# EFFECTS OF SELECTED NANOPARTICLES ON THE FRESHWATER FISH, *OREOCHROMIS MOSSAMBICUS* (PETERS, 1852)

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**DOCTOR OF PHILOSOPHY IN ZOOLOGY**

***Under the Faculty of Sciences***

**BY**


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## CERTIFICATE

This is to certify that Ms. Vidya Balakrishnan P.V. has carried out the research work embodied in the present thesis under my supervision and guidance for the full period prescribed under the Ph. D ordinance of this University. I recommend her thesis entitled "Effects of selected nanoparticles on the freshwater fish, *Oreochromis mossambicus* (Peters, 1852)" for submission for the degree of Doctor of Philosophy in this University.

I further certify that this thesis represents the independent work of the candidate under my supervision and no part of the thesis has been presented for the award of any other degree, diploma or associateship in any University.

**Dr. K. C. Chitra**
Supervisor

# DECLARATION

I hereby declare that the work presented in the thesis entitled "Effects of selected nanoparticles on the freshwater fish, *Oreochromis mossambicus* (Peters, 1852)" is a genuine record of research work done carried out by me under the guidance and supervision of Dr. K.C. Chitra, Assistant Professor, Department of Zoology, University of Calicut. To the best of my knowledge, no part of this thesis has been previously submitted for the award of any degree, diploma or associateship in any other University.

**(Vidya Balakrishnan P.V.)**
Candidate

C. U. Campus
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DEDICATED TO MY ANGELS.....

LOPAMUDRA AND RITUPARNA

# ABSTRACT

The toxic effects of selected nanoparticles namely aluminium oxide ($Al_2O_3$-NPs), iron oxide ($Fe_3O_4$-NPs), silicon dioxide ($SiO_2$-NPs) and titanium dioxide ($TiO_2$-NPs) were evaluated in the fish, *Oreochromis mossambicus*. Chapter 1 of the thesis determined acute toxicity of nanoparticles using Probit analysis, and the median lethal concentration or $LC_{50}$-96 h obtained was $Al_2O_3$-NPs - 40 mg/ L, $SiO_2$-NPs - 120 mg/ L and $TiO_2$-NPs - 164 mg/ L. Median lethal concentration was not found for $Fe_3O_4$-NPs, therefore, based on agglomeration the test concentration was selected as 150 mg/ L. In Chapter 2, the effects of selected nanoparticles on the antioxidant status of gill, liver and brain tissues were examined. One-tenth of $LC_{50}$ value was selected as sublethal concentration, and each nanoparticles were exposed for short-term (24, 72 and 96 h) and long-term (15, 30 and 60 days) durations maintaining the control group. The study revealed the induction of oxidative stress in all tissues but more profound effect was noted in gill tissues. Treatment withdrawal for 60 days showed that nanoparticles-induced oxidative stress was irreversible and permanent in gill, liver and brain tissues. Chapter 3 deals with histological examinations of gill, liver and brain tissues exposed to selected nanoparticles at sublethal concentrations. The results showed morphological alterations in the gill, liver and brain tissues of the fish where the severity of damages were increased with increase in exposure duration stating the effects of nanoparticles are time-dependent. Nanoparticles exerted permanent and irrecoverable pathological lesions in all tissues that were obvious after the treatment withdrawal. The genotoxic potential of selected nanoparticles were analysed in Chapter 4, which concluded that nanoparticles act as genotoxins, and exerted genetic damage and cytotoxicity both *in vivo* and *in vitro*.

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# CHAPTER 1

## Acute toxicity of selected nanoparticles in the fish, *Oreochromis mossambicus*

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| µg | Microgram |
| µm | Micrometer |
| $Al_2O_3$-NPs | Aluminium oxide nanoparticles |
| APHA | American Public Health Association |
| Au | Gold |
| cm | Centimeter |
| DNA | Deoxyribonucleic acid |
| E | East |
| $EC_{50}$ | Median effective concentration |
| $ED_{50}$ | Median effective dose |
| EPA | Environmental Protection Agency |
| E-Wastes | Electronic wastes |
| $Fe_3O_4$ | Magnetite |
| $Fe_3O_4$-NPs | Iron oxide nanoparticles |
| FeO | Wustite |
| g | Gram |
| *g* | Gravity |
| GC/MS | Gas chromatography /Mass spectrometry |
| h | Hours |
| HR-TEM | High Resolution Transmission Electron Microscope |
| $IC_{50}$ | Median inhibitory concentration |
| ICP/MS | Inductive Couples Plasma/ Mass Spectrometry |
| K | Potassium |
| kg | Kilo gram |
| KHz | Kilo hertz |
| L | Litre |
| $LC_{50}$ | Median lethal concentration |
| $LD_{50}$ | Median lethal dose |
| mg | Milligram |
| min | Minute |

| | |
|---|---|
| ml | Milliliter |
| mol | Mole |
| MRI | Magnetic Resonance Imaging |
| N | North |
| Na | Sodium |
| nm | Nanometer |
| NOEL | No Observed Effect Level |
| $^{o}C$ | Degree Celsius |
| OECD | Organization of Economic Co-operation and Development |
| P | Probability |
| Ppb | Parts per billion |
| ppm | Parts per million |
| ppt | Parts per trillion |
| r | Correlation coefficient |
| $SiO_2$-NPs | Silicon dioxide nanoparticles |
| $TiO_2$-NPs | Titanium dioxide nanoparticles |
| XRD | X-Ray diffraction |
| $\alpha$-$Fe_2O_3$, $\beta$-$Fe_2O_3$, | Hematite |
| $\gamma$-$Fe_2O_3$, $\varepsilon$-$Fe_2O_3$ | Maghemite |

# Introduction

## 1.1 Toxicology

Toxicology is the branch of science acknowledged as 'science of poisons' that focus on the adverse effects of toxicants on the living organisms. Basically, toxicology is defined as the study of the adverse effects of any chemical or physical agents that cause harmful effects on the animals, humans and the environment. On the other hand, toxicology is also referred as the 'science of safety' because it apply the power of science to predict the harmful effects of the toxicants, and shares the impact of toxic substances in order to protect the health status of the ecosystem. Toxicity of any physical or chemical agents is the relative ability of the compounds to cause some adverse effects in the living system. The term ability depend upon many factors such as dose, duration, frequency of exposure, route of exposure, composition of toxicant along with intra- and inter-specific variations, such as age, gender, health condition of exposed organism, and other environmental factors (Casarett and Klaassen, 2008).

Toxicants enter into the body through four possible routes namely ingestion, injection, dermal exposure and inhalation. Toxic compounds once entered into the body of organisms circulate through the blood stream and get translocated to different systems and organs. There are three possible fates of toxicants inside the body namely metabolism, storage and excretion. Many toxicants are metabolized or transformed into either highly toxic or less toxic compounds, which are stored to increase the persistence of the chemicals inside the body or excreted over a period of time, to reduce the toxicity. If the elimination rate is slow, then the chemicals may accumulate deleterious effects in the animals and cause damage to the target organs (Hayes and Kruger, 2014). Besides, the synergistic or antagonistic effects of single toxicant with other compounds also have profound effects on the properties of toxicants.

The dose-response relationship is the fundamental concept in toxicology, which is defined as the mathematically and biologically plausible correlation between the responses to a compound at certain dose over an exposure period, usually expressed as cumulative percentage (Moffett *et al.*, 2015). Dose-response relationship primarily depends on the exposure period and route, which is used to establish safe, hazardous and beneficial levels of any substances to organisms,

including human. The toxic responses or health effects caused by toxicants come under two categories, namely acute and chronic effects. Acute toxic effects are the quick response to a single chemical or brief exposure to a relatively high dose for a short period of time. However, chronic effects are caused by repeated or long-term exposure of toxicants to lower concentrations over a prolonged period. Acute toxic effects may be local or systemic whereas the health effects of chronic exposure may vary according to species and cell or tissue type (Casarett and Klaassen, 2008).

Thus the toxicity ranges from tissue or organ level, sometimes may extend to organism or genetic level thereby leading to severe damage to the entire ecosystem. Most of the toxicants have specific targets for their action, which may vary from fundamental molecules to organ systems. Organ level of toxicity impairs various systems including respiratory, immune, endocrine, digestive, cardiovascular, musculo-skeletal, central nervous system, renal and reproductive systems. Cellular or tissue level of toxicity includes interference with cell signalling pathway, metabolic energy inhibition, disruption in endocrine receptor function, enzymatic inhibition or induction, alteration in normal protein function, DNA cross linking formations, changing genetic code, degradation and fragmentation of genetic materials and proteins, destructing cellular integrity and so on. Adverse effects at molecular level include alteration in transcription, translation, specific cytoplasmic and nuclear receptor binding and the related modifications in the gene and gene products (Zhao and Liu, 2012).

The toxic effects within an organism are generally associated with the alterations in the tissue morphology, biochemistry, and physiology. However, many toxicants can induce cell death, cytotoxicity and DNA level damages, and based on the type of effects, the toxicants are categorized as teratogenic, mutagenic or carcinogenic. The toxic effects of compounds that lead to the congenital malformations are termed as teratogenicity. The toxicants that cause cellular or genetic level abnormalities are termed as mutagens or carcinogenic. Mutagens are an agent that forms sources of alteration in the genetic material that pass over generations, and may sometimes lead to carcinogenesis. At organism level, the toxic effects could impair several key organs such as liver, kidney, brain, lung, heart, blood, sense organs, gonads and other major organs. Thus it is difficult to ascertain

the effects of toxic compounds on particular factor alone as there are profound toxic testing strategies. Toxicants are the chemicals that contaminate various environmental compartments such as air, water, land, and the flora and fauna, and have potential to cause adverse effects on the health of animals, including humans, and the surrounding environment thus also referred as environmental contaminants.

## 1.2 Environmental contaminants

Environment consists of both abiotic and biotic components that interact continuously for the exchange of energy and materials for adaptation and survival. Environmental contaminants are the pollutants with undesirable properties, which are intentionally or deliberately introduced to air, water and soil by natural process or as a result of human activities. The rapid advancement in the field of technology, urbanization, improper agricultural practices, inappropriate waste management system are some of the factors responsible for the cause of environmental contamination faced by many countries worldwide. Contaminants can be categorized as physical, chemical or biological pollutants, ranging from oil spills, plastics, E-wastes, atomic wastes, biological wastes, industrial wastes to pesticides, chemicals and most recently added nanowastes. It is a well-known fact that most of the chemicals end up in the aquatic ecosystems due to inappropriate method of disposal in the wastewater treatment plants. Thus the quality of wastewater effluents are responsible for the spread of various waterborne diseases, decreased levels of dissolved oxygen, changes in water quality, release of toxic substances, bioaccumulation or biomagnification in aquatic life, and increased nutrient loads (Akpor, 2011).

Fish species are known to tolerant severe contamination in water at some extent but show a variety of morphological, biochemical, physiological, cellular, tissue or organism level modifications that appear to reflect the level of water pollution. Sometimes the uptake of certain toxicants by aquatic organisms like fish may be followed by metabolism of the toxicants into more toxic derivatives than the parent compound. In some cases, aquatic organisms attempt to concentrate toxic solutes from the environment without any apparent damages to themselves. Recently, ecotoxicological studies raise new concern on the possibility of

synthetic nanoparticles as environmental contaminant where the impacts on health and stability in local ecosystems are difficult to predict. Naturally-occurring nanoparticles possess several application in biological and geochemical processes whereas the mimic or synthetic nanoparticles though drawing attention in several fields makes distinction from natural nanoparticles because of the harmful effects on aquatic organisms (Nowack and Bucheli, 2007).

There are several types of nanoparticles that can harm aquatic faunas including plankton, bacteria, and fish in the aquatic ecosystem because they facilitate biotransformation reactions. Nanoparticles are released into the environment by anthropogenic activities including large-scale mining, burning fossil fuels or demolition, automobile traffic and so forth. Recently, metal and metal oxide nanoparticles have been generated as a result of mining and metal refinery operations thereby contaminate aquatic ecosystems. Nanoparticles-contaminated water that is discharged from the manufacturing units usually endures several purification processes, including mechanical filtering, settling treatments, digestion with microbes, and finally chemical disinfection process. Unfortunately, these treatment stages are not specifically designed to completely remove nanoparticles from the waste water effluents thus it may remain in the water even after the treatment for long period, and get released directly into the aquatic environment. Some nanoparticles sometimes remain in the leftover of microbe-bearing sludge released from the purification process and leach into soil in and around the landfill area or enter into ground water or other aquatic sources (Nel *et al.*, 2006). Thus exposure of nanoparticles into aquatic ecosystems through several sources may harm aquatic animals or humans, through the food chain.

## 1.3 Nanoparticles

Recent development in science and technology contributed the engineered nanoparticles to mankind. The term nanotechnology is essentially the science of understanding and controlling molecular materials in nano scale range between 0.1 - 100 nm. Nanoparticles are the particles having at least one dimension in nanoscale, most preferably less than 100 nm sizes. Nanoparticles are classified into three types based on the dimensions of a material. All nanoparticles are zero dimensional if all

the dimensions are measured within the nanoscale. In one-dimensional nanomaterials, one dimension is outside the nanoscale, which includes nanotubes, nanorods and nanowires. However, three-dimensional nanomaterials are not confined to nanoscale in any dimension and this class includes bulk powders, dispersion of nanoparticles, bundles of nanotubes, nanowires and multi-nanolayers (Oberdorster *et al.*, 2005). Nowadays, nanoparticles are used in different areas, such as cosmetics, pharmaceuticals, electronics, medical applications, and environmental processes. Due to the widespread applications, the production rate of nanoparticles has been expected to increase from 2,000 to 58,000 tons by the year 2020 (Maynard, 2006).

Increased relative surface area and quantum effects are the two principal factors that cause the properties of nanoparticles to differ from the bulk materials. These two properties enhance the strength, reactivity and electrical characteristics of nanoparticles. Reduction in the size of nanoparticles enable high proportion of atoms at the surface than those found inside which makes the particle more reactive than the same mass of material made up of larger particles. Similar to the surface area effects, the quantum effects of nanoparticles provides the electrical, optical and magnetic behaviour that was exploited in some nanoscale materials such as quantum dots and quantum well lasers. The shape of the nanoparticles may be spherical, tubular or irregular shaped and are categorized as natural or synthetic nanoparticles. The naturally occurring with geogenic or pyrogenic origin are the natural nanoparticles whereas the nanoparticles that are engineered or manufactured by using specific processes and released deliberately into the environment are called synthetic nanoparticles (Nowack and Bucheli, 2007). Based on the chemical composition, nanoparticles are further divided into organic and inorganic nanoparticles. Examples of organic nanoparticles include liposomes, dendrimers, carbon nanomaterials and polymeric micelles. Quantum dots, superparamagnetic iron oxide nanoparticles, gold nanoparticles, paramagnetic lanthanide ions are some examples of inorganic nanoparticles (Caruso *et al.*, 1998).

There are two fundamentally different approaches for the controlled synthesis and characterization of nano-based products namely bottom-up and top-down approaches. Bottom-up approach is characterized by the growth and self-

assembly of single atoms and molecules or it can be explained as the synthesis of nanoparticles from atomic level. In top-down approach, nanostructured materials are made from large uniform pieces of materials with the help of technologies like lithography and etching, which is used to reduce the size from bulk materials (Pomogailo, 2006). It can be assumed that the application of nanotechnology is advantageous to individuals and organizations as the newly produced nano-scale materials provide radically different properties and new phenomena, which are associated with very large surface area to volume ratios, and can be experienced with its dimensions and quantum-effects. Nano-based products have wide range of applications in the field of science, technology, and industrial as well as in consumer products. However, environmental exposure to such materials with phenomenal properties may pose risk to the exposed organisms.

### 1.4 Sources of nanoparticles

Nano-scale materials exist naturally in the atmosphere generated by volcanic eruptions, desert surfaces, and from dust and cosmic sources (Buzea *et al*., 2007). Carbon black or soot is the best example of fossil fuels and vegetation, which is the partial combustion product (Nowack and Bucheli, 2007). Volcanic eruption also produces nanoparticles into the environment by Aiken-mode nucleation process (Aiken, 1884). Minerals and ores of metals, forest fires, pollen fragments, and meteorites are the other few examples of the natural source of nanoparticles. Nano-scale materials are present in organisms like magnetotactic bacteria, mollusks, arthropods, fish, birds and even in human brain. Besides the natural sources, humans have created nanoparticles as by-products of simple combustion, chemical manufacturing, oil refining and smelting, combustions of treated sewage sludge, coal and fuel oil, vehicle and airplane engines and during welding process. These are collectively referred as nanoparticles of anthropogenic origin.

Newly designed particles and functionally modified particles widely used in semiconductors, metal oxides, carbon-based products, polymers, nanospheres, cosmetics, fabrics, electronics, optics, displays and in cleaning agents are the intentional sources of nanoparticles. Recently, some of the engineered nanoparticles are commonly used in sporting goods, stain-resistant clothing, tires, food additives,

sunscreens, toothpaste and so on, which constitute the environmental nanomaterials of recent health concern (Oberdorster, 2001). The nanoparticles that are released into the environment from the production and transportation sites, emissions or during the conversions of products forms the unintentional sources. Fumes and smokes from internal combustion engines, power plants, incinerators, jet engines, polymers, frying and fumes formed during welding, smelting are some examples of unintentional sources of nanoparticles. Nanoparticles, either natural or engineered that are released intentionally or unintentionally may reach the main three compartments of environment such as air, land and water. Thus the exposures to nanoparticles are not limited to targeted organisms, but may reaches the non-targets that include plants, animals and humans.

Nanoparticles that reach humans or other targeted or non-targeted organisms are of endogenous or exogenous origin. Nanoparticles that are derived endogenously are released from intestinal calcium and phosphate secretion in the gastrointestinal tract (Lomer *et al*., 2004). Exogenous sources are particles that are derived from outside the body such as water, food, pharmaceuticals, cosmetics, inhaled particles and dental prosthesis debris (Takenaka *et al*., 2001; Ballestri *et al*., 2001). It has been reported that the dietary consumption of nanoparticles in developed countries is estimated around $10^{12}$ particles per person per day (Oberdorster, 2004). The main particles that enter through food are titanium dioxide and mixed silicates as they are widely used as colourants and preservatives in food products and pharmaceuticals. Such nanoparticles do not undergo degradation in time and may accumulate in macrophages. Even a small fraction of inhaled nanoparticles pass into the gastrointestinal tract, which is highly influenced by the size, charge, surface chemistry, dose and duration of administration (Hoet *et al*., 2004). Nanoparticles of less than 14 nm size can cross the colonic mucous layer of gastrointestinal tract within two minutes, when the particle size is 415 nm it takes 30 minutes to cross the layer, and particles do not pass the barrier if the size is 1000 nm. After penetration through the mucous layer it reaches the enterocytes and then enters the lymphatic systems and capillaries. Translocation of nanoparticles also occurs through dermal, injection and by implants thereby making accessible to various organs in the body (Hoet *et al*., 2004).

## 1.5 Properties of nanoparticles

The exceptional property of nanoparticles is having the high surface area to volume ratio that makes even the simplest nanoparticles to have distinct surface chemistry from the core material. Engineered nanoparticles designed to interact with the biological systems possess suitable functional groups attached to the surface such as short chain peptides. Besides, the surface of nanoparticles may also be functionalised with a range of metal ions, small molecules, surfactants or polymers. Thus surface functionalization is critical to produce nanoparticles exhibiting unique properties for its applications in numerous consumer products (Oberdorster *et al.,* 2005). The second layer of the nanoparticles is called the shell that completely differs from that of the core material. Core is the centre of the nanoparticles usually referred as the nanoparticles itself that determine the fate and the environmental behaviour of the nanoparticles. Most of the researchers in physics and chemistry concentrate only on the core of the nanoparticles. Morphology is another distinct property of nanoparticles where the most common is the simple rod or wire, such as a carbon nanotube, however, tetrapod, tear drop, dumbbell and dendrite structures are readily available. The control over the shapes is related to the phase of the components of the system, and also related to changes in composition of the materials themselves (Mana *et al*., 2000). Surface characteristics like surface charge and area affect agglomeration of nanoparticles in dispersions, and its uptake and translocation inside the biological system (Hoshino *et al.,* 2004). The surface charge of nanoparticles has significant role in altering integrity and permeability of blood-brain barrier (Lockman *et al*., 2004).

Particle mobility, surface energy and colloid stabilization, optical and catalysis properties are some of the intrinsic properties of nanoparticles that determines the fate, behaviour and reaction in the environment. The size of nanoparticle is one of the important properties that decide various interactions like absorption, distribution, metabolism, and excretion in the biological systems (Borm *et al*., 2006; Choi *et al*., 2007). Nanoparticles generally form colloids or nanodispersions in aquatic media rather than solutions with solvents and may contain same particles of different sizes. Therefore, aggregation or agglomeration is the characteristic behaviour of nanoparticles that contribute to behave in different

way. Solubility and stability are the other properties utilized to evaluate the behaviour and utility of nanoparticles. Hydrophilic and well-dispersed nanoparticles generate more biological responses when compared to less dispersed ones. At the same time stability seems to be indirectly proportional to the toxicity where the particles with less stability are likely to be more toxic (Warheit *et al*., 2004). Apart from the all described and known properties of nanoparticles, sometimes they possess unexpected and unique properties on interacting to the biological system, which enable them to enhance the utility range as well as the growing concern regarding its toxicity.

## 1.6 Applications of nanoparticles

Nanoparticles have wide range of applications in almost all fields including biomedical, energy and electronics, consumer products, manufacturing, and in environmental perspectives. In biomedical field, the applications of nanoparticles are colossal, and the major applications include tissue engineering, MRI contrast enhancement (Weissleder *et al*., 1990), in dental and joint implants, probing of DNA structure (Mahtab *et al*., 1995), fluorescent biological labels (Bruchez *et al*., 1998), anti-bacterial material and immune system stimulant (Wang *et al*., 2002), for the detection of proteins, manipulation of biomolecules (Nam *et al*., 2003), targeted drug and gene delivery, drug discovery, adjuvant in chemotherapy, stem cell therapy, antioxidant in traumatic conditions, treatment of tumours, separation and purification of biomolecules (Salata, 2004). Applications of nanoparticles in electronics and energy includes photocatalyst, semiconductors, fuel cells, batteries, photovoltaic devices, storage devices in cell phones and computers, sensors, optical devices, power plants, solar cells, biofuels and artificial photosynthesis.

Nanoparticles have been used in numerous consumer products including cosmetics, paints, enamels, cleaning agents like detergents and soaps, pharmaceuticals, food products and preservatives, coating for self cleaning surfaces, textiles and clothing. In the environmental point of view, nanoparticles are used in the disinfection processes, photocatalysis and removal of organic pollutants, heavy metals, pathogens in water, phytoremediation and stabilization of the soil, treatment

of greenhouse gases, volatile organic compounds, and bioaerosols in air (Ibrahim *et al.*, 2016).

## 1.7 Nanoparticles in the environment

Above and beyond the applications of nanoparticles, there are many hazards for the environment right from the production site to the disposal of particles. Thus along with promoting the use and benefits of nanoparticles in various fields, proper measure should be taken to ensure that no harmful effects result from the use of nanoparticles. Nanoparticles can enter into the environment in three possible scenarios such as release during production of raw material and nano-enabled products, release during use, and discharge from untreated wastewater effluents (Tolaymat *et al.*, 2017). Direct or indirect emissions from the wastewater treatment plants discharge nanoparticles into the environmental compartments, mainly on soil, landfills, sediments, air and groundwater. Some of the nanoparticles undergo aggregation leading to the increased concentration in groundwater and soil. Global estimation of nanoparticles discharge have reported as 63-91% in the landfills followed by 8-25% in soil and 7% and 1.5% in aquatic environment and air, respectively (Keller *et al.*, 2013).

Risk assessment of nanoparticles in the environment mainly based upon the reactivity, mobility, ecotoxicity and persistent nature of the particles. Detection of environmental concentrations of nanoparticles in the natural ecosystem can be measured by several analytical methods and computational modeling. Concentration and size of metal-based nanoparticles can be determined by single particle inductively coupled plasma mass spectrometry or fractionation technique in combination with light scattering and elemental detection (Philippe and Schaumann, 2014). Nanoparticles evade most of natural barriers and may turn even reserved rare elements into ubiquitous ones. Prolonged exposure of organisms to nanoparticles may potentially lead to unforeseen health and environmental hazards. The organisms like algae, fungi, plants, prokaryotes, planktons etc., which are included in the primary consumer groups that interact immediately with the environment are the primary targets of nanoparticles exposure. Bioaccumulation of nanoparticles inside the body of the primary consumers enables the trophic transfer of nanoparticles

through the food chain and finally results in biomagnifications in higher organisms, including human (Cedervall *et al*., 2012).

## 1.8 Effects of nanoparticles in human

Although nanoparticles bring significant benefit to industry and consumers, it also creates potential health hazards to humans. Nanoparticles find way into human through several routes such as dermal, food, inhalation, and ingestion. Nanoparticles penetrate into the human skin by two possible mechanisms namely intercellular transepidermal mechanism or through simple diffusion on skin pores and hair cavities (Bennat and Muller-Goymann, 2000). Dermal exposure of nanoparticles mainly occurs in therapeutics, personal care products, occupational exposure, contact with medical devices or during clinical procedures (Borm and Kreyling, 2004). The main route of entry of nano-sized particles into the gut of human is through food and are translocated to different organs and tissues, leading to severe health effects such as oxidative damage and inflammatory reactions (Donaldson *et al*., 2004).

Many millions of organic and inorganic nanoparticles are inhaled and ingested by humans every day through food and drinking water. It was estimated that in every breathe people inhale around 10 million nanometre scale particles, and even many traditional foods such as dairy products are known to contain naturally occurring nanoparticles mainly as protein structures (Bouwmeester *et al*., 2009). Many studies have suggested that nanoparticles are more toxic than the equivalent bulk material of the same substance. This was evident by several adverse effects of nanoparticles on human such as antioxidant depletion and formation of free radicals, loss of cell viability, pro-inflammatory response, tumour formation, skin irritation, mutagenicity, genotoxicity and so on (Handy and Shaw, 2007). For instance, titanium dioxide nanoparticles induced oxidative stress and cellular damage followed by indirect induction of genotoxicity in human lung cells (Bhattacharya *et al*., 2009).

Nanoparticles exposure resulted in cytotoxicity by the induction of oxidative stress and DNA adduct formation in embryonic kidney cells (Wang *et al*., 2009), human lymphoblast and embryonic epithelial cells (Magdolenova *et al*., 2012). The

ability of nanoparticles to cross the blood-brain barrier is one of the advantageous in the pharmaceutical formulations, but simultaneously nanoparticles exert brain toxicity due to the surface charge modification (Lockman *et al*., 2004). Reduction in mitochondrial activity along with the induction of cytotoxic and genotoxic responses were observed in human erythrocytes and lymphocytes (Ghosh *et al*., 2013). However, the systemic toxicity of nanoparticles including acute and chronic toxicity and the consequences to human health is still limited, and only the cell line studies are available to extrapolate the results in human model. As human beings are at the top of food chain as the ultimate consumers, toxicants may enter through food or water as there is always a high chance of biomagnification. Among the aquatic food, freshwater fish forms an important part of diet to humans around the world. According to the handbook of Fishery Statistics 2014, in India, Kerala occupies third position in fish consumption where rural areas consume about 2.1 kg of fish per month and 1.9 kg of fish was consumed by urban area people. Thus any level of contamination that affects the fish population also known to cause significant effects on humans.

## 1.9 Nanoparticles in the aquatic ecosystem

Direct applications of nanoparticles to aquatic environment for remediation, direct disposal from point and non-point sources, accidental and unintentional spillage are the major routes for the entry of nanoparticles into the aquatic ecosystem. Besides, some, airborne nanoparticles also settle in terrestrial and aquatic ecosystem by wet deposition and gravitational settling. Run-off and leach out from contaminated terrestrial ecosystem could also be a significant source of water contamination (MacCormack and Goss, 2008). In aquatic ecosystem, they may settle in sediments or form dispersions with water, and undergo reactions and modifications with other organic and inorganic materials that are already present in water. The fate of nanoparticles in aquatic ecosystems depends on several properties such as agglomeration and aggregation, dissolution, redox reactions and transformation into new solid phases (Nowack and Bucheli, 2007). Nanoparticles are only weakly bound by agglomeration, whereas aggregation forms strong chemical bonds between particles (Jiang *et al*., 2009). Agglomeration of

nanoparticles can be determined by the medium composition, mostly at low pH, high ionic strength and the concentration of natural organic matter.

Solubility, stability and penetrance are the other factors responsible for the induction of toxicity in the aquatic organisms. Bioavailability is one of the important properties of nanoparticles that enable to modify the characteristics according to the surroundings. Engineered nanoparticles are bioavailable, and thus responsible for the induction of toxic responses in aquatic organisms (Johnston *et al.*, 2010; Behra *et al.*, 2013). Nanoparticles toxicity in aquatic ecosystems are affected by salinity, water chemistry, dissolved oxygen matter and ionic strength of water, which in turn affect the stability of nanoparticles dispersion, sedimentation processes and final size of nanoparticles aggregates (Brunelli *et al.*, 2013). In the aquatic ecosystem, organisms uptake the nanoparticles by two principal steps namely first by the uptake through environment-organism barrier, and second by translocation through the barrier tissue into the organism from where the internal distribution can prevail.

### 1.10 Effects of nanoparticles in aquatic organisms

Nanoparticles limit its accessibility in aquatic organisms to some extent due to the unique property of aggregation and poor water solubility (Maynard *et al.*, 2004). However, the occurrence and toxicity of nanoparticles inside the body of aquatic animals was reported in many studies suggesting the exceptional physico-chemical properties that facilitate for internalisation. However, studies using engineered nanoparticles in laboratory conditions have shown that nanoparticles are bioavailable and get bioaccumulated which then transferred from one trophic level to another (Pakrashi *et al.*, 2014). Adequate toxicity studies have evaluated the adverse effects of nanoparticles in different aquatic animal models ranging from invertebrates to vertebrates. In invertebrates, the most studied species was *Daphnia magna*, the crustacean, due to its feeding traits, general behavioural habits and position in food chain that forms an immediate food source for fish, which recognise as a relevant test organism (Baun *et al.*, 2008a). *Daphnia magna* is a filter-feeder where it filters water to catch algae in the size ranging between 0.4 and 40 µm where along with algae nanoparticles of size 0.4-40 µm also get ingested into the organism (Baun *et al.*, 2008b). Similar to *Daphnia magna* there are several other aquatic

invertebrates that ingest nanoparticles from water or sediments through multiple routes of exposure.

Functionalization and uptake of nanoparticles in fish is very important as it occupies the higher trophic level as a secondary consumer, and also as targeted model organism in aquatic ecosystem. Nanoparticles get accumulated in the food web through trophic transfer and the health hazards reported in fishes are remarkable (Gottschalk *et al.,* 2009). Uptake of nanoparticles in fish occurs through multiple routes such as by gill, engulping of water, dermal uptake and diet (Handy *et al.*, 2008). Trophic transfer of nanoparticles demonstrated more adverse toxic effects at higher level consumers than that of primary consumers showing biomagnifications of particles at each trophic level (Schwarzenbach *et al.*, 2002).

## 1.11 Selected nanoparticles for the toxicity tests

The present study focussed on four different nanoparticles namely three metal oxide nanoparticles - aluminium oxide ($Al_2O_3$-NPs), iron oxide ($Fe_3O_4$-NPs) and titanium dioxide ($TiO_2$-NPs), and the metalloid nanoparticles - silicon dioxide ($SiO_2$-NPs). Metal oxide nanoparticles have exceptional properties as they exhibit better durability, higher stability and selectivity, and also possess remarkable applications in drug delivery, diagnosis, water treatment, catalysis, semiconductors, cosmetics, sensing and solid oxide fuels (Corr, 2013). Metalloid nanoparticles possess characteristics of mixture of metals and non-metals, and have various biological effects on cells and tissues. All the nanoparticles selected are recognised as man-made and intentionally produced nanoparticles that could cause severe impact on the aquatic ecosystems. The properties and significance of the selected nanoparticles are discussed below:

### 1.11.1 Aluminium oxide nanoparticles ($Al_2O_3$-NPs)

| | | |
|---|---|---|
| Molecular formula | : | $Al_2O_3$ |
| Molecular weight | : | 101.96 g/mol |
| Purity | : | 99.2% |
| Size | : | 20-30 nm |
| CAS No. | : | 1344-28-1 |

Among the engineered nanoparticles, metal oxide nanoparticles have many important applications in various fields and are produced in large scales. $Al_2O_3$-NPs are most abundantly produced that account for about 20% of world production (Rittner, 2002). It is commonly called as nano-alumina and naturally occurs as crystalline corundum, which is widely used in jewellery and ornament industry. Engineered $Al_2O_3$-NPs are used as chemosensors, adsorbent in chromatography and removal of hydrocarbons impurities from air, sorbent in nuclear power plant, abrasive for polishing optics and jewellery, catalyst in chemical reactions, polymer modification, waste water treatment, biosensors, biofiltration, heat transfer fluids, nanoenergetics, diesel fuel additive and so on (Colvin, 2003; Prakash *et al.*, 2011).

### 1.11.2 Iron oxide nanoparticles ($Fe_3O_4$-NPs)

| | | |
|---|---|---|
| Molecular formula | : | $Fe_3O_4$ |
| Molecular weight | : | 231.53 g/mol |
| Purity | : | 97% |
| Size | : | 50-100 nm |
| CAS No. | : | 1317-61-9 |

Iron oxide nanoparticles was selected in the study mainly for two reasons: First, it occurs in the natural environment as a result of volcanic eruption, forest fire and so forth where it exist in different forms such as magnetite ($Fe_3O_4$), hematite ($\alpha$-$Fe_2O_3$), $\beta$-$Fe_2O_3$, maghemite ($\gamma$-$Fe_2O_3$), $\varepsilon$-$Fe_2O_3$ and wustite (FeO). Second reason which attracted to the study is due to the unique properties such as superparamagnetism, greater surface area, surface-to-volume ratio, and bioengineering, industrial and commercial applications. The main applications include magnetic resonance imaging, gene and drug delivery, hyperthermia, molecular imaging, *in vitro* bioseparation, cellular labelling, protein immobilization, removal of metals from aqueous solutions and environmental remediation (Sun *et al.*, 2016). As the iron oxide nanoparticles are persistent in nature, they may enter into aquatic ecosystem as effluents from wastewater treatment plants, spillage during production, transportation and usage, and deposition from air (Garner and Keller, 2014).

### 1.11.3 Silicon dioxide nanoparticles ($SiO_2$-NPs)

| | | |
|---|---|---|
| Molecular formula | : | $SiO_2$ |
| Molecular weight | : | 60.08 g/mol |
| Purity | : | 99% |
| Size | : | <100nm |
| CAS No. | : | 7631-86-9 |

Silicon dioxide nanoparticles are commonly known as nano-silica, which exists in two forms namely crystalline and amorphous. Quartz and porosil are the best examples for crystalline silica, in which quartz may be natural or manmade, but porosil is completely manmade. Amorphous silica can be natural resembling opal and silica glass or manmade. $SiO_2$-NPs are synthesized in large scale for various biomedical and industrial applications including targeted drug and gene delivery, biosensors, cancer therapy, enzyme immobilization and in nano-agroproducts (Barik *et al*., 2008). $SiO_2$-NPs attracted great attention in the present study for the wider plausible applications in nanomedicine, polishing, varnish, cosmetic and food industries. However, the targeted as well as the passive exposure to nanoparticles also reported to have undesirable biological and toxicological responses in organisms (Oberdorster *et al*., 2005). The toxicity, tissue distribution, metabolism, mode of action and excretion of $SiO_2$-NPs depends on the route, duration, size, solubility and reactivity of nanoparticles (Kim *et al*., 2015).

### 1.11.4 Titanium dioxide nanoparticles ($TiO_2$-NPs)

| | | |
|---|---|---|
| Molecular formula | : | $TiO_2$ |
| Molecular weight | : | 79.87 g/mol |
| Purity | : | 99.5% |
| Size | : | < 100nm |
| CAS No. | : | 13463-67-7 |

$TiO_2$-NPs is the one among the most discussed nanoparticles in recent nanotoxicological research, which exist as both natural and manmade nanoparticles. Naturally, $TiO_2$-NPs exist as ores and minerals in three forms called anatase, rutile and brookite. Manmade nanoparticles are more reactive in biological system as it is engineered for several targeted uses (Sharma, 2009). It is widely used in various

consumer goods and products of daily use such as cosmetics, dispersion paints, dyes and varnishes, textiles, toothpaste and drugs, paper and plastics, food additives, wood preservatives and even as photocatalyst. Earlier $TiO_2$-NPs have been used as negative control and considered as non-toxic in both *in vitro* and *in vivo* toxicological studies. However, the concept was challenged by numerous toxic reports addressed in various cell lines, mammalian models, aquatic invertebrates, fish and other vertebrates. Wide range of applications confers suspected environmental release and possible potential health risk to humans, live-stocks and the entire ecosystem. The present study was also focused to evaluate the adverse effects of $TiO_2$-NPs in the exposed fish at sublethal concentration.

### 1.12 Fish as model organism

Model organisms are non-human species widely bred and maintained in laboratory condition, having particular experimental advantages to understand biological processes (Hunter, 2008). For the past few decades, fish is widely used as ecotoxicological model, as it is sensitive and respond to diverse groups of contaminants in the aquatic ecosystem (Naigaga *et al.*, 2011). Range of fish model systems includes zebrafish, rainbow trout, cod, Atlantic salmon, killi fish, puffer fish and several others. Since 1950s, zebrafish has been used as toxicological model to study carcinogenesis, developmental biology and genetics. It is followed by Medaka, rainbow trout and fathead minnow, among which, the latter was chosen by the Environmental Protection Agency for standardized tests of acute, chronic, early-life, life cycle and endocrine system toxicity as a valuable model (Ballatori and Villalobos, 2002). Nowadays, fish models are widely accepted in toxicology but still a question of why the use of fish models when there are sufficient and perfect rodent models arise among a very few researchers in toxicology. Thus it is noteworthy to justify the response before any ecotoxicological research was conducted in fish models.

One of the reasons are fish occupy an important place in human food chain, so any negative effects on fish population may directly influence the human as well as other depending organisms and the entire ecosystem. Another advantage of fish model is the high rate of fecundity, easy handling and well-described immune,

nervous, endocrine, circulatory and osmoregulatory systems (Song *et al.*, 2012). Moreover, fishes are exposed to mixtures of chemicals in the natural environment, therefore, effects of multiple chemicals on laboratory study seems more reliable in fish model system. More recently, tilapian fishes substitute other freshwater fishes as toxicological model. In the present study a tilapian fish, *Oreochromis mossambicus* is used as model organism in studying the toxicological effects of nanoparticles.

### 1.12.1 *Oreochromis mossambicus* (Peters, 1852) – an ideal model

*Classification:*

| | | |
|---|---|---|
| Kingdom | : | Animalia |
| Phylum | : | Chordata |
| Subphylum | : | Vertebrata |
| Class | : | Pisces |
| Subclass | : | Teleostomi |
| Super order | : | Acanthopterygii |
| Order | : | Perciformes |
| Family | : | Cichlidae |
| Subfamily | : | Pseudocrenilabrinae |
| Tribe | : | Tilapiini |
| Genus | : | *Oreochromis* |
| Species | : | *mossambicus* |

*Oreochromis mossambicus* is an important invasive species commonly called as Tilapia, native to southern Africa, the Mediterranean, and the Middle East. Tilapia is well recognized as capture fish and culture fish, as they occupy ninth position as an important aquaculture species worldwide, which are also widely introduced to several tropical and subtropical countries. Tilapia can be cultured in either fresh or salt water in tropical and subtropical climates mainly for its nutritious flesh. Tilapia is the second most important group of wild-captured fish, after carps, with a global capture and harvest reaching 769,936 metric tonnes from 2007. In many countries, it was cultured to control mosquitoes, which then compete with the

native species for food and space, and eventually become the most abundant species of that region.

The body of tilapia are laterally compressed with long dorsal fins, and their robust nature helps them to survive in slow flowing as well as in fast flowing water bodies. They are generally considered as freshwater species, but can tolerate salinity up to 25 ppt so that also inhabit in brackish water (Suresh and Lin, 1992). Tilapia are omnivorous and filter feeder, where it consume variety of food ranging from small algae and diatoms to small invertebrates, eggs and fries of other fish and rooted plants (Fryer and Iles, 1972). Tilapian fish exhibit cannibalism on overcrowding with the same or different species in order to meet their nutritional requirements. They exhibit high parental care and are recognized as maternal mouth brooders where male excavate nest during spawning and attract female towards the nest in which the female lay her eggs. The male fertilizes the egg and female carry the eggs in her mouth in expense of her health and fitness. The male guards the nest and this high level of parental care and social organization are critical behind the reproductive success and invasive tendencies.

Tilapia is a rich source of protein, phosphorus, potassium, selenium, niacin, vitamin B-12, omega-3 fatty acids, and low in saturated fat, calories, carbohydrates and sodium. Tilapia is known to resist diversity of pollutants in the natural ecosystem hence it is widely used as model organism to monitor the sublethal effects of diverse environmental contaminants. The present ecotoxicological study used *Oreochromis mossambicus* as an ideal model for studying effects of nanoparticles using several endpoints such as acute toxicity, antioxidant defense system, histopathological changes and genotoxicity in laboratory condition.

### 1.13 Acute toxicity studies

Toxic potential of any toxicant, either natural or synthetic, can be determined by various toxicity evaluation tests. Organization of Economic Co-operation and Development (OECD), an international agency, design, standardize and approve all toxicity evaluation procedures. OECD has several guidelines for testing the toxicity endpoints in terrestrial and aquatic organisms either in time-response or dose-response manner. Acute, sub-acute and chronic toxicity are the commonly

performed methods for determining the toxic responses of organisms. Acute toxicity is the preliminary test carried out for the initial screening of toxicity of an unknown chemical (Lorke, 1983). Acute toxicity provides the immediate effects, hazards and lethality of chemicals to the test animal either exposed in a single dose or in multiple doses. Commonly used acute toxicity indices as per OECD guidelines are median lethal dose ($LD_{50}$) and median lethal concentration ($LC_{50}$). $LD_{50}$ and $LC_{50}$ are the values that indicate the amount of toxicant required to kill half (50%) of the test population at specific period of time, usually 96 h (Finney, 1971). In case of terrestrial animals, $LD_{50}$ is commonly used for determining toxicity in which the definite dose of chemical or toxicant is administered to the test animal by several modes such as dermal, injection, oral or nasal applications. In this case, amount of toxicant given to the test animal is known and are expressed as dose in microgram or milligram per kilogram body weight.

In some terrestrial animals and aquatic animals, acute toxicity is also determined by median lethal concentration, $LC_{50,}$ in which the test animals are exposed to toxicant-mixed medium (Brungs and Mount, 1978). In case of $LC_{50}$ the amount of chemical taken by the animals remains unknown and the concentration present in the medium can only be acknowledged. The $LC_{50}$ values are usually expressed as microgram/ litre (ppb) or milligram/ litre (ppm). $LC_{50}$ values and toxicity are inversely proportional, which means lower the $LC_{50}$ value for a tested chemical represent higher toxicity to the test animal. Besides $LD_{50}$ and $LC_{50}$, other indices used for determining acute toxicity are median effective dose ($ED_{50}$), median effective concentration ($EC_{50}$) and no observed effect level (NOEL). $ED_{50}$/ $EC_{50}$ is the dose or concentration of a chemical required to elicit effective toxic responses in half (50%) of the tested population (Holt *et al.*, 2002). NOEL is the exposure level of compounds which does not exhibit any statistically or biologically significant toxic responses in the exposed groups when compared to the unexposed control groups (Dorato and Engelhardt, 2005). Acute toxicity data are commonly used to find the lethal or responsive dose of a compound from which the sublethal dose can be selected for further toxicological studies. Chronic toxicity studies make use of sublethal concentration of toxicant exposed for long-term durations. Acute toxicity usually result in sudden responses as death or lethality and immobilization, but the

endpoints of chronic exposure include changes in growth, behaviour, reproduction, and development of pathological symptoms, loss of function of organs and even carcinogenesis (Pope *et al*., 2002).

## 1.14 Objectives of the study

In the present study both short-term and long-term effects of selected nanoparticles are evaluated at sublethal concentration of selected nanoparticles. The objectives selected for present study steadily followed the approved guidelines for fish acute toxicity, OECD-203 (OECD, 1992) and the objectives are as follows:

1. To study the effects of selected nanoparticles on the antioxidant status and lipid peroxidation in gill, liver and brain tissues of the freshwater fish, *Oreochromis mossambicus*.
2. To assess the histopathological changes induced by the selected nanoparticles.
3. To evaluate the genotoxic potential of the selected nanoparticles in the fish.

The above objectives are discussed in different chapters as follows:

Chapter 2: Impact of the selected nanoparticles on the antioxidant defense system of the fish, *Oreochromis mossambicus*.

Chapter 3: Histopathological changes in the fish, *Oreochromis mossambicus* exposed to the selected nanoparticles.

Chapter 4: Evaluation of genotoxic potential of the selected nanoparticles.

# Review of Literature

There is an increasing concern that anthropogenic materials released from domestic, urban, agricultural and industrial byproducts into aquatic bodies without any safety measures raise risk of health hazards in aquatic organisms. Recently, advances in nanotechnology resulted in the continuous deposition of nanoparticles into the environment, which was being newly added in the list of environmental pollutant that deserve special attention. An alarming fact that concerns ecotoxicologists are the production of engineered nanoparticles has been expected to rise by 58,000 tons during 2011-2020 (Maynard, 2006). In order to extrapolate the existing toxicological data on the unexplored nanoparticles, comprehensive reviews on toxicological classification and adverse effects of the nano-scale products on organisms is necessary. Lack of data on the toxicological studies of nanoparticles in the biological system has made researcher to pave great attention in the area of nanotoxicological research. Some of the nanoparticles are known to occur in nature, while others are the engineered nanoparticles. Both natural and engineered nanoparticles have been released continuously into various environmental compartments as soil, air, water and sediments. Accumulation of nanoparticles has been documented in some non-targeted aquatic organisms such as blue green algae, fish and other aquatic vertebrates (Navarro *et al*., 2008; Handy *et al*., 2008)

The ability of nanoparticles to spread in the environment or penetrate into the organisms depends on its unique structure and modification. Moreover, nanoparticles have been shown to be more toxic than its equivalent micron-sized particle at similar doses or concentrations (Colvin, 2003). The mechanism for cellular entry of nanoparticles follow three routes namely endocytosis, diffusion and through ion channels. Nano-sized particles have been reported to cross the cell membrane by receptor mediated endocytosis, the process by which the transport of materials across the membrane as cargo enveloping inside lipid bilayer forming a vesicle (Chan and Nie, 1998). Diffusion is the mechanisms by which nanoparticles enter into cell passively across the cell membrane and the peculiar properties of nanoparticles have been known to undergo diffusion (Verma *et al.,* 2008; Nel *et al.,* 2009). Entry of nanoparticles into the cell by the ion channels mediated through receptor proteins occurs for a certain extent. In case of prokaryotes, like bacteria, lack structures for the bulk transport of supramolecular and colloidal particles across

the cell wall and thus the entry of nanoparticles occur by simple diffusion (Torchilin, 2009). In aquatic animals, the routes of entry of nanoparticles are known to occur through ingestion, direct passage across gills and external surface epithelia (Dowling, 2004; Warheit, 2004). Several experiments on the toxicity of engineered nanoparticles have been demonstrated in aquatic organisms.

There are a number of methodologies adopted to evaluate the complex issues of nanoparticles in the environment and the organisms. The Organization for Economic Co-operation and Development (OECD) along with other national and international organization had developed guidelines to investigate the impact of nanoparticles on the environment and health. Acute toxicity test is one of the most widely accepted protocols for determining the toxic potential of any substances, including nanoparticles, on any organisms (Finney, 1971). Most of the acute toxicity studies conducted determines the effects of a single toxicant on an animal in order to address the toxicity of that particular test substance. Acute toxicity tests follow an unusual protocol in which repeated dose toxicity with the predetermined doses of test substances was conducted sequentially with an interval of 24 h of dosing between subsequent groups so as to reduce the use of number of animals (Gad, 2014). Another remarkable aspect of acute toxicity studies has been illustrated by sustaining the animal without food prior to dosing, which allows maintaining the dose of test chemical within the body of organisms so as to make biochemical or functional balance to the exposed animal (Weingand *et al.*, 1996). The results of well-designed acute toxicity study serve to determine the serious toxicological effects of the compound because it determines mortality as the endpoint.

The toxicity of nanoparticles depends on physical and chemical characteristics, in particular, chemical composition such as diameter and shape, high area or surface ratio, and the possible state of aggregation or agglomeration (Nel *et al.,* 2006). The toxic responses of nanoparticles has been extensively studied in rat models, however, acute toxicity studies in mammalian models remains scanty. Acute toxicity of copper nanoparticles of 23.5 nm size has reported 413 mg/ kg as the $LD_{50}$ value in mice along with other adverse effects such as heavy injuries on kidney, spleen and liver tissues thereby discussed that the large surface area, ultrahigh reactivity, exceeding consumption of hydrogen ion as the cause of grave

nanotoxicity (Chen *et al.*, 2006). Administration of nano-sized titanium dioxide particles of 25 and 80 nm sizes has reported no obvious acute toxicity in adult mice, however, a fixed large dose of 5 g/ kg body weight has been shown to undergo massive effects on serum biochemical parameters and also showed biodistribution in liver, spleen, kidney and lung tissues (Wang *et al.*, 2007). Median lethal dose or $LD_{50}$-24 h of silica nanoparticles of 20 and 50 nm size has been observed as 80.2 µg/ ml and 140.3 µg/ ml, respectively in human embryonic kidney cells (Wang *et al.*, 2009). Besides the acute toxicity tests of nanoparticles in rodents, in one of the *in vitro* studies of silica nanoparticles with 15 and 30 nm size treated on HaCaT cells has demonstrated 50 percent inhibition in cell growth ($IC_{50}$) values as 23 and 27.3 µg/ ml, respectively (Yang *et al.*, 2010). Thus several mammalian *in vivo* and *in vitro* studies conducted in laboratory established that the engineered nanomaterials have been shown to cause a wide variety of toxic effects.

The toxicity of nanoparticles has been reported in aquatic organisms. In the green algae, *Desmodesmus subspicatus* the concentration-effect relationship of titanium dioxide nanoparticles of 25 nm size has been reported as 40 mg/ L, which was size-dependent where the large particles of 100 nm size were found to be less toxic (Hund-Rinke and Simon, 2006). Nanoparticles exposure has been associated to induce ecological imbalance, which was evident by the toxicity of double-walled nanotubes on earthworm, *Eisenia veneta* by impairing nutrient and reproductive cycle (Scott-Fordsmand *et al.*, 2008). The bioaccumulation and acute toxicity has been reported in *Chlamydomonas reinhardtii*, green alga, and *Daphnia magna* treated with zinc oxide nanoparticles and fullerene $C_{60}$ even at the low concentration of 1ppm (Luo, 2007). Nanosilver and nanocopper has been shown to cause toxicity in aquatic organisms as zebrafish, daphnids, and an algal species with 48-h median lethal concentrations as low as 40 and 60 µg/ L, respectively, in *Daphnia pulex* adults, whereas titanium dioxide did not caused toxicity in any of the tests. The study confirmed that the susceptibility to nanometal toxicity has been shown to differ among species, where the filter-feeding invertebrates being markedly more susceptible to nanometal exposure when compared with zebrafish (Griffitt *et al.*, 2008).

The acute toxicity of nano-sized titanium dioxide, boron nanoparticles, and two types of aluminum nanoparticles namely ALEX and L-ALEX has been evaluated using Microtox toxicity test and the acute toxicity test with *Daphnia magna* reported titanium dioxide with low toxicity. However, 50% effective concentration ($EC_{50}$) of boron nanoparticles has reported to range between 56 to 66 mg/ L and classified as harmful to aquatic microorganisms than that of titanium dioxide and boron nanoparticles (Strigul *et al*., 2009). Nanoparticles once when entered into the aquatic environment has been known to cause harmful effects on aquatic organisms, including fish (Owen and Depledge, 2005). Nanoparticles have been shown to enter into the fish mainly through the epithelial surfaces such as gills and skin, or by direct ingestion through engulping of water (Moore *et al.,* 2006). In an ecological perspective, nanoparticles have the ability to bioaccumulate in organisms and undergo biomagnifications through different trophic levels so as it readily reaches human (EPA, 2007). The environmental concentrations of nanoparticles has been estimated around $10^{-5}$ to $10^{1}$ µg/ L concentrations for surface waters and the concentrations of $10^{-2}$ to $10^{4}$ µg/ L for aquatic sediments and the predicted concentrations are expected to increase based on the continuous discharge (Gottschalk *et al*., 2009).

Acute toxicity of copper nanoparticles in zebrafish for 48 h has been determined as 1.5 mg/ L where rapid aggregation of nanoparticles have been observed after suspension in water producing different histological changes and patterns of gene expression in the gill tissue (Griffitt *et al*., 2007). $LC_{50}$ value of nanosilver for 48 h in zebrafish has been reported as 7.07 mg/ L concentration (Griffitt *et al*., 2008). Titanium dioxide nanoparticles exposed to rainbow trout for 14 days showed no haematological disturbances, however, erosion on the intestinal epithelium and respiratory distress has been reported (Federici *et al.,* 2007). In another study, dietary exposure of titanium dioxide nanoparticles at 10 and 100 mg/ kg doses for 8 weeks has been observed with particle accumulation in gill, liver, gut, brain, and spleen without affecting the growth rate and haematological parameters (Ramsden *et al.,* 2009). Median lethal concentration of silver nanoparticles for 24 h in zebrafish has been reported as 250 mg/ L showing induction of oxidative stress and apoptosis in the liver tissue (Choi *et al*., 2010).

The acute toxicity of nano-silver, 81 nm size observed for 48 h in zebrafish under static renewal study has reported median lethal concentration or $LC_{50}$ value for nano-silver and silver ions as 84 and 25 µg/ L, respectively. The observations also revealed the increased rate of operculum movement and surface respiration after nanoparticles exposure thereby suggested respiratory toxicity in zebrafish (Bilberg *et al.*, 2012). A comprehensive study on the acute toxicity of 31 different nanoparticles has been conducted in adulthood and early life stages of zebrafish, *Danio rerio* using evaluation of 48 h and 96 h $LC_{50}$ values. The report suggested that six kinds of nanoparticles such as calcium oxide, copper, magnesium oxide, copper in the form of oxide, copper zinc iron oxide, and nickel resulted in cumulative mortality. However, acutely toxicity or $LC_{50}$ value of copper nanoparticles in zebrafish has been observed as 1-1.5 mg/ L concentrations whereas the toxicity of aluminium, cobalt and titanium dioxide nanoparticles was comparatively less to adult zebrafish. The study also revealed that 48 h and 96 h lethal concentration or $LC_{50}$ values of nanosilver as 2.9 mg/ L (Kovriznych *et al.*, 2013). Administration of titanium dioxide nanoparticles of 10-30 nm size at waterborne levels of 10 and 100 mg/ L concentrations has been shown to increase the uptake and organ level accumulation of nanoparticles in gold fish, *Carrasius auratus* as evidenced by impaired growth, metabolism and physiology (Ates *et al.*, 2013). Similarly, fish exposed to 2.5, 5, 7.5, 10, 12.5 mg/ L concentrations of nano-titanium dioxide has been shown to exhibit respiratory damage as well as depletion in total protein and glycogen in zebrafish (Vutukuru *et al.*, 2013).

Nano-silicon dioxide of 15 and 50 nm size exposed at concentration gradient of 50, 200, 350, 500, 650, 800 and 1000 µg/ ml for 3 days showed lowest observed effect concentration (LOEC) of 15 nm sized nanoparticles at 350 µg/ ml and for 50 nm it was 650 µg/ ml, which has been proved by analyzing the behavioural patterns such as the rest total, rest bout length, total activity and waking activity in the larvae of zebrafish (Xue *et al.*, 2013). The size of silicon dioxide nanoparticles between 68 and 100 nm has demonstrated the median lethal concentration ($LC_{50}$-96 h) as 50 mg/ L and at sublethal concentrations altered antioxidant enzyme level and induced DNA damage in the fish, *Danio rerio* (Ramesh *et al.*, 2013). The 24, 48, 72 and 96 h median lethal concentration ($LC_{50}$) values of silver nanoparticles of 61 nm particle

size for silver carp has been estimated at 0.810, 0.648, 0.383 and 0.202 mg/ L, respectively and 20% and 10% of the 96-h $LC_{50}$ values resulted in erythrocyte reduction, hematological disturbances, leucocytosis and stress response in silver carp, *Hypophthalmichthys molitrix* (Shaluei *et al*., 2013). In one of the studies, silver nanoparticles were prepared differently, one was freshly prepared and the other used was aged nanoparticles, which has been exposed to the embryos of Japanese medaka. The study reported that the aged silver nanoparticles exhibited more toxic effects since the $LC_{50}$ value observed was 1.44 mg/ L than the freshly prepared nanoparticles having 3.53 mg/ L as the median lethal concentration. The study further confirmed that the acute toxicity of aged silver nanoparticles was due to the release of silver ions as established by kinetic analysis (Kim *et al*., 2013). Different shaped zinc oxide nanoparticles such as nanospheres, nanosticks, cuboidal submicron particles has been shown to exhibit the median lethal concentrations at a range of 7.1 to 11.9 mg/ L and the median effective concentration ranged between 1 and 2.2 mg/ L where the nanosticks of zinc oxide has been regarded as more toxic as revealed by high mortality and hatching inhibition in the embryos of zebrafish, *Danio rerio* (Hua *et al*., 2014). For the assessment of iron oxide nanoparticles toxicity, different concentrations ranged from 2, 10, 100, 250, 500, 750 and 1000 ppm exposed to *Labeo rohita* has accounted 50% mortality at 500 ppm concentration and its chronic exposure resulted in altered haematological, ion regulatory and gill $Na^+/K^+$ activity in the fish (Remya *et al*., 2014).

Silver nanoparticles and silver nanowires are basically synthesized from silver element but the toxic effects of nanoparticles has been shown to vary among different aquatic organisms as fish, *Daphnia* and algae thus categorizing nanoparticles as acute 1 for *Daphnia magna and Raphidocelis subcapitata*, acute 2 for *Oryzias latipes* whereas nanowires has been categorized as acute 1 for *Daphnia magna*, acute 2 for *Oryzias latipes* and *Raphidocelis subcapitata* (Sohn *et al*., 2015). Acute toxicity of spherical 50 nm sized copper nanoparticles demonstrated the lowest observed effect concentration values as 0.17, 0.023 and less than 0.023 mg/ L in three freshwater fishes namely rainbow trout, fathead minnow and zebrafish, respectively, and the study also reported that the fate of the nanoparticles was temperature-dependent (Song *et al*., 2015). Larvae of zebrafish when exposed to

different concentrations of newly engineered nanoparticles namely nano-titanium dioxide on gold nanoparticles ($TiO_2$/Au) and nano-gold coated titanium dioxide nanoparticles (Au/$TiO_2$) showed neither mortality nor sublethal effects in the fish embryo toxicity test which was evidenced by several endpoints such as viability, larval growth, brain morphology, pharyngeal arches and jaw, other craniofacial structures, heart, fins, notochord, somites, tail, body shape, cardiovascular function, yolk sac and locomotor function and touch response (Brundo *et al.*, 2016). Assessment of 96 h median lethal concentration of iron oxide nanoparticles in *Labeo rohita* at the concentrations of 100, 1500 and 3000 ppm for four days showed $LC_{50}$ value as 3000 ppm, and its sublethal concentrations altered haematological parameters and decreased serum total protein, carbohydrate and lipids (Keerthika *et al.*, 2017).

Bioavailability of silver nanoparticles by exposure through different routes has revealed waterborne exposure of the test chemical resulted in high concentrations on gill tissue. The study also revealed that the bioavailability remained high in the gastrointestinal tract on dietary exposure consequently proving short lifetime of silver nanoparticles in water which then get transferred to sediment, feed, or sediment-dwelling food sources such as larvae and worms thereby making diet as a significant long-term exposure route (Kleiven *et al.*, 2018). Synthesis of biogenic silver nanoparticles using infusion of the extracts of *Althaea officinalis* as reducing agent has been reported as more toxic than those synthesized by the infusion of roots of the plant thereby proved the toxicity of nanoparticles in the juvenile zebrafish, *Danio rerio* (Rheder *et al.*, 2018). Acute and transgenerational effects of zinc oxide nanoparticles have been studied in the nervous and vascular systems of zebrafish reporting the persistent neurotoxic effects from one generation to the next, however, the effects has been known to be mitigated by the dissolved organic material (Kteeba *et al.*, 2018)

# Materials and Methods

### 3.1 Characterization of test chemicals

Aluminium oxide nanoparticles - $Al_2O_3$NPs (Cat. No: 0140408) and silicon dioxide nanoparticles - $SiO_2$NPs (Cat. No: 1940323) were obtained from SISCO Research Laboratory (SRL), India. Titanium dioxide nanoparticles - $TiO_2$NPs (titanium-IV oxide, mix of anatase and rutile, Cat. No. 634662) and iron oxide nanoparticles - $Fe_3O_4$NPs (Cat. No. 637106) were obtained from Sigma Aldrich, Germany. The purity of the selected nanoparticles was confirmed using X-ray diffraction (XRD- Rigaku Miniflux) and the average particle size was calculated using Scherrer's formula. The size and crystalline structure were confirmed using High Resolution Transmission Electron Microscope (HR-TEM- Jeol/JEM 2100) having resolution point 0.23 nm and lattice 0.14 nm at magnification 2000 X-1500000 X. The nanodispersions were prepared just before the exposure by ultra-sonication at 100 kHz for 30 min (for $SiO_2$ NPs- 10 min) using double distilled water. All other chemicals were of analytical grade and were obtained from local commercial sources.

### 3.2 Test animal

*Oreochromis mossambicus* weighing 6±1.5g and length 6.5±1cm were collected from local fish farm, Safa Aquarium, Kozhikode, Kerala (11º22'N, 75º85'E). Fish were acclimatized in dechlorinated water for two weeks in the laboratory conditions prior to experiment in glass tanks of 40 L capacity provided with good aeration and light (12: 12h; light: dark).

### 3.3 Preliminary screening

The physico-chemical features of the tap water were estimated as per the guidelines of American Public Health Association (APHA, 1998). Water samples free from chlorine, fluorine, iron and other metals, organic compounds and traces of radioisotopes were detected using GC/MS or ICP/MS. Water temperature in the experiment was maintained as 28±2°C, oxygen saturation of water ranged between 70% and 100 % and pH was retained between 7.4 and 7.6, which were monitored continuously using standardized procedures throughout the experiments.

## 3.4 Determination of median lethal concentrations

The standard measure to determine the acute toxicity effects of selected nanoparticles in the surrounding medium that kill 50 percent of the test animal at specified time interval was performed by analysing median lethal concentration or $LC_{50}$ values for 96 h using Probit analysis, with a confident limit of 5% level (Finney, 1971). In order to assess $LC_{50}$ of the nanoparticles, fish were not fed a day prior to and during the test period to reduce faecal and excess food contaminating the test solution. Ten specimens were maintained in each treatment tanks and aerated using tubed motorized pumps. Control tank without toxicants were also maintained along with the treatment groups. The movement and the behaviour along with the mortality of fishes were continuously monitored throughout the study. For determining $LC_{50}$ concentration of nanoparticles, it is necessary to understand that how much concentration of a toxicant is required to cause 50% mortality. For this, four different treatment groups were formed and in each group fishes were exposed to different concentrations of nanoparticles as given below:

Group I: $Al_2O_3$-NPs at seven different concentrations, ie., 10, .20, 30, 40, 50, 60 and 70 mg/ L for 96 h.

Group II: $Fe_3O_4$-NPs at seven different concentrations, ie., 5, 25, 50, 75, 100, 125 and 150 mg/ L for 96 h.

Group III: $SiO_2$-NPs at eight different concentrations, ie., 5, 25, 50, 75, 100, 125, 150 and 175 mg/ L for 96 h

Group IV: $TiO_2$-NPs at nine different concentrations ie., 25, 50, 75, 100, 125, 150, 175, 200 and 225 mg/ L for 96 h.

All treatment groups at different concentrations of nanoparticles were tanked separately along with control animal. Fishes without any movement for long period were considered as dead and were removed from the tanks immediately to prevent contamination.

## 3.5 Selection of treatment concentrations and durations

Treatment concentrations for *in vivo* studies were selected according to median lethal concentrations. Fishes were exposed only to sub-lethal concentrations of all the four nanoparticles. For this the $1/10^{th}$ of $LC_{50}$ (except $Fe_3O_4$NPs) were taken as the test concentrations. Fishes were exposed to all the four nanoparticles ($Al_2O_3$NPs, $Fe_3O_4$NPs, $SiO_2$NPs and $TiO_2$NPs) for both short-term and long-term durations. Short-term durations include 24, 72 and 96 h, whereas long-term durations include 15, 45 and 60 days. Biochemical parameters, genotoxicity analysis, and histopathological studies were performed in the selected concentrations and durations.

## 3.6 Experimental design

<table>
<tr><th>Test animal and sample size</th><th>Test chemicals and test concentrations</th><th>Treatment durations</th><th>Parameters tested</th><th>Tissues analysed</th></tr>
<tr><td rowspan="3">Oreochromis mossambicus<br><br>N=10/ treatment group</td><td rowspan="3">$Al_2O_3$NPs – 4 mg/ L<br>$Fe_3O_4$NPs – 15 mg/ L<br>$SiO_2$NPs – 12 mg/ L<br>$TiO_2$NPs – 16.4 mg/ L<br><br>and<br><br>Control group (without toxicant)</td><td rowspan="3">Short-term – 24, 72 and 96 h<br><br>Long-term - 15, 45 and 60 days<br><br>Treatment withdrawal – 60 days</td><td>Biochemical studies</td><td rowspan="2">Gill<br>Liver<br>Brain</td></tr>
<tr><td>Histopathology studies</td></tr>
<tr><td>Genotoxicity analysis</td><td>Whole blood</td></tr>
</table>

### 3.7 Statistical analysis

All experiments of acute toxicity tests were performed in triplicates for the accuracy of the results. Total number of animal used in the experiments, the exposure concentrations and the mortality rate in each experiment were fit to a Probit model using log10 concentration transformation using the statistical package SPSS 17.0. The correlation between mortality on Y-axis and concentrations on X-axis and the best-fit line was obtained by plotting graph using MS Excel 2007.

# Results

## 4.1 Characterization of nanoparticles

Characterization of the four selected nanoparticles namely $Al_2O_3$-NPs, $Fe_3O_4$-NPs, $SiO_2$-NPs and $TiO_2$-NPs were confirmed using X-ray diffraction (Rigaku Miniflux) and it was found that the particles are pure and free from impurities (Figure 1a-d). TEM images of nanoparticles indicated crystalline or amorphous, irregular and roughly symmetrical structure of nanoparticles, and the size ranged between 1 and 20 nm. The size of the nanoparticles derived using Scherrer's formula was $Al_2O_3$-NPs - 16.7 nm, $Fe_3O_4$-NPs - 15.65 nm, $SiO_2$-NPs- 1 nm and $TiO_2$-NPs- 11.4 nm (Figure 2a-d). The results confirmed that the nanoparticles used in the present study have the size almost similar to the range as specified in the manufacturers' details (Vidya and Chitra, 2017).

## 4.2 Median lethal concentrations of selected nanoparticles

The mortality of fishes in each nanoparticles exposed groups were continuously monitored throughout the experiment period. No mortality was observed in the untreated control group throughout the experiment. It was observed that in Group I - $Al_2O_3$-NPs exposed group, at 10 and 20 mg/ L concentrations for 96 h showed no mortality. At the next three concentrations namely 30, 40 and 50 mg/ L, fish exhibited 20, 50 and 80% mortality, respectively. When the concentrations were further increased to 60 and 70 mg/ L showed 100% mortality of fish at 96 h and 24 h, respectively (Table 1a and 1b; Figure 3). In Group II - $Fe_3O_4$-NPs exposed group, no mortality was observed at 10, 50 and 100 mg/ L concentrations for 96 h. However, only 10% mortality was observed at 150 mg/ L concentration exposed for 96 h, and above the concentration showed agglomeration of nanoparticles (Table 2).

In Group III - $SiO_2$-NPs exposed group, demonstrated no mortality at 5, 25, 50 and 75 mg/ L concentrations for 96 h. However, 40 and 50% mortality was observed at 100 and 125 mg/ L concentrations for 96 h and at 150 and 175 mg/ L concentrations showed 70 and 100% mortality after 96 h and 48 h, respectively (Table 3a and 3b; Figure 4). In Group IV- $TiO_2$-NPs exposed group, fish showed no mortality after 25, 50, 75 and 100 mg/ L concentrations of nanoparticles exposure for 96 h. $TiO_2$-NPs at 125, 150, 175 and 200 mg/ L concentrations showed 20, 40,

50, and 80% of mortality, respectively for 96 h. Increase in the concentration at 225 mg/ L was observed with 100% mortality within 24 h of exposure (Table 4a and 4b; Figure 5). The mortality values observed after each nanoparticles exposure were analysed by plotting mortality in Y-variable against concentrations of nanoparticles in X-variable. The values are then computed using Probit analysis at 95% confidence limit were 40 mg/ L for $Al_2O_3$-NPs, 120 mg/ L for $SiO_2$-NPs and 164 mg/ L for $TiO_2$-NPs. The results of Probit analysis indicated that the percentage of mortality showed high degree of positive correlation (r = +0.97, +0.947 and +0.91) against the concentrations of $Al_2O_3$-NPs, $SiO_2$-NPs and $TiO_2$-NPs, respectively (Figures 3-5).

Based on the median lethal concentrations ($LC_{50}$-96 h) observed for the selected nanoparticles, one-tenth of $LC_{50}$-96 h was selected as sublethal concentrations for the toxicity studies in the following chapters as follows:

| **Selected nanoparticles** | **Median lethal concentration ($LC_{50}$-96 h)** | **Sublethal concentration (One-tenth of $LC_{50}$-96 h)** |
|---|---|---|
| $Al_2O_3$-NPs | 40 mg/L | 4 mg/L |
| $Fe_3O_4$-NPs | 150 mg/L (agglomeration concentration) | 15 mg/L |
| $SiO_2$-NPs | 120 mg/L | 12 mg/L |
| $TiO_2$-NPs | 164 mg/L | 16.4 mg/L |

**Figure 1 XRD (Rigaku Miniflux) images showing structural and crystalline nature of the powdered samples of nanoparticles**

(a) - XRD peaks correspond to $Al_2O_3$-NPs with particle size of 16.7 nm; (b) - XRD peaks correspond to $Fe_3O_4$-NPs with particle size of 15.65 nm; (c) - XRD showing the characteristic peak of $SiO_2$-NPs with particle size of 1nm; (d) - XRD peaks of $TiO_2$-NPs denoting the peaks of both anatase and rutile at an angle of 27.6º and 28.9º, respectively with particle size of 11.4 nm.

