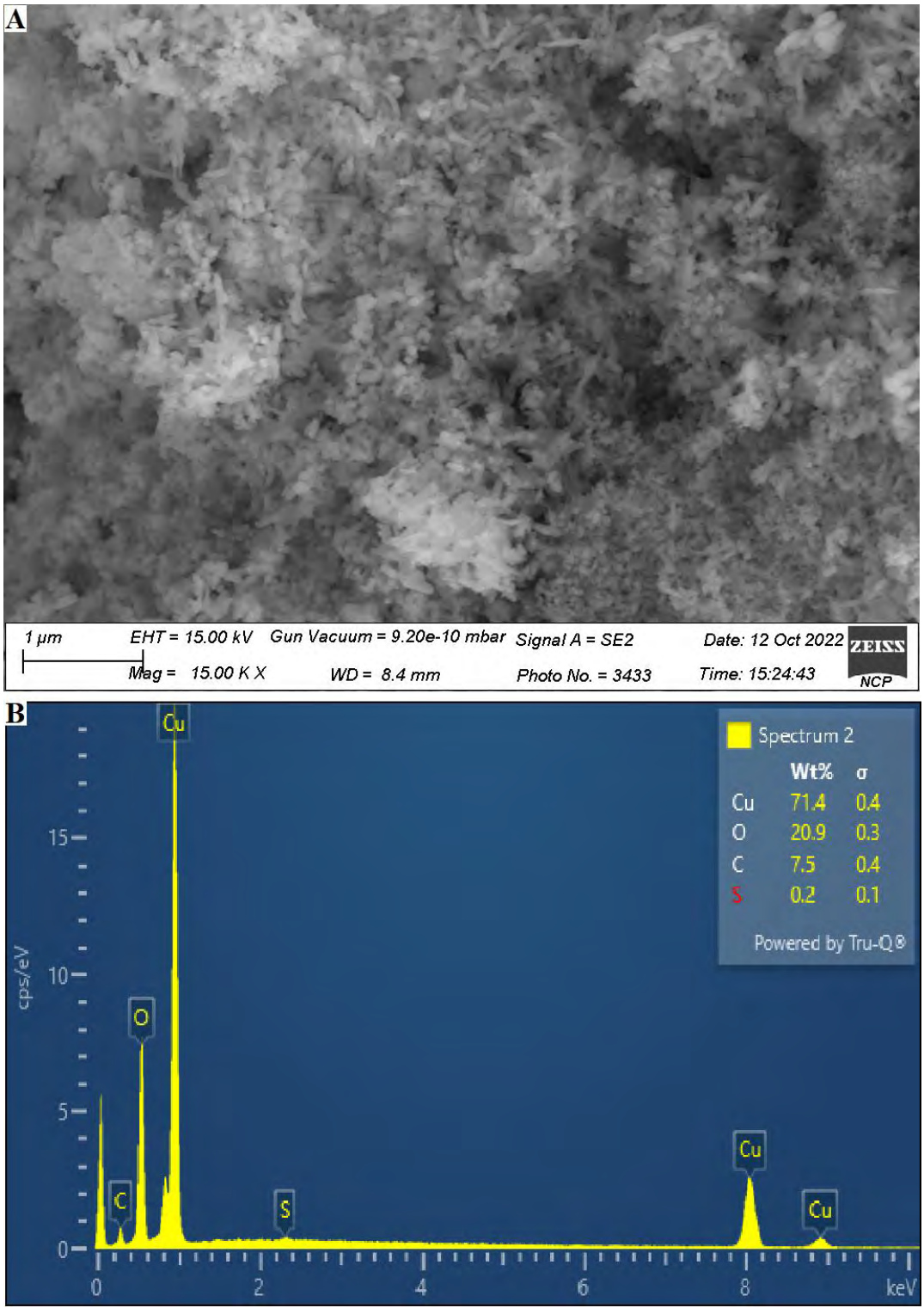


Figure 20: (A) Scanning Electron Micrograph of Copper Oxide (CuO) Nanoparticles; (B) EDX of Copper Oxide Nanoparticles

3.1.2 Fourier Transform Infrared Spectroscopy (FTIR)

The FT-IR spectral peak of CuO-NPs fell in the range of 4000–400 cm^{-1} (see Figure. 21). The broad spectral band at 3421.1 cm^{-1} corresponds to OH stretching of the O-H hydroxy group. This characteristic of absorbed water molecules on nanocrystalline may be due to their large surface area. The sharp peak at 1112.72 cm^{-1} corresponds to C-N stretching vibrations of the amine group. The intense peaks at 597.82, 524.54 and 512.97 cm^{-1} 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 due to the Cu-O bond formation.

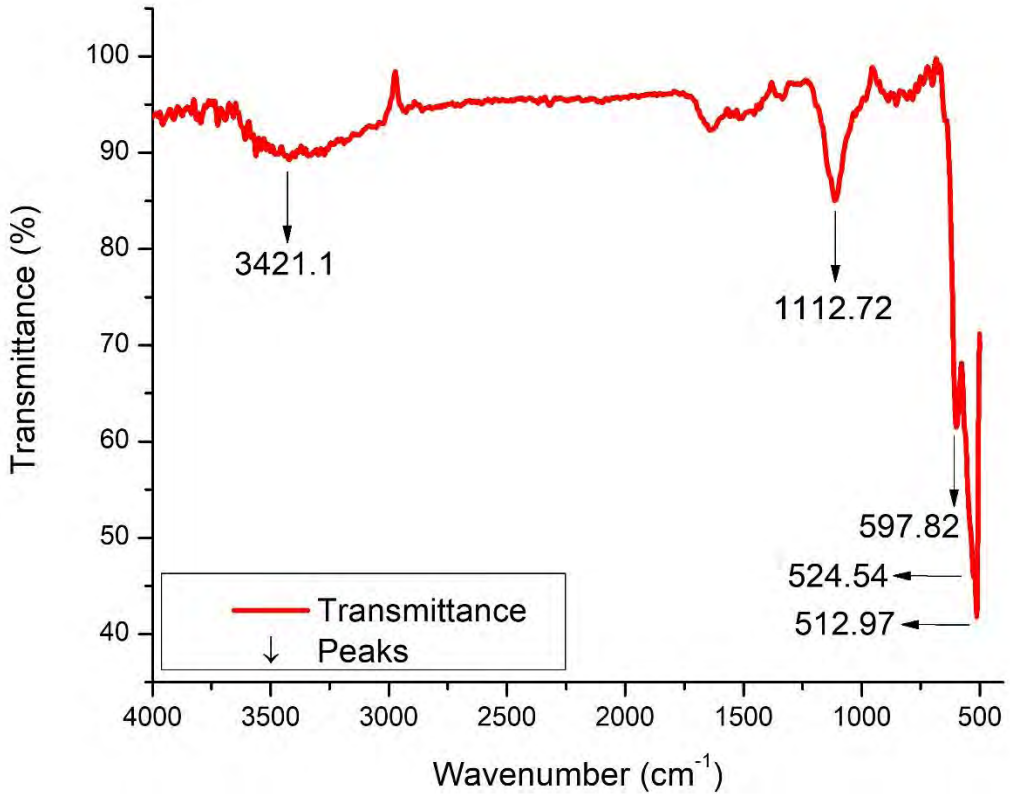


Figure 21: FT-IR Spectrum of Copper Oxide Nanoparticles. Intense peaks indicate purity of the nanostructures.

3.1.3 X-Ray Diffraction (XRD)

The X-ray diffraction pattern showed crystalline structure of CuO-NPs used in the current study as shown in figure 22. The XRD pattern exhibited intense peaks at 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 CuO-NPs (JCPDS card no. 45-0937) (Ahamed et al., 2014). Lack of impurity peaks indicates high quality of CuO-NPs.

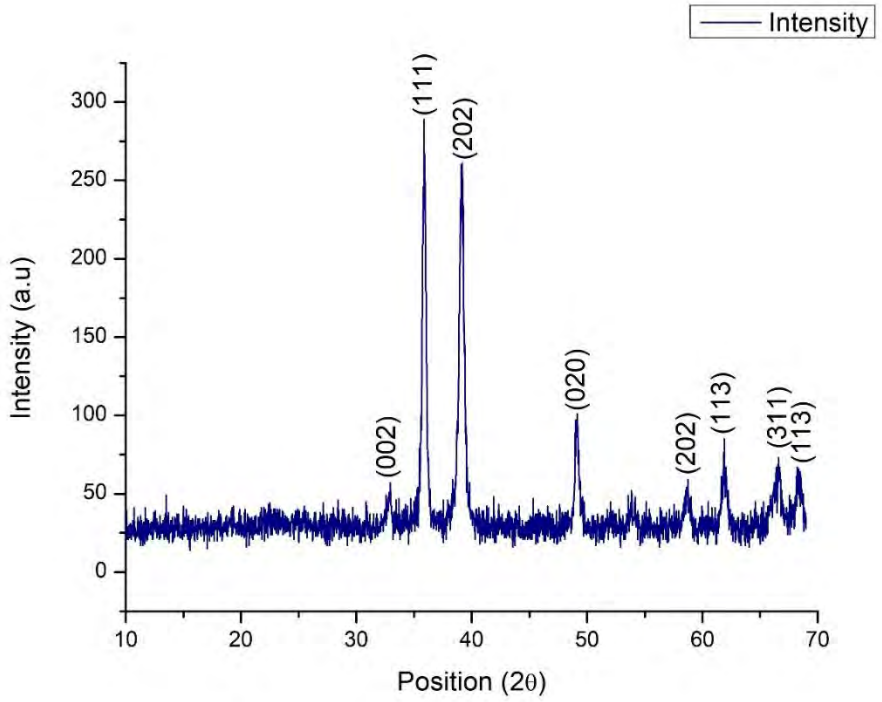


Figure 22: XRD Pattern of Copper Oxide Nanoparticles

3.2 Water Quality Parameters

In present studies the parameters such as pH, electrical conductivity (EC), temperature, dissolved oxygen (DO) and total dissolved solids (TDS) were analyzed at different intervals randomly over the experiment period of 28 days. A slight difference has been observed in the TDS and EC values of control and exposed treatment groups. The values for pH ranged between 7.37 and 7.45. Mean values of temperature and electrical conductivity were found to be 25ºC and 375 µS/cm, respectively. Whereas, TDS and DO were 266.5 mg/L and 6.75 mg/L, respectively. The values of all parameters were found in the optimum range suggested for zebrafish maintenance in the laboratory by Avdesh et al. (2012).

Table 5: Physicochemical parameters of aquarium water exposed to different treatment groups over 28 days of experiment.

Treatments	Temperature (C°)			EC (µS/cm)			TDS (mg/L)			DO (mg/L)			pH		
	Min	Max	Mean	Min	Max	Mean	Min	Max	Mean	Min	Max	Mean	Min	Max	Mean
CT	23.3	26.9	25.1	367	388	377.5	257	276	266.5	6.2	7	6.6	7.26	7.5	7.38
T1	23.3	26.8	25.05	358	389	373.5	253	273	263	6.2	7.3	6.75	7.14	7.6	7.37
T2	23.4	27.5	25.45	365	382	373.5	257	272	264.5	6.1	7.2	6.65	7.2	7.7	7.45
T3	23.2	26.8	25	358	382	370	255	272	263.5	6	7.1	6.55	7.2	7.7	7.45

CT: Control

T1:$NaAsO_2$ (300µg/L)

T2: CuO-NPs (1mg/L)

T3: $NaAsO_2$ (300µg/L) + CuO-NPs (1mg/L)

3.3 Total Protein Estimation

Total protein (TP) content is an essential biochemical parameter used to estimate the adverse effects of toxic pollutants in aquatic organisms (Kayhan et al., 2022).

3.3.1 Calibration curve for total protein estimation using Bovine Serum Albumin

Bovine Serum Albumin (BSA) solution was used to prepare serial dilutions as standards (Table 7). Absorbance was recorded at 595 nm and the values were used to plot a standard calibration curve (Fig 23) for estimating the total protein values in unknown samples.

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

BSA Standard Dilutions (mg/L = µg/mL)	Absorbance at 595 nm
0	0
10	0.019
20	0.028
40	0.048
60	0.059
80	0.071
100	0.081

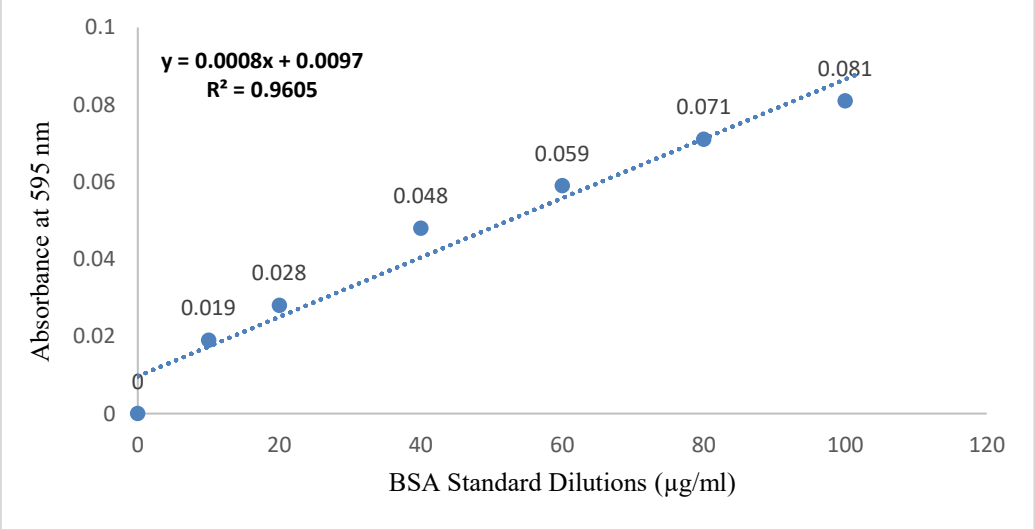


Figure 23: Standard Calibration Curve for Total Protein Estimation in *Danio rerio* gill samples at 595 nm.

3.4 Total Protein Content in Zebrafish Gills

Total protein content was measured in zebrafish gills to evaluate oxidative stress during the experiment. Across all sampling intervals, CT (control) had the highest evaluated total protein content than the treatment groups T1 ($NaAsO_2$), T2 (CuO-NPs) and T3 ($NaAsO_2$+CuO-NPs). On day 7, 14 and 28 of exposure, sampling intervals, CT (control group) showed highest total protein content compared to exposed groups at a significant difference at the order CT>T2>T1>T3. However, a different trend was observed after 21 days of exposure with CT and T2 showing the highest and lowest total protein content at a significant difference, respectively. Treatment groups T1 and T3 showed no significant difference ($p > 0.05$). The estimated total protein content has been plotted in a graph shown in Figure 24.

Table 7: Assessment of Total Protein content in *Danio rerio* exposed to singular and joint toxicity of $NaAsO_2$ and CuO-NPs.

Treatments	**Exposure Days**			
	D7	**D14**	**D21**	**D28**
CT (Control)	15.1 ± 1.1^{a}	15.4 ± 0.95^{a}	15.7 ± 0.90^{a}	15.9 ± 1.1^{a}
T1 ($NaAsO_2$)	13.5 ± 0.9^{c}	13.5 ± 1.05^{c}	13.33 ± 1.05^{c}	13.6 ± 0.9^{c}
T2 (CuO-NPs)	14.3 ± 0.95^{b}	14.6 ± 0.9^{b}	14.8 ± 0.7^{b}	14.8 ± 0.95^{b}
T3 (NaAsO2+CuO-NPs)	12.8 ± 1.1^{d}	12.86 ± 0.65^{d}	13.1 ± 0.9^{c}	13.2 ± 1.1^{d}

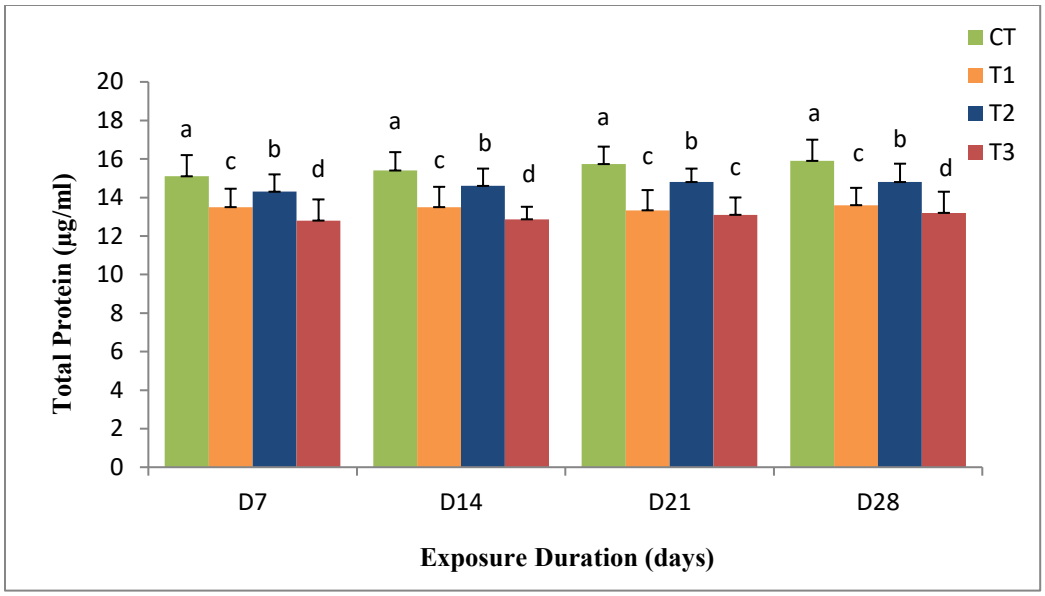


Figure 24: Total protein levels in *Danio rerio* gills after single and joint exposure to $NaAsO_2$+ CuO-NPs. Treatments are presented as following; CT: Control, T1: $NaAsO_2$, T2: CuO-NPs, and T3: NaAsO2+ CuO-NPs. Vertical bars represent mean ± SD of triplicates (n=3). Significant difference ($p<0.05$) in treatments is denoted by lowercase alphabet.

3.5 Oxidative Parameters

The reactive oxygen species levels and malondialdehyde content were measured in order to analyze the oxidative induced in zebrafish upon exposure to low doses of $NaAsO_2$ and CuO-NPs in a time dependent manner as shown in Figures 29 and 30.

3.5.1 Reactive Oxygen Species

Reactive oxygen species (ROS) was measured in zebrafish gills to evaluate oxidative stress during the experiment. Across all sampling intervals, CT (control) had the lowest evaluated reactive oxygen species than the treatment groups T1 ($NaAsO_2$), T2 (CuO-NPs) and T3 ($NaAsO_2$+CuO-NPs). On day 7 of exposure, all the treatment groups were significantly higher than CT whereas T1 and T3 were significantly different from each other. However, T2 was not significantly different than T1 and T3 ($p<0.05$). At 14-day and 21-day interval, CT was significantly lower than the treatment groups whereas T3 showed highest levels of ROS than T1 and T2 that were not significantly different compared to each other. On the 28th day of exposure, T3 was significantly highest among other treatment groups. In this interval, T2 was significantly lower than T3 and T1 but had higher levels of reactive oxygen

species than CT as shown in figure 25. The trend was similar during the experiment with CT showing lowest levels of reactive oxygen species compared to the treatment groups hence exhibiting an increasing order CT<T2<T1<T3 in a significant fashion ($p<0.05$).

Table 8: Assessment of Reactive Oxygen Species in *Danio rerio* gills exposed to singular and joint toxicity of $NaAsO_2$ and CuO-NPs.

Treatments	**Exposure Days**			
	D7	**D14**	**D21**	**D28**
CT (Control)	21.11 ± 0.93^{c}	23.86 ± 0.90^{c}	25.87 ± 1.06^{c}	23.70 ± 1.22^{d}
T1 ($NaAsO_2$)	31.38 ± 1.49^{b}	33.16 ± 0.94^{b}	34.24 ± 1.41^{b}	36.64 ± 1.03^{c}
T2 (CuO-NPs)	28.25 ± 1.07^{ab}	31.59 ± 1.18^{b}	31.85 ± 1.06^{b}	33.53 ± 1.07^{b}
T3 ($NaAsO_2$+CuO-NPs)	33.64 ± 1.10^{a}	36.97 ± 1.49^{a}	38.56 ± 0.87^{a}	40.66 ± 0.96^{a}

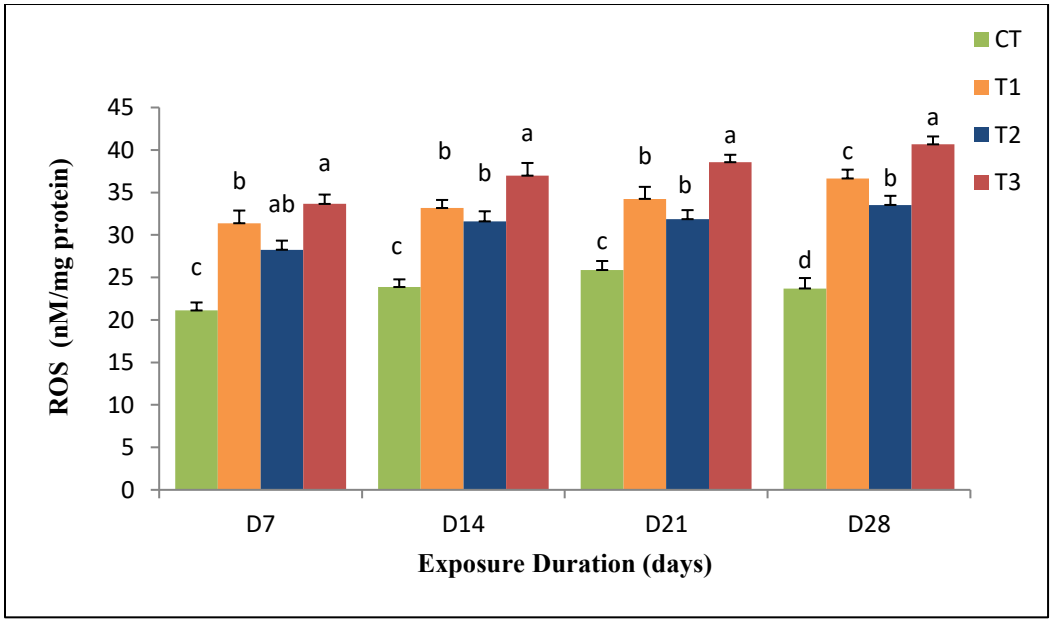


Figure 25: Levels of reactive oxygen species in *Danio rerio* gills following single and joint exposure to NaAsO2 and CuO-NPs. Treatments are presented as following; CT: Control, T1: $NaAsO_2$, T2: CuO-NPs, and T3: $NaAsO_2$+ CuO-NPs. Vertical bars represent mean ± SD of triplicates (n=3). Significant difference ($p<0.05$) in treatments is denoted by lowercase alphabet.

3.5.2 Malondialdehyde Content (MDA)

The Malondialdehyde levels were analyzed in gills of zebrafish to estimate the oxidative damage induced in CT (control) and T1 ($NaAsO_2$), T2 (CuO-NPs) and T3 ($NaAsO_2$+CuO-NPs) the treatment groups to measure significant difference between them ($p<0.05$). At first interval i.e., after 7 days exposure, CT was significantly different from T1 and T3 but T2 showed no significant variance in this interval ($p<0.05$). Treatment group T3 was significantly higher than T2 however there was no significant difference between T1 and T2. In the second interval of 14 days of exposure, T1 and T3 were not significantly different but showed significantly higher MDA content than T2 and CT with T2 being significantly different to CT. In the last two intervals of 21 and 28 days of exposure, a similar trend was observed with T3 and CT showing the highest and lowest MDA content in a significant manner, respectively (Figure 26). Treatment T2 was lower than both T1 and T4 but higher than CT with significant difference ($p<0.05$). The evaluated values showed the order CT<T2<T1<T3 throughout the experiment. The values for MDA content have been plotted in a graph shown in figure 26.

Table 9: Assessment of Malondialdehyde levels in *Danio rerio* gills exposed to singular and joint toxicity of $NaAsO_2$ and CuO-NPs.

Treatments	**Exposure Days**			
	D7	**D14**	**D21**	**D28**
CT (Control)	13.20 ± 1.02^{c}	13.49 ± 1.03^{c}	14.82 ± 0.59^{d}	15.08 ± 0.70^{d}
T1 ($NaAsO_2$)	17.56 ± 0.96^{ab}	19.50 ± 0.83^{a}	20.71 ± 1.02^{b}	22.40 ± 1.08^{b}
T2 (CuO-NPs)	15.44 ± 0.76^{bc}	16.75 ± 0.47^{b}	17.59 ± 0.58^{c}	18.70 ± 1.30^{c}
T3 ($NaAsO_2$+CuO-NPs)	19.69 ± 0.92^{a}	20.47 ± 1.14^{a}	23.65 ± 1.16^{a}	25.75 ± 1.28^{a}

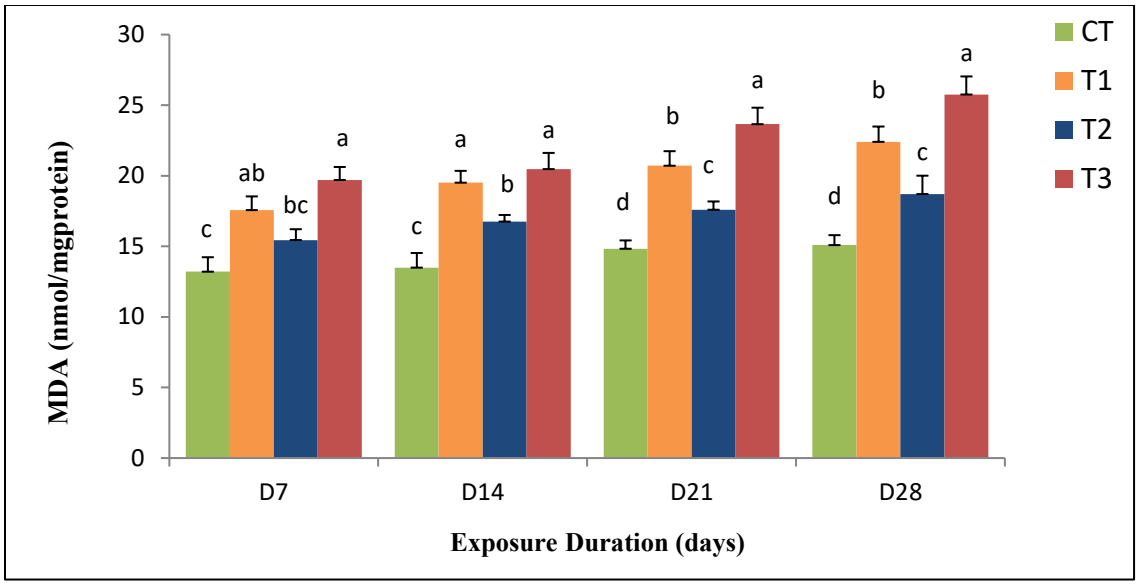


Figure 26: Malondialdehyde levels in *Danio rerio* gills following single and joint exposure to $NaAsO_2$ and CuO-NPs. Treatments are presented as following; CT: Control, T1: $NaAsO_2$, T2: CuO-NPs, and T3: $NaAsO_2$+ CuO-NPs. Vertical bars represent mean ± SD of triplicates (n=3). Significant difference ($p<0.05$) in treatments is denoted by lowercase alphabet.