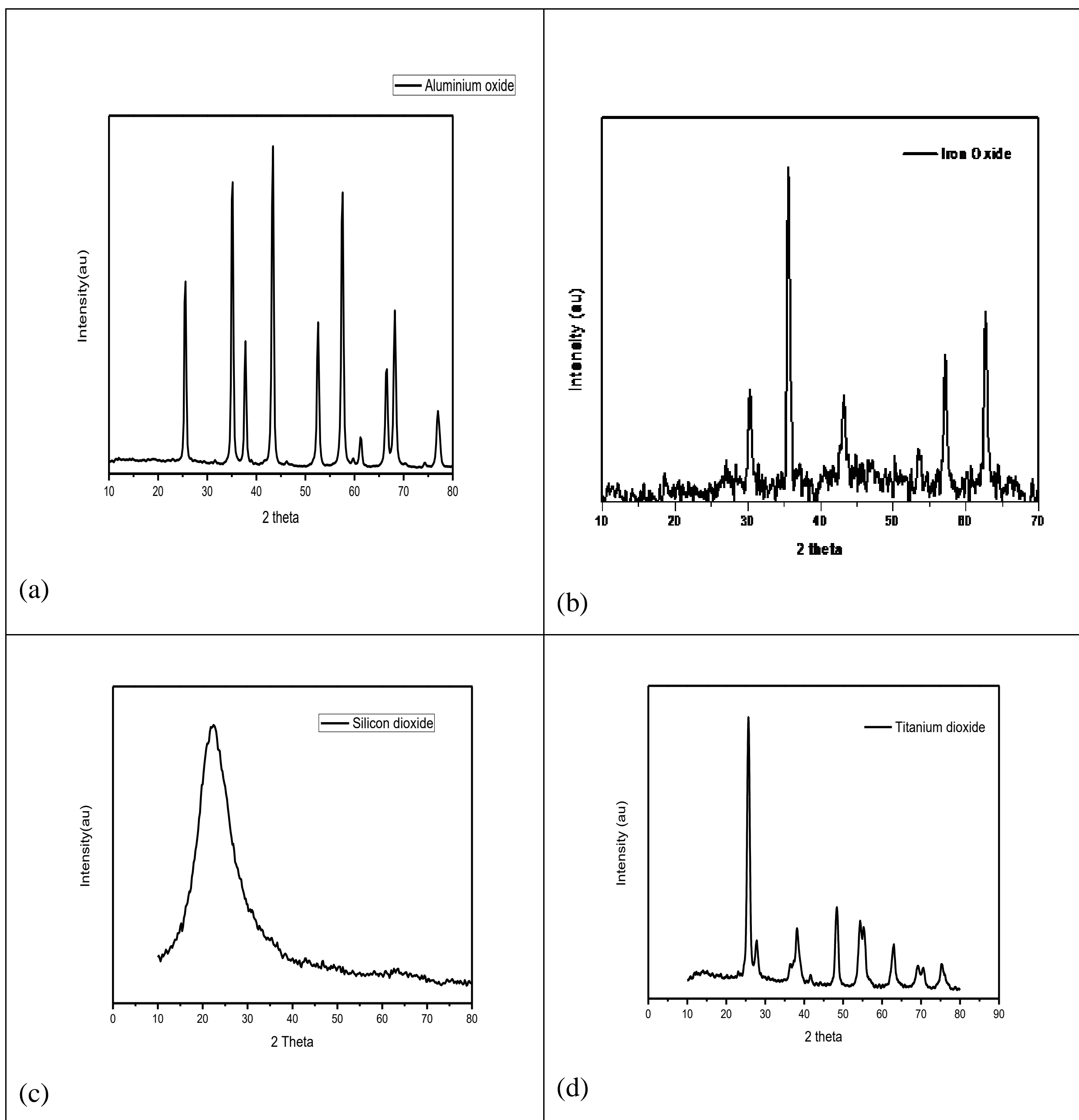


**Figure 2** **TEM images showing the morphology of a) $Al_2O_3$-NPs; b) $Fe_3O_4$-NPs; c) $SiO_2$-NPs and d) $TiO_2$-NPs aggregates**

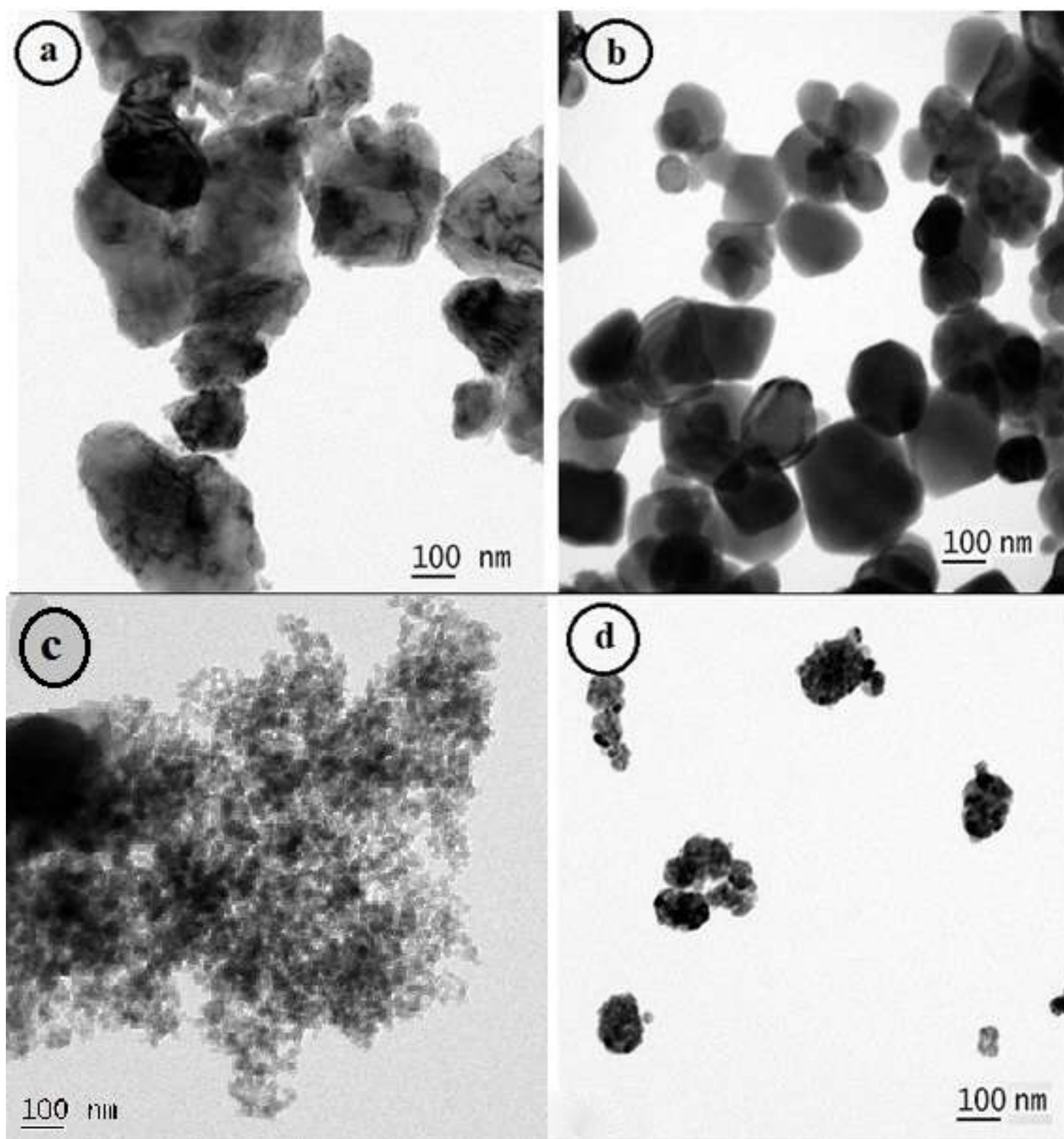


**Table 1a Percentage of fish mortality exposed at different concentrations of $Al_2O_3$-NPs in *Oreochromis mossambicus* for 96 h**

| Concentrations (mg/L) | Total (No. of animals) | Mortality (%) | Hour of mortality |
|---|---|---|---|
| 10.00 | 10.00 | 0 | 96 h |
| 20.00 | 10.00 | 0 | 96 h |
| 30.00 | 10.00 | 20 | 96 h |
| 40.00 | 10.00 | 50 | 96 h |
| 50.00 | 10.00 | 80 | 96 h |
| 60.00 | 10.00 | 100 | 96 h |
| 70.00 | 10.00 | 100 | 24 h |

**Table 1b Probit analysis of 95% confidence limits for effective concentrations of $Al_2O_3$-NPs in *Oreochromis mossambicus***

| Prob | Concentration (mg) | 95% Confidence Limits | |
|---|---|---|---|
| | | Lower | Upper |
| .01 | 17.85793 | 0.34029 | 25.58665 |
| .02 | 20.45254 | 4.15094 | 27.51176 |
| .03 | 22.09874 | 6.98513 | 28.74855 |
| .04 | 23.33712 | 9.10728 | 29.68883 |
| .05 | 24.34444 | 10.82603 | 30.46114 |
| .06 | 25.20182 | 12.28286 | 31.12458 |
| .07 | 25.95359 | 13.55501 | 31.71150 |
| .08 | 26.62670 | 14.68943 | 32.24165 |
| .09 | 27.23887 | 15.71694 | 32.72801 |
| .10 | 27.80237 | 16.65887 | 33.17959 |
| .15 | 30.13542 | 20.51079 | 35.09719 |
| .20 | 31.98966 | 23.50084 | 36.69257 |
| .25 | 33.58043 | 25.99746 | 38.12984 |
| .30 | 35.00899 | 28.16990 | 39.49015 |
| .35 | 36.33276 | 30.11054 | 40.82314 |
| .40 | 37.58889 | 31.87595 | 42.16408 |
| .45 | 38.80422 | 33.50441 | 43.54106 |
| .50 | 40.00027 | 35.02475 | 44.97850 |
| .55 | 41.19632 | 36.46139 | 46.49963 |
| .60 | 42.41165 | 37.83759 | 48.12886 |
| .65 | 43.66778 | 39.17782 | 49.89498 |
| .70 | 44.99155 | 40.51014 | 51.83628 |
| .75 | 46.42011 | 41.86986 | 54.00932 |
| .80 | 48.01088 | 43.30662 | 56.50646 |
| .85 | 49.86512 | 44.90154 | 59.49697 |
| .90 | 52.19817 | 46.81872 | 63.34930 |
| .91 | 52.76167 | 47.27023 | 64.29131 |
| .92 | 53.37384 | 47.75650 | 65.31890 |
| .93 | 54.04696 | 48.28658 | 66.45340 |
| .94 | 54.79872 | 48.87342 | 67.72562 |
| .95 | 55.65611 | 49.53678 | 69.18254 |
| .96 | 56.66343 | 50.30900 | 70.90137 |
| .97 | 57.90180 | 51.24920 | 73.02361 |
| .98 | 59.54800 | 52.48589 | 75.85789 |
| .99 | 62.14262 | 54.41089 | 80.34924 |

**Figure 3** **Median lethal concentration ($LC_{50}$-96 h) of $Al_2O_3$-NPs in *Oreochromis mossambicus* (n = 10 fish per group)**

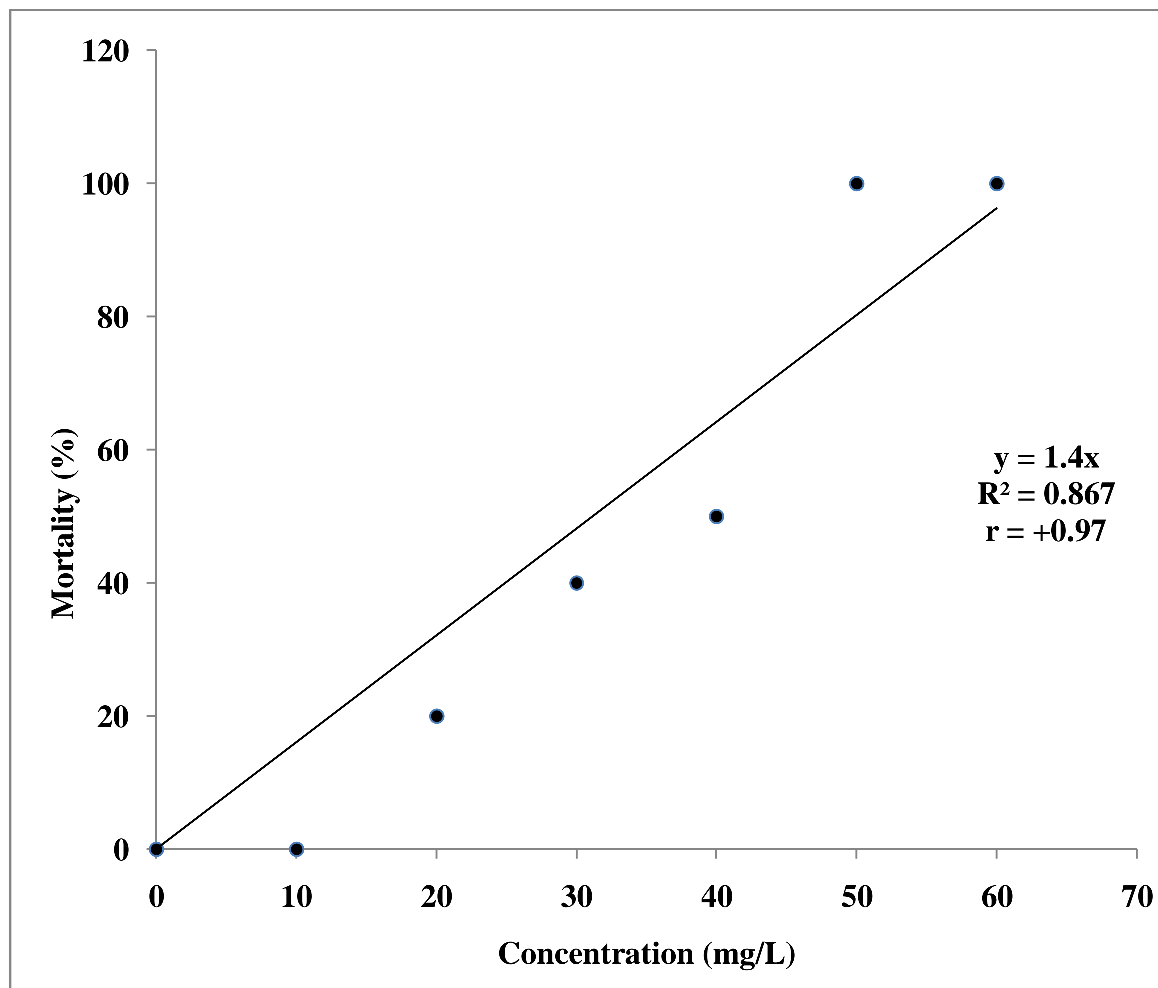


**Table 2** **Percentage of fish mortality exposed at different concentrations of $Fe_3O_4$-NPs in *Oreochromis mossambicus* for 96 h**

| Concentrations (mg/L) | Total (No. of animals) | Mortality (%) | Hour of mortality |
|---|---|---|---|
| 5.00 | 10.00 | 0 | 96 h |
| 25.00 | 10.00 | 0 | 96 h |
| 50.00 | 10.00 | 0 | 96 h |
| 75.00 | 10.00 | 0 | 96 h |
| 100.00 | 10.00 | 0 | 96 h |
| 125.00 | 10.00 | 0 | 96 h |
| 150.00 | 10.00 | 10 | 96 h |

**Table 3a** **Percentage of fish mortality exposed at different concentrations of $SiO_2$-NPs in *Oreochromis mossambicus* for 96 h**

| Concentrations (mg/ L) | Total (No. of animals) | Mortality (%) | Hour of mortality |
|---|---|---|---|
| 5.00 | 10.00 | 0 | 96 h |
| 25.00 | 10.00 | 0 | 96 h |
| 50.00 | 10.00 | 0 | 96 h |
| 75.00 | 10.00 | 0 | 96 h |
| 100.00 | 10.00 | 40 | 96 h |
| 125.00 | 10.00 | 50 | 96 h |
| 150.00 | 10.00 | 70 | 96 h |
| 175.00 | 10.00 | 100 | 48 h |

**Table 3b Probit analysis of 95% confidence limits for effective concentrations of $SiO_2$-NPs in *Oreochromis mossambicus***

| Prob | Concentration (mg) | 95% Confidence Limits | |
|---|---|---|---|
| | | Lower | Upper |
| .01 | 62.74245 | 32.99795 | 79.67651 |
| .02 | 67.75591 | 38.16381 | 84.13493 |
| .03 | 71.14254 | 41.83916 | 87.12121 |
| .04 | 73.80127 | 44.82570 | 89.45680 |
| .05 | 76.03706 | 47.40370 | 91.41789 |
| .06 | 77.99336 | 49.70766 | 93.13367 |
| .07 | 79.75001 | 51.81357 | 94.67580 |
| .08 | 81.35643 | 53.76904 | 96.08849 |
| .09 | 82.84548 | 55.60601 | 97.40110 |
| .10 | 84.24024 | 57.34712 | 98.63424 |
| .15 | 90.26922 | 65.07972 | 104.02869 |
| .20 | 95.36712 | 71.83233 | 108.72215 |
| .25 | 99.96951 | 78.03545 | 113.12803 |
| .30 | 104.29158 | 83.88921 | 117.47702 |
| .35 | 108.46326 | 89.49669 | 121.93910 |
| .40 | 112.57596 | 94.91051 | 126.66699 |
| .45 | 116.70341 | 100.15529 | 131.81541 |
| .50 | 120.01315 | 105.24370 | 137.55034 |
| .55 | 125.27474 | 110.19154 | 144.05462 |
| .60 | 129.86778 | 115.03202 | 151.53713 |
| .65 | 134.79210 | 119.82688 | 160.25490 |
| .70 | 140.18380 | 124.67484 | 170.56019 |
| .75 | 146.24449 | 129.72339 | 182.99327 |
| .80 | 153.30221 | 135.19818 | 198.47538 |
| .85 | 161.95987 | 141.48462 | 218.77992 |
| .90 | 173.55114 | 149.38724 | 248.00626 |
| .91 | 176.47298 | 151.30907 | 255.72014 |
| .92 | 179.70294 | 153.40629 | 264.40444 |
| .93 | 183.32274 | 155.72553 | 274.32992 |
| .94 | 187.45174 | 158.33449 | 285.89723 |
| .95 | 192.27452 | 161.33721 | 299.73505 |
| .96 | 198.09943 | 164.90621 | 316.91156 |
| .97 | 205.50279 | 169.36134 | 339.46452 |
| .98 | 215.77438 | 175.41100 | 372.07509 |
| .99 | 233.01591 | 185.27462 | 430.21187 |

**Figure 4** Median lethal concentration ($LC_{50}$-96 h) of $SiO_2$-NPs in *Oreochromis mossambicus* (n = 10 fish per group)

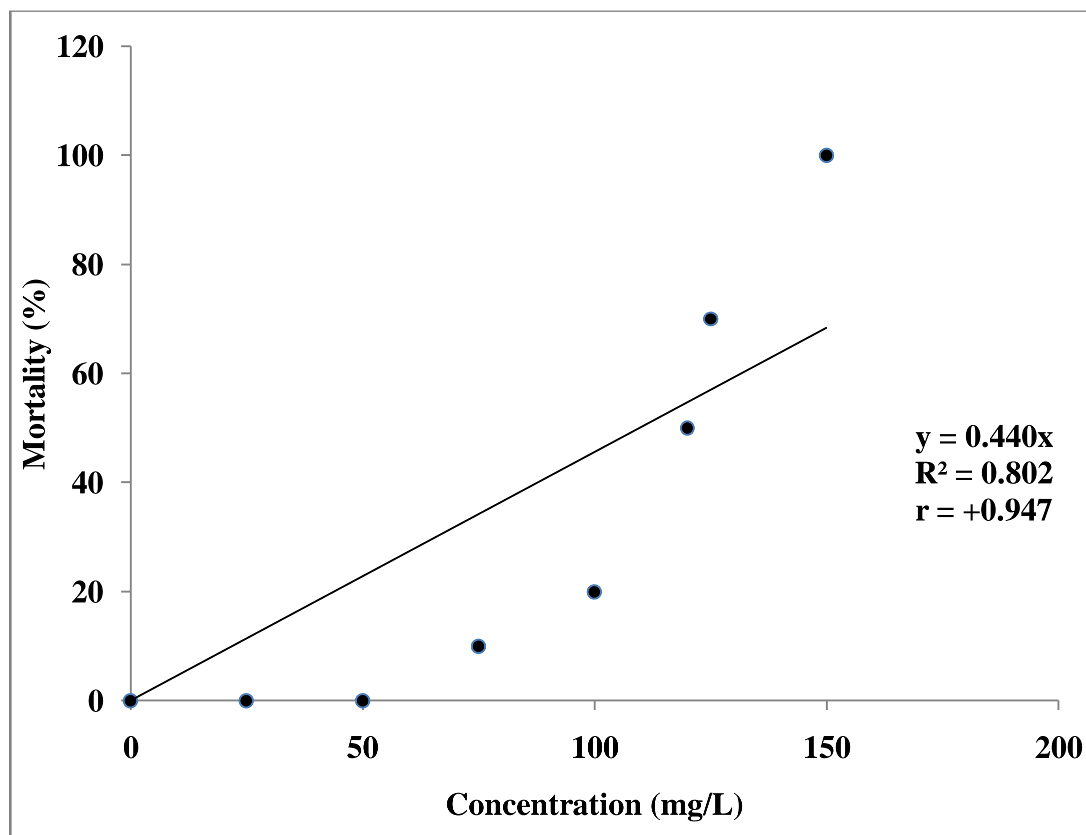


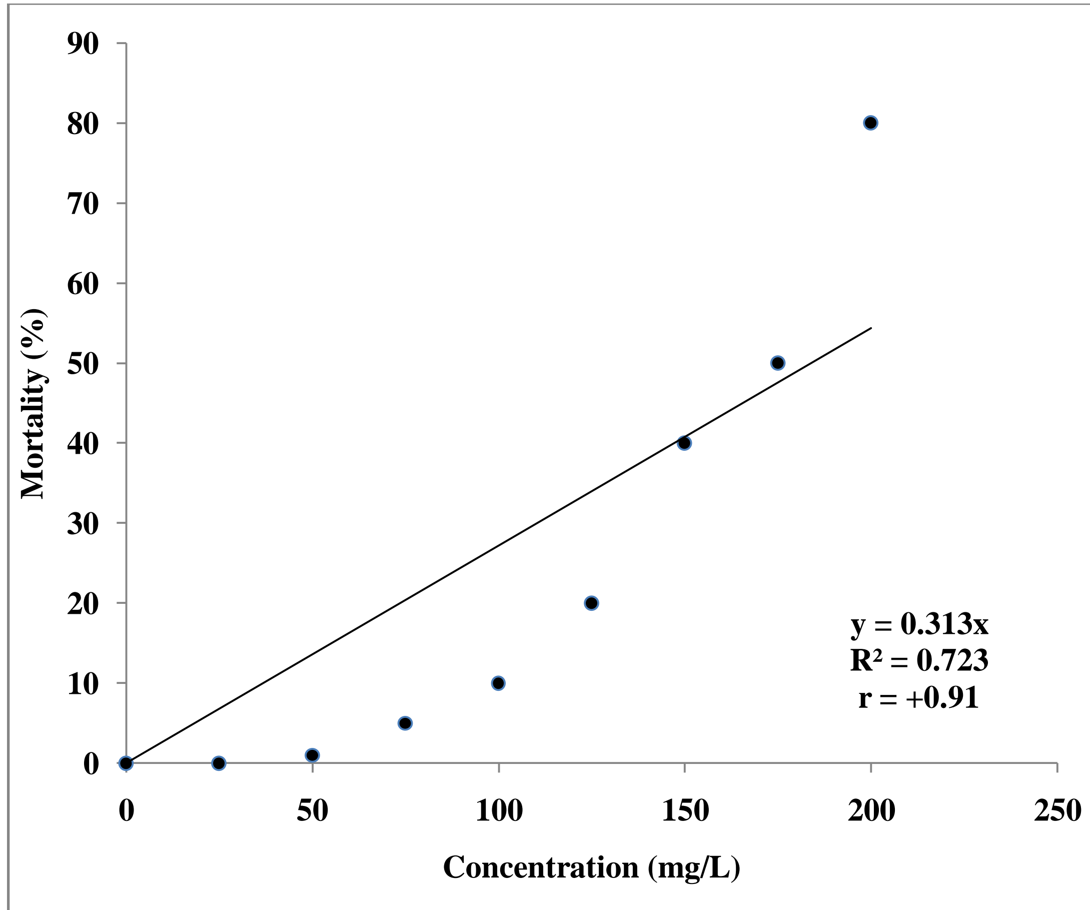
**Table 4a Percentage of fish mortality exposed at different concentrations of $TiO_2$-NPs in *Oreochromis mossambicus* for 96 h**

| Concentrations (mg/ L) | Total (No. of animals) | Mortality (%) | Hour of mortality |
|---|---|---|---|
| 25.00 | 10.00 | 0 | 96 h |
| 50.00 | 10.00 | 0 | 96 h |
| 75.00 | 10.00 | 0 | 96 h |
| 100.00 | 10.00 | 0 | 96 h |
| 125.00 | 10.00 | 20 | 96 h |
| 150.00 | 10.00 | 40 | 96 h |
| 175.00 | 10.00 | 50 | 96 h |
| 200.00 | 10.00 | 80 | 96 h |
| 225.00 | 10.00 | 100 | 24 h |

**Table 4b Probit analysis of 95% confidence limits for effective concentrations of $TiO_2$-NPs in *Oreochromis mossambicus***

| Prob | Concentration (mg) | 95% Confidence Limits | |
|---|---|---|---|
| | | Lower | Upper |
| .01 | 79.36112 | 11.13292 | 106.02350 |
| .02 | 89.30182 | 29.37677 | 113.22481 |
| .03 | 95.60887 | 40.88573 | 117.86000 |
| .04 | 100.35343 | 49.49879 | 121.39155 |
| .05 | 104.21275 | 56.46989 | 124.29916 |
| .06 | 107.49765 | 62.37387 | 126.80351 |
| .07 | 110.37786 | 67.52439 | 129.02545 |
| .08 | 112.95674 | 72.11224 | 131.03876 |
| .09 | 115.30213 | 76.26246 | 132.89203 |
| .10 | 117.46107 | 80.06164 | 134.61907 |
| .15 | 126.39964 | 95.51704 | 142.04371 |
| .20 | 133.50374 | 107.36139 | 148.38369 |
| .25 | 139.59842 | 117.06739 | 154.27823 |
| .30 | 145.07163 | 125.30259 | 160.05278 |
| .35 | 150.14338 | 132.43471 | 165.90280 |
| .40 | 154.95597 | 138.70534 | 171.95095 |
| .45 | 159.61220 | 144.30158 | 178.27325 |
| .50 | 164.19462 | 149.38457 | 184.91986 |
| .55 | 168.77704 | 154.09873 | 191.93529 |
| .60 | 173.43327 | 158.57529 | 199.37729 |
| .65 | 178.24586 | 162.93704 | 207.33432 |
| .70 | 183.31761 | 167.30727 | 215.94622 |
| .75 | 188.79082 | 171.82537 | 225.43788 |
| .80 | 194.88550 | 176.67621 | 236.18758 |
| .85 | 201.98960 | 182.15581 | 248.89231 |
| .90 | 210.92817 | 188.86256 | 265.06559 |
| .91 | 213.08711 | 190.45871 | 268.99566 |
| .92 | 215.43250 | 192.18412 | 273.27374 |
| .93 | 218.01138 | 194.07191 | 277.98711 |
| .94 | 220.89159 | 196.16980 | 283.26169 |
| .95 | 224.17649 | 198.55038 | 289.28943 |
| .96 | 228.03581 | 201.33276 | 296.38575 |
| .97 | 232.78037 | 204.73472 | 305.12841 |
| .98 | 239.08742 | 209.23020 | 316.77708 |
| .99 | 249.02812 | 216.26592 | 335.18653 |

**Figure 5** **Median lethal concentration ($LC_{50}$-96 h) of $TiO_2$-NPs in *Oreochromis mossambicus* (n = 10 fish per group)**



# Discussion

## 5.1 Importance of characterization of nanoparticles

Nanotechnology is a rapidly emerging multidisciplinary technology with the development of new nanoscale products in various shapes. Due to the widespread application of the engineered nanoparticles in various fields, they are released continuously in large scale into the aquatic environment. Toxicological studies using engineered nanoparticles concluded that nanoparticles are potentially harmful due to the specific physico-chemical properties (Hoshino *et al.*, 2004). It is therefore, crucial to demonstrate the characterization of nanoparticles in toxicological studies in order to correlate the toxic potential and responses of nanoparticles with their properties, and to ensure the reproducibility and accuracy of results and observations (Oberdorster *et al.*, 2005; Jiang *et al.*, 2009). Behaviour of nanoparticles depends on several unique characteristics including size, shape, surface area and surface reactivity. Thus agglomeration, adsorption, uptake, metabolism and excretion of nanoparticles in humans or other organisms and their environment are greatly influenced by its physico-chemical properties. There is a growing agreement on the necessity of performing suitable and accurate characterization of commercially available nanoparticles in environmental media and biological systems before conducting any toxicity tests. Execution of any nanotoxicity tests without characterization of nanoparticles possesses inadequate value to the experiments due to the inconsistency in physico-chemical properties (Warheit, 2008). There are many test batteries available for adequate characterization of nanoparticles, however, they are undoubtedly costly and time-consuming. Some of the principal characteristics tests conducted before performing toxicity studies include size, shape, surface area, state of dispersion and surface chemistry (Cong *et al.*, 2011).

In the present study, characterization of commercially available selected four nanoparticles was performed before conducting the acute toxicity tests. Only the two principal characteristics namely the size and shape of the nanoparticles were carried out in the study. The results obtained using X-ray diffraction confirmed that the selected nanoparticles are pure and free from impurities (Figures 1a-d) and the TEM images indicated that the size of nanoparticles used in the study are almost in the range as specified by the manufacturers. Characterization of $Al_2O_3$-NPs showed the size of the nanoparticles at 16.7 nm, and the size mentioned in the manufacturers

label was 20-30 nm (Figure 2a). The size of $Fe_3O_4$-NPs observed after characterization was 15.65 nm, which was below the size as mentioned in the manufacturer's information as 50-100 nm (Figure 2b). The particle size of $SiO_2$-NPs mentioned by the manufacturer was below 100 nm and the size observed after characterization was only 1 nm (Figure 2c). Characterization of $TiO_2$-NPs showed the particle size as 11.4 nm, and the size mentioned in the manufacturer's tag was below 100 nm (Figure 2d). Thus characterization of nanoparticles performed in the selected nanoparticles provides the exact particle size and shape which is very essential for conducting any toxicity tests. A slight difference in size observed between the manufacturer details and that measured in the laboratory may be most likely due to batch-to-batch variation during production, changes in material properties between synthesis and initial characterization, and also could be variations in certain experimental conditions such as pH, ionic strength, and temperature (Cong *et al*., 2011).

After characterization, dispersion of nanoparticles is the other important factor for the preparation of evenly suspended stock of nanoparticles. In the present study the selected nanoparticles were suspended in double distilled water by performing sonication as the external mixing force. Such characterization and dispersion of nanoparticles according to the standard prescribed methods may sometimes change the properties of nanoparticles as what was expected to be released into the environment. Therefore, mimicking the environmentally released nanoparticles in the laboratory condition is unrealistic to certain extent. However, the physico-chemical features of tap water such as water pH, salinity and temperature, dissolved organic material, and natural competing cations were estimated using the standard APHA guidelines. These environmental factors are likely to play important roles in determining the dispersion and toxic consequences of nanoparticles in the exposed fish. The toxicity and reactivity of nanoparticles are highly influenced by the features as size and shape of the particles because internalization of nanoparticles into the biological system depends on the size and shape. Thus characterizations of the selected four nanoparticles were performed in the laboratory before conducting the toxicity tests in the fish, *Oreochromis mossambicus*.

## 5.2 Median lethal concentrations of selected nanoparticles

Determination of median lethal concentration is the direct measure of acute toxicity testing in classical toxicological studies. Acute toxicity comprises any adverse effects of the exposed toxicants within a short-term period after administration of a single dose. Adverse toxicity effects includes the signs of intoxication, time to onset and the duration of toxic effects, dose-response relationship, reversibility of toxic effects, sex-specific and organ or tissue-specific toxic effects, mode of action, lowest toxic, lowest lethal dose and highest non-toxic effects of the toxicant and median lethal dose or concentration. Among which, median lethal concentration is one of the simplest, most convenient and statistically precise methods approved by Organization of Economic Co-operation and Development (OECD) for studying inherent property of toxicant. In the present study, the median lethal concentrations of the selected nanoparticles namely $Al_2O_3$-NPs, $Fe_3O_4$-NPs, $SiO_2$-NPs and $TiO_2$-NPs were determined for 96 h using Probit analysis with 95% confidence limits. The experiment was divided into four separate groups of each nanoparticles exposed at several different concentrations along with control group by maintaining ten animals per group. Each experiment was repeated twice under identical conditions and the statistical analysis was performed for obtaining high degree of precision.

Group I includes $Al_2O_3$-NPs exposed group in which exposure at 10 and 20 mg/ L concentrations of nanoparticles for 96 h did not kill the animal. However, at the concentrations of 30, 40 and 50 mg/ L showed 20, 50 and 80% mortality, respectively, further at 60 and 70 mg/ L concentrations were observed with 100% mortality at 96 h and 24 h, respectively (Table 1a and 1b; Figure 3). The data obtained was then used for calculating the slope of dose-response curve and the confidence limit. The results showed a high degree of positive correlation (r = +0.97) when a graph was plotted between the concentrations of nanoparticles and percentage of mortality of animal, and the Probit analysis determined the median lethal concentration of $Al_2O_3$-NPs as 40 mg/ L concentration.

Group II comprise $Fe_3O_4$-NPs exposed group, were no mortality was observed at 10, 50 and 100 mg/ L concentrations for 96 h. However, only 10% mortality was observed at 150 mg/ L concentration exposed for 96 h, and above

which showed agglomeration of nanoparticles (Table 2). Therefore, the median lethal concentration of $Fe_3O_4$-NPs was not determined and the concentration of 150 mg/ L, which showed the maximum dispersion, was considered for the present study. Another study was found in agreement with the present results where the toxicity assessment of uncoated alpha iron oxide nanoparticles ($\alpha$-$Fe_2O_3$-NPs) of 30 nm size at concentration above 10 mg/ L has been shown to cause aggregates on the surface of zebrafish embryo resulting in developmental abnormalities (Zhu *et al.*, 2012).

Group III consist of $SiO_2$-NPs exposed group at eight different concentrations were observed without mortality at 5, 25, 50 and 75 mg/ L concentrations for 96 h. Concentrations at 100, 125 and 150 mg/ L showed 40, 50 and 70% mortality for 96 h whereas 175 mg/ L concentration showed 100% mortality after 48 h (Table 3a and 3b; Figure 4). The results showed an increase in the mortality after increase in the test concentrations with a high degree of positive correlation (r = +0.947), and $LC_{50}$-96 h calculated from the Probit log plot was 120 mg/ L concentration. In one of the acute toxicity studies, silica nanoparticles of 68.06 nm size has been reported to have the median lethal concentration as 50 mg/ L in zebrafish (Ramesh *et al.*, 2013). The variations observed in the results could be due to the difference in size and internalization of nanoparticles into the organism or due to the species diversity.

Group IV contain $TiO_2$-NPs exposed at nine different concentrations namely 25, 50, 75, 100, 125, 150, 175, 200 and 225 mg/ L for 96 h. No mortality of animal was observed after 25, 50, 75 and 100 mg/ L concentrations of nanoparticles exposure for 96 h. However, after 125, 150, 175 and 200 mg/ L concentrations of $TiO_2$-NPs exposure showed 20, 40, 50, and 80% mortality, respectively for 96 h, and at 225 mg/ L showed 100% mortality within 24 h of exposure (Table 4a and 4b; Figure 5). Correlation graph plotted against concentrations and mortality showed a high degree of positive correlation (r = +0.91) and the calculated 95% confidence limit using Probit analysis indicated the median lethal concentration of $TiO_2$-NPs as 164 mg/ L. The median lethal concentration obtained in the experiments can be considered as its acute toxic effects of the selected nanoparticles as they provide the dose-response relationship. In another study, $Al_2O_3$-NPs of 40 nm size has been

shown to cause 50% mortality in the fish, *Oreochromis mossambicus* in between 235 and 245 ppm concentration (Murali *et al.*, 2017). The disparity in the lethality and toxic responses of $Al_2O_3$-NPs observed within the same species might be due to the difference in particle size or change in water parameters such as pH, turbidity, temperature, salinity and oxygen demand that highly influence the toxicity of nanoparticles.

The $LC_{50}$-96 h obtained in the present experiment such as 40 mg/ L for $Al_2O_3$-NPs, 150 mg/ L for $Fe_3O_4$-NPs, which was based on the dispersion limit, 120 mg/ L for $SiO_2$-NPs and 164 mg/ L for $TiO_2$-NPs indicate variability in the acute toxicity of the selected nanoparticles. This could be due to the change in physico-chemical properties of the nanoparticles and the results clearly demonstrated that $Al_2O_3$-NPs showed $LC_{50}$ value at 40 mg/L, which is lowest among the selected nanoparticles thereby indicating $Al_2O_3$-NPs as more toxic to the fish *Oreochromis mossambicus*. However, no median lethal concentration was observed for $Fe_3O_4$-NPs because it began to agglomerate at the concentration of 150 mg/ L which made further dispersion impossible, and it was considered as the test concentration for the study, which also showed less mortality. Thus the results conclude that beside the physico-chemical properties, life stage, sex, season and health of the animal along with the rate of internalization, accumulation, metabolism or excretion of nanoparticles highly influence the toxicity of the chemical. As a result, diverse results of acute toxicity of nanoparticles could occur among the same or different species of fishes.

# Conclusions

1. X-ray diffraction patterns of selected nanoparticles confirmed that the particles are pure and free from impurities.

2. The size of the nanoparticles derived using Scherrer's formula was $Al_2O_3$-NPs - 16.7 nm, $Fe_3O_4$-NPs - 15.65 nm, $SiO_2$-NPs- 1 nm and $TiO_2$-NPs- 11.4nm.

3. The median lethal concentrations ($LC_{50}$-96 h) observed using Probit analysis was $Al_2O_3$-NPs - 40 mg/L, $SiO_2$-NPs - 120 mg/L and $TiO_2$-NPs - 164 mg/L in the fish, *Oreochromis mossambicus*.

4. Median lethal concentration of $Fe_3O_4$-NPs was not determined and the concentration of 150 mg/ L showed maximum dispersion with low mortality.

5. The disparity observed on the median lethal concentrations of the selected nanoparticles could be due to the difference in the physico-chemical properties.

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VIDYA BALAKRISHNAN P.V. “EFFECTS OF SELECTED NANOPARTICLES ON THE FRESHWATER FISH, OREOCHROMIS MOSSAMBICUS “. THESIS. DEPARTMENT OF ZOOLOGY, UNIVERSITY OF CALICUT, 2018.

# CHAPTER 2

## Impact of the selected nanoparticles on the antioxidant defense system of the fish, *Oreochromis mossambicus*

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| µg | Microgram |
| µm | Micrometer |
| µmol | Micromol |
| AChE | Acetylcholinesterase |
| $Al_2O_3$-NPs | Aluminium oxide nanoparticles |
| ALP | Alkaline phosphatase |
| BSA | Bovine serum albumin |
| CAT | Catalase |
| cm | Centimeter |
| DNA | Deoxyribo nucleic acid |
| DTNB | Dithiobisnitrobenzoic acid |
| EDTA | Ethylenediaminetetraacetic acid |
| eNOS | Epithelial Nitric Oxide Synthase |
| $Fe_3O_4$-NPs | Iron oxide nanoparticles |
| g | Gram |
| *g* | Gravity |
| GPx | Glutathione peroxidase |
| GR | Glutathione reductase |
| GSH | Glutathione |
| h | Hour |
| HSI | Hepatosomatic index |
| iNOS | Inflammatory NOS |
| K | Potassium |
| Kg | Kilogram |
| L | Levo |
| L | Litre |
| $LC_{50}$ | median lethal concentration |
| $LD_{50}$ | median lethal dose |
| M | Molar |
| MDA | Malondialdehyde |
| mg | Milligram |
| min | Minute |
| ml | Milliliter |
| mM | Milli molar |
| N | Normal |
| Na | Sodium |

| | |
|---|---|
| NADH | Nicotinamide adenine dinucleotide |
| NADPH | Nicotinamide adenine dinucleotide phosphate |
| nM | Nano molar |
| nm | Nanometer |
| nNOS | Neuronal NOS |
| $NO^{\bullet}$ | Nitrous oxide free radical |
| NOS | Nitric oxide synthase |
| Nox | NADPH oxidases |
| $O_2^-$ | Superoxide |
| $^oC$ | Degree Celsius |
| P | Probability |
| ppm | Parts per million |
| PUFA | Polyunsaturated fatty acid |
| RNS | Reactive nitrogen species |
| ROS | Reactive oxygen species |
| SD | Standard deviation |
| $SiO_2$-NPs | Silicon dioxide nanoparticles |
| SOD | Superoxide dismutase |
| TBARS | Thiobarbituric reactive substance |
| $TiO_2$-NPs | Titanium dioxide nanoparticles |
| UV | Ultra violet |
| XDH | Xanthine dehydrogenase |
| XO | Xanthine oxidase |
| XOR | Xanthine oxidoreductase |

# General Introduction

## 1.1 Free radicals and reactive oxygen species

Free radicals are independent group of atoms or molecules having one or more unpaired electrons, which are formed by the loss or gain of electron from non-radicals (Martinez-Alvarez *et al.*, 2005). In normal cells, free radicals are classified into two types namely reactive nitrogen species (RNS) and reactive oxygen species (ROS), produced in a controlled manner in order to maintain the homeostasis of the cell (Harman, 1981). RNS is the collective term for the nitrogen containing reactive molecules derived from nitrous oxide free radical ($NO^{\cdot}$), which has one unpaired electron in its orbit. ROS are oxygen-derived radicals generated constantly in normal aerobic life. But the unregulated production of free radicals inside the cells is involved in the etiology of various degenerative diseases, mutagenesis, metabolic disorders and cellular damages in organisms (Halliwell, 1997).

Molecular oxygen, often called free oxygen, is a bi-radical that perform oxidation and reduction reactions in the cell, and the bi-radical nature of oxygen contributed towards free radical generation thereby leading to oxygen toxicity in biological system (Davies, 2000). The free radicals, non-radicals and reactive molecules of molecular oxygen are collectively called as reactive oxygen species. Free radicals include superoxide anion, hydroxyl radical, hydroxyl ion, alkoxyl radicals, peroxyl radicals, whereas non-radicals include molecular and singlet oxygen, hydrogen peroxide, ozone, organic peroxide, hypochlorous acid and so on (Halliwell, 2001; Kohen and Nyska, 2002). Superoxide anion, hydroxyl ion and hydrogen peroxide have got much importance in the biological systems as they are involved in the regulation of various biological activities, development of certain pathological conditions including cancers and for the induction of oxidative stress (Covarrubias *et al.*, 2008). Superoxide anions are the most abundant and important group of free radicals, which are produced both in enzymatic and non-enzymatic processes inside the mitochondria by auto-oxidation and electron transfer reactions, respectively (Michelson *et al.*, 1977; Riley, 1994). Superoxide anions act both as reducing and oxidizing agent in the biological system and regulate various conversion reactions. Dismutation reaction combines two superoxide molecules, in

which one molecule is oxidized to oxygen and the other molecule, undergo reduction to hydrogen peroxide (Bielski *et al*., 1985).

Hydroxyl radical is a highly reactive free radical, which is the neutral form of hydroxyl ion formed in Fenton reaction that reacts with both organic and inorganic molecules (Bedwell *et al*., 1989). In stressed condition, excessive production of superoxide triggers the Fenton reaction so as to produce the highly reactive hydroxyl radical. Haber-Weiss reaction also leads to the formation of hydroxyl radical from superoxide and hydrogen peroxide (Haber and Weiss, 1934; Kehrer, 2000). Hydroxyl radicals react with vital biomolecules including carbohydrates, lipids, proteins and even DNA to cause severe cellular damages. Hydrogen peroxide is another biologically important ROS, which is not a free radical but indirectly induce oxidative stress and DNA damage by forming hydroxyl radical. Hydrogen peroxide can easily cross the biological membranes and damage the cellular components even at very low concentrations (Halliwell *et al*., 2000).

## 1.2 Sources of reactive oxygen species

There are various sources of ROS in biological system, which may be derived either from exogenous or endogenous sources, or both. Exogenous sources of ROS include several drugs like doxorubicin, paracetamol, bleomycine, and the rest includes heavy metals like iron, copper, cobalt, tobacco smoke, plastic, air and water pollution, pesticides, industrial solvents, high temperature, radiation and so on (Phaniendra *et al*., 2015). In some cases, ROS are formed endogenously inside every cell during normal cellular activities, especially as a by product of aerobic respiration (Loschen *et al*., 1971; Schieber and Chandel, 2014). Mitochondria are the key organelle responsible for the endogenous generation of ROS at cellular level, either by enzymatic or non-enzymatic processes (Fridovich, 1987). The mitochondrial electron transport chain consist of four complexes called complex I, II, III and IV, in which electron transfer occurs from complex I to IV (Turrens, 2003). At the end of electron transport chain, cytochrome oxidase, the final electron acceptor, catalyzes the tetravalent reduction of oxygen which results in the formation of water. But in between the electron transport chain, some electrons leak

out and undergo monovalent reduction of oxygen to produce reactive intermediates (Martinez-Alvarez *et al*., 2005). Superoxide is the predominant reactive intermediate produced in the electron transport chain, which is later converted to hydrogen peroxide and hydroxyl radical. Among the four complexes, the redox centres of complex I and III are the major site of ROS generation (Murphy, 2009).

Apart from mitochondria, endoplasmic reticulum, peroxisomes, immune cells like phagocytic cells and non-phagocytic cells, excessive exercises, mental stress, inflammation, aging, and cancer are some endogenous contributors to generate ROS. In endoplasmic reticulum, cytochrome P450 enzyme system and diamine oxidase are the major enzymes that facilitate ROS generation (De Duve and Baudhuhuin, 1966; Bhattacharyya *et al*., 2014). The enzymes involved in the β-oxidation of fatty acids in peroxisomes are also responsible for the production of ROS (Cheeseman and Slater, 1993). The phagocytic cells perform its bactericidal activity by the stimulated production of ROS thereby increase the consumption of oxygen by the cells, which is generally termed as 'respiratory burst' (Babior *et al*., 1973; Murphy and DeCoursey, 2006). In non-phagocytic cells, several cytokines like tumour necrosis factor-α, interleukins, interferons, and growth factors like tumour growth factor-β are known to trigger the production of ROS in order to stimulate the signal transduction cascades for various cellular activities (Crapo, 2003). The stimuli received from other factors such as angiotensin, bradykinin, endothelin, serotonin found in different cell type bind with the specific receptors to undergo signal transduction for the generation of ROS (Jain *et al*., 2013; Tabima *et al*., 2012).

Endogenous production of ROS also occurs from enzymatic sources. The major cellular enzymes involved in the generation of ROS are nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, xanthine oxidase, nitric oxide synthases and mitochondrial oxidases, cyclooxygenases and lipoxygenases (Soberman and Christmas, 2003; Phaniendra *et al*., 2015). NADPH oxidase is membrane bound enzyme complex present and expressed in different cell types to mediate various cellular functions by the production of free radicals, especially superoxide (Sumimoto *et al*., 2005). ROS generating NADPH oxidases are collectively called

NOX family, consisting of 7 members namely Nox 1, Nox 2, Nox 3, Nox 4, Nox 5, Duox 1 and Duox 2 (Sumimoto *et al.*, 2005). In an unexcited state, the different subunits of the enzymes remain unassembled at membrane bound and cytosolic regions. After activation by certain stimuli, the assembly of subunits occurs and the activated enzyme generates superoxide by transferring electrons from the substrate to the molecular oxygen (Boveris, 1980). NADPH oxidase is one of the well expressed ROS generating enzymes found distributed in cell membrane, mitochondria, peroxisome, and endoplasmic reticulum of the phagocytic and non-phagocytic cells (Lambeth, 2004). In phagocytic cells, NOX helps to evade microbes and also forms a major enzymatic source of ROS in endothelial cells, smooth muscles and fibroblast (Sumimoto *et al.*, 2005). Altogether, generation of ROS by NADPH oxidase is important in the defense mechanism of the body as well as in the regulation of certain vital functions inside the organism such as intercellular communication and apoptosis (Covarrubias *et al.*, 2008).

Xanthine oxidase (XO), xanthine dehydrogenase (XDH) and xanthine oxidoreductase (XOR), are complex metalloflavoenzymes involved in many biological processes like biotransformation reactions in the liver, degradation of nucleic acids, metabolism of iron and so on (Harrison, 2004). XO catalyses the conversion of hypoxanthine to xanthine, which is then converted to uric acid accompanied by the generation of ROS. In the presence of NADH or xanthine as substrate, XO catalyse the reduction of nitrate to nitrite, and later converted to the biological messenger, nitric oxide (Nishino *et al.*, 2008). The interconversion of XDH to XO also produce large amount of reactive intermediates such as superoxide, hydrogen peroxide and nitric oxide in the cells.

Nitric oxide synthase (NOS) is another major enzymatic source of reactive intermediate production, which catalyses the conversion of L-arginine to L-citrulline and nitric oxide by a coupled reaction. However, in uncoupled reaction, the enzyme produces superoxide, generated from oxygenase domain by dissociation of ferrous-dioxygen complex (Vasquez-Vivar *et al.*, 1998). NOS exist in three isoforms namely neuronal NOS (nNOS), epithelial NOS (eNOS) and inflammatory NOS (iNOS). Nitric oxide thus formed by the enzymatic action of NOS diffuse through the cell

without binding to the receptor and helps in the signal transmission to maintain certain cellular activities like vasodilation, cell proliferation, inhibition of platelet aggregation, cell to cell adhesion and in immune responses by the macrophages against pathogens (Radomski *et al*., 1987).

Monoamine oxidase and cytochrome P450 oxidase are the other class of mitochondrial oxidases enzymes that contribute to endogenous generation of ROS in the cell. Monoamine oxidase present in the outer membrane of mitochondria functions as an inhibitor of neurotransmitters, and also has a significant role to catalyse the oxidative deamination of amines along with the production of hydrogen peroxide (Cadenas and Davies, 2000). Cytochrome P450 oxidase is the key enzyme found in the inner membrane of mitochondria responsible for the intramolecular transfer of oxygen during the metabolism and hydroxylation of several biocompounds like cholesterol, hormones, vitamins etc along with the reduction of oxygen to produce the free radical, superoxide.

The generation of ROS is predestined inside the biological system as it is essential for performing various biological functions like cell differentiation, apoptosis, cell signalling, necrosis, gene expression, inflammatory responses and defense against precancerous cells and pathogens (Gutteridge, 1995; Salganik, 2001) DNA, RNA, lipids and proteins are considered as the major targets of ROS, which oxidise the vital molecules to cause oxidative damages in various cells and tissues (Phaniendre *et al*., 2015). Cells are continuously exposed to minimal level of ROS however any increase in the level of ROS may lead to cellular damage, oxidative stress and DNA damage. The involvement of ROS and induction of oxidative stress is associated with the development and progress of several diseases like diabetes mellitus (Oberlay, 1988; Ahmad, 2005), cataract, asthma (Phaniendra *et al*., 2015), development of tumours including colorectal, prostate, breast, lungs and bladder cancers (Goldstein and Witz, 1990; Dreher and Junod, 1996), several neurodegenerative diseases like Alzheimer’s, Parkinson’s and Multiple sclerosis (Uttara *et al*., 2009), which are reported in various literatures. Normally ROS formed inside the cells are effectively scavenged by antioxidant system, which play a crucial role in maintaining proper biological activities and redox state of the cell.

## 1.3 Antioxidant defense system

Antioxidant defense system is the principal defense system against the reactive oxygen species and reactive nitrogen species in the biological system. All aerobic organisms have well-developed antioxidant systems that effectively scavenge the harmful effects of free radicals and ROS to slow down the lipid peroxidation (Halliwell *et al*., 2000). Under normal conditions, the formation of oxyradicals is counterbalanced by the production and activities of antioxidant system, consisting of low molecular weight radical scavengers which can act directly or indirectly on radicals. Based on the mode of action, antioxidants are broadly classified as extracellular antioxidants, membrane bound antioxidants and cellular antioxidants. Extracellular antioxidants are usually plasma proteins and other molecules such as transferrin, lactoferrin, ceruloplasmin, bilirubin, mucous and urate that actively scavenge free radicals (Gutteridge *et al*., 1981; Gutteridge, 1995). The free radicals formed on the surface of membrane are lipophilic and are different from those seen inside the cell. Therefore, different antioxidant system is required for the removal of free radicals, which makes the membrane bound antioxidants important and unique. Tocopherol (Vitamin-D), β-carotene, coenzyme-Q are the few important antioxidants found on the surface of membrane (Gutteridge, 1995). Cellular antioxidant system found inside the cells that effectively remove the harmful reactive metabolites formed inside the cell are known to cause intracellular damages (Li, 1999).

The mechanism of action of antioxidant defense system follows different methods as it prevents the free radical formation, interrupt once formed free radicals, and restore the oxidative damages caused by radicals. But in case of excessive ROS production, the oxidative damage formed in the molecules are not restored thereby leading to the accumulation of mutations in the cells (Gutteridge, 1995). Cellular antioxidants are highly specific as they are mostly enzymes, which include superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR) and glutathione peroxidase (GPx). The activities of all the enzymes are interrelated and operate in combined or series of cascade phenomenon in order to convert oxygen free radical to molecular oxygen and other harmless by products.

Superoxide ($O_2$-) is a radical that is formed during normal oxygen metabolism and as a by product of various physiological processes (McCord and Fridovich, 1969; Droge, 2002). Dismutation of superoxide is catalyzed by SOD, the potential antioxidant enzyme, which efficiently convert it into oxygen and hydrogen peroxide (Marklund *et al*., 1982). Thus SOD functions as the first line of defense against ROS, especially to the exogenously formed superoxides (Faraci and Didion, 2004). The hydrogen peroxide formed as a result of dismutation is more harmful to cells as it has the potential to interact with cellular compartments and even with DNA molecule thereby causing cellular and genetic damages (Halliwell and Gutteridge, 1999). Thus, the generated hydrogen peroxide is immediately eliminated by another important and kinetically most active enzyme called catalase into water and oxygen.

Glutathione, one of the stress reducing molecules found inside the cell, often called as ‘mother of all antioxidants’ help to protect the cell from oxidative damage (Schaffer and Buettner, 2001). Glutathione reductase maintains the level of glutathione in the cell, and along with glutathione peroxidase enzymes it removes free hydrogen peroxide in the form of water, and also reduce the level of lipid hydroperoxides (Di Giulio *et al*., 1989). Any disturbance in the redox state of cells as a result of generation of peroxides and free radicals could result in a condition called oxidative stress, which ultimately leads to cell death by carbohydrate oxidation, protein carbonylation, lipid peroxidation and nucleic acid oxidation. The major changes includes oxidation of enzymes or cofactors, amino acids of proteins, fatty acids in lipids, oxidative damages and strand breaks in the DNA and RNA, and also disrupts the cellular signalling system (Gutteridge, 1995). Thus during oxidative the imbalance of pro-oxidant and antioxidant status in cellular system occurs, which is effectively repaired by the action of enzymatic and non-enzymatic antioxidant enzymes. Therefore, evaluating the activities of antioxidant enzymes by suitable methods give the insight of oxidative and redox status of the cell or tissue. Lipid peroxidation is the renowned measure to detect oxidative damage in cells, in which malondialdehyde or 4-hydroxy-2-nonenal is the potent products of omega-3 and omega-6 polyunsaturated fatty acids formed during the reaction (Gutteridge, 1988).

Many environmental pollutants are known to induce the production of ROS, and its overproduction beyond the capacity of antioxidant defense system of organism leads to several deleterious effects on the components of cells such as proteins, lipids and DNA (Hofer *et al.*, 2005). Despite the large number of studies on the toxic effects of environmental contaminants on the organisms, the use of antioxidant defense system as endpoint to detect oxidative stress helps to monitor the health status of organisms. Aquatic ecosystem is highly sensitive and dynamic, where oxygen is the major limiting factor. Thus the health status and existence of the aquatic organisms depends on the availability of oxygen in the environment for respiration, and also play a role to maintain redox status within the cells or tissues of the aquatic organisms (Davies, 2000).

### 1.4 Antioxidant defense system in fishes

Similar to other aerobic organisms, fish also possess elaborate and efficient antioxidant defense system to escape from the attack of reactive oxygen species. On analyzing through the evolutionary scale, the generic name 'fish' refers to many different species that occupy different habitat, either freshwater or marine. There are many studies available to compare the similarities and differences between the antioxidant defense system of the most primitive fish species and the recently evolved one. Reports have correlated the relationship between phylogenetic position of fish and the activities of antioxidant enzymes. The primitive elasmobranch dogfish, *Squalus acanthias* has a very primitive antioxidant system where there was lack of catalase activity and relatively little SOD activity in the erythrocytes. However, the limited enzymatic antioxidant system was compensated by the high level of glutathione, urea and vitamin K (Rudneva, 1997). Antioxidant defense system of fish consists of both enzymatic and non-enzymatic antioxidants as in the case of other vertebrates. The major antioxidant defensive enzymes detected in fish include superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR) and glutathione peroxidase (GPx) (Rudneva, 1997). It seems that the activities of SOD, CAT and GPx is lower in fish than mammals as during the evolutionary

process low molecular weight antioxidants have appeared earlier than the enzymatic antioxidants (Martınez-Alvarez *et al*., 2005).

The fish antioxidant defense system also depends on many other factors such as age, nutritional status, fish behaviour, feeding behaviour, health status, environmental factors, exposure to pollutants and so on (Rueda-Jasso *et al*., 2004). According to the free radical theory of ageing, ROS generation increases with age that ends up in functional deterioration of antioxidant defense system in fish. The study of activities of catalase, SOD and the level of lipid peroxidation in liver and erythrocytes of different freshwater and marine fish species showed that the younger fish possess more antioxidant activity than the aged fish (Wdzieczak *et al*., 1982). Nutritional studies have revealed the crucial role of some minerals such as manganese, zinc, selenium, and copper in preventing oxidative stress where the deficiency of these minerals altered antioxidant defense system in rainbow trout, *Onchorhynchus mykiss* (Hidalgo *et al*., 2002). Feeding behaviour of fish varies among the species and is broadly classified as herbivores, carnivores, omnivores and limnivores. The antioxidant enzyme activities showed modifications in herbivores than omnivorous fish with the induction of lipid peroxidation, therefore feeding behaviour also known to influence the antioxidant status of fish (Radi *et al*., 1987).

Besides age, nutrition and feeding behaviour, certain environmental factors such as light, temperature, salinity, oxygen saturation and pH in water influence the health status and behaviour of fish, which in turn affect the potential of antioxidant status. In some case, fish experience food deprivation during the lifecycle, and the partial or complete food restriction has reported to increase lipid peroxidation in rainbow trout (Hidalgo *et al*., 2002). It was experimentally proved in fishes such as *Sparus aurata* and *Dentex dentex* when kept under food restriction or fasting for several weeks were detected with new isoforms of SOD and induction of oxidative stress (Pascual *et al*., 2003; Morales *et al*., 2004). Generally two types of SOD have been identified in fish namely cytosolic and mitochondrial SOD having similar functions like that of other vertebrates. The non-enzymatic antioxidants like vitamin C, E and K, carotinoids, peptides, and amino acids are also detected in fish. Both

enzymatic and non-enzymatic antioxidants act together for eliminating free radicals from the fish body.

It is well-known that fish is a thermo-dependent organism that adjusts the body temperature to the surrounding aquatic environment by cooling or warming actions. Thermal tolerance is also linked to aerobic capacity of fish where it involves the adjustment of both density and functional property of mitochondria thereby affecting ROS generation and antioxidant defenses (Portner, 2002). In addition, tissue of fish is made up of high levels of polyunsaturated fatty acid (PUFA), which are prone to oxidative damage. Dietary supplement of antioxidant such as vitamin E is also found to increase the radical scavenging action, and reduce the rate of oxidation of PUFA in the tissues of the fish (Watanabe *et al.*, 1981).

The body of fish with pathogenic attack, diseases, tumours etc are highly vulnerable to develop free radical accumulation and alterations in antioxidant system. In the aquatic ecosystem, the presence of xenobiotics is a major problem causing not only oxidative damage but also challenges the life of the aquatic organisms. Therefore, most of the ecotoxicological studies focused on the effects of different xenobiotic compounds on antioxidant enzyme activities, and proposed the responses are based on several aspects such as diversity in species, tissues, antioxidant parameters and duration or dose levels. The present study focused on the toxic effects of different nanoparticles at sublethal concentration in gill, liver and brain tissues for various durations in the fish, *Oreochromis mossambicus* by evaluating the antioxidant status. Xenobiotics when enter into the fish tempt to undergo generation of ROS as metabolic by products thereby interfere with the antioxidant system (Livingstone, 2001). Phase I and phase II metabolism are important for the degradation and elimination of xenobiotics from the body of organism. Fish also possesses a well-developed metabolic process to eradicate the free radicals formed during contaminant exposure. However, the endogenous functions of metabolizing enzymes are suppressed by the continuous exposure of environmental pollutants, which in turn permanently degrades the lipids in cell membrane by the induction of lipid peroxidation. Pathogenesis of several diseases

and irreversible toxic effects of xenobiotics on organism shows the synergistic effect of lipid peroxidation (Wilhelm, 2000).

## 1.5 Lipid peroxidation

Lipid peroxidation is a free radical oxidation leading to oxidative deterioration of polyunsaturated fatty acids that occurs frequently in the biological system (Gutteridge, 1995). Lipid peroxidation is a chain reaction and it occurs through chain initiation, propagation and termination reactions consisting of four main steps. Firstly, the chain initiation of peroxidation of PUFA occurs by hydrogen atom abstraction, which means the attack of any reactive species namely hydroxyl, peroxyl, or alkoxyl radical, and not by hydrogen peroxide and superoxide, to the hydrogen atom forming a carbon centred lipid radical. Secondly, the lipid radical undergo some molecular rearrangement to form conjugated diene, which then combines with molecular oxygen forming lipid peroxyl radical. In next stage, the reversal of second step occurs in which fragmentation of lipid peroxyl radical into lipid radical and molecular oxygen take place. In the fourth step, the rearrangement of peroxyl radical followed by the cyclization of peroxyl radical happens to end the propagation process (Porter *et al.*, 1995). The chain termination occurs when some antioxidant molecules or enzymes are added to break the chain, and the final end products of peroxidation are mainly hydroperoxides and cyclic peroxides (Gutteridge, 1995).

The products of lipid peroxidation also results in the formation of aldehydes where as many as 32 were identified, among which malondialdehye have been considered as the most important lipid peroxidation metabolite. The other highly reactive product is 4-hydroxynonenal, which is known to interact with biomolecules such as protein, DNA and phospholipids thereby generating inter- and intramolecular adducts (Esterbauer, 1996). Lipid peroxidation also decreases the membrane fluidity and disrupts the barrier functions of the membranes which seriously lead to oxidative stress-induced tissue damages. Therefore, evaluation of tissue specific damages induced by toxicant is essential in order to recognize specific effects of the chemicals on the target tissues. Many biochemical changes

induced by toxicants can be identified by assessing tissue specific marker enzymes, which help to interpret heterogenicity of toxic chemicals.

### 1.6 Tissue-specific markers

Tissue-specific markers are the enzymes that are peculiar for one type of cell or tissue where its activity is mainly restricted in the specific tissue only. The altered level or activity of such markers is the indication of abnormal metabolism and malfunctioning of the tissue, hence used as a tool for toxicity testing (Campion *et al.*, 2013). The major tissues or cells widely used for toxicity studies in fish comprise gill, liver, muscle, brain, kidney and erythrocytes. Several enzymes are known to be specific for certain tissues thereby recognized as indicator of environmental contamination (Magurie and Watkin, 1975; Walker, 1995). Commonly used tissue markers in toxicological studies are acid phosphatase, alkaline phosphatase, alanine aminotransferase, aspartate aminotransferase, glutamate oxaloacetate transaminases, glutamate pyruvate transaminase, lactate dehydrogenase, acetylcholinesterase and so on. The level of tissue markers can be either elevated or lowered according to the mechanism of action of the toxicants exposed. In aquatic organisms, the response observed in tissue specific markers is correlated to either the stressed condition due to toxicants or by the deterioration in the quality of water as a result of toxicant exposure (Oikari and Soivio, 1977; Sanders, 1993). Two major tissue specific markers evaluated in the present study were discussed in the sections below.

#### 1.6.1 Alkaline phosphatase

Alkaline phosphatase (ALP) is the enzyme predominantly found in gills, liver, serum, and muscle. ALP is a homodimeric enzyme optimally active at alkaline pH, and provided with 5 cysteine residues, 2 zinc atoms and 1 magnesium atom in the catalytic centre that play a key role in the metabolism within the liver (Milan, 2006). It is a large superfamily of enzymes found in prokaryotes and eukaryotes with the functions ranging from embryogenesis to bone mineralization in different tissues and life stages of organisms (Milan, 2006). ALP has phosphotransferase and

phosphodiesterase activities, and the catalytic action of the superfamily involves phosphorylation, sulfatation and phosphonation of the conserved serine, threonine and cysteine amino acid residues (Kuzminova, 2016). Therefore, the use of ALP as biomarker for ichthyomonitoring has been increased in recent research.

### 1.6.2 Acetylcholinesterase

Acetylcholinesterase (AChE) is the key brain marker enzyme responsible for the breakdown of acetylcholine and other choline ester neurotransmitters (Quinn, 1987). AChE is the integral enzyme that terminates synaptic transmission of stimulus by catalysing the breakdown of neurotransmitter, and found abundant in nerves and tissues of central and peripheral nervous system, muscles, sensory, motor, cholinergic and non-cholinergic fibers (Massoulie *et al*., 1993). It is the enzyme of carboxylesterase family and target for inhibition of many environmental pollutants and drugs. Thus AChE is widely used as a crucial neurotoxicity marker in most of the toxicology studies, and the variation in the activity of enzyme is also associated to change in the behaviour of the animal.

The use of tissue specific biochemical markers provides a functional tool to identify adverse effects of any toxicant on the organism. Furthermore, it is necessary to understand that biochemical markers are not always more sensitive than whole organism responses because the sensitivity or response of an organism towards the toxicant depends on several factors such as the mode of action, duration of exposure and test species. Therefore, the present study investigated the combined battery of biomarkers such as antioxidant status as well as tissue specific enzyme activities to analyse different levels of toxic effects of selected nanoparticles in the freshwater fish, *Oreochromis mossambicus*.

# Review of Literature

In recent years, increased use of nanoparticles pave way to discharge them in large quantities from various sources into the environment and creates major concern to the ecotoxicologists. Metal and metal oxide nanoparticles are widely used in many fields ranging from industrial sectors, biomedicine, pharmaceuticals, cosmetics etc. The toxic effects of various metal and metal oxide nanoparticles have been addressed in several scientific literatures. Nanoparticles exert toxicity on humans, animals and in the ecosystems but still controversy remains regarding the toxic effects and mechanism of nanoparticles. Nanoparticles are known to generate reactive oxygen species at a greater extent than the micro-sized particles, resulting in several adverse effects such as oxidative stress, pro-inflammation, tumorigenesis, neurodegenerative disorders etc. All organisms experience oxidative stress during the course of evolution, which can be prevailed by the appropriate defensive strategies so as to prevent or inactivate reactive intermediates and terminate lipid peroxidation chain reaction. In aquatic ecosystem, the generation of ROS by contaminants is largely associated with cellular injures due to alterations in DNA, proteins and membranes (Leonard *et al*., 2004). The alteration in the activities of antioxidant enzymes are crucial and very sensitive in revealing pro-oxidant condition, and have been used as biomarkers of oxidative stress in fish (Ahmad *et al*., 2006; Oliveira *et al*., 2010).

There are several literature studies portraying the toxic effects of different nanoparticles in various organs and test species, where the mechanism of action are known to vary based on the species, dose and duration of exposure. Changes in the biochemical parameters characterized by the alteration in the level of glutathione and induction of lipid peroxidation thereby provoking oxidative stress has been reported on the sublethal exposure of titanium dioxide nanoparticles for 14 days in the rainbow trout (Federici *et al*., 2007). Chronic sublethal exposure of fullerene nanoparticles aggregates has demonstrated to induce oxidative stress in liver and gill tissues of the freshwater fish, *Carassius auratus* (Zhu *et al*., 2008). In rainbow trout, dietary exposure to titanium dioxide nanoparticles for eight weeks resulted in the accumulation of nanoparticles in the tissues like gill, liver, brain, spleen and gut without clearance of particles in brain tissue even after the recovery period

(Ramsden *et al.*, 2009). Single intravenous injection of titanium dioxide nanoparticles in rainbow trout has resulted in high accumulation in kidney tissue with minimal disturbance to renal functioning along with tissue clearance after 90 days (Scown *et al.*, 2009). Changes in the activities of alanine aminotransferase and hydroxyproline have been observed in the liver of the mice injected with 70 nm sized silica twice a week for 4 weeks. In addition, histopathological alterations in liver and spleen tissues have been noted after silica nanoparticles exposure for four weeks in rats (Nishimori *et al.*, 2009). Nano-silica exposure in human kidney cell lines, HEK293 has shown to reduce cell viability and GSH level, increased ROS generation and TBARS level thereby suggested cytotoxicity and oxidative stress in the cell line (Wang *et al.*, 2009). Titanium dioxide nanoparticles exposed up to eight days in juvenile carp, *Cyprinus carpio* has shown to cause depletion of antioxidant enzyme activities and elevated lipid peroxidation in gill, liver and brain tissues along with histopathological changes indicated the potential risk of nanoparticles (Hao *et al.*, 2009).

Gold and silver nanoparticles have been shown to induce ROS generation, loss of membrane integrity and cellular metabolic activities in the hepatocytes of rainbow trout (Farkas *et al.*, 2010). Accumulation of nanoparticles in the brain tissue and induction of neurotoxicity has been addressed in many literatures. Gold nanoparticles has been shown to get absorbed into the systemic circulation, and accumulated in various tissues including brain in the rat model, suggesting the non-saturable uptake of nanoparticles by brain tissue (Lasagna-Reeves *et al.*, 2010). Zebrafish exposed to silver nanoparticles has been shown to induce oxidative stress and also caused DNA damage as demonstrated by the upregulation of p53-related pro-apoptotic genes such as Bax, Noxa and p21 in the liver of adult zebrafish (Choi *et al.*, 2010). Nano-scale titanium dioxide, zinc oxide and their bulk counterparts in zebrafish has exhibited acute toxicity by the generation of oxygen free radicals in gill, liver and gut tissues that lead to oxidative stress and oxidative damage to the fish (Xiong *et al.*, 2011).

Juvenile common carp, *Cyprinus carpio*, on exposure to silver nanoparticles altered antioxidant defense system and reported histological abnormalities in gill,

liver and brain tissues (Lee *et al.*, 2012). Exposure of synthesized silver nanoparticles at 25, 50 and 75 mg/L concentrations for eight days reduced the activities of antioxidant enzymes and lowered the contents of antioxidants in the gill and liver tissues of the fish, *Oreochromis mossambicus* (Govindasamy and Rahuman, 2012). The activities of antioxidant enzymes such as superoxide dismutase, catalase and glutathione peroxidase, and non-enzymatic antioxidant namely reduced glutathione content has been decreased with elevated level of lipid peroxidation in gill, liver, brain and intestine of juvenile carp, *Cyprinus carpio* after exposure to zinc oxide nanoparticles (Hao and Chen, 2012). Mechanism of silver nanoparticles-induced developmental toxicity studies revealed the production of free radicals and reactive oxygen species with generation of high thiobarbituric acid reactive substances in embryonic medaka, *Oryzias latipes* (Wu and Zhou, 2012). Intraperitoneal injection of silicon-based quantum dots to *Carassius auratus gibelio* for one week durations induced oxidative stress in renal tissue followed by structural damages, however, renal tissue showed restoration through nephron development (Petrache *et al.*, 2012). In freshwater snail *Lymnaea luteola*, the acute exposure to the transition metal oxide zinc oxide nanoparticles for 96 h resulted in oxidative stress and genotoxic responses by alteration in the level of glutathione, decreased activities of antioxidant enzymes and increased level of malondialdehyde (Ali *et al.*, 2012). Fullerene $C_{60}$ nanoparticles has been shown to induce redox disruption in brain and gill tissues through thiol-disulfide pathway leading to loss of antioxidant competence and oxidative damage in *Cyprinus carpio* (Ferreira *et al.*, 2012).

Acute exposure to titanium dioxide nanoparticles has resulted in respiratory inhibition by significant change in oxygen consumption, and dose-dependent decrease in total glycogen and total protein in the gill and muscle tissue of the fish *Danio rerio* (Vutukuru *et al.*, 2013). Exposure to sublethal concentrations of silica nanoparticles has been shown to alter the antioxidant enzymes and induced lipid peroxidation in the gill, liver and muscle of zebrafish after an exposure period of 7 days (Ramesh *et al.*, 2013). Nano-scale zinc oxide exposed to zebrafish embryos for 144 hour post-fertilization has been shown to enhance the transcriptional expression of mitochondrial inner membrane genes, Bcl-2, related to reactive oxygen species

production thereby contributed to oxidative stress and DNA damage (Zhao *et al.*, 2013).

Subacute exposure of nickel nanoparticles for 14 days has been shown to decline the activities of antioxidant enzymes thereby caused oxidative stress in liver and gill of the fish, *Oreochromis mossambicus* (Jayaseelan *et al.*, 2014). Silver and titanium dioxide nanoparticles exposed to fish cell line, BF-2, and zebrafish embryos showed oxidative stress-dependent cytotoxicity and embryonic toxicity that has been increased upon exposure to simulated solar light, and the elevated toxicity observed was due to surface oxidation and physicochemical modification of nanoparticles (George *et al.*, 2014). Titanium dioxide nanoparticles on exposure to the embryos of zebrafish also showed overproduction of reactive oxygen species and reported sublethal oxidative stress (Faria *et al.*, 2014). Increased lipid peroxidation and decreased level of glutathione, catalase, and superoxide dismutase has been observed in heart and gill cell lines of *Catla catla* and gill cell line of *Labeo rohita* after exposure to silver nanoparticles (Taju *et al.*, 2014). Induction of reactive oxygen species and elevated expression of transcripts of pro-apoptotic genes tumour necrosis factor-α has been reported when indium and indium tin oxide nanoparticles were exposed to zebrafish liver cells and in the embryos of zebrafish. The results provided first evidence of endoplasmic reticulum stress with stronger effects on cell lines than the embryo of fish (Brun *et al.*, 2014). Induction in the expression of antioxidant genes of catalase, superoxide dismutase and glutathione sulfo-transferase has been observed in the liver of the fish *Oreochromis niloticus* after intraperitoneal injection of titanium dioxide nanoparticles for 24 h (Varela-Valencia *et al.*, 2014).

The toxicity of $Al_2O_3$-NPs has been demonstrated in Chinook salmon cells, CHSE-214, where it induced cytotoxicity and oxidative stress as evidenced by the decline in cell viability, reduction in the activities of superoxide dismutase, catalase and glutathione with increase in glutathione sulfo-transferase and lipid peroxidation in dose-dependent manner (Srikanth *et al.*, 2015). Toxic effects of nano-sized zinc oxide particles at 1 and 10 mg/L concentrations has reported with accumulation of nano-sized particles in various tissues such as liver, gill, intestine, brain, kidney and

muscle, along with elevated oxidative stress in tilapia, *Oreochromis niloticus* on comparison with the large-zinc oxide particles (Kaya *et al*., 2015). Titanium dioxide and zinc oxide nanoparticles exposure has been shown to cause a dose-dependent increase in DNA damage, lipid peroxidation and protein carbonylation along with a significant decrease in the activities of superoxide dismutase, catalase, total glutathione levels and total antioxidant capacity thus indicated that the exposed WAG cell lines from gill tissue of *Wallago attu* were under oxidative stress (Dubev *et al*., 2015). Similarly the exposure of zinc nanoparticles for 96 h induced deleterious biochemical alterations and lipid peroxidation in the brain tissues of *Tilapia zillii* and *Oreochromis niloticus* (Saddick *et al*., 2015).

Sublethal exposure to silicon dioxide nanoparticles at 5 mg/L concentration for 96 h has been shown to induce oxidative damage in the hepatocytes of the fish *Oreochromis mossambicus* (Vidya and Chitra, 2015). Five different nanoparticles namely europium-doped hydroxyapatite, europium-doped yttrium oxide, bismuth germinate, multi walled carbon nanotubes and silver on exposure to zebrafish for 48 h has resulted in protein oxidation and lipid peroxidation in a dose-dependent manner (Carillo *et al*., 2015). Ability of nanoparticles to cross the placenta has been demonstrated in a study where carbon-14 labeled fullerene $C_{60}$ when administered to pregnant and lactating rats showed the radioactivity in the placenta, fetuses of pregnant rats, milk of lactating rats and in the pups (Snyder *et al*., 2015). Embryos of zebrafish exposed to environmentally relevant doses of cobalt ferrite nanoparticles for 96 h has induced severe oxidative stress, apoptosis of cells in the head, heart and tail region thus contributed to decreased survival rate, malformation, hatching delay, heart dysfunction and tail flexure of larvae (Ahmad *et al*., 2015).

Exposure to silver nanoparticles has been shown to induce cytotoxicity, oxidative stress and alterations of cellular structures, particularly the mitochondria, in the fish hepatic cell line, PLHC-1 (Bermejo-Nogales *et al*., 2016). Sublethal acute exposure of copper oxide nanoparticles has been shown to induce oxidative stress and triggered apoptosis in the zebrafish embryo stating uncharted connection between oxidative stress and teratogenicity during the developmental process of the exposed embryo (Ganesan *et al*., 2016). Exposure to silver nanoparticles at 4 mg/L

concentration has been shown to induce oxidative stress in fish models, *Tilapia zilli* and *Oreochromis niloticus* (Afifi *et al.*, 2016). Eco-relevant concentrations of copper nanoparticles has been shown to elevate the activities of antioxidant enzymes along with histological changes and protein down-regulations in the liver, kidney and gill of the juvenile carp, *Cyprinus carpio* (Gupta *et al.*, 2016). In the fish *Labeo rohita*, sublethal exposure to silver nanoparticles for 7 days resulted in the depletion of antioxidant enzymes namely superoxide dismutase, catalase and glutathione S-transferase activities in the gill, liver and muscle tissues (Rajkumar *et al.*, 2016). Exposure of zinc oxide nanoparticles has experienced biochemical and redox balance disturbances associated with alteration in ethoxy-resorufin-*O*-deethylase activity and induced oxidative stress in gill tissue thus shown to interfere with cytochrome P450 metabolic processes (Connolly *et al.*, 2016).

Iron oxide nanoparticles at five different sublethal concentrations exposed for 96 h showed mild oxidative damage and high level of genetic damage in zebrafish (Villacis *et al.*, 2017). Silver nanoparticles has been shown to cause osmoregulatory impairment and oxidative stress in Caspian kutum, *Rutilus kutum* as demonstrated by alteration in heat shock protein 70, mRNA expression, $Na+/K+$-ATPase activity in gill tissue, and decreased enzymatic activities of superoxide dismutase, glutathione peroxidase, lactate dehydrogenase and alkaline phosphatase in liver tissue, along with decreased level of whole-body cortisol and thyroid hormones (Masouleh *et al.*, 2017). Treatment of silver nanoparticles for 28 days induced alterations in the antioxidant enzymes and caused oxidative stress in the liver and gill tissues of the fish, *Labeo rohita* (Khan *et al.*, 2017). Subchronic exposure of silver nanoparticles for 21days has been shown to produce oxidative stress as evidenced by a diminution in antioxidant enzymes activity and an increase in thiobarbituric acid reactive species and oxidized proteins. In addition, the levels of macromolecules such as proteins, carbohydrates and lipids has been decreased, and generated a high-energy consumption as reflected in the reduction of glucose levels in the goodeid fish *Chapalichthys pardalis* (Valerio-Garcia *et al.*, 2017). Silica-coated iron oxide nanoparticles has been shown to induce cytotoxicity and oxidative stress in Chinook salmon cells, CHSE-214, derived from *Oncorhynchus*

*tshawytscha* embryos as confirmed by significant dose-dependent reduction in total glutathione content, activities of catalase and glutathione reductase enzymes with a concomitant increase in the levels of lipid peroxidation and protein carbonyl (Srikanth *et al*., 2017).

Induction of oxidative stress has been reported in the muscle tissue of Nile tilapia after zinc oxide nanoparticles exposure at 1 and 2 mg/ L concentrations for 15 days (Abdelazim *et al*., 2018). In a recent study, copper oxide nanoparticles exposure caused alteration in aerobic metabolic rate, gill osmoregulatory physiology and mitochondrial function, oxidative stress markers, and morphological damage in two Amazon fish species namely, dwarf cichlid, *Apistogramma agassizii* and cardinal tetra, *Paracheirodon axelrodi* (Braz-Mota *et al*., 2018). The role of nanoparticles as toxicants, and the ability of nanoparticles to generate ROS and induction of oxidative stress were offered in several literatures. The present study was aimed to evaluate the potential acute and chronic toxic effects of the selected nanoparticles on the antioxidant status in the gill, liver and brain tissues of the fish, *Oreochromis mossambicus*.

# Materials and Methods

### 3.1 Test concentrations and durations of selected nanoparticles

After the determination of median lethal concentrations of selected nanoparticles, one-tenth of $LC_{50}$-96 h was selected as test concentrations for the present study. Control group, without nanoparticles, was also maintained along with the treatment groups retaining ten animals in each group. Thus the sublethal concentrations used in the study was $Al_2O_3$-NPs – 4 mg/ L, $Fe_3O_4$-NPs – 15 mg/ L (based on agglomeration), $SiO_2$-NPs – 12 mg/ L and $TiO_2$-NPs – 16.4 mg/ L. Nanoparticles were exposed for short-term i.e., 24, 72 and 96 h, and long-term durations i.e., 15, 30 and 60 days, respectively.

### 3.2 Collection of tissues and preparation of samples

After the every treatment durations, fish were caught gently by using a small dip net, one at a time, with least disturbances so as to avoid stress to the animal. Fish were weighed and recorded, then the tissues such as gill, liver and brain were dissected out, weighed, and stored at 4ºC until the biochemical analyses were performed.

### 3.3 Hepatosomatic index (HSI)

Hepatosomatic index of the selected nanoparticles was determined using the formula as given below:

Calculation of HSI = Liver weight (mg)/ Total body weight (mg) X 100

### 3.4 Preparation of samples

A 1% (w/ v) homogenate of tissues were prepared in ice-cold normal saline using a motor-driven glass Teflon homogenizer on crushed ice for a minute. The homogenates were centrifuged at 800*g* for 15 min at 4ºC to obtain the supernatants of gill, liver and brain tissues, and then used for the biochemical analyses. Protein was estimated by the method of Lowry *et al* (1951) with bovine serum albumin (BSA) as the standard and all other biochemical assays were performed by the following methods:

### 3.5 Assay of antioxidant enzymes

Activities of superoxide dismutase, catalase, glutathione reductase and glutathione peroxidase enzymes were analyzed in gill, liver and brain tissues as described below:

#### 3.5.1 Superoxide dismutase enzyme

Superoxide dismutase (EC 1.15.1.1) was assayed by the method of Marklund and Marklund (1974). Briefly, Tris hydrochloric acid buffer (50 mM, pH 7.6) containing 1 mM EDTA, 0.2 mM pyrogallol was taken. To the assay mixture, 100 µl of enzyme source was added and the increase in the absorbance was measured immediately at 420 nm against enzyme blank for 3 min at 10 sec intervals on a Shimadzu UV-Visible Spectrophotometer. Activity of enzyme was expressed as nmol pyrogallol oxidised/ min/ mg protein.

#### 3.5.2 Catalase enzyme

Catalase (EC. 1.11.1.6) was assayed by the method of Claiborne (1985). Total assay mixture of 3 ml contained phosphate buffer (50 mM, pH 7.0), hydrogen peroxide (19 mM) and 50 µl enzyme source. Decrease in absorbance was measured immediately at 240 nm against enzyme blank at 10 sec intervals for 3 min on a Shimadzu UV-Visible Spectrophotometer. Activity of enzyme was expressed as µmol of hydrogen peroxide consumed/ min/ mg protein.

#### 3.5.3 Glutathione reductase enzyme

Glutathione reductase (EC. 1.6.4.2) was assayed by the method of Carlberg and Mannervik (1985). The assay mixture consists of phosphate buffer (100 mM, pH 7.6), NADPH (0.2 µM), Glutathione oxidised (2 mM) and EDTA (0.01 M). Enzyme was added with the assay mixture and the disappearance of NADPH was measured immediately at 340 nm against enzyme blank at 10 sec intervals for 3 min on a Shimadzu UV-Visible Spectrophotometer. Activity of enzyme was expressed as nmol of NADPH oxidised/ min/ mg protein.

### 3.5.4 Glutathione peroxidase enzyme

Glutathione peroxidase (EC.1.11.1.9) was assayed by the method of Mohandas *et al* (1984) using hydrogen peroxide and NADPH as substrates. The assay mixture consists of phosphate buffer (100 mM, pH 7.6), EDTA (0.01 M), sodium azide, glutathione reductase, glutathione reduced and NADPH (0.2 μM). Enzyme was added and the disappearance of NADPH was measured immediately at 340 nm against enzyme blank at 10 sec intervals for 3 min on a Shimadzu UV-Visible Spectrophotometer. Activity of enzyme was expressed as nmol of NADPH oxidised/ min/ mg protein.

## 3.6 Determination of lipid peroxidation

The level of lipid peroxidation was measured using thiobarbituric acid colour reaction for malondialdehyde (MDA) at 535 nm, according to the method of Ohkawa *et al* (1979). For the assay, 15% w/v of trichloroacetic acid, 0.37% of 2-thiobarbituric acid and 0.25 N hydrochloric acid were mixed in the ratio 1:1:1 and kept as working solution. Enzyme was added to the working solution in the ratio 1:2 and incubated in boiling water bath for 10 min and absorbance was measured. MDA solution was used as a standard, and the results were expressed as nmol of MDA produced/ mg protein.

## 3.7 Determination of hydrogen peroxide generation

Level of hydrogen peroxide generation was measured by the method of Pick and Keisari, 1981. The assay was based on the $H_2O_2$-mediated and horseradish peroxidise-dependent oxidation of phenol red to a product. Briefly, the incubation mixture contained phosphate buffer (50 mM, pH 7.6), horseradish peroxidase (8.5 unit), 0.28 nM phenol red, 5.5 nM dextrose and 100 μl of enzyme source. The reaction was carried out at room temperature for 30 min and terminated by the addition of sodium hydroxide (10 N), and the absorbance was read at 610 nm against the blank. Standard curve was prepared using known concentrations of $H_2O_2$ and expressed as nmol hydrogen peroxide generated/ mg protein.