3.6 Antioxidant Defense System

The superoxide dismutase and catalase levels were measured in order to analyze the antioxidant defense system in zebrafish upon exposure to low doses of $NaAsO_2$ and CuO-NPs in a time dependent manner as shown in figure 29 and figure 30.

3.6.1 Superoxide Dismutase (SOD)

The superoxide dismutase (SOD) activity was observed to evaluate the antioxidant system in gills of the zebrafish in CT (control treatment), T1 ($NaAsO_2$), T2 (CuO-NPs) and T3 ($NaAsO_2$+CuO-NPs) the treatment groups to measure significant difference between them. In the first interval on day 7 of exposure, there was no significant difference in CT and the treatment groups however T3 was significantly lowest in the treatment groups ($p<0.05$). On day 14 of exposure, there was no significant difference in SOD activity in CT, T1 and T2 whereas T3 showed the lowest SOD activity in a significant manner (shown in figure 27). At the 21 and 28-day interval, a clear decreasing trend was observed in CT and treatment groups upon exposure to singular and joint toxicity of $NaAsO_2$ and CuO-NPs. It

was observed that CT and T3 showed the highest and lowest SOD activity, respectively. The order observed in this case was CT>T2>T1>T3. The complete SOD activity across the sampling intervals has been plotted in a graph shown in Figure 27.

Table 10: Assessment of superoxide dismutase activity in *Danio rerio* gills exposed to singular and joint toxicity of $NaAsO_2$ and CuO-NPs.

Treatments	**Exposure Days**			
	D7	**D14**	**D21**	**D28**
CT (Control)	25.39 ± 1.40^{ab}	25.37 ± 1.09^{a}	24.99 ± 1.54^{a}	26.75 ± 0.95^{a}
T1 ($NaAsO_2$)	27.71 ± 1.27^{a}	23.50 ± 1.30^{a}	16.50 ± 0.90^{c}	14.50 ± 0.90^{c}
T2 (CuO-NPs)	27.47 ± 1.21^{a}	24.73 ± 1.33^{a}	20.34 ± 1.29^{b}	20.60 ± 0.67^{b}
T3 ($NaAsO_2$+CuO-NPs)	22.52 ± 0.66^{b}	18.06 ± 1.19^{b}	12.44 ± 0.70^{d}	11.42 ± 0.92^{d}

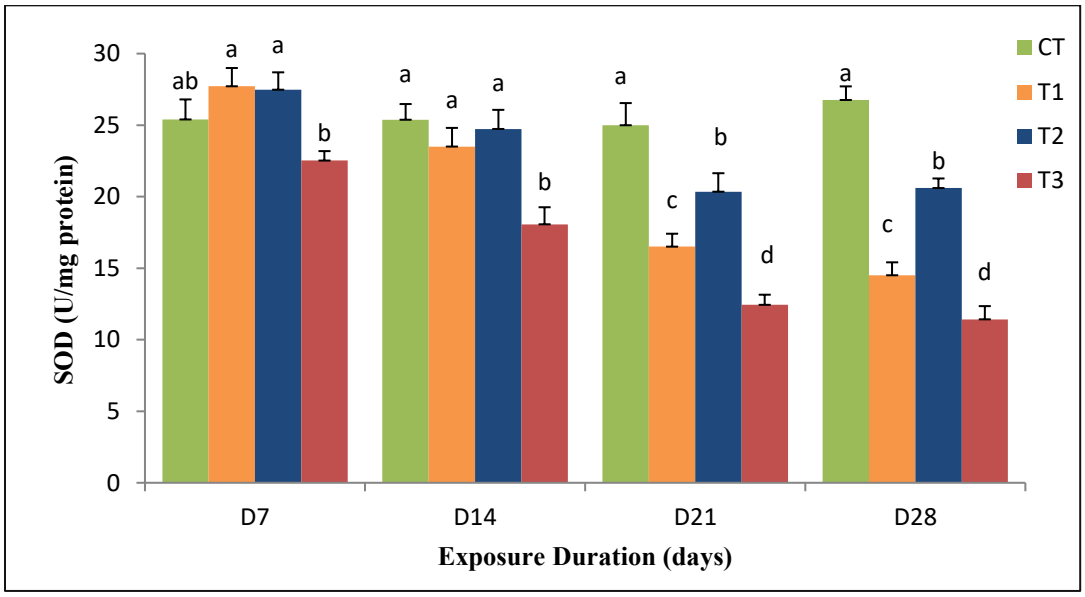


Figure 27: Superoxide dismutase activity in *Danio rerio* gills following single and joint exposure to $NaAsO_2$ and CuO-NPs. Treatments are presented as following; CT: Control, T1: $NaAsO_2$, T2: CuO-NPs, and T3: $NaAsO_2$+ CuO-NPs. Vertical bars represent mean ± SD of triplicates (n=3). Significant difference ($p<0.05$) in treatments is denoted by lowercase alphabet.

3.6.2 Catalase (CAT)

The CAT activity in each interval has been shown in Figure 28. The catalase (CAT) activity was estimated to evaluate the antioxidant system in gills of the zebrafish in CT (control), T1 ($NaAsO_2$), T2 (CuO-NPs) and T3 ($NaAsO_2$+CuO-NPs) the treatment groups to measure significant difference between them. At the 7-day and 14-day sampling there was no significant difference between CT and treatment group T2 however T1 and T3 were significantly different in this matter. Whereas treatment group T3 showed the lowest CAT activity than T2 at statistical significance($p<0.05$). After 21 days of exposure, CT showed significantly highest CAT activity than the treatment groups. The treatment groups in this interval were not significantly different but T3 showed the lowest CAT activity than T1 and T2 as shown in figure 28. After 28 days of exposure, CT and the treatment groups showed significant variance. In this interval, a decreasing trend in CAT activity was observed in the order CT>T2>T1>T3.

Table 11: Assessment of Catalase activity in *Danio rerio* gills exposed to singular and joint toxicity of $NaAsO_2$ and CuO-NPs.

Treatments	**Exposure Days**			
	D7	**D14**	**D21**	**D28**
CT (Control)	36.65 ± 0.91^{a}	35.41 ± 1.54^{a}	35.66 ± 1.70^{a}	36.2 ± 1.31^{a}
T1 ($NaAsO_2$)	29.46 ± 1.72^{b}	27.71 ± 1.50^{b}	25.25 ± 1.25^{bc}	22.23 ± 1.25^{c}
T2 (CuO-NPs)	34.48 ± 1.10^{a}	32.98 ± 1.35^{a}	28.32 ± 1.23^{b}	26.43 ± 1.23^{b}
T3 ($NaAsO_2$+CuO-NPs)	24.75 ± 1.34^{c}	23.21 ± 1.20^{c}	22.21 ± 1.20^{c}	18.78 ± 1.21^{d}

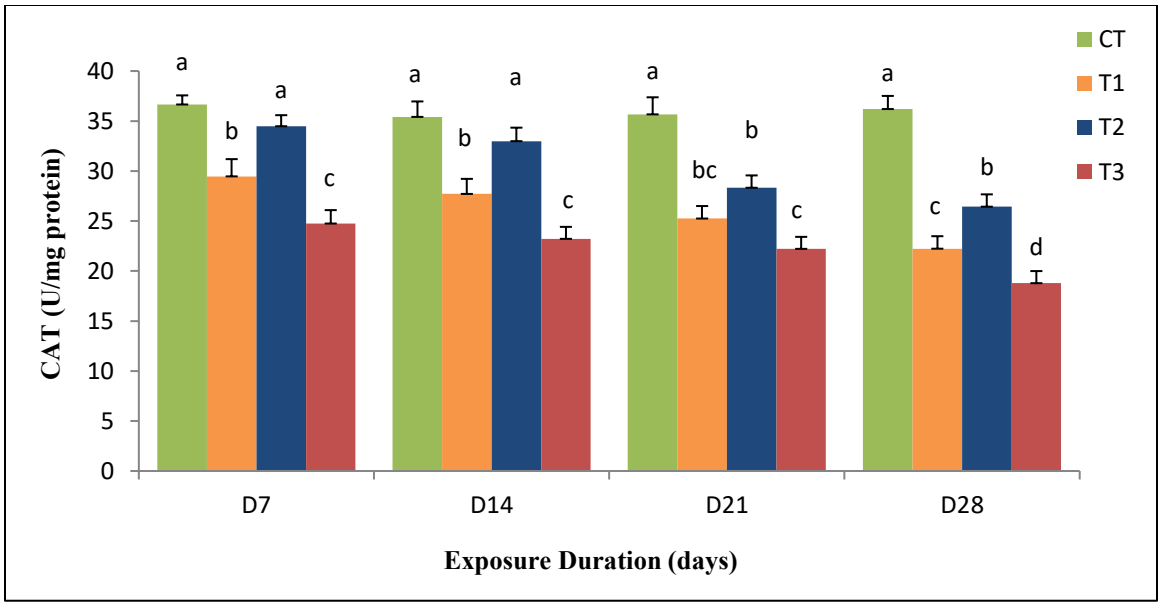


Figure 28: Catalase activity in *Danio rerio* gills following single and joint exposure to $NaAsO_2$ and CuO-NPs. Treatments are presented as following; CT: Control, T1: $NaAsO_2$, T2: CuO-NPs, and T3: $NaAsO_2$+ CuO-NPs. Vertical bars represent mean ± SD of triplicates (n=3). Significant difference ($p<0.05$) in treatments is denoted by lowercase alphabets.

3.7Acetylcholinesterase Activity (AChE)

The activity of acetylcholinesterase was evaluated in the brain tissues of zebrafish exposed for 7, 14, 21 and 28 days from all treatment groups. The continuous inhibition in AChE activity was observed in a time dependent manner. AChE activity was significantly higher in CT (control) compared to the treatment groups at each sampling interval during the experiment. The inhibition of AChE was significantly higher in T1 ($NaAsO_2$), T2 (CuO-NPs) and T3 ($NaAsO_2$+CuO-NPs) than CT (control) at each interval during the experiment ($p<0.05$). At each sampling interval, there was significant inhibition of AChE activity in each treatment group with the highest decline in T3 as shown in figure 29. The trend of inhibition of AChE remained similar throughout the experiment. Furthermore, among the treatment groups, T2 showed the lowest inhibition of AChE compared to other treatment groups in a significant manner. The order of AChE activity was found to be CT> T2 > T1>T3 at each interval. AChE activity for the experiment is shown in Figure 29.

Table 12: Assessment of Acetylcholinesterase activity in *Danio rerio* gills exposed to singular and joint toxicity of $NaAsO_2$ and CuO-NPs.

Treatments	Exposure Days			
	D7	**D14**	**D21**	**D28**
CT (Control)	26.36 ± 0.41^{a}	25.53 ± 0.85^{a}	24.83 ± 0.65^{a}	26.38 ± 0.67^{a}
T1 ($NaAsO_2$)	18.83 ± 0.41^{c}	17.61 ± 0.94^{c}	16.53 ± 0.85^{c}	14.53 ± 0.94^{c}
T2 (CuO-NPs)	23.88 ± 0.80^{b}	22.93 ± 0.49^{b}	22.66 ± 0.86^{b}	21.13 ± 1.13^{b}
T3 ($NaAsO_2$+CuO-NPs)	14.45 ± 0.82^{d}	14.26 ± 0.72^{d}	12.96 ± 0.70^{d}	11.71 ± 1.08^{d}

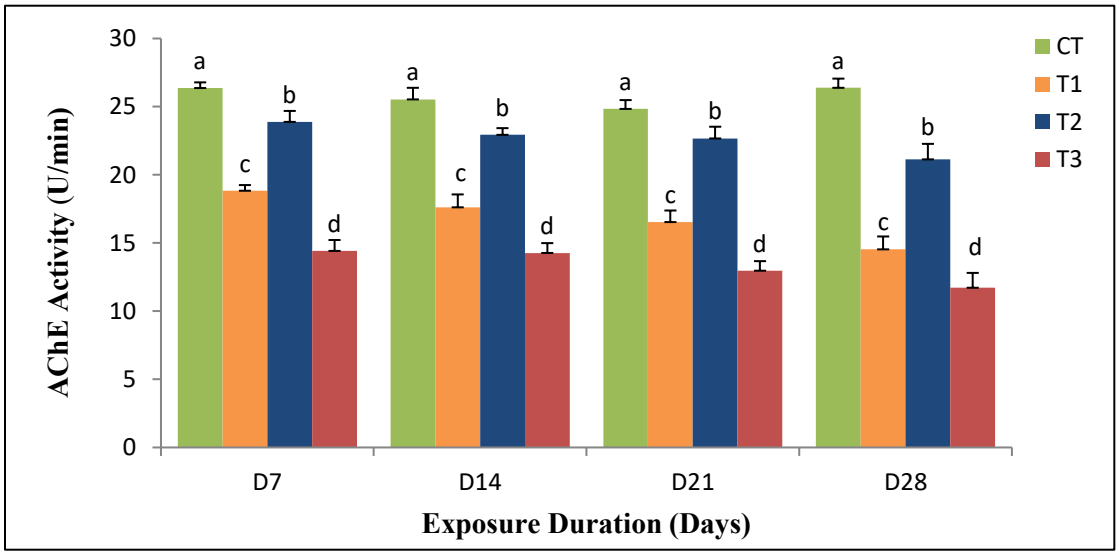


Figure 29: Acetylcholinesterase activity in brain tissues of *Danio rerio* upon single and joint exposure to $NaAsO_2$ and CuO-NPs. Treatments are presented as following; **CT**: Control, **T1**: $NaAsO_2$, **T2**: CuO-NPs, and **T3**: $NaAsO_2$+ CuO-NPs. Vertical bars represent mean ± SD of triplicates (n=3). Significant difference ($p<0.05$) in treatments is denoted by lowercase alphabets.