### 3.8 Assay of tissue-specific marker enzymes

#### 3.8.1 Assay of alkaline phosphatase enzyme

The activity of alkaline phosphatase (EC.3.1.3.1) in gill and liver tissues were measured by the method of Bessey *et al.*, 1946. Preincubation of *p*-nitrophenyl phosphate and glycine buffer, pH 10.5 was done for 5 min at 37ºC. To the incubation mixture, sample and 0.02 N NaOH was added and incubated for 30 min. The colour developed was read at 420 nm on a Shimadzu UV-Visible Spectrophotometer against the blank. Then 0.1 ml of concentrated hydrochloric acid was added, mixed and the differences in absorbance were taken as the measure of enzyme activity. The alkaline phosphatase activity was calculated from the calibration curve obtained using *p*-nitrophenol standard. Activity of enzyme was expressed as µmol of *p*-nitrophenol liberated/ min/ mg protein.

#### 3.8.2 Assay of acetylcholinesterase enzyme

Activity of acetylcholinesterase in brain was assayed by the method of Ellman *et al.*, 1961. The enzyme activity was measured by following the increase of yellow colour produced from thiocholine when it reacts with dithiobisnitrobenzoate ion. Dithiobisnitrobenzoic acid (DTNB) 0.01 M is the reagent used for this assay. The tissue was homogenized, and dissolved in phosphate buffer, 0.1 M, pH 8.0 and 15 mg of sodium bicarbonate and DTNB were added. The reagent was made up in buffer of pH 7. The activity of enzyme was expressed as nmol acetylthiocholine hydolysed/ min/ mg protein.

### 3.9 Statistical analysis

Statistical Package for the Social Sciences (SPSS, version 17.0) was used to analyse the significance of the results. One-way analysis of variance (ANOVA) followed by Duncan's Multiple Range test as Post-hoc test was performed in the experiment. Differences were set significant at $p<0.05$ against the control group and are denoted as asterisk (*) symbol in the Tables and Figures. Data are presented as mean ± SD for ten animals per group. All biochemical estimations were carried out in duplicate to avoid statistical errors.

# Results

## 4.1. Effects of nanoparticles on the body weights, organ weights and hepatosomatic index in the fish, *Oreochromis mossambicus*

**Effect of $Al_2O_3$-NPs**

The body weights of $Al_2O_3$-NPs exposed fish, for short-term (24, 72 and 96 h) and long-term (15, 30 and 60 days) durations, showed no signification changes when compared to the control group (Table 1). Exposure to $Al_2O_3$-NPs showed significant ($P<0.05$) increase in the weight of gill tissues after 96 h onwards (Table 1). Increase in the gill tissue weight was retained in the treatment withdrawal group as well (Table 1). Significant reduction in the hepatosomatic index (HSI) was observed after 60 days of $Al_2O_3$-NPs treatment, whereas HSI of the treatment withdrawal group was found similar to that of the control group (Table 1). Weight of brain tissue increased significantly ($P<0.05$) after 96 h and continued up to 30 days whereas subsequent significant ($P<0.05$) reduction was observed in 60 days of $Al_2O_3$-NPs exposure group (Table 1). Treatment withdrawal group showed significant changes only in the weights of gill and brain tissues (Table1).

**Effect of $Fe_3O_4$-NPs**

$Fe_3O_4$-NPs exposure did not alter the body weight of fish throughout the experiment period (Table 2). The weights of gill increased significantly ($P<0.05$) in 30 and 60 days exposure groups, and also in treatment withdrawal group (Table 2). Hepatosomatic index (HSI) decreased significantly ($P<0.05$) after 30 and 60 days of $Fe_3O_4$-NPs treatment (Table 2). Brain weights after $Fe_3O_4$-NPs exposure showed no significant changes throughout the experiment (Table 2). The reversal of treatment for 60 days showed no significant changes in the brain weight and hepatosomatic index when compared to the control group (Table 2).

**Effect of $SiO_2$-NPs**

Exposure to $SiO_2$-NPs showed significant ($P<0.05$) reduction in the body weights of fish after 30 days onwards whereas no significant changes were observed after the treatment withdrawal (Table 3). Weight of gill tissues showed significant ($P<0.05$) reduction in 30 and 60 days of $SiO_2$-NPs exposure groups, without any

significant changes in the treatment withdrawal group though a slight increase was noted (Table 3). Hepatosomatic index of $SiO_2$-NPs exposed groups showed significant ($P<0.05$) and time-dependent decrease from 96 h and continued throughout the treatment period of 60 days, and the treatment withdrawal regained the effects with slight increase in the percentage of HSI (Table 3). The weight of the brain tissue decreased significantly ($P<0.05$) after 30 and 60 days of $SiO_2$-NPs exposure, and slight increase in the brain weight observed after the treatment withdrawal was not significant (Table 3).

**Effect of $TiO_2$-NPs**

$TiO_2$-NPs exposed for short-term and long-term durations did not alter the weight of the animal when compared to the control fish (Table 4). Gill weights of fish exposed to $TiO_2$-NPs showed significant ($P<0.05$) increase in 96 h of short-term exposure group, and the time-dependent significant ($P<0.05$) increases were observed in all groups of long-term exposure (Table 4). However, the hepatosomatic index and brain weight remained unchanged throughout the experimental period (Table 4). The reversal of treatment for 60 days showed significant increase in the weights of gill and brain tissues without any significant changes in the body weight and hepatosomatic index when compared to the corresponding control group (Table 4).

**Table 1** **Effect of aluminium oxide nanoparticles ($Al_2O_3$-NPs) on the body weight and tissue weights of the fish, *Oreochromis mossambicus* (Mean ± SD; n = 10/ group; *P<0.05 against the control group)**

| Parameters | $Al_2O_3$-NPs (4 mg/ L) | | | | | | | Treatment withdrawal (60 days) |
|---|---|---|---|---|---|---|---|---|
| | Short-term exposure | | | | Long-term exposure | | | |
| | Control | 24 h | 72 h | 96 h | 15 days | 30 days | 60 days | |
| **Body weight (g)** | 6.71±0.19 | 6.54±0.33 | 6.09±0.21 | 6.15±0.15 | 6.58±0.32 | 6.76±0.25 | 6.78±0.19 | 7.03±0.13 |
| **Gill weight (mg)** | 143±11.9 | 145±2.87 | 159±3.72 | 161±4.09* | 174±3.16* | 175±2.71* | 179±2.28* | 193±3.23* |
| **Hepatosomatic index (%)** | 13.53±1.86 | 12.42±1.61 | 13.84±2.29 | 13.39±1.85 | 12.50±1.12 | 12.09±0.83 | 10.84±0.93* | 13.19±0.59 |
| **Brain weight (mg)** | 16.5±1.81 | 15.8±1.31 | 16.3±3.77 | 19.5±3.30* | 19.6±2.22* | 19.7±3.80* | 13.9±1.37* | 19.9±0.87* |

**Table 2** **Effect of iron oxide nanoparticles ($Fe_3O_4$-NPs) on the body weight and tissue weights of the fish, *Oreochromis mossambicus* (Mean ± SD; n = 10/ group; *P<0.05 against the control group)**

| Parameters | $Fe_3O_4$-NPs (15 mg/ L) | | | | | | | Treatment withdrawal (60 days) |
|---|---|---|---|---|---|---|---|---|
| | Short-term exposure | | | | Long-term exposure | | | |
| | Control | 24 h | 72 h | 96 h | 15 days | 30 days | 60 days | |
| **Body weight (g)** | 6.71±0.09 | 6.25±0.40 | 6.31±0.35 | 6.51±0.26 | 6.59±0.38 | 6.47±0.38 | 6.63±0.40 | 6.99±0.13 |
| **Gill weight (mg)** | 143±11.9 | 146±3.25 | 152±3.88 | 152±1.19 | 159±3.20 | 161±2.98* | 172±1.41* | 186±1.50* |
| **Hepatosomatic index (%)** | 13.53±1.86 | 13.78±1.75 | 13.22±1.06 | 12.06±1.42 | 11.98±1.53 | 11.90±1.75* | 9.84±1.26* | 13.22±0.48 |
| **Brain weight (mg)** | 16.5±1.81 | 16.6±2.23 | 16.3±3.56 | 15.2±1.61 | 15.1±0.90 | 15.3±2.83 | 15.0±1.66 | 19.5±1.08 |

**Table 3** **Effect of silicon dioxide nanoparticles ($SiO_2$-NPs) on the body weight and tissue weights in the fish, *Oreochromis mossambicus* (Mean ± SD; n = 10/ group; *P<0.05 against the control group)**

| Parameters | $SiO_2$-NPs (12 mg/ L) | | | | | | | Treatment withdrawal (60 days) |
|---|---|---|---|---|---|---|---|---|
| | Short-term exposure | | | | Long-term exposure | | | |
| | Control | 24 h | 72 h | 96 h | 15 days | 30 days | 60 days | |
| **Body weight (g)** | 6.71±0.09 | 6.68±0.07 | 6.63±0.08 | 6.64±0.06 | 6.68±0.04 | 6.59±0.04* | 6.52±0.05* | 6.81±0.04 |
| **Gill weight (mg)** | 143±11.9 | 142±7.71 | 141±4.89 | 140±4.86 | 138±6.90 | 135±5.93* | 132±4.06* | 151±4.32 |
| **Hepatosomatic index (%)** | 13.53±1.86 | 13.0±0.13 | 12.8±0.14 | 12.4±0.22* | 12.1±0.20* | 12.0±0.23* | 11.6±0.14* | 13.7±0.11 |
| **Brain weight (mg)** | 16.5±1.81 | 16.5±1.38 | 16.4±0.62 | 16.4±0.88 | 16.1±0.45 | 15.9±1.21* | 15.6±0.76* | 17.2±1.12 |

**Table 4** **Effect of titanium dioxide nanoparticles ($TiO_2$-NPs) on the body weight and tissue weights of the fish, *Oreochromis mossambicus* (Mean ± SD; n = 10/ group; *P<0.05 against the control group)**

| Parameters | $TiO_2$-NPs (16.4 mg/ L) | | | | | | | Treatment withdrawal (60 days) |
|---|---|---|---|---|---|---|---|---|
| | Short-term exposure | | | | Long-term exposure | | | |
| | Control | 24 h | 72 h | 96 h | 15 days | 30 days | 60 days | |
| **Body weight (g)** | 6.71±0.19 | 6.58±0.32 | 6.39±0.31 | 6.13±0.18 | 6.76±0.31 | 6.56±0.38 | 6.98±0.26 | 7.02±0.16 |
| **Gill weight (mg)** | 143±11.9 | 140±4.90 | 146±6.74 | 157±3.31* | 160±2.88* | 162±5.49* | 192±3.42* | 185±2.69* |
| **Hepatosomatic index (%)** | 13.53±1.86 | 13.46±1.42 | 13.73±0.61 | 14.52±1.57 | 13.00±1.94 | 14.07±1.57 | 13.45±1.12 | 13.14±0.61 |
| **Brain weight (mg)** | 16.5±1.81 | 15.5±3.30 | 14.8±3.96 | 13.5±2.27 | 15.4±4.35 | 15.1±4.33 | 13.5±2.27 | 19.3±0.82* |

## 4.2. Effects of nanoparticles on the antioxidant status in gill tissue of the fish, *Oreochromis mossambicus*

**Effect of $Al_2O_3$-NPs**

$Al_2O_3$-NPs when exposed to fish for short-term (24, 72 and 96 h) and long-term (15, 30 and 60 days) durations showed significant (P<0.05) reduction in the activities of antioxidant enzymes such as superoxide dismutase, catalase, glutathione reductase and glutathione peroxidase when compared to the corresponding control group (Figures 1a-d). The levels of hydrogen peroxide generation and lipid peroxidation increased significantly (P<0.05) after $Al_2O_3$NPs exposure than that of the control group (Figures 1e and 1f). In the treatment withdrawal group, the activities of all antioxidant enzymes, and the levels of hydrogen peroxide generation and lipid peroxidation were found similar to that of the treatment groups (Figures 1a-f).

**Effect of $Fe_3O_4$-NPs**

In the gill tissue of the fish treated with $Fe_3O_4$-NPs, the activities of antioxidant enzymes namely superoxide dismutase, catalase, glutathione reductase and glutathione peroxidase showed significant (P<0.05) decrease in both short-term and long-term exposure groups when compared to the control group (Figures 2a-d). There was a concomitant time-dependent and significant (P<0.05) increase in the levels of hydrogen peroxide generation and lipid peroxidation in $Fe_3O_4$-NPs exposed groups (Figures 2e and 2f). The alterations in the antioxidant defense status of gill tissues that were observed after the treatment withdrawal for 60 days appear similar to the treatment groups (Figures 2a-f).

**Effect of $SiO_2$-NPs**

Activities of superoxide dismutase, catalase, glutathione reductase and glutathione peroxidase in the gill tissue of $SiO_2$-NPs exposed groups showed significant (P<0.05) reduction after short-term and long-term exposure when compared to the control group (Figures 3a-d). The levels of hydrogen peroxide generation and lipid peroxidation increased significantly (P<0.05) after $SiO_2$-NPs treatment in time-dependent manner when compared with the corresponding control

group (Figures 3e and 3f). When $SiO_2$-NPs exposure was withdrawn for the period of 60 days also showed decrease in the activities of antioxidant enzymes with simultaneous increase in the levels of hydrogen peroxide generation and lipid peroxidation in gill tissue of fish (Figures 3a-f).

**Effect of $TiO_2$-NPs**

Fish exposed to $TiO_2$-NPs for short-term durations showed no significant change in the activity of superoxide dismutase, however, the activity of enzyme increased significantly ($P<0.05$) after 15 days and then consequently decreased in time-dependent manner with significant ($P<0.05$) reduction after 60 days of nanoparticles treatment (Figure 4a). Catalase enzyme activity in gill tissue increased significantly ($P<0.05$) in 96 h treatment group, while the changes in the long-term group were found similar to the activity of superoxide dismutase (Figure 4b). The activities of glutathione reductase and glutathione peroxidase showed significant ($P<0.05$) reduction after short-term and long-term $TiO_2$-NPs exposure in time-dependent manner (Figures 4c and 4d). Level of hydrogen peroxide generation increased significantly ($P<0.05$) after 96 h onwards in time-dependent mode (Figure 4e), however, the level of lipid peroxidation showed significant ($P<0.05$) increase only after 30 and 60 days of $TiO_2$-NPs exposure (Figure 4f). The changes observed in all groups of $TiO_2$-NPs exposed gill tissues were maintained as such in the treatment withdrawal group also (Figures 4a-f).

**Figure 1a** Effect of $Al_2O_3$-NPs on the activity of superoxide dismutase in the gill of the fish, *Oreochromis mossambicus* (Mean ± SD; * P<0.05)

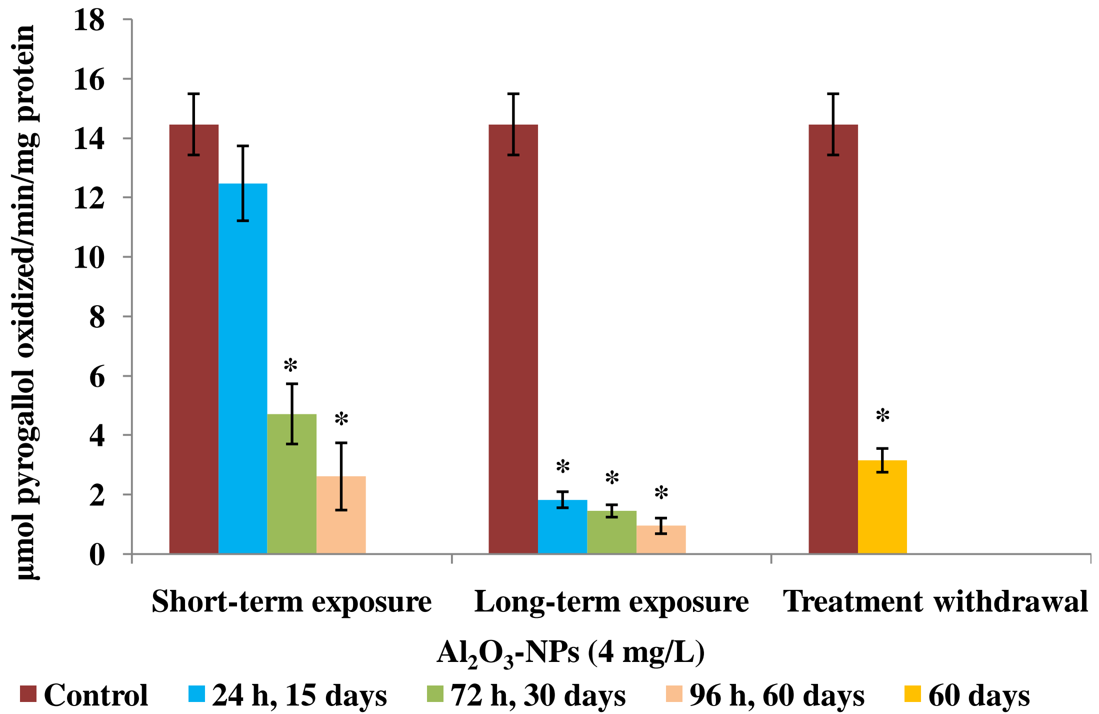



**Figure 1b** Effect of $Al_2O_3$-NPs on the activity of catalase in the gill of the fish, *Oreochromis mossambicus* (Mean ± SD; * P<0.05)

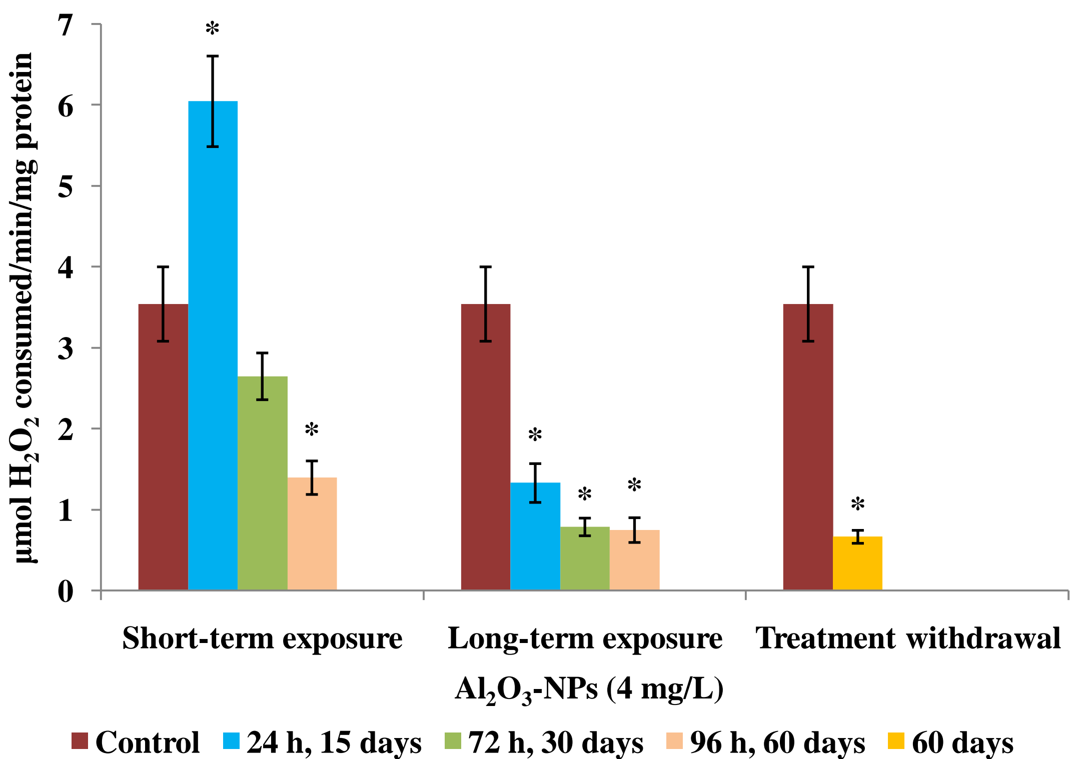


**Figure 1c** Effect of $Al_2O_3$-NPs on the activity of glutathione reductase in the gill of the fish, *Oreochromis mossambicus* (Mean ± SD; * P<0.05)

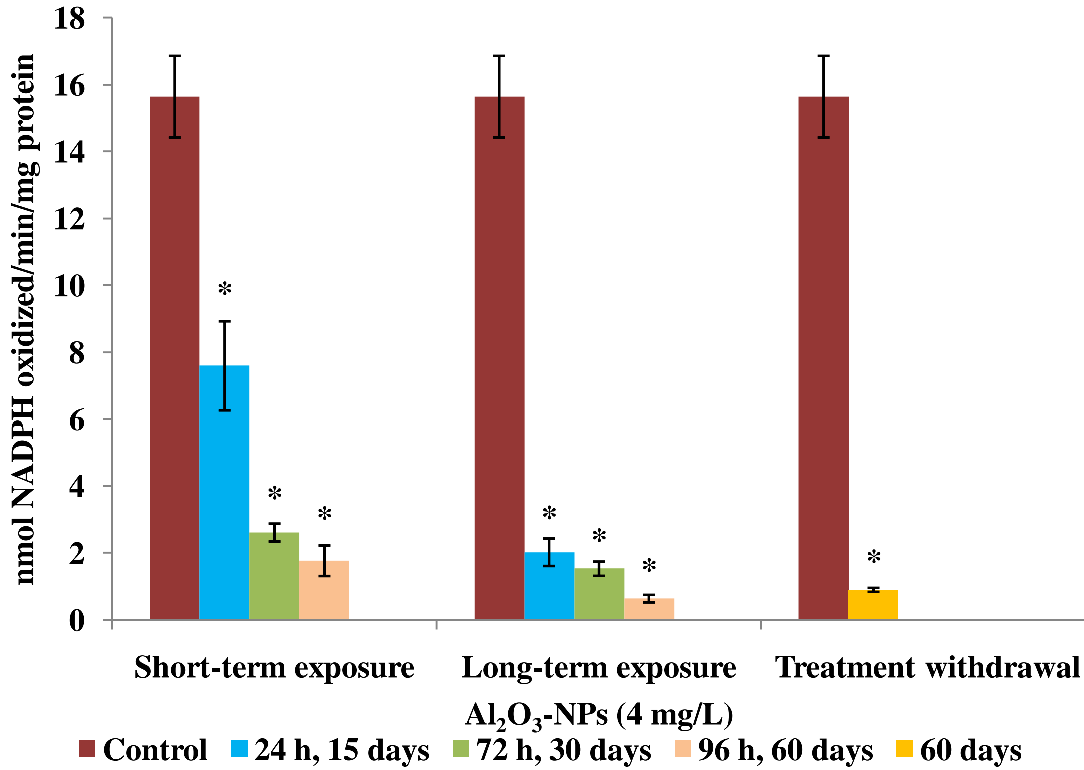



**Figure 1d** Effect of $Al_2O_3$-NPs on the activity of glutathione peroxidase in the gill of the fish, *Oreochromis mossambicus* (Mean ± SD; * P<0.05)

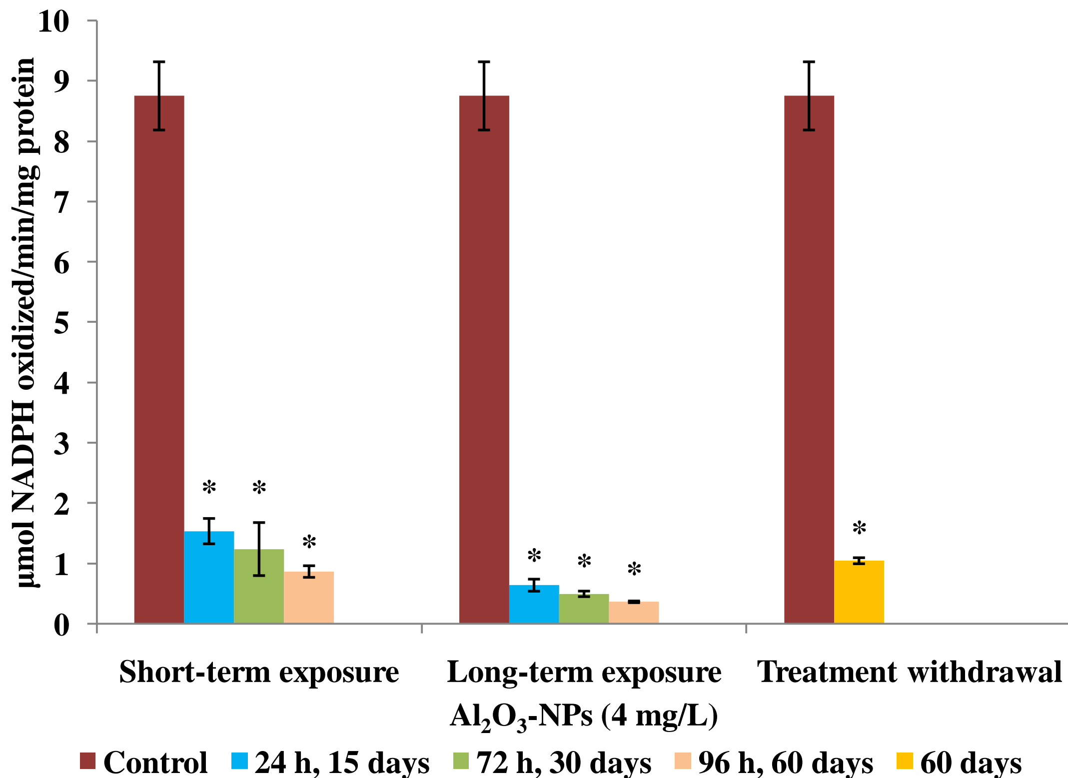


Figure 1e Effect of $Al_2O_3$-NPs on the level of hydrogen peroxide generation in the gill of the fish, *Oreochromis mossambicus* (Mean ± SD; * $P<0.05$)

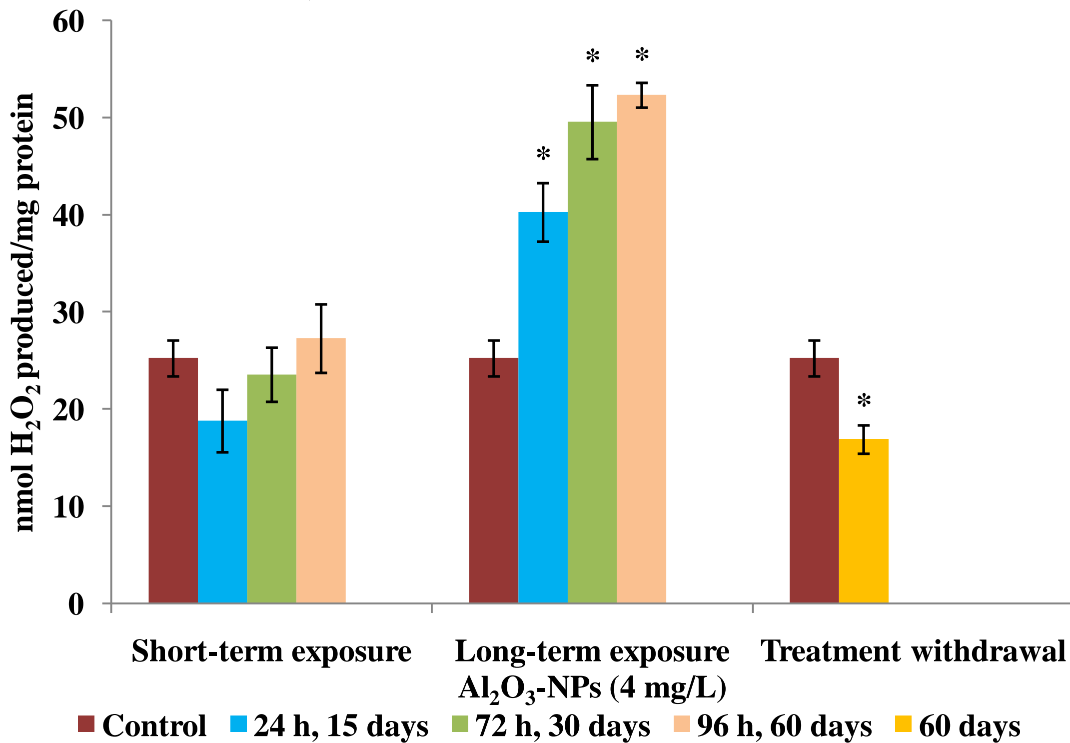



Figure 1f Effect of $Al_2O_3$-NPs on the level of lipid peroxidation in the gill of the fish, *Oreochromis mossambicus* (Mean ± SD; * $P<0.05$)

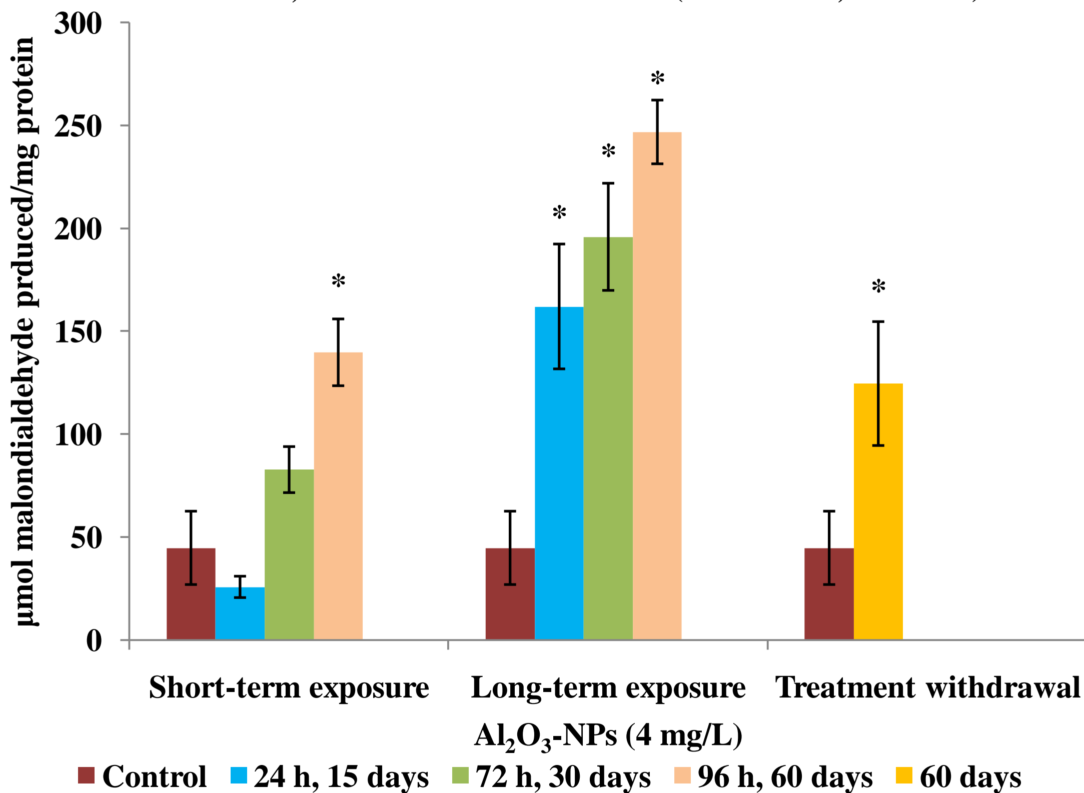


**Figure 2a** Effect of $Fe_3O_4$-NPs on the activity of superoxide dismutase in the gill of the fish, *Oreochromis mossambicus* (Mean ± SD; * P<0.05)

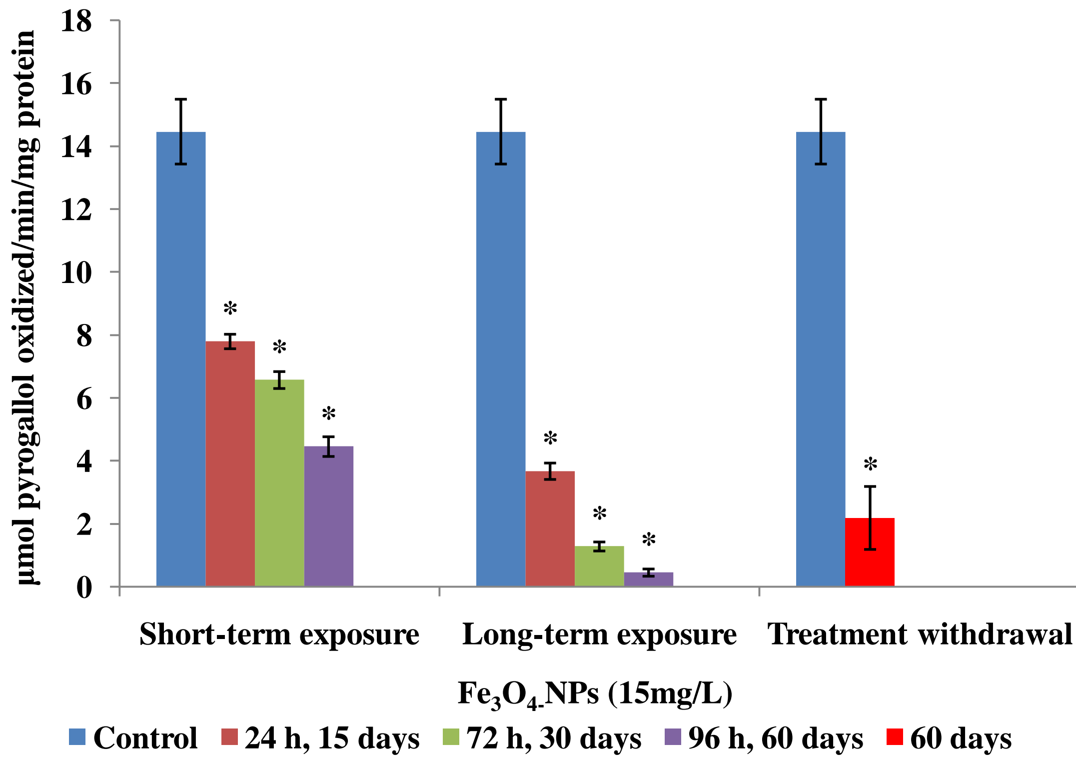



**Figure 2b** Effect of $Fe_3O_4$-NPs on the activity of catalase in the gill of the fish, *Oreochromis mossambicus* (Mean ± SD; * P<0.05)

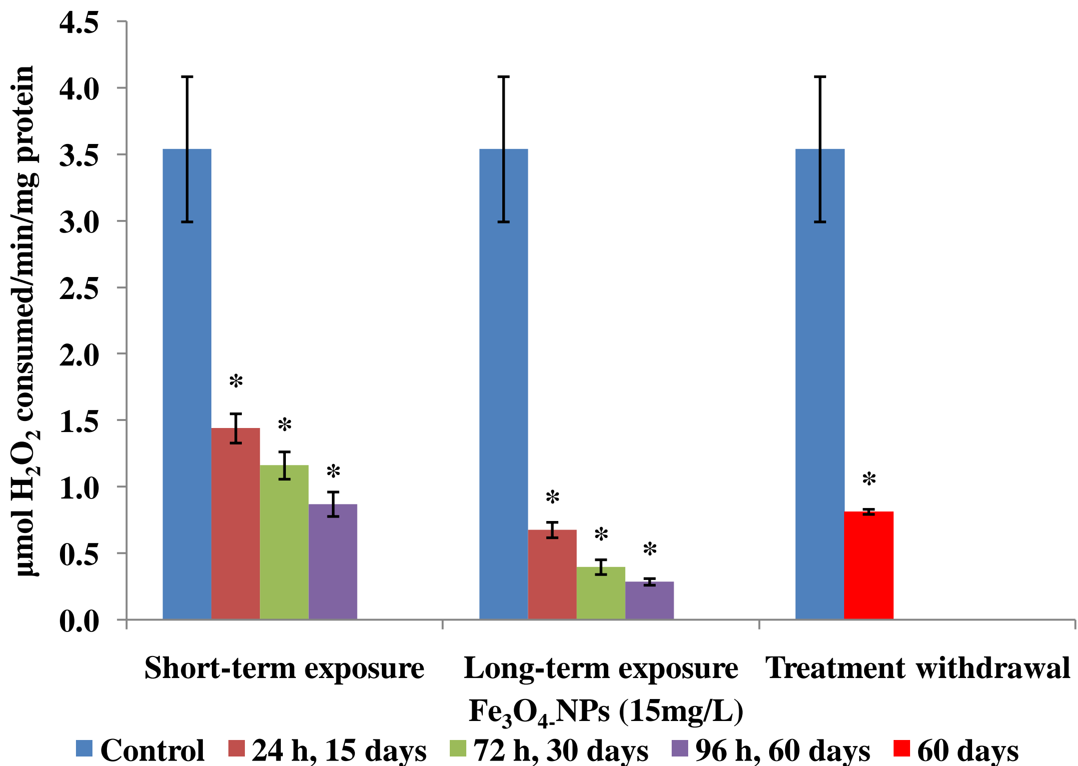


**Figure 2c** Effect of $Fe_3O_4$-NPs on the activity of glutathione reductase in the gill of the fish, *Oreochromis mossambicus* (Mean ± SD; * P<0.05)

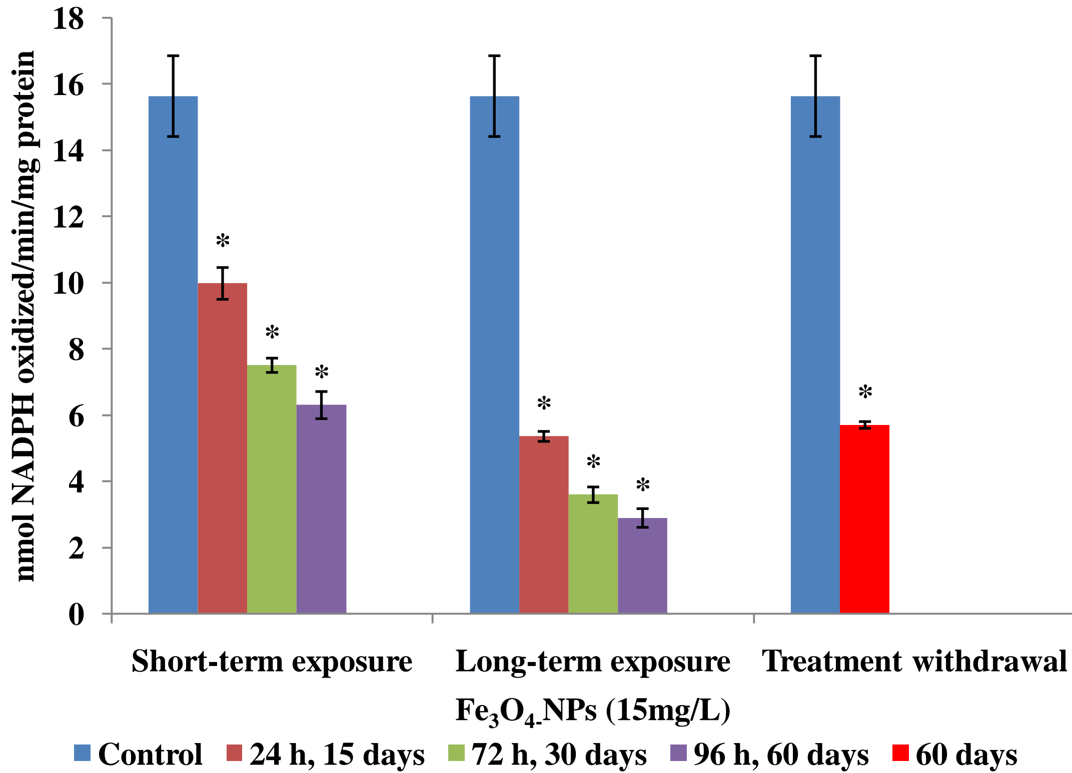



**Figure 2d** Effect of $Fe_3O_4$-NPs on the activity of glutathione peroxidase in the gill of the fish, *Oreochromis mossambicus* (Mean ± SD; * P<0.05)

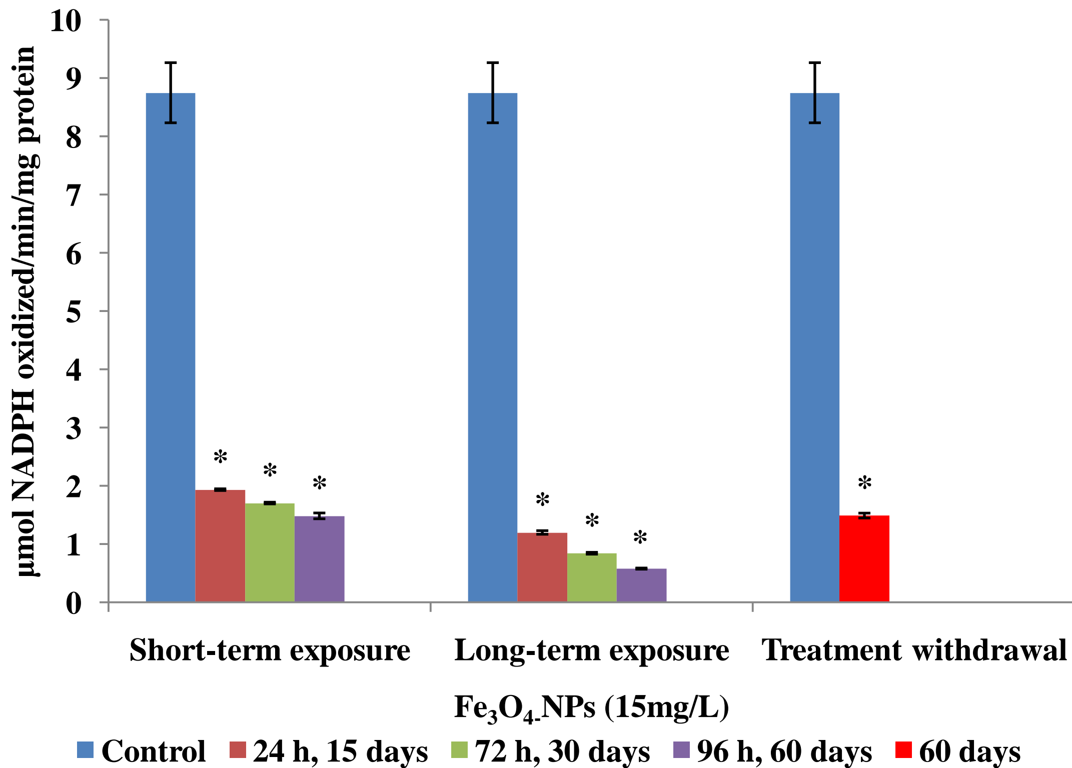


**Figure 2e** Effect of $Fe_3O_4$-NPs on the level of hydrogen peroxide generation in the gill of the fish, *Oreochromis mossambicus* (Mean ± SD; * P<0.05)

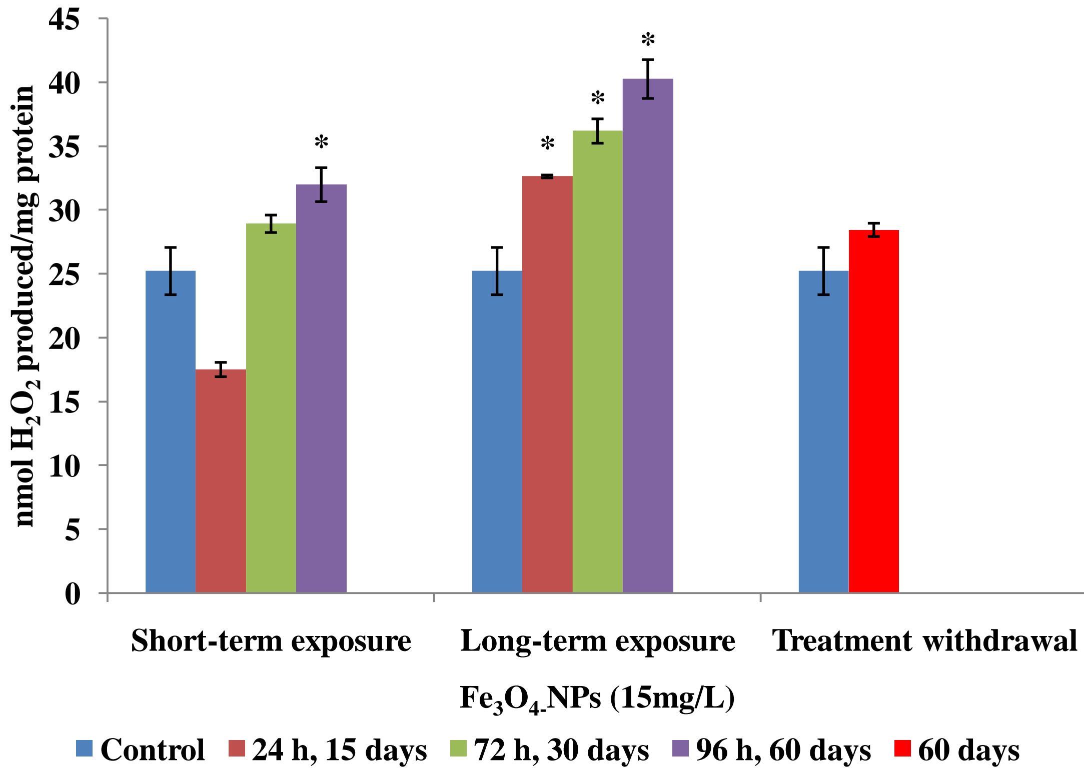



**Figure 2f** Effect of $Fe_3O_4$-NPs on the level of lipid peroxidation in the gill of the fish, *Oreochromis mossambicus* (Mean ± SD; * P<0.05)

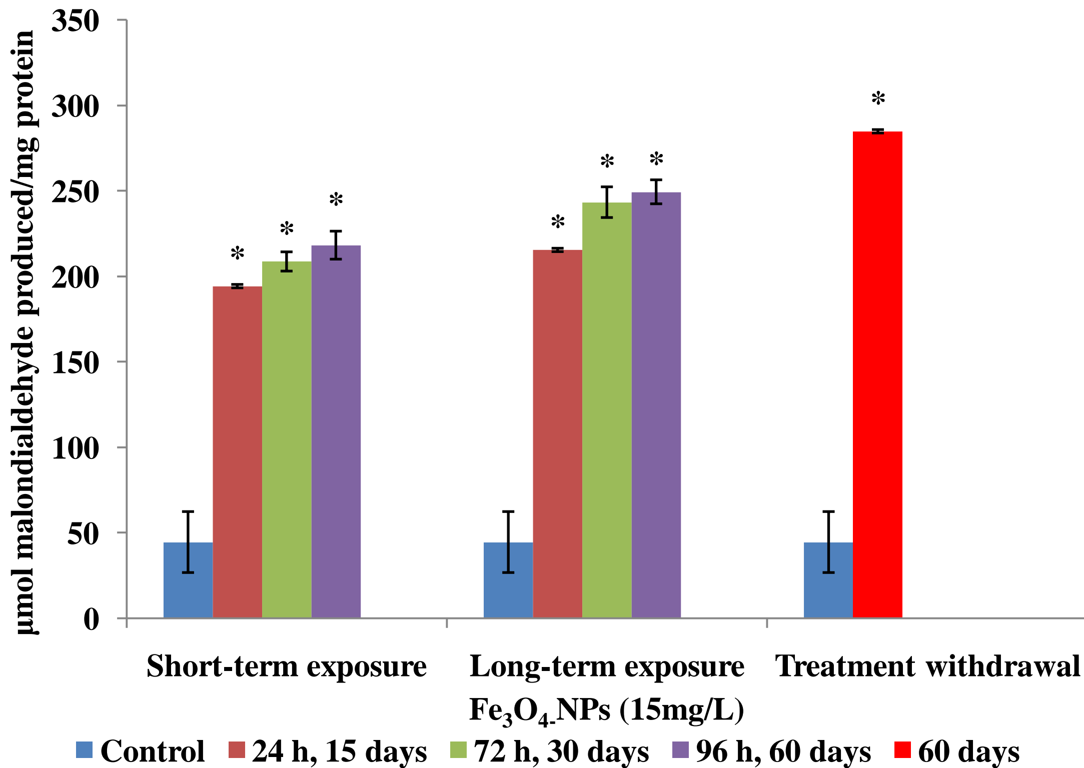


**Figure 3a** Effect of $SiO_2$-NPs on the activity of superoxide dismutase in the gill of the fish, *Oreochromis mossambicus* (Mean ± SD; * P<0.05)

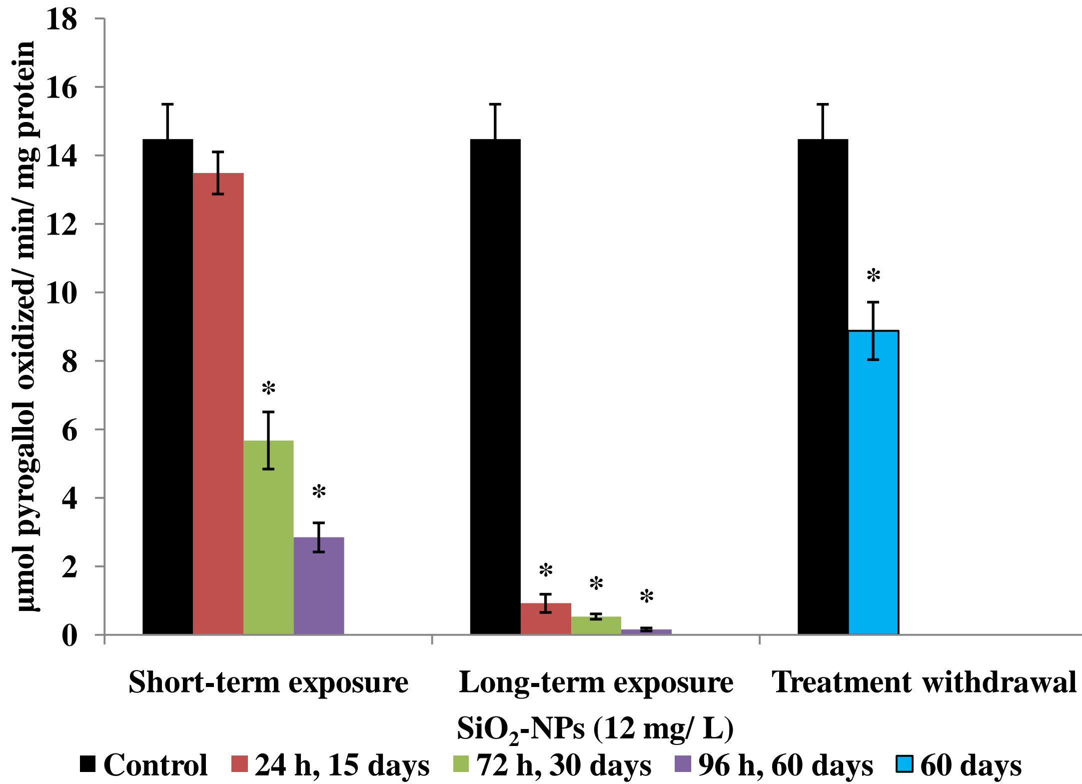



**Figure 3b** Effect of $SiO_2$-NPs on the activity of catalase in the gill of the fish, *Oreochromis mossambicus* (Mean ± SD; * P<0.05)

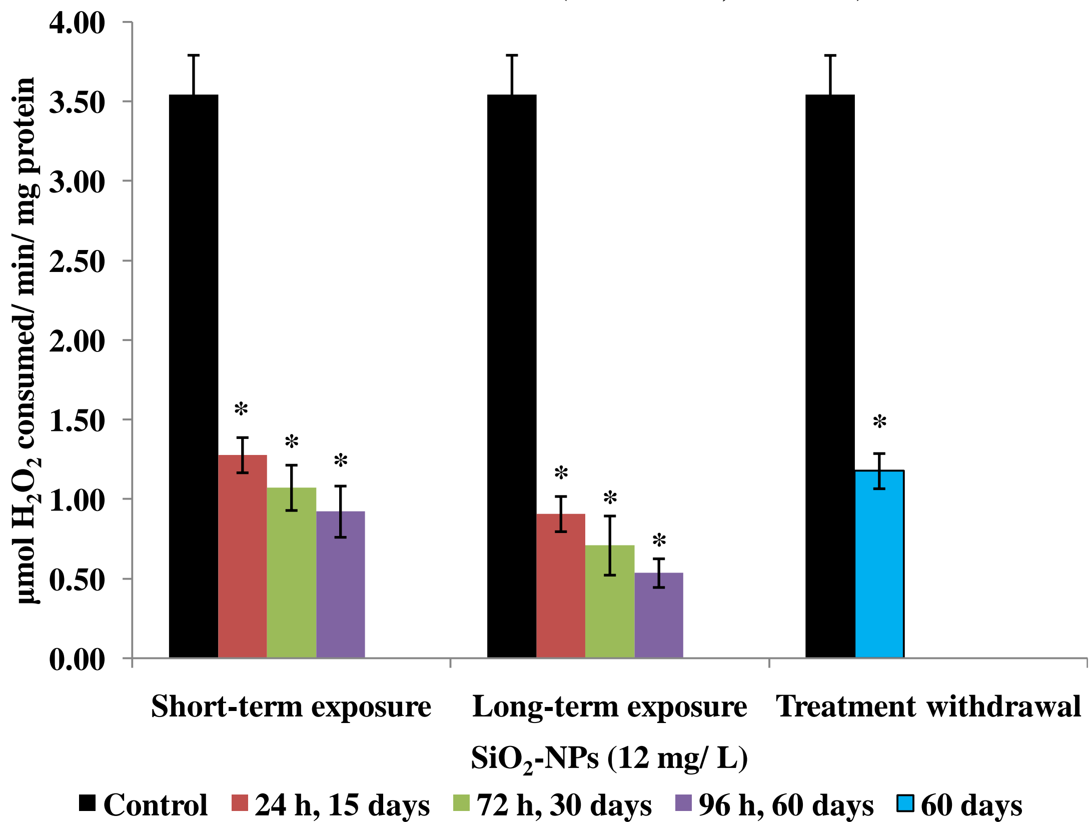


Figure 3c Effect of $SiO_2$-NPs on the activity of glutathione reductase in the gill of the fish, *Oreochromis mossambicus* (Mean ± SD; * P<0.05)

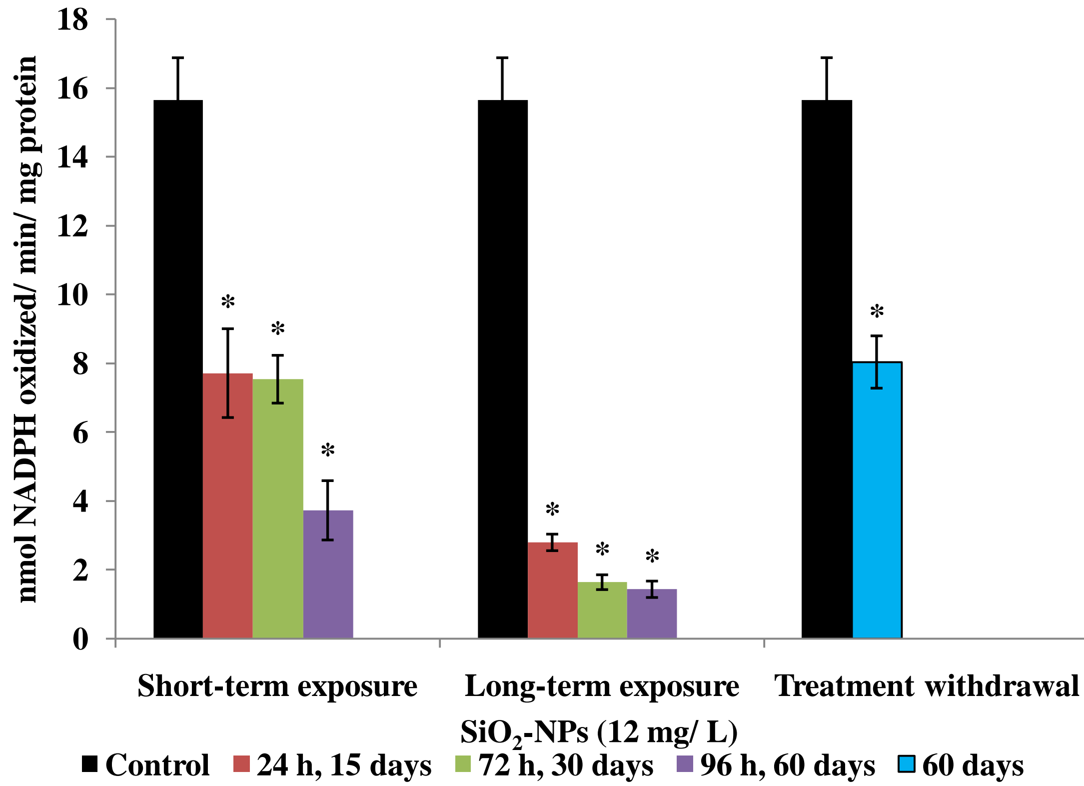



Figure 3d Effect of $SiO_2$-NPs on the activity of glutathione peroxidase in the gill of the fish, *Oreochromis mossambicus* (Mean ± SD; * P<0.05)

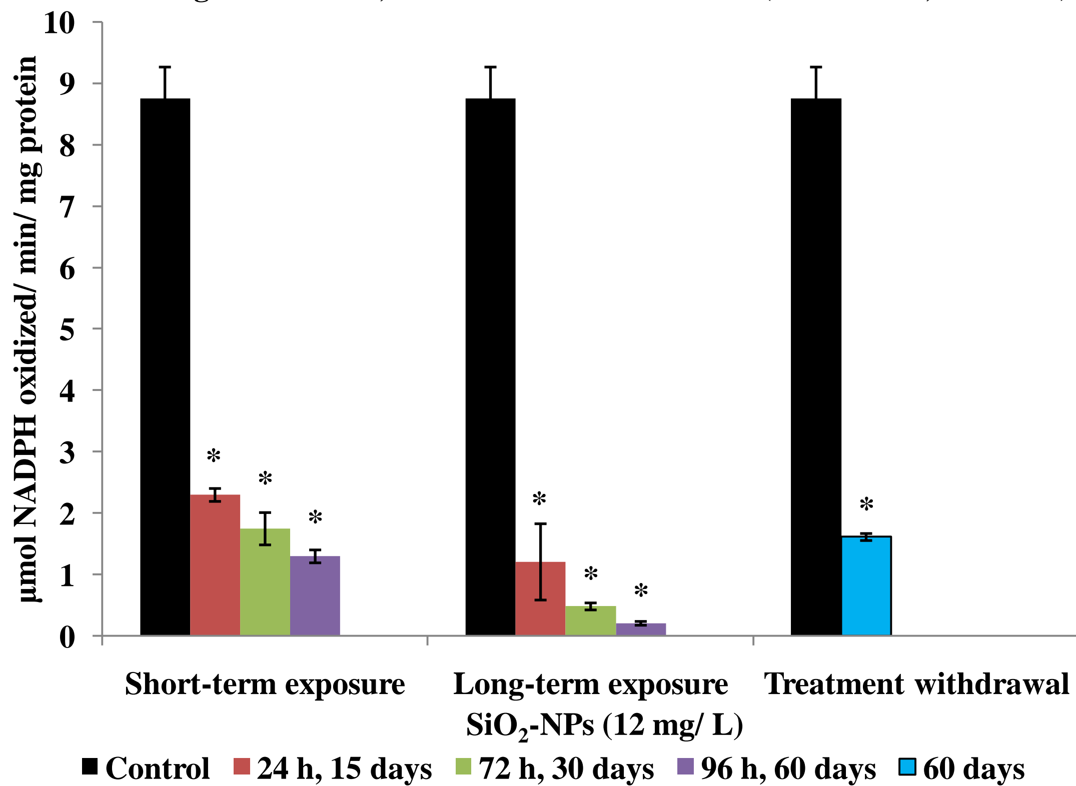


**Figure 3e** Effect of $SiO_2$-NPs on the level of hydrogen peroxide generation in the gill of the fish, *Oreochromis mossambicus* (Mean ± SD; * P<0.05)

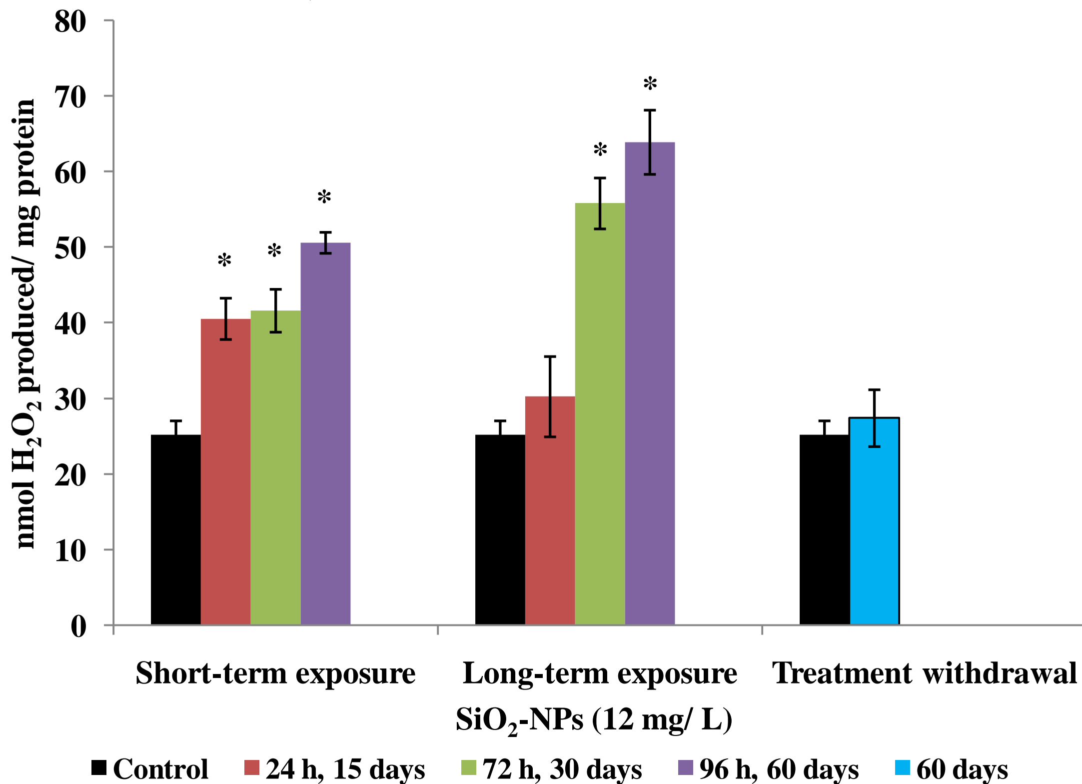



**Figure 3f** Effect of $SiO_2$-NPs on the level of lipid peroxidation in the gill of the fish, *Oreochromis mossambicus* (Mean ± SD; * P<0.05)

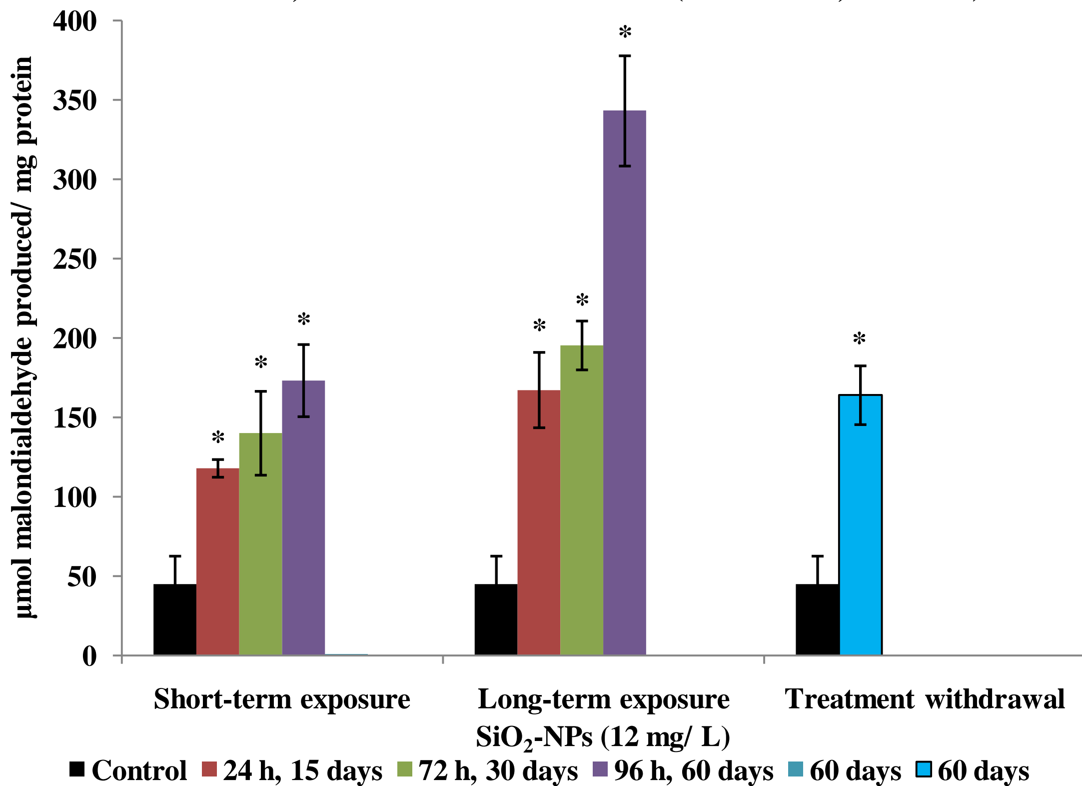


**Figure 4a** **Effect of $TiO_2$-NPs on the activity of superoxide dismutase in the gill of the fish, *Oreochromis mossambicus* (Mean ± SD; * P<0.05)**

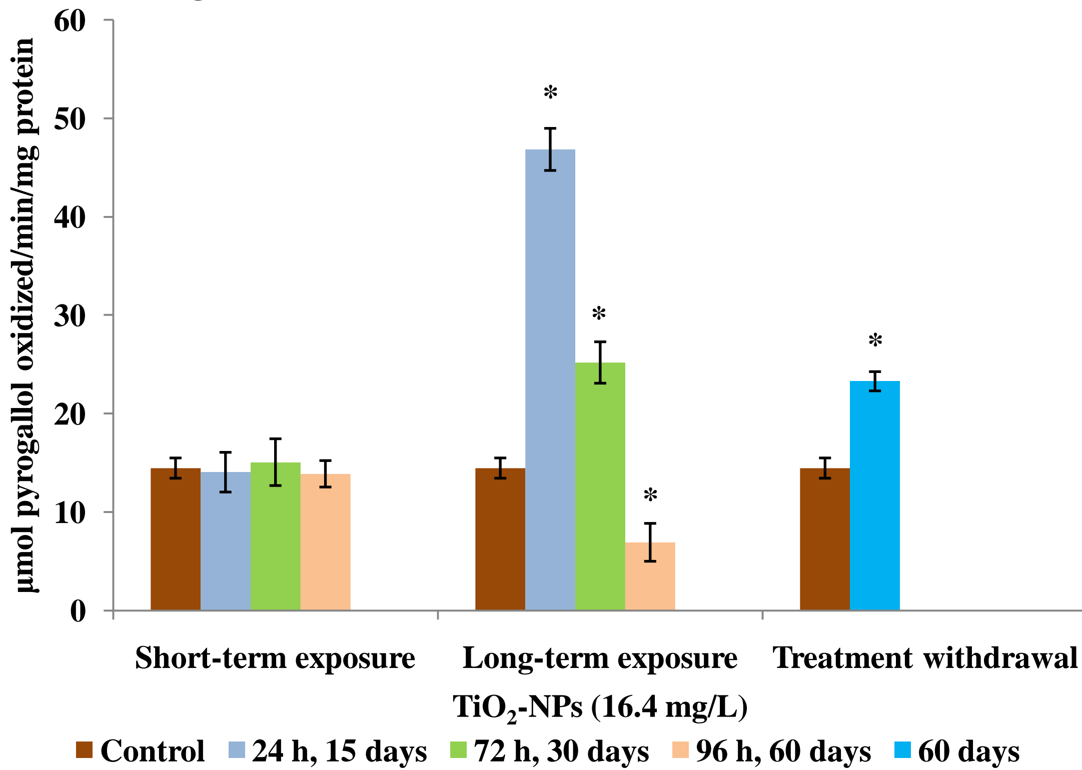



**Figure 4b** **Effect of $TiO_2$-NPs on the activity of catalase in the gill of the fish, *Oreochromis mossambicus* (Mean ± SD; * P<0.05)**

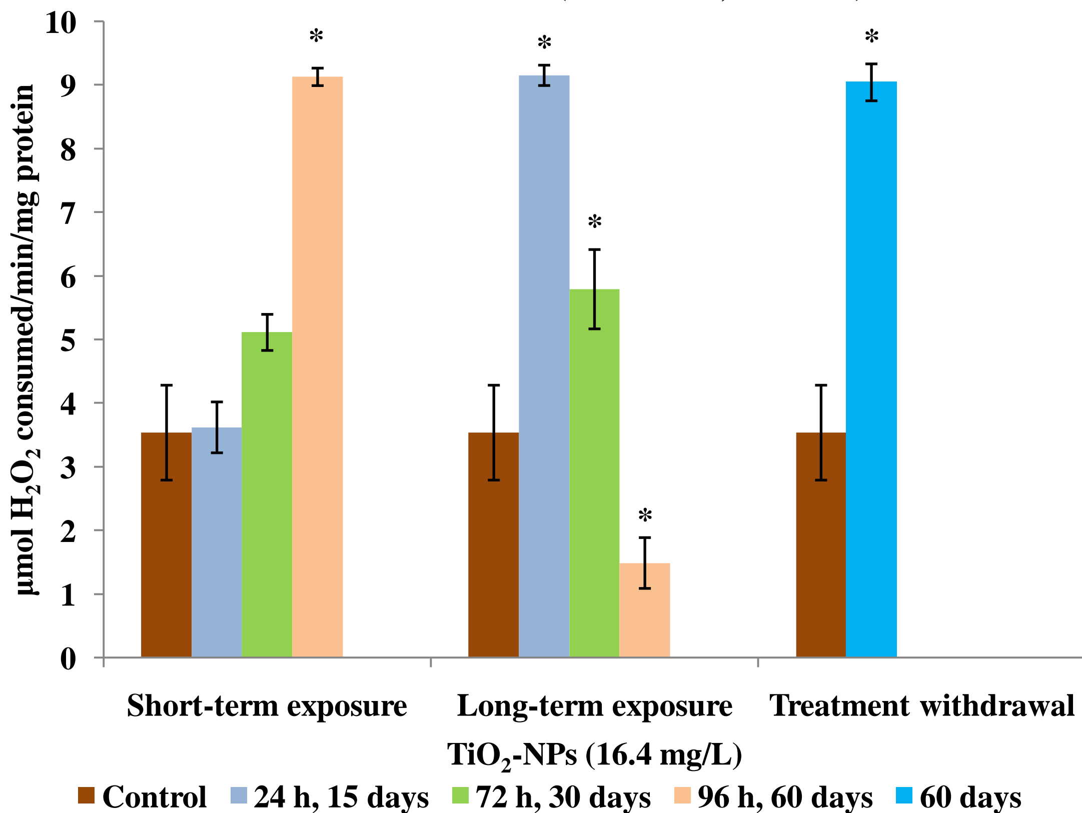


**Figure 4c** Effect of $TiO_2$-NPs on the activity of glutathione reductase in the gill of the fish, *Oreochromis mossambicus* (Mean ± SD; * P<0.05)

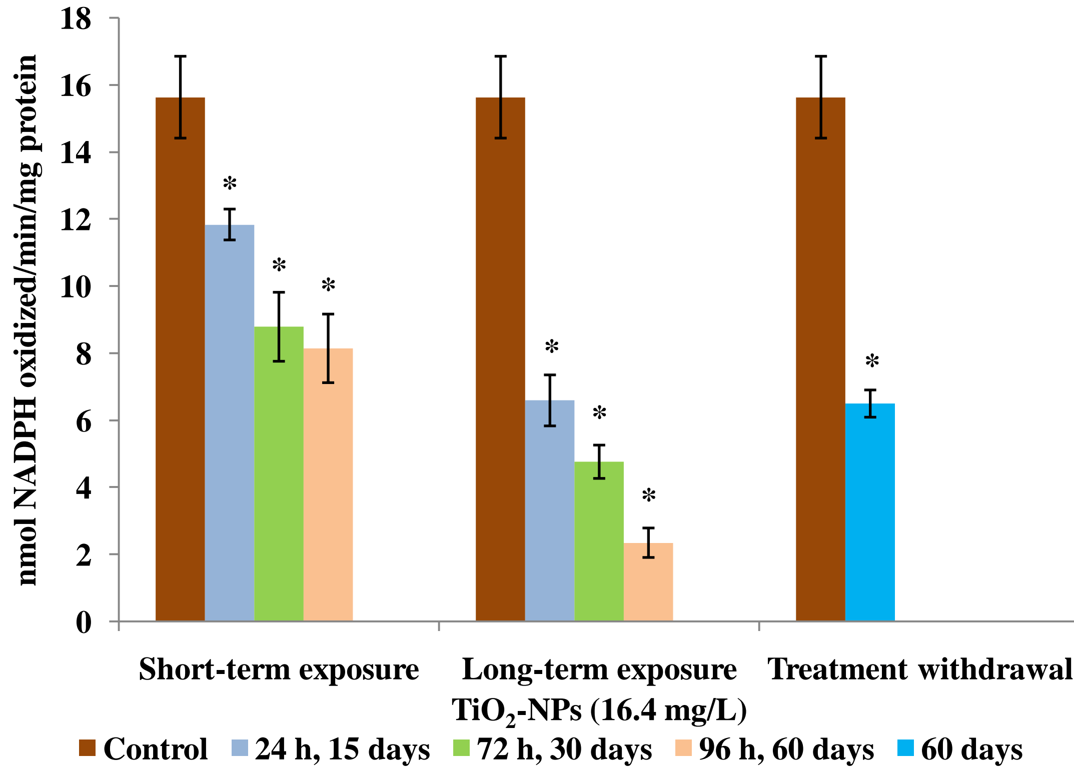



**Figure 4d** Effect of $TiO_2$-NPs on the activity of glutathione peroxidase in the gill of the fish, *Oreochromis mossambicus* (Mean ± SD; * P<0.05)

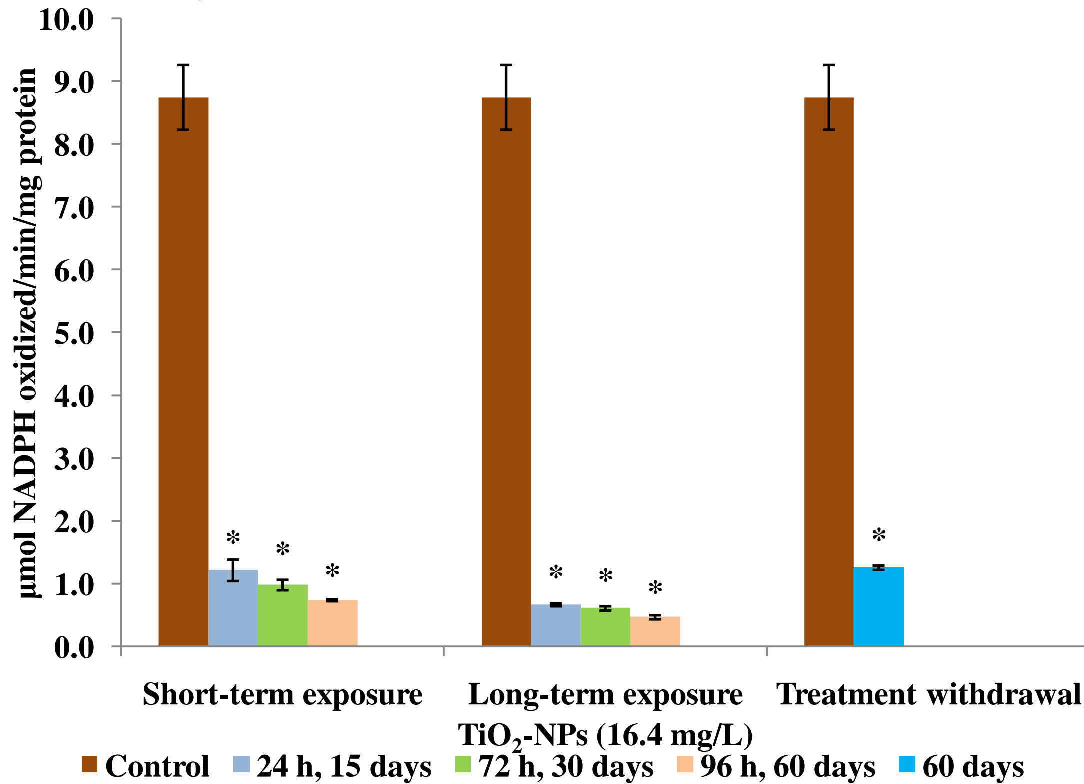


**Figure 4e** Effect of $TiO_2$-NPs on the level of hydrogen peroxide generation in the gill of the fish, *Oreochromis mossambicus* (Mean ± SD; * P<0.05)

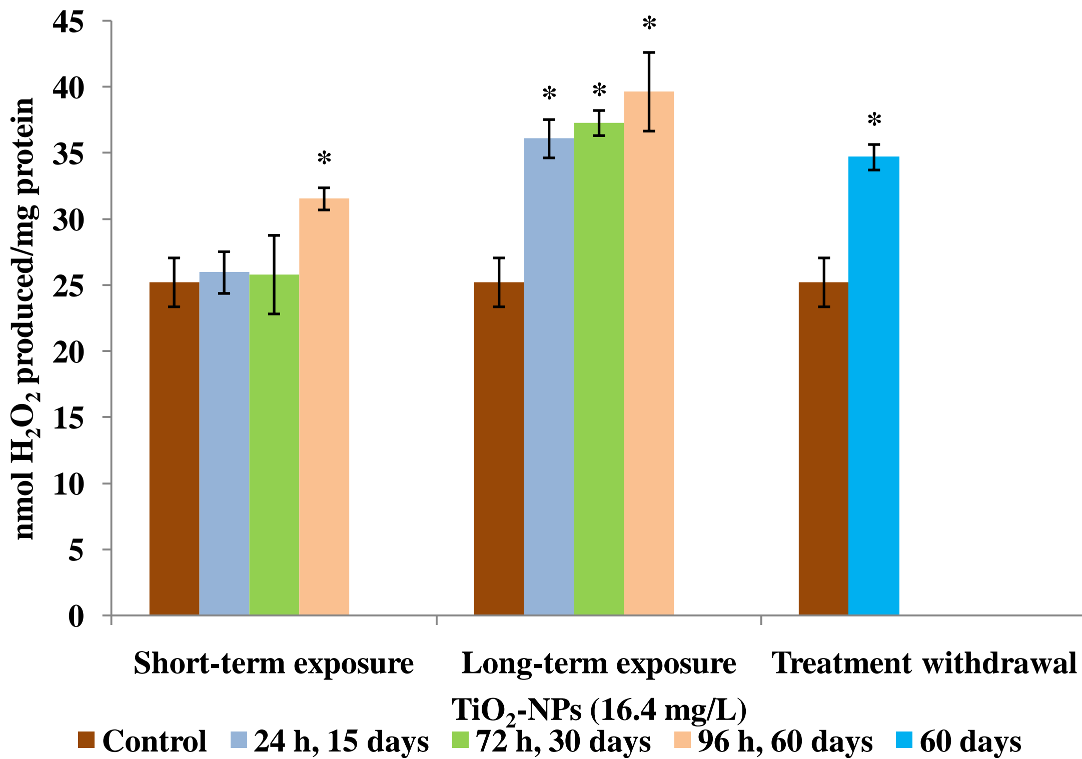
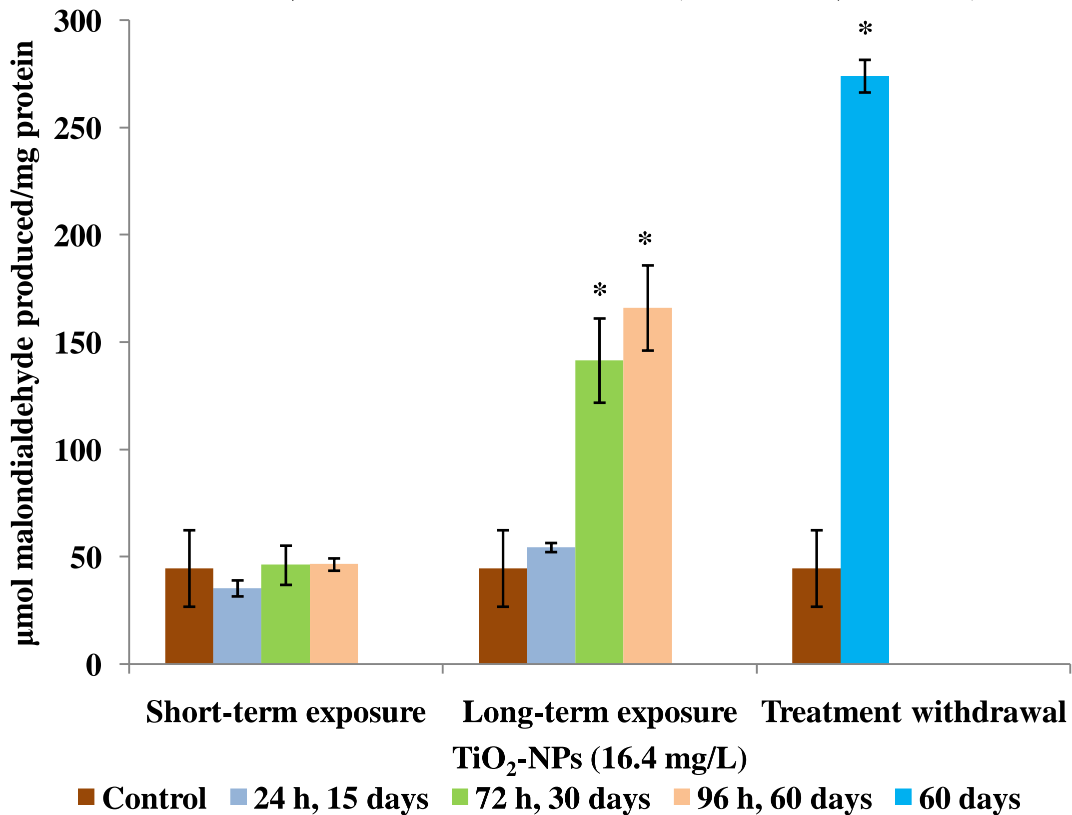



**Figure 4f** Effect of $TiO_2$-NPs on the level of lipid peroxidation in the gill of the fish, *Oreochromis mossambicus* (Mean ± SD; * P<0.05)



## 4.3 Effects of nanoparticles on the antioxidant status in liver tissue of the fish, *Oreochromis mossambicus*

**Effect of $Al_2O_3$-NPs**

The antioxidant enzymes in liver tissue after exposure to $Al_2O_3$-NPs showed no remarkable changes in short-term exposure groups, however, long-term exposure to the nanoparticles significantly ($P<0.05$) decreased the activities of superoxide dismutase, catalase, glutathione reductase and glutathione peroxidase in time-dependent manner (Figures 5a-d). Similarly, the levels of hydrogen peroxide and lipid peroxidation remained unchanged after $Al_2O_3$-NPs exposure for short-term durations in liver tissues, but when the nanoparticles exposed for long-term durations resulted in significant ($p<0.05$) increase (Figures 5e and 5f). In the treatment withdrawal group, the activities of superoxide dismutase and glutathione peroxidase decreased significantly ($P<0.05$) similar to the treatment groups whereas the activities of other enzymes, and the levels of hydrogen peroxide generation and lipid peroxidation remained unchanged and was found similar to that of the corresponding control group (Figures 5a-f).