3.8 DNA Damage Assessed by Comet Assay

To assess the DNA damage in zebrafish upon exposure to $NaAsO_2$ and CuO-NPs in CT (control) and the treatment groups T1 ($NaAsO_2$), T2 (CuO-NPs) and T3 ($NaAsO_2$+CuO-NPs), the comet assay was used. At each interval (i.e., day 7, 14, 21, and 28), a gradual

decrease in head length was observed across 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 intervals, DNA in the head ranged between 83.95% and 86.07% in all control groups however it decreased gradually in the treatment groups. Furthermore, the DNA percentage in the tail increased steadily across all treatment groups with T3 showing the highest percentage compared to all treatment groups after 28 days of exposure. In the control group, the DNA percentage in the tail remained between 13.92% and 16.09% at each sampling interval.

Table 13: DNA damage assessment in liver tissue of Zebrafish by comet assay parameters.

Treatment Groups	Comet length (µm)	Head length (µm)	Tail length (µm)	% DNA in head	% DNA in tail	Tail moment (µm)
Day 7						
CT	41.2±3.0	35.3±2.2	5.9±0.8	85.67±4.2	14.32±3.1	0.11±0.03
T1	41.5±2.2	34.8±3.0	6.1±0.7	85.08±3.9	14.91±2.2	0.11±0.03
T2	40.9±2.8	34.8±3.0	6.1±0.7	85.08±3.9	14.91±2.2	0.11±0.03
T3	39.8±3.2	33.1±2.8	6.7±1.0	83.16±3.7	16.84±1.7	0.13±0.04
Day 14						
CT	39.5±2.7	34.0±2.8	5.5±0.7	86.07±3.3	13.92±1.3	0.10±0.03
T1	42.1±2.8	35.3±2.5	6.8±1.3	83.84±2.9	16.15±4.1	0.17±0.05
T2	39.1±2.9	33.7±3.2	5.4±1.2	86.18±3.1	13.81±1.8	0.11±0.03
T3	40.7±3.2	31.8±2.3	8.9±1.1	78.13±3.7	21.86±2.1	0.21±0.07
Day 21						
CT	40.3±3.3	34.2±2.7	6.1±1.3	84.86±3.2	15.13±2.1	0.11±0.03
T1	41.2±3.0	29.7±3.0	11.5±1.2	72.08±3.2	27.91±2.9	0.24±0.15
T2	39.2±2.7	30.7±2.9	8.5±1.7	78.31±3.1	21.68±2.8	0.15±0.03
T3	42.2±2.8	27.9±3.1	14.3±1.6	66.11±3.9	33.88±2.7	0.39±0.06
Day 28						
CT	39.9±3.1	33.5±2.9	6.4±1.8	83.95±3.9	16.09±1.7	0.13±0.05
T1	42.1±3.5	27.3±3.3	14.8±1.5	64.22±3.1	35.77±3.2	0.48±0.22
T2	40.3±2.7	30.2±3.1	10.1±1.5	74.93±3.1	25.05±1.8	0.17±0.02
T3	40.7±2.7	23.2±3.8	17.5±2.0	57.09±4.2	42.10±3.1	0.98±0.12

CT: Control; **T1**: $NaAsO_2$ (300µg/L); **T2**: CuO-NPs (1mg/L); **T3**: $NaAsO_2$ (300µg/L)+CuO-NPs (1mg/L)

3.8.1. Tail Moment

Tail moment (TM) was measured in zebrafish liver to assess DNA damage in CT (control), T1 ($NaAsO_2$), T2 (CuO-NPs) and T3 ($NaAsO_2$+CuO-NPs) by measuring significant difference ($p<0.05$) at each interval during the experiment. In the first two sampling intervals, no significant differences in tail movement between the treatment groups and CT were observed. Whereas, after 21 days of exposure, tail movement in T3 increased significantly compared to CT and T2, however T1 was not significantly different than T2 and T3. At 28-day interval, treatment T3 was the highest among other treatment groups and CT at a statistically significant variance (Figure 30). Treatment T1 was significantly different than CT but showed no significant difference compared to T2. Tail moment in liver tissues across all sampling intervals has been shown in figure 30 and 31.

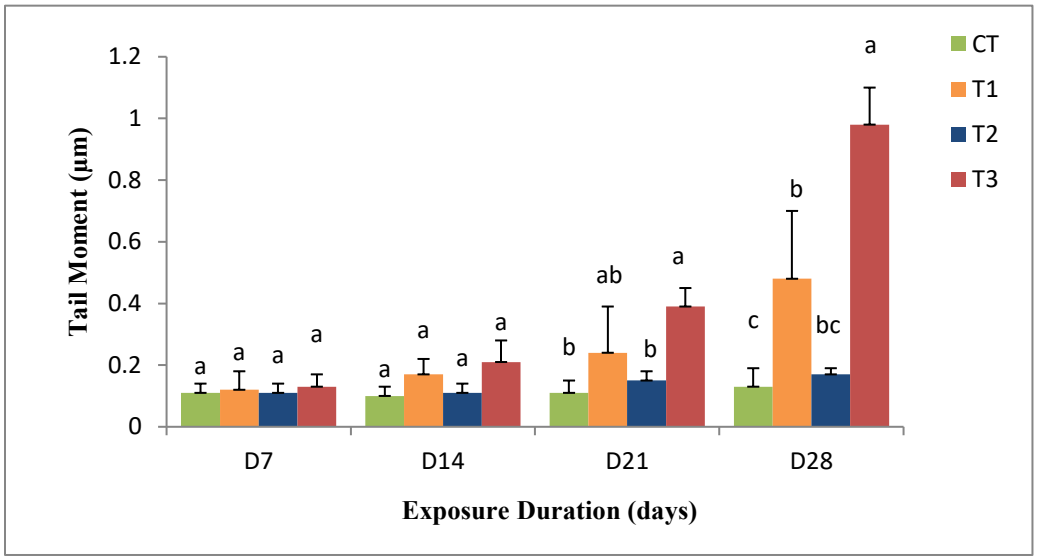


Figure 30: Tail moment in liver tissues of zebrafish upon single and joint exposure to $NaAsO_2$ and CuO-NPs. Treatments are presented as following; **CT**: Control, **T1**: $NaAsO_2$, **T2**: CuO-NPs, and **T3**: $NaAsO_2$+ CuO-NPs. Vertical bars represent mean ± SD of triplicates (n=3). Significant difference ($p<0.05$) in treatments is denoted by lowercase alphabets.

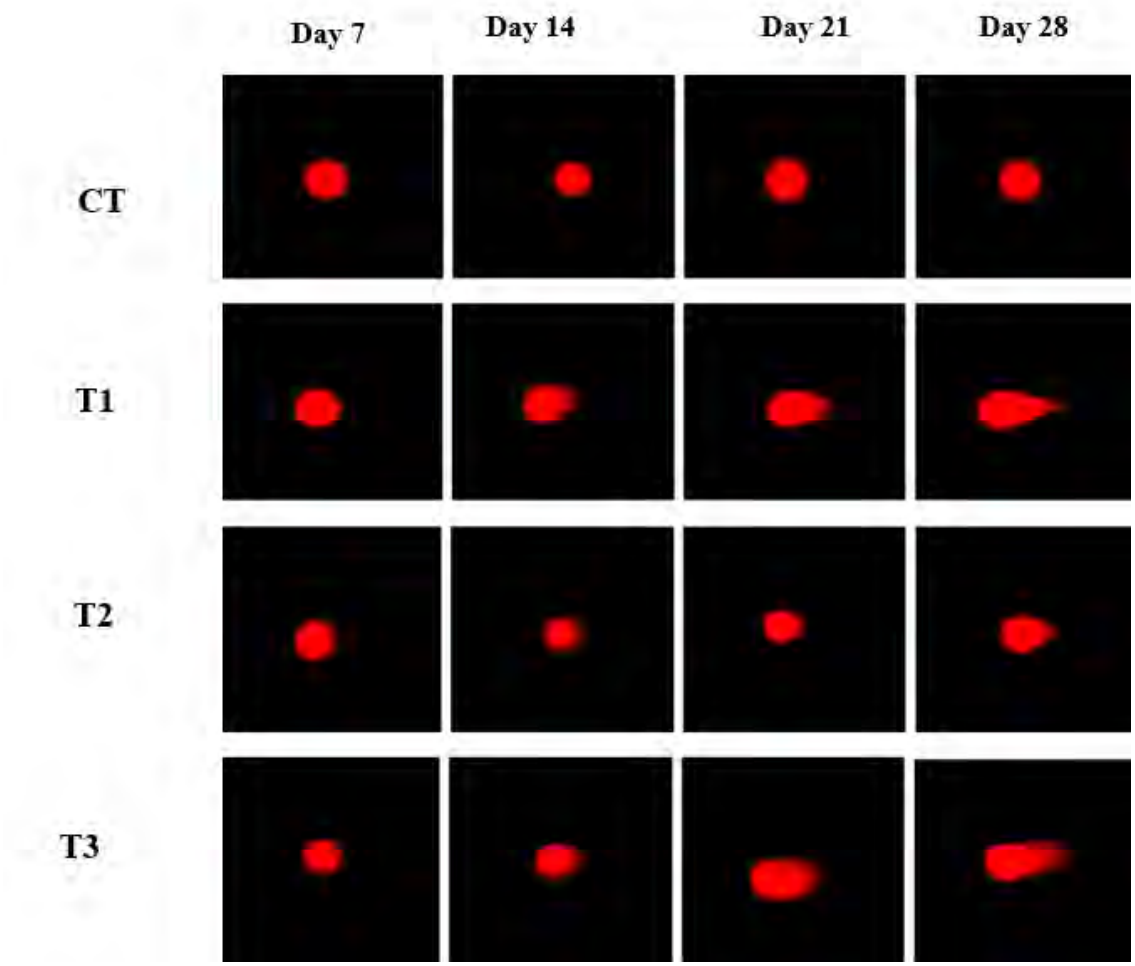


Figure 31: Fluorescence photomicrographs of DNA damage in *Danio rerio* liver following single and joint exposure to $NaAsO_2$ and CuO-NPs. Treatments are presented as following; **CT**: Control; **T1**: $NaAsO_2$; **T2**: CuO-NPs; and **T3**: $NaAsO_2$+ CuO-NPs.

3.9 Histopathological Changes in Skeletal Muscle of Zebrafish

The histology of zebrafish skeletal muscles was examined in CT (control) and the treatment groups T1 ($NaAsO_2$), T2 (CuO-NPs) and T3 ($NaAsO_2$+CuO-NPs). The histological examination showed normal morphology of the muscles of the control groups along with the nucleus at the periphery of the fibers at all sampling intervals. However, during the first sampling interval, necrosis was observed in T1, splitting of muscle fibers was observed in T2 and degeneration of muscle fibers was found in T3 as shown in figure 32. Treatment group T1 showed segmental necrosis after 14 days of exposure. Furthermore, treatment group T2 showed degeneration of muscle fibers whereas treatment group T3 showed splitting of muscle fibers (Figure 33). At 21-days, segmental necrosis and shortening of

fiber size were observed in treatment groups T1 and T2, respectively. Whereas, splitting of muscle fibers was observed in treatment group T3 (Figure 34). After 28 days of prolonged exposure severe muscle damage was observed in all treatment groups as shown in figure 35. Treatment T1 showed segmental necrosis in muscle fibers. Whereas, the shortening and degeneration of muscle bundles were observed in T2, and the splitting of muscle fibers was found in T3.