**Effect of $Fe_3O_4$-NPs**

Exposure to $Fe_3O_4$-NPs for short-term and long-term durations showed significant ($P<0.05$) decrease in the activities of antioxidant enzymes such as superoxide dismutase, catalase, glutathione reductase and glutathione peroxidase when compared to the respective control groups (Figures 6a-d). The levels of hydrogen peroxide generation and lipid peroxidation in the liver tissue increased significantly ($P<0.05$) in all treatment groups when compared to the control tissue (Figures 6e and 6f). The reversal of treatment for 60 days showed significant decrease in the activities of antioxidant enzymes and increase in the levels of hydrogen peroxide and lipid peroxidation in liver tissues (Figures 6a-f).

**Effect of $SiO_2$-NPs**

The activities of superoxide dismutase and catalase increased after 24 h of $SiO_2$-NPs exposure, which then decreased significantly ($P<0.05$) at the end of 72 h and a time-dependent reduction was noticed up to 60 days of nanoparticles exposure

(Figures 7a and 7b). The other antioxidant enzymes namely glutathione reductase and glutathione peroxidase showed significant (P<0.05) decrease in all treatment durations when compared to the corresponding control group (Figures 7c and 7d). There was a significant (P<0.05) and concurrent increase in the levels of generation of hydrogen peroxide and lipid peroxidation in the liver tissue after $SiO_2$-NPs treatment (Figures 7e and 7f). The results of treatment withdrawal group were found similar to the treatment groups in the liver tissues of fish (Figures 7a-f).

**Effect of $TiO_2$-NPs**

Liver tissue of the $TiO_2$-NPs exposed fish showed significant (P<0.05) increase in the activity of superoxide dismutase in short-term exposure, which then decreased in time-dependent manner with significant (P<0.05) reduction after 60 days (Figure 8a). Catalase, glutathione reductase and glutathione peroxidase enzyme activities showed no significant changes in the short-term exposure group, but the activity of catalase decreased significantly (P<0.05) after long-term exposure (Figure 8b). Glutathione reductase and glutathione peroxidase enzyme activities showed significant (P<0.05) decrease only after 60 days of treatment (Figures 8c and 8d). Increase in the levels of hydrogen peroxide generation and lipid peroxidation was noticed only after prolonged exposure (Figures 8e and 8f). Treatment withdrawal for 60 days showed significant (P<0.05) increase in the activity of superoxide dismutase and in the levels of hydrogen peroxide generation and lipid peroxidation, while the activities of catalase, glutathione reductase and glutathione peroxidase decreased in the liver tissue of fish (Figures 8a-f).

**Figure 5a** Effect of $Al_2O_3$-NPs on the activity of superoxide dismutase in the liver of the fish, *Oreochromis mossambicus* (Mean ± SD; * P<0.05)

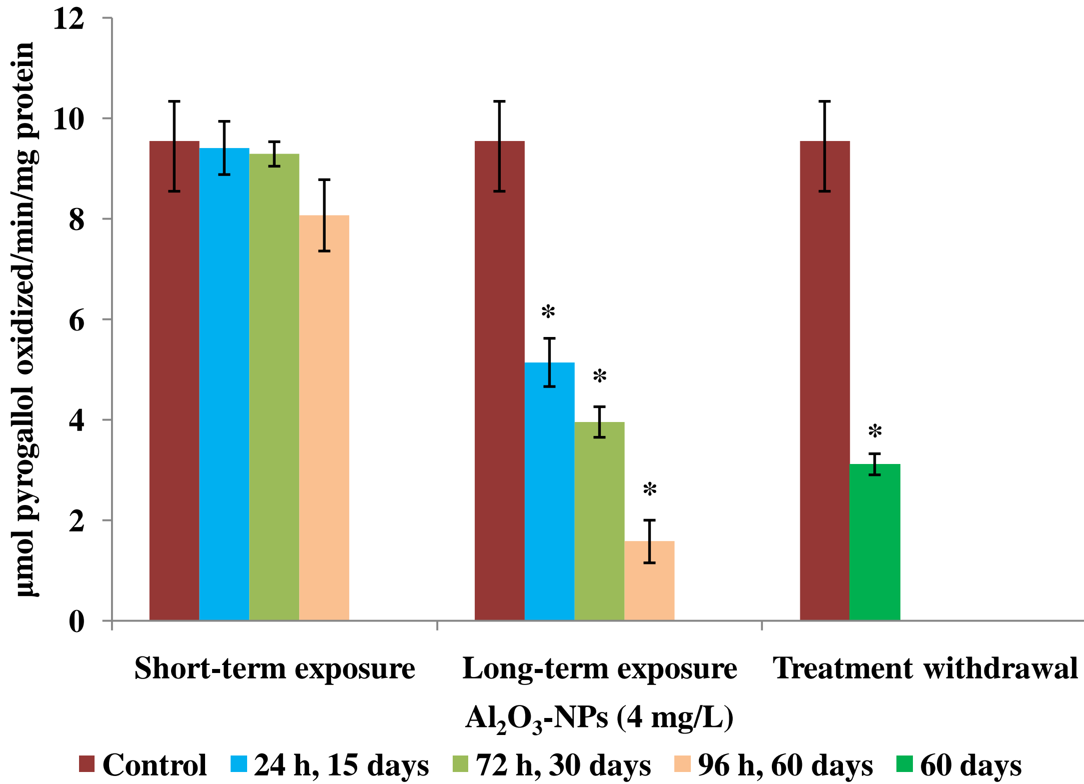



**Figure 5b** Effect of $Al_2O_3$-NPs on the activity of catalase in the liver of the fish, *Oreochromis mossambicus* (Mean ± SD; * P<0.05)

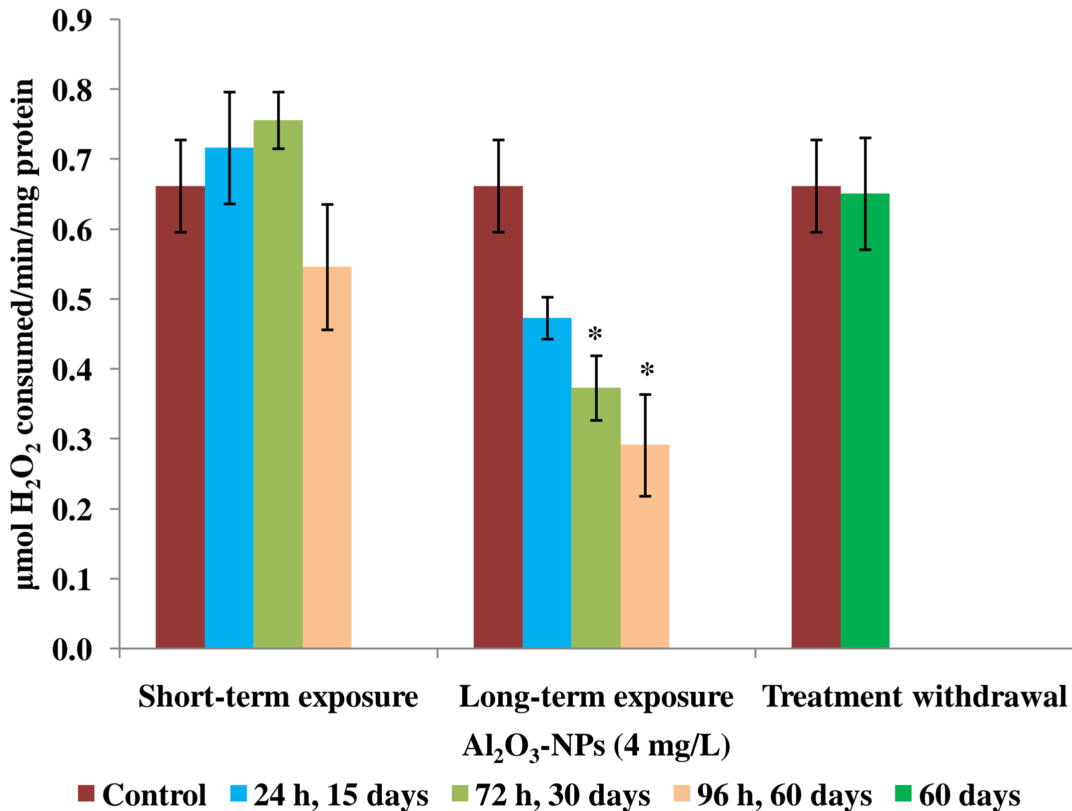


**Figure 5c** Effect of $Al_2O_3$-NPs on the activity of glutathione reductase in the liver of the fish, *Oreochromis mossambicus* (Mean ± SD; * P<0.05)

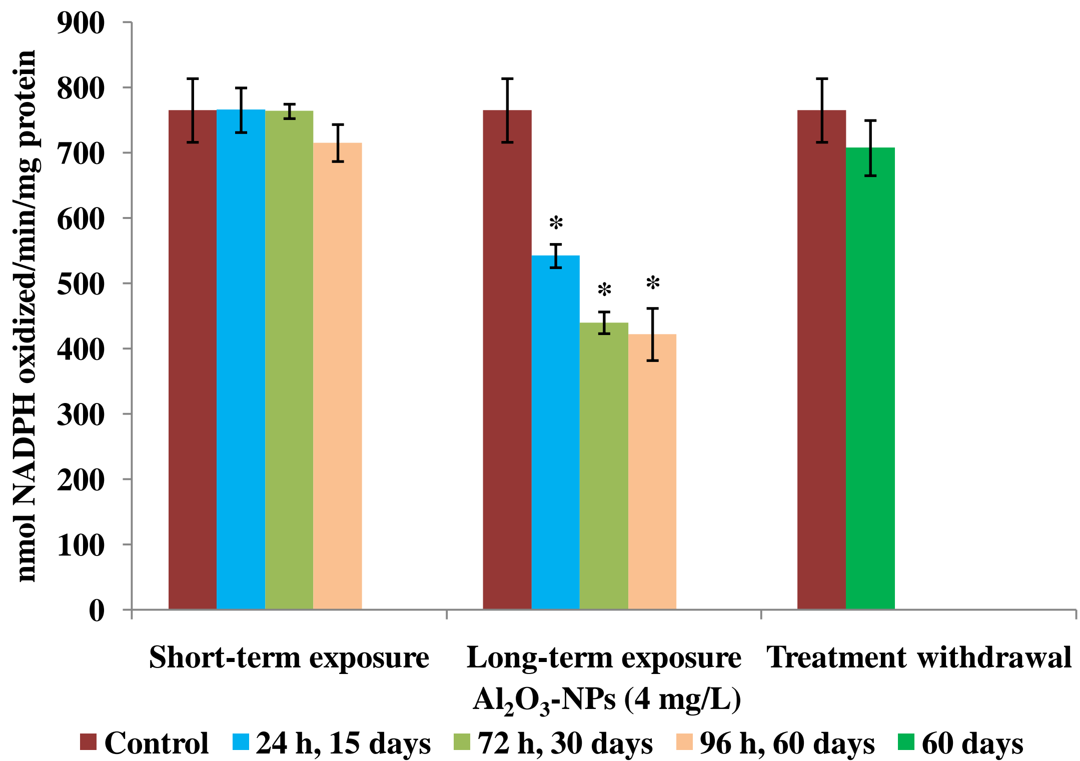



**Figure 5d** Effect of $Al_2O_3$-NPs on the activity of glutathione peroxidase in the liver of the fish, *Oreochromis mossambicus* (Mean ± SD; * P<0.05)

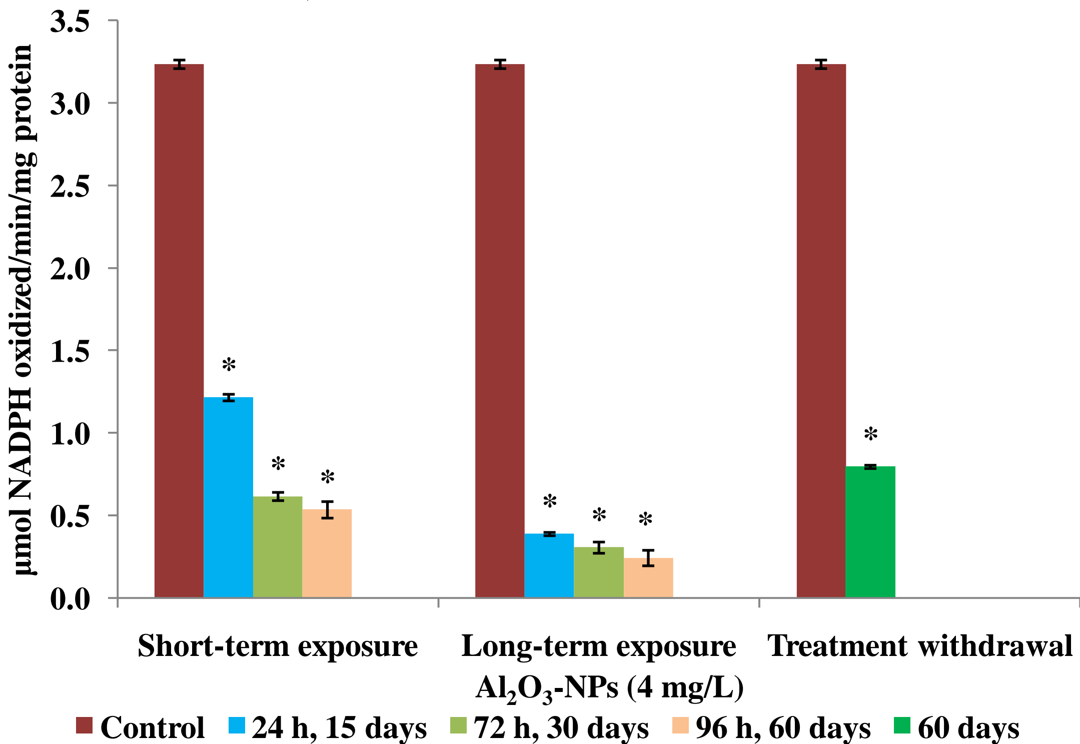


**Figure 5e** Effect of $Al_2O_3$-NPs on the level of hydrogen peroxide generation in the liver of the fish, *Oreochromis mossambicus* (Mean ± SD; * P<0.05)

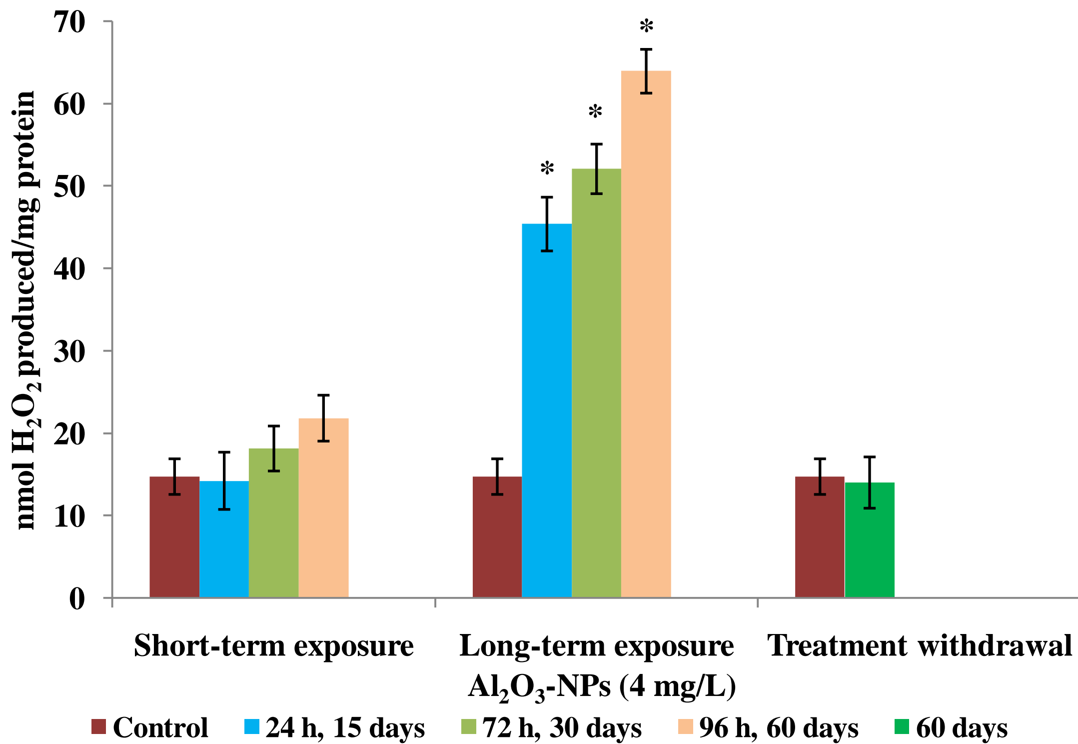



**Figure 5f** Effect of $Al_2O_3$-NPs on the level of lipid peroxidation in the liver of the fish, *Oreochromis mossambicus* (Mean ± SD; * P<0.05)

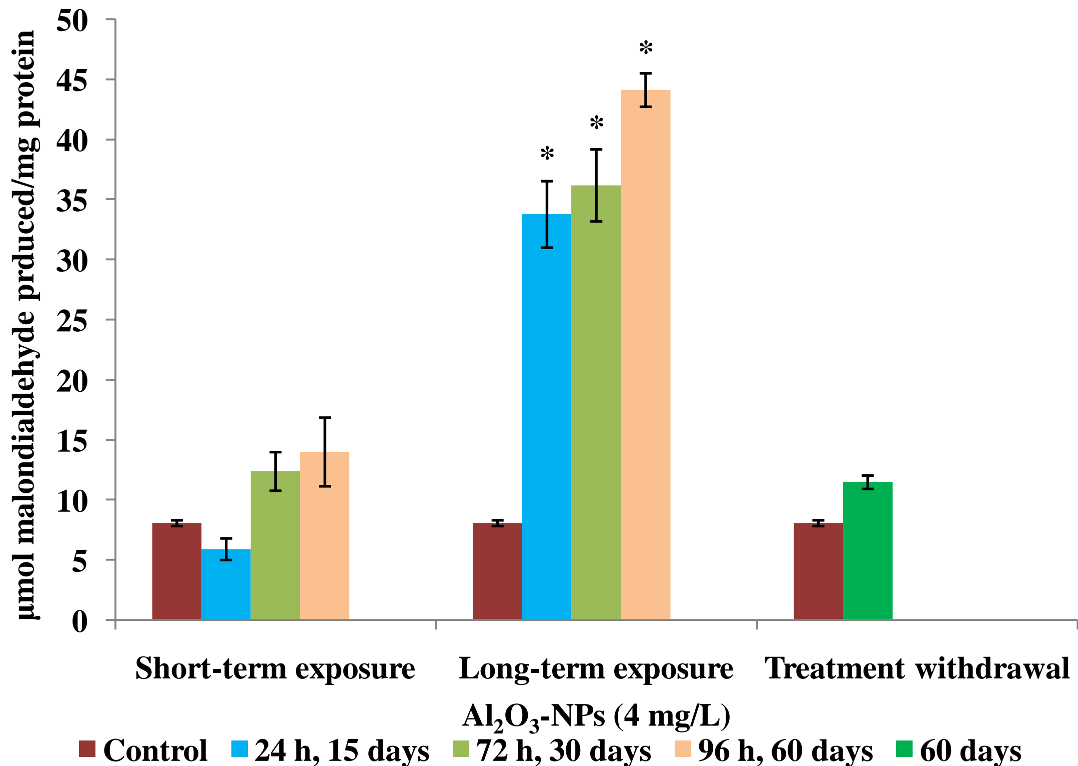


**Figure 6a** Effect of $Fe_3O_4$-NPs on the activity of superoxide dismutase in the liver of the fish, *Oreochromis mossambicus* (Mean ± SD; * P<0.05)

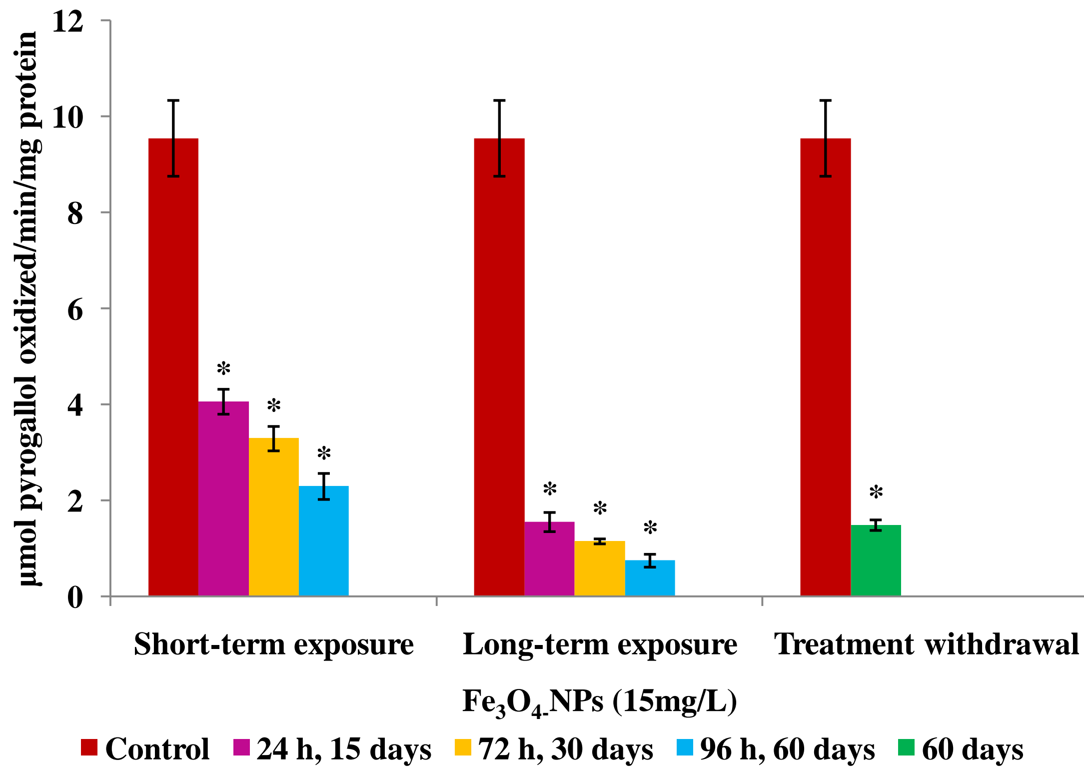



**Figure 6b** Effect of $Fe_3O_4$-NPs on the activity of catalase in the liver of the fish, *Oreochromis mossambicus* (Mean ± SD; * P<0.05)

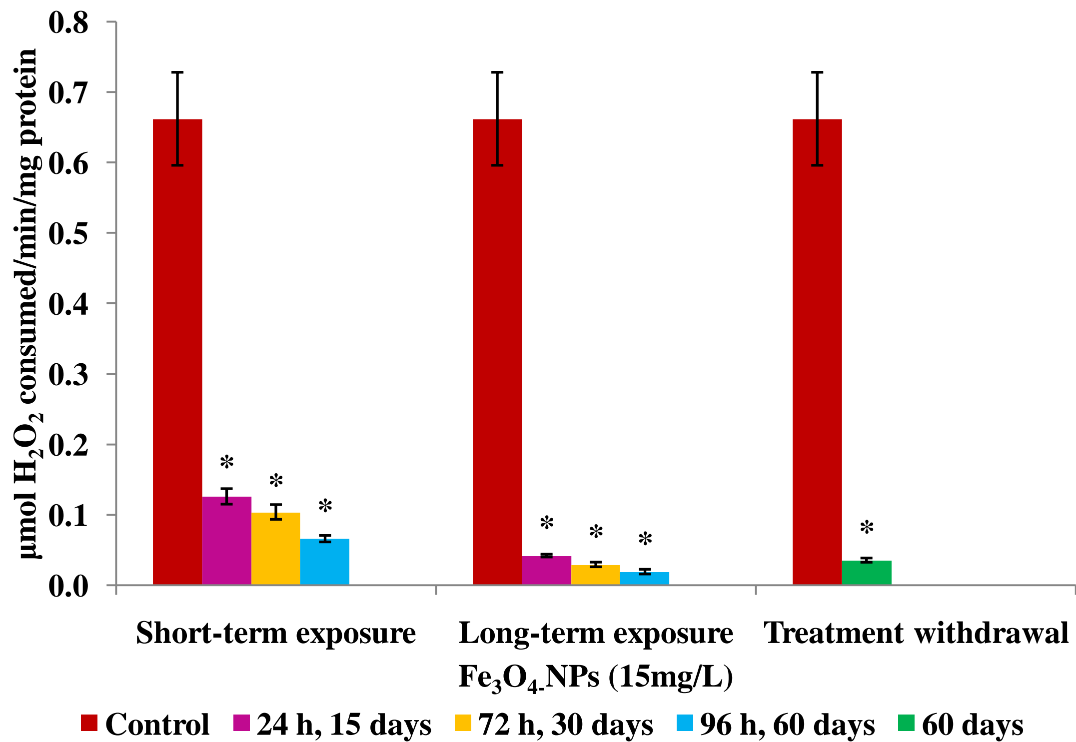


**Figure 6c** Effect of $Fe_3O_4$-NPs on the activity of glutathione reductase in the liver of the fish, *Oreochromis mossambicus* (Mean ± SD; * P<0.05)

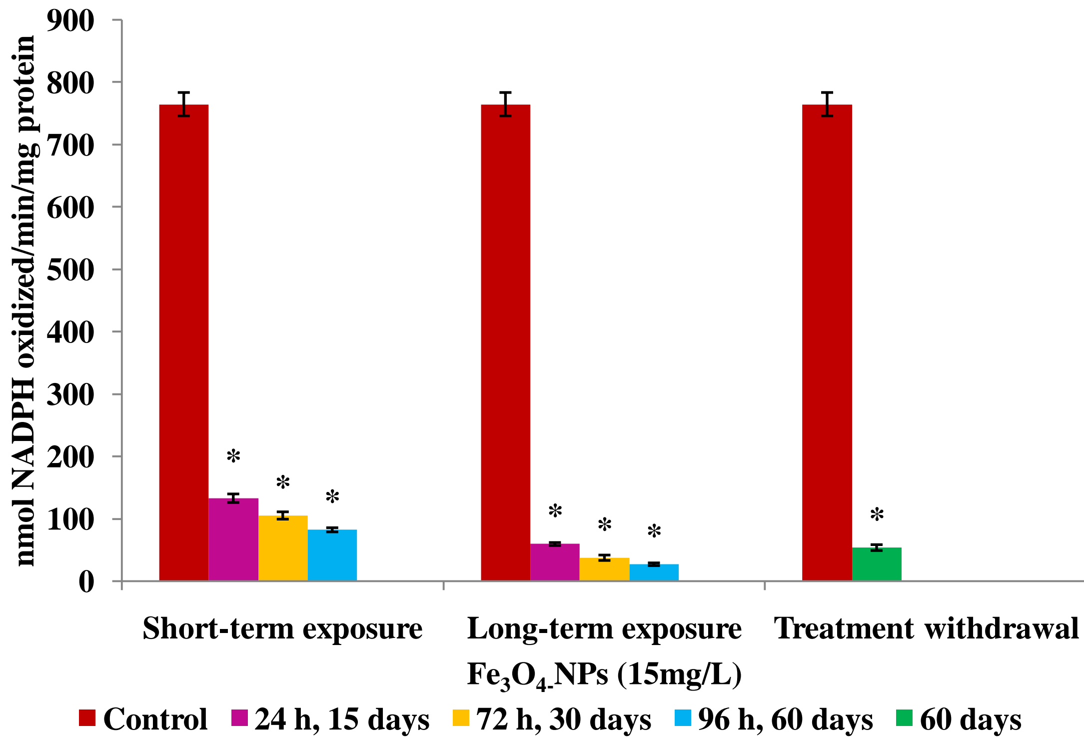



**Figure 6d** Effect of $Fe_3O_4$-NPs on the activity of glutathione peroxidase in the liver of the fish, *Oreochromis mossambicus* (Mean ± SD; * P<0.05)

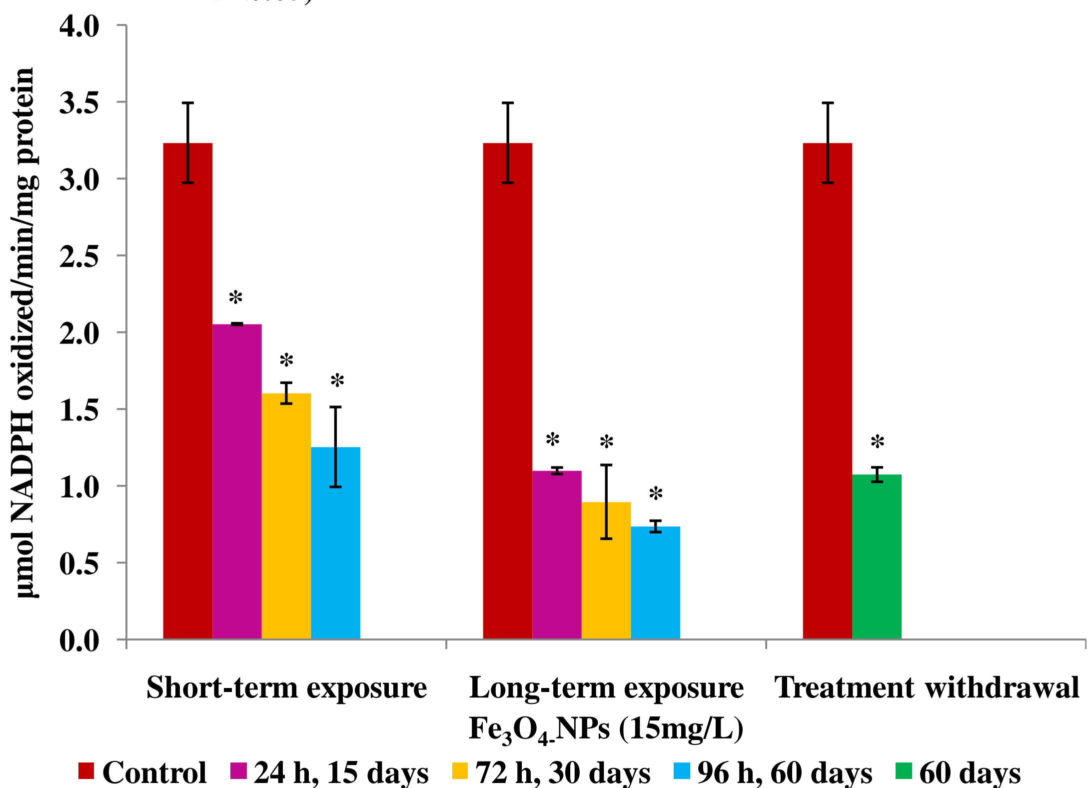


**Figure 6e** Effect of $Fe_3O_4$-NPs on the level of hydrogen peroxide generation in the liver of the fish, *Oreochromis mossambicus* (Mean ± SD; * P<0.05)

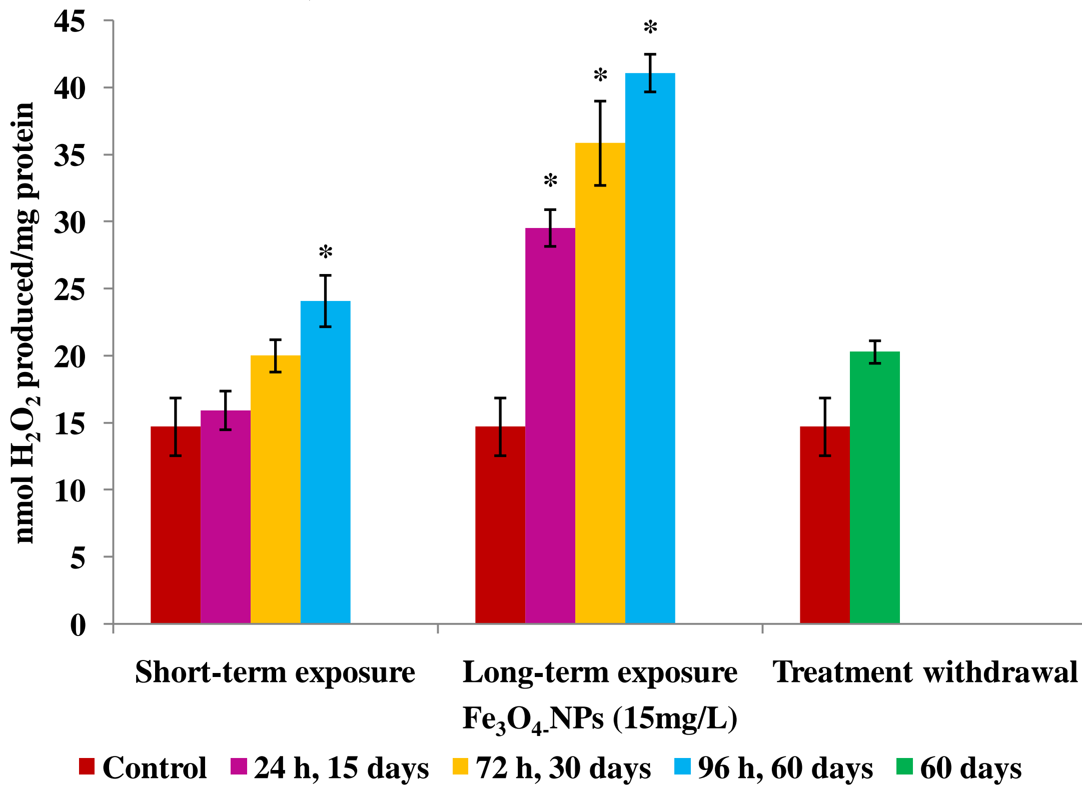



**Figure 6f** Effect of $Fe_3O_4$-NPs on the level of lipid peroxidation in the liver of the fish, *Oreochromis mossambicus* (Mean ± SD; * P<0.05)

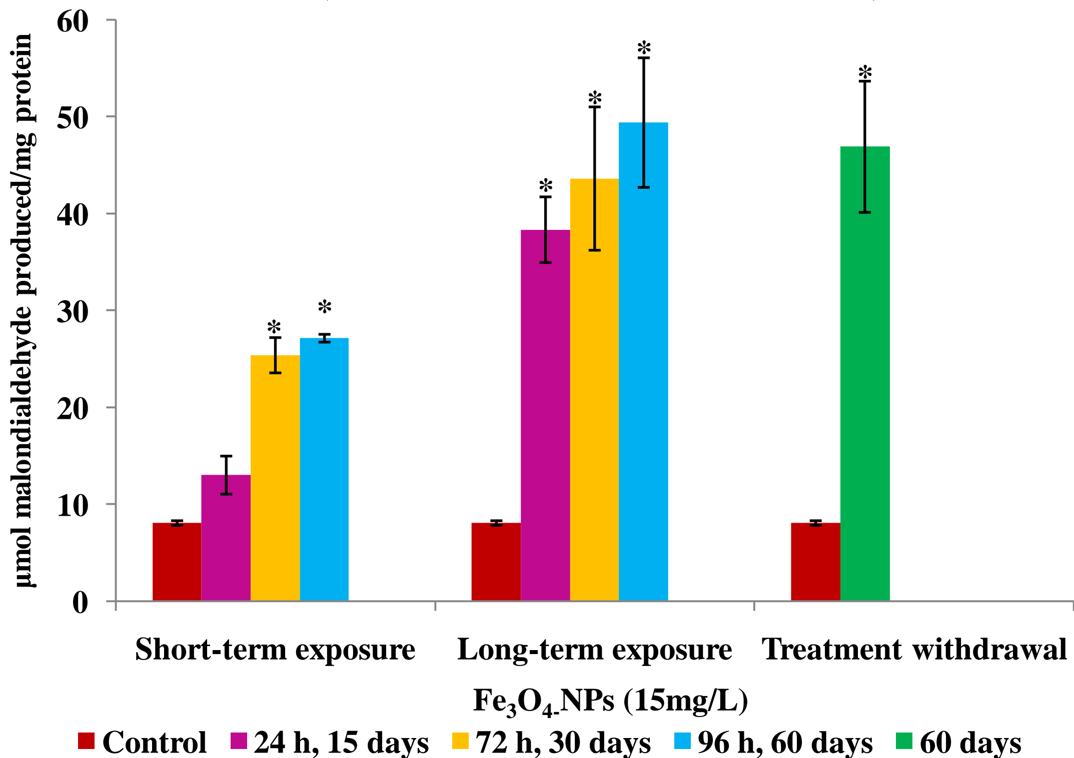


**Figure 7a** Effect of $SiO_2$-NPs on the activity of superoxide dismutase in the liver of the fish, *Oreochromis mossambicus* (Mean ± SD; * P<0.05)

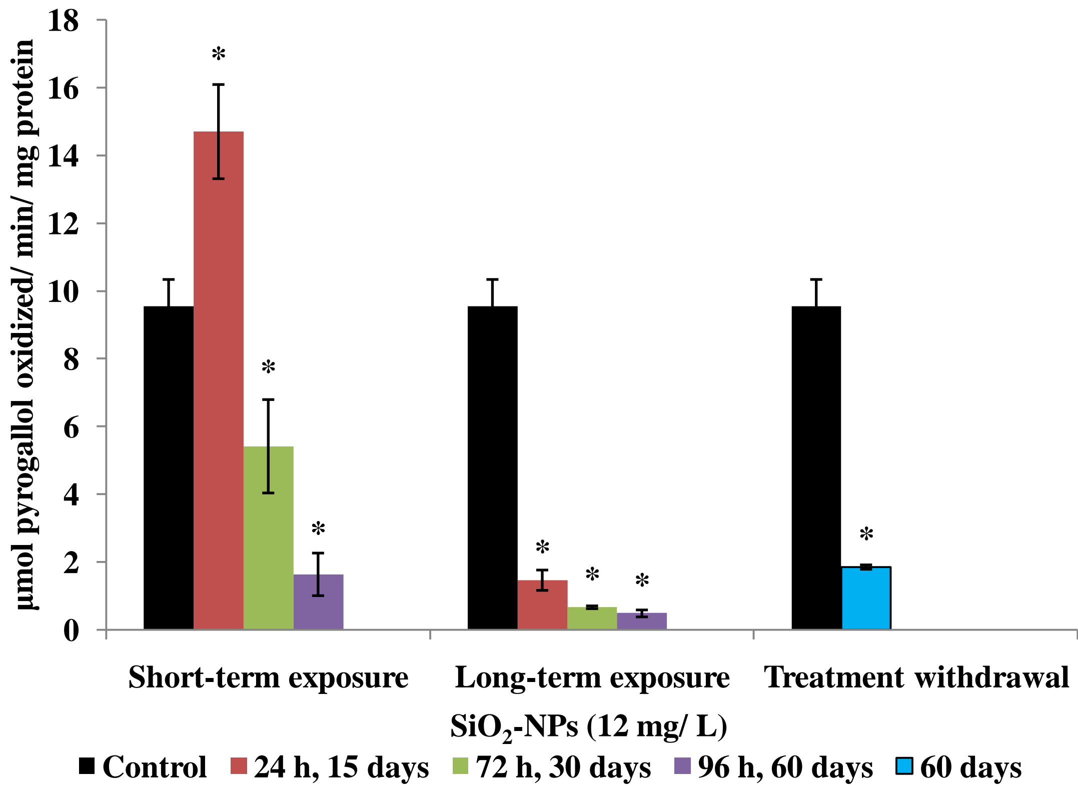



**Figure 7b** Effect of $SiO_2$-NPs on the activity of catalase in the liver of the fish, *Oreochromis mossambicus* (Mean ± SD; * P<0.05)

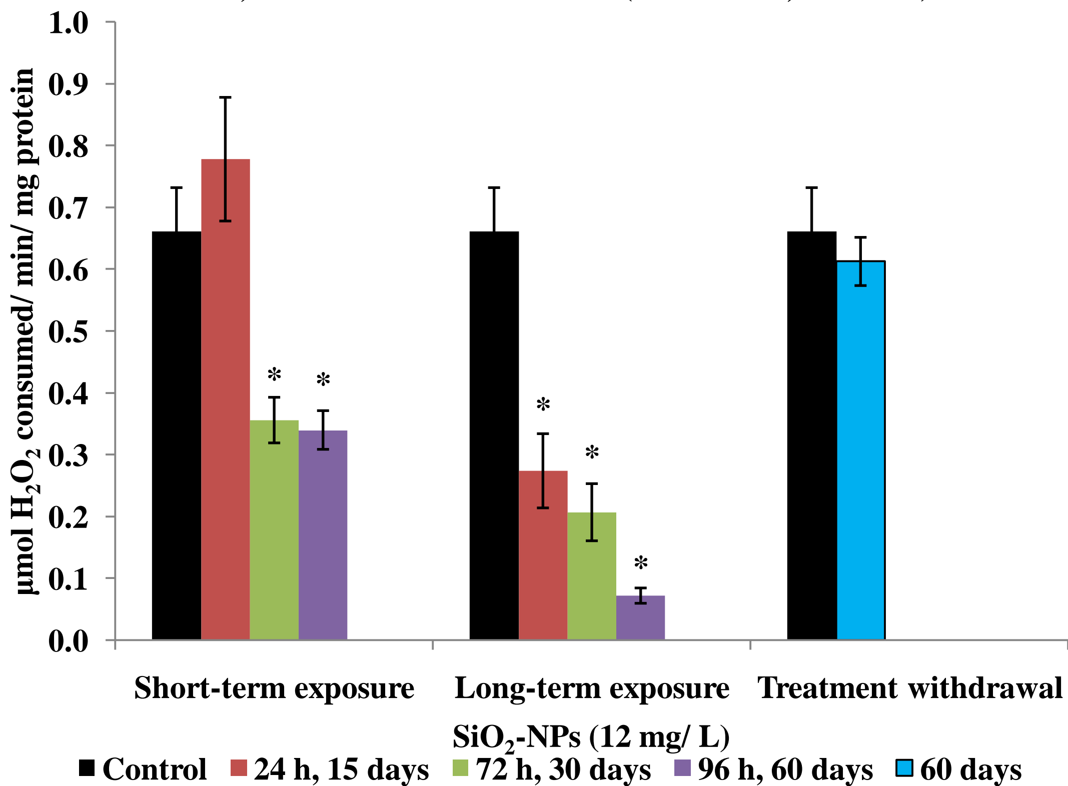


Figure 7c Effect of $SiO_2$-NPs on the activity of glutathione reductase in the liver of the fish, *Oreochromis mossambicus*

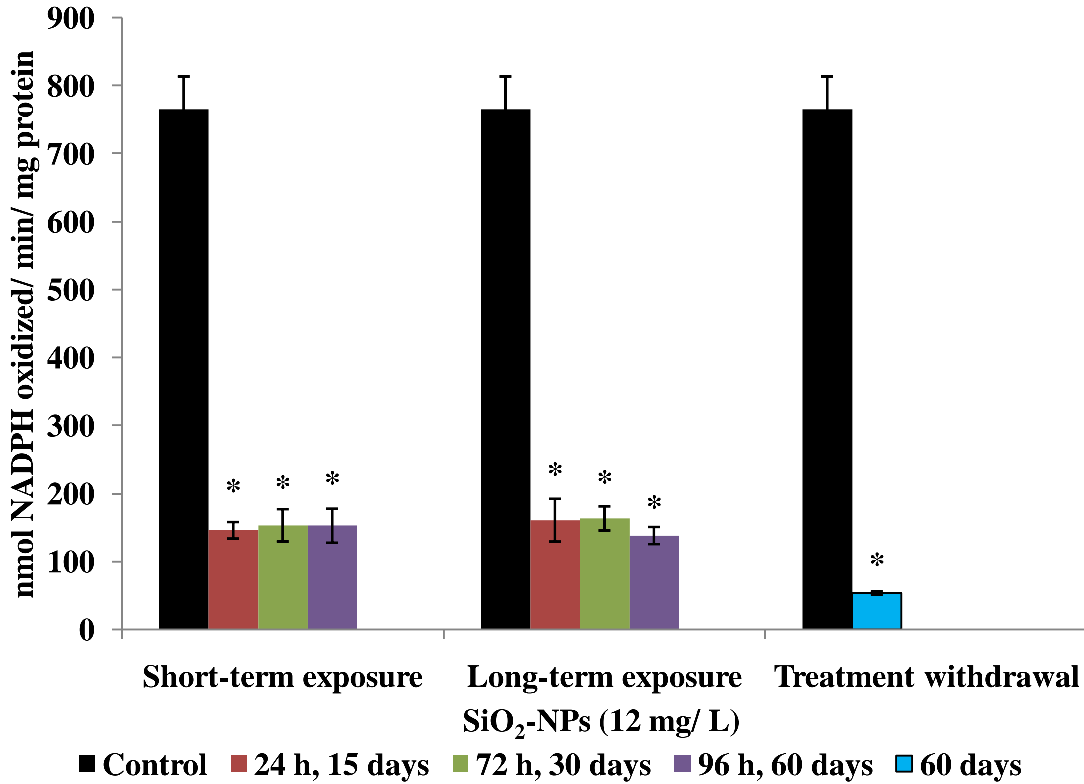



Figure 7d Effect of $SiO_2$-NPs on the activity of glutathione peroxidase in the liver of the fish, *Oreochromis mossambicus* (Mean ± SD; * $P<0.05$)

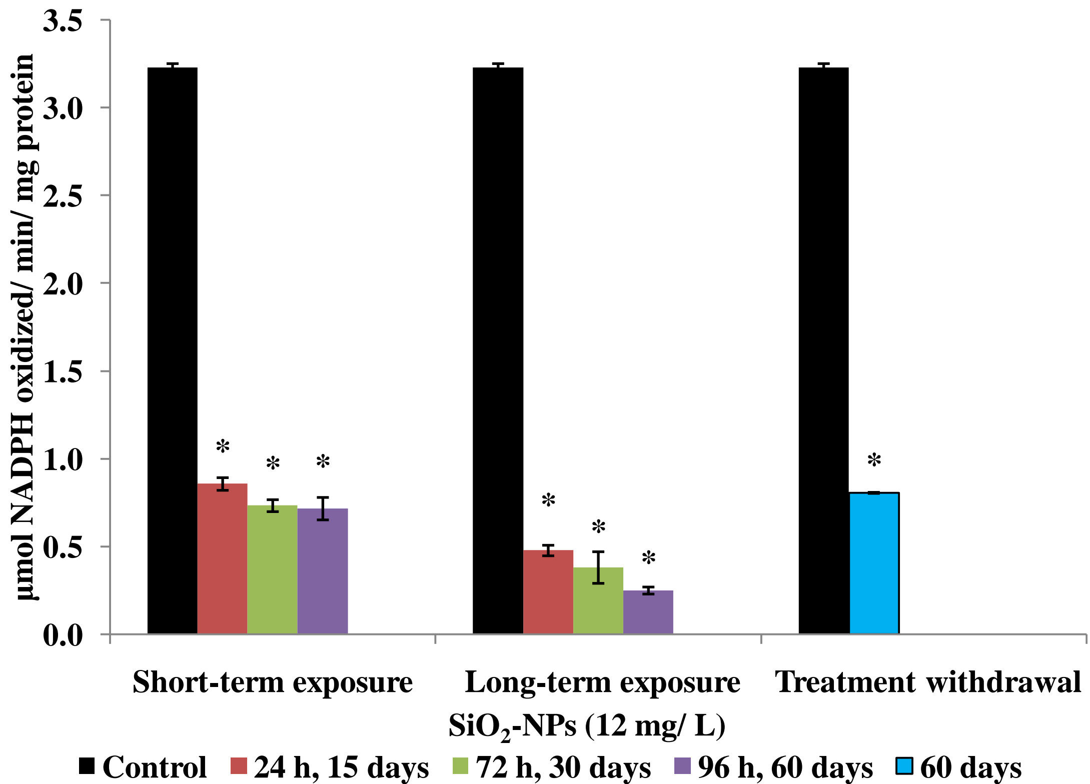


**Figure 7e** **Effect of $SiO_2$-NPs on the level of hydrogen peroxide generation in the liver of the fish, *Oreochromis mossambicus* (Mean ± SD; * P<0.05)**

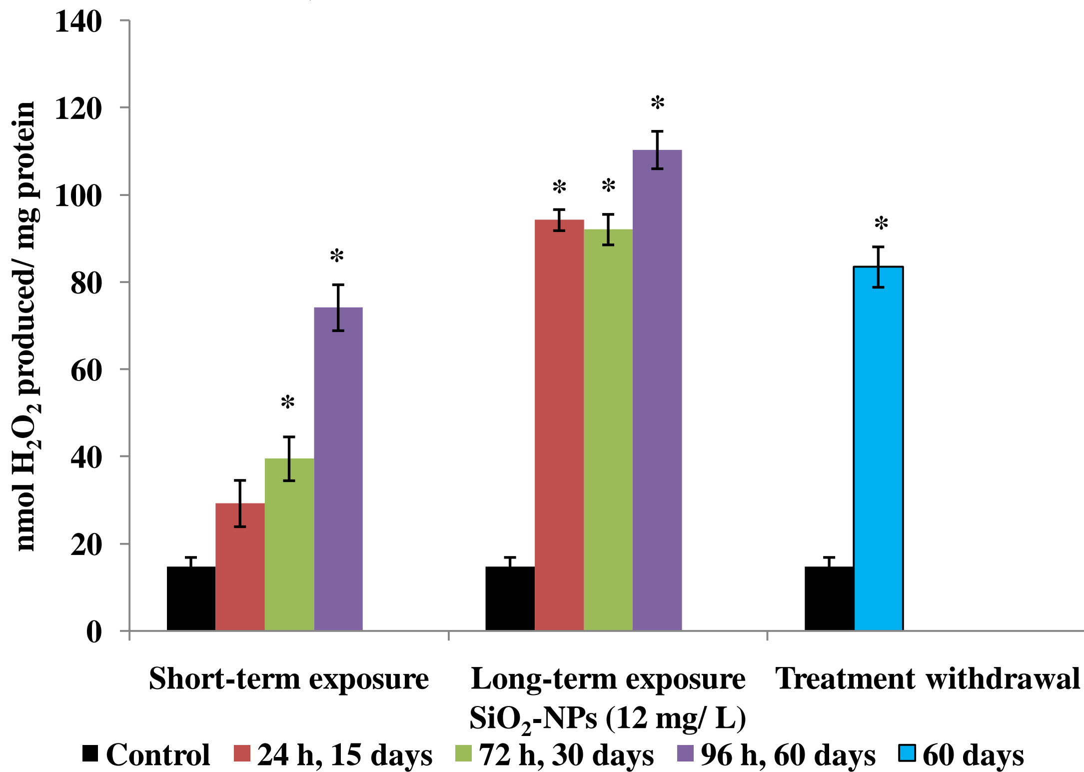



**Figure 7f** **Effect of $SiO_2$-NPs on the level of lipid peroxidation in the liver of the fish, *Oreochromis mossambicus* (Mean ± SD; * P<0.05)**

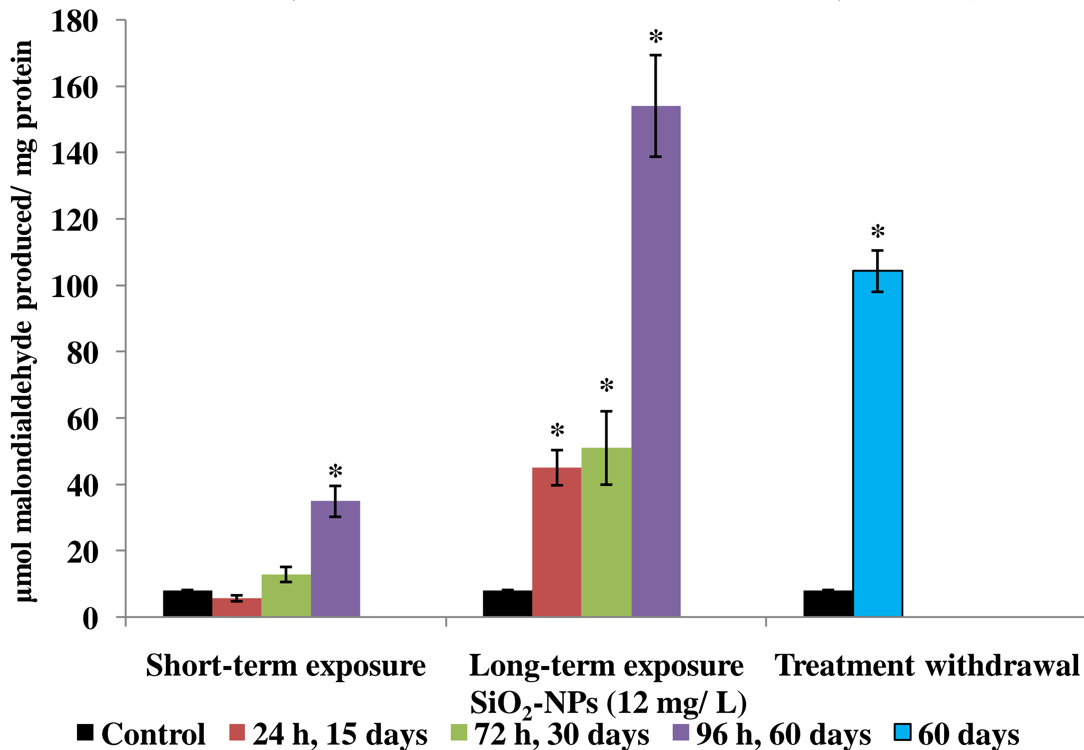


**Figure 8a** Effect of $TiO_2$-NPs on the activity of superoxide dismutase in the liver of the fish, *Oreochromis mossambicus* (Mean ± SD; * P<0.05)

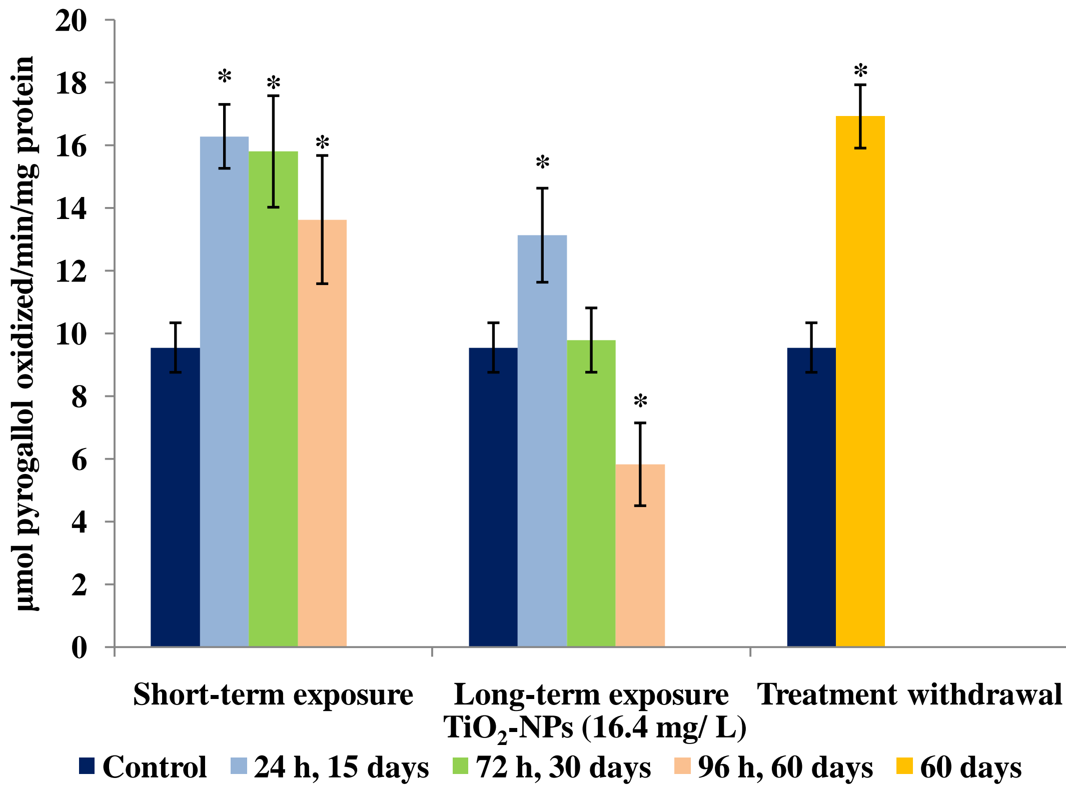



**Figure 8b** Effect of $TiO_2$-NPs on the activity of catalase in the liver of the fish, *Oreochromis mossambicus* (Mean ± SD; * P<0.05)

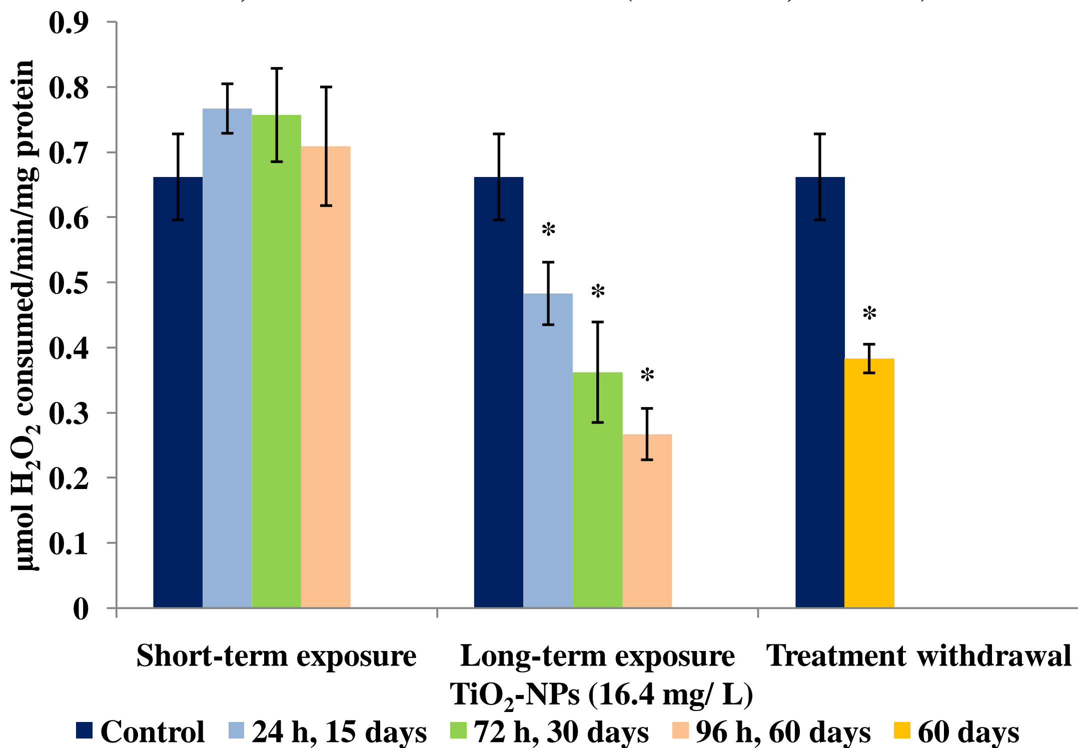


**Figure 8c** **Effect of $TiO_2$-NPs on the activity of glutathione reductase in the liver of the fish, *Oreochromis mossambicus* (Mean ± SD; * P<0.05)**

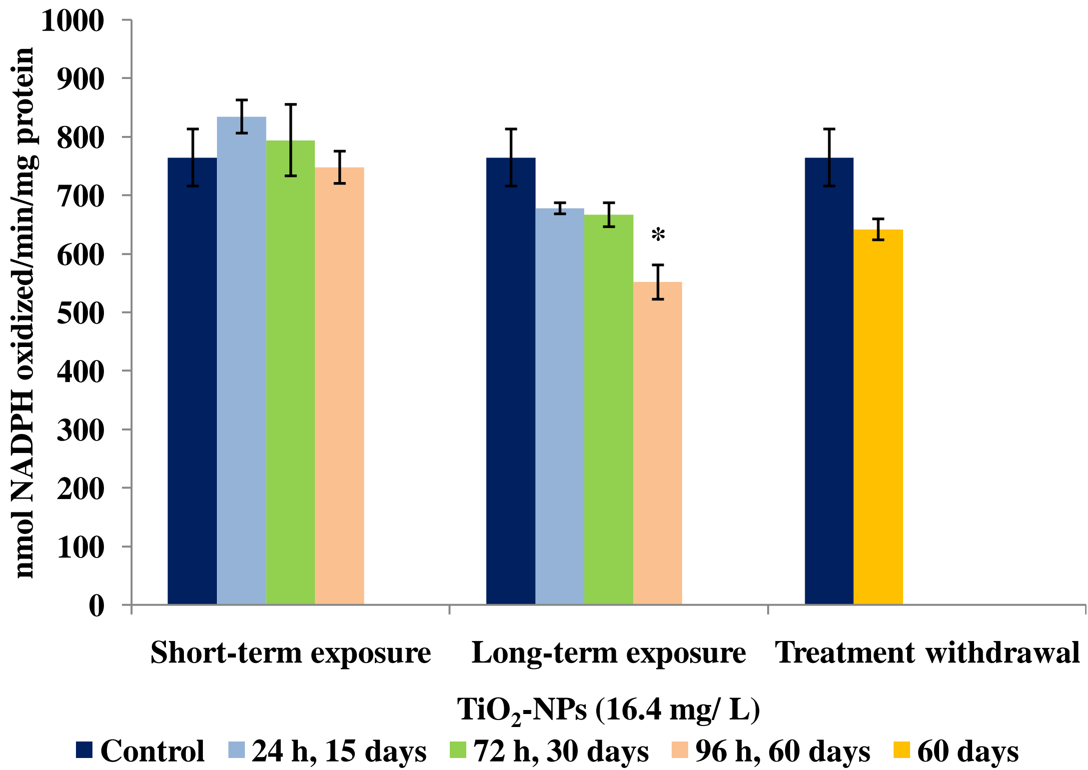



**Figure 8d** **Effect of $TiO_2$-NPs on the activity of glutathione peroxidase in the liver of the fish, *Oreochromis mossambicus* (Mean ± SD; * P<0.05)**

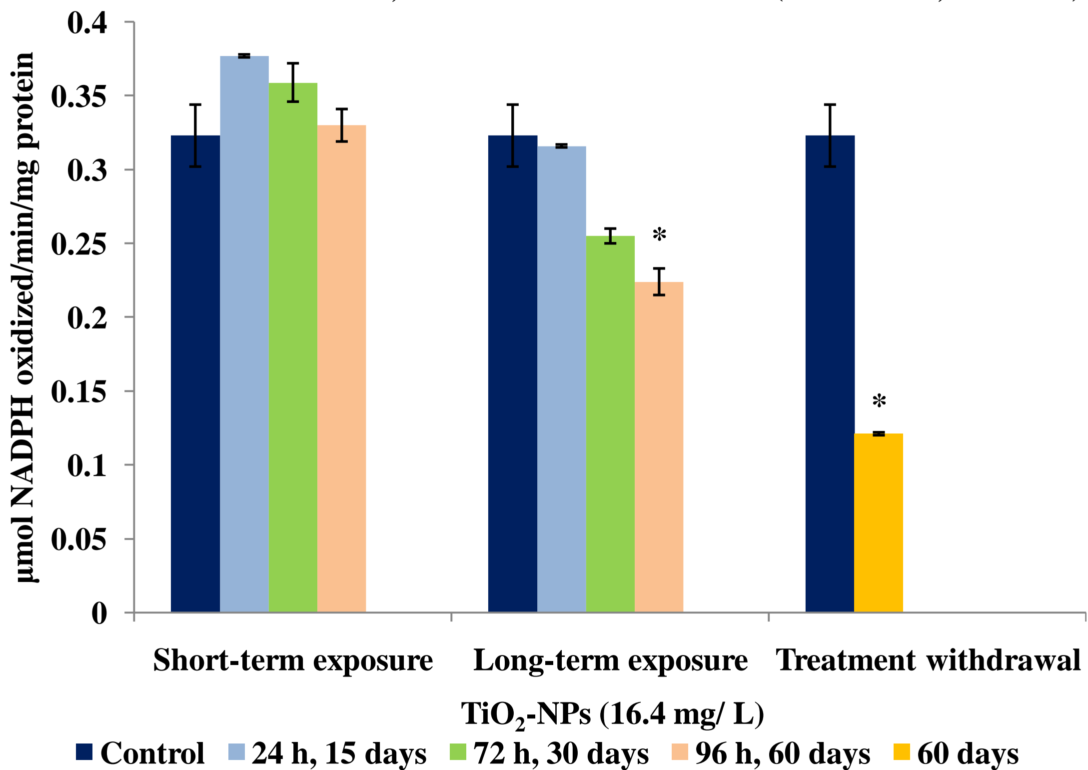


**Figure 8e** Effect of $TiO_2$-NPs on the level of hydrogen peroxide generation in the liver of the fish, *Oreochromis mossambicus* (Mean ± SD; * P<0.05)

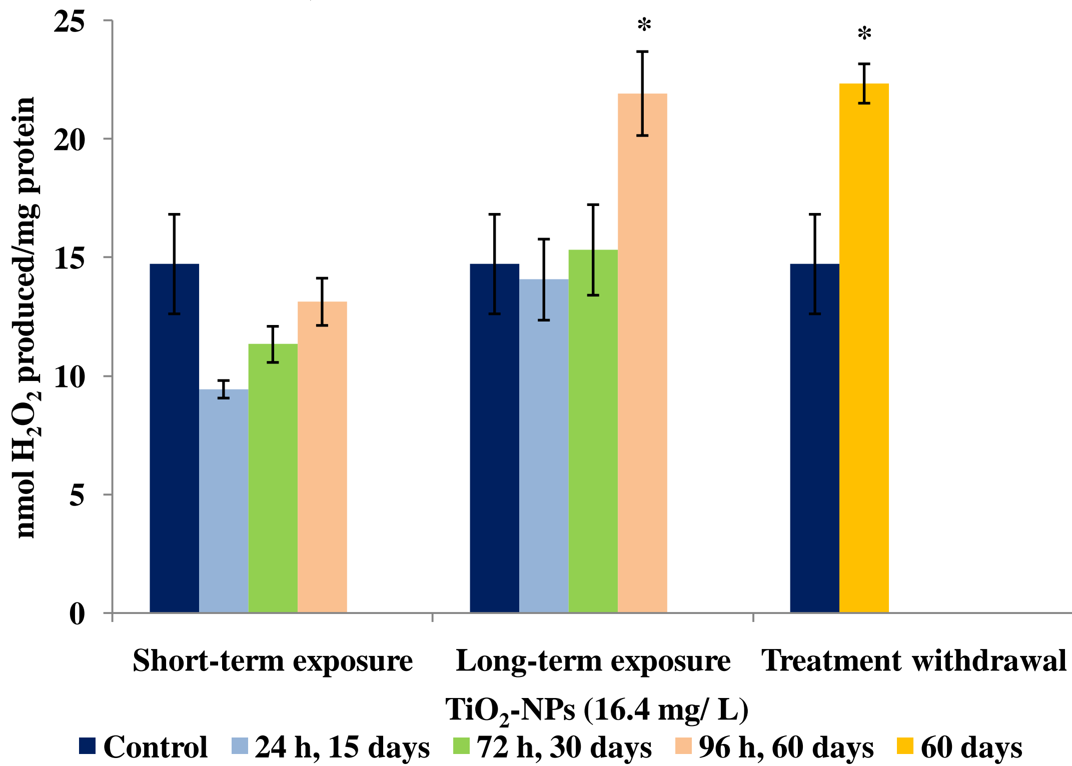
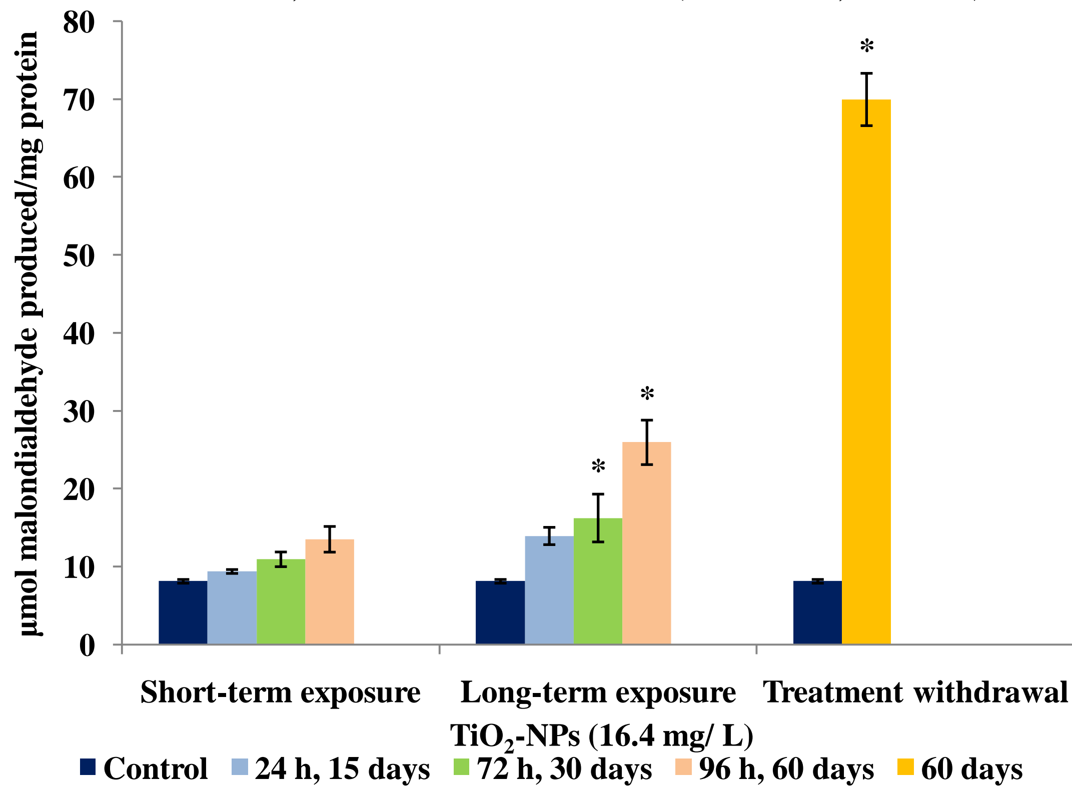



**Figure 8f** Effect of $TiO_2$-NPs on the level of lipid peroxidation in the liver of the fish, *Oreochromis mossambicus* (Mean ± SD; * P<0.05)



## 4.4 Effects of nanoparticles on the antioxidant status in brain tissue of the fish, *Oreochromis mossambicus*

**Effect of $Al_2O_3$-NPs**

Short-term and long-term exposure of $Al_2O_3$-NPs showed significant ($P<0.05$) decrease in the activities of superoxide dismutase, catalase, glutathione reductase and glutathione peroxidase in the brain tissue of the fish *Oreochromis mossambicus* (Figures 9a-d). However, the levels of hydrogen peroxide generation and lipid peroxidation increased significantly ($P<0.05$) only in the long-term exposure groups (Figures 9e and 9f). Withdrawal of $Al_2O_3$-NPs for 60 days showed significant ($P<0.05$) decrease in the activities of all antioxidant enzymes, and increase in the levels of hydrogen peroxide generation and lipid peroxidation in brain tissue (Figures 9a-f).

**Effect of $Fe_3O_4$-NPs**

$Fe_3O_4$-NPs exposed fish showed significant ($P<0.05$) decrease in the activities of superoxide dismutase, catalase, glutathione reductase and glutathione peroxidase enzymes throughout the experimental period in time-dependent manner in the brain tissue (Figures 10a-d). The levels of hydrogen peroxide generation and lipid peroxidation increased significantly ($P<0.05$) throughout the experiment (Figures 10e and 10f). Treatment withdrawal group showed similar effects as in the case of treatment groups (Figures 10a-f).

**Effect of $SiO_2$-NPs**

Brain tissue of $SiO_2$-NPs treated fish showed significant ($P<0.05$) decrease in the activities of superoxide dismutase, glutathione reductase and glutathione peroxidase after 96 h as well as in all durations of long-term exposure groups (Figures 11a, c and d). The activity of catalase remained unchanged in short-term exposed groups, while a time-dependent significant ($P<0.05$) increase was observed in all durations of long-term exposure groups (Figure 11b). In 96 h of short-term exposure group and in all durations of long-term exposure groups of brain tissue showed significant ($P<0.05$) increase in the level of hydrogen peroxide generation (Figure 11e). However, the induction of lipid peroxidation showed significant

($P<0.05$) increase only after 30 and 60 days of $SiO_2$-NPs exposure (Figure 11f). The effects were found similar in the treatment withdrawal of $SiO_2$-NPs for 60 days (Figures 11a-f).

**Effect of $TiO_2$-NPs**

The activities of superoxide dismutase, catalase, glutathione reductase and glutathione peroxidase in the brain tissue decreased significantly ($P<0.05$) in all treatment groups in a time-dependent manner after $TiO_2$-NPs exposure (Figures 12a-d). Conversely, the levels of hydrogen peroxide generation remained unchanged in short-term exposure group with tremendous time-dependent increase in the long-term exposure group (Figure 12e). After 30 and 60 days of $TiO_2$-NPs exposure, the level of lipid peroxidation increased significantly ($P<0.05$) in brain tissue without much changes in other treatment groups (Figure 12f). The results observed in the treatment withdrawal group were found similar to that of the $TiO_2$-NPs exposed group (Figures 12a-f).