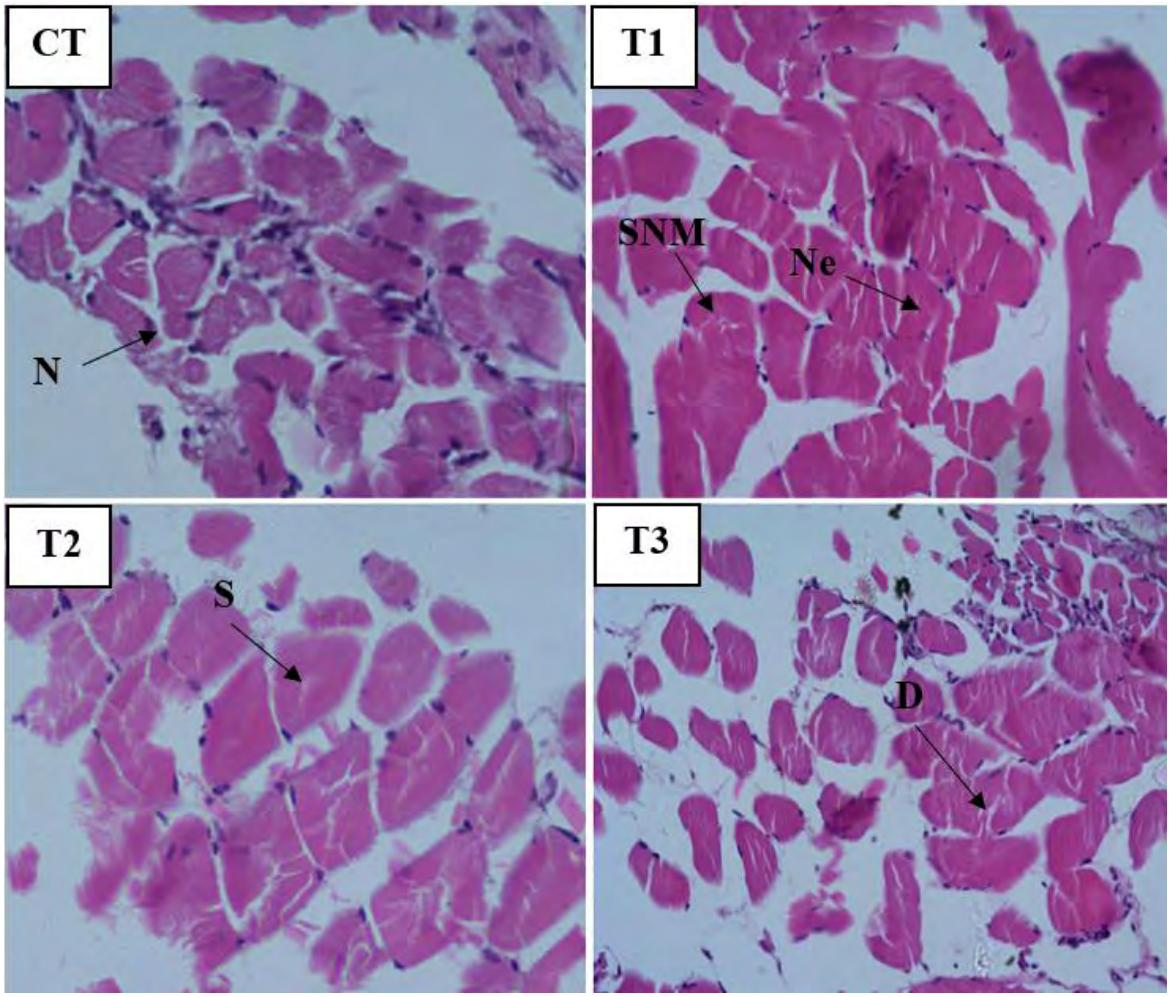


Figure 32: Photomicrographs of zebrafish skeletal muscle after 7 days of exposure at 40x magnification. **CT**: Control Treatment, **T1**: $NaAsO_2$, **T2**: CuO-NPs, and **T3**: $NaAsO_2$+ CuO-NPs.

N → represents the Nucleus located at the periphery of muscle fibers
SNM → represents Segmental Necrosis in muscle fiber
Ne → represents Necrosis
D → represents Degeneration in muscle bundles
S → represents the Splitting of muscle fibers

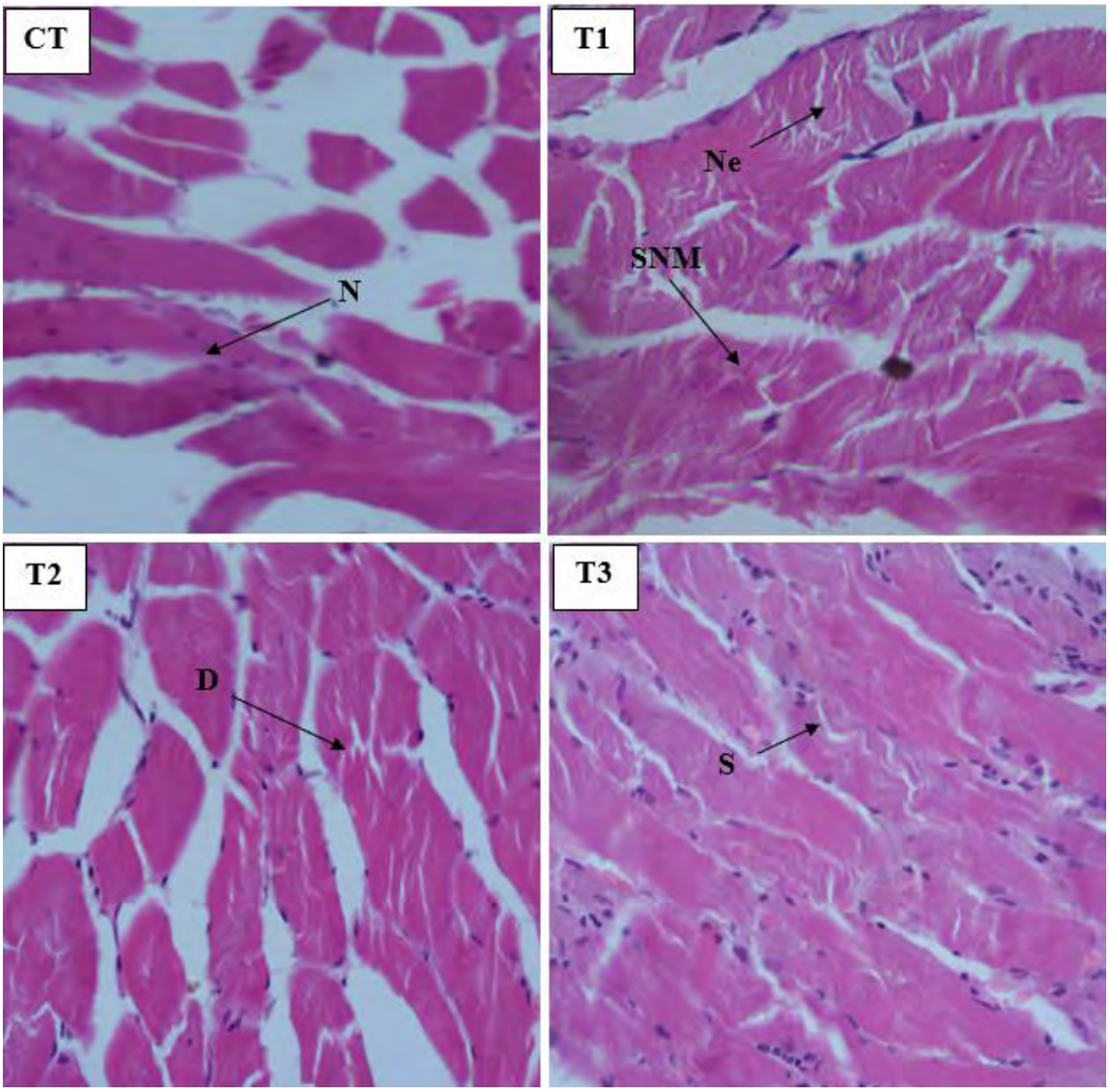


Figure 33: Photomicrographs of zebrafish skeletal muscle after 14 days of exposure at 40x magnification. **CT**: Control Treatment, **T1**: $NaAsO_2$, **T2**: CuO-NPs, and **T3**: $NaAsO_2$+ CuO-NPs.

N → represents the Nucleus located at the periphery of muscle fibers
SNM → represents Segmental Necrosis in muscle fiber
Ne → represents Necrosis
D → represents Degeneration in muscle bundles
S → represents the Splitting of muscle fibers

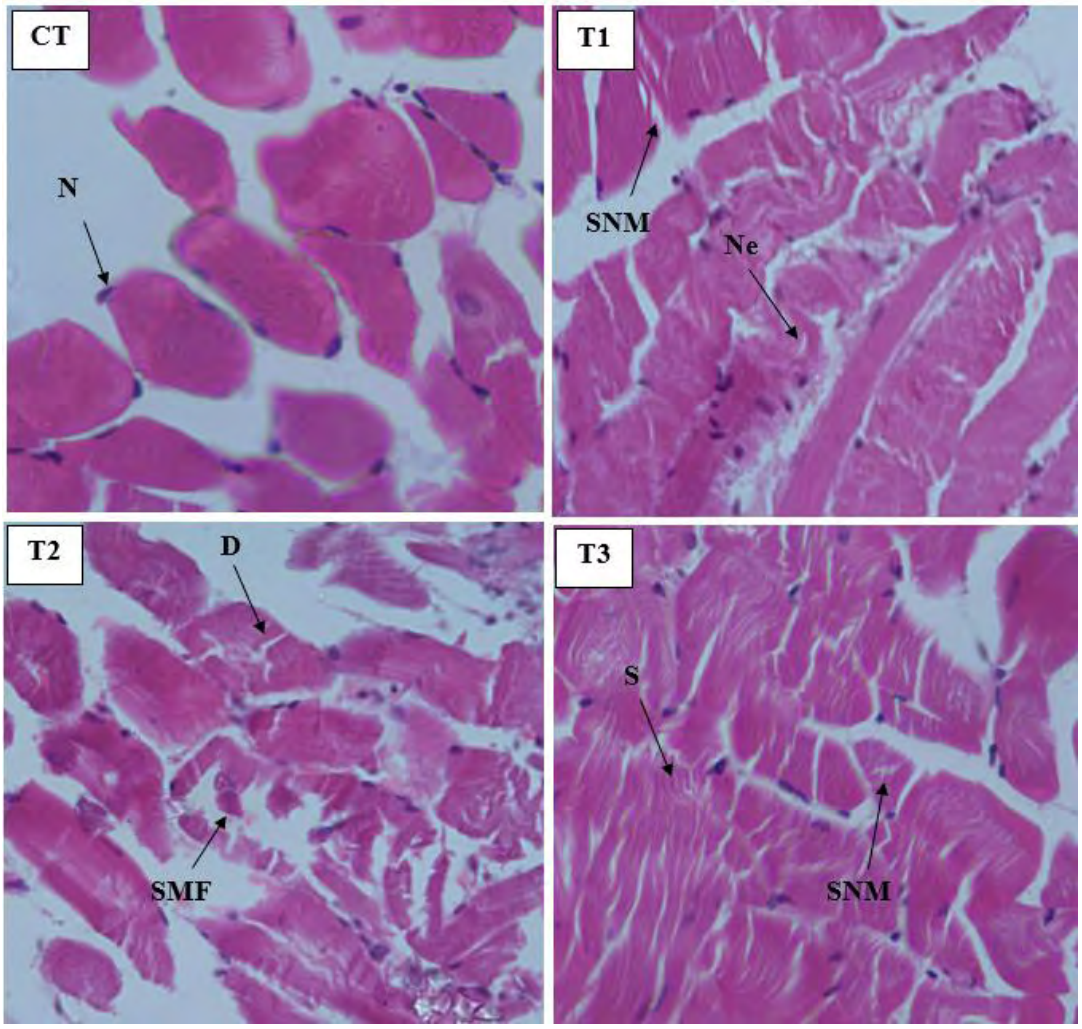


Figure 34: Photomicrographs of zebrafish skeletal muscle after 21 days of exposure at 40x magnification. **CT**: Control Treatment, **T1**: $NaAsO_2$, **T2**: CuO-NPs, and **T3**: $NaAsO_2$+ CuO-NPs.

N	→ represents the Nucleus located at the periphery of muscle fibers
SNM	→ represents segmental Necrosis of muscle fiber
Ne	→ represents Necrosis
D	→ represents Degeneration in muscle bundles
SMF	→ represents the Shortening of fiber size with overall reduction
SNM	→ represents segmental necrosis of muscle
S	→ represents the Splitting of muscle fibers

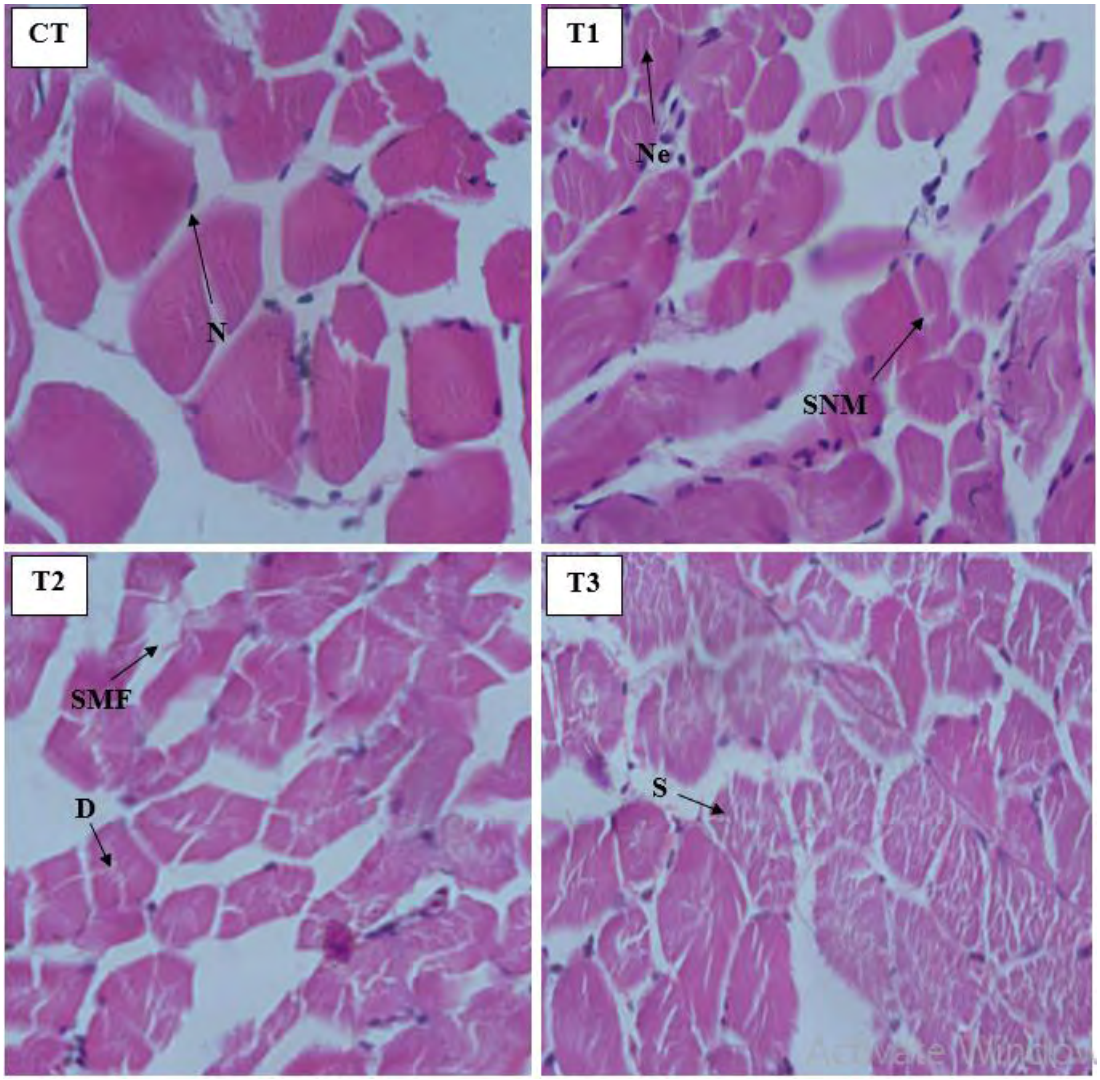


Figure 35: Photomicrographs of zebrafish skeletal muscle after 28 days of exposure at 40x magnification. **CT**: Control Treatment, **T1**: $NaAsO_2$, **T2**: CuO-NPs, and **T3**: $NaAsO_2$+ CuO-NPs.