**Figure 9a** Effect of $Al_2O_3$-NPs on the activity of superoxide dismutase in the brain of the fish, *Oreochromis mossambicus* (Mean ± SD; * P<0.05)

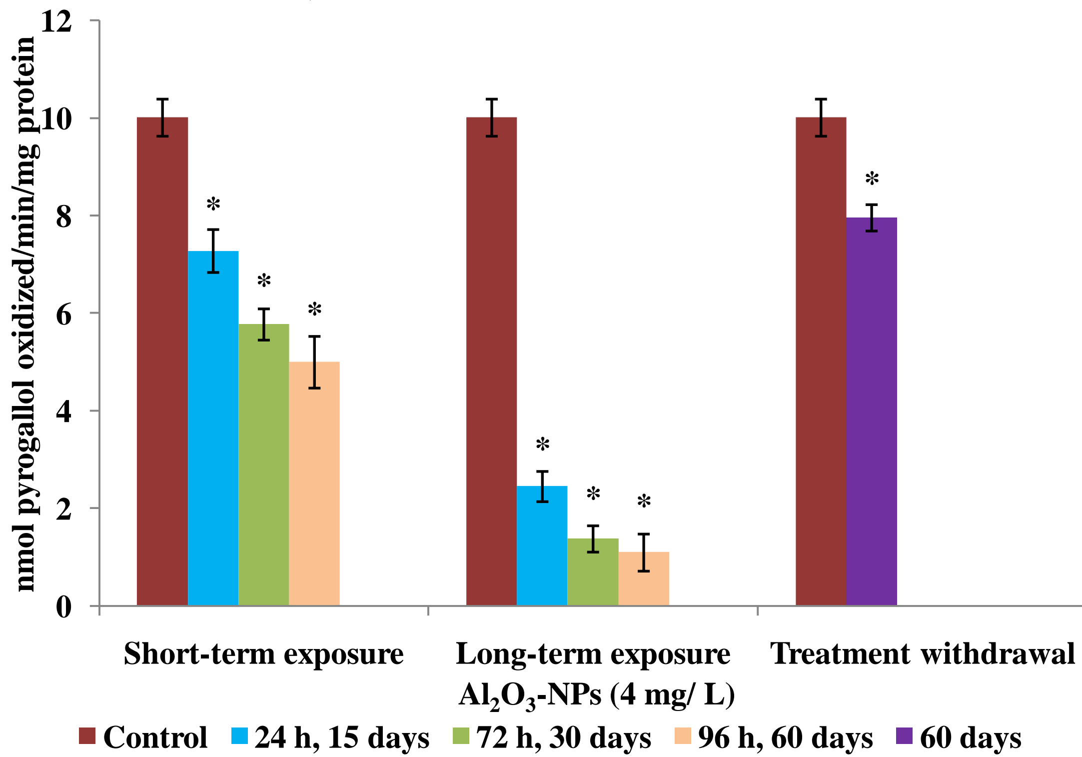



**Figure 9b** Effect of $Al_2O_3$-NPs on the activity of catalase in the brain of the fish, *Oreochromis mossambicus* (Mean ± SD; * P<0.05)

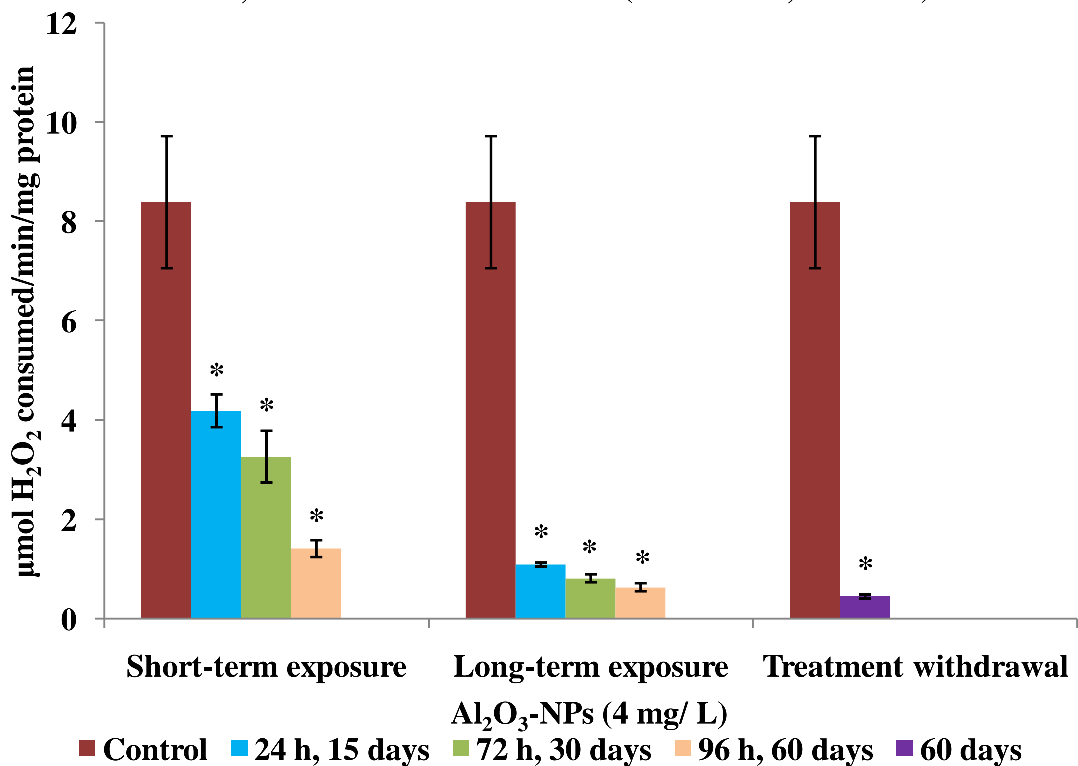


Figure 9c Effect of $Al_2O_3$-NPs on the activity of glutathione reductase in the brain of the fish, *Oreochromis mossambicus* (Mean ± SD; * P<0.05)

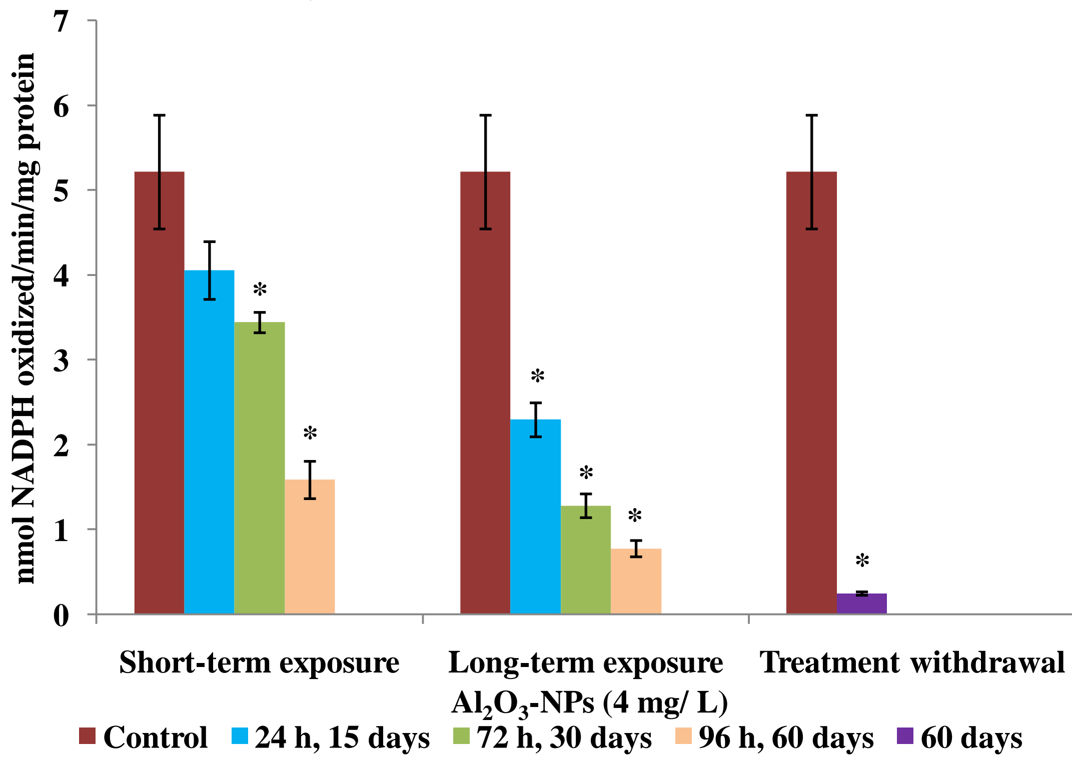



Figure 9d Effect of $Al_2O_3$-NPs on the activity of glutathione peroxidase in the brain of the fish, *Oreochromis mossambicus* (Mean ± SD; * P<0.05)

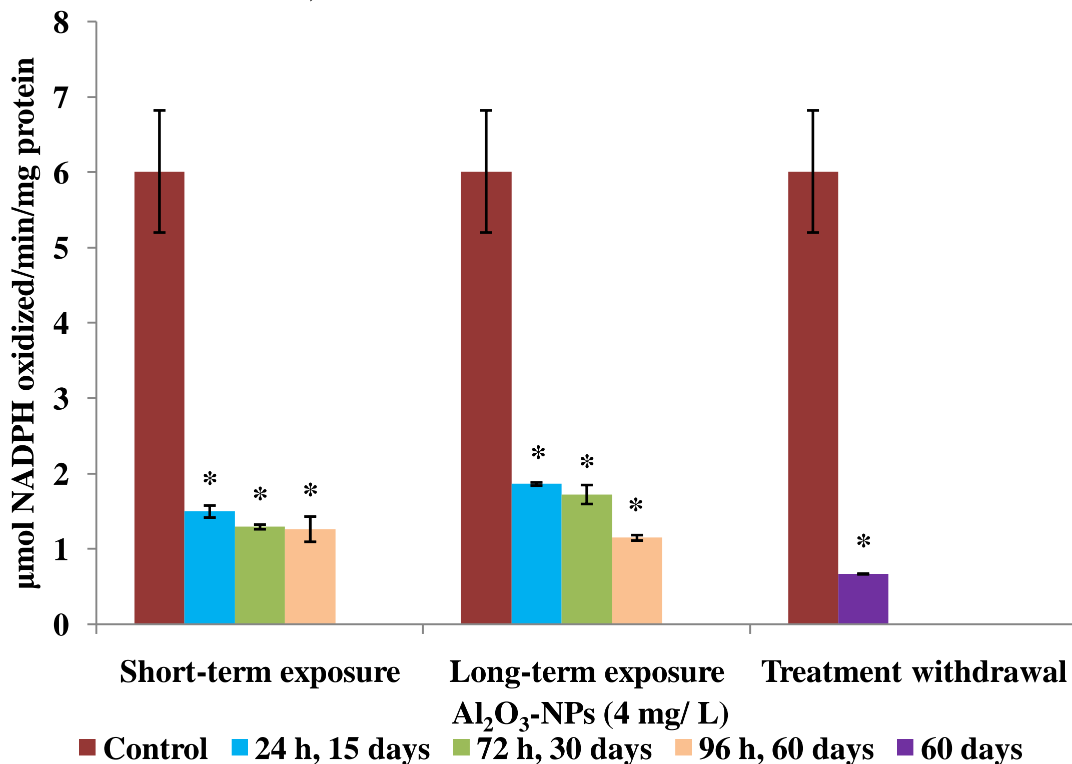


**Figure 9e** Effect of $Al_2O_3$-NPs on the level of hydrogen peroxide generation in the brain of the fish, *Oreochromis mossambicus* (Mean ± SD; * P<0.05)

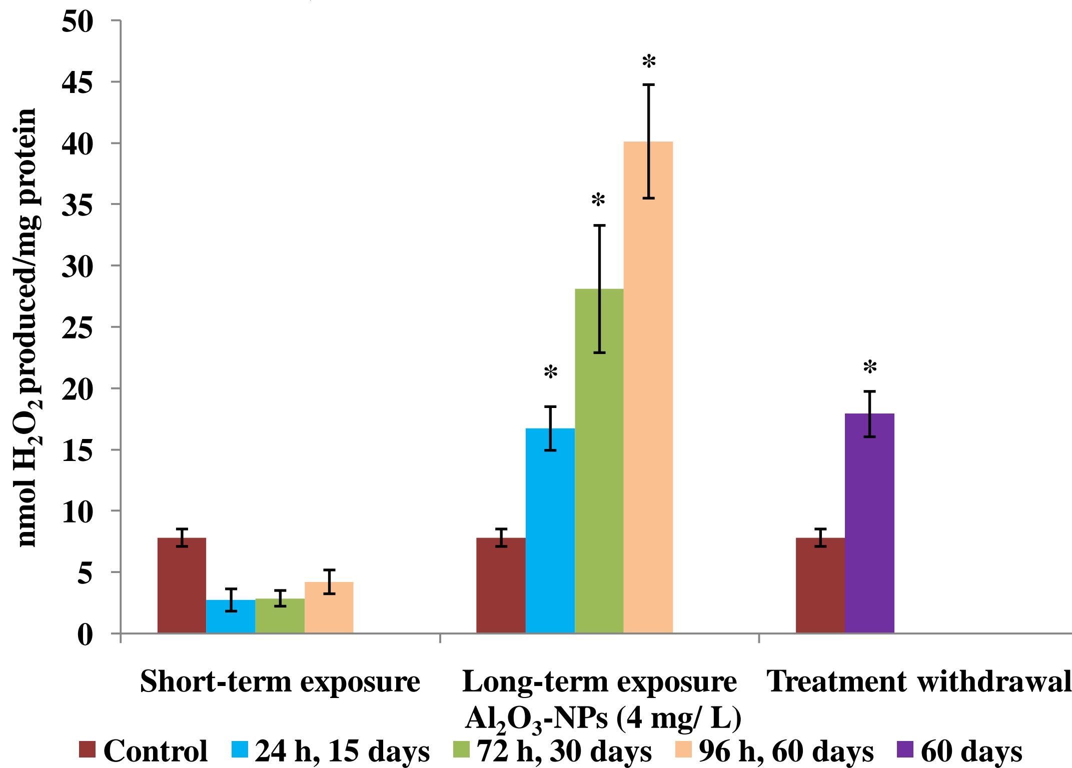



**Figure 9f** Effect of $Al_2O_3$-NPs on the level of lipid peroxidation in the brain of the fish, *Oreochromis mossambicus* (Mean ± SD; * P<0.05)

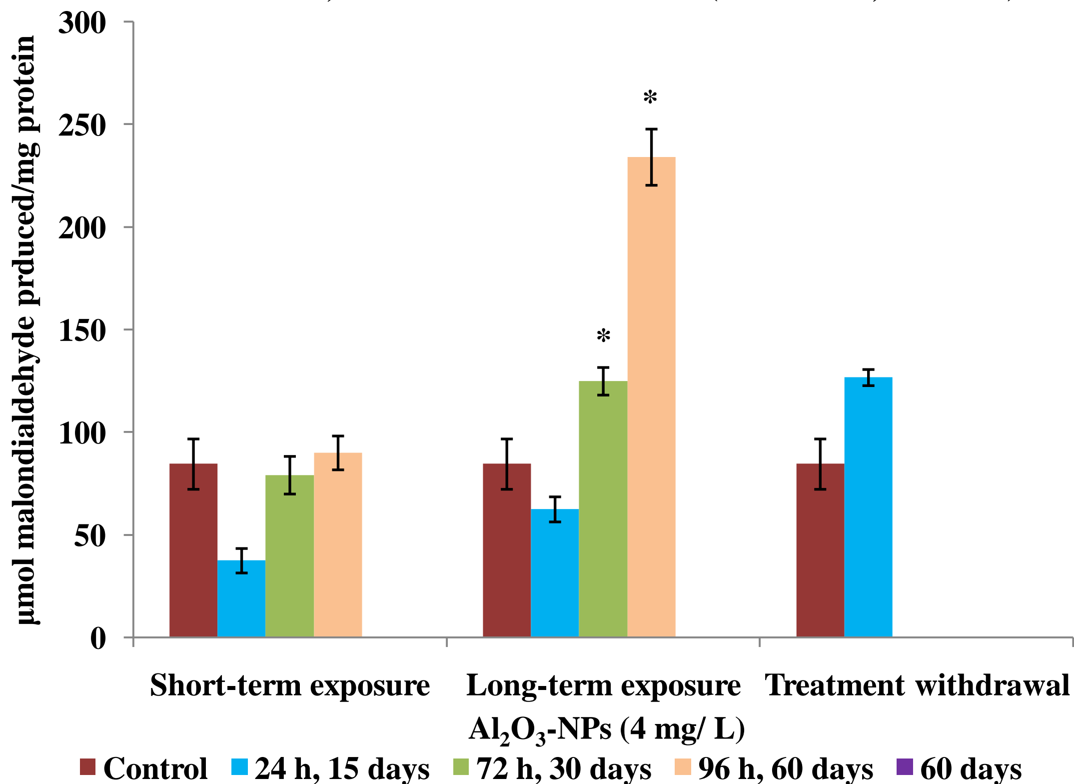


**Figure 10a** Effect of $Fe_3O_4$-NPs on the activity of superoxide dismutase in the brain of the fish, *Oreochromis mossambicus* (Mean ± SD; * P<0.05)

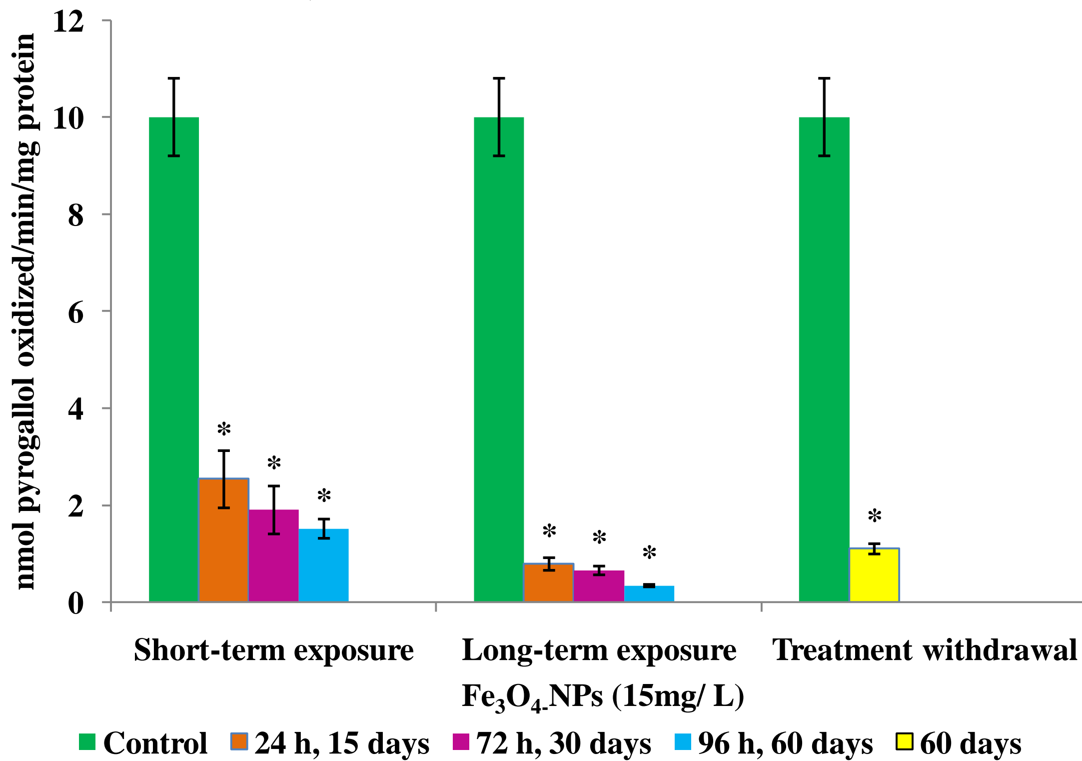



**Figure 10b** Effect of $Fe_3O_4$-NPs on the activity of catalase in the brain of the fish, *Oreochromis mossambicus* (Mean ± SD; * P<0.05)

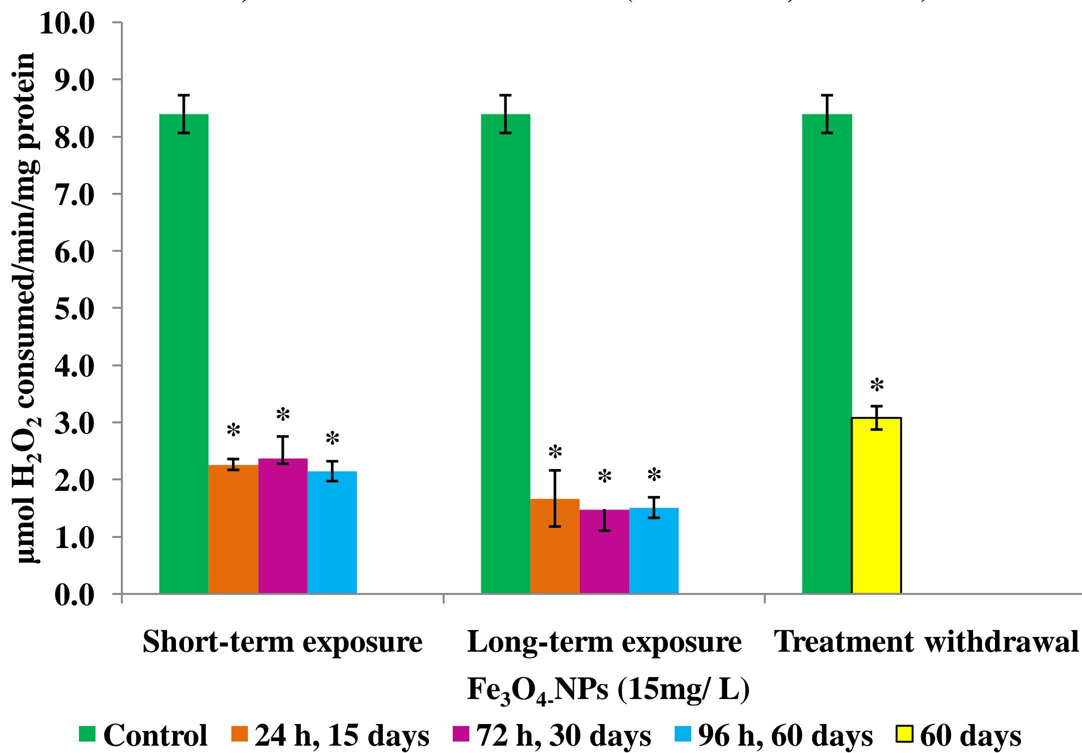


**Figure 10c** Effect of $Fe_3O_4$-NPs on the activity of glutathione reductase in the brain of the fish, *Oreochromis mossambicus* (Mean ± SD; * P<0.05)

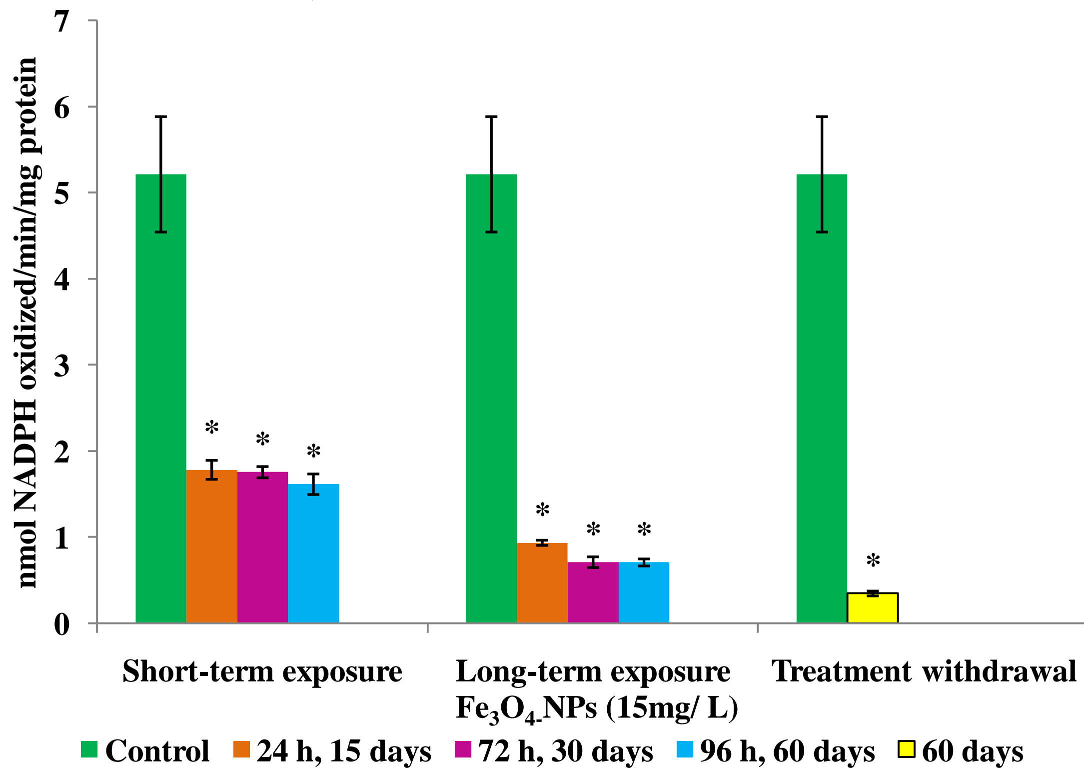



**Figure 10d** Effect of $Fe_3O_4$-NPs on the activity of glutathione peroxidase in the brain of the fish, *Oreochromis mossambicus* (Mean ± SD; * P<0.05)

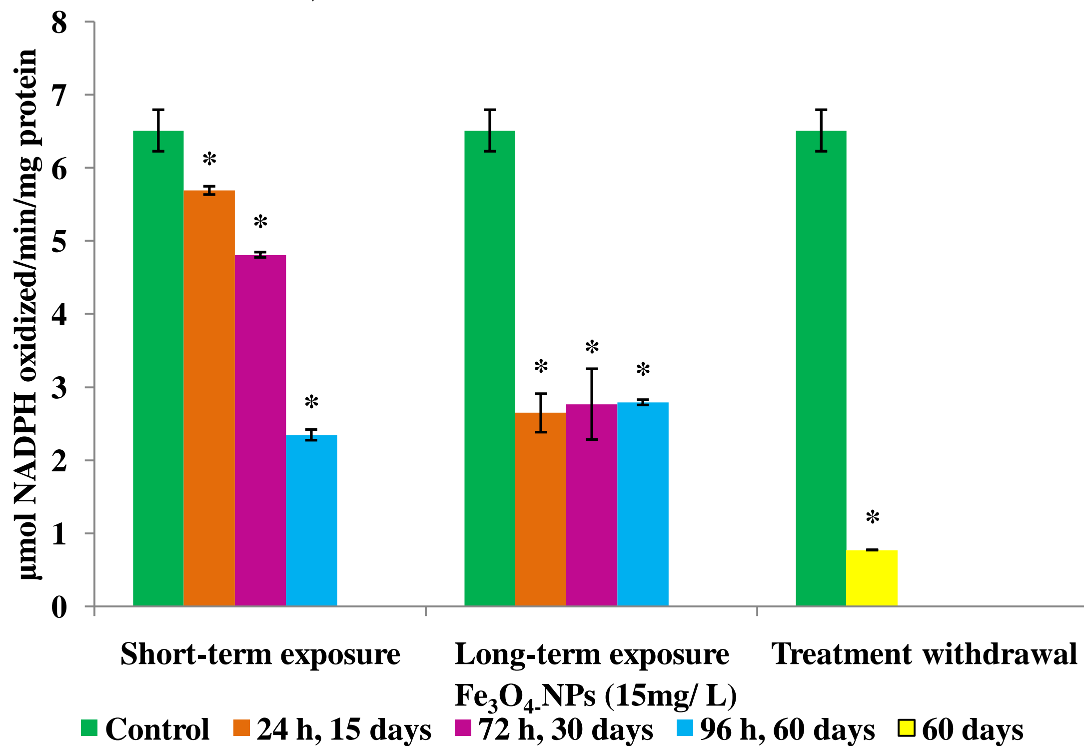


**Figure 10e** Effect of $Fe_3O_4$-NPs on the level of hydrogen peroxide generation in the brain of the fish, *Oreochromis mossambicus* (Mean ± SD; * P<0.05)

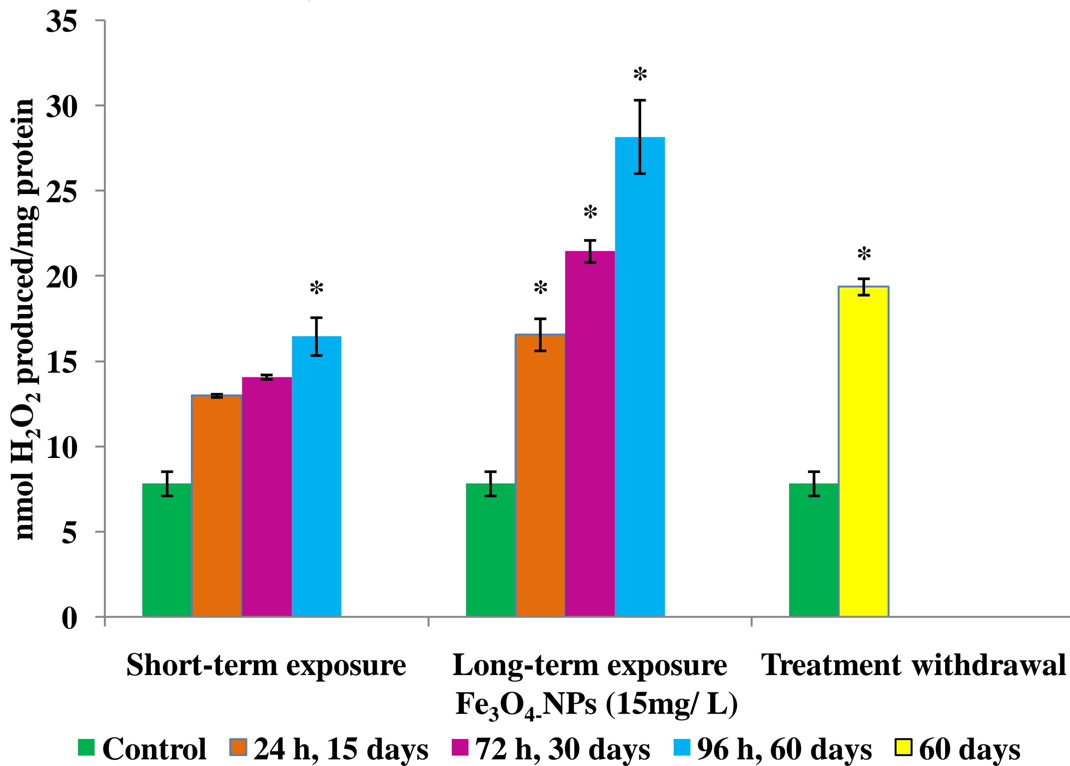



**Figure 10f** Effect of $Fe_3O_4$-NPs on the level of lipid peroxidation in the brain of the fish, *Oreochromis mossambicus* (Mean ± SD; * P<0.05)

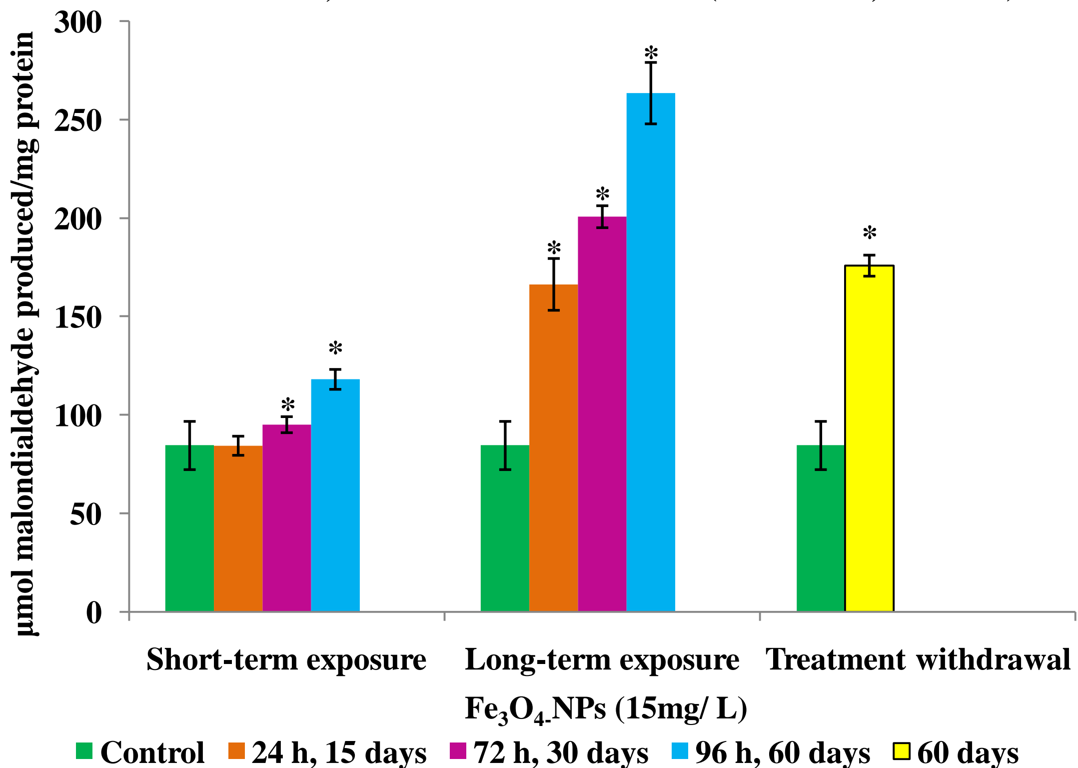


**Figure 11a** Effect of $SiO_2$-NPs on the activity of superoxide dismutase in the brain of the fish, *Oreochromis mossambicus* (Mean ± SD; * P<0.05)

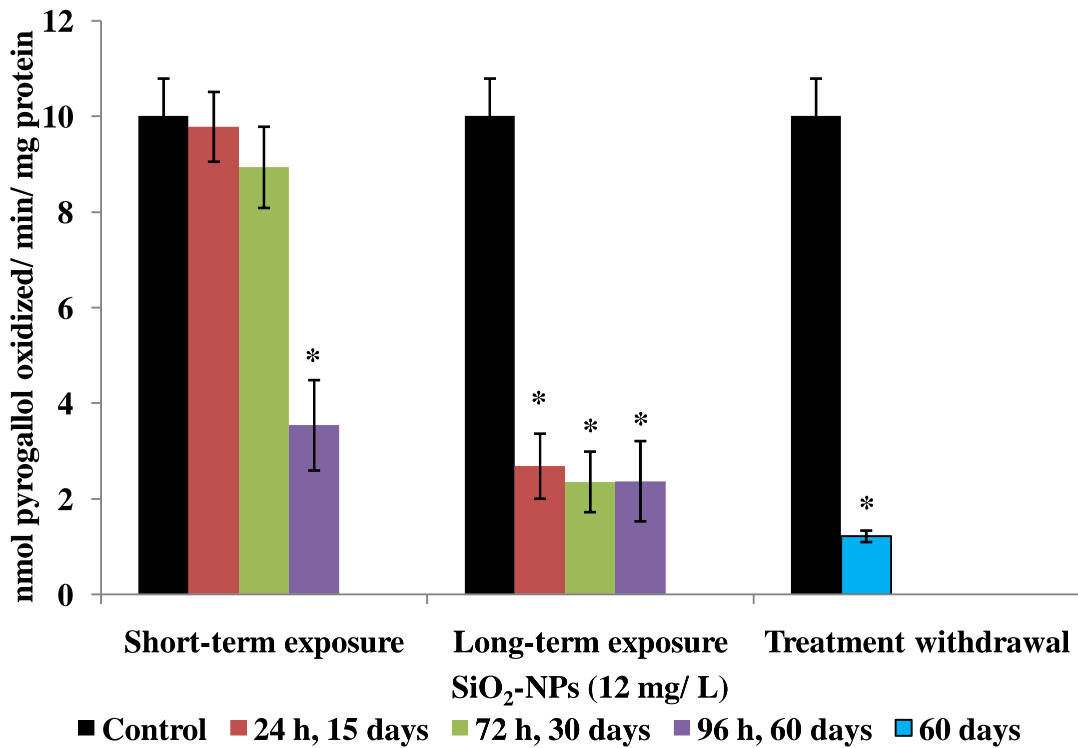



**Figure 11b** Effect of $SiO_2$-NPs on the activity of catalase in the brain of the fish, *Oreochromis mossambicus* (Mean ± SD; * P<0.05)

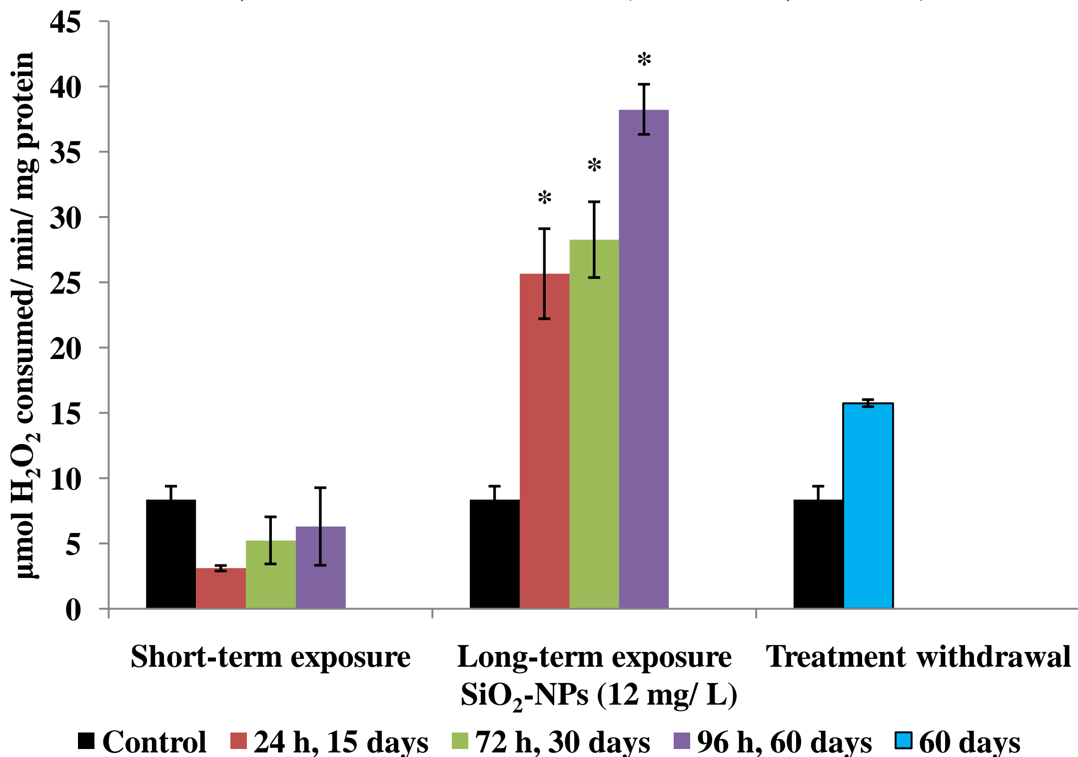


**Figure 11c** Effect of $SiO_2$-NPs on the activity of glutathione reductase in the brain of the fish, *Oreochromis mossambicus* (Mean ± SD; * P<0.05)

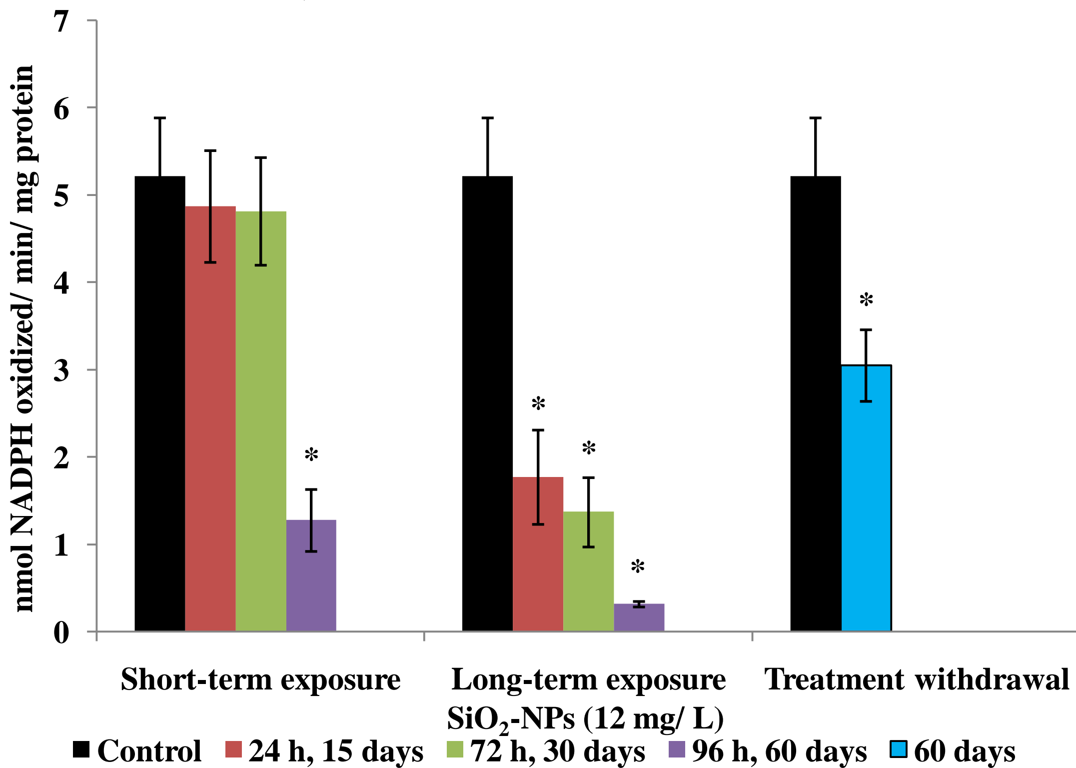



**Figure 11d** Effect of $SiO_2$-NPs on the activity of glutathione peroxidase in the brain of the fish, *Oreochromis mossambicus* (Mean ± SD; * P<0.05)

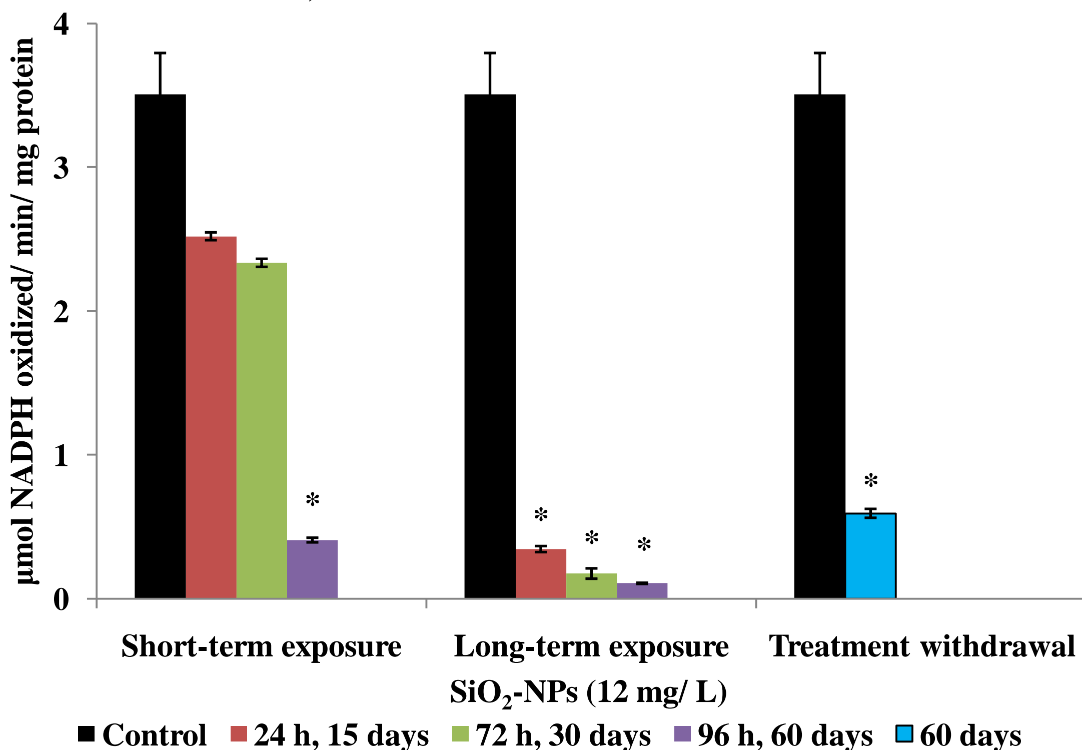


**Figure 11e** Effect of $SiO_2$-NPs on the level of hydrogen peroxide generation in the brain of the fish, *Oreochromis mossambicus* (Mean ± SD; * P<0.05)

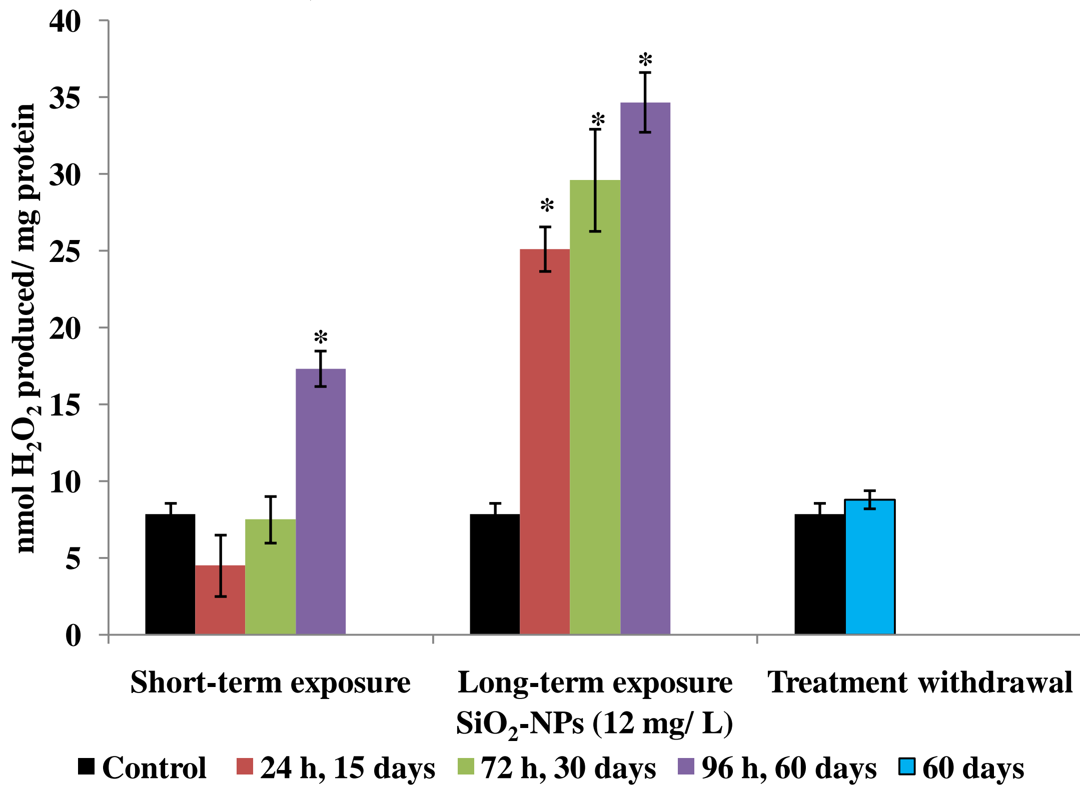



**Figure 11f** Effect of $SiO_2$-NPs on the level of lipid peroxidation in the brain of the fish, *Oreochromis mossambicus* (Mean ± SD; * P<0.05)

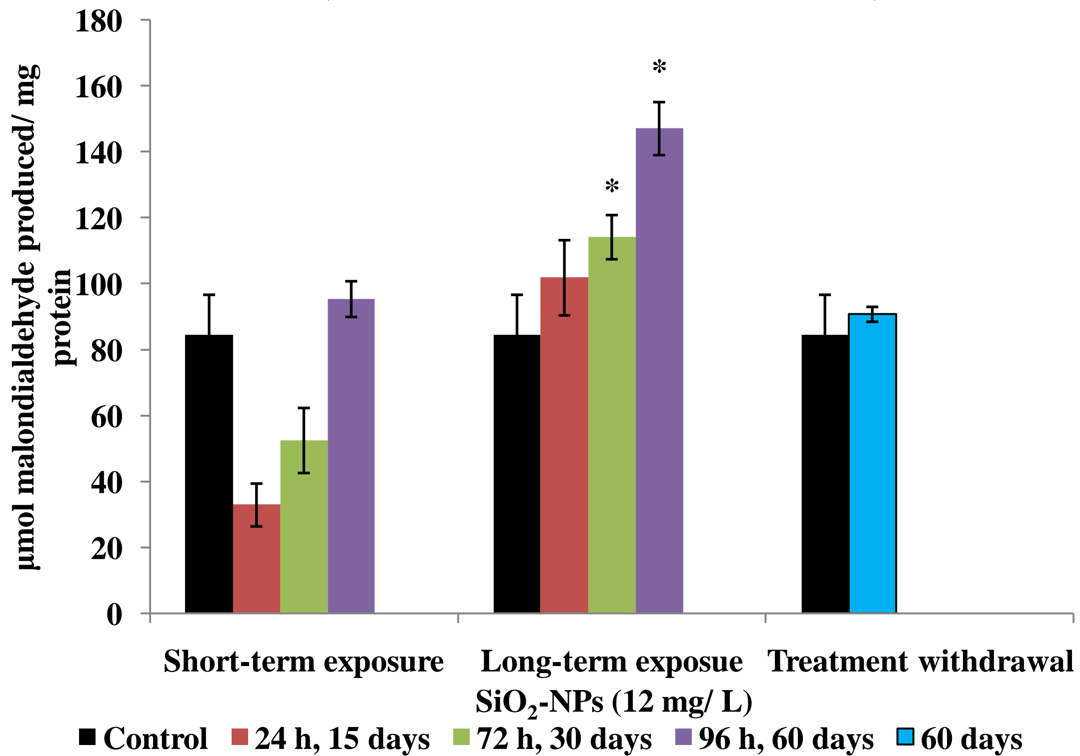


**Figure 12a** Effect of $TiO_2$-NPs on the activity of superoxide dismutase in the brain of the fish, *Oreochromis mossambicus* (Mean ± SD; * P<0.05)

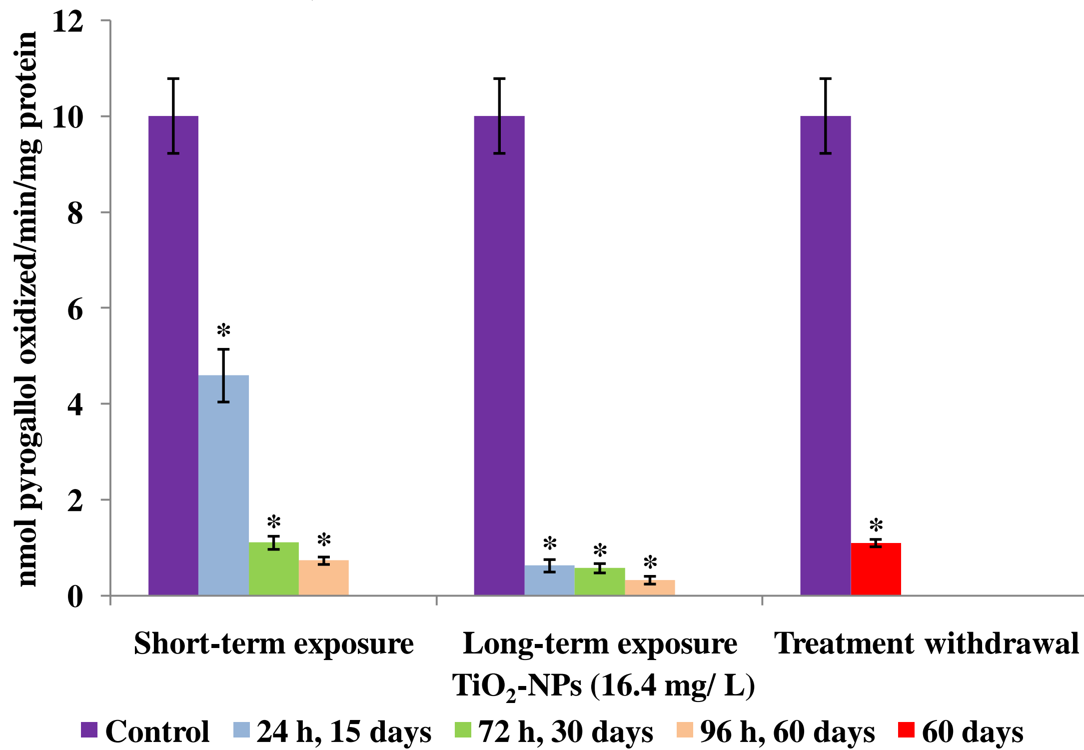



**Figure 12b** Effect of $TiO_2$-NPs on the activity of catalase in the brain of the fish, *Oreochromis mossambicus* (Mean ± SD; * P<0.05)

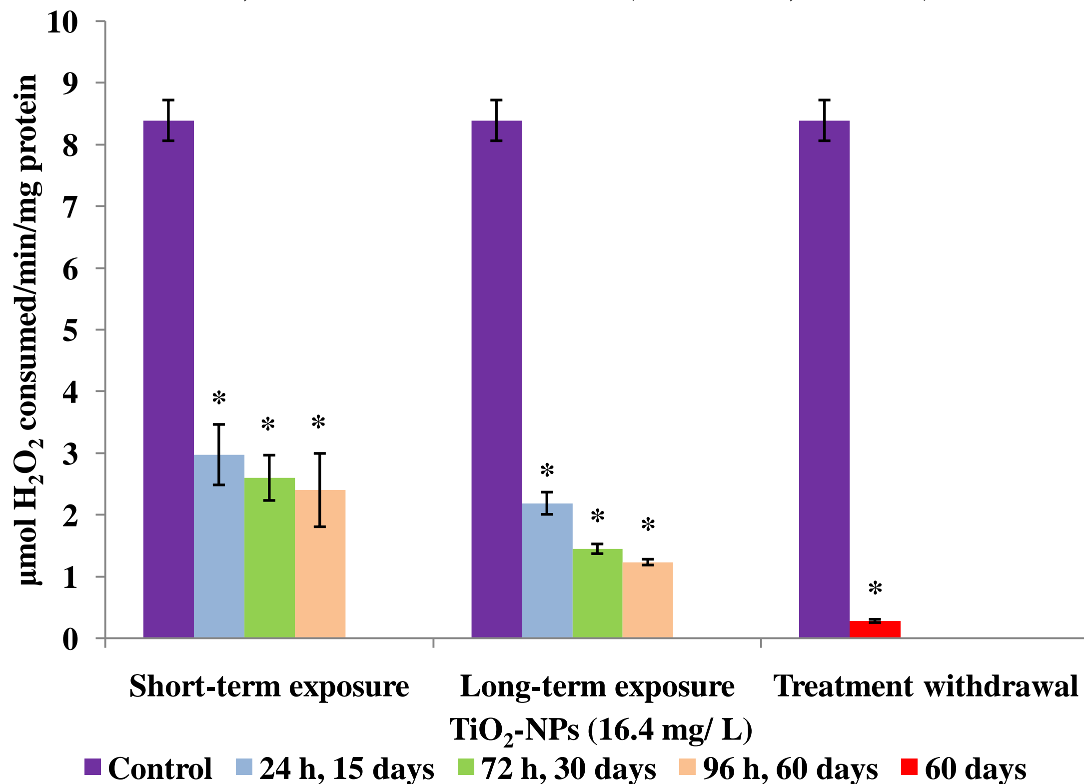


**Figure 12c** Effect of $TiO_2$-NPs on the activity of glutathione reductase in the brain of the fish, *Oreochromis mossambicus* (Mean ± SD; * P<0.05)

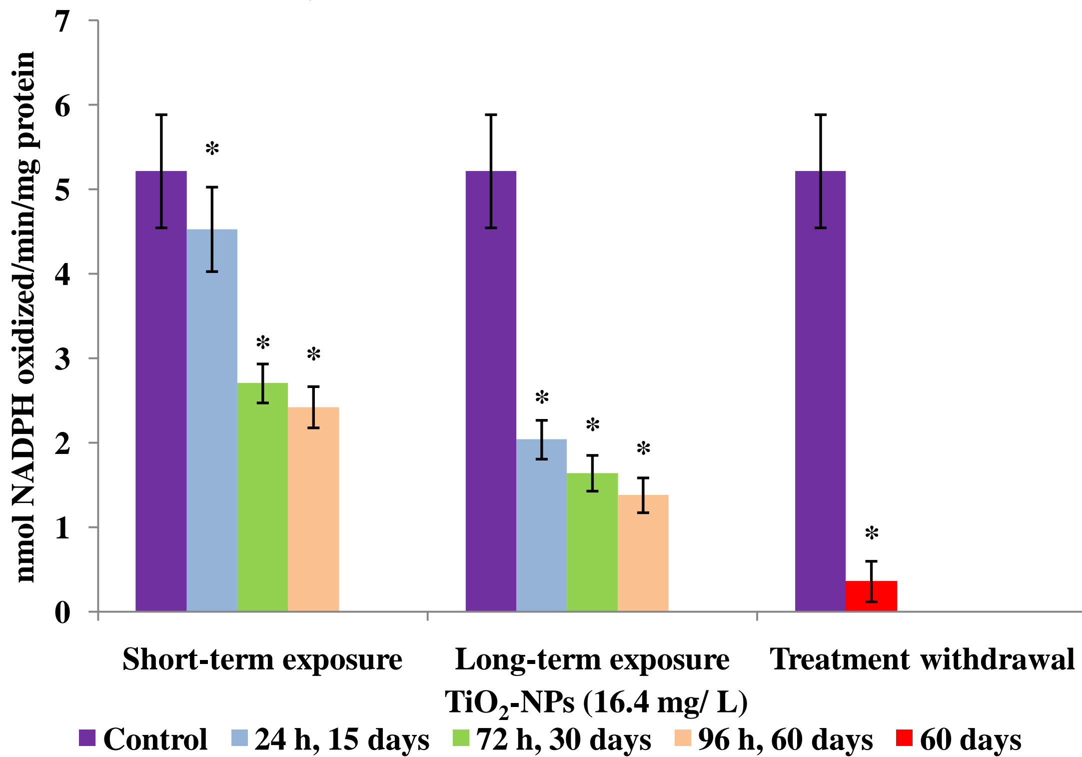



**Figure 12d** Effect of $TiO_2$-NPs on the activity of glutathione peroxidase in the brain of the fish, *Oreochromis mossambicus* (Mean ± SD; * P<0.05)

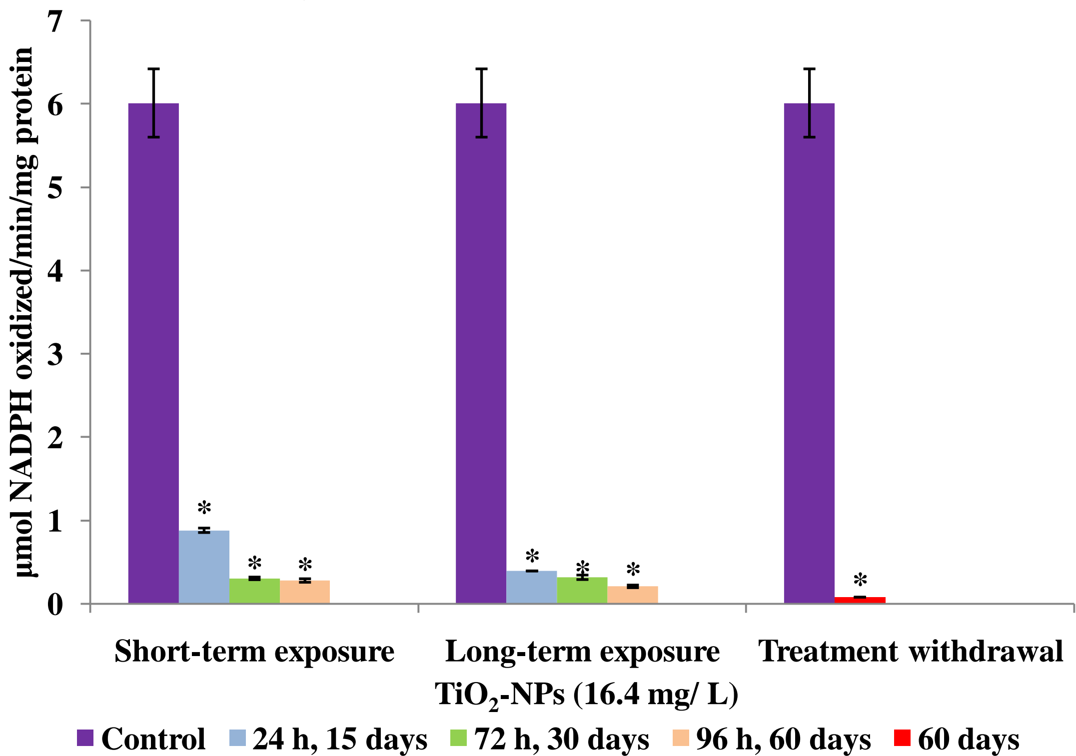


**Figure 12e** Effect of $TiO_2$-NPs on the level of hydrogen peroxide generation in the brain of the fish, *Oreochromis mossambicus* (Mean ± SD; * P<0.05)

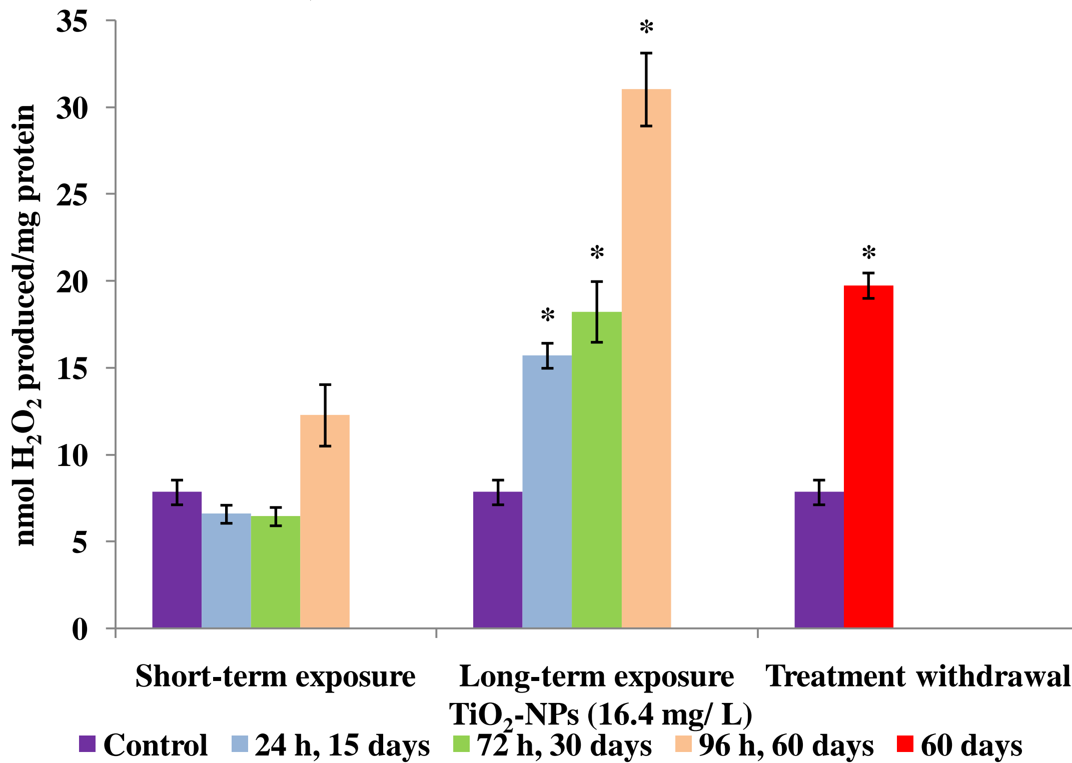
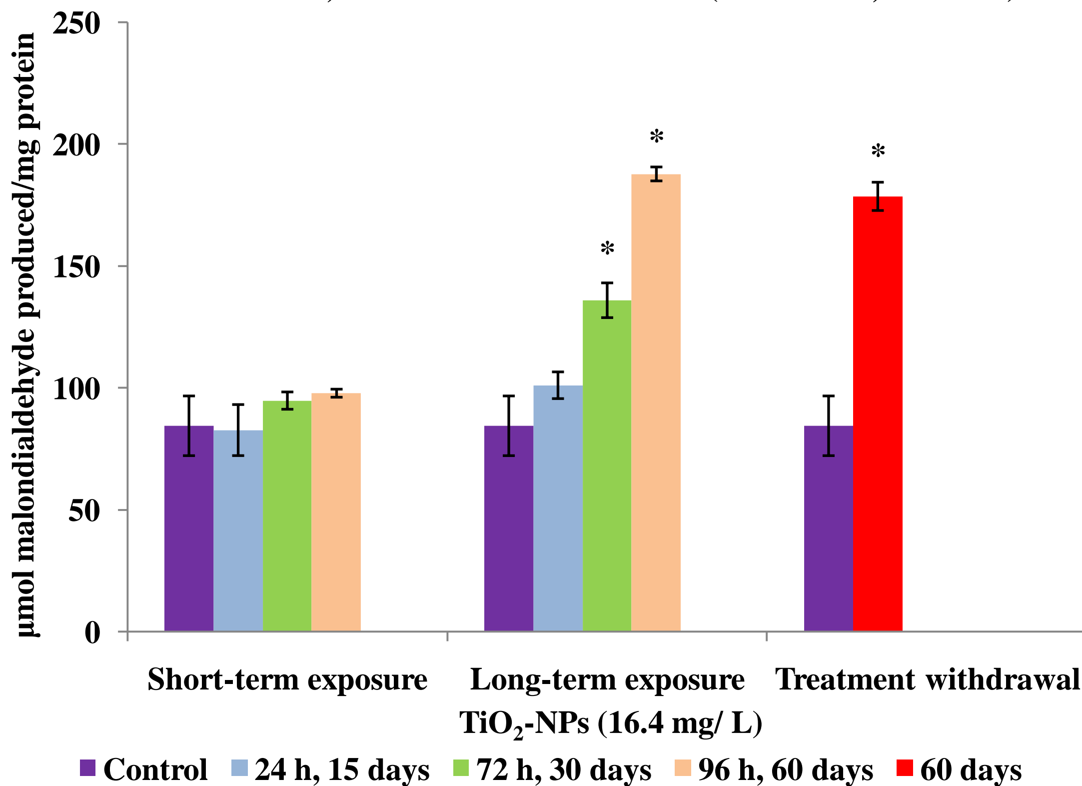



**Figure 12f** Effect of $TiO_2$-NPs on the level of lipid peroxidation in the brain of the fish, *Oreochromis mossambicus* (Mean ± SD; * P<0.05)



## 4.5 Effects of nanoparticles on the tissue-specific marker enzymes in the fish, *Oreochromis mossambicus*

**Effect of $Al_2O_3$-NPs**

The activity of alkaline phosphatase showed significant ($P<0.05$) decrease in gill tissue, however, the activity of enzyme increased in liver tissues followed by $Al_2O_3$-NPs treatment (Figures 13a and 13b). The activity of brain marker enzyme, acetylcholinesterase decreased significantly ($P<0.05$) in all exposure groups when compared to the control group (Figure 13c). The activities of all tissue specific marker enzymes remained the same like that of $Al_2O_3$-NPs exposed groups after the treatment withdrawal (Figures 13a-c).

**Effect of $Fe_3O_4$-NPs**

There was a significant ($P<0.05$) decrease in the activity of alkaline phosphatase enzyme in both gill and liver tissues after $Fe_3O_4$-NPs exposure (Figures 14a and 14b). Similarly, the activity of acetylcholinesterase also showed significant ($P<0.05$) decrease in brain tissue of all experimental groups than that of control tissue (Figure 14c). In the reversal of $Fe_3O_4$-NPs treatment, the enzyme activities of all tissue specific markers were found similar to the treatment groups (Figures 14a-c).

**Effect of $SiO_2$-NPs**

In gill tissue, the activity of alkaline phosphatase showed significant ($P<0.05$) reduction from 96 h of $SiO_2$-NPs exposure onwards in a time-dependent manner (Figure 15a). In liver tissue, the activity of alkaline phosphatase showed no significant changes during short-term exposure, however, the activity of enzyme significantly ($P<0.05$) decreased in all treatment durations of long-term exposure in a time-dependent manner (Figure 15b). The brain marker enzyme, acetylcholinesterase showed no changes after short-term exposure, while a drastic significant ($P<0.05$) decrease was observed in long-term exposure groups of $SiO_2$-NPs (Figure 15c). No reversal effects on the activities of alkaline phosphatase and acetylcholinesterase were observed when the treatment was withdrawn for 60 days (Figures 15a-c).

**Effect of $TiO_2$-NPs**

$TiO_2$-NPs exposure for short-term and long-term durations significantly ($P<0.05$) decreased the activity of alkaline phosphatase in gill tissue (Figure 16a). However, the activity of alkaline phosphatase enzyme in the liver tissue remained unchanged in short-term treatment group with significant ($P<0.05$) time-dependent reduction in long-term exposure group (Figure 16b). Activity of acetylcholinesterase showed significant ($P<0.05$) decrease throughout the experiment period in the brain tissue (Figure 16c). The activities of all tissue specific marker enzyme activities decreased significantly ($P<0.05$) after treatment withdrawal for 60 days (Figures 16a-c).

**Figure 13a** **Effect of $Al_2O_3$-NPs on the activity of alkaline phosphatase in the gill of the fish, *Oreochromis mossambicus* (Mean ± SD; * P<0.05)**

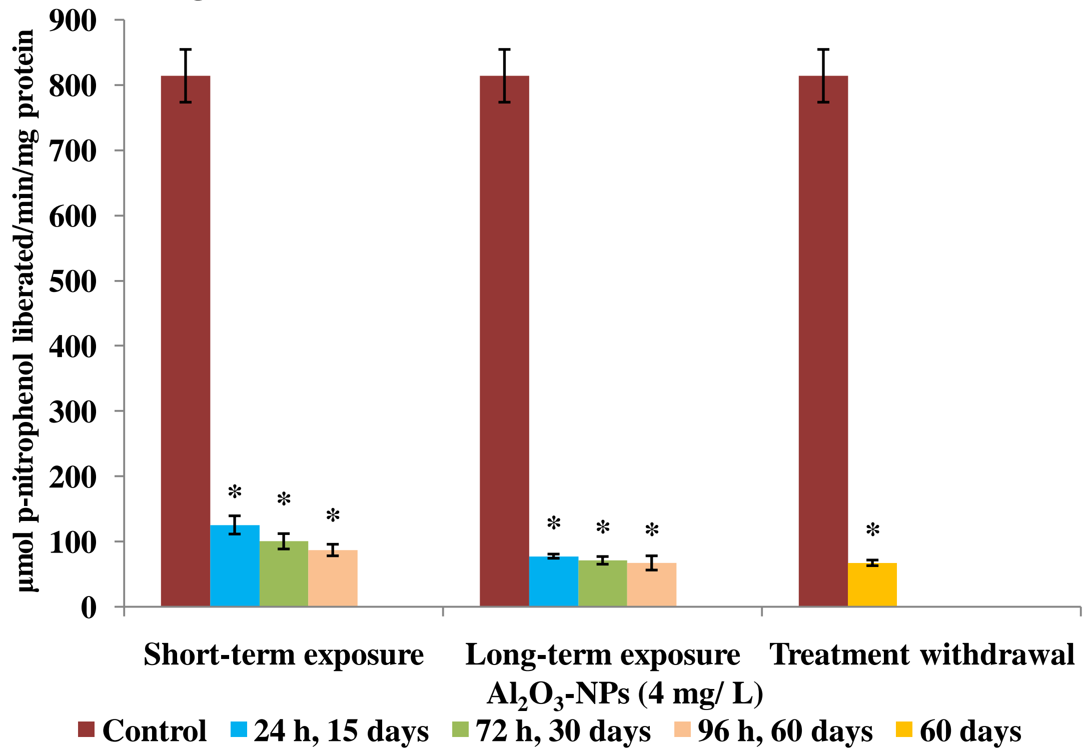



**Figure 13b** **Effect of $Al_2O_3$-NPs on the activity of alkaline phosphatase in the liver of the fish, *Oreochromis mossambicus* (Mean ± SD; * P<0.05)**

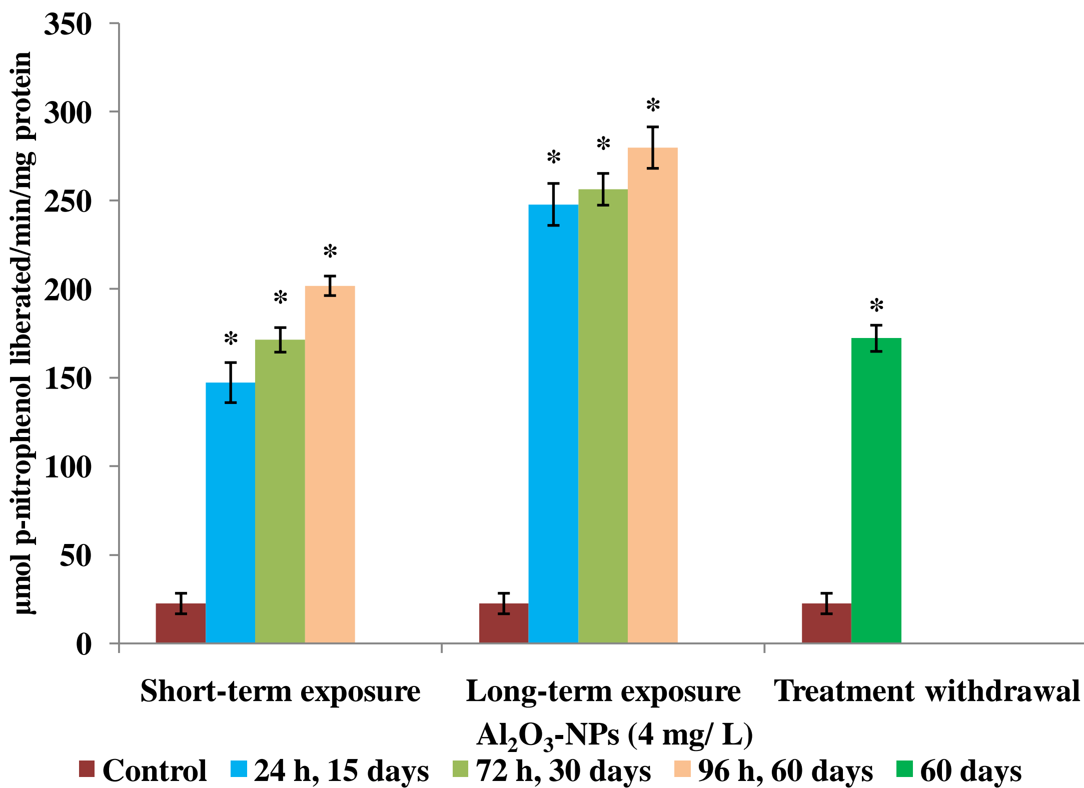


**Figure 13c** Effect of $Al_2O_3$-NPs on the activity of acetylcholinesterase in the brain of the fish, *Oreochromis mossambicus* (Mean ± SD; * P<0.05)

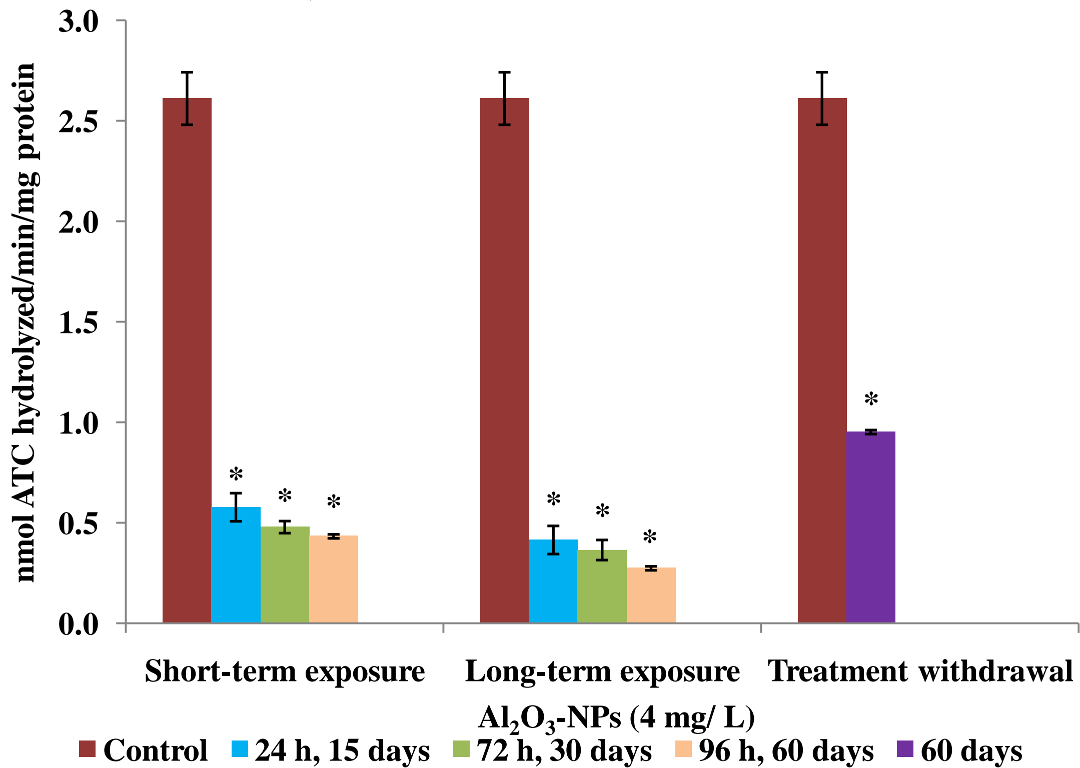



**Figure 14a** Effect of $Fe_3O_4$-NPs on the activity of alkaline phosphatase in the gill of the fish, *Oreochromis mossambicus* (Mean ± SD; * P<0.05)

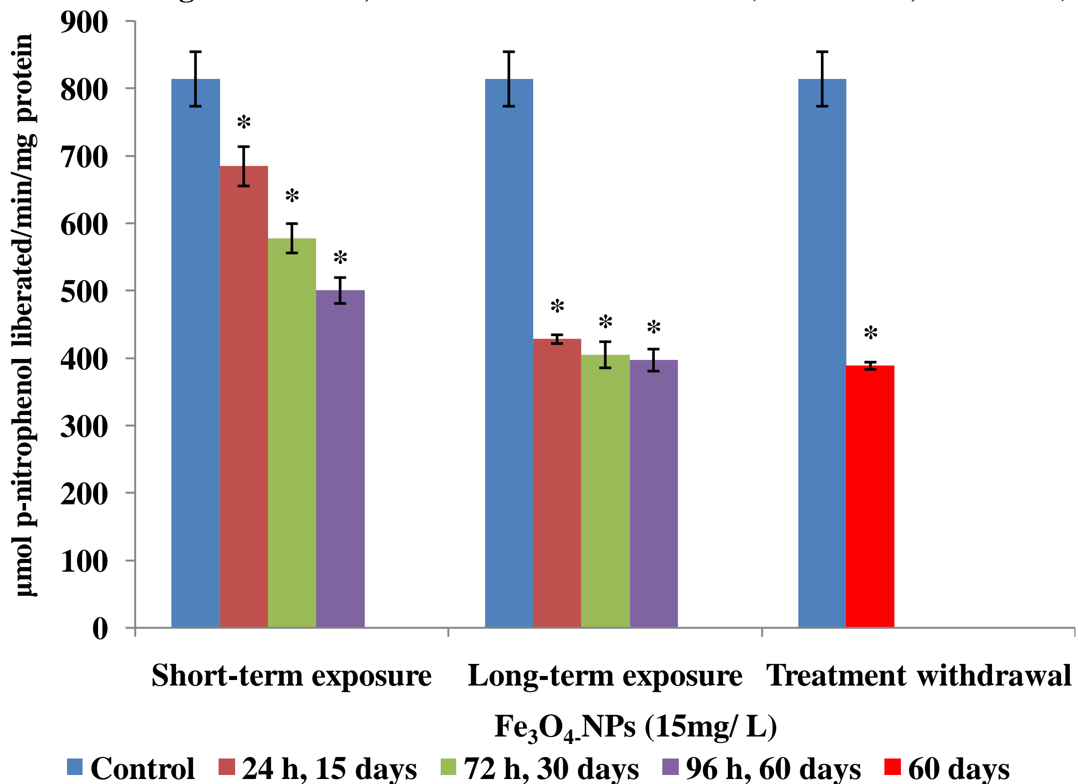


**Figure 14b** Effect of $Fe_3O_4$-NPs on the activity of alkaline phosphatase in the liver of the fish, *Oreochromis mossambicus* (Mean ± SD; * P<0.05)

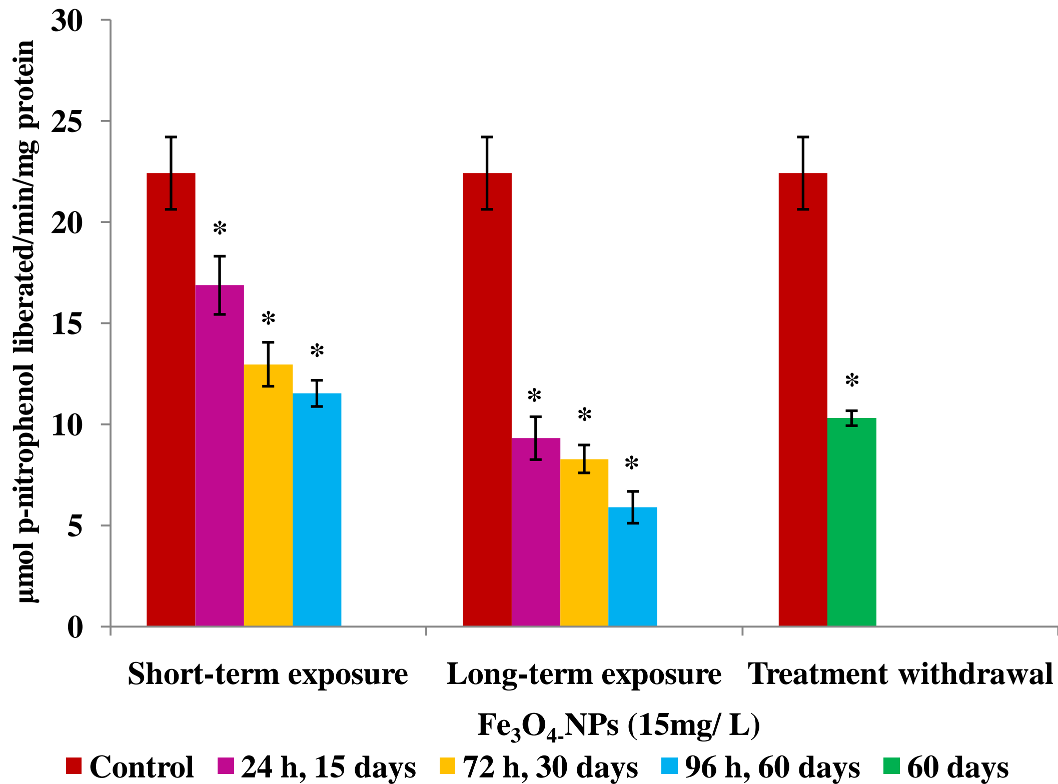



**Figure 14c** Effect of $Fe_3O_4$-NPs on the activity of acetylcholinesterase in the brain of the fish, *Oreochromis mossambicus* (Mean ± SD; * P<0.05)

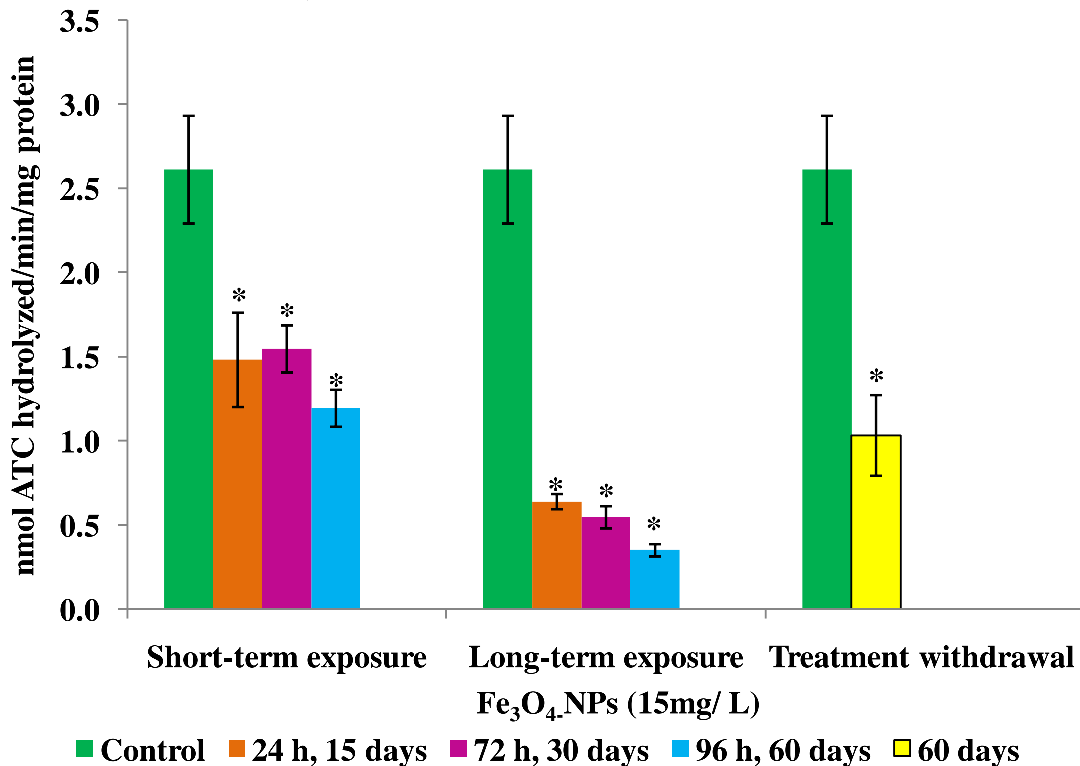


**Figure 15a** Effect of $SiO_2$-NPs on the activity of alkaline phosphatase in the gill of the fish, *Oreochromis mossambicus* (Mean ± SD; * P<0.05)

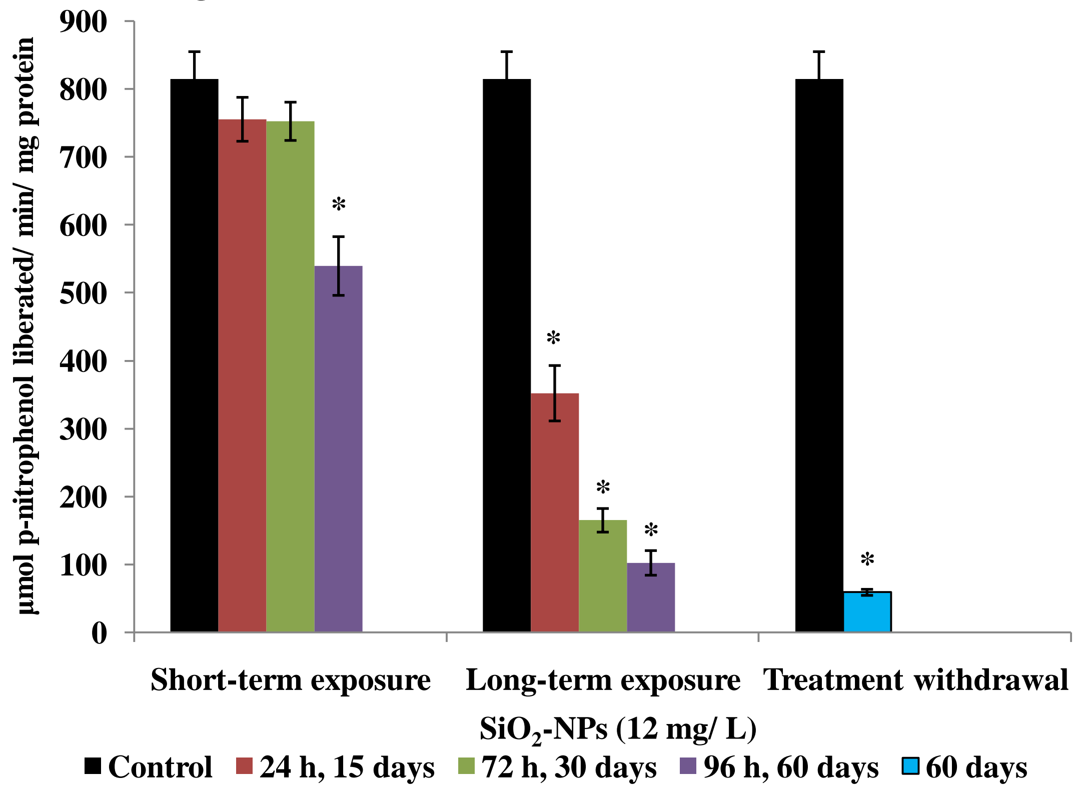



**Figure 15b** Effect of $SiO_2$-NPs on the activity of alkaline phosphatase in the liver of the fish, *Oreochromis mossambicus* (Mean ± SD; * P<0.05)

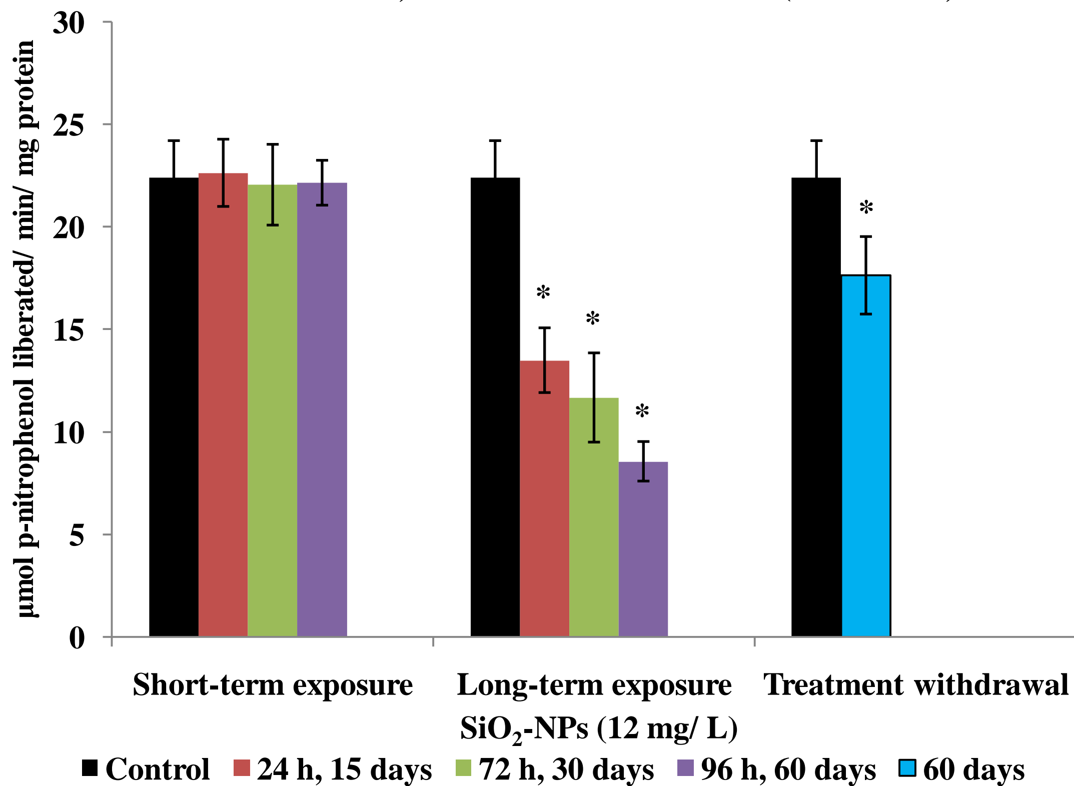


**Figure 15c** Effect of $SiO_2$-NPs on the activity of acetylcholinesterase in the brain of the fish, *Oreochromis mossambicus* (Mean ± SD; * P<0.05)

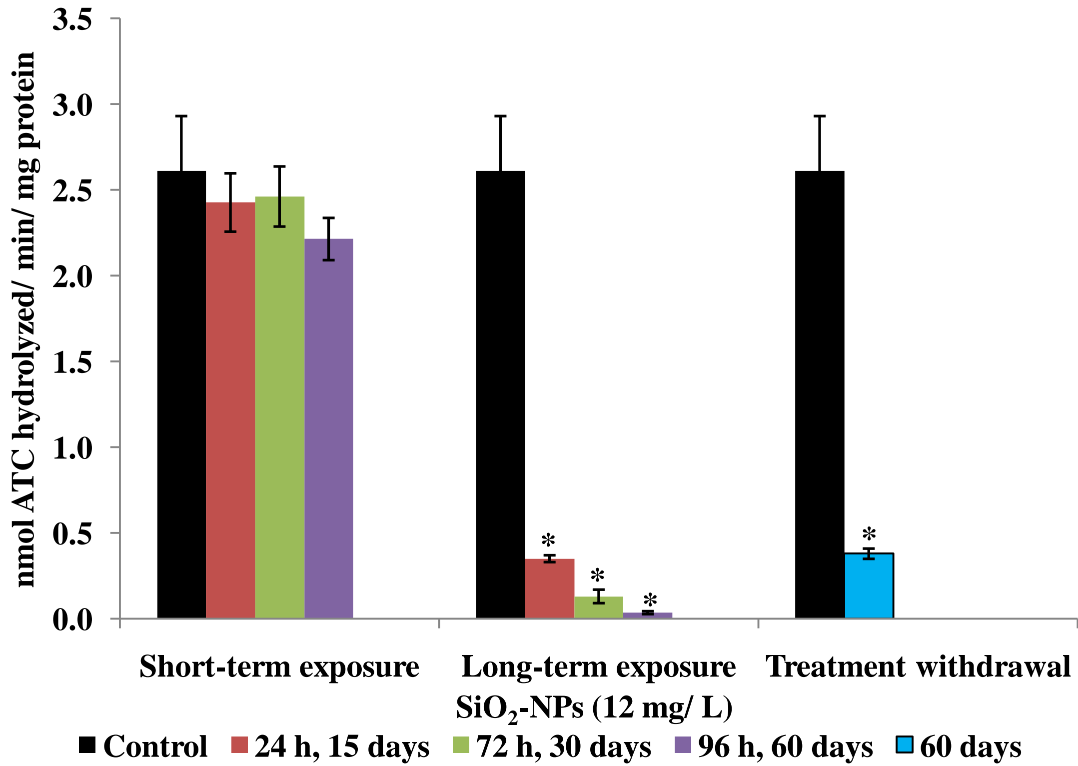



**Figure 16a** Effect of $TiO_2$-NPs on the activity of alkaline phosphatase in the gill of the fish, *Oreochromis mossambicus* (Mean ± SD; * P<0.05)

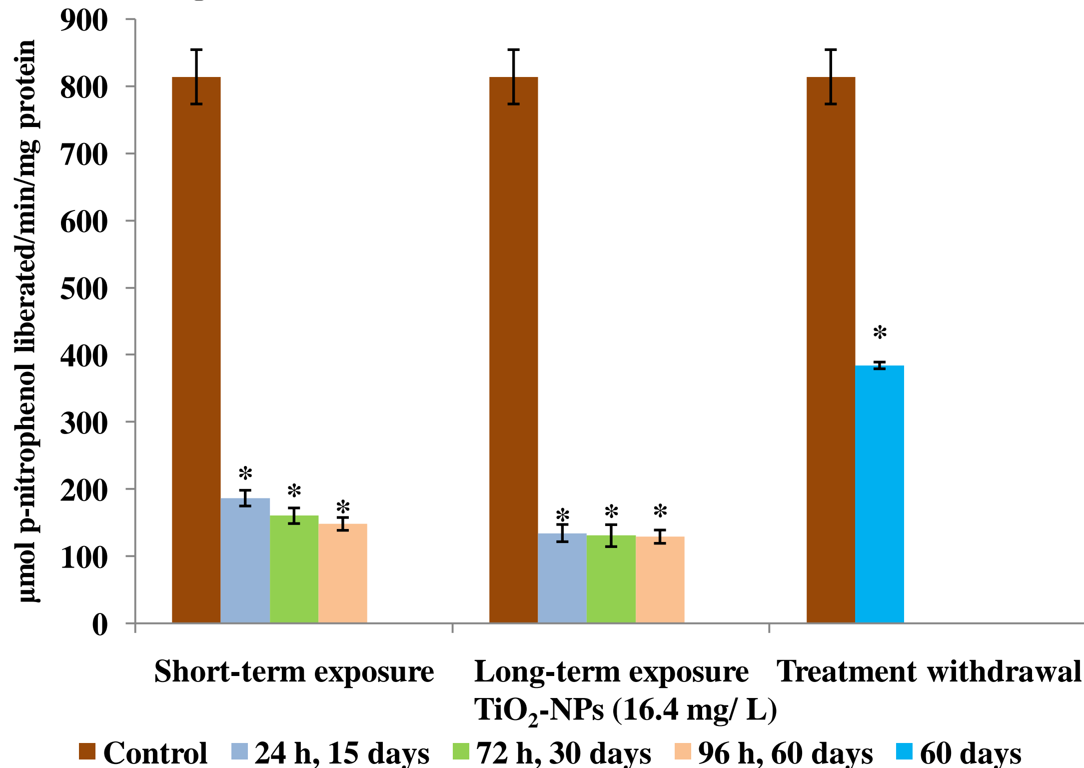


**Figure 16b** Effect of $TiO_2$-NPs on the activity of alkaline phosphatase in the liver of the fish, *Oreochromis mossambicus* (Mean ± SD; * P<0.05)

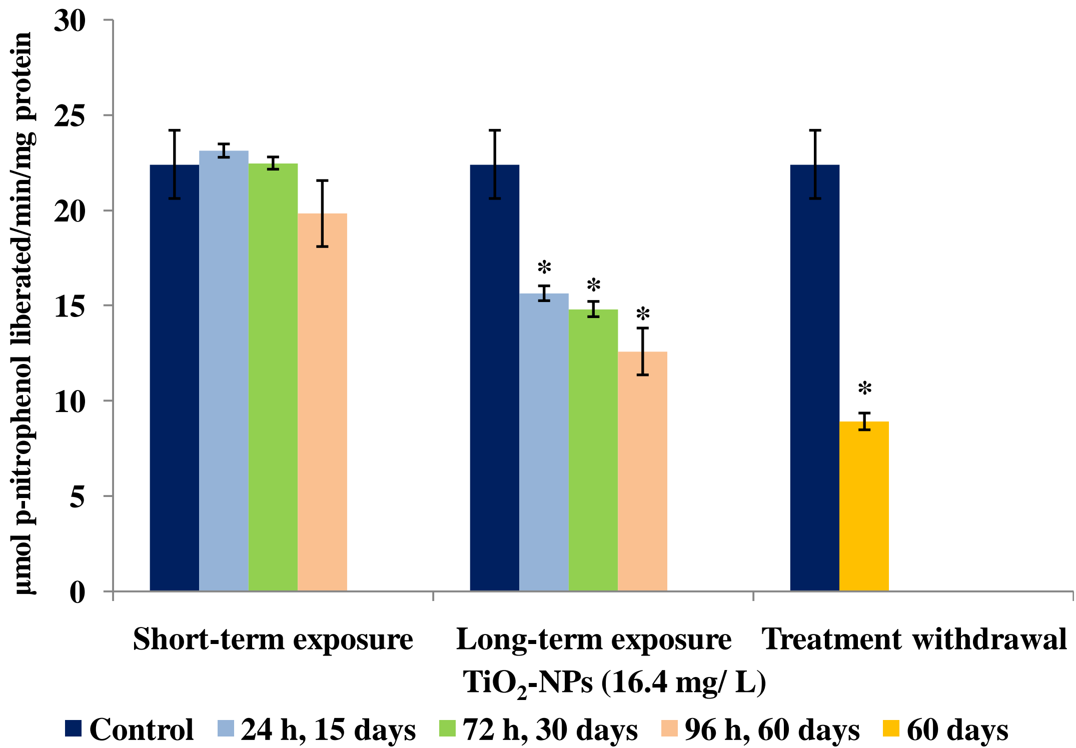
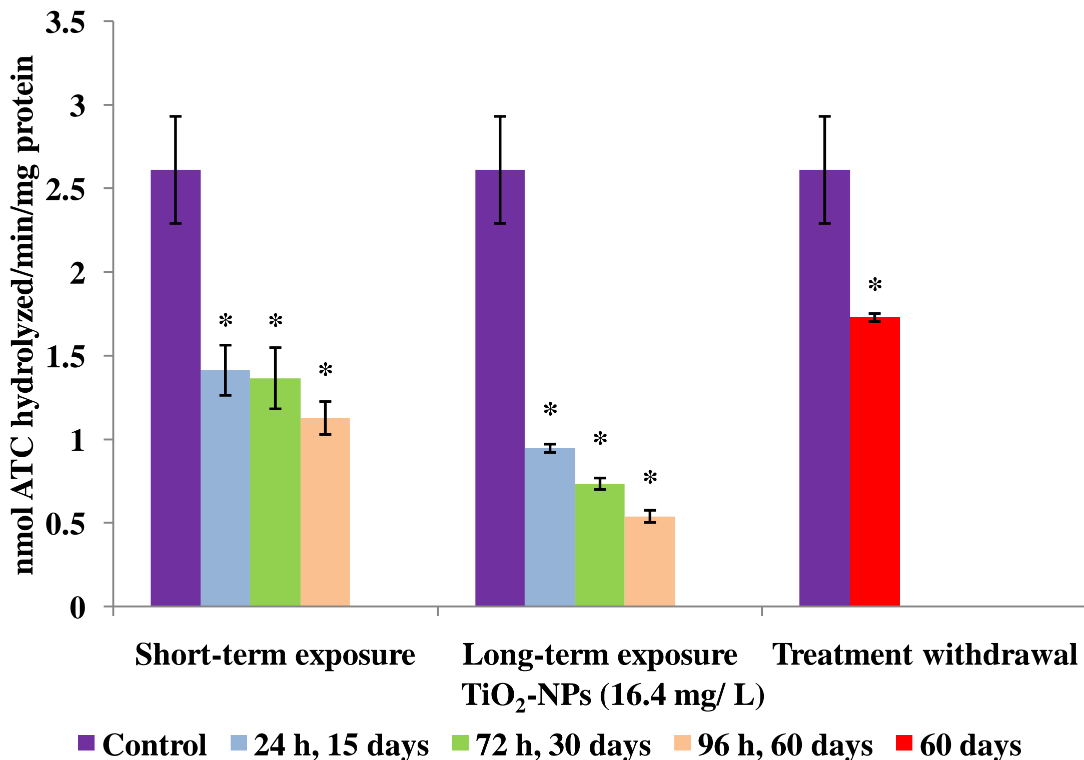



**Figure 16c** Effect of $TiO_2$-NPs on the activity of acetylcholinesterase in the brain of the fish, *Oreochromis mossambicus* (Mean ± SD; * P<0.05)



# Discussion

## 5.1 Effects of selected nanoparticles on the body weights, organ weights and hepatosomatic index in the fish, *Oreochromis mossambicus*

Fish are commonly used sentinel species for biomonitoring contamination in aquatic environment. Fish are very sensitive to the surrounding contaminants, thus reflects the health status of entire aquatic ecosystem. Fish also try to survive in extreme adverse conditions by modifying the behaviour, morphology, biochemistry, reproduction and even the gene expression. Nano-scaled materials that are produced globally over metric tonnes every year may create serious threat to the exposed animals and its environment. Although nanoparticles have several useful applications, the improper handling and disposal allow direct or indirect entry into the body of organisms (Oberdoster *et al.*, 2005). The response of each tissue towards nanoparticles and its mechanism of action to exert toxicity in various animals differ, which has been illustrated in ample of scientific literatures as explored in review section of this chapter. In almost all cases, the nature of nanotoxicity was known to exert through the generation of reactive oxygen species and induction of oxidative stress (Ahmed *et al.*, 2010). However, the toxicity data of nanoparticles remain controversial and imprecise as most of the studies were conducted in cell lines and embryo of the fishes. The present study was evaluated in order to assess the antioxidant status of gill, liver and brain tissues exposed to the selected nanoparticles in the freshwater fish, *Oreochromis mossambicus*.

In toxicological studies, the body weight of animal was measured routinely as the end product, as it provides direct evidence of toxicity of the compound. The alteration in body weight also occurs due to other conditions like diseases, age, and stress, hormonal or nutritional status. Therefore, the use of control animals along with the treatment groups helps to minimize the influence of other factors thereby provide direct evidence of systemic toxicity of the compound. In the present study, fish body weights remained unchanged after sublethal exposure to the selected nanoparticles namely $Al_2O_3$-NPs, $Fe_3O_4$-NPs and $TiO_2$-NPs throughout the exposure period of 60 days. However, after 30 days of $SiO_2$-NPs exposure showed reduction in the body weight of the fish and this could be due to the influence of nanoparticles exposure (Tables 1-4).

Tissue weights are considered as the most sensitive indicator to evaluate the effect of any toxic compound between the control and experimental groups as the change in tissue weights may also occur irrespective of any morphological changes like reduction or increase in the body weight of animal. Exposure to $Al_2O_3$-NPs and $TiO_2$-NPs increased the weight of gill tissue after 96 h and showed time-dependent increase till the end of 60 days, and also in the treatment withdrawal group (Table 1 and 4). The increase in weight of gill could be correlated with mucous deposition over the surface of gill lamellae in order to escape from the direct contact of nanoparticles. Gill tissue of $Fe_3O_4$-NPs exposed fish also increased after 30 and 60 days, as well as in the treatment withdrawal group, and this might be due to excessive mucous secretion on the gill surface as observed in $Al_2O_3$-NPs and $TiO_2$-NPs exposure groups (Table 2). On contrary, the gill tissue of $SiO_2$-NPs exposed group showed significant decrease after 30 and 60 days (Table 3), and this could be due to treatment related necrosis or tissue damage as evidenced in histopathological modifications, which will be discussed in detail in chapter 3.

Brain weight of $Al_2O_3$-NPs increased after 96 h, 15 days and 30 days of treatment, and decreased thereafter without decrease in the treatment withdrawal group (Table 1). The results were found associated to the histological changes as severe edema in the cerebral tissue after the initial exposure of nanoparticles, followed by necrotic and neurodegenerated brain tissue after 60 days of nano-aluminium oxide treatment. However, the weight of brain showed significant reduction in all durations after long-term exposure of $SiO_2$-NPs without any prominent changes in $Fe_3O_4$-NPs and $TiO_2$-NPs exposed groups (Tables 2-4). The changes observed in the weight of tissue coincide with histological alteration and will be discussed briefly in chapter 3.

Comparison of tissue weights in the treatment groups sometimes complicate the results as there occur some variations in the body weight of animal in each group. In order to overcome the uncertainty, the present study also chosen the parameter of liver weight expressed relative to the body weight of animal, which is referred as hepatosomatic index. The results stands alone to hold other observations such as gill and brain weights measured in the present study. Hepatosomatic index

(HSI) is the ratio of liver weight to body weight generally described as relative tissue weight. A significant reduction in HSI was observed after long-term exposure of all nanoparticles exposure except $TiO_2$-NPs and the results coincides with decrease in the brain tissue weights of respective nanoparticles treatment (Tables 1-4). Similar observations of decrease in the relative tissue weights have been reported after silver nanoparticles exposure for eight weeks in the fish, rainbow trout (Monfared and Soltani, 2013).

## 5.2 Effects of selected nanoparticles on the antioxidant status in gill tissue of the fish, *Oreochromis mossambicus*

In order to face the inevitable consequences of oxygen toxicity such as generation of reactive oxygen species (ROS) inside the body, fish are equipped with well-defined and functional prooxidant-antioxidant defense system. ROS are the intermediate reactive metabolites formed inside the body of aerobic organisms, and the commonly found ROS are superoxide radical, hydrogen peroxide and the hydroxyl radical (Martinez-Alvarez *et al.*, 2005). A range of enzymatic and non-enzymatic antioxidant compounds are included in the defense system of fish. Superoxide dismutase, catalase, glutathione reductase, and glutathione peroxidase are the key enzymes involved in scavenging oxygen free radicals formed inside the cells (Rudneva, 1997). Superoxide radical accumulated in the cells or tissues are instantly removed by superoxide dismutase enzyme whereas catalase and glutathione related enzymes catalyzes the conversion of more toxic hydrogen peroxide radical into water and molecular oxygen. In fish, several exogenous and endogenous factors are known to create imbalance in the formation of free radicals, and failure of scavenging the generated free radicals leads to oxidative stress (Sikka, 2001). Oxidative stress is one of the mechanisms through which the cells or tissues undergo oxidative damage thereby responsible for stress-mediated tissue injuries (Rahal *et al.*, 2014). Oxidative stress can be measured by analysing the activities of antioxidant enzymes and by the determination of levels of hydrogen peroxide generation and lipid peroxidation. In the present study the antioxidant status of gill

tissue was evaluated after exposure to the selected nanoparticles in the fish, *Oreochromis mossambicus*.

Sublethal exposure to $Al_2O_3$-NPs at 4 mg/ L, $Fe_3O_4$-NPs at 15 mg/ L, $SiO_2$-NPs at 12 mg/ L and $TiO_2$-NPs at 16.4 mg/ L concentrations showed alteration in the antioxidant defense system of gill tissue (Figures 1a-4f). Fish gill is considered as the first organ exposed to the contaminants, providing route for the entry of nanoparticles into the body as it forms the link between the internal environment of the organism and the external medium. Besides, gill tissue also involved as the primary centre for gaseous exchange, ion exchange, osmoregulation and excretion in fish (Evans, 1987). In $Al_2O_3$-NPs exposed group, after 24 h there was a significant increase in the activity of superoxide dismutase and this could be defensive mechanism of gill tissue to scavenge the free radical, but the activity was not successful. It was proved by the elevated levels of hydrogen peroxide generation and lipid peroxidation in gill tissues (Figures 1a-f). In another study, $Al_2O_3$-NPs either in single, or in combination with zinc oxide nanoparticles has been shown to increase lipid peroxidation in gill and liver tissues of the freshwater fish, *Carrasius auratus* (Benavides *et al*., 2016).

$Fe_3O_4$-NPs exposed to the fish showed significant decrease in the activities of superoxide dismutase, catalase, glutathione reductase and glutathione peroxidase in both short-term and long-term exposure groups (Figures 2a-d). This could be due to the excessive production of ROS as a result of nanoparticles exposure. The levels of hydrogen peroxide generation and lipid peroxidation were increased after prolonged exposure (Figures 2e and 2f), thereby indicated the induction of oxidative stress in the gill tissues. When the exposure of nanoparticles was withdrawn for 60 days the alterations persisted showing the permanent toxicity of the nanoparticles.

Gill tissue of $SiO_2$-NPs exposed groups showed significant reduction in the activities of superoxide dismutase, catalase, glutathione reductase and glutathione peroxidase in all treatment groups with concomitant increase in the levels of hydrogen peroxide generation and lipid peroxidation (Figures 3a-f) thereby proved imbalance of pro-oxidant and antioxidant defense system. When $SiO_2$-NPs exposure was withdrawn for the period of 60 days did not reversed the effect to normal

(Figures 3a-f) thus proved that the nanotoxicity of silica-nanoparticles are irreversible. Alteration in gill antioxidant system also indicates nanoparticles could alter osmoregulation, ionic balance, circulatory and respiratory systems (Vidya *et al.*, 2016). Exposure to $TiO_2$-NPs showed time-dependant significant decrease in the activities of glutathione reductase and glutathione peroxidase enzymes, but superoxide dismutase and catalase enzyme activities decreased only after 60 days of exposure (Figures 4a-f). The result designates that the antioxidant system of gill initially acquired scavenging efficiency to remove the oxygen radicals formed, but prolonged exposure to nanoparticles could have imbalanced the antioxidant defense system.

Lipid peroxidation is the process by which oxidants such as free radicals or non-radical species attack lipids, especially the polyunsaturated fatty acids thereby causing membrane damage (Lushchak, 2011). Thus lipid peroxidation is used as an important biomarker of tissue damage in toxicology. Malondialdehyde, the primary product of lipid peroxidation, increased in gill tissue after all nanoparticles exposure. The continuous oxidation of fatty acid side chains produce aldehydes, which eventually lead to loss of membrane integrity and change in the permeability of gill tissues (Negre-Salvayre *et al.*, 2008). In one of the studies, silver nanoparticles exposure has been shown to induce oxidative stress as evidenced by the increased level of lipid peroxidation and its associated gill tissue damages in the fish, *Labeo rohita* (Rajkumar *et al.*, 2016). Similarly, one of the nanoparticles fullerene $C_{60}$ has been shown to induce oxidative damages in gill tissue by the alteration of antioxidant enzymes and induction of lipid peroxidation in the fish, *Etroplus maculatus* (Sumi and Chitra, 2016).

## 5.3 Effects of selected nanoparticles on the antioxidant status in liver tissue of the fish, *Oreochromis mossambicus*

Antioxidant defense system of liver tissue showed dissimilar performance to eradicate the free radicals formed after the selected nanoparticles exposure. Liver tissues are mainly involved in metabolism, biotransformation, detoxification and elimination of toxicants so as to protect the animal from drastic effects, and for the

better survival of organism in the polluted environment. Thus liver is the major organ more vulnerable to toxic responses, and highly susceptible to endogenous oxidative damage along with other cell organelles as mitochondria, peroxisomes and endoplasmic reticulum (Wu *et al*., 2014). $Al_2O_3$-NPs exposure did not alter the activities of antioxidant enzymes in the liver tissues after short-term exposure, and the effects were prominent only after long-term exposure of nanoparticles (Figures 5a-d). The present observations clearly demonstrated that liver tissue could have actively involved in detoxification process to eliminate the toxic nanoparticles, simultaneously antioxidant defense system could have participated in the elimination of free radicals formed during the short-term exposure of $Al_2O_3$-NPs. But due to the continuous exposure of nanoparticles for 60 days could have upset the pro-oxidant and antioxidant balance in the liver tissue, which was evidenced by the decrease in antioxidant enzymes and concomitant increase in the hydrogen peroxide generation and lipid peroxidation. The results showed agreement with another observation where exposure to fullerene ($C_{60}$) nanoparticles has been shown to alter the antioxidant status in liver tissues of the fish, *Pseudetroplus maculatus* (Sumi and Chitra, 2017a). The treatment withdrawal group showed irreversible alteration in the antioxidant defense system thereby proved persistent toxic effects of $Al_2O_3$-NPs in the liver of fish, *Oreochromis mossambicus*.

The effects of $Fe_3O_4$-NPs was more prominent as the exposure decreased the activities of antioxidant enzymes, and increased the levels of hydrogen peroxide generation and lipid peroxidation in all experimental groups in time-dependent manner (Figures 6a-f). The results clearly indicated the toxicity of nanoparticles since the liver tissue failed to scavenge the free radicals formed and resulted in oxidative stress. The reversal of treatment for 60 days without $Fe_3O_4$-NPs exposure did not restored the activities of antioxidant enzymes, which suggests the toxicity of nano-iron particles remained stable and permanent in the liver tissue of *Oreochromis mossambicus*.

Exposure of $SiO_2$-NPs increased the activities of superoxide dismutase and catalase immediately after the treatment with decrease in the activities of glutathione reductase and glutathione peroxidase enzymes (Figures 7a-d). This could be the first

line of defensive mechanism of liver tissue to scavenge the reactive oxygen species formed because superoxide dismutase and catalase equally involved in the removal of superoxide radical and hydrogen peroxide. But the failure of glutathione related enzymes to scavenge hydrogen peroxide into water and oxygen resulted in the elevated level of hydrogen peroxide in the liver tissue (Figure 7e). Similarly, due to the consequence of nanoparticles exposure, peroxidation of membrane lipids occurred as evidenced by increase in the level of lipid peroxidation (Figure 7f). The present results showed an agreement with results on exposure to silicon dioxide nanoparticles for seven days in the zebrafish (Ramesh *et al.*, 2013). Decrease in the activities of all antioxidant enzymes and induction of lipid peroxidation noticed in the treatment withdrawal group reflects irreversible damage to the liver antioxidant system in the fish, *Oreochromis mossambicus*.

$TiO_2$-NPs treated liver showed significant alteration in the activities of antioxidant enzymes only in the long-term exposure groups (Figures 8a-d). Similarly increase in the levels of hydrogen peroxide generation and lipid peroxidation occurred only after prolonged exposure of nanoparticles (Figures 8e and 8f). Nanoparticles exposure was withdrawn for 60 days after long-term exposure in toxicant-free water and the results of antioxidant enzymes were found similar to the treatment groups. Thus the present observations suggested the failure of antioxidant defense system thereby leading to stable and persistent nanotoxic effects in the liver tissue of *Oreochromis mossambicus*. To brief, all the exposed nanoparticles generated reactive oxygen species in the liver tissue, though several attempts were made to scavenge the oxygen radicals resulted ineffective, leading to irreversible oxidative damage.

## 5.4 Effects of nanoparticles on the antioxidant status in brain tissue of the fish, *Oreochromis mossambicus*

Brain is the master of all organs in an organism known by its structural and functional complexity. It has been reported that blood-brain barrier, an effective system, protect the brain from the entry of molecules. However, nanoparticles are known to cross the blood-brain barrier and get accumulated in the brain tissue (Kim

*et al*., 2006). Highly rich polyunsaturated fatty acids present in the brain tissue make the tissue more vulnerable to oxidative damage. The antioxidant defense system of brain is well equipped with several enzymatic and non-enzymatic antioxidants to eradicate the reactive metabolites. In the present study, exposure to the selected nanoparticles resulted in the alteration of antioxidant enzymes activities with tremendous rise in the generation of hydrogen peroxide resulting in the induction of lipid peroxidation in the brain tissue of the fish, *Oreochromis mossambicus*.

The exposure to the nanoparticles such as $Al_2O_3$-NPs, $Fe_3O_4$-NPs and $TiO_2$-NPs showed significant reduction in the activities of antioxidant enzymes namely superoxide dismutase, catalase, glutathione reductase and glutathione peroxidase in both short-term and long-term exposure groups (Figures 9a-d; 10a-d; 12a-d). The present observations coincided while zinc oxide nanoparticles were exposed in *Oreochromis niloticus* and *Tilapia zilli*, thereby induced oxidative stress in the brain tissue (Saddick *et al*., 2015). However, on exposure to $SiO_2$-NPs, all antioxidant enzymes except catalase decreased in all treatment groups (Figures 11a-d). The contradictory increase in the activity of catalase enzyme could be due to the efficiency of enzyme to eradicate hydrogen peroxide radical formed, however, due to the fall in the activities of other antioxidant enzymes failed to remove hydrogen peroxide from the brain tissues. Similar results like increased activity of catalase with reduction in the activities of all other antioxidant enzymes has been reported in mussels after exposure to lead (Almedia *et al*., 2004) and zinc oxide nanoparticles in the snail, *Lymnaea luteola* (Ali *et al*., 2012).

After the exposure of all selected nanoparticles, the levels of hydrogen peroxide generation and lipid peroxidation were found increased in the brain tissues (Figures 9e and 9f; 10e and 10f; 11e and 11f; 12e and 12f). The results indicated that brain tissue experienced severe oxidative stress due to nanoparticles exposure. Oxidative stress developed by the nanoparticles in the brain tissue of the fish *Oreochromis mossambicus* was found persistent and irreversible even after the treatment withdrawal for 60 days (Figures 9-12). Oxidative stress in brain has been considered as one of the major reasons for the development of several neurodegenerative diseases and the role of nanoparticles as neurotoxicant is

undoubtedly proven in several studies (Li *et al.*, 2014; Afifi *et al.*, 2016; Sheng *et al.*, 2016). The bulk material of aluminium has been already proved as neurotoxicant responsible for several neurodegenerative diseases such as progression of Alzheimer's disease in human and rats (Campbell and Bondy, 2000; Fattoretti *et al.*, 2003). Since nano-sized particles are more toxic than the large-sized particles, and its ability to cross blood-brain barrier could have prompted the nanoparticles to behave as neurotoxicant.

The present findings concluded that brain tissue was equally targeted like gill and liver tissues by the attack of free radicals generated by nanoparticles. Though all the three tissues responded differentially according to the efficiency of their defensive mechanism, prolonged exposure of nanoparticles imbalanced pro-oxidant and antioxidant balance thereby resulted in oxidative stress in gill, liver and brain tissues irrespective of the physicochemical distinction among the nanoparticles.

## 5.5 Effects of nanoparticles on the tissue-specific marker enzymes in the fish, *Oreochromis mossambicus*

Tissue-specific marker enzymes are widely used in toxicology to detect organ specific effects of toxicants as they are confined to specific cells or tissues or organs. The magnitude of response of the marker enzymes to the toxicants often represents the severity of tissue damage. Alkaline phosphatase is the lysosomal enzyme that regulates physical properties of membranes or in absorption of lipids. It is also involved in the hydrolysis of exogenous materials, transphosphorylation and membrane transport (Dyhrman and Palanik, 1999). Hence the activity of alkaline phosphatase is taken as a marker for stress under diseases and toxicant exposure in various tissues like liver, gill, muscle etc. Therefore, any change in the activity of alkaline phosphatase can affect the metabolism of fish.

In the present study, after exposure to the selected nanoparticles namely $Al_2O_3$-NPs, $Fe_3O_4$-NPs, $SiO_2$-NPs and $TiO_2$-NPs, the activity of alkaline phosphatase was measured in gill and liver tissues and the activity of acetylcholinesterase was evaluated in brain tissue. Exposure of all selected nanoparticles showed decreased activity of alkaline phosphatase enzymes in the gill

tissue in time-dependant manner (Figures 13a, 14a, 15a and 16a). This could be due to the inhibition of enzyme by nanoparticles or damage in gill tissue thereby indicated impaired membrane transport and cellular toxicity. Similar observations have been observed in the gill tissue of *Etroplus maculatus* exposed to fullerene $C_{60}$ nanoparticles (Sumi and Chitra, 2016). The results of treatment reversal groups of respective nanoparticles exposure were found similar to that of the treatment groups (Figures 13a, 14a, 15a and 16a) thereby proving irreversible damage of gill tissue after nanoparticles exposure.

In the liver tissue of *Oreochromis mossambicus,* exposure to $Fe_3O_4$-NPs, $SiO_2$-NPs and $TiO_2$-NPs resulted in the reduction of alkaline phosphatase activity when compared with the respective control groups in time-dependent manner (Figures 14b, 15b and 16b). The inhibition of alkaline phosphatase often coincided with hepatic dysfunction, which ultimately leads to loss of membrane integrity, hydrolysis impairment and disruption in the transport of materials across the hepatic cell membrane (Simko, 1991). Present findings are in accordance with another study, in which the decreased activity of alkaline phosphatase has been observed in the liver of fish, *Pseudetroplus maculatus* on exposure to fullerene $C_{60}$ nanoparticles (Sumi and Chitra, 2017a). Exceptionally, treatment of $Al_2O_3$-NPs elevated the activity of alkaline phosphatase enzyme throughout the experimental period (Figure 13b). This could be due to the lysosomal mobilization and hepatic necrosis induced by nano-alumina as evidenced in histological analysis (discussed in chapter 3). One of the organophosphate pesticides, RPR-II has been shown to increase the activity of alkaline phosphatase in the liver of fish, *Oreochromis mossambicus* (Rao, 2006). Similar results have been observed in rat model, on chronic exposure to silica nanoparticles increased the activity of alkaline phosphatase and simultaneously decreased the activities of antioxidant enzymes (Parveen *et al*., 2012). Treatment withdrawal study conducted for 60 days without exposing to nanoparticles showed similar changes in the liver tissue marker enzymes like the treatment groups reflecting the liver damage was stable and persistent.

Acetylcholinesterase (AChE) is the key enzyme involved in the hydrolytic breakdown of the neurotransmitter acetylcholine, released from presynaptic nerve in

response to an action potential into acetate ion and choline (Quinn, 1987). Besides neurotransmission, AChE also performs several functions such as development of nervous system, synapse assembly, amyloid precursor protein biosynthesis, muscle development and so forth. The pivotal role of AChE as neurotransmitter in the nervous and muscular system has long been considered AChE as biomarker for neurotoxicity. Usually some toxicants like organophosphate pesticides and certain drugs used for the treatment of neurodegenerative diseases has been known to inhibit the activity of AChE, thereby leading to hyper stimulation of nicotinic and muscarinic receptors (Colovic *et al*., 2013). In the present study, irreversible inhibition in the activity of AChE was observed after exposure of all selected nanoparticles when compared to the control group (Figures 13c, 14c, 15c and 16c). The irreversibility of brain damage was confirmed after the treatment withdrawal for 60 days. Inhibition of acetylcholinesterase activity in the brain tissue has been associated with behavioural modifications in the fish *Etroplus maculatus* after sublethal exposure of chlordecone (Asifa and Chitra, 2015). Similarly, one of the nanoparticles, fullerene $C_{60}$ at sublethal concentration has shown to decrease the activity of AChE and also altered the behaviour of the fish *Pseudetroplus maculatus* (Sumi and Chitra, 2017b). The present findings interpret the neurotoxic effects of nanoparticles exposed even at sublethal concentration, and the damages caused were found irrecoverable.

The study, therefore, concluded that though there exist ambiguity regarding the use and safety of nanoparticles due to the inconclusive and contradictory endpoints observed from the available literatures, the present findings proved the fact that nanoparticles at sublethal concentration induced oxidative stress to vital tissues as gill, liver and brain. In addition, the observations clearly illustrated those nanoparticles irrespective of any unique physicochemical properties, exerted toxicity to the tissues explored, which was evident by serious tissue damages in the fish *Oreochromis mossambicus*. Moreover, all the response observed remained stable, static and irreversible, deriving to the conclusion that nanoparticles, if exposed chronically, to non-target organisms are harmful, and appropriate guidelines should be established for the safe handling and disposal of nanoparticles.

# Conclusions

1. Exposure to nanoparticles at one-tenth of $LC_{50}$ concentration ($Al_2O_3$ - 4 mg/ L, $SiO_2$ - 12 mg/ L and $TiO_2$ - 16.4 mg/ L. $Fe_3O_4$ - 15 mg/ L) did not caused remarkable changes in the body weight of the animal, and this reflects that nanoparticles did not induced treatment related anorexia throughout the experiment.

2. Weight of the tissues such as gill, brain, and hepatosomatic index showed noticeable changes only after prolonged exposure of nanoparticles.

3. All selected nanoparticles caused imbalance in the antioxidant status of the gill, liver and brain tissue after short-term and long-term of exposure, but the effects are more prominent in long-term exposed group thereby indicating the toxicity of nanoparticles is time-dependant.

4. The concomitant increase in the levels of hydrogen peroxide generation and lipid peroxidation in the gill, liver and brain tissues observed after nanoparticles exposure indicated the oxidative damages in vital tissues.

5. Toxicity of nanoparticles was further evidenced by significant decrease in the activities of specific tissue marker enzymes such as alkaline phosphatase in gill and liver, and acetylcholinesterase in the brain of fish.

6. The selected nanoparticles, equally contributed to the induction of oxidative stress, however, the effects are well-noted in gill and brain tissues when compared to liver, as the first line of defense, the hepatic tissue attempted to detoxify the nanoparticles by its detoxifying mechanism, but failed, during prolonged exposure.

7. All tissues were equally targeted by the nanoparticles; however, more profound effect was evident in gill tissue followed by liver and brain.

8. The treatment withdrawal for a period of 60 days showed that the nanoparticles induced oxidative stress was irreversible and permanent in gill, liver and brain tissues.

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VIDYA BALAKRISHNAN P.V. “EFFECTS OF SELECTED NANOPARTICLES ON THE FRESHWATER FISH, OREOCHROMIS MOSSAMBICUS “. THESIS. DEPARTMENT OF ZOOLOGY, UNIVERSITY OF CALICUT, 2018.

# CHAPTER 3

## Histopathological changes in the fish, *Oreochromis mossambicus* exposed to the selected nanoparticles

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| µg | Microgram |
| A | Aneurism |
| $Al_2O_3$-NPs | Aluminium oxide nanoparticles |
| ATP | Adenosine triphosphate |
| D | Degeneration |
| DNA | Deoxyribonucleic acid |
| E | Edema |
| E | Enucleated cells |
| $Fe_3O_4$-NPs | Iron oxide nanoparticles |
| H | Hyperplasia |
| h | Hour |
| L | Litre |
| M | Mucous deposition |
| mg | Milligram |
| $SiO_2$-NPs | Silicon dioxide nanoparticles |
| $TiO_2$-NPs | Titanium dioxide nanoparticles |
| V | Vacuolization |

# General Introduction

## 1.1 Fish histopathology – a bioassessment tool

Histology is the Latin word in which ‘histo’ means tissue and ‘logos’ means study thus histology is the scientific study of fine details on various tissues and are viewed under the microscope. Histopathology is another term analogous to histology, which means the microscopic studies of the manifestation in the tissue morphology caused by contaminants or diseases in organisms (Bernet *et al.*, 1999). Number of anthropogenic contaminants discharged into the aquatic ecosystem could cause toxicity in the dwelling organisms, especially to fishes (Sindermann, 1979; Bukola *et al.*, 2015). Fish behave as primary, secondary and tertiary level of consumer of pollutants and it promotes bioaccumulation as well as biomagnifications of the contaminants in different trophic levels. Therefore, fish are chosen widely as an effecter organism as well as ecological tool to monitor toxicity in the aquatic environment.

The endpoints of toxicity vary according to the nature and duration of exposure, composition and properties of contaminant, age, species and resistance of organisms exposed. In an aquatic ecosystem, scrutinizing the morphological endpoints in the form of histological analysis provides details on the alteration in structure and function of tissues or organs exposed to toxicants even at sublethal concentrations, and thus appears more relevant than any lethality measures (Wester and Canton, 1991). Therefore, histopathological assessment of fish is considered as one of the effective methods to assess adverse biological and ecological effects caused by the toxicants (Bernet *et al.*, 1999; Reddy and Kusum, 2013).

Histopathological studies are critical and sensitive in toxicology, as it gives the direct evidence on the impact of toxins on morphology and biochemistry of cells or tissues. Hence, histopathology is used widely as a biomarker for various biomonitoring programmes intended to assess the effects of contaminants in aquatic organisms (Johnson *et al.*, 1993). The mechanism of toxicity may be different for different contaminants, such as generation of reactive intermediates and oxidative stress, biochemical alterations, DNA and cellular damages etc, and all of which could end up in structural alterations of tissues. However, prolonged alterations in

the morphology also lead to the loss of function of the tissue and affect the survival of the organisms (Myers *et al.*, 1994).

Histopathology permits for the assessment of organs or tissues from fish of any size, age or type, so that allows for diagnoses of gross changes as well as functions as biomarker for the mechanism of toxicity (Marchand *et al.*, 2009). Histology is a sensitive tool to assess both short-term and long-term effects of the toxicants and remains as an important reliable bio-indicator to environmental stressors (Hinton, 1995). According to the reaction patterns and alterations, histopathological lesions are mainly classified under five categories, namely circulatory disturbances, regressive changes, progressive changes, inflammatory responses and development of tumours (Sindermann, 1979). Circulatory disturbance is the pathological condition that occurs as a result of altered flow of blood or tissue fluid. Haemorrhages, aneurysm, edema are some of the examples that come under the category of circulatory disturbances.

Regressive changes are the reaction patterns that are responsible for the loss of function of an organ or the total loss of organ itself. It includes irreversible alterations such as atrophy, necrosis, degenerations, malfunctions, structural deformities and so on (Takashima and Hibiya, 1995). In contrast, progressive changes are the reaction patterns that are responsible for the increased activity and secretions in the cell type or tissue, which includes hypertrophy and hyperplasia. Inflammatory responses are the normal pattern of reaction against any foreign molecule, usually found associated with other reaction patterns, and consist mainly of exudates and infiltration (Bernet *et al.*, 1999). Tumours are formed as a result of uncontrolled proliferation of cells or tissues, and the reaction pattern includes benign-type where the differentiated cells lack the property of metastasis, and malignant-type in which the poorly differentiated cells undergo metastasis (Bernet *et al.*, 1999).

Based on the intensity of alteration and severity of damages, histological lesions are classified as minimal, moderate and marked pathological lesions. In minimal pathological lesions, the damages are reversible when the exposure of toxicants is withdrawn. In moderate pathological lesions, reversible effects can be

seen on neutralizing the stressor with some other agents. However, in marked pathological lesions, the damages are usually irreversible that could be due to partial or complete loss of organ function (Genten *et al*., 2009). In the aquatic ecosystem, histopathological lesions in fish are studied by selecting suitable target organs, which determines the biological effects of the toxicants. Many toxicants like pesticides, metals, dyes, biphenyls, plasticizers, nanomaterials and so on are known to alter the normal architecture of various tissues in fishes (Murray *et al*., 2003). The major tissues widely used for histopathological analysis of fish include liver, gill, spleen, muscle, intestine, kidney and brain tissues. The present study mainly focused on the effects of selected nanoparticles at sublethal concentration on the histological aspects of gill, liver and brain tissues in the freshwater fish, *Oreochromis mossambicus*.

### 1.2 Morphology of fish gill

Gill is an important primary target tissue where the toxicants attack, as it is directly exposed to water, and any alterations in gill morphology could affect the respiratory status and even the life of fish. Gill of fish is not a simple tissue, but considered as a whole organ provided with different cell types, supplied by various blood vessels and systems, and are under nervous and hormonal control (Evans *et al*., 2005). Gill is composed of various structures that include gill epithelium, gill arches, primary lamellae and secondary lamellae. Epithelium of fish gill is very thin with large surface area and highly rich gill capillaries so as to have high level of gaseous exchange across the water. The highly rich blood capillaries in gill epithelium allows for the exchange of oxygen and carbon dioxide, but also results in the vulnerability to toxicant exposure, pathogen invasion and irritation. Gill surface forms more than half of the entire body surface area where the epithelial layer alone forms few microns that separate the interior of fish to the external environment (Cengiz and Unlu, 2002). As there is close association between water and gill tissue, makes gill most susceptible organ to the toxicants exposed in their immediate environment. Gill is the system that brings blood haemoglobin in close contact with water for the exchange of oxygen and carbon dioxide. Besides respiration, gill also

plays major role in osmoregulation, ionic balance, and excretion of nitrogenous waste products, primarily ammonia (Hoar and Randell, 1984).

Fish gill is bilaterally arranged on either sides of pharynx consisting of arch like structures called gill arches in which the gill filament or primary lamellae are attached (Wilson and Laurent, 2002). In different groups of fish, the respiratory network arrangements and structure of supporting tissues show considerable variations (Laurent and Dunel, 1980). Teleost fishes usually have five pairs of gill arches, among which four pairs in the front are active in respiration and other functional responses of the gill. The four pairs of arches situated in the front are joined to each other at the base by a gill septum. The fifth pair of gill arches generally transforms into the pharangeal bone and does not play a role in respiratory functions (Wilson and Laurent, 2002).

The supporting structure for gill is the branchial arch, in which the primary lamella is arranged posterior-laterally. Primary lamellae are provided with salt-secreting chloride cells at the centre that are found numerous at the basal part of the lamellae, provided with blood vessels on both sides. Perpendicular to the primary lamellae, secondary lamellae or respiratory lamellae are arranged, which are highly vascularised and lined with two thin layers of epithelium separated by a space through which blood flows. The space found between two adjacent secondary lamellae is known as inter lamellar region (Wilson and Laurent, 2002). Apart from the epithelial cells and chloride cells, primary and secondary lamella also provided with mucous cells, pillar cells, non-differentiated cells, neuroepithelial cells and pavement cells (Wilson and Laurent, 2002).

Chloride cells or the mitochondrial rich cells (MRC's) are the large epithelial cells located largely at the proximal ends of lamellae and in the interlamellar regions, where they are irregularly spaced and functions as osmoregulatory, acid-base regulatory and ion regulatory unit (Perry, 1997). They are largely supplied with mitochondria to generate more ATP for driving the transporter proteins (Wilson and Laurent, 2002). Chloride cells are large sized that exist in huge number in the salinity adapted fishes rather than freshwater fishes to assist in the transport of sodium and chloride ions (Laurent and Dunel, 1980). Chloride cells also

participate in ionic transport and also possess possible role in detoxification process. Pavement cells or respiratory cells that cover more than 90% of total gill surface area consist of either columnar, cuboidal or squamous cells having low number of mitochondria, and usually found associated with the chloride cells which together forms the outermost lining of epithelium (Laurent and Dunel, 1980). The nucleus of columnar or cuboidal pavement cells is spherical shaped whereas the squamous pavement cells possess compressed type of nucleus (Wilson and Laurent, 2002). Mucous cells or goblet cells are the most common cell type present in almost all fish species, except hag fish (Mallat and Paulsen, 1986). They are large ovoid cells provided with flattened nucleus, where cytoplasm contains cellular machinery that includes endoplasmic reticulum, Golgi and mitochondria to produce mucin or mucous. Mucous cells are present throughout the epithelium and lamellae that secretes mucous for normal gill functioning, and excess mucous secretion in case of any stress or infection (Laurent, 1984).

Pillar cells are the modified endothelial cells unique to fish gills, which characterize the blood spaces within the lamellae (Wilson and Laurent, 2002). The non-differentiated or undifferentiated cells are the progenitor cells of epithelium that consists of both pavement cells and chloride cells. Undifferentiated cells contains high nuclear to cytoplasm ratio with large number of free ribosomes, and the mitosis of undifferentiated cells are known to occur common in filaments than in lamellar region (Chretien and Pisam, 1986; Laurent *et al.*, 1994). Neuroepithelial cells are the specialized cells found deep inside the filament epithelium and are largely presented with dense cored vesicles (Laurent, 1984). Neuroepithelial cells form subepithelial network of unmyelinated nerve fibers that function as oxygen sensor and also involved in the regulation of blood flow (Sudin and Nilsson, 2002). The different cell types makes gills a complete and complicated organ to perform various vital functions like respiration, regulation of ionic, acid-base and osmotic balances, excretion of waste and also provide defense against exogenous materials and invading microbes. Exposure to environmental toxicants causes injury to gill tissue and the level of damages depends on the concentration and duration of exposure. Thus fish gill is considered as the most suitable histopathological target tissue used

as promising biomarker for the assessment of toxic effects of environmental stressors, and also to assess health status of the entire ecosystem.

### 1.3 Morphology of fish liver

Liver is the largest gland found outside the alimentary canal and functions in production of bile, assimilation of nutrients, detoxification of several endogenous and exogenous toxicants, maintenance of carbohydrate, protein, fat and vitamin metabolism (Brusle and Anadon, 1996; Vicentini *et al.*, 2005). Fish liver also synthesizes large plasma proteins including albumin, fibrinogen and complement factors (Genten *et al.*, 2009). The structure of fish liver differ from the mammalian liver as there are only two lobules, and the basic units of liver tissue are the hepatocyte-sinusoid centre and the portal triad. The parenchyma of fish liver is contained within a thin capsule of fibrous connective tissue. Hepatocytes are polyhedral cells arranged as chords between the two adjacent sinusoids that supply blood to the cells (Hinton and Lauren, 1990). The lumen of sinusoids is known to contain macrophages and erythrocytes whereas the large cells that rest on the luminal surface of sinusoidal epithelium are called Kupffer cells and they are relatively small and few in number. Normal hepatocytes possess a central spherical nucleus and homogenous cytoplasm with a single nucleolous that occupy around the sinusoids (Vicentini *et al.*, 2005).

The hepatocytes consist of the basic cell organelles such as mitochondria, Golgi apparatus, endoplasmic reticulum, and ribosomes (El-Bab, 2006). The ultrafine structures of hepatic cells reveal that the chromatin is granular with more condensed heterochromatin located at the periphery of the nucleus. The cytoplasm of hepatocytes is irregularly filled with lipid droplets and vacuoles, nucleolus is more homogenous while the shape of mitochondria varies as round to elongate, and are usually found associated to the rough endoplasmic reticulum, which assembles as lamellar structure parallel to the nuclear membrane (Huang *et al.*, 2018).

The bile duct of fish liver usually located near the portal vein and is lined with cuboidal epithelium. Bile duct are formed by the joining of bile canaliculi, and carries bile from the liver, joins with the hepatic duct and deliver the bile to the gall

bladder for storage (Boyer, 2013). Bile canaliculi exist as either intracellular bile canaliculi or extracellular bile canaliculi. The intracellular bile canaliculi collect the bile secreted by the hepatic cells and gives to the extracellular bile canaliculi from where bile is carried out (El-Bab, 2006). According to the formation of bile duct and the associated blood vessels, the bile tract structure of fish liver is classified into four types, namely biliary-venous tract, biliary-arteriolar tract, portal tract and isolated type. Major functions of liver include intermediate metabolism, bile production, synthesis of several proteins and metabolites, storage of metabolites like glycogen and lipid, detoxification and drug metabolism (Takashima and Hibiya, 1995).

Exposure to environmental pollutants is known to impair the structure and functions of liver tissue. Most common histological lesions found in the fish liver includes hepatocyte hypertrophy, hyperplasia, edema, vacoular degeneration, atrophy, necrosis, fatty degeneration, bile stagnation, congestion, melanomacrophage accumulation and so on (Takashima and Hibiya, 1995). Thus in the present study, fish liver was selected as one of the target organs as it provides valuable information on the effects of selected nanoparticles in the physiological and biochemical functions as well as the tissue can be used as bio-indicator to study the health status of the fish.

## 1.4 Morphology of fish brain

Brain of fish is structurally similar to higher vertebrates except in complexity. Morphology of brain shows slight modifications among the different species of fish based on their adaptations, behaviour and habitat. In teleost fish, brain accounts for almost 0.3% of total body weight and is divided into three main parts namely forebrain, midbrain and hindbrain (Lisney and Collin, 2006). Forebrain is formed of two regions namely the anterior most, telencephalon composed of the major brain centre, the cerebrum, and diencephalon, olfactory lobes and many interconnected neurons. The cerebrum consists of two cerebral hemispheres which are covered by non-membranous sheath called pallium and the two hemispheres are connected together by the nerve bundles called anterior commissure (Speare and

Frasca, 2006). Olfactory lobes are projected anteriorly into the bulbous structures and with nerve masses that helps to sense the smell. Diencephalon includes the major centre for coordination between the different regions of brain namely epithalamus, hypothalamus, thalamus and pineal body (Speare and Frasca, 2006). The dorsal wall of diencephalon is epithalamus, the lateral wall forms thalamus, ventrally a pouch like growth called infundibulum occur, and at the floor of diencephalon is hypothalamus. All the regions of diencephalon together functions as the relay centre, and also maintain homeostasis and regulate the endocrine system. Thus the forebrain functions as the centre for olfaction, colour vision, sexual characters, reproduction, feeding behaviour, memory and so on (Hibiya, 1982).

Midbrain or mesencephalon is the largest region in brain provided with optic lobes, the functional centre for vision (El-Bab, 2006). Hind brain is formed of two regions called metencephalon and myelencephalon. Metencephalon consists of pons and cerebellum that serves as the important centre for balancing, orientation, swimming, and muscular tone by receiving stimulus from lateral line so as to maintain equilibrium, and help in swimming. Myelencephalon is composed of medulla oblongata, which continues caudally as spinal cord and functions as reticulomotor system and centre for involuntary actions. Myelencephalon also contains two lobes namely facial lobe and vagal lobe, in which the former stimulates the cutaneous chemoreceptor and the later stimulates the chemoreceptors from skin of head, gills and the oral cavity (El-Bab, 2006).

Brain tissue is considered as the most protected tissue against the toxicants as it is well served with effective barriers to prevent the entry of chemicals. However, some of the nanotoxicants pave way into the brain through the blood-brain barrier owing to its nano-size. Thus evaluation of brain histology serves as an important parameter to assess the fish health status after exposure to the selected nanoparticles. The present study determines the toxic effects of the nanoparticles on the histology of vital tissues namely gill, liver and brain thereby provides the reflection of the well-being of fish population. Multiple focussing on the impact of chronic exposure of selected nanoparticles at sublethal concentration against the acute exposure period also provides various toxicological end-points of the toxicants on the vital tissues.

# Review of Literature

Histopathology has been considered as structural science and serves to compliment the knowledge gained from the anatomy, physiology and pathology. The advantage of using histopathology as the marker for environmental pollution is it provides unbiased results in a rapid manner (Bernet *et al.*, 1999). Histopathology can also be used in natural as well as laboratory studies, where fish is one of the most sensitive organisms, usually exposed to several range of contaminants. Thus histology represents useful tool for assessing the degree of pollution in the aquatic ecosystem. One of the pollution studies conducted in Egyptian canal has revealed histological changes in gill, liver, kidney and gonadal tissues of the fishes *Oreochromis niloticus*, *Tilapia zillii* and, *Synodoniis schall.* The notable changes includes proliferative, degenerative and inflammatory changes in all the tissues, which showed variations in season and the test species, in which winter was most affected and *Oreochromis niloticus* as the most susceptible species (Mohamed, 2003). Similarly, remarkable histological changes has been observed in liver and kidney of the fish *Channa punctatus*, captured from two different sites of Tung Dhab drain in Amritsar, India (Kaur and Dua, 2015). Effects of water borne pollutants analysed in Tamis river, Serbia on eight different fish species has reported gill as the most susceptible tissue exposed to pollutants that showed alteration in gill morphology of all fish species examined (Lujic *et al.*, 2013). Thus exposures to pollutants are likely to induce several histological lesions in different tissues of the fish (Sindermann, 1979; Mallat, 1985; Hinton and Lauren, 1990).

Nanoparticles, engineered or natural, are also known to induce morphological alterations in various tissues and test species. In terrestrial animals especially in rat and mice models, histopathological changes induced by several nanoparticles have been extensively studied. In one of the studies, chronic injection of silica particles for four weeks resulted in histological alterations of major organs such as the liver, spleen, lung, kidney, brain and heart of mice (Nishimori *et al.*, 2009). Hepatic microgranulation and splenic megakaryocyte accumulation were the prominent damages reported in the study while kidney, lung, brain and heart remained unaffected thereby confirmed liver and spleen tissues as the major target organs for nanotoxicity in mice (Nishimori *et al.*, 2009). In another study, gold

nanoparticles when administered to rats has been shown to alter hepatocytes, portal triads and the sinusoids and the major lesions includes hydropic degeneration, cloudy swelling, fatty degeneration, portal and lobular infiltrate by chronic inflammatory cells and congestive dilated central veins (Abdelhalim and Jarrar, 2012). Similarly, nickel nanoparticles has been shown to induce liver damages in mice with significant lesions such as hyperemia and expansions of central veins, secretions of inflammatory cells including neutrophils and lymphocytes and increased bile ductules, swollen Kupffer cells and dispersed apoptosis (Ajdari and Ghahnavieh, 2014). Several degenerative changes have been reported in spleen, liver and lung tissues of mice on administration of nickel nanoparticles (Ajdari and Ghahnavieh, 2014).

Histopathological damages in aquatic organisms are also more prominent after nanoparticles exposure. In a study using semi-static test system, titanium dioxide nanoparticles was exposed to rainbow trout at 0.1, 0.5 and 1.0 mg/ L concentrations for 14 days reported that nanoparticles caused lesions such as edema and thickening of the lamellae in gill tissue, while liver tissues has been observed with minor fatty change, lipidosis and condensed nuclear or apoptotic bodies (Federici *et al.*, 2007). In a comparative study of three different nanoparticles tested namely nanocopper, nanosilver and nano-titanium dioxide in zebrafish has reported accumulation of nanoparticles in the gill followed by morphological and transcriptional responses, and the toxicity of nanocopper was more prominent than the other nanoparticles exposed (Griffitt *et al.*, 2009).

Exposure of titanium dioxide nanoparticles exhibited histopathological changes such as edema in gill lamellae along with accumulation of nanoparticles in gill, liver, heart and brain tissues thereby represented translocation among organs, and penetration through blood-brain and blood-heart barrier after long-term exposure in zebrafish (Chen *et al.*, 2011). Hepatic damages like cloudy swelling of hepatocytes, congestion, vacuolar degeneration, karyolysis, karyohexis, dilation of sinusoids and nuclear hypertrophy have been reported in liver, and mild congestion of blood vessels, fusion of primary lamellae, marked hyperplasia of the branchial arch, desquamation, necrosis of epithelium, epithelial lifting, edema, lamellar fusion,

collapsed and curling of secondary lamellae have been reported in the gill tissue of the fish *Oreochromis mossambicus* treated with 50 mg/ L concentration of silver nanoparticles (Govindasamy and Rahuman, 2012). Copper nanoparticles exposed to fish caused organ injuries as indicated by hyperplasia, aneurisms, and necrosis in the secondary lamellae of the gills, swelling of goblet cells, necrosis in the mucosa layer and vacuole formation in the gut, injury to hepatocytes with pyknotic nuclei in the liver, damage to the renal epithelial tubules and increased Bowman's space in the kidney, mild changes in the nerve cell bodies of telencephalon, alteration in the thickness of the mesencephalon, and enlargement of blood vessel on the ventral surface of the cerebellum of brain along with degenerated skeletal muscle fibres in the rainbow trout, *Oncorhynchus mykiss* (Al-Bairuty *et al*., 2013).

Sublethal exposure of nickel nanoparticles have been shown to cause nuclear hypertrophy, degeneration, necrosis and irregular-shaped nuclei in liver tissue. Hyperplasia of the gill epithelium, lamellar fusion of secondary lamellae, dilated marginal channel, epithelial lifting and epithelial rupture has also been reported in gill tissue. While muscle tissue showed degeneration in muscle bundles, focal area of necrosis, vacuolization in muscle bundles, edema, and splitting of muscle fibers after the nanoparticles exposed for 14 days in the fish *Oreochromis mossambicus* (Jayaseelan *et al*., 2014). *Cyprinus carpio* exposed to zinc oxide nanoparticles has shown pathological lesions such as epithelial lifting, desquamation, necrosis, alteration in secondary structure, loss of secondary lamellae, acute cellular swelling, blood congestion, hyperplasia of epithelial cells, lamellar fusion, aneurism, lamellar disorganization and lamellar curling in the gill tissue (Subashkumar and Selvanayagam, 2014).

Sublethal exposure of silver nanoparticles has reported mild, moderate to severe histopathological lesions in gill tissues in the form of proliferation and inflammation, whereas intestines experienced necrosis and inflammation, and melanomacrophage aggregation has been observed in liver tissues of rainbow trout (Johari *et al*., 2015). Silver nanoparticles exposed at 3, 300 and 1000 mg/ L concentrations for eight weeks has been shown to induce hyperplasia of epithelium, aneurism and adhesion in the secondary lamellae of gill tissues, whereas kidney

showed lesions including hyaline cast formation, decrease in the glomerular diameter and formation of intra-cytoplasmic vacuoles in the common carp (Monfared *et al*., 2015). In a study of acute toxicity, histopathological alterations of copper nanoparticles has been assessed in three species of fish namely juvenile rainbow trout *Oncorhynchus mykiss*, fathead minnow *Pimephales promelas* and zebrafish *Danio rerio*. The major damages has been observed in gill tissue showing damages to gill filaments and gill pavement cells, where the intensity of damages has been shown to vary among the fish species studied (Song *et al*., 2015). Zinc oxide nanoparticles exposure has been shown to disrupt the normal tissue morphology showing damages to gill, muscle, intestine, brain and ovary of the fish *Oreochromis mossambicus* and the lesions explored was concentration-dependent (Suganthi *et al*., 2015).

*Labeo rohita* exposed to silver nanoparticles has been reported with proliferation of bronchial chloride cells, lamellar fusion, aneurism and severe haemorrhage in gill tissue, whereas liver showed congestive enlargement of liposomes, vacuolar degenerations, and necrosis while muscle tissue was observed with severe vacuolization and inflammations in muscle fibres (Rajkumar *et al*., 2016). Exposure to lead, carbon nanotubes and mixture of lead and carbon nanotubes for four days has been observed with alterations in gill tissues including hyperplasia and displacement of epithelial cells, occurrence of aneurisms in the secondary lamella, which was more prominent in the mixture of lead and carbon nanotubes in the fish, *Oreochromis niloticus* (Barbieri *et al*., 2016).

One of the electron microscopic studies revealed that titanium nanoparticles caused mitochondrial degeneration with swelling and cristae loss in Sertoli cells, and spermatogonial cells. In addition, accumulation of autophagic vacuole and necrosis has also been observed in the cytoplasm of Sertoli cell in zebrafish, *Danio rerio* (Kotil *et al*., 2017). *Oreochromis mossambicus* exposed at varying concentrations of aluminium oxide nanoparticles for 96 h resulted in the accumulation of nanoparticles in the liver tissue and major hepatic anomalies like structural alterations in the portal vein, necrotic hepatocytes, vacuolization, aggregation of blood cells and melanomacrophages aggregation (Murali *et al*., 2017). Histological

alterations including hyperplasia, fusion and detachment of secondary lamellae, blood congestion in vascular axis of primary filaments, reduced secondary lamellae and cellular degeneration in the gill tissue, blood congestion in the central veins, cytoplasmic vacuolation, cellular degeneration and congestion in the blood sinusoids, and necrosis of the hepatocytes in the liver tissue, and glomerular shrinkage, severe degeneration in the tubules cells, increase in interstitial tissue and glomerulus, and macrophages aggregation in the kidney tissue have been observed in the fish, *Rutillus rutillus caspicus* treated with sublethal levels of copper nanoparticles (Aghamirkarimi *et al.*, 2017). Subchronic exposure to zinc nanoparticles has been shown to induce histopathological tubular deformations and mononuclear cell infiltrations in kidney tissues, aggregation of melanomacrophages and mononuclear cell infiltrations in liver tissues whereas hyperplasia, lamellar fusion has been observed in gill tissues of *Oreochromis niloticus* (Kaya *et al.*, 2017).

Amine-coated silver nanoparticles have been shown to induce histological alterations in gill tissues, which included fusion of secondary lamellae, separation of gill epithelium, fusion and necrosis of lamellar cells, hyperplasia, deformed cartilaginous skeleton, separation and lifting of epithelium, and curling of lamellae in a dose-dependent manner. In the liver tissue, silver nanoparticles induced abnormalities such as reduction in the size of hepatocytes and nuclei, and stimulated the production of necrotic and apoptotic bodies in 28 days treated *Labeo rohita* (Khan *et al.*, 2018). Copper oxide nanoparticles exposed from low to high concentrations, ranging from 0.5 to 1.5 mg/ L concentrations showed alterations in gill tissues as edema, curved tips, fusion of gill lamellae, and thickening of primary and secondary gill lamellae. At the highest concentration of nanoparticles, necrosis and apoptosis with condensed nuclear bodies and pyknotic nuclei has been observed in the liver tissues thereby confirming organ toxicity of nanoparticles (Shahzad *et al.*, 2018). Titanium dioxide nanoparticles exposed at 1, 5, 10, and 50 mg/ L concentrations showed hyperplasia, cellular hypertrophy, proliferation of mitochondria-rich cells, and lamellar stasis in gill tissues whereas histology of kidneys showed cellular and nuclear hypertrophy, focal tubule degeneration,

necrosis, and melanomacrophage proliferation in Neotropical freshwater fish, *Prochilodus lineatus* (do Carmo *et al*., 2018).

*Oreochromis mossambicus* when exposed to sublethal concentrations i.e., 120, 150 and 180 ppm, of aluminium oxide nanoparticles for 96 h showed histological abnormalities in the brain, gill, intestine, kidney and muscle tissues where the severity of damages and extensive architectural loss has been more pronounced at 180 ppm concentration (Murali *et al*., 2018). Silver nanoparticles when exposed at 1 mg/ kg through diet showed alteration in histoarchitecture of liver such as pyknotic nuclei, leucocyte infiltration, hemorrhage and karyokinesis, blood vessels with infiltration and lipid vacuoles in the liver tissue. Histology of gill displayed hyperplasia, aneurism, blood congestion, severe telengiectiasis, epithelial lifting, curling of secondary lamella, hyperplasia of epithelial cell of secondary lamella in the fish, *Channa striatus* (Kumar *et al*., 2018). Female guppies, *Poecilia reticulata*, exposed to citrate-functionalized iron oxide nanoparticles has been shown to increase the number, area, and perimeter of melanomacrophage center response, and the increase in the frequency of histopathological changes in time-dependent manner showed micro- and macro-vesicular steatosis, melanomacrophage aggregates, exudates, and hemorrhagic foci (Qualhato *et al*., 2018).

The available literatures reviewed on histopathological alterations induced by nanoparticles enlightened the fact that several morphological modifications have been induced due to the exposure of nano-sized particles. However, the reversal of histopathological effects of nanoparticles was lacking among the literature reviewed. Thus the present study attempted to evaluate the histological changes induced by the selected nanoparticles on the chief vital tissues such as gill, liver and brain of the fish, *Oreochromis mossambicus*. In addition, the study also evaluated the withdrawal effects of nanoparticles thereby provide additional information to fill the gap of knowledge that if organ toxicity induced by nanoparticles can be reversed or persevered, thereby affecting the fish population.

# Materials and Methods

### 3.1 Histopathological analysis

After the end of short-term (96 h) and long-term (60 days) exposure periods of nanoparticles, and from the treatment reversal (60 days) and the respective control groups, the tissues such as gill, liver and brain were collected by sacrificing the fish. The tissues were then fixed in 10% buffered formalin for 24 h, and dehydrated in ascending grades of alcohol and cleared in xylene until they became translucent. Tissues were then transferred to molten paraffin wax for 1 h to remove xylene completely, and then impregnated with wax and blocks were made. Blocks were then cut in a rotary microtome to prepare sections of thickness 4 to 6 microns. The sections were double stained with haematoxylin and eosin, and mounted in DPX mountant. The structural alterations of gill, liver and brain tissues were observed under light microscope and were compared with those of control tissues. Photomicrographs were taken using Canon shot camera fitted to the Carl Zeiss Axioscope 2 Plus Trinocular Research Microscope.

# Results

## 4.1 Effects of nanoparticles on the histology of gill tissues

### Effect of $Al_2O_3$-NPs

Gill of control tissue showed normal histoarchitecture with compact gill epithelium, gill arches, primary and secondary lamellae (Figure 1a). Exposure to $Al_2O_3$-NPs for 96 h resulted in hyperplasia of gill arches, upliftment of gill epithelium, hypertrophy and aneurism, lamellar curling and loss of secondary lamella (Figure 1b). Long-term exposure of nanoparticles for 60 days showed histological lesions as severe mucous deposition, vacuolization, hyperplasia, aneurism, and absence of secondary lamellae (Figure 1c). The severity of damages observed after the nanoparticles exposure was time-dependent. Reversal of treatment for 60 days in toxicant-free medium showed similar lesions as that of treatment groups such as severe mucous deposition, vacuolization, hyperplasia of gill arches, aneurism, blebbing of primary lamellae and absence of secondary lamellae (Figure 1d).

### Effect of $Fe_3O_4$-NPs

Gill tissue of control group showed normal architecture with distinct gill epithelium, gill arches, primary and secondary lamellae (Figure 2a). Exposure to $Fe_3O_4$-NPs for 96 h resulted in mucous deposition, vacuolization, aneurism, hyperplasia and absence of secondary lamellae (Figure 2b). $Fe_3O_4$-NPs treatment for 60 days showed blebbing of epithelium, aneurism, hyperplasia and absence of secondary lamellae (Figure 2c). The structural alterations observed in the treatment group persisted in the treatment withdrawal group also (Figure 2d).

### Effect of $SiO_2$-NPs

Control gill tissue was found normal without any abnormalities (Figure 3a). Exposure to $SiO_2$-NPs for 96 h showed excess mucous deposition, upliftment of gill epithelium, vacuolization of gill arches, aneurism, absence and curling of secondary lamellae (Figure 3b). Long-term exposure of $SiO_2$-NPs for 60 days showed severe damages such as total injury of gill filaments, mucous deposition, vacuolization, aneurism, loss of secondary lamella and loss of chloride cells when compared to the

control tissue (Figure 3c). Morphological alteration in the treatment withdrawal group did not recover to normal, and was found similar to the treatment groups (Figure 3d).