N → Nucleus located at the periphery of muscle fibers
SNM → Segmental Necrosis of muscle fiber
Ne → represents Necrosis
D → represents degeneration in muscle bundles
SMF → shows the shortening of fiber size with overall reduction
S → represents the Splitting of muscle fibers.

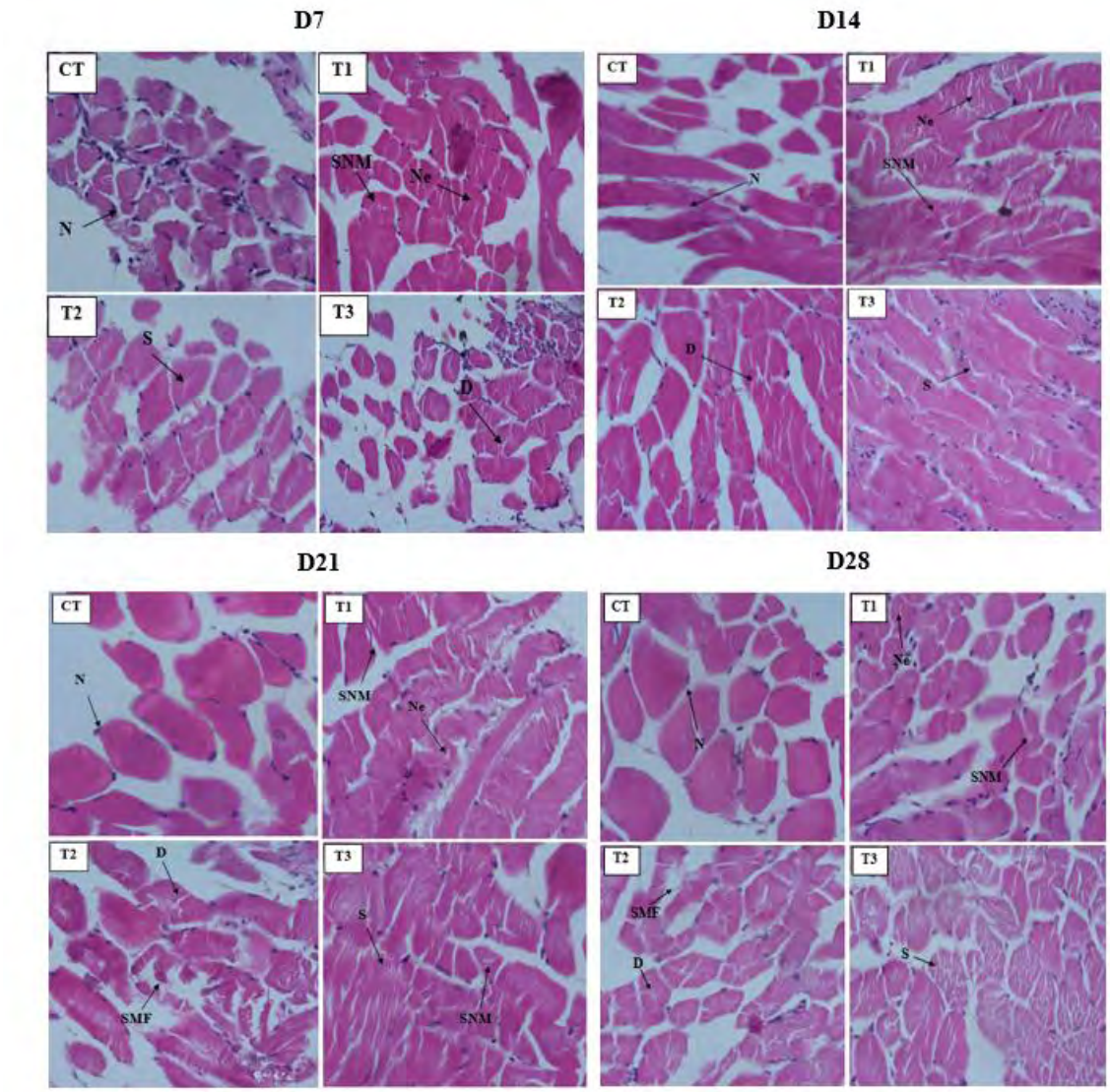


Figure 36: Photomicrographs of zebrafish skeletal muscle across all sampling intervals at 40x magnification. **CT**: Control Treatment, **T1**: $NaAsO_2$, **T2**: CuO-NPs, and **T3**: $NaAsO_2$+ CuO-NPs.

Chapter 4

Discussion

4. DISCUSSION

4.1 Characterization of Copper Oxide Nanoparticles

Copper oxide nanoparticles (CuO-NPs) have been extensively reported to be more toxic than their bulk counterparts. To understand toxicological effects of CuO-NPs, it is important to understand the characterization of these nanoparticles, their routes of exposure and mechanism or pathways involved in their toxicity (Naz et al., 2020; Chang et al., 2012). 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 utilized in the present study ranged between 50-100nm in size and showed structures resembling rice grains that assembled into aggregates, as exhibited by scanning electron microscope (SEM) as shown in Figure 20. Likewise, this morphology of CuO-NPs has previously been reported in studies by Ananth et al. 2015 and Sukumar et al. 2020. Previously, aggregation and agglomeration tendency of CuO-NPs from the same manufacturer have been observed by multiple authors (Ślosarczyk et al., 2023). The absence of uniformity in size or shape can be attributed to improper control on nanoparticle growth during synthesis such as capping agent, however when surfactants are absent, nanoparticles form aggregates to reduce Gibb's free energy (Sonnahalli and Chowdhary, 2020; Wang et al., 2002). The elemental composition assessed by energy dispersive x-ray (EDX) exhibited prominent peaks of copper (Cu) and oxygen (O) with weight percentages 71.4 and 20.9%, respectively, as shown in Figure 20. The presence of sharp and narrow peaks indicates high crystallinity of CuO-NPs (Sabeena et al., 2022; Sukumar et al., 2020).

Fourier Transform Infrared (FT-IR) Spectroscopy can detect the presence of any surface biomolecules or functional groups present on the nanoparticles (Sukumar et al., 2020; Badaway et al., 2021). Sharp peaks at 512.97, 52454 and 597.82 cm^{-1} are characteristic of pure CuO nanostructure formation (Hemalatha and Makeswari, 2017; Quirino et al., 2018), 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., 2018). No additional peaks were observed for commonly occurring impurities (Cu_2O or $Cu(OH)_2$), suggesting high purity (Buledi et al., 2020).

4.2 Water Quality Parameters

The growth and maintenance of fish in its external environment depends on the quality and physicochemical factors of water that hold a variety of effects on the biotic elements of an aquatic environment (Adeogun et al., 2005). The physicochemical parameters pH, EC, Temperature, DO, and TDS were monitored and checked at random intervals throughout the experiment and no significant differences were observed in the mean values of these parameters in control and treatment groups. The values obtained fell in the optimum range reported by Avdesh et al. (2012) for the maintenance of zebrafish in the laboratory.

4.3 Total Protein (TP)

Total protein (TP) content is a biomarker to critically analyze cellular disturbances caused in various organisms in response to chemical stressors in toxicological studies (Shahsavani et al., 2009). Changes in total protein levels can occur due to reduced capacity of protein synthesis, and over-consumption of nutritional values due to stressful cellular conditions as a result of a toxicity test (Patriche et al., 2011). In the current study, total protein levels declined upon singular and joint toxicity of sodium arsenite ($NaAsO_2$) and copper oxide nanoparticles (CuO-NPs). Previously, a study by Tuncsoy and Erdem (2021) reported reduction in total protein content in trout *Oreochromis niloticus* exposed to CuO-NPs. Furthermore, $NaAsO_2$ reduced total protein levels in zebrafish *larvae* (Perumal et al., 2021). Reduction in total protein levels can be attributed to the consumption of protein content as substitute energy source to meet energy requirements in exposed fish due to cellular destruction. The appetite is reduced in exposed fish that further reduces serum protein values (Raza et al., 2021).

4.4 Oxidative Stress

Evaluation of oxidative stress in zebrafish will help understand the toxicological pattern of $NaAsO_2$ and CuO-NPs. Reactive Oxygen Species (ROS) are redox products that initiate oxidative stress in cells upon exposure to chemical stressors. Through excessive accumulation of ROS in cells, an imbalance occurs in the antioxidant defense system that

further propagates oxidative stress in the organism (Schieber and Chandel, 2014). Malondialdehyde (MDA) is produced as a result of lipid peroxidation as lipids are easily oxidized by ROS causing damage to cellular structures such as mitochondria, endoplasmic network etc. (Lu et al., 2014). The increase in ROS levels and MDA content in an organism can cause toxic modifications to ribonucleic acids, proteins and lipids as free radicals therefore damaging cellular processes (Hallauer et al., 2016). In our study, the exposure of sodium arsenite ($NaAsO_2$) and copper oxide nanoparticles (CuO-NPs) caused a significant increase in the ROS levels and MDA content as compared to the control group. Arsenic is widely known to induce oxidative stress in various organisms. The significant increase in ROS levels and MDA content can be due to the continuous exposure to $NaAsO_2$ till the end of the exposure. Our results were in accordance with a study by Sarkar et al. (2014) in which Arsenite (AsIII) caused excessive oxidative stress in zebrafish brain through generation of ROS hence compromising antioxidant defense system in a time dependent manner. Moreover, upon exposure to arsenic trioxide, Indian catfish showed a significant increase in products of lipid peroxidation such as MDA (Bhattacharya and Bhattacharya, 2007). On the other hand, CuO-NPs caused significant increase in the treatment group compared to control group ($p<0.05$). Copper oxide nanoparticles have been reported to cause oxidative stress by disrupting cellular processes through generation of ROS and production MDA (Wang et al., 2013). The significant increase ROS and MDA indicated the production of toxic hydroxyl radicals ($^{\cdot}OH$) hence causing oxidative damage to cellular components (Yamakoshi et al., 2003). Similarly, CuO-NPs were found to have caused oxidative stress in adult zebrafish by increasing ROS levels and MDA content at low doses of 1 and 3 mg/l of CuO-NPs in a dose dependent manner (Mani et al., 2019). Likewise, CuO-NPs caused oxidative damage in the cellular components of zebrafish embryos by a significant increase in ROS levels and MDA content (Ganesan et al., 2015). The combined toxic exposure of $NaAsO_2$ and CuO-NPs showed a significant increase in oxidative stress than individual toxic groups hence causing synergistic toxic effect in zebrafish. Synergistic effect of copper and arsenic was observed in *Gallus gallus* upon exposure by increased oxidative stress (Sun et al., 2018). However, single exposure of arsenite caused increased MDA content in common carp which was alleviated by co-exposure of zinc (Wang et al., 2021). Our present study indicates that CuO-NPs promote $NaAsO_2$ oxidative stress in a

synergistic manner which can be attributed to the adsorption of arsenic on CuO-NPs (Zhang et al., 2023).

4.5 Antioxidant Response

Antioxidant defense system is initiated to counter increased ROS levels and MDA content in an organism (Zhu et al., 2008). It consists of enzymatic response including SOD, CAT and non-enzymatic response consisting of vitamin C and glutathione. These antioxidants scavange free radicals produced as one of the ROS species to lessen the eliminate the threat of oxidative damage (Melegari et al., 2012). SOD is released as the first defense line enzyme in response to the ROS species by converting the highly toxic free radical O_2^- into its less toxic form H_2O_2 which is further converted into water and molecular oxygen by CAT enzyme (Mani et al., 2019). The present study analyzed the enzymes SOD and CAT to evaluate the antioxidant response in zebrafish to oxidative stress induced by singular toxicity and joint toxicity of $NaAsO_2$ and CuO-NPs. In the singular exposure groups, SOD levels were slightly increased in the first interval of the experiment whereas it significantly decreased in the rest of the experiment compared to control group ($p<0.05$). However, CAT levels showed a significant decrease than control group throughout the experiment. Sayit Altikat et al. (2014) investigated the toxic effect of $NaAsO_2$ on antioxidant enzymes in mirror carp *(Cyprinus carpio)* and observed a decrease in SOD and CAT activities compared to control groups. A study on adult zebrafish chronically exposed to CuO-NPs showed a significant decrease in SOD and CAT levels compared to control group (Mani et al., 2019). Compared with SOD levels, CAT levels showed a different pattern showing that initially SOD primarily countered ROS by converting O_2^- into H_2O_2 but was overwhelmed by the continuous exposure of contaminants till the end of the experiment. Whereas the significant decrease in catalase activity could be a result of the overproduction of H_2O_2 exceeding its scavenging ability hance resulting in oxidative stress (Atli e t al., 2006). The same phenomenon was observed in a study by Adeyemi et al. (2015) in which arsenic was observed to reduce catalase activity in zebrafish embryos (Adeyemi et al., 2015). Arsenic significantly reduced CAT activity in zebrafish at low doses hence showing incapability of CAT to scavenge free radicals therefore promoting oxidative stress (Sunainna et al., 2016). On the contrary, enhanced CAT activity was observed in *D. rerio* exposed to sodium arsenite at its reference doses (Sun et al., 2020). These different

responses of antioxidant enzymes in different organisms are mainly because arsenic toxicity is dependent on species and their metabolism and detoxification in different tissues (Ventura-Lima et al., 2009). The combined exposure of $NaAsO_2$ and CuO-NPs was significantly lower as compared to control and singular treatment groups, therefore indicating synergistic effect in this case. The same phenomenon was observed in a study by Sun et al. (2018), a combined exposure of wild chicken *Gallus gallus* to copper sulfate ($CuSO_4$) and arsenic trioxide (As_2O_3) showed reduced activity of antioxidant enzymes compared to singular treatment groups. Inactivation of antioxidant enzymes can be attributed to the overwhelming production of ROS and MDA which is mostly observed during chronic toxicity tests resulting in gradual decrease of antioxidant levels (Zhao and Wang, 2011). Low amount of antioxidant enzymes adversely affects metabolic enzymes and causes cellular injury under oxidative stress (Raza et al., 2021).

4.6 Acetylcholinesterase Activity

Acetylcholinesterase (AChE) is a neurological enzyme which is found at nerve and postsynaptic junctions. The primary purpose of AChE is to degrade acetylcholine, a natural neurotransmitter, converting it into choline and acetic acid (Nayak and Patnaik, 2021). Environmental contaminants can adversely affect AChE activity by inhibiting it, which is commonly analyzed in toxicological research as a neurotoxicity biomarker. AChE inhibition causes neurotoxicity by accumulating acetylcholine in the synaptic region, hence disrupting the normal functioning of nervous system (Bertrand et al., 2000). In the present study, the AChE activity was evaluated in zebrafish brain exposed to singular and joint toxicity of $NaAsO_2$ and CuO-NPs. Moreover, the time-dependent inhibition of AChE activity in all treatment groups is evident in our study throughout the experiment in a significant manner as compared to control group. Previously, it has been reported in a research study by Kanungo et al. (2022) that $NaAsO_2$ significantly inhibited AChE activity in larvae of zebrafish in a dose-dependent manner. Furthermore, arsenic induced significant inhibition of AChE activity in iridescent shark *Pangasianodon hypophthalmus* (Kumar et al., 2020). Previous studies indicate that CuO-NPs are neurotoxic to adult zebrafish and zebrafish embryos as a study by Ganesan et al. (2015) reported that CuO-NPs reduced AChE activity in zebrafish embryos in a dose-dependent manner. Furthermore, Haverroth

et al. (2015b) reported the decline in AChE activity in zebrafish muscles but not in brain exposed to copper suggesting the reduction in muscles may be due to locomotor alterations. In line with our study, inhibition of AChE was reported in zebrafish exposed to CuO-NPs for 30 days which (Mani et al., 2019). According to Wang et al. (2021), the combined toxicological effects of arsenic and zinc in a chronic experiment observed reduced AChE activity in common carp *Cyprinus carpio* compared to control group however zinc alleviated the reduction AChE caused in the treatment group exposed to arsenic. Moreover, CuO-NPs and ZnO-NPs significantly inhibited AChE activity in goldfish *Carassius auratus* in a dose dependent manner (Xia et al., 2013). This is relevant to our study as our co-exposure group having $NaAsO_2$ and CuO-NPs showed synergistic effects.

4.7 DNA Damage by Comet Assay

DNA damage has been an important biomarker in ecotoxicological studies for many years which is assessed by using the Comet Assay. Oliveira et al. (2009) emphasized that toxic contaminants may directly cause DNA damage by the toxic action of parent chemical compounds or their metabolites or it is done indirectly through the production of ROS. For the detection of DNA single and double strand breaks, the Comet assay has been reported to be a sensitive, quick, and cost-effective assay that can readily employed for the testing of genotoxic parameters (Žegura and Filipič, 2019). The assay applies an electric field to the exposed DNA that causes the damaged DNA to move toward anode which is then analyzed by quantifying it (Muazzam et al., 2019). Our study observed an increase in DNA damage induced by the co-exposure treatment of $NaAsO_2$ and CuO-NPs as compared to the control group treatment. With enhanced oxidative stress in the singular treatment groups of $NaAsO_2$ and CuO-NPs, DNA damage was clearly higher than the control group with combined treatment group ($NaAsO_2$+CuO-NPs) showing the highest DNA damage than other treatment groups in a significant manner ($p<0.05$). In a previous study, $NaAsO_2$ significantly increased the comet tail DNA (%) in liver, and gills of tilapia *Oreochromis mossambicus*, indicating genotoxic potential of $NaAsO_2$ (Ahmed et al., 2011). Kousar and Javed (2014) exposed four different types of fish (*Labeo rohita, Cirrhina mrigala, Catla catla, Ctenopharyngodon Idella)* to arsenic and observed adverse genotoxic effects in peripheral blood erythrocytes of all fish species, such as increased tail lengths and damaged cells. Furthermore, exposure to arsenic reported increased tail moment in zebrafish

embryos, hence indicating genotoxicity due to increased oxidative stress (Adeyemi et al., 2015). In another study, CuO-NPs induced DNA damage with increasing concentration and exposure time in *Labeo rohita* (Aziz and Abdullah, 2023). Nanoplastics induced DNA damage in red blood cells of goldfish *(Carassius auratus*) upon chronic exposure (Brandts et al., 2022). TiO_2-NPs were reported to damage the DNA by breaking strands in rainbow trout *Oncorhyncus mykiss* through single exposure and combined exposure with UVA irradiation that was facilitated by induced oxidative stress (Vevers and Jha, 2008). Endosulfan caused significant DNA damage in a dose-dependent manner in zebrafish (Shao et al., 2012). The combined toxicity of nanoplastics and arsenic caused DNA damage in zebrafish brain due to enhanced ROS accumulation in mitochondria (Zhang et al., 2023). Furthermore, a study on combined toxicity of metal oxide nanoparticles concluded that exposure to CuO-NPs showed higher comet tail length as compared to ZnO-NPs in zebrafish. Whereas the same study reported that CuO-NPs and nanoplastics showed higher percentage of DNA in comet tail as compared to co-exposure of ZnO-NPs and nanoplastics (Singh et al., 2021). The same results were observed in the co-exposure treatment group of our study with the highest significant increase in tail length indicating highest DNA damage compared to other treatment groups.

4.8 Histopathological Alterations in Muscle Tissues

Histology is the assessment of tissues which involves examining tissue fragments under a microscope to have a detailed understanding of tissues. Whereas histopathology method is a reliable tool for microscopic assessment of tissues to evaluate toxicological effects of pollutants. Histopathological alterations are one of the best biomarkers of toxicological effects of chemical species (Mansouri et al., 2016). Previously it has been shown that animal tissues have the ability to accumulate heavy metals depending on dose and time. Aquatic organisms could accumulate heavy metals in the tissues of their skeletal muscles and gills upon exposure to xenobiotics (Annabi et al., 2011). Moreover, this method can elucidate injuries caused to skeletal muscles and evaluate them in a quantitative manner in affected organisms under exposure (Mansouri et al., 2017). The histological examination of fish shows a response to chemical stressors on a cellular level to contaminants in order to determine their health and well-being, therefore, it is an essential parameter for better

understanding of the toxicological impacts of contaminants (Azize et al., 2017). In zebrafish the gills are primary target of contaminants whereas muscles are the secondary target as they are not directly exposed to the contaminants (Ferrandino et al., 2022). The histopathological alterations of zebrafish skeletal muscles have a crucial role in evaluating the anomalies caused by environmental contaminants on muscle tissues (Azize et al., 2017). In our study, microscopic examination of skeletal muscle tissues exhibited significant histopathological alterations in singular and co-exposure groups as compared to the control group. Histopathological examination revealed significant disruption of muscle fiber, characterized by loss of striations, irregular fiber size, necrosis, apoptosis, and muscle fiber degeneration hence damaging histoarchitecture in exposed zebrafish. One of the key observations in our study is the presence of segmental necrosis in muscle fibers and the shortening and degeneration of fiber bundles in zebrafish exposed to $NaAsO_2$ and CuO-NPs in zebrafish. Ahmed et al. (2013) investigated histopathological alterations in tilapia *Oreochromis mossambicus* exposed to $NaAsO_2$ and reported necrosis, vacuolar degeneration, and oedema in the muscles of gills in both time and dose dependent manner. The gill tissues of zebrafish *Danio rerio* were adversely affected by low doses of $NaAsO_2$, which resulted in damage to the surface of epithelial cells through loss of mucus and desquamation (Sun et al., 2020). Our results were in line with a study by Mani et al. 2019 that exposed zebrafish skeletal muscles to CuO-NPs and reported defective muscle histology in a dose dependent manner. Similar findings were reported in zebrafish skeletal muscles exposed to cadmium showing necrosis and swelling of muscle fibers in time dependent manner concluding that the adverse histological effects had worsened as time progressed (Azize et al., 2017). Polystyrene nanoparticles caused histological lesions in gills, liver, and intestine of goldfish *Carassius auratus* (Abarghouei et al., 2021). Furthermore, titanium dioxide nanoparticles (TiO_2-NPs) caused significant damage to the histology of gills and intestine in common carp *Cyprinus carpio* (Mansouri et al., 2016). In another study on mosquitofish *Gambusia affinis,* cadmium caused histopathological alterations in gills, kidney, and liver in a time dependent manner (Annabi et al., 2011). Mansouri et al. (2017) assessed the combined exposure of TiO_2-NPs and CuO-NPs on histology of gills and intestine tissues of common carp *Cyprinus carpio* and concluded that TiO_2-NPs promoted the effects of CuO-NPs on the histopathological abnormalities by

causing necrosis, degeneration, and erosion, hence concluding a synergistic effect. These findings from the previous studies are in accordance with our results as histopathological anomalies were observed in all treatment groups, with synergistic effect in co-exposure treatment group showing the highest significant damage than the singular treatment groups.

Chapter 5

Conclusions and Recommendations

5 CONCLUSIONS AND RECOMMENDATIONS

The current study is the first one to examine the combined toxicity of sodium arsenite ($NaAsO_2$) and copper oxide nanoparticles (CuO-NPs) on zebrafish *Danio rerio*. This study explores the singular and joint toxicity of both contaminants to evaluate their toxic effects on living beings. The CuO-NPs were found to be in tenorite phase with monoclinic crystal system and slightly aggregated with size ranging between 50 and 100 nm. In the 28-day experiment period, assessments were made on total protein levels, oxidative stress markers ROS and MDA, and activities of antioxidant enzymes SOD and CAT in gills, AChE in brain, DNA damage in the tissues of liver, histopathological alterations in skeletal muscles at all the sampling intervals. The contaminants induced oxidative stress by significantly increasing ROS levels and MDA content. On the other hand, the activity of antioxidant enzymes SOD and CAT declined throughout the experiment due to continuous exposure. Moreover, both contaminants significantly reduced AChE activity and caused DNA damage. The singular and joint toxicity of both contaminants caused segmental necrosis in muscle fiber, degeneration and splitting of muscle fibre, atrophy, reduction in the size of muscle fiber and vacuolar degeneration. The observed toxicity effects in the current study can be attributed to increased oxidative stress leading to tissue damage, neurotoxicity, and genotoxicity. The combined exposure of both contaminants caused comparatively more toxicity than singular treatment groups, hence suggesting synergistic toxicity effect of $NaAsO_2$ and CuO-NPs. Sodium arsenite and copper oxide nanoparticles co-exist in water bodies and there is a lack of information on how these two contaminants interact with each other and cause toxicity to the exposed organisms. Nanoparticles are emerging pollutants due to their advanced use in various fields. Therefore, it is essentially important to not only study their toxic effects upon interactions with organisms but also their combined toxicity with other emerging pollutants should be examined in depth.

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Annexures

Annexure 1

Stock Solution Preparation for $NaAsO_2$ and CuO-NPs

- **$NaAsO_2$ Stock Solution** = 100 mg/L in tap water
- **CuO-NP Stock Solution** = 50 mg/L in tap water

1. Exposure Solution of *Sodium Arsenite* $NaAsO_2$ (300 µg/L in 15 Liters)

➢ **Preparation of a Stock Solution of 100 ppm**

$C_1V_1 = C_2V_2$

$100mg/L \times V_1 = 0.3mg/L \times 15000mL$

$1\cancel{00mg/L} \times V_1 = 0.3\cancel{mg/L} \times 150\cancel{00}mL$

$1 \times V_1 = 0.3 \times 150mL$

$V_1 = 45mL$

45mL from the stock solution will be added in the tanks to attain the final concentration of 300µg/L.

2. Exposure Solution of *Copper Oxide Nanoparticles* CuO-NPs (1mg/L in 15 Liters)

➢ **Preparation of a Stock Solution of 50ppm**

$C_1V_1 = C_2V_2$

$50mg/L \times V_1 = 1mg/L \times 15000mL$

$5\cancel{0mg/L} \times V_1 = 1\cancel{mg/L} \times 1500\cancel{0}mL$

$5 \times V_1 = 1 \times 1500mL$

$V_1 = 1500mL/5$

$V_1 = 300mL$

300mL from the stock solution will be added in the tanks to attain the final concentration of 1mg/L.