**Effect of $TiO_2$-NPs**

In control group, the gill tissue showed normal architecture having well-defined epithelium, gill arches, primary and secondary lamellae (Figure 4a). Exposure to $TiO_2$-NPs for 96 h resulted in epithelial upliftment, vacuolization, hyperplasia, aneurism and absence of secondary lamella (Figure 4b). Long-term exposure of $TiO_2$-NPs for 60 days resulted in severe structural lesions than observed during short-term exposure (Figure 4c). The gill tissue of the treatment reversal group also showed alteration in gill morphology like epithelial damages, vacuolization, hyperplasia, aneurism and absence of secondary lamella (Figure 4d).

**Figure 1** **Histomorphology of gill tissue exposed to $Al_2O_3$-NPs in *Oreochromis mossambicus*. 1a-Gill control; 1b: $Al_2O_3$NPs at 4 mg/ L exposed for 96 h showing aneurism (A), absence of secondary lamellae (→); 1c: $Al_2O_3$NPs at 4 mg/ L exposed for 60 days showing mucous deposition (M), vacuolization (V), hyperplasia (H); 1d: Treatment withdrawal showing mucous deposition (M), absence of secondary lamellae (→)**

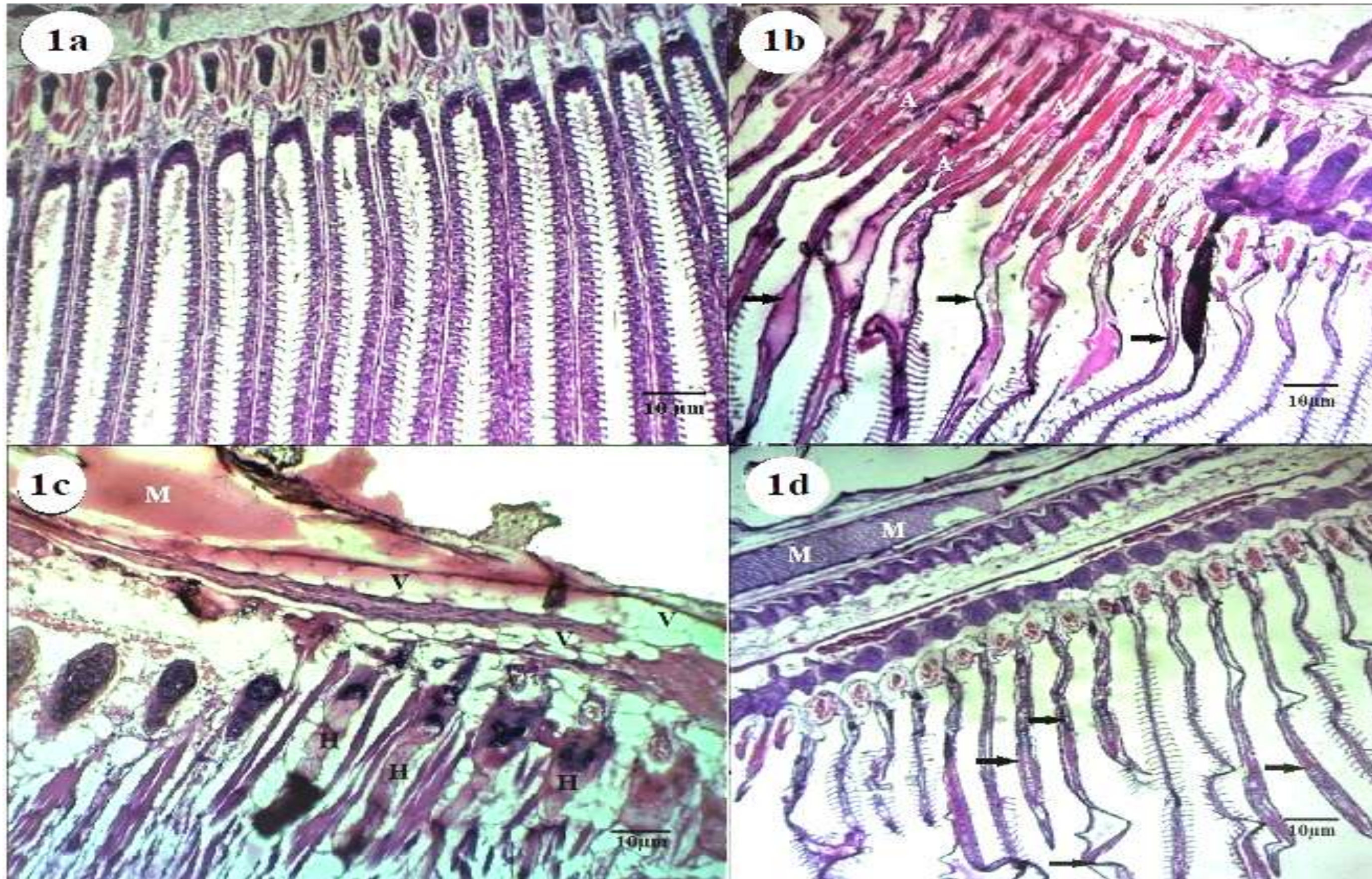


**Figure 2** **Histomorphology of gill tissue exposed to $Fe_3O_4$-NPs in *Oreochromis mossambicus*. 2a-Gill control; 2b: $Fe_3O_4$NPs at 15 mg/ L exposed for 96 h showing mucous deposition (M), vacuolization (V), hyperplasia (H), absence of secondary lamellae (→); 2c: $Fe_3O_4$NPs at 15 mg/ L exposed for 60 days showing blebbing, aneurism (A), hyperplasia (H), absence of secondary lamellae (→); 2d: Treatment withdrawal showing aneurism (A), hyperplasia (H), absence of secondary lamellae (→)**

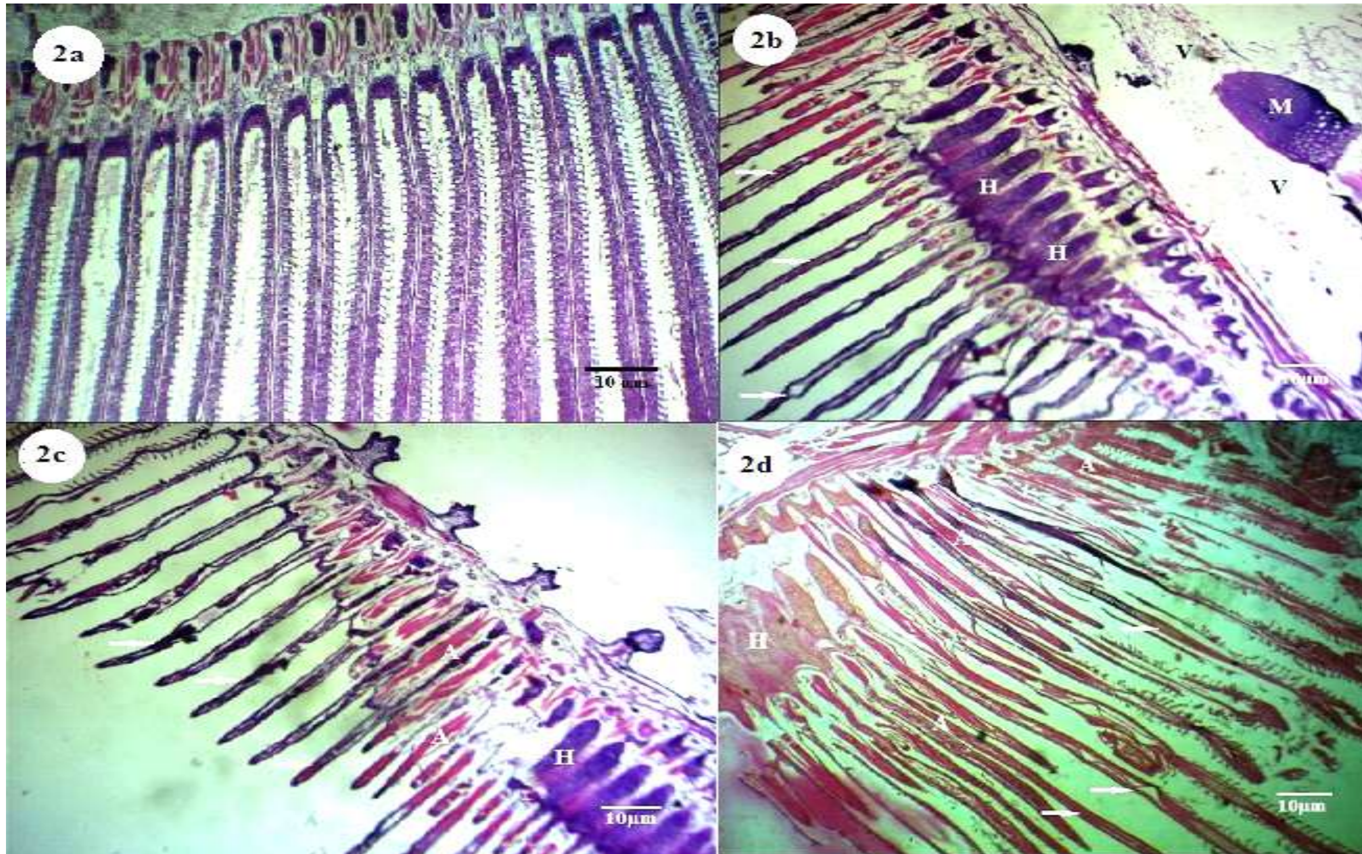


**Figure 3** **Histomorphology of gill tissue exposed to $SiO_2$-NPs in *Oreochromis mossambicus*. 3a-Gill Control; 3b: $SiO_2$NPs at 12 mg/ L exposed for 96 h showing aneurism (A), absence of secondary lamellae (→), curling of secondary lamellae (*); 3c: $SiO_2$NPs at 12 mg/ L exposed for 60 days showing mucous deposition (M), vacuolization (V), absence of secondary lamellae (→); 3d: Treatment withdrawal showing mucous deposition (M), vacuolization (V), absence of secondary lamellae (→)**

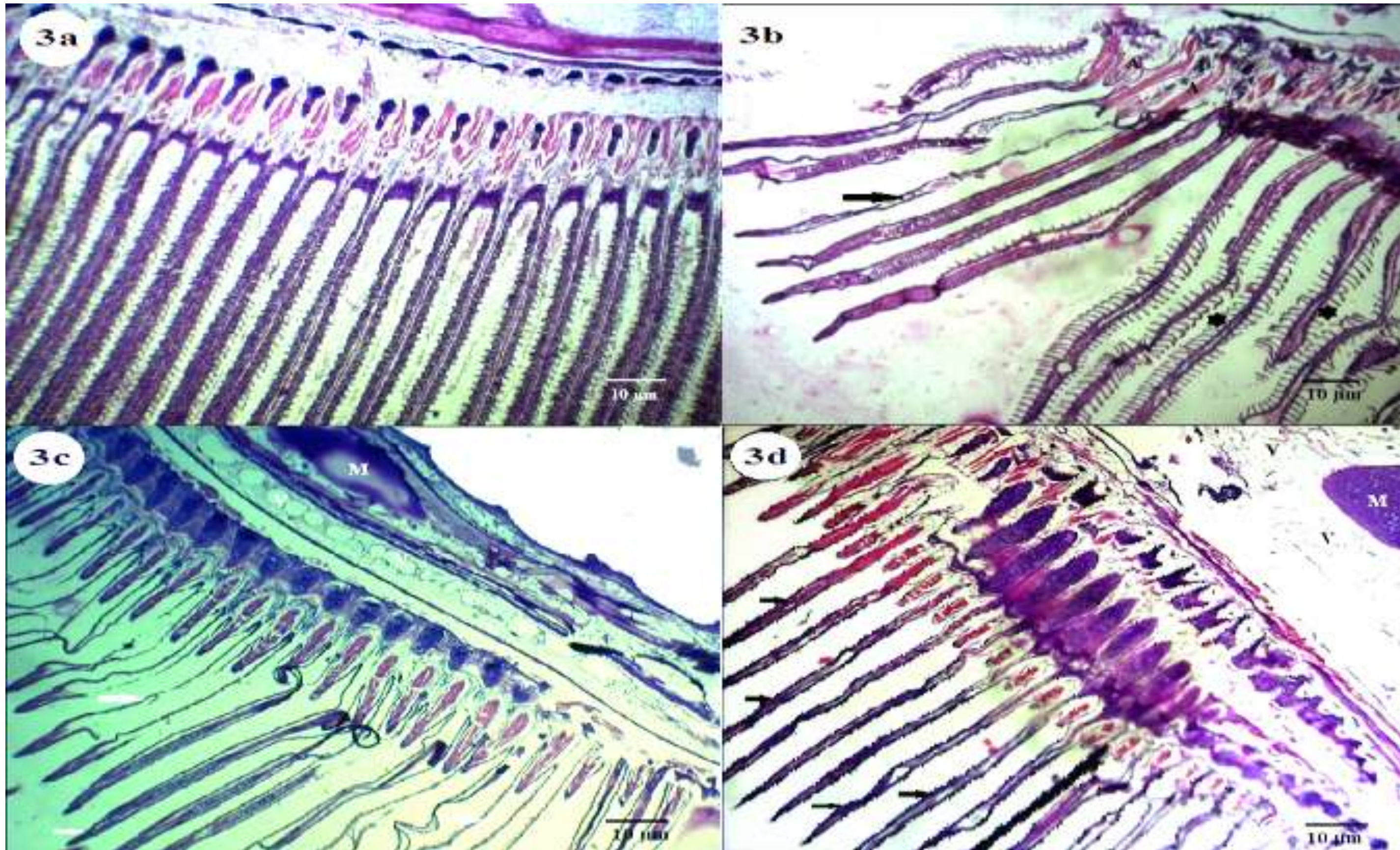
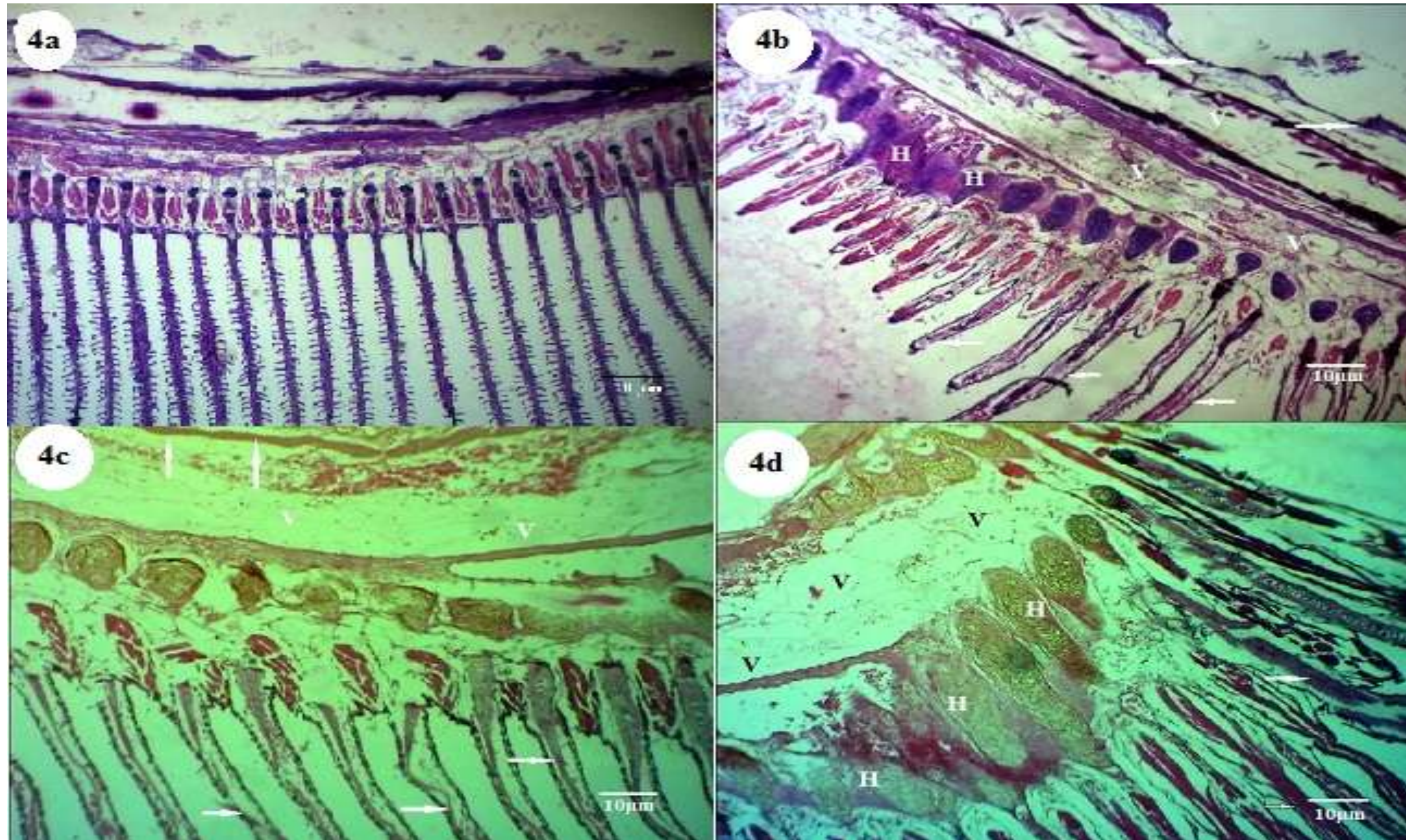


**Figure 4** **Histomorphology of gill tissue exposed to $TiO_2$-NPs in *Oreochromis mossambicus*. 4a-Gill control; 4b: $TiO_2$NPs at 16.4 mg/ L exposed for 96 h showing epithelial upliftment (→), vacuolization (V), hyperplasia (H), absence of secondary lamellae (←); 4c: $TiO_2$NPs at 16.4 mg/ L exposed for 60 days showing epithelial upliftment (↑), vacuolization (V), absence of secondary lamellae (→); 4d: Treatment withdrawal showing vacuolization (V), hyperplasia (H), absence of secondary lamellae (→)**



### 4.2 Effects of nanoparticles on the histology of liver tissues

**Effect of $Al_2O_3$-NPs**

Control liver tissue showed normal pattern of hepatocytes having homogenous cytoplasm and spherical nucleus (Figure 5a). However, exposure to $Al_2O_3$-NPs for 96 h showed pathological changes as segmentation and degeneration of hepatocytes with spindle shaped nucleus (Figure 5b). After 60 days of nanoparticles exposure, histology of liver tissues showed severe vacuolization and necrosis (Figure 5c). In the treatment withdrawal group, the histological lesions remained unchanged and found similar to the treatment groups (Figure 5d).

**Effect of $Fe_3O_4$-NPs**

Control liver tissue showed normal histomorphology having hepatocytes with homogenous cytoplasm and a large central spherical nucleus (Figure 6a). Exposure to $Fe_3O_4$-NPs for 96 h showed notable lesions such as segmentation of hepatocytes and spindle shaped nucleus (Figure 6b). After 60 days of $Fe_3O_4$-NPs treatment showed aggregation of melanomacrophages followed by severe necrosis (Figure 6c). Treatment reversal group also showed degenerated cytoplasm with deposition of melanomacrophages (Figure 6d).

**Effect of $SiO_2$-NPs**

Liver tissue obtained from the control group was found normal having homogenous cytoplasm and centrally placed spherical nucleus (Figure 7a). Liver tissue exposed to $SiO_2$-NPs for 96 h showed segmentation in hepatocytes and spindle shaped nucleus (Figure 7b). $SiO_2$-NPs exposed for 60 days resulted in severe vacuolization, absence of nucleus and complete disorganization of hepatocytes (Figure 7c). When the treatment was withdrawn for 60 days showed similar histological lesions in liver tissues as observed in the treatment groups (Figure 7d).

**Effect of $TiO_2$-NPs**

Control liver tissue exhibited normal architecture as observed with homogenous cytoplasm and a large central spherical nucleus (Figure 8a). $TiO_2$-NPs

exposure for 96 h showed severe vacuolization and aggregation of melanomacrophages (Figure 8b). Long-term exposure for 60 days showed histological modifications such as severe necrosis and spindle shaped nucleus (Figure 8c). Liver tissue of the treatment reversal group remained similar like that of treatment groups (Figure 8d).

**Figure 5** **Histomorphology of liver tissue exposed to $Al_2O_3$-NPs in *Oreochromis mossambicus*. 5a-Liver control; 5b: $Al_2O_3$NPs at 4 mg/ L exposed for 96 h showing segmented hepatocytes and spindle nucleus; 5c: $Al_2O_3$NPs at 4 mg/ L exposed for 60 days showing vacuolization and necrosis; 5d: Treatment withdrawal showing severe degenerated cytoplasm and spindle shaped nucleus**

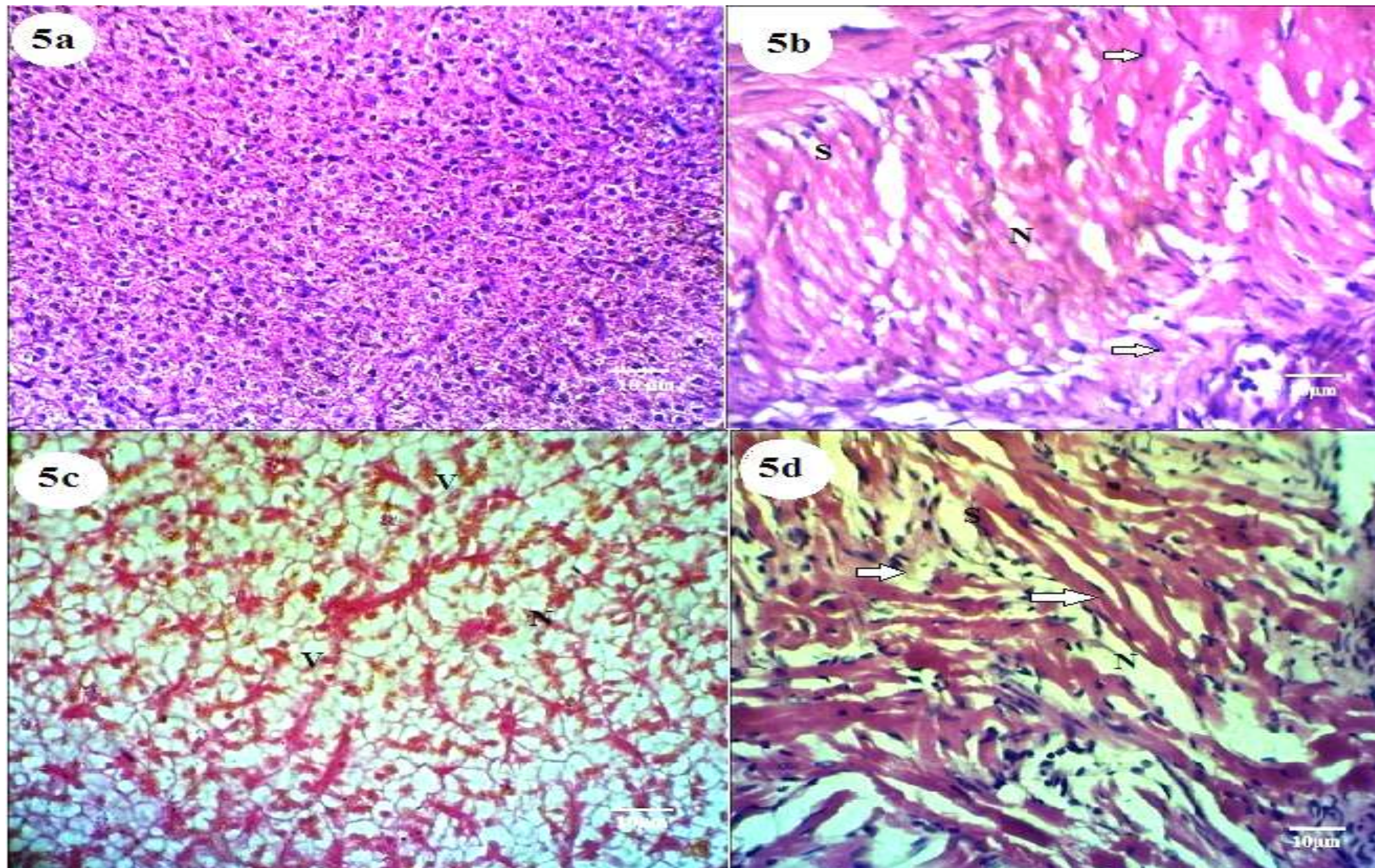


**Figure 6** **Histomorphology of liver tissue exposed to $Fe_3O_4$-NPs in *Oreochromis mossambicus*. 6a-Liver control; 6b: $Fe_3O_4$NPs at 15 mg/ L exposed for 96 h showing segmentation of hepatocytes and spindle shaped nucleus; 6c: $Fe_3O_4$NPs at 15 mg/ L exposed for 60 days showing severe necrosis and aggregation of melanomacrophages; 6d: Treatment withdrawal showing severe degenerated cytoplasm with deposition of melanomacrophages**

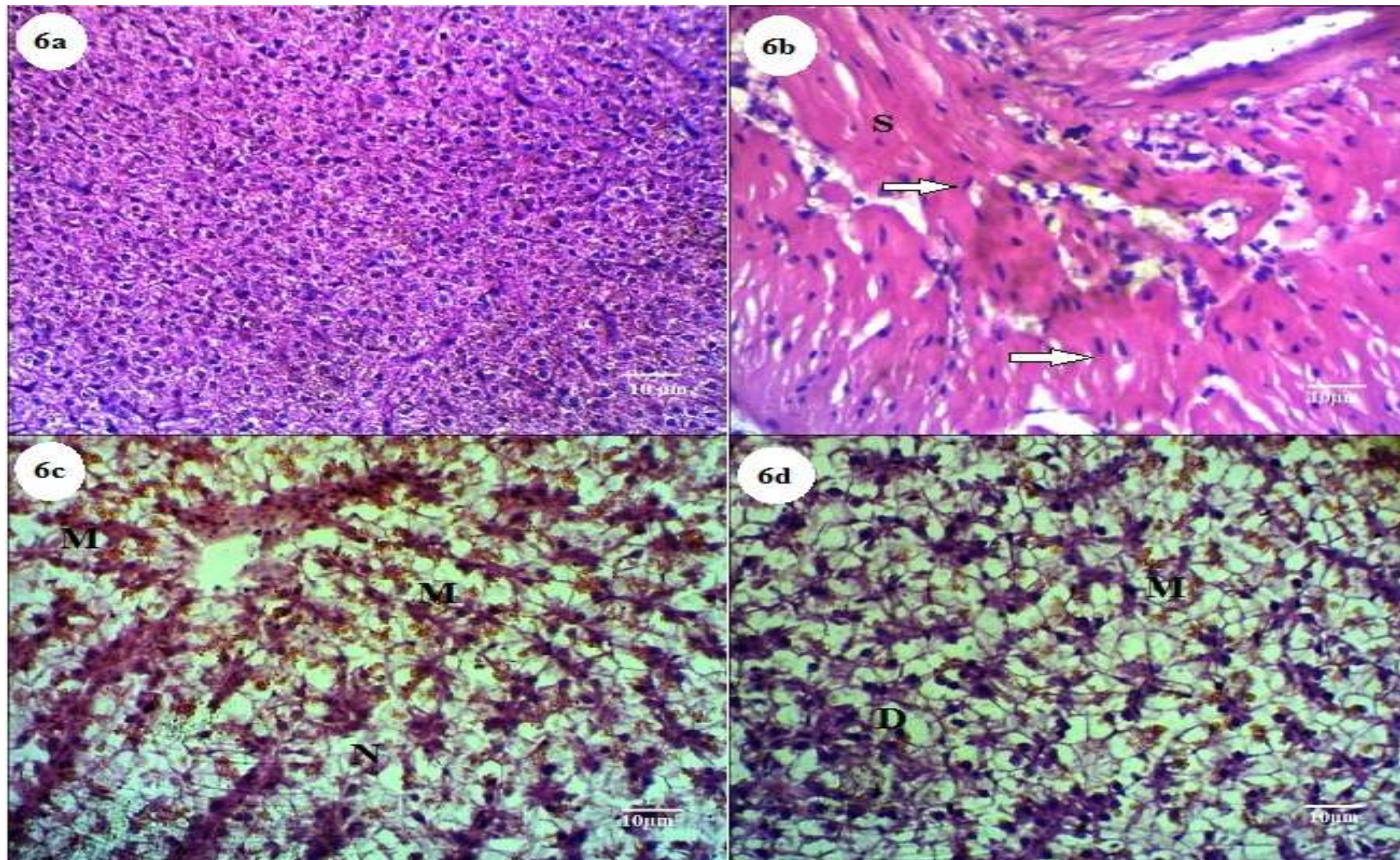


**Figure 7** **Histomorphology of liver tissue exposed to $SiO_2$-NPs in *Oreochromis mossambicus*. 7a-Liver Control; 7b: $SiO_2$NPs at 12 mg/ L exposed for 96 h showing segmented hepatocytes and spindle nucleus; 7c: $SiO_2$NPs at 12 mg/ L exposed for 60 days showing vacuolization; 7d: Treatment withdrawal showing severe necrosis**

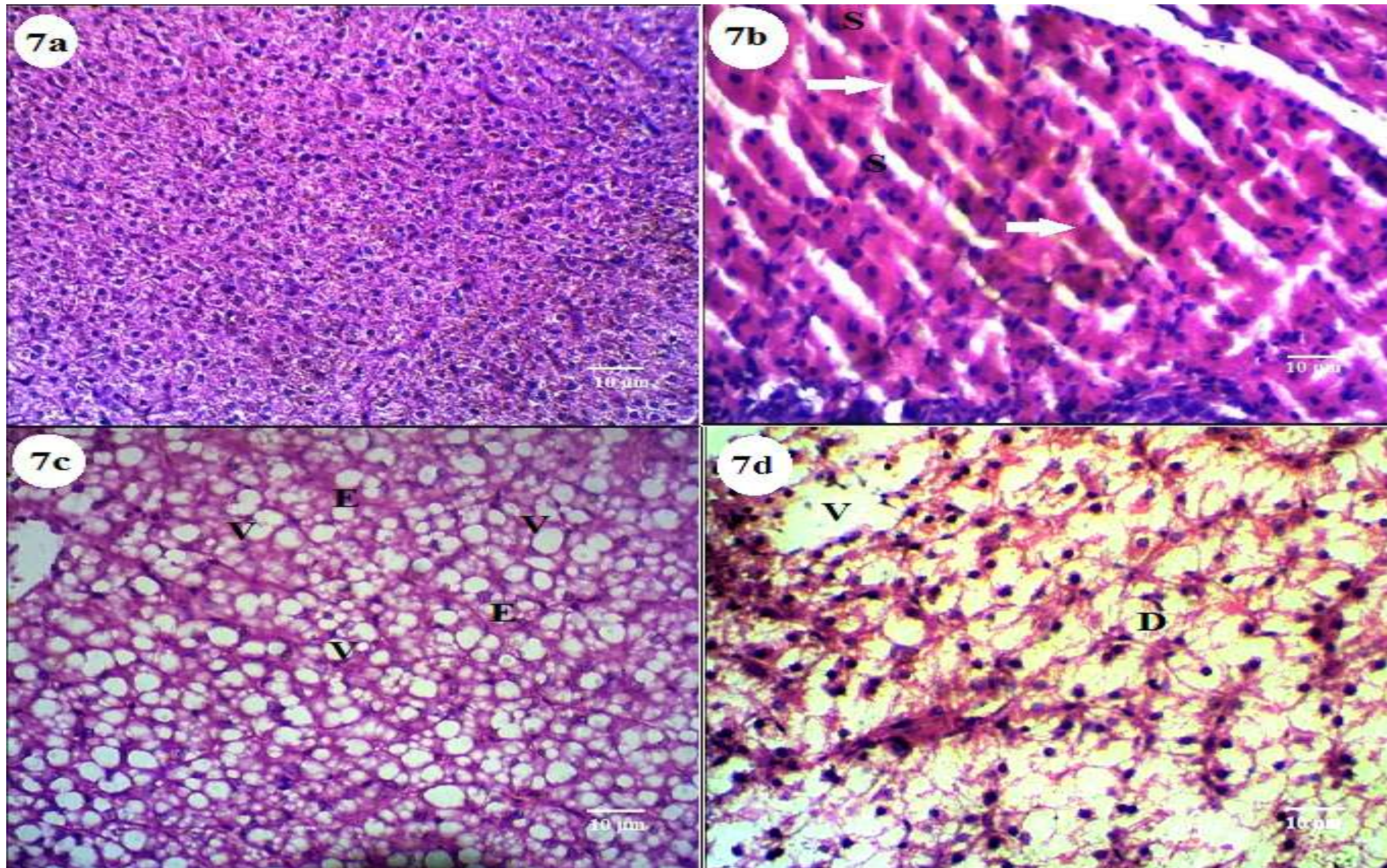
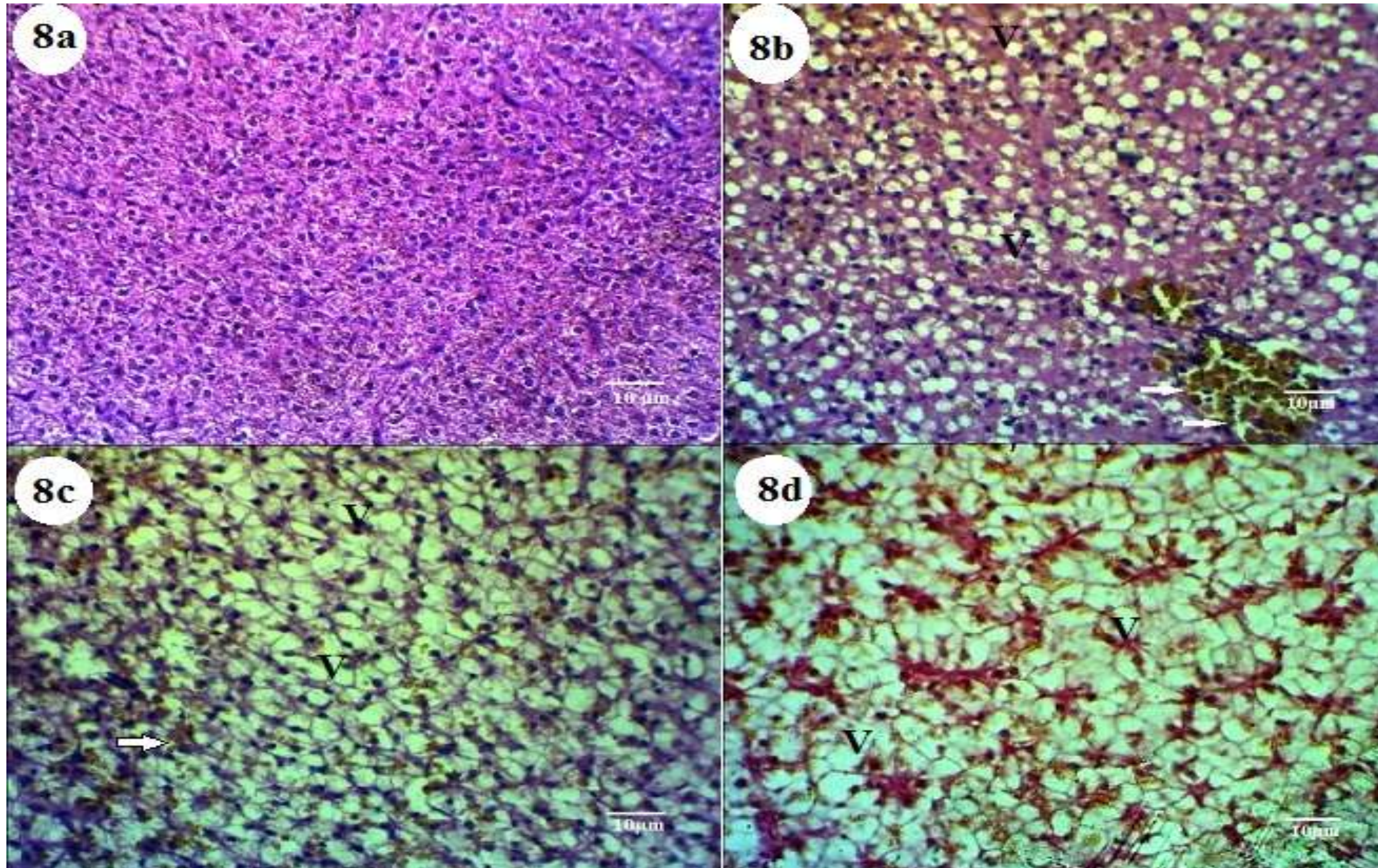


**Figure 8** **Histomorphology of liver tissue exposed to $TiO_2$-NPs in *Oreochromis mossambicus*. 8a-Liver control; 8b: $TiO_2$NPs at 16.4 mg/ L exposed for 96 h showing vacuolization and deposition of melanomacrophages; 8c: $TiO_2$NPs at 16.4 mg/ L exposed for 60 days showing severe necrosis and spindle shaped nucleus; 8d: Treatment withdrawal showing severe degenerated cytoplasm and spindle shaped nucleus.**



## 4.3 Effects of nanoparticles on the histology of brain tissues

**Effect of $Al_2O_3$-NPs**

Histology of control brain tissue was found normal, where the cerebral hemispheres or cerebrum viewed under the microscope showed outer granular layer, middle basophilic layer and inner ganglionic layer possessing neuronal cells (Figure 9a). $Al_2O_3$-NPs exposure for 96 h resulted in pathological lesions as evidenced by mild degenerative changes on all regions, and mild vacuolization in neural cells (Figure 9b). Long-term exposure of nanoparticles for 60 days showed severe degenerative changes along with intracellular edema (Figure 9c). Treatment of nanoparticles when withdrawn for 60 days showed similar pathological abnormalities like that of treatment groups (Figure 9d).

**Effect of $Fe_3O_4$-NPs**

Brain tissue from control group showed normal histoarchitecture without any indication of lesions (Figure 10a). Nanoparticles exposure for 96 h showed degeneration of neurons, vacuole formation and severe loss of granular cells in the brain tissues (Figure 10b). After 60 days of $Fe_3O_4$-NPs exposure, the severity of damages was more prominent as evidenced by more distinct changes as vacuolization, intracellular edema, congestion of neural cells and aggregation of gliosis (Figure 10c). Vacuolization and neuronal degeneration was also observed in the treatment reversal group (Figure 10d).

**Effect of $SiO_2$-NPs**

Control brain tissue showed normal histoarchitecture throughout the experiment (Figure 11a). Brain tissue exposed to $SiO_2$-NPs for 96 h resulted in the mild degeneration of nerve cells whereas when the treatment period was increased to 60 days showed severe histological abnormalities such as neurodegeneration, formation of vacuolization, cerebral edema, necrosis of neurofibrillar region and lesion in choroid plexus (Figures 11b and 11c). In the treatment withdrawal group, the modifications observed in the nanoparticles treated group persisted in brain tissue (Figure 11d).

**Effect of $TiO_2$-NPs**

No histopathological lesions were observed in the control brain tissues (Figure 12a). Short-term exposure of nanoparticles for 96 h resulted in degeneration of neurons in the cerebral region (Figure 12b). Severity of damages was observed after long-term exposure of nanoparticles for 60 days showing vacuolar changes with empty sacs in ganglionic region of cerebrum (Figure 12c). Nanoparticles exposure when withdrawn for 60 days showed pathological lesions such as neuronal degeneration and vacuolization in brain tissues (Figure 12d).

**Figure 9** **Histomorphology of brain tissue exposed to $Al_2O_3$-NPs in *Oreochromis mossambicus*. 9a-Brain control; 9b: $Al_2O_3$NPs at 4 mg/ L exposed for 96 h showing mild neurodegeneration and vacuolization; 9c: $Al_2O_3$NPs at 4 mg/ L exposed for 60 days showing severe neurodegeneration; 9d: Treatment withdrawal showing vacuolization and severe neurodegeneration**

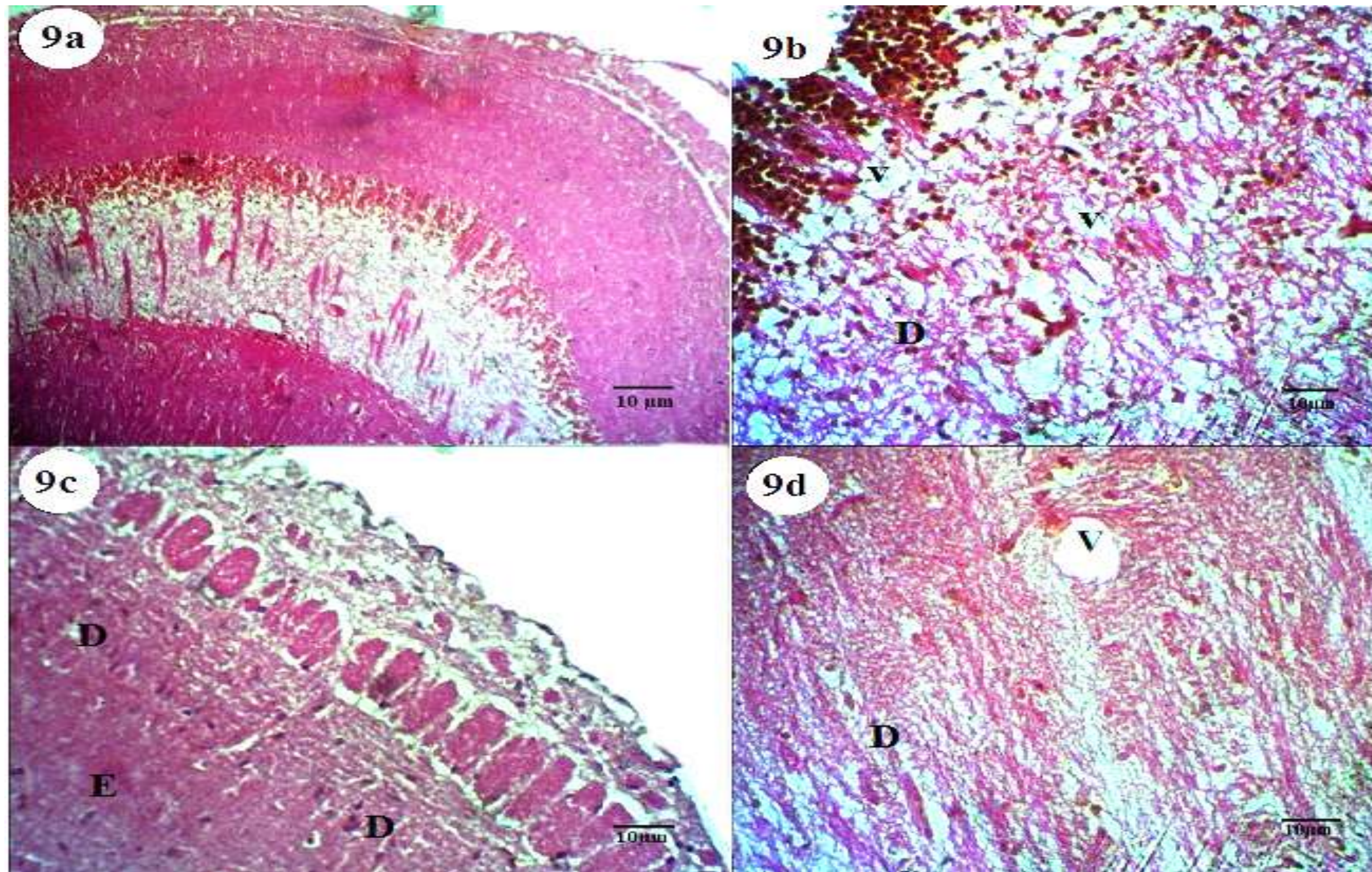


**Figure 10** Histomorphology of brain tissue exposed to $Fe_3O_4$-NPs in *Oreochromis mossambicus*. 10a-Brain control; 10b: $Fe_3O_4$NPs at 15 mg/ L exposed for 96 h showing degeneration of neural cells and vacuole formation; 10c: $Fe_3O_4$NPs at 15 mg/ L exposed for 60 days showing vacuolization and intracellular edema; 10d: Treatment withdrawal showing severe neurodegeneration

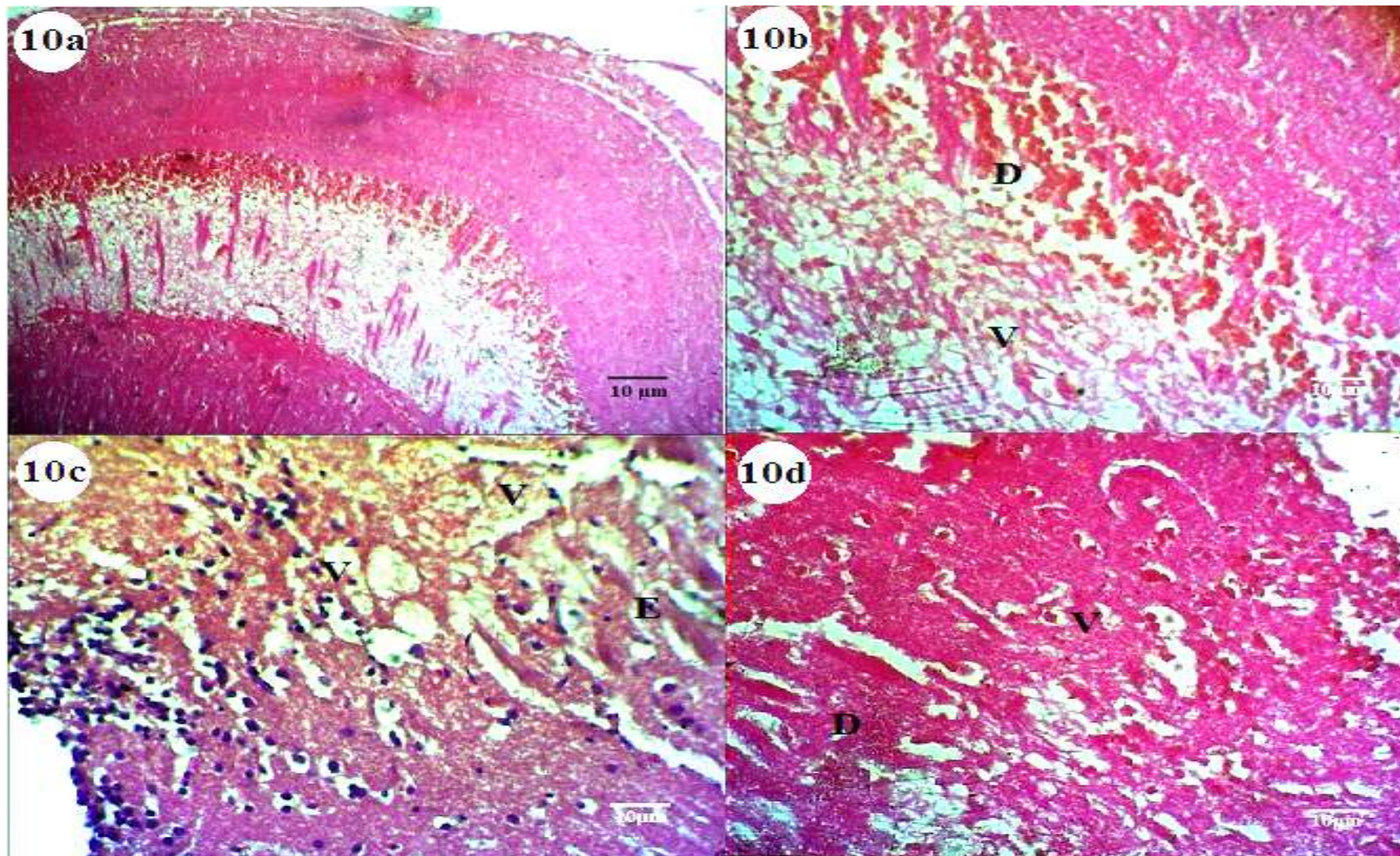


**Figure 11** **Histomorphology of brain tissue exposed to $SiO_2$-NPs in *Oreochromis mossambicus*. 11a-Brain Control; 11b: $SiO_2$NPs at 12 mg/ L exposed for 96 h showing mild neurodegeneration; 11c: $SiO_2$NPs at 12 mg/ L exposed for 60 days showing severe neurodegeneration as lesion of choroid plexus; 11d: Treatment withdrawal showing vacuolization and severe neurodegeneration**

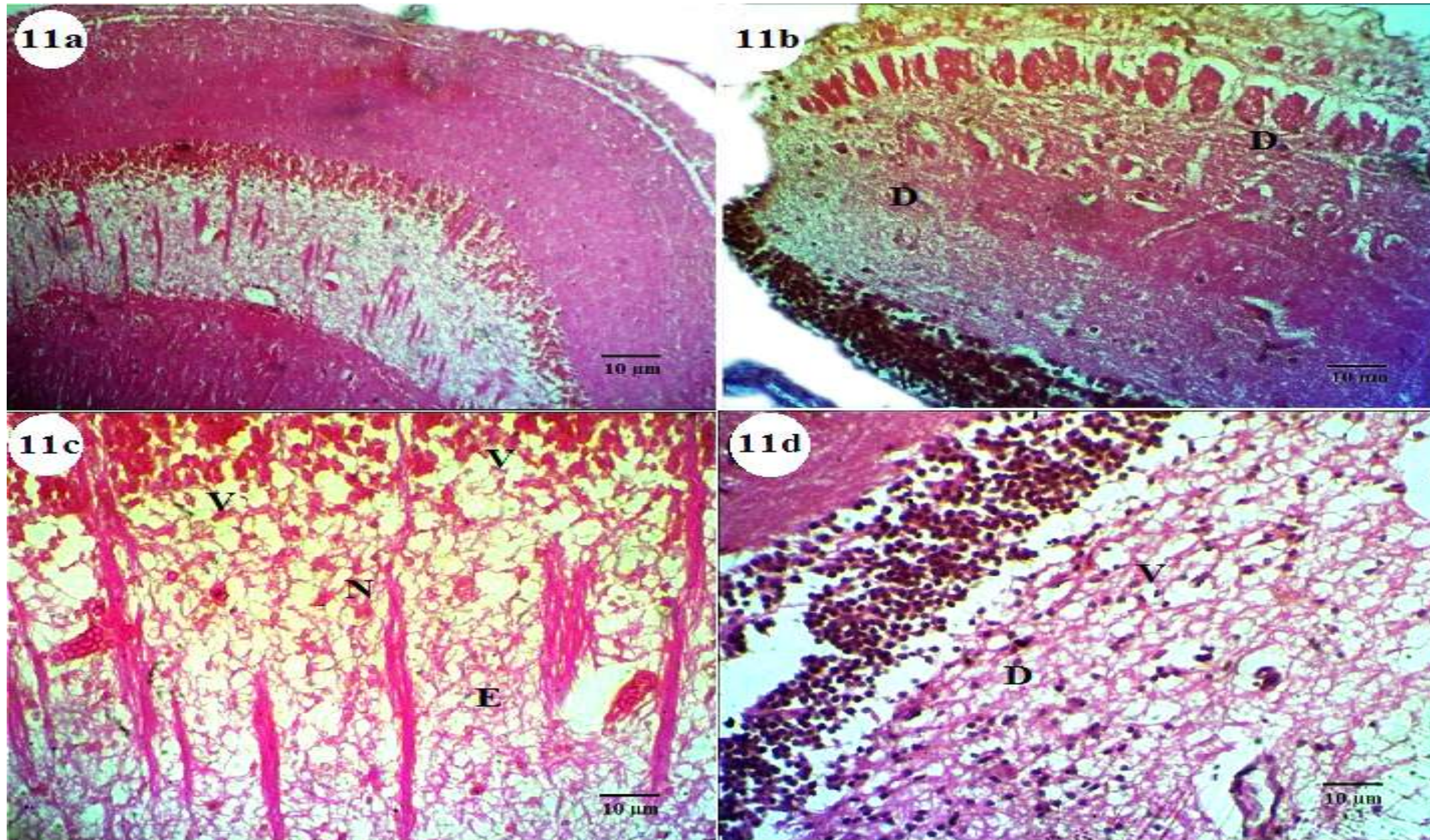
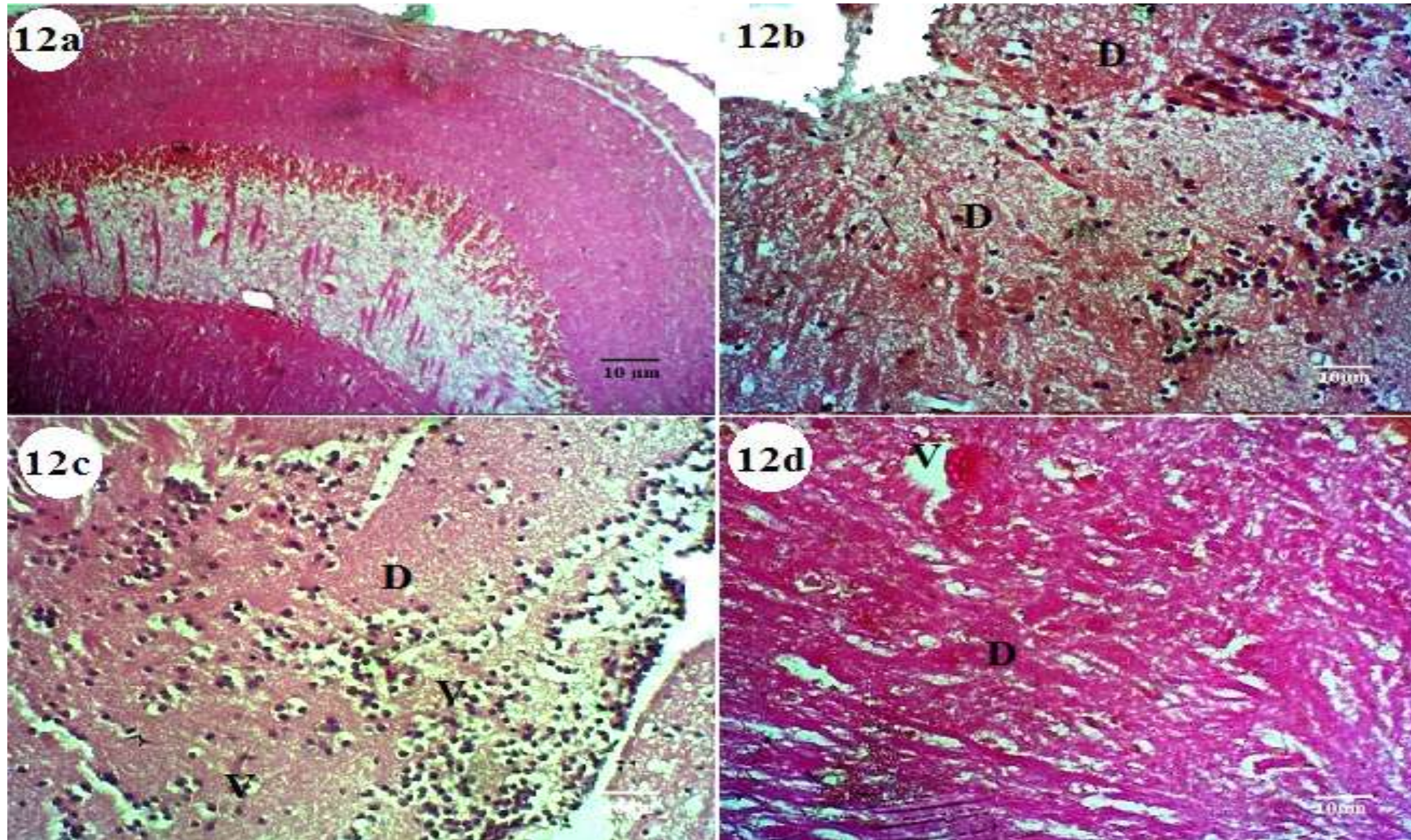


**Figure 12** **Histomorphology of brain tissue exposed to $TiO_2$-NPs in *Oreochromis mossambicus*. 12a-Brain control; 12b: $TiO_2$NPs at 16.4 mg/ L exposed for 96 h showing neurodegeneration; 12c: $TiO_2$NPs at 16.4 mg/ L exposed for 60 days showing severe neurodegeneration and vacuolar changes with empty sacs; 12d: Treatment withdrawal showing vacuolization and severe neurodegeneration**



# Discussion

## 5.1 Significance of the study

Health and well-being of fish population is essential to manage fitness and biotic integrity of aquatic ecosystems. Continuous discharge of many anthropogenic contaminants from various intentional and unintentional sources is the major threat faced by the aquatic ecosystems. Aquatic organisms, particularly fish, are very sensitive to the changes in the water quality. However, fish possess some adaptations to protect against the effects of environmental contaminants. The adaptations include modification in behavioural, biochemical, physiological and histological parameters. In the environmental monitoring, histology is considered as an important tool and biomarker to examine specific target organs of the organisms as it also remains the most reliable and unbiased parameter in biological and medical sciences (Bernet *et al*., 1999). The prolonged exposure of a single or multiple toxicants even at sublethal concentration produce drastic effects on the exposed organisms, which eventually affect the fitness and survival of the species. Thus histopathology is widely used technique to investigate the effects of toxicants in various organs, especially on gill, liver, muscle, intestine, brain, kidney and gonads.

In the current study tilapian fish, *Oreochromis mossambicus* was challenged with selected nanoparticles at sublethal concentration by fixing acute (96 h), chronic (60 days) and withdrawal (60 days) periods. Alteration in the morphology of vital tissues such as gill, liver and brain was examined after exposing the animal to the selected nanoparticles namely aluminium oxide ($Al_2O_3$-NPs), iron oxide ($Fe_3O_4$-NPs), silicon dioxide ($SiO_2$-NPs) and titanium dioxide ($TiO_2$-NPs). Comparison of tissues treated with nanoparticles against the control tissues gives the impact of toxicants at organ-specific level. Various morphological deformities have been recorded in the different tissues of fish exposed to the selected nanoparticles where noticeable changes are observed even after the short-term exposure for 96 h, and much pronounced effects were noted after 60 days of chronic exposure. Treatment withdrawal effects were also carried out for 60 days after the long-term exposure of nanoparticles in order to assess if the organism recovered from the toxic effects within the time interval. Thus the study provides the lethal consequences of selected nanoparticles and also explored different range of organ toxicity in fish.

## 5.2 Effects of nanoparticles on the histology of gill tissues

It is crucial to determine the histological changes in gill tissue exposed to toxicants for many reasons. Gill is the first exposed organ that keeps constant contact with the environment, and serves as warning signs of damage to animal health (Evans, 1987). Gills are equipped with specialized structures that are capable to eliminate the entry of toxicant into the body to some extent by the secretion of mucous. However, continuous exposure of toxicants insists gill tissue for the uptake through the circulating blood vessels. Hence, histological analysis of gill tissue is very valuable as it provides the clear picture of the effects of toxicants in the aquatic ecosystem and the adaptations developed by the tissue to escape from such harmful effects. Gill tissue consists of epithelium with large number of filaments or primary lamellae, secondary lamellae, mucous cells, chloride cells and pillar cells, each with specific peculiar functions (Laurent and Dunel, 1980). Any alterations in the structural integrity of the tissue reflect as improper functioning or loss of function of the whole tissue. Sublethal exposure to the selected nanoparticles resulted in modifications of gill tissue of the fish *Oreochromis mossambicus*, and discussed in detail below:

Control gill tissues showed no remarkable changes in the histoarchitecture throughout the experiment period (Figure 1a). Fish exposed to $Al_2O_3$-NPs for 96 h showed lesions in gill histology, which includes epithelial upliftment, hypertrophy and hyperplasia of gill arches, aneurism, lamellar curling and loss of secondary lamella (Figure 1b). On exposure to the nanoparticles, as the first line of defensive mechanism, the upliftment of gill epithelium occurred so as to increase the surface area thereby made an attempt to prevent the entry of toxicant inside the body of animal. Gill epithelium also secretes excess of mucous which form a thick protective layer against the nanoparticles exposed. The regular elimination of mucous layer from the gill surface into the aquatic media helps to remove the bounded pathogens or toxicants that remain trapped to the gills (Powell *et al*., 1992). Similarly, in another study when silver nanoparticles have been exposed to the rainbow trout resulted in excess mucous deposition (Johari *et al*., 2015). The increased secretion of mucous as a result of nanoparticles exposure could be one of the reasons for the

increase in the weight of gill tissue after nanoparticles exposure, which was discussed earlier in chapter 2.

Long-term exposure of nanoparticles for 60 days also showed severe mucous deposition, vacuolization, hyperplasia, aneurism, and absence of secondary lamellae (Figure 1c). Hyperplasia is another common lesion observed in both short-term and long-term exposure groups. Hyperplasia is the progressive reaction pattern as adaptation to protect the underlying cell types from the entry of pollutants (Evans *et al.*, 2005). However, hyperplasia also cause impediment in the respiratory and regulatory pathways of the gill. The gill lesions such as vacuolization and hyperplasia has been observed after sublethal exposure to the mixtures of essential and toxic metals such as cadmium, nickel, copper and iron in the gill of fish, *Channa punctatus* (Pandey *et al.*, 2008). There are several frequently occurring toxicant induced gill damages such as curling and absence of secondary lamellae, which can be recognized as non-specific damages (Mallatt, 1985). Such lamellar anomalies were observed after the nanoparticles exposure in the fish, *Oreochromis mossambicus*. Among which aneurism and edema are the most prominent form of lesions that was categorized under circulatory disturbance due to the altered flow of circulatory or body fluid (Bernet *et al.*, 1999). The severity of morphological alterations observed after $Al_2O_3$-NPs exposure was found time-dependent and the reversal of treatment for 60 days without toxicant also showed similar lesions like the treatment groups (Figure 1d). Thus nanoparticles induced irreversible tissue damage was evident by the alterations in gill structure after treatment withdrawal.

$Fe_3O_4$-NPs exposure for 96 h showed mucous deposition, vacuolization, aneurism, hyperplasia and absence of secondary lamellae in gill tissue whereas control tissue showed normal architecture (Figures 2a and 2b). Occurrence of such abnormalities as a result of nanoparticles exposure could be due to the altered blood flow and other body fluids in the gill tissue of the fish. It is well-known that secondary gill lamellae play an important role in the transport of respiratory gases and any damages in the lamellae could result in the reduction of oxygen transport, which in turn could influence the metabolic system of the fish. $Fe_3O_4$-NPs treatment for 60 days showed blebbing of epithelium, aneurism, hyperplasia and absence of

secondary lamellae (Figure 2c). Loss of secondary lamellae lead to impairment in circulation, respiration, osmoregulation and in ion regulatory mechanisms of the fish and ultimately affect the total functioning of the gill tissue (Fernandes and Mazon, 2003).The structural alterations observed in the treatment group persisted in the treatment withdrawal group also (Figure 2d). The present results illustrates that $Fe_3O_4$-NPs at 15 mg/ L concentration caused pronounced permanent and irreversible tissue damage to the fish, *Oreochromis mossambicus*.

Histological analysis of short-term and long-term $SiO_2$-NPs exposure groups also showed similar morphological alterations in the gill tissue as evidenced by excess mucous deposition, upliftment of gill epithelium, vacuolization of gill arches, necrosis, aneurism, absence and curling of secondary lamellae when compared to control tissue without any abnormalities (Figures 3a-3c). Lifting of the epithelial layer is known to increase the distance across the tissue and the outer surface through which waterborne toxicants diffuse to reach the bloodstream. Even though, epithelial upliftment is an adaptive modification for reducing the entry of exogenous materials, it also decreases the respiratory rate and thus affects the fitness and survival of the fish. Vacuolization and necrosis related gill tissue damage observed in $SiO_2$-NPs exposure groups could have contributed to the decrease in the weight of gill tissue as discussed in chapter 2. Similar degenerative changes and disturbed circulatory systems have been identified in the gill tissue after exposure to titanium dioxide nanoparticles in rainbow trout (Federici *et al*., 2007). Nanoparticles toxicity was found to be irreversible when the fish was maintained in toxicant-free medium for 60 days (Figure 3d). Thus $SiO_2$-NPs exposure exerted permanent pathological damages in the gill tissue of the fish, *Oreochromis mossambicus*.

On exposure to $TiO_2$-NPs for 96 h and 60 days resulted in remarkable time-dependent structural alterations in gill tissues when compared to the control gill without recovery to normal state on the treatment withdrawal for 60 days (Figures 4a-4d). The major lesions include epithelial upliftment, vacuolization, hyperplasia, aneurism and absence of secondary lamella. The absence or fusion of secondary lamella might be due to the stress induced by nanoparticles exposure or as a result of transformation of electrically charged properties of the epithelial cells in the

secondary lamellae (Bonga, 1997). The lifting of the respiratory epithelium is one of the common histological damages found in fish. It is characterized by displacement of gill epithelium from secondary lamellae, which could result in the formation of a space called edema. The observed lesions could be due to the exposure of nanoparticles, which ultimately leads to the reduction of the gill surface and impair the gas exchange process. Hyperplasia also leads to the proliferation of adjacent lamellar cells thereby reduce the inter-lamellar space and fusion of secondary lamellae (Fracario *et al.*, 2003). Nanoparticles induced gill lesions has also been observed in *Oreochromis mossambicus* exposed to different concentrations of zinc oxide nanoparticles for a period of 96 h (Suganthi *et al.*, 2015). The permanent and irreversible morphological tissue damage induced by $TiO_2$-NPs exposure even at sublethal concentration was confirmed by the treatment withdrawal for 60 days.

### 5.3 Effects of nanoparticles on the histology of liver tissues

Liver is the major organ involved in detoxification and also essential for the important physiological activities including anabolism and catabolism of biological compounds (Hinton and Lauren, 1990). Hence histopathological examination of liver tissue in fish was found crucial in the current study. Control liver tissue examined in the present study was found normal possessing homogenous polyhedral parenchyma cells with clear cytoplasm and centrally placed spherical nucleus (Figure 5a). Exposure to the selected nanoparticles namely $Al_2O_3$-NPs at 4 mg/ L, $Fe_3O_4$-NPs at 15 mg/ L, $SiO_2$-NPs at 12 mg/ L and $TiO_2$-NPs at 16.4 mg/ L concentrations resulted in remarkable destruction of liver morphology. Vacuolization, swelling of hepatocytes, focal necrosis, disorganization of parenchyma, changes in shape of nuclei are some of the common histological liver lesions found in fishes (Hibiya, 1982).

In the present study exposure to $Al_2O_3$-NPs for 96 h showed pathological changes as segmentation and degeneration of hepatocytes with spindle shaped nucleus (Figure 5b). Liver is the first organ to face any toxicants through the portal circulation, the appearance of cytoplasmic degeneration in hepatocytes of fish indicated liver damage and failure of hepatocytes to breakdown into respective

metabolites. This could be reason for $Al_2O_3$-NPs induced hepatic toxicity and induction of oxidative stress in liver tissue as discussed in chapter 2. $Al_2O_3$-NPs exposed for 60 days induced severe vacuolization and necrosis in the liver tissues (Figure 5c). Vacuolization is one of the degenerative changes found in hepatocytes due to the deposition of glycogen granules, and thus important for normal metabolism (Camargo and Martinez, 2007). However, vacuolization is the indication of imbalance between rate of synthesis and rate of release of substances to the circulation, and considered as a degenerative process that leads to enucleation, necrosis and metabolic damage to liver tissues (Pacheco and Santos, 2002). The present results revealed that $Al_2O_3$-NPs exposure could have altered normal metabolic process and finally directed to necrosis of liver tissues. This could be one of the reasons behind the increase in the activity of alkaline phosphatase in liver tissue as discussed in chapter 2. Reversibility of histological lesions was not observed in the treatment withdrawal group (Figure 5d), and the results proved irrevocable degeneration of hepatocytes in the fish, *Oreochromis mossambicus*. Similar histological anomalies like necrosis, vacuolization, aggregation of blood cells and melanomacrophages have been reported after $Al_2O_3$-NPs exposure for 96 h at 120, 150 and 180 ppm concentrations in *Oreochromis mossambicus* (Murali *et al.*, 2017).

The liver of fish maintained under normal conditions, without nanoparticles exposure showed a typical regular structure (Figure 6a). $Fe_3O_4$-NPs exposed for 96 h showed segmentation of hepatocytes and spindle shaped nucleus (Figure 6b) and this could be due to the loss of structural integrity as a result of nanoparticles exposure. When the treatment period was extended to 60 days, $Fe_3O_4$-NPs showed aggregation of melanomacrophages followed by severe necrosis (Figure 6c). Melanomacrophages are the highly pigmented phagocytes found in liver and other tissues such as kidney and spleen, which play a role in adaptive immune response and regarded as histological indicator of immune functions (Aguis and Roberts, 2003; Steinel and Bolnick, 2017). Aggregation of melanomacrophages after $Fe_3O_4$-NPs indicated failure of toxicant clearance and storage of highly indigestible nanoparticles in the liver tissue. The present findings on hepatic lesions have been

reported in *Tilapia zilli* exposed at 25, 50 and 100 µg/ L concentrations of aluminium for 96 h (Hadi and Alwan, 2012). In the treatment withdrawal group, degenerated cytoplasm with deposition of melanomacrophages was retained in liver tissue (Figure 6d), which indicated irrecoverable liver damage in the fish, *Oreochromis mossambicus*.

Histological study of liver tissues showed a typical structural organization in the untreated fish (Figure 7a). However, exposure of $SiO_2$-NPs for 96 h showed segmentation in hepatocytes and spindle shaped nucleus (Figure 7b). Spindle shaped nuclei are associated with altered liver metabolism, and failure of biotransformation into the less toxic metabolites in the liver tissue (Braunbeck *et al*., 1990). Spindle shaped nuclei also designates degenerative change in liver as well as indication of change in chromatin structure (Bernet *et al*., 1999). Change of size and shape of liver nucleus has been previously reported after sublethal exposure of organophosphate pesticide Dimethoate 500 in the liver tissue of fish, *Brachydanio rerio* (de Lara Rodrigues and Fanta, 1998). Prolonged exposure of $SiO_2$-NPs for 60 days resulted in severe vacuolization, absence of nucleus and complete disorganization of hepatocytes (Figure 7c), which indicated metabolic dysfunction of the liver tissue. The present results coincided with another study on exposure of silver nanoparticles to rainbow trout, *Oncorhynchus mykiss* for 21 days (Johari *et al*., 2015). Disorganization of hepatocytes noted after nanoparticles exposure retained in the treatment withdrawal group also (Figure 7d) thereby represented irreversible tissue damage in the fish, *Oreochromis mossambicus*.

Normal architecture of liver was observed with homogenous polygonal cells and centrally placed spherical nucleus in the control tissue (Figure 8a). On exposure of $TiO_2$-NPs for 96 h showed severe vacuolization and aggregation of melanomacrophages (Figure 8b). Increase in vacuolization could result in fatty change of hepatocytes in response to toxicant exposure, which forces the nucleus to the periphery of the cell (van Dyk *et al*., 2007) and such degenerations was observed after 60 days of $TiO_2$-NPs exposure (Figure 8c). Necrosis is considered as a direct effect of toxicants, they are generally irreversible, and the persistence or progression could lead to a partial or total loss of organ function (Agamy, 2012). Withdrawal of

nanoparticles also resulted in similar lesions as observed after nanoparticles treatment (Figure 8d), which indicated irreversible toxic effects of $TiO_2$-NPs.

### 5.4 Effects of nanoparticles on the histology of brain tissues

The study of brain pathology serves to learn about the structure and function of central nervous system. Histopathological reports on the effects of nanoparticles induced alterations in brain tissue remains scanty. Thus the current study provides new insight on the adverse effects of nanoparticles in brain tissue. The architecture of brain tissue from the control group showed no lesions but with clear neural cells and distinct nucleus (Figure 9a). $Al_2O_3$-NPs at 4 mg/ L concentration for 96 h showed mild degenerative changes on all regions of cerebral cortex, and mild vacuolization in neural cells (Figure 9b). The results suggested that acute exposure of nanoparticles caused neurodegeneration, which could be the result of decreased activity of acetylcholinesterase activity in brain tissues, which was discussed in chapter 2. Prolonged exposure of nanoparticles for 60 days showed severe degenerative changes such as intracellular edema (Figure 9c) and the results indicated that nanoparticles caused structural and functional alterations to the brain tissue. Similar pathological abnormalities were observed in the treatment withdrawal group (Figure 9d) thereby indicated irreversible effect of $Al_2O_3$-NPs in brain tissue of *Oreochromis mossambicus*.

Brain is the controlling center of all functions and movements in the body of organisms and serves as a relay station. There was no histoarchitectural changes noted in the control tissue (Figure 10a). $Fe_3O_4$-NPs exposed for 96 h was observed with degeneration of neurons, vacuole formation and severe loss of granular cells in the brain tissues (Figure 10b). The results can be correlated to possible inhibition of the activity of acetylcholinesterase enzyme in the brain tissue as mentioned in chapter 2. $Fe_3O_4$-NPs when exposed for 60 days resulted in severity of damages as evidenced by more distinct changes as vacuolization, intracellular edema, congestion of neural cells and aggregation of gliosis (Figure 10c), which could be one of the causes for the decreased brain weight as discussed in chapter 2. The treatment reversal for 60 days did not reversed the effects of nanoparticles (Figure

10d) thereby suggested permanent destruction of brain tissue in the fish, *Oreochromis mossambicus*. The present results were in agreement with another study on exposure to organophosphate pesticide phorate for four days, which has been shown to induce structural degeneration of brain tissues in *Cyprinus carpio* (Lakshmaiah, 2017).

In the present study, histopathological analysis of control brain tissue showed normal histological features (Figure 11a). However, exposure to $SiO_2$-NPs for 96 h resulted in the mild degeneration of nerve cells whereas when the treatment period was increased to 60 days showed severe histological abnormalities such as neurodegeneration, formation of vacuolization, cerebral edema, necrosis of neurofibrillar region and lesion in choroid plexus (Figures 11b and 11c). The present findings indicated the progressive toxicity of $SiO_2$-NPs, which was time-dependent. Necrosis of neurofibrillar region and lesion in choroid plexus noted after nanoparticles exposure could be the basis for the reduction in the weight of brain tissue as observed and explained in chapter 2. The pathological lesions observed in the treatment groups were maintained as such even after the treatment withdrawal (Figure 11d) thereby indicated the neurotoxic effect of $SiO_2$NPs in the fish, *Oreochromis mossambicus*. Similar observations on nanoparticles induced brain lesions, neurotoxicity and impaired behaviour has been observed in *Oreochromis mossambicus* after zinc oxide nanoparticles treatment for 96 h (Suganthi *et al*., 2015).

Structural alterations that occur in brain tissue are generally irreversible as the neuronal cells lack the capacity of regeneration therefore examination of brain morphology is inevitable in toxicological studies. The present study observed $TiO_2$-NPs exposed for 96 h showed neurodegeneration changes and vacuolization in the brain without any histological lesions in the control tissue (Figures 12a and 12b). Pathological lesions observed after long-term exposure were more prominent as indicated by vacuolar changes with empty sacs in ganglionic region of cerebrum (Figure 12c). In one of the studies, the herbicide glyphosate exposed to catfish, *Clarias gariepinus* has been shown to cause severe neurodegeneration including edema and vacuolization in cerebral tissue (Erhunmwunse *et al*., 2014). The present

findings also correlated to reduction in brain weight, imbalance in antioxidant defense system and inhibition of acetylcholinesterase enzyme activity in $TiO_2$-NPs exposed brain tissue as discussed in chapter 2. Among the selected nanoparticles, $Al_2O_3$-NPs are more widely accepted as neurotoxicant, as aluminium and its derivatives has been recognized to cause neurodegeneration in different test organisms (Chen *et al*., 2008; Li *et al*., 2009).

On the basis of the abovementioned discussion, it can be concluded that all selected nanoparticles used in the present study even at sublethal concentration caused irrecoverable and permanent histomorphological damages in gill, liver and brain tissues of the fish, *Oreochromis mossambicus*. It may be suggested that nanoparticles exposure caused marked pathological lesions due to partial or complete loss of organ functions, imbalanced the normal functioning of vital organs thereby altered normal respiration, excretion, osmoregulation, ionic balance, metabolism, biotransformation and behaviour of fish. If all the vital activities are disturbed that could finally affect the normal well-being of organism. Simultaneously, it is alarming to understand the failure of treatment reversal of nanoparticles, and the persistent toxic effects could finally result in the decline of fish population and ecological imbalance.

# Conclusions

1. Exposure of selected nanoparticles resulted in morphological alterations in the gill, liver and brain tissues of the fish.
2. The major histopathological changes observed in gill tissue includes upliftment of gill epithelium, hyperplasia of gill arches, aneurism, curling and absence of secondary lamellae, thereby proved nanoparticles challenge normal gill functions such as respiration, excretion and osmoregulation.
3. Liver tissue damages are mainly characterized by segmentation, vacuolization and necrosis, which indicated exposure of nanoparticles could affect metabolism and detoxification process.
4. Nanoparticles-induced brain tissue damage was identified as severe degeneration, vacuolization and necrosis of neural cells thereby represented internalization of nanoparticles across the blood-brain barrier to exert the toxic effects.
5. All tissues equally exhibited mild to severe damages, where the severity of damages increased with increase in exposure duration stating the effects of nanoparticles are time-dependent.
6. Nanoparticles exerted permanent and irreversible marked pathological damages as evident by persistent lesions of gill, liver and brain tissues even after the treatment withdrawal.
7. The severity of morphological damage noticed after the nanoparticles exposure could cause several physiological and biochemical alterations that ultimately leads to permanent organ damage.
8. To brief, the present results contributed to environmental biomonitoring and safety assessment to reduce the excessive production, use and release of nanoparticles nearby the aquatic ecosystem so as to protect the fish population and also humans, which indirectly enter through food chain.