3. Mixture of Exposure Solutions comprising of $NaAsO_2$ + CuO-NPs (300µg/L+1mg/L) in 15 Liters

For $NaAsO_2$ (300µg/L)

$C_1V_1 = C_2V_2$

$100mg/L \times V_1 = 0.3mg/L \times 15000mL$

$1\cancel{00mg/L} \times V_1 = 0.3\cancel{mg/L} \times 150\cancel{00}mL$

$1 \times V_1 = 0.3 \times 150mL$

$V_1 = 45mL$

45mL will be added in the tanks to attain the final concentration of 300µg/L.

For CuO-NPs (1mg/L)

$C_1V_1 = C_2V_2$

$50mg/L \times V_1 = 1mg/L \times 15000mL$

$5\cancel{0mg/L} \times V_1 = 1\cancel{mg/L} \times 1500\cancel{0}mL$

$5 \times V_1 = 1 \times 1500mL$

$V_1 = 1500mL/5$

$V_1 = 300mL$

300mL from the stock solution will be added in the tanks to attain the final concentration of 1mg/L.

Annexure 2

Formulae for Calculations of AChE, Oxidative Stress and Different Antioxidant Enzyme by Mr. Muhib

Formula for Calculation of AChE:

Acetylcholine Esterase activity (AChE) = $\frac{(\mathbf{T}f - \mathbf{T}i)}{\mathbf{0.01}}$ = U/min

Whereas:

T_f = Final Absorbance

T_i = Initial Absorbance

0.01=Constant (Time Constant for 1 minute)

Total Protein Estimation:

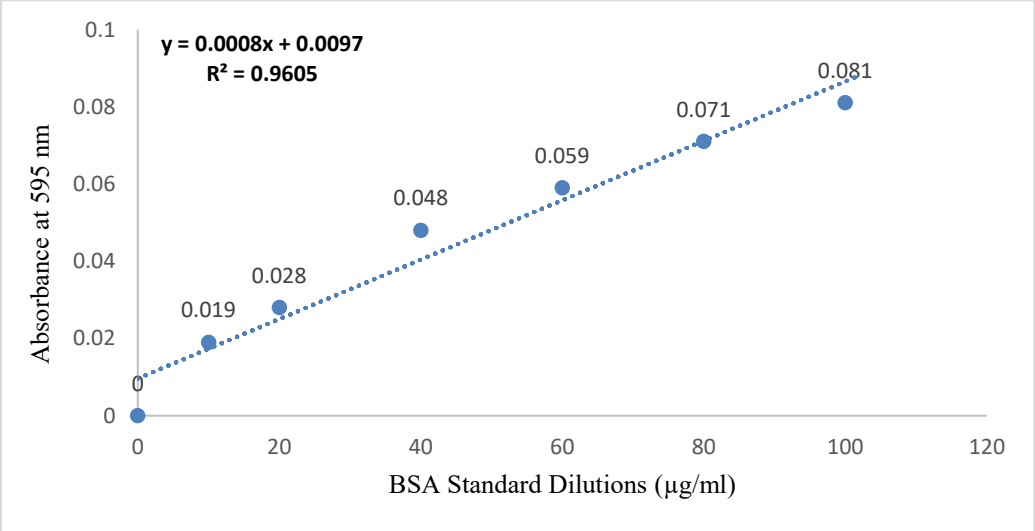


Figure: Standard Calibration Curve for Total Protein Estimation in *Danio rerio* gill samples at 595 nm.

Slope Equation: *Y=0.008X + 0.0097*

X = Y - 0.0097/0.008

While Y represents the samples' absorbance and X estimates the amount of total protein.

Formula for Calculation of ROS:

$$\text{Reactive Oxygen Species (ROS)} = \frac{(\boldsymbol{Sample\ OD} \times \boldsymbol{Cuvette\ Vol.} \times \mathbf{10\text{^}6})}{\boldsymbol{Protein} \times \boldsymbol{Sample\ Vol.} \times \Delta\boldsymbol{Time} \times \boldsymbol{Constant}\ \mathbf{15600}}$$

Units = nanomoles/mg protein

Where:

- Sample OD = Sample Absorbance
- Cuvette Volume = Volume of Cuvette
- Protein = Total Protein in Sample (mg/mL)
- Sample Volume = Volume of Enzyme Supernatant
- Time = Measurement Time
- 15600 =Extinction Co-efficient (M^{-1} cm^{-1})

Formula for Calculation of MDA:

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

Units = nanomoles/mg protein

Where:

- ΔSample = Change in Sample Absorbance
- ΔBlank = Change in Blank Sample Absorbance
- Cuvette Volume = 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:

$$\text{Superoxide Dismutase (SOD)} = \frac{((\Delta\text{Sample} - \Delta\text{Blank}k)\times \textit{Cuvette Vol.}\times 10\text{^}6)}{(\text{Protein} \times \text{Sample Vol.}\times \Delta\text{time} \times \text{Constant}(6.22))}$$

Units = U/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
- **Δ**time = Measurement Time
- 6.22 =Extinction Co-efficient (M^{-1} cm^{-1})

Formula for Calculation of CAT:

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

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
- **Δ**time = Measurement Time
- 43.1 =Extinction Co-efficient (M^{-1} cm^{-1})

Annexure 3

Calculation of Enzyme by Dr. Majid

Acetylcholinesterase (AChE) $= \frac{T3-T1}{0.01}$ = U/min

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

Reactive Oxygen Species (ROS) $= \frac{(Sample\ OD \times Cuvette\ Vol. \times Protein)}{Constant \times Sample\ Vol. \times Time \times 15600}$ = nM/mL

Malondialdehyde (MDA) $= \frac{(\text{Sample OD} \times Cuvette\ Vol. \times Protein)}{(Constant \times Sample\ Vol. \times Time \times 15600)}$ = nM/mL

Superoxide Dismutase (SOD) $= \frac{((\Delta\text{Sample} - \Delta\text{Blank}) \times Cuvette\ Vol. \times protein)}{(\text{Protein} \times \text{Sample Vol.} \times \text{time} \times 6.22)}$ = U/min

****

α-Amylase inhibition formula

% enzyme inhibition = [(ODs – ODn) ÷ (ODb – ODn)] × 100

ODs, ODn and ODb = absorbance value of sample, negative control and blank, respectively.

Trypsin inhibition formula

BAEE Units/mL enzyme $= \frac{\Delta A253nM/min(Test) - \Delta A253nM/min\ (Blank))(10.0)(df)}{(0.001)(0.10)(0.50)}$

Where:

df = Dilution factor

0.001 = The change in A_{253nM}/minute per unit of Trypsin at pH 7.6 at 25 ºC in a 3.2 mL reaction mix

0.10 = Volume (in milliliters) of Enzymatic Reaction Mixture used in step 7.4.5

10.0 = Total volume (in milliliters) of Inhibition Reaction in step 7.4.1

0.50 = Volume (in milliliters) of Trypsin

Exploring the Interactive Toxicological Impacts of Sodium Arsenite and Copper Oxide Nanoparticles on Zebrafish *Danio rerio* (H.)

Aqsa Huma and Mazhar Iqbal Zafar

Affiliations
Quaid-i-Azam University, Islamabad

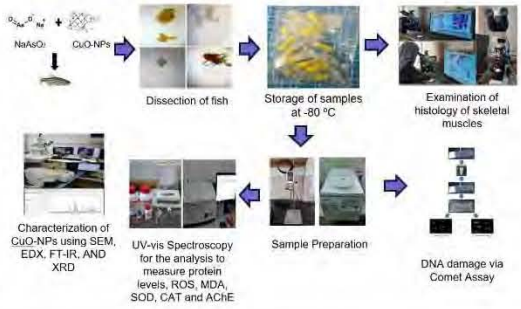
1 Introduction

- Sodium arsenite is widely known for its use as herbicide in agriculture and has been found to be highly toxic to exposed organisms.
- Copper oxide nanoparticles (CuO-NPs) are emerging pollutants that can affect the toxicological risks associated with the already existing contaminants after interacting with them.
- Nanoparticles due to their variety of unique properties can impact the toxicity of sodium arsenite when jointly exposed.
- Several research studies have reported the emergence of sodium arsenite and CuO nanoparticles in the aquatic environment
- The present study aims at evaluating the individual toxic effects of sodium arsenite ($NaAsO_2$), copper oxide nanoparticles (CuO-NPs) and their joint toxicity on zebrafish *Danio rerio*.

2 Objectives

- Assess the combined toxicity of $NaAsO_2$ and CuO-NPs on oxidative stress and activity of antioxidant response in zebrafish gills.
- Examine the activity of acetylcholinesterase (AChE) in brain.
- Analyze DNA damage in liver tissue of zebrafish through comet assay.
- Examine muscle damage by histopathological alterations in muscle tissue of zebrafish.

3 Methodology



4 Results

- Both contaminants significantly ($p<0.05$) increased ROS levels and MDA content, hence inducing oxidative stress in the exposed fish.
- The activities of SOD and CAT were inhibited significantly throughout the experiment.
- The exposure to both contaminants significantly decreased AChE activity in the brain and synergistic effect was observed in the co-exposure treatment group.
- The DNA damage was caused by both $NaAsO_2$ and CuO-NPs inducing increase in tail length and movement.
- Histopathological changes including segmental necrosis, degeneration and splitting of muscle fibers, necrosis and shortening of muscle fiber were observed in all treatment groups other than the control group at all sampling intervals.
- Co-exposure of $NaAsO_2$ and CuO-NPs induced greater toxic effect than singular action of these pollutants.

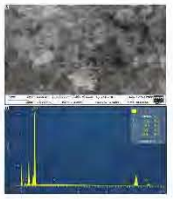


Figure 1 SEM-EDX of CuO-NPs

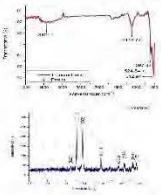


Figure 2: FT-IR & X-RD of CuO-NPs

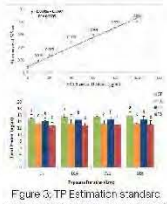


Figure 3: TP Estimation standard curve; TP levels during experiment

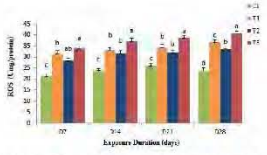


Figure 4: ROS levels in zebrafish gills during experiment

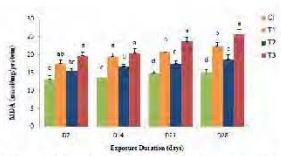


Figure 5: MDA conten. in zebrafish gills during experiment

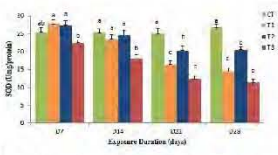


Figure 6: SOD activity in zebrafish gills during experiment

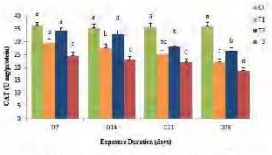


Figure 7: CAT activity in zebrafish gills during experiment

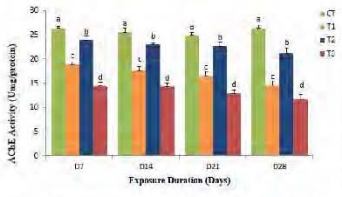


Figure 8: AChE activity in zebrafish brain during experiment

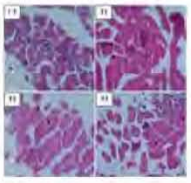


Figure 9: Histological alterations in muscle after 7 days

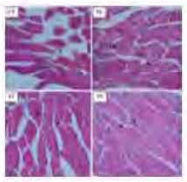


Figure 10: Histological alterations in muscle after 14 days

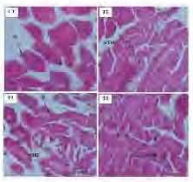


Figure 11: Histological alterations in muscle after 21 days

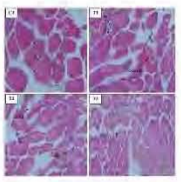


Figure 12: Histological alterations in muscle after 28 days

6 Conclusion

- The current study is the first one to examine the combined toxicity of $NaAsO_2$ and CuO-NPs on zebrafish *Danio rerio*.
- The CuO-NPs were found to be in tenorite phase with monoclinic crystal system and slightly aggregated with size ranging between 50 and 100 nm
- $NaAsO_2$ and CuO-NPs induced oxidative stress by increasing ROS levels and MDA content and declined activity of antioxidant enzymes SOD and CAT.
- Co-exposure resulted in reduced AChE activity, greater DNA damage and histopathological alterations in muscle segments.
- It is essentially important to not only study the toxic effects $NaAsO_2$ and CuO-NPs upon interactions with organisms but also their combined toxicity with other emerging pollutants should be examined in depth.

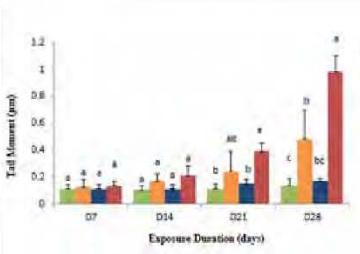


Figure 13: Tail moment in zebrafish liver during experiment

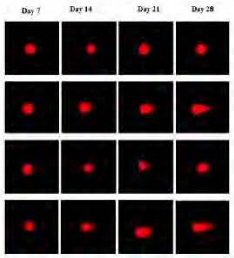


Figure 14: DNA damage in zebrafish liver during experiment

7 Acknowledgement

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Related Literature

- Kumari, B., Kumar, V., Sinha, A. K., Ahsan, J., Ghosh, A. K., Wang, H., and DeBoeck, G. (2016). Toxicology of arsenic in fish and aquatic systems. *Environmental Chemistry Letters*, 15(1), 43–64.
- Mani, R., Balasubramanian, S., Raghunath, A., and Perumal, E. (2019). Chronic exposure to copper oxide nanoparticles causes muscle toxicity in adult zebrafish. *Environmental Science and Pollution Research*, 27(22), 27358–27369.
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