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# CHAPTER 4

# Evaluation of genotoxic potential of the selected nanoparticles

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| µg | Microgram |
| µl | Microlitre |
| µM | Micro molar |
| µmol | Micromol |
| 3T3 | 3-day transfer, inoculum $3\times10^5$ cells |
| 8-OHdG | 8- hydroxy-2′-deoxyguanosine |
| A549 | Adenocarcinoma 549 |
| AFM | Atomic force microscopy |
| Ag | Silver |
| AgNPs | Silver nanoparticles |
| $Al_2O_3$-NPs | Aluminium oxide nanoparticles |
| BRL 3A | Buffalo Rat Liver 3A |
| CAT | Catalase |
| CBMN | Cytokinesis block micronucleus |
| CBPI | Cytokinesis Block Proliferation index |
| $CeO_2$ | cerium dioxide |
| CHS-20 | Chediak-Higashi Syndrome-20 |
| cm | Centimeter |
| CoNP | Cobalt nanoparticles |
| CuNPs | Copper nanoparticles |
| CuO | Copper oxide |
| CYP1A | Cytochrome P450 1 A |
| DMSO | Dimethyl sulphoxide |
| DNA | Deoxyribonucleic acid |
| EDTA | Ethylenediaminetetraacetic acid |
| FBS | Foetal bovine serum |
| $Fe_3O_4$-NPs | Iron oxide nanoparticles |
| g | Gram |
| GFSk-S1 | Goldfish skin cells |
| GPx | Glutathione peroxidase |
| GR | Glutathione reductase |
| GSH | Glutathione |
| h | Hours |
| HaCaT | Human keratinocyte |
| HeLa | Henrietta Lacks |
| HEp-2 | Human epithelial type 2 |
| $IC_{50}$ | Half-maximal inhibitory concentration |
| Kg | Kilo gram |
| L | Litre |
| LMPA | Low melting point agarose |
| M | Molar |
| mg | Milligram |
| MI | Mononucleated cells |
| MII | Binucleated cells |
| MIII | Trinucleated cells |
| MIV | Tetranucleated cells |
| ml | Milliliter |

| | |
|---|---|
| mM | Milli molar |
| MMC | Mitomycin C |
| mmol | Millimol |
| MNi | Micronucleus |
| mol | Mol |
| mt2 | Metallothionin 2 |
| N2a | Neuro2a |
| NaCl | Sodium chloride |
| NaOH | Sodium hydroxide |
| Nbud | Nuclear bud |
| NDI | Nuclear division index |
| Ni-NPs | Nickel nanoparticles |
| nm | Nanometer |
| NMA | Normal melting point agarose |
| NPB | Nucleoplasmic bridge |
| NPs | Nanoparticles |
| Nrf2 | Nuclear factor (erythroid-derived 2)-like 2 |
| $^oC$ | Degree Celsius |
| OECD | Organization of Economic Co-operation and Development |
| P | Probability |
| p53 | Tumour protein 53 |
| PI | Propidium iodide |
| POR | P450 oxidoreductase |
| PVA | Poly vinyl alcohol |
| qRT-PCR | Quantitative reverse transcription polymerase chain reaction |
| RBC | Red blood corpuscles |
| RLE-6TN | Rat Lung Epithelial-6-T-antigen Negative. |
| RNA | Ribonucleic acid |
| ROS | Reactive oxygen species |
| RT gill-W1 | Rainbow trout gill W1 |
| RTG-2 | Rainbow Trout Gonad-2 |
| RTL-W1 | Rainbow trout liver W1 |
| SD | Standard deviation |
| SDS | Sodium dodecyl sulphate |
| $SiO_2$-NPs | Silicon dioxide nanoparticles |
| SOD | Superoxide dismutase |
| SPION | Superparamagnetic iron oxide nanoparticle |
| SPSS | Statistical package for social sciences |
| $TiO_2$NFs | Titanium dioxide nano fibers |
| $TiO_2$-NPs | Titanium dioxide nanoparticles |
| UV | Ultraviolet |
| UVA | Ultraviolet A |
| V | Volt |
| WAG | Wallagu attu gill |
| ZnO | Zinc oxide |
| $\gamma$-H2AX | Gamma H2AX |

# General Introduction

### 1.1 Genotoxicology

During the past few decades, toxicological studies had undergone considerable advancements, and much attention was given to the genetic level damages caused by toxicants, hence genotoxicity studies gained greater interest. Genotoxicity or genetic toxicology is the general term given based on the ability of any compound to induce genetic damages, both in DNA and RNA, thereby affect the cellular integrity in the exposed organism (Friedberg, 2008). Genotoxicity is any responses that are measured in nucleic acids, which includes either damage to the genome or modifications in the gene expression (Lam and Gray, 2003). Any physical or chemical agents that cause genetic damages are referred as genotoxin, which also known to possess carcinogenic properties and lead to genetic diversity in natural population (Mohamed *et al*., 2017). Genetic damage can occur at gene level as point mutations, insertion or deletion, and at chromosome level in the form of aneuploidy, translocations and so on (Herceg and Hainaut, 2007). Thus exposure to genotoxicants can undergo DNA lesions, and if not repaired, can initiate a cascade of biological outcomes that affect at molecular, cellular, tissue or organism level, and finally influence the population or community.

Many researchers focused attention on the genotoxic effects of many substances on mammalian models. However, the effects of genotoxicants on aquatic organisms, particularly on fish, achieved significance only in recent years. Fish possess great economic value as it serve as direct food for human and also holds recreational, nutritional, industrial, and medicinal rates. Thus biomonitoring of genotoxicity in aquatic organisms enable to detect genotoxins-induced mutation in the natural environment as well as ensure to prevent such mutagens from entering the food chain to human. Besides, genotoxicity studies are well employed for industrial purposes, especially on the production sites of new personal care products or drugs around the globe, before they are released into the market. The endpoints of genotoxicity differ based on the testing animal, type of chemicals, exposure period and testing system. Several chemicals with genotoxic potential are released into the aquatic environment accordingly several genotoxicity tests are developed to detect the nature of genotoxins and its associated genetic risks on the exposed organisms.

### 1.2 Classification of genotoxins

Genotoxins are generally categorized into mutagen, carcinogen or teratogen based on the effects on various organisms. Mutagens are the agents such as radiation or chemical substances that cause genetic mutations in both somatic and germ cells. Mutagens can alter the structure or sequence of DNA leading to various genetic damages, loss or gain of gene functions, create genetic variations or instability and impair biological functions (Huang *et al*., 2003; Janssen and Medema, 2013). Some of the chemical mutagens exert their toxic effects by several mechanisms that include base analogs, intercalating agents, and mutagens that alter the pairing properties of DNA bases. Bromouracil and aminopurines are the well-known examples of base analogs that cause transitions and spontaneous tautomerization events (Friedberg *et al*., 2006). Acridine orange, proflavin and ethidium bromide are some genotoxins that interact and intercalate with bases of DNA resulting in frameshifts. Some of the known mutagens are physical agents like X-rays, gamma rays, alpha particles, radioactive elements, UV radiation etc that produce a range of damages to cells and organisms. Most of the effects results in protein and DNA damage, which may leads to organelle failure, blockage in cellular checkpoints, inflammation and lesions (Glass *et al*., 2003).

Genotoxins are also carcinogenic, both in somatic and germ cells causing uncontrolled differentiations of cells. Tobacco, aflatoxin, asbestos, dioxin, ionizing radiations and viruses are some examples of genotoxins that functions as carcinogens. In cancer cells, several hundreds of genes have been identified in the past three decades as recurrent site for genotoxin-induced genetic damages. Some genotoxins induce point mutations in specific genes and also alter gene methylation, induce aneuploidy and cause specific chromosome rearrangements (Wogan *et al*., 2004). Teratogenic properties of genotoxins are illustrated by the delay in the development of embryo and birth defects in children (Rogers and Kavlock, 1996). Teratogenic hazards include malformations, growth retardation, functional imperfections and death in embryos (Rogers and Kavlock, 1996). Certain chemicals such as benazepril, tetracycline, warfarin, thalidomide are the major classes of teratogens (Chiodo *et al*., 1993). However, not all genotoxic compounds are

mutagenic, carcinogenic or teratogenic, but can exert the toxicity based on the duration and dose of exposure.

### 1.3 Eco-genotoxicology

Eco-genotoxicology or genetic ecotoxicology is an emerging field in toxicology to assess the potential effects of environmental genotoxins on the health of the ecosystem. Potency of genotoxins is evidenced by DNA damage, mutation and induction of various types of cancer. Besides the formation of tumour, genotoxins also induce several inflammatory or autoimmune diseases, neurodegenerative disorders, and disrupt the balance between the correct and mismatched DNA synthesis (Kunkel, 2004). There are several endpoints to evaluate the effects of environmental genotoxicants such as frequencies of gametes loss due to cell death, mortality of embryo due to lethal mutations, abnormal development, detection of tumour formation, and rate of mutation frequencies in the gene pool of exposed organisms (Anderson and Wild, 1994).

Test animals that are widely used for ecological risk assessment are mostly rat, mice, and hamster to extrapolate the results to human, however the use of aquatic representatives such as daphnia, algae or fish model are more appropriate to demonstrate environmental monitoring. Genotoxins are responsible for genetic damage in aquatic organisms, particularly in fish, causing abnormal development, reduced growth, and mortality in embryos, larva and adults, and development of cancer thereby affecting fitness in fish population. Genotoxicity in fish also affects the economy of fish production and indirectly cause risk to human health as fish reaches through the food chain. Recently, fish is used as ecogenotoxicological model because fish can perform similar response to genotoxicants like that of higher vertebrates. Fish are more sensitive to the changes in the immediate environment, and also possess well-developed adaptive mechanisms by the biotransformation of toxicants through cytochrome-P450-dependent oxidative metabolism (Hebling *et al.*, 1999). Thus eco-genotoxicology is one of the appropriate measures for the early detection and monitoring of genotoxins in aquatic environment where fish are used as genetic model.

## 1.4 Nano-genotoxicology

There are many reasons for the concern that nanoparticles-induced genotoxicity are associated with different mechanisms. In general, genotoxicity of nanoparticles occur mainly by two mechanisms namely primary and secondary genotoxicity. The direct nano-specific effect of the particles are termed as primary genotoxicity whereas the induction of toxicity occurs through other mode of actions such as by the generation of reactive oxygen species are referred as secondary genotoxicity (Schins and Knaapen, 2007). Evidence suggests that nanoparticles can enter into the cell nucleus either through the nuclear pore complex or by direct interaction with DNA during mitosis (Nabiev *et al*., 2007). Besides, the localization of nanoparticles into the nucleus and DNA interaction depends on the size and charge of the particles. The indirect mechanism or secondary genotoxicity includes the stimulation of cells to release reactive oxygen species or induction of cytochrome-P450 enzymes involved in electron transport chain thereby impair cellular DNA repair (Magdolenova *et al*., 2012). Another possible mechanism of nanoparticles induced genotoxicity is by the interaction with the mitotic spindle apparatus, disruption of centrioles and their associated proteins to result in the loss or gain of chromosomes in the daughter cells (Sargent *et al*., 2010).

Organization for Economic Co-operation and Development (OECD) has established several regulatory test batteries for genotoxicity testing *in vivo* and *in vitro*. Nanoparticles are known to induce substantial amount of DNA damage thereby inducing genotoxicity. The most commonly used genotoxicity assays of nanoparticles includes comet assay and analysis of micronuclei *in vitro*, however the assay was also developed to test in several organisms, including fish. Chromosomal aberrations, cell transformation assays, bacterial and mammalian mutagenicity tests are the other applicable methods for testing nanoparticles induced genotoxicity. Administration route, selection of target tissues, dosage and duration, size and charge of the nanoparticles are some factors that affect the uptake of nanoparticles in organisms exposed. Thus bacterial or mammalian cells are recommended for easy comparison and for the positive findings of nanoparticles-induced genotoxicity (Braakhuis *et al*., 2015).

## 1.5 Strategy for genotoxicity tests

Genotoxicity studies can be performed on either *in vivo* or *in vitro* model systems, or both. *In vivo* genotoxicity studies use both terrestrial and aquatic organisms as animal models whereas *in vitro* studies are conducted in cell lines of different origins. There are different endpoints for *in vivo* and *in vitro* studies hence the genotoxicity test batteries also differ. The commonly used *in vivo* methods are chromosomal aberration test, sister chromatid exchange assay, erythrocyte micronucleus test and comet assay. Chromosomal aberration or chromosomal mutation occurs as a result of structural or numerical abnormalities in one or more chromosomes, and may affect more than a single gene, which finally leads to genetic instability (Rieger *et al*., 1968; Santaguida and Amon, 2015). Karyotyping is the technique used for the detection of chromosomal anomalies such as deletions, substitutions, inversions, insertions, translocations, rings, breaks and dicentric chromosomes. Besides the structural aberrations, aneuploidy in animals can also be detected using karyotyping, which includes gain or loss of single or a pair of chromosome. The major sources for the formation of chromosomal aberrations include exposure to ionizing radiations, genotoxins or other related pollutants (Snustad and Simmons, 2015). Thus chromosomal aberration is considered as an important biomarker in toxicological studies and is evaluated using terrestrial and aquatic animal models.

Sister chromatid exchange occurs during DNA replication in which two sister chromatids break and rejoin thus physical exchange of regions occurs in the duplicated chromosomes of the parental strands. It is highly conserved and error-free process because it is not generally altered during reciprocal interchange by homologous recombination (Wilson and Thompson, 2007). Exchange of sister chromatids can be visualized in growing cells in the presence of 5'-bromo-deoxyuridine for two rounds of DNA replication followed by collecting metaphase spreads on glass slides, and using appropriate stain. Sister chromatid exchange is also considered as a classic toxicology assay for genotoxicity as it facilitate in detecting alterations in biochemistry essential for cellular homologous recombination (Stults *et al*., 2014).

*In vitro* studies also use similar endpoints like chromosomal aberration test, sister chromatid exchange assay, erythrocyte micronucleus test and comet assay for genotoxicity analysis. Besides, OECD has approved various standard protocols for *in vitro* genotoxicity studies. It includes bacterial reverse mutation test or Ames test, mammalian chromosome aberration test, mammalian cell gene mutation test, mammalian erythrocyte micronucleus test, mammalian bone marrow chromosome aberration test, and genetic toxicology tests using reverse assay in *Escherichia coli*, sex-linked recessive lethal test in *Drosophila melanogaster*, dominant lethal test in rodents and sister chromatid exchange assay in mammalian cells (Evans, 1976; Galloway *et al*., 1987; Aaron *et al*., 1994; Clements, 2000; Mortelmans and Zeiger, 2000).

Although ranges of assay methods are available for genotoxicity analysis, erythrocyte micronucleus assay and comet assay are the popularly used techniques both *in vivo* and *in vitro* studies. The simplicity, reliability and sensitivity of the test protocol for detecting DNA damage makes the assay methods more popular in various organisms as well as in cell cultures. In the present chapter, genotoxic potential of the selected nanoparticles was evaluated *in vivo* and *in vitro* using micronucleus test and comet assay. The teleost fish, *Oreochromis mossambicus* is used as animal model for *in vivo* genotoxicity tests by emphasizing ecological risk assessment of exposed nanoparticles. Simultaneously, *in vitro* studies are also performed in human peripheral blood to evaluate the direct effects of nanoparticles.

### 1.5.1 Micronucleus test in aquatic organism

Multiple ranges of contaminants from different sources, including nanoparticles end up in aquatic ecosystem causing multiple consequences at organ or organism level to ecosystem level hence the health and safety of aquatic organisms are of growing concern. Among the various contaminants, chemicals possessing carcinogenic, mutagenic and teratogenic properties are most hazardous as the effects are beyond individual level and pass into several generations. A number of fish species are used for genotoxicity studies like carcinogenicity (Metcalfe, 1988) and teratogenicity of chemicals (Wisk and Cooper, 1990), as fish cells are

unique and sensitive to respond to pollutants and have low rate of repair mechanism (Yang *et al.*, 1990; Stegeman and Lech, 1991). Micronucleus test using fish erythrocyte is the most suitable and integrated genotoxicity test in aquatic organisms because it forms an index of genetic damages accumulated throughout the life span of a cell (Bolognesi and Hayashi, 2011). Micronuclei are formed by the condensation of acentric chromosomal fragment or a whole chromosome during the process of cell division. After DNA damage, the event of micronuclei formation is expressed at different times depending upon the cell cycle kinetics of the organism and the mechanism by which DNA damage is induced (Heddle *et al.*, 1983; Bolognesi and Hayashi, 2011). The chromosome without centromere are not incorporated into the daughter nuclei formed after anaphase which are left behind as small nuclear portions or micronuclei with size ranging from one-third to one-twentieth of the normal nuclei (Heddle, 1973; Schmid, 1975).

Metaphase analysis in fish by performing chromosomal aberration and sister chromatid assays are laborious, and provide only low levels of DNA, large number of small chromosomes and low mitotic activity (Ojima *et al.*, 1976). Hence, micronucleus test has got wide acceptability in genotoxicity studies since the test can be done in any proliferating cell population without karyotyping. In micronucleus test, along with micronuclei certain other nuclear anomalies are also considered as genotoxicity endpoints. Frequently occurring nuclear anomalies in fish includes formation of nuclear buds, nucleoplasmic bridges, binucleated cells, blebbed, lobed or notched nucleus, deformed nucleus, vacuolated nucleus, karyolytic nuclei and so on (Bolognesi *et al.*, 2006). The scoring criteria for micronuclei and other nuclear anomalies was first introduced by Carrasco *et al.* (1990), which was widely accepted and applied even in recent genotoxicity studies. In the present study, genotoxicity of selected nanoparticles are evaluated using fish as a model organism. Fish are non-target organisms of nanoparticles that are exposed unintentionally from point and non-point sources. Humans are direct consumers of fish so nanoparticles may reach through the food chain. Besides, nanoparticles are abundant in various personal care products and biomedical devices

hence, human are also direct targets. Thus the present study evaluated the toxic responses of selected nanoparticles in human peripheral blood cells.

### 1.5.2 Micronucleus test in human blood cells

Micronucleus test can be performed in cultured cells of any organism including human and other mammals. In case of human micronucleus test erythrocytes are not used because human RBC is devoid of nucleus. Hence, nucleated blood cells like lymphocytes are commonly used for micronucleus test. Moreover, while performing *in vitro* studies, the cell cycle is interrupted using suitable mitogens and the formation of micronucleus is examined at different stages of cell cycle for concluding the mechanism of genotoxicity. Human micronucleus test are usually carried out by a technique called cytokinesis block micronucleus (CBMN) assay, in which the cytokinesis is blocked using cytokinesis blockers like cytochalasin-B and micronucleus of once divided cells are evaluated (Fenech, 2007). CBMN is an established cytogenetic method for the measurement of chromosome breakage and loss in nucleated cells (Fenech *et al.*, 2003).

The test enables to measure the DNA damage, cytostasis and cytotoxicity. Micronucleus found in binucleated cells alone are considered for DNA damage induced by the exposed chemical in order to prevent the confounding effects caused by scoring errors and altered cell cycle kinetics (Fenech and Morley, 1985; Fenech, 2007). Micronucleus, nuclear bud and nucleoplasmic bridge are the damages usually measured during CBMN assay. In addition, cytotoxicity was measured by taking the ratio of normal and apoptotic or necrotic cells, and cytostatic properties was estimated by nuclear division index (NDI) from the ratios of mono, bi, tri and multinucleated cells (Fenech, 2000, Fenech, 2007). Hence CBMN assay is widely used in pharmaceutical industries, preventive medicine, nutritional studies (Fenech *et al.*, 2005), and in biomonitoring of genotoxic response in human (Bonassi *et al*, 2006; El-Zein *et al.*, 2006). The formation of micronuclei is used as biomarker for the chromosomal aberrations, which forms casual pathway to several diseases, including cancer. Besides toxicants, micronuclei formation also occurs due to lifestyle factors, various diseases and dietary deficiencies.

### 1.5.3 Comet assay or single cell gel electrophoresis assay

Single cell gel electrophoresis assay, otherwise called comet assay, was first developed by Ostling and Johanson in 1984 to analyze the DNA damage in cells. The alkaline version of comet assay, the most sensitive version, was introduced to detect even small levels of DNA damages by Singh and co-workers in 1988. Later comet assay was approved by OECD as the suitable method for testing genotoxicity in animals since 30 years (Moller, 2018). Generally in comet assay, the DNA damages in single cells are measured and it is interpreted for genotoxicity endpoints. The major advantage of using comet assay in genotoxicity studies is that it can be used in both *in vitro* and *in vivo* studies. Moreover, comet assay is a high through-put screening assay helpful in differentiating cytotoxicity and genotoxicity in cells and also in distinguishing genotoxic or non-genotoxic carcinogenesis (Tice *et al.*, 2000). The main procedure for comet assay is the alkaline unwinding of DNA followed by lysis, neutralization, electrophoresis and visualization. Thus the genetic damages can be visualized by the appearance of a comet-shaped tail composed of DNA fragments. Tail length, tail percent DNA, Olive moment, tail moment, tail intensity, head diameter are some of the significant parameters used for measuring genetic damage. *In vivo* studies are carried out in the blood or tissues obtained from the test animal, while *in vitro* studies use the cells or cell lines that are initially isolated from the culture media. Comet assay enables to identify the grades of DNA damages including strand breaks formed as a result of incomplete repair mechanism or alkali liable sites, and also used as a marker of oxidative stress in the toxicant-exposed cells (Karlsson, 2010).

The present experiment concentrates on the genotoxic potential of the selected nanoparticles *in vivo* in erythrocytes of the fish, *Oreochromis mossambicus*, and *in vitro* using human peripheral blood lymphocytes. The intensity of genetic damages induced by the nanoparticles was evaluated using micronucleus test and comet assay focusing on the mode of action of the particles.

# Review of Literature

Nucleic acids, DNA and mRNA, along with other cellular components are under the constant risk by the exposure of several environmental toxicants. Structural alterations occur in both genomic and mitochondrial DNA as a result of physical, chemical and infectious agents. Among the genotoxic agents, nanoparticles have evolved recently that are known to alter the structural integrity of DNA, and subsequently leading to heritable or non-mutagenic effects. In genoecotoxicological studies fish is considered as a sensitive bioindicator of toxicants in aquatic ecosystem. Genotoxic effects in fish could lead to genetic diversity in the exposed organism, which can be efficiently eliminated to certain extent by detoxification process or through DNA repair mechanism. The precise mechanism by which nanoparticles induced genotoxicity is unclear as there are two possible ways such as the behaviour and unique physiochemical properties or through reactive oxygen species induction. Extreme small size, unique behaviour and properties makes nanoparticles to easily enter into the cellular components causing cytotoxicity and DNA level damages. The review of literatures helps to explore the knowledge on the genotoxic potential of various nanoparticles at different endpoints in certain range of organisms.

Genotoxicity studies are adopted both *in vivo* and *in vitro*, using suitable animal models and cell lines, respectively. It has been estimated that more than 4000 studies are available in nanotoxicology, in which above 100 studies evaluated both *in vitro* and *in vivo* genotoxicity in various models. In more than 60 studies comet assay was used as the tool for detecting genetic damage whereas 40 studies used micronucleus test (Magdelenova *et al*., 2014). Toxic responses of metal oxide nanoparticles have been reviewed largely both *in vivo* and *in vitro*. In a comparative study using different cell lines such as HEp-2, A549, RLE-6TN and N2a cell lines have reported that silicon dioxide induced genotoxicity characterized by aberrant clusters of topoisomerase I in the nucleoplasm and intranuclear protein aggregates, which inhibited the replication, transcription, and cell proliferation (Chen and von Mikecz, 2005). In another comparative study of different nanoparticles such as silver, molybdenum oxide, aluminum, iron oxide, cadmium oxide, manganese oxide, tungsten and titanium dioxide in rat liver derived cell lines, BRL 3A has reported

silver nanoparticles as highly cytotoxic, molybdenum oxide with moderate cytotoxicity and the remaining nanoparticles exhibited less cytotoxicity (Hussain *et al.*, 2005).

The genotoxicity and cytotoxicity effects of titanium dioxide alone and in combination with ultraviolet A analysed in goldfish skin cells, GFSk-S1 has been observed with significant DNA damage in single as well as combined exposure (Reeves *et al.*, 2008). Intrinsic genotoxic and cytotoxic potential of titanium dioxide nanoparticles evaluated in fish gonadal cell line, RTG-2, derived from rainbow trout, *Oncorhyncus mykiss* has showed reduction in lysosomal integrity, DNA damage, strand breaks and cytotoxicity (Vevers and Jha, 2008). Cobalt nanoparticles exposed to peripheral blood leukocytes has been shown to induce genotoxicity, which was evidenced by the increased frequency of micronuclei and dose-dependent increase in the percent of tail DNA (Colognato *et al.*, 2008).

The genotoxic effects of aluminium oxide nanoparticles exposed at 500, 1000 and 2000 mg/ kg body weight in female Wistar rats showed significant dose-related increase in percent tail DNA and frequency of micronuclei (Balasubramanyam *et al.*, 2009). Genotoxic and ecotoxic responses of three nanoparticles namely cerium dioxide, silicon dioxide and titanium dioxide in the freshwater crustacean *Daphnia magna* and the larva of the aquatic midge *Chironomus riparius* has been shown to induce DNA strand breaks and mortality in both species (Lee *et al.*, 2009). A study on mice model when exposed to titanium dioxide nanoparticles in drinking water has been shown to induce DNA damage, genetic instability and DNA lesions, which was determined using comet assay, micronucleus test, γ-H2AX immuno-staining assay and by measuring 8- hydroxy-2′-deoxyguanosine levels (Trouiller *et al.*, 2009). The ability of silver nanoparticles in the induction of cyto- and genotoxicity has been explored in the cell line of *Oryzias latipes* substantiated the results by chromosomal aberrations and aneuploidy (Wise *et al.*, 2010). Investigation of the relationship between the toxicity and surface chemistry has been evaluated using zinc oxide nanoparticles coated with oleic acid, polymethacrylic acid and medium soaked, has reported that uncoated zinc oxide

nanoparticles induced ROS production, cytotoxicity and genotoxicity whereas surface coating reduced the toxicity of the nanoparticles (Yin *et al*., 2010).

Exposure of titanium dioxide nanoparticles in the human epidermal cells A431 has been shown to induce mild cytotoxic response, oxidative stress, DNA damage and micronuclei formation (Shukla *et al*., 2011). Size of nanoparticles has been shown to influence genotoxicity as evidenced by exposure to different sized silver nanoparticles in cell lines of murine fibroblasts and macrophages as well as in mouse embryonic cell lines. The results revealed that small sized silver nanoparticles exerted severe cytotoxicity, inflammation, genotoxicity and developmental toxicity than the large sized particles (Park *et al*., 2011). In mice model, exposure to five different metal nanoparticles such as silver, iron oxide, maghmite, titanium dioxide and copper oxide has been shown to induce genotoxicity in the liver tissue and induced micronuclei formation in erythrocytes (Song *et al*., 2012).

Genotoxicity of silver nanoparticles of 5 nm size has been assessed using two standard assays namely *Salmonella* reverse mutation assay using *Salmonella* strains of TA102, TA100, TA1537, TA98, and TA1535, and micronucleus assay in human lymphoblastoid TK6 cells. The observation indicated that *Salmonella* reverse mutation assay showed negative result whereas induction of micronuclei was observed only in TK6 cells (Li *et al*., 2012). Cytotoxicity of titanium dioxide nanoparticles was reported in HeLa cells exposed at 2 µg/ ml concentration as revealed by elevated levels of ROS and lipid peroxidation, reduction in antioxidant levels, induction of apoptosis, cell cycle arrest, increased expression of pro-apoptotic proteins and inhibition of anti-apoptotic enzymes (Ramkumar *et al*., 2012).

Genotoxic effects of different forms of titanium oxide nanoparticles such as anatase, rutile, and an anatase–rutile mixture, micrometer-sized-anatase, and polyacrylate-coated nano-titanium dioxide tested in Chinese hamster lung fibroblast-V79 cells showed decreased cell viability and DNA damage in all forms of nanoparticles except in polyacrylate-coated nano-titanium dioxide (Hamzeh and Sunahara, 2013). Several test batteries of cytotoxic, genotoxic, hemolytic and

morphological parameters such as comet assay, PI/Annexin V staining and atomic force microscopy used to prove the cytotoxic effects of titanium dioxide nanoparticles in human lymphocyte cells revealed the induction of genotoxicity occurred in dose-dependent manner (Ghosh *et al.*, 2013). Fresh water algae, when treated with two different concentrations of aluminium nanoparticles has been shown to induce cytotoxicity, morphological changes and cell wall damages (Pakrashi *et al.*, 2013).

In one of the preliminary study, when human mesenchymal stem cells were exposed to aluminium nanoparticles, it has been shown to cause genotoxicity, characterized by decreased membrane potential, down regulation of expression of antioxidant enzyme genes such as superoxide dismutase, glutathione-S-transferase, glutathione reductase and glutathione peroxidase, however upregulated the stress responsive genes namely CYP1A and POR (Alshatwi *et al.*, 2013). Genotoxic and cytotoxic properties of cadmium sulphide quantum dots and silver sulphide coated with methyl polyethylene glycol investigated in a rainbow trout cell line, RTG-2 has revealed cadmium sulphide quantum dots as highly cytotoxic at 10 and 50 µg/ ml concentrations, and exhibited a concentration-dependent genotoxicity after 24 h exposure whereas silver sulphide showed neither genotoxic nor cytotoxic effects (Munari *et al.*, 2014).

The cytotoxic and genotoxic effects of three metal based nanoparticles namely iron oxide, aluminium oxide and copper nanoparticles were evaluated using Ames test, *in vitro* cytotoxicity assay, micronucleus test and comet assay in bacteria and monkey kidney cell lines CHS-20, respectively, which showed all exposed nanoparticles induced cyto- and genotoxicity while copper nanoparticles exhibited the highest toxicity (Sadiq *et al.*, 2014). Dose-dependent increase in DNA damage, protein carbonylation, lipid peroxidation, reduction in the activities of antioxidant enzymes and levels of glutathione has been observed in WAG cell lines when treated with titanium dioxide and zinc oxide nanoparticles (Dubey *et al.*, 2015). Repeated oral administration of silicon dioxide, silver and iron oxide nanoparticles have been shown to induce genotoxicity in rat model characterized by DNA damage and cellular instability (Yun *et al.*, 2015). Titanium dioxide nanoparticles exposed at

1 and 10 µg/ L for 5, 7, 14, 21 and 28 days has been shown to induce cytotoxicity, genotoxicity and carcinogenic response in zebrafish *in vitro* and *in vivo* (Rocco *et al*., 2015). In another study, fish exposed to two different doses of titanium dioxide nanoparticles at 1.5 and 3 µg/ L concentrations intraperitoneally showed genotoxicity, and potential cytotoxicity by causing DNA damage, induction of micronuclei and other erythrocyte nuclear abnormalities along with decreased erythrocyte viability in the marine fish *Trachinotus carolinus* (Vignardi *et al*., 2015).

Occurrence of micronuclei and nuclear abnormalities in peripheral erythrocytes, and genomic instability in muscle tissue has been observed after the co-exposure of titanium dioxide and cadmium chloride nanoparticles at 1 and 0.1 mg/ L concentrations in the marine European sea bass *Dicentrarchus labrax* thereby reporting genomic, DNA and chromosomal damage (Nigro *et al*., 2015). Cytogenotoxicity studies of zinc oxide nanoparticles has elucidated the toxic effects in the form of primary DNA damage as measured with alkaline comet assay suggesting the increase of cytogenetic damage in cultured human lymphocytes, which could possibly become mutation or chromosomal aberrations (Branica *et al*., 2016). Genetic toxicity of engineered silica nanoparticles has been screened using 3D *in vitro* skin model in human B cells has revealed decrease in cell viability and induction of genotoxicity (Wills *et al*., 2016). Silver nanoparticles exposed at 400 and 800 µg/ L for seven days has been shown to induce DNA damage as evidenced by single cell gel electrophoresis, and documented as dose-dependent genotoxic effects in the liver of *Labeo rohita* (Sharma *et al*., 2016). Genotoxic effects of zinc oxide nanoparticles has revealed that the changes in the expression levels of stress-related genes namely p53, rad51, mt2 analyzed using qRT-PCR and the related DNA strand breaks was due to the induction of stress-related genes in zebrafish (Boran and Ulutas, 2016).

Comet and micronucleus assays revealed the exposure of iron oxide nanoparticles ranging from 4.7 to 74.4 mg/ L concentrations for 96 h induced DNA lesions with associated underrepresentation of RNA biogenesis, translation, ribosomes, and several metabolic processes in liver cells of zebrafish (Villacis *et al*., 2017). Differential genotoxic and mutagenic effects of iron oxide nanoparticles has

been reported in the female guppy *Poecilia reticulate* exposed at environment relevant concentration for 3, 7, 14 and 21 days. The assessment reported that long-term exposure of iron oxide nanoparticles increased the DNA damage and the total erythrocyte nuclear abnormalities, indicating a higher induction rate of clastogenic and aneugenic effects in the erythrocytes of fish (Qualhato *et al*., 2017). Human keratinocytes HaCaT cell line on exposure to well-characterized 30 nm citrate coated silver nanoparticles at 10 and 40 µg/ mL concentrations has been shown to reduce cell viability and proliferation, decreased nuclear division index but increased DNA fragmentation and damage, induced the frequency of micronuclei formation and cytostaticity (Bastos *et al*., 2017).

Silver nanoparticles exposed at 10 and 25 µg/ L concentration has been shown to cause excessive metal burdens, alterations in oxidative stress markers and genotoxicity including DNA damage in the gill, liver and brain tissues of the juvenile fish *Piaractus mesopotamicus* (Bacchetta *et al*., 2017). In another study, a dissimilar cytotoxic effect has been observed when metallic nanoparticles such as gold, silver and platinum exposed in the human bronchial epithelial cells (Lebedova *et al*., 2018). Similarly exposure of titanium dioxide nanoparticles at 1 to 100 µg/ ml concentrations in gill cell lines RTL-W1 and RTG-W1 has reported non-cytotoxic effects in rainbow trout, *Onchorynchus mykiss* (Lammel and Sturve, 2018). However, exposure to nano, micro and crystalline forms of titanium dioxide increased the frequency of micronucleus formation, induced DNA strand breaks and 8-oxo-7,8-dihydro-2′-deoxyguanosine in the peripheral blood mononuclear cells, mixed population of lymphocytes and monocytes (Andreoli *et al*., 2018). Gold nanoparticles exposed at 0.5 and 50 µg/ L concentration for 96 h demonstrated increase in DNA strand breaks in red blood cells of fish, *Sparus aurata* (Teles *et al*., 2018).

The literatures reviewed represented different mechanism of nanogenotoxicity in different animal models and cell lines. Genotoxic effects of nanoparticles vary in different species thereby stating that the mechanisms behind the induction of genetic damage are influenced by its unique physicochemical properties.

# Materials and Methods

### 3.1 Genotoxicity tests in the erythrocytes of *Oreochromis mossambicus*

#### 3.1.1 Micronucleus test

Blood of fish was collected by cardiac puncture, and used immediately for the genotoxicity tests. Micronucleus test was performed according to the method of Heddle (1973) and Schimd (1975). Blood smear of fish was prepared by mixing a drop of blood with a drop of foetal bovine serum (FBS) in a clean glass slide. Thin smear was prepared which was air dried. After drying, the slide was fixed in absolute methanol for 10 min then stained using 5% Giemsa for 10 min, dried again and used for scoring nuclear and cellular anomalies. Scoring was performed according to the procedure of Carrasco *et al.* (1990) with slight modification. A total of 1000 cells were scored from both control and experimental groups, which was viewed under the microscope at 100x magnification.

#### 3.1.2 Comet assay *in vivo*

Comet assay was performed according to the method of Singh *et al.* (1988) with slight modification. Microscopic slides were cleaned using alcohol and cleared in flame. Then the slides were frosted and coated with 1% normal melting point agarose (NMA) and stored at $4^oC$. Whole blood sample (30 µl) was mixed with 50 µl of 0.5 % low melting point agarose (LMPA) and spread uniformly over the first layer of pre-coated agarose. Finally, NMA (80 µl) was used as a final protective layer. After each step, the slides were covered with cover slip and incubated at $4^oC$ for 10 min to allow agarose to set. After setting, the slides were placed in cold and freshly prepared lysing solution (NaCl, 2.5 M; $Na_2EDTA$, 100 mM; Tris 10 mM and SDS at pH 10) followed by DMSO (10 %) and Triton-X 100 (1 %) and incubated for 2 h. After lysis, slides were placed in electrophoresis buffer (NaOH and $Na_2$ EDTA, 300 mM at pH 13) for 20 min to allow unwinding of DNA.

Electrophoresis was conducted in the same buffer by applying an electric current of 0.8 V/cm for 20 min. After electrophoresis, slides were washed in neutralization buffer (0.4 µL Tris, pH 7.5) for three times, each 5 min. Slides were then dried and stained with 50 µl ethidium bromide and observed under Epifluorescent inverted microscope (Olympus CKX41) using green filter (Excitation

filter BP480-550C) and the images were captured using C-mount camera (Optika pro5 CCD camera). Randomly 50 cells were analysed from each group using software (Comet imager V 2.2. 1, MetaSystems, GmbH, Germany). Tail length and % tail DNA are the parameters selected for evaluating DNA damage. According to the percent of DNA in the Comet tail, damages are categorized under 5 grades. Grade 0 - no damage (% tail DNA was less than 5%), grade 1 - slight damage (% tail DNA was within 5–20%), grade 2 - medium damage (% tail DNA was within 20–40%), grade 3 - high damage (% tail DNA was within 40–95%), and grade 4 - severe damage (% tail DNA was more than 95%). Then the overall score was calculated by using the formula as given below:

Total score = (% of cells in grade 0 X 0) + (% of cells in grade 1 X 1) + (% of cells in grade 2 X 2) + (% of cells in grade 3 X 3) + (% of cells in grade 4 X 4)

## 3.2 Genotoxicity tests in human peripheral lymphocytes

### 3.2.1 Collection of human blood

According to OECD guidelines, peripheral blood must be obtained from non-smoker, healthy volunteer below 30 years, confidentially known as not exposed to ionising radiations, mutagenic agents, and drug or chemical therapy, and also with no history of chromosomal fragility or viral infections. In the present study, 5 ml of own peripheral blood was drawn to make triplicates of each treatment group.

### 3.2.2 Trypan blue exclusion assay

The cell viability was determined by trypan blue exclusion test as described by Strober (2015). To demonstrate, the dead cells intake the dye and stains blue, whereas viable cells exclude dye and did not intake stain. Briefly, blood cells were cultured in 24 well plates in Hikaryo XL (HiMedia) ready mix media. Nanoparticles stock solutions were prepared at a concentration of 500 µg/ ml. From the stock solution, different concentrations of nanoparticles suspensions such as $Al_2O_3$-NPs at 5, 10, 15, 20, 25, 30, 35, 40 µg/ ml concentrations; $Fe_3O_4$-NPs at 30, 45, 60, 75, 90, 105, 120 µg/ ml concentrations; $SiO_2$-NPs at 10, 20, 40, 60, 80, 100, 120 and 140 µg/ ml concentrations; and $TiO_2$-NPs at 20, 40, 60, 80, 100, 120, 140 and 160 µg/ ml

concentrations were added to the culture, and viability was determined at 3 h and 24 h. According to 50 percent viability of cells observed, the half-maximal inhibitory concentration or $IC_{50}$ values of four nanoparticles were determined. In the present study, one-third, one-half and one-fourth of $IC_{50}$ values of each selected nanoparticles were chosen as three sublethal concentrations, respectively, and used for all genotoxicity evaluation in human peripheral lymphocytes.

### 3.2.3 Cytokinesis block micronucleus (CBMN) assay

The cytokinesis-block micronucleus assay was done according to the method of Fenech (2007). Culture of blood were set up by mixing 0.5 ml of heparinised whole blood in 4.5 ml of Hikaryo XL (HiMedia) ready mix media and maintained at $37^{o}$C. Selected test concentrations of the nanoparticles, along with the negative and positive controls, were used to treat peripheral blood cell culture. The CBMN assay was performed by adding the nanoparticles or mitomycin C, positive control, at $44^{th}$ h and cytochalasin B at $48^{th}$ h to demonstrate the frequency of genotoxic effects such as micronucleus (MNi), nucleoplasmic bridges (NPBs) and nuclear buds (Nbuds). Carbon dioxide was released at every 24 h and the cells were harvested at the end of 72 h. The cells were then treated hypotonically with cold 0.075 M KCl for 5-7 min, including centrifugation and followed by fixation in methanol-acetic acid (3: 1) mixture. Subsequently, slides were stained with Giemsa solution (4%) and scored for abnormalities following the criteria as specified by Fenech *et al.* (2003). Photomicrophotographs of selected nuclear anomalies were taken using Carl Zeiss Axioscope 1.

### 3.2.4 Scoring for nuclear abnormalities by cytokinesis block proliferation index (CBPI)

DNA damage is scored specifically in formerly divided binucleated cells that include micronucleus, nucleoplasmic bridges and nuclear buds. A minimum of 1000 binucleated cells were analyzed for each individual using the standard scoring criteria (Fenech *et al.*, 2003). Cytokinesis block proliferation index was performed to check the cell cycle kinetics and it was calculated using the frequencies of mono-,

bi-, tri- and tetra-nucleated cells obtained/ 500 cells according to the standardized formula (Eastmond and Tucker, 1989).

CBPI = [MI + 2MII + 3 (MIII +MIV)]/[(MI + MII) + (MIII +MIV)]

MI - mononucleated cell, MII - binucleated cell, MIII - trinucleated cell, MIV - tetranucleated cell.

### 3.2.5 Comet assay *in vitro*

Comet assay was performed in human peripheral blood lymphocytes according to the method of Singh *et al*. (1988) as described in section 3.1.2. Finally slides prepared were dried and stained with 50 µl acridine orange and images were observed and captured using Carl Zeiss Axioscope camera with green filter (Excitation filter BP480-550C). Randomly 50 cells were analysed from each group using software (Comet imager V 2.2. 1, MetaSystems, GmbH, Germany).

## 3.3 Statistical analysis

Statistical analyses were performed using statistical package SPSS 17.0. One-way analysis of variance (ANOVA) using Duncan's multiple range test as post-hoc test was employed to compare mean differences between different concentrations within the durations. Statistical significance was set at $p<0.05$ against the control group, and are denoted as asterisk (*) symbol in the Tables and Figures. Data are presented as mean ± SD for triplicates in each concentration groups.

# Results

## 4.1 Effects of selected nanoparticles on genotoxicity tests in erythrocytes of the fish, *Oreochromis mossambicus*

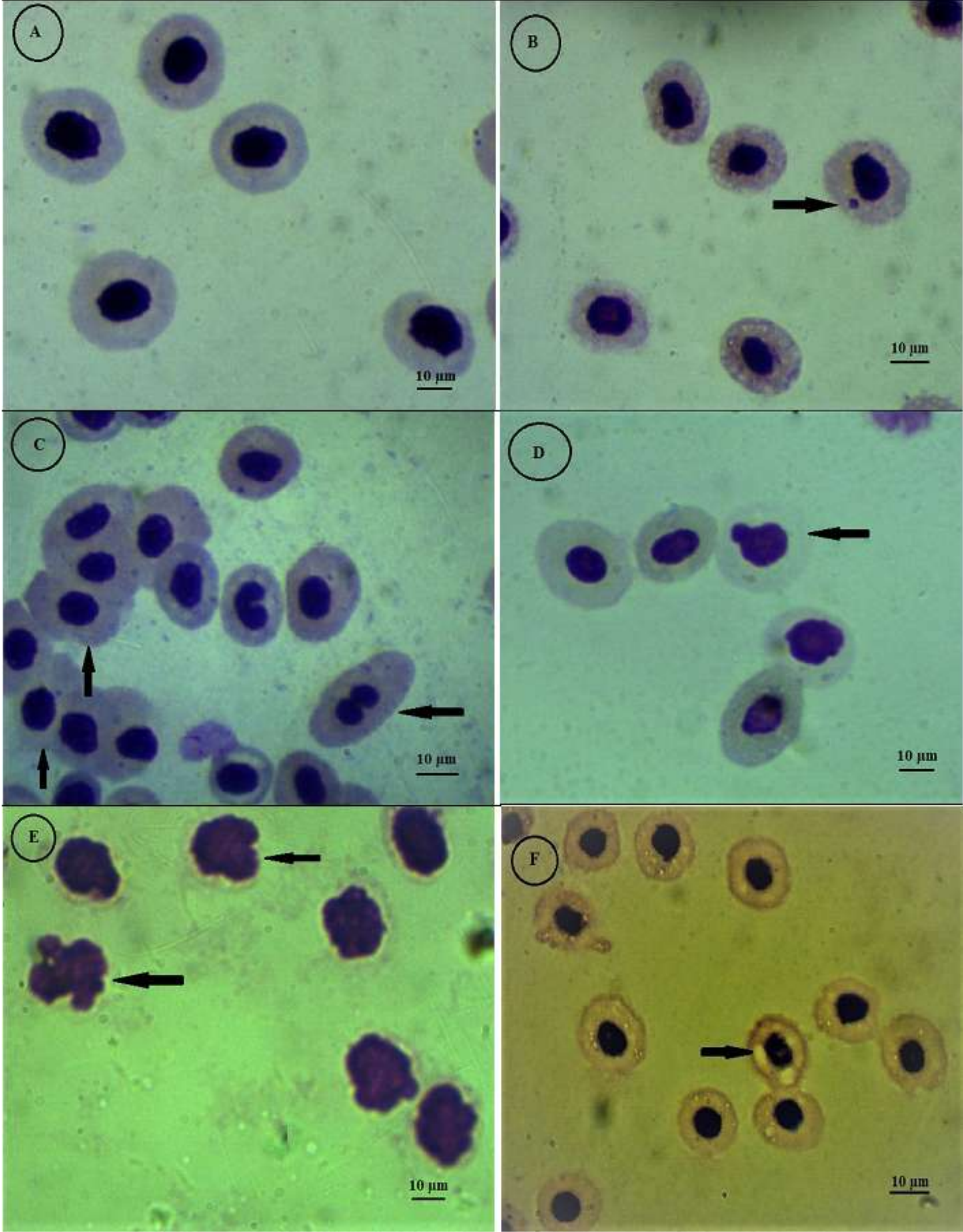
### 4.1.1 Effects of selected nanoparticles on micronucleus test

The frequencies of micronuclei along with other nuclear anomalies such as binucleated cells, nuclear bud, irregular nuclei, and cellular anomalies such as sticky cells and vacuolated cytoplasm was observed in the erythrocytes of nanoparticles exposed groups (Table 1; Figure 1). Short-term and long-term exposure of selected nanoparticles namely $Al_2O_3$-NPs at 4 mg/ L, $Fe_3O_4$-NPs at 15 mg/ L, $SiO_2$-NPs at 12 mg/ L and $TiO_2$-NPs at 16.4 mg/ L showed significant ($P<0.05$) increase in nuclear and cytoplasmic abnormalities in the erythrocytes of the fish, *Oreochromis mossambicus* in time-dependent manner when compared to the control group (Table 1; Figure 1).

**Table 1** **Effects of selected nanoparticles on micronuclei formation and other nuclear and cytoplasmic abnormalities in erythrocytes of the fish, *Oreochromis mossambicus* (Mean ± SD; * $P<0.05$ against the control group)**

| Treatment groups | | Micronucleus | Binucleated | Nuclear Bud | Irregular nucleus | Sticky cells | Vacuolated cytoplasm |
|---|---|---|---|---|---|---|---|
| $Al_2O_3$-NPs (4 mg/ L) | Control | 2 ± 1.41 | 5 ± 0.01 | 2 ± 0.01 | 0.5 ± 0.7 | 1 ± 0.01 | 1.5 ± 0.7 |
| | 24 h | 4 ± 0.03* | 4.5 ± 0.16 | 4 ± 0.01 | 6.5 ± 0.11* | 3.5 ± 1.06* | 4 ± 0.01* |
| | 72 h | 6 ± 1.14* | 4 ± 1.12 | 6 ± 1.01* | 9.5 ± 0.21* | 5.5 ± 0.45* | 3 ± 0.1* |
| | 96 h | 8 ± 0.02* | 5 ± 0.34 | 8 ± 0.41* | 15 ± 0.03* | 8 ± 0.13* | 7 ± 0.01* |
| | 15 days | 15.5 ± 0.70* | 8 ± 0.02 | 14 ± 1.34* | 16.5 ± 0.7* | 10 ± 0.05* | 14 ± 2.82* |
| | 30 days | 29.5 ± 1.13* | 11 ± 0.21* | 20 ± 0.04* | 43 ± 0.56* | 19 ± 1.44* | 16 ± 1.21* |
| | 60 days | 52 ± 2.82* | 15 ± 1.65* | 23.5 ± 0.63* | 106 ± 1.41* | 20.5 ± 0.6* | 24.5 ± 0.77* |
| $Fe_3O_4$-NPs (15 mg/ L) | Control | 2 ± 1.41 | 5 ± 0.01 | 2 ± 0.01 | 0.5 ± 0.7 | 1 ± 0.01 | 1.5 ± 0.7 |
| | 24 h | 5.5 ± 0.78* | 5 ± 0.04 | 8.5 ± 0.71* | 7.5 ± 0.66* | 3 ± 0.01* | 8.5 ± 0.67* |
| | 72 h | 8 ± 0.05* | 4.5 ± 0.65 | 11 ± 0.03* | 11 ± 0.07* | 4 ± 0.32* | 11 ± 0.21* |
| | 96 h | 13 ± 0.65* | 7.5 ± 0.55 | 12 ± 0.56* | 14 ± 0.35* | 6 ± 0.07* | 14.5 ± 0.78* |
| | 15 days | 19 ± 2.82* | 9 ± 1.41* | 17.5 ± 0.71* | 17.5 ± 0.67* | 8 ± 0.62* | 19 ± 0.43* |
| | 30 days | 32.5 ± 0.77* | 12 ± 0.07* | 27 ± 0.03* | 26 ± 0.04* | 11 ± 1.12* | 31 ± 1.21* |
| | 60 days | 68.5 ± 0.82* | 19 ± 2.84* | 38 ± 1.71* | 34.5 ± 1.06* | 13.5 ± 1.2* | 36 ± 1.25* |
| $SiO_2$-NPs (12mg/ L) | Control | 2 ± 1.41 | 5 ± 0.01 | 2 ± 0.01 | 0.5 ± 0.7 | 1 ± 0.01 | 1.5 ± 0.7 |
| | 24 h | 11 ± 1.05* | 5 ± 0.01 | 2 ± 1.41 | 3 ± 0.02* | 1.5 ± 0.7 | 12 ± 1.41* |
| | 72 h | 13 ± 0.02* | 8 ± 1.82 | 4 ± 0.05 | 6.5 ± 1.12* | 3 ± 0.01* | 15 ± 0.001* |
| | 96 h | 14 ± 2.82* | 9 ± 1.41* | 4 ± 1.05 | 10 ± 0.01* | 4.5 ± 0.71* | 20.5 ± 0.67* |
| | 15 days | 22 ± 0.07* | 13 ± 1.34* | 7.5 ± 0.67* | 11 ± 0.75* | 6 ± 0.02* | 25 ± 0.43* |
| | 30 days | 38 ± 1.42* | 20.5 ± 0.66* | 8 ± 0.001* | 11± 0.01* | 7 ± 0.45* | 40.5 ± 0.71* |
| | 60 days | 59 ± 0.02* | 20 ± 0.03* | 17.5 ± 0.17* | 17.5± 0.16* | 8 ± 1.42* | 54.5 ± 3.53* |
| $TiO_2$-NPs (16.4 mg/ L) | Control | 2 ± 1.41 | 5 ± 0.01 | 2 ± 0.01 | 0.5 ± 0.7 | 1 ± 0.01 | 1.5 ± 0.7 |
| | 24 h | 3.5 ± 0.67* | 5 ± 0.03 | 6.5 ± 0.12* | 9 ± 0.24* | 3 ± 0.01* | 7 ± 0.18* |
| | 72 h | 6 ± 0.04* | 4.5 ± 0.07 | 7 ± 0.02* | 11.5 ± 0.18* | 3.5 ± 0.11* | 9 ± 0.001* |
| | 96 h | 8 ± 1.41* | 6 ± 1.21 | 10 ± 1.24* | 18 ± 1.12* | 6 ± 0.21* | 12 ± 0.42* |
| | 15 days | 17.5 ± 1.71* | 11 ± 0.77* | 17.5 ± 1.76* | 21.5 ± 1.32* | 7 ± 0.42* | 21 ± 0.01* |
| | 30 days | 30.5 ± 0.81* | 17 ± 0.76* | 28 ± 0.01* | 28.5 ± 0.67* | 12 ± 0.05* | 27.5 ± 2.78* |
| | 60 days | 55 ± 0.35* | 23.5 ± 0.65* | 32 ± 0.27* | 38 ± 1.25* | 16 ± 1.84* | 32.5 ± 0.81* |

**Figure 1** Nanoparticles exposed fish erythrocytes showing A - Normal erythrocytes (control); B - erythrocytes with micronucleus (→); C - binucleated (←) and sticky cells (↑); D - Nuclear bud (←); E - irregular nucleus (←); F - cytoplasmic vacuolization (→)



### 4.1.2 Effects of selected nanoparticles on comet assay in erythrocytes of the fish, *Oreochromis mossambicus*

$Al_2O_3$-NPs, $Fe_3O_4$-NPs, $SiO_2$-NPs and $TiO_2$-NPs exposure for short-term (24, 72 and 96 h) and long-term (15, 30 and 60 days) durations showed significant ($P<0.05$) and time-dependant increase in the tail length and percent tail DNA in the erythrocytes of *Oreochromis mossambicus* (Table 2). The grades of DNA damage and the total scores showed in Table 3 and Figure 2 represents all nanoparticles equally contributed in the induction of DNA damage.

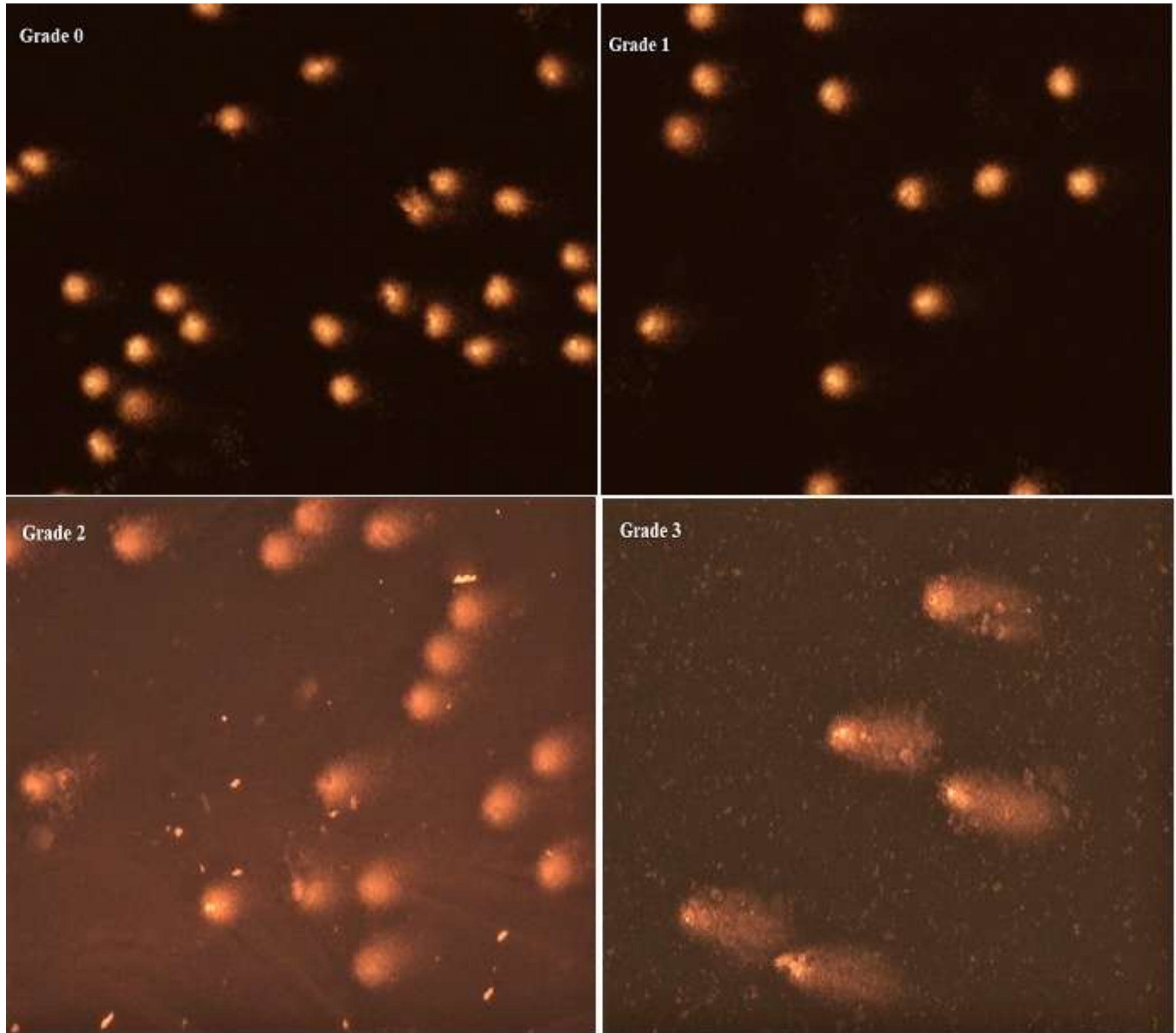
**Table 2 Variation in tail length and tail DNA (%) after nanoparticles exposure in the fish, *Oreochromis mossambicus***
**(Mean ± SD; * significance at P<0.05 against the control group)**

| Treatment groups | | Tail length | Tail DNA (%) |
|---|---|---|---|
| $Al_2O_3$-NPs (4 mg/ L) | Control | 9.71 ± 3.74 | 4.60 ± 1.96 |
| | 24 h | 18.78 ± 3.72* | 13.05 ± 5.00* |
| | 72 h | 24.12 ± 6.30* | 22.26 ± 4.29* |
| | 96h | 33.32 ± 5.26* | 27.96 ± 4.18* |
| | 15 days | 35.85 ± 5.19* | 31.72 ± 3.54* |
| | 30 days | 44.94 ± 6.33* | 35.84 ± 3.76* |
| | 60 days | 86.69 ± 4.43* | 40.20 ± 4.66* |
| $Fe_3O_4$-NPs (15 mg/ L) | Control | 9.71 ± 3.74 | 4.60 ± 1.96 |
| | 24 h | 9.11 ± 1.97 | 13.06 ± 5.89* |
| | 72 h | 18.95 ± 3.64* | 19.00 ± 6.40* |
| | 96h | 29.17 ± 4.58* | 24.76 ± 7.41* |
| | 15 days | 40.14 ± 5.98* | 28.25 ± 6.86* |
| | 30 days | 45.22 ± 5.69* | 32.14 ± 6.57* |
| | 60 days | 82.91 ± 3.81* | 38.81 ± 5.72* |
| $SiO_2$-NPs (12 mg/ L) | Control | 9.71 ± 3.74 | 4.60 ± 1.96 |
| | 24 h | 10.42 ± 3.04 | 9.77 ± 2.38 |
| | 72 h | 15.59 ± 2.93* | 11.17 ± 2.83* |
| | 96h | 21.63 ± 3.64* | 15.59 ± 5.74* |
| | 15 days | 28.05 ± 3.55* | 17.86 ± 3.58* |
| | 30 days | 33.37 ± 2.68* | 20.06 ± 4.49* |
| | 60 days | 41.71 ± 4.07* | 32.93 ± 8.49* |
| $TiO_2$-NPs (16.4 mg/ L) | Control | 9.71 ± 3.74 | 4.60 ± 1.96 |
| | 24 h | 17.39 ± 3.85* | 10.07 ± 3.15* |
| | 72 h | 25.78 ± 3.35* | 13.58 ± 3.59* |
| | 96h | 31.88 ± 3.94* | 18.95 ± 5.88* |
| | 15 days | 43.52 ± 3.43* | 26.26 ± 6.68* |
| | 30 days | 53.87 ± 4.02 * | 32.39 ± 4.45* |
| | 60 days | 78.82 ± 4.93* | 36.62 ± 7.52* |

**Table 3** **Grades of DNA damage in the fish exposed to selected nanoparticles**

| Treatment groups | | Grades of DNA damage | | | | | Score |
|---|---|---|---|---|---|---|---|
| | | **Grade 0** | **Grade 1** | **Grade 2** | **Grade 3** | **Grade 4** | |
| | Control | 43 | 7 | 0 | 0 | 0 | 14 |
| **$Al_2O_3$-NPs (4 mg/ L)** | 24h | 4 | 40 | 6 | 0 | 0 | 104 |
| | 72h | 4 | 14 | 32 | 0 | 0 | 156 |
| | 96h | 4 | 3 | 43 | 0 | 0 | 178 |
| | 15 days | 3 | 32 | 45 | 0 | 0 | 188 |
| | 30 days | 1 | 1 | 18 | 30 | 0 | 254 |
| | 60 days | 0 | 0 | 12 | 38 | 0 | 278 |
| **$Fe_3O_4$-NPs (15 mg/ L)** | 24h | 7 | 43 | 0 | 0 | 0 | 86 |
| | 72h | 7 | 30 | 13 | 0 | 0 | 112 |
| | 96h | 6 | 12 | 32 | 0 | 0 | 152 |
| | 15 days | 4 | 4 | 40 | 2 | 0 | 180 |
| | 30 days | 1 | 2 | 41 | 6 | 0 | 204 |
| | 60 days | 0 | 0 | 33 | 17 | 0 | 234 |
| **$SiO_2$-NPs (12 mg/ L)** | 24h | 5 | 45 | 0 | 0 | 0 | 90 |
| | 72h | 4 | 46 | 0 | 0 | 0 | 92 |
| | 96h | 4 | 35 | 11 | 0 | 0 | 114 |
| | 15 days | 2 | 25 | 23 | 0 | 0 | 142 |
| | 30 days | 2 | 16 | 29 | 3 | 0 | 166 |
| | 60 days | 0 | 4 | 38 | 8 | 0 | 208 |
| **$TiO_2$-NPs (16.4 mg/ L)** | 24h | 7 | 43 | 0 | 0 | 0 | 86 |
| | 72h | 4 | 44 | 2 | 0 | 0 | 96 |
| | 96h | 3 | 12 | 34 | 1 | 0 | 166 |
| | 15 days | 2 | 3 | 42 | 3 | 0 | 192 |
| | 30 days | 0 | 1 | 44 | 5 | 0 | 208 |
| | 60 days | 0 | 2 | 32 | 16 | 0 | 228 |

**Figure 2** **Comet morphology showing different grades of DNA damage after nanoparticles exposure in erythrocytes of *Oreochromis mossambicus***



### 4.2 Effects of nanoparticles on genotoxicity tests in human peripheral blood lymphocytes

#### 4.2.1 Selection of test concentrations using trypan blue exclusion test

The $IC_{50}$ values of each selected nanoparticles, and its respective sublethal concentrations in the human peripheral blood cells were selected based on the cell viability using trypan blue exclusion test (Figure 3) as given below:

$Al_2O_3$-NPs : 20 µg/ ml ($IC_{50}$); and its sublethal concentrations - 15 µg/ ml (three-fourth of $IC_{50}$), 10 µg/ ml (one-half of $IC_{50}$) and 5 µg/ ml (one-fourth of $IC_{50}$).

$Fe_3O_4$-NPs : 75 µg/ ml ($IC_{50}$); and its sublethal concentrations - 56.25 µg/ ml (three-fourth of $IC_{50}$), 37.5 µg/ ml (one-half of $IC_{50}$) and 18.75 µg/ ml (one-fourth of $IC_{50}$).

$SiO_2$-NPs : 60 µg/ ml ($IC_{50}$); and its sublethal concentrations 45 µg/ ml (three-fourth of $IC_{50}$), 30 µg/ ml (one-half of $IC_{50}$) and 15 µg/ ml (one-fourth of $IC_{50}$).

$TiO_2$-NPs : 80 µg/ ml ($IC_{50}$); and its sublethal concentrations - 60 µg/ ml (three-fourth of $IC_{50}$), 40 µg/ ml (one-half of $IC_{50}$) and 20 µg/ ml (one-fourth of $IC_{50}$).

According to the OECD guidelines of genotoxicity testing, minimum of three sublethal concentrations, showing low to moderate toxicity among the whole cell population exposed were selected as test concentrations for the following genotoxicity assays. Along with untreated negative control, mitomycin C (0.2 µg/ ml) was used as positive control in cytokinesis block micronucleus (CBMN) assay, and hydrogen peroxide as positive control in comet assay throughout the experiment.

#### 4.2.2 Effects of nanoparticles on nuclear anomalies in human peripheral blood lymphocytes using cytokinesis block micronucleus assay

Human peripheral blood lymphocytes exposed to sublethal concentrations of nanoparticles such as $Al_2O_3$-NPs, $Fe_3O_4$-NPs, $SiO_2$-NPs and $TiO_2$-NPs showed significant ($P<0.05$) induction of nuclear anomalies such as micronuclei, nucleoplasmic bridge and nuclear buds in concentration-dependent manner (Table 4; Figure 4). However, the formation of nuclear bud occurred only in $Al_2O_3$-NPs at three-fourth of $IC_{50}$ concentration (Table 4). The formation of nucleoplasmic bridge

was found prominent in $Fe_3O_4$-NPs exposed at 56.25 µg/ ml concentration (Table 4). $SiO_2$-NPs treated cultures at highest concentration showed the maximum induction of micronuclei formation when compared to the positive and negative controls (Table 4). In mitomycin C treated positive control group, the frequency of micronuclei formation and other nuclear abnormalities remained highest when compared to the three sublethal concentrations of nanoparticles whereas in the untreated control group there was no remarkable nuclear abnormalities (Table 4). Cytokinesis block micronucleus assay showed a significant ($P<0.05$) concentration-dependant increase in total nuclear anomalies after exposure to the selected nanoparticles (Table 4).

### 4.2.3 Cytokinesis block proliferation index (CBPI) in human peripheral blood lymphocyte exposed to the selected nanoparticles

Culture of human peripheral blood lymphocyte in the selected nanoparticles exposed groups showed increase in the cytokinesis block proliferation index when compared with untreated culture group (Figure 5). Mitomycin C exposed culture group showed significant increase in the index rate, similarly among the selected nanoparticles, $Al_2O_3$-NPs at high sublethal concentration showed significant ($P<0.05$) increase in the proliferation index. However, the other nanoparticles cultured groups such as $Fe_3O_4$-NPs, $SiO_2$-NPs and $TiO_2$-NPs showed concentration-dependent increase in the proliferation index (Figure 5).

**Figure 3 Trypan blue exclusion test showing a - 100 % viable cells and; b - 50 % non-viable cells (←) of human peripheral lymphocytes**

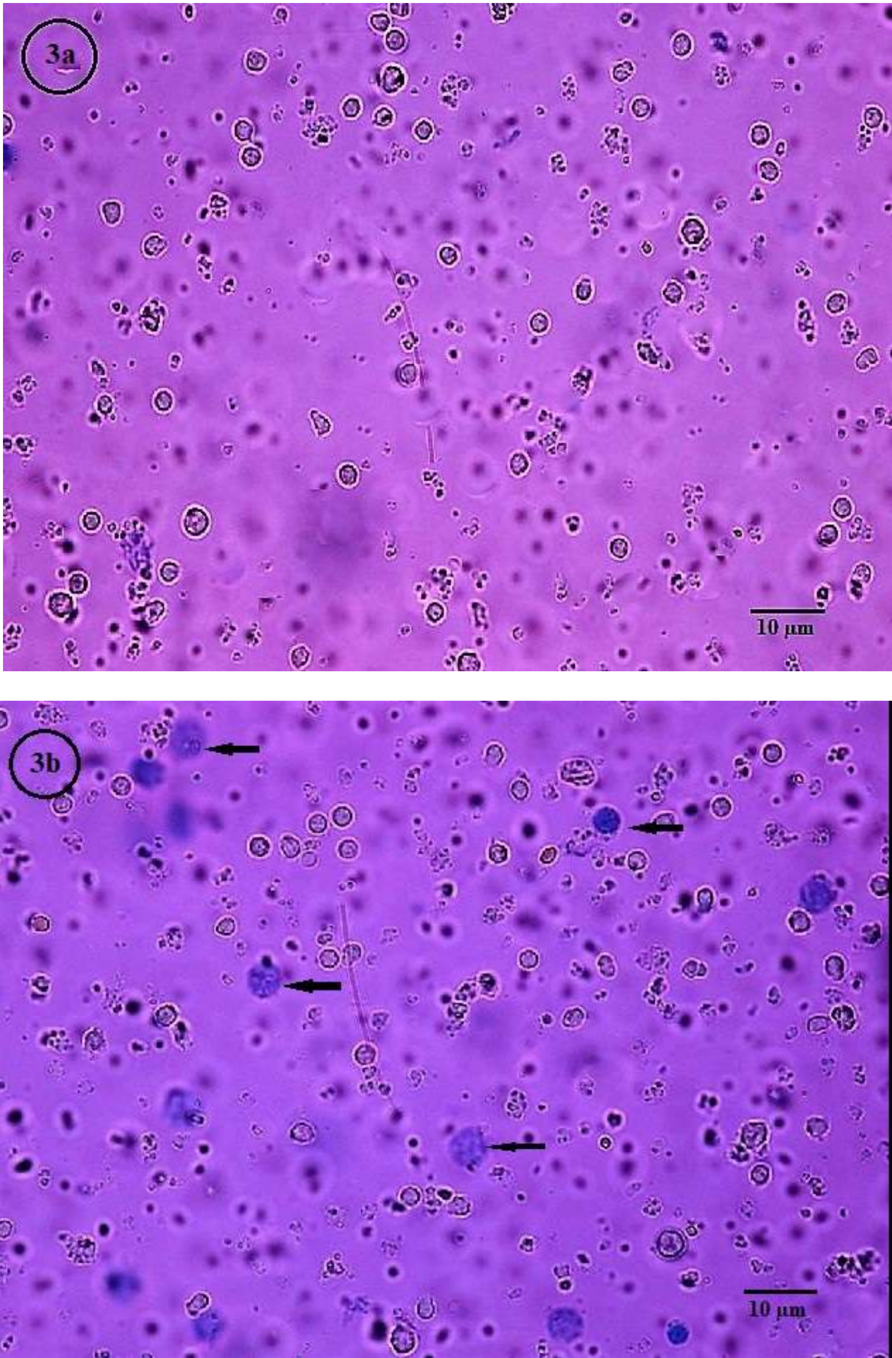


**Table 4** **Effects of selected nanoparticles on micronuclei frequency and other nuclear abnormalities in human peripheral blood lymphocytes *in vitro* (Mean ± SD; * denotes statistically significant at $p<0.05$ against the control; MMC - Mitomycin C)**

| Concentration (µg/ ml) | Micronucleus | Nucleoplasmic bridges | Nuclear buds |
|---|---|---|---|
| **$Al_2O_3$-NPs** | | | |
| Untreated control | 2.33 ± 1.86 | 0.17 ± 0.04 | 0 |
| 5 | 24.0 ± 2.52* | 2.83 ± 0.16* | 0 |
| 10 | 36.3 ± 3.67* | 3.33 ± 0.63* | 0 |
| 15 | 37.5 ± 2.88* | 3.66 ± 0.36* | 0.16 ± 0.08 |
| MMC | 87.50 ± 6.41* | 27.83 ± 2.32* | 14.33 ± 2.34* |
| **$Fe_2O_3$-NPs** | | | |
| Untreated control | 2.33 ± 1.86 | 0.17 ± 0.04 | 0 |
| 18.75 | 28.6 ± 5.92* | 10.3 ± 1.36* | 0 |
| 37.50 | 61.3 ± 6.50* | 13.0 ± 2.28* | 0 |
| 56.25 | 61.8 ± 5.90* | 21.2 ± 3.12* | 0 |
| MMC | 87.50 ± 6.41* | 27.83 ± 2.32* | 14.33 ± 2.34* |
| **$SiO_2$-NPs** | | | |
| Untreated control | 2.33 ± 1.86 | 0.17 ± 0.04 | 0 |
| 15 | 39.5 ± 4.27* | 2.83 ± 0.75* | 0 |
| 30 | 90.33 ± 4.58* | 3.50 ± 0.40* | 0 |
| 45 | 96.16 ± 8.35* | 4.83 ± 0.47* | 0 |
| MMC | 87.50 ± 6.41* | 27.83 ± 2.32* | 14.33 ± 2.34* |
| **$TiO_2$-NPs** | | | |
| Untreated control | 2.33 ± 1.86 | 0.17 ± 0.04 | 0 |
| 20 | 44.5 ± 6.47* | 0.16 ± 0.04 | 0 |
| 40 | 45.5 ± 5.16* | 1.50 ± 0.36* | 0 |
| 60 | 67.2 ± 7.30* | 2.66 ± 0.81* | 0 |
| MMC | 87.50 ± 6.41* | 27.83 ± 2.32* | 14.33 ± 2.34* |

**Figure 4** **Nanoparticles treated human blood peripheral lymphocytes showing A - micronucleus (↓); B - nucleoplasmic bridge (↓); C - nuclear bud (←)**

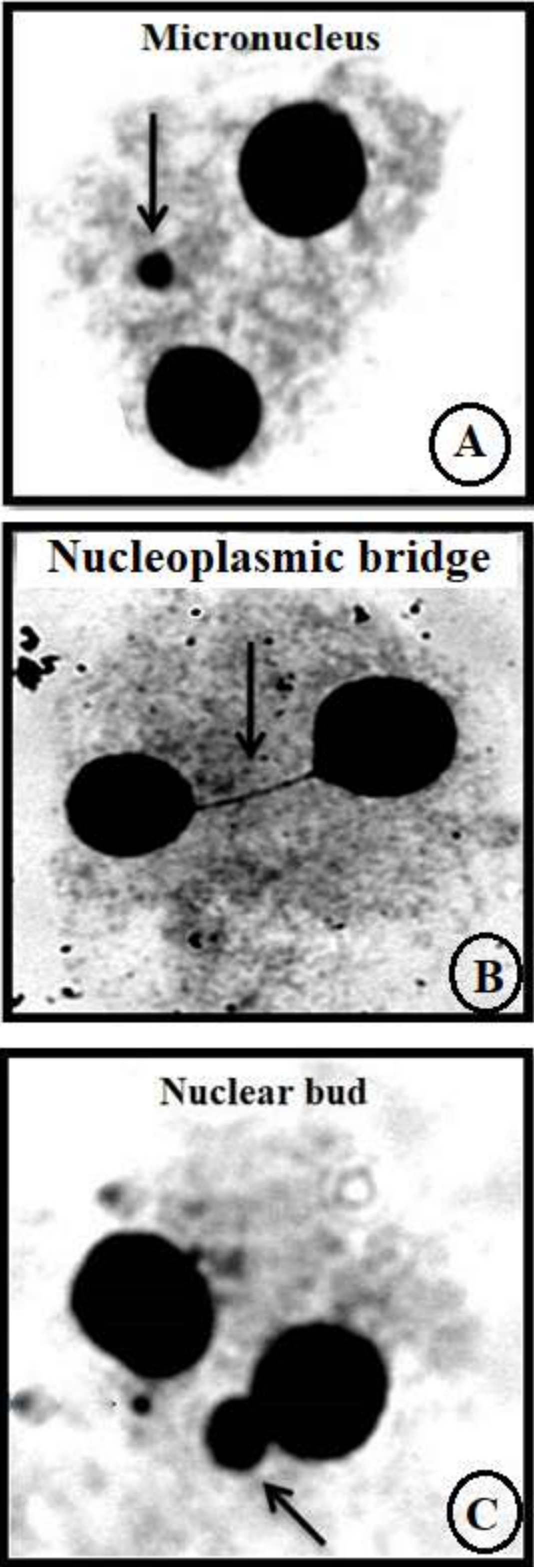
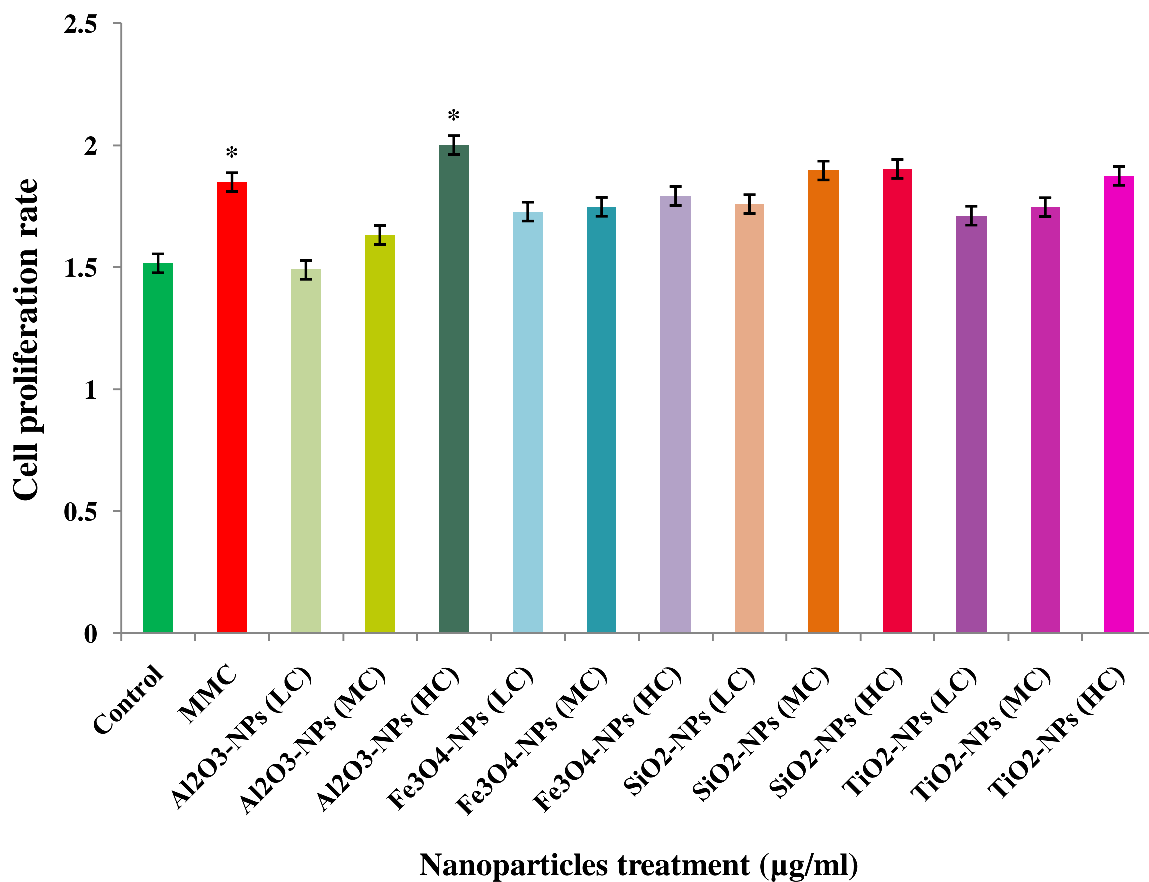


**Figure 5** **Cytokinesis block proliferation index (CBPI) in nanoparticles-treated human peripheral lymphocytes**
**MMC – mitomycin C, positive control**
**HC – High concentration (three-fourth of $IC_{50}$)**
**MC – Medium concentration (one-half of $IC_{50}$)**
**LC – Low concentration (one-fourth of $IC_{50}$)**



### 4.2.4 Effects of nanoparticles on DNA damage in human peripheral blood lymphocytes using comet assay

Sublethal exposure to selected nanoparticles showed alterations in the comet parameters such as tail length and percent tail DNA on the human peripheral lymphocytes. The total score of DNA damage and grades are represented in Tables 5, 6 and Figure 6. The untreated control culture did not show comet tail and the grade of DNA damage was observed as grade 0 (Figure 6). Culture of human peripheral lymphocytes at one-fourth of $IC_{50}$ concentration of the selected nanoparticles showed low grade of DNA damage, but the grade was found above the untreated controls (Figure 6). However, in $TiO_2$-NPs exposed culture group, the grade of DNA damage observed at one-fourth of $IC_{50}$ concentration was grade 1 (Table 6; Figure 6). At one-half of $IC_{50}$ concentration of all selected nanoparticles, the tail length was found increased and the DNA damage observed was grade 1, and in $TiO_2$-NPs exposed culture group showed grade 2 (Table 6; Figure 6). When the concentration of nanoparticles was increased to three-fourth of $IC_{50}$ concentration, the grade of DNA damage was found further increased to grade 2 while $TiO_2$-NPs exposed culture group showed DNA damage as grade 3 (Table 6; Figure 6). In mitomycin C exposed positive control culture group, the DNA damage observed was grade 3, and the results of sublethal exposure to selected nanoparticles showed significant ($P<0.05$) increase in DNA damage in concentration-dependant manner (Table 6).

**Table 5** **Variation in tail length and tail DNA (%) after nanoparticles exposure in human peripheral blood lymphocytes *in vitro***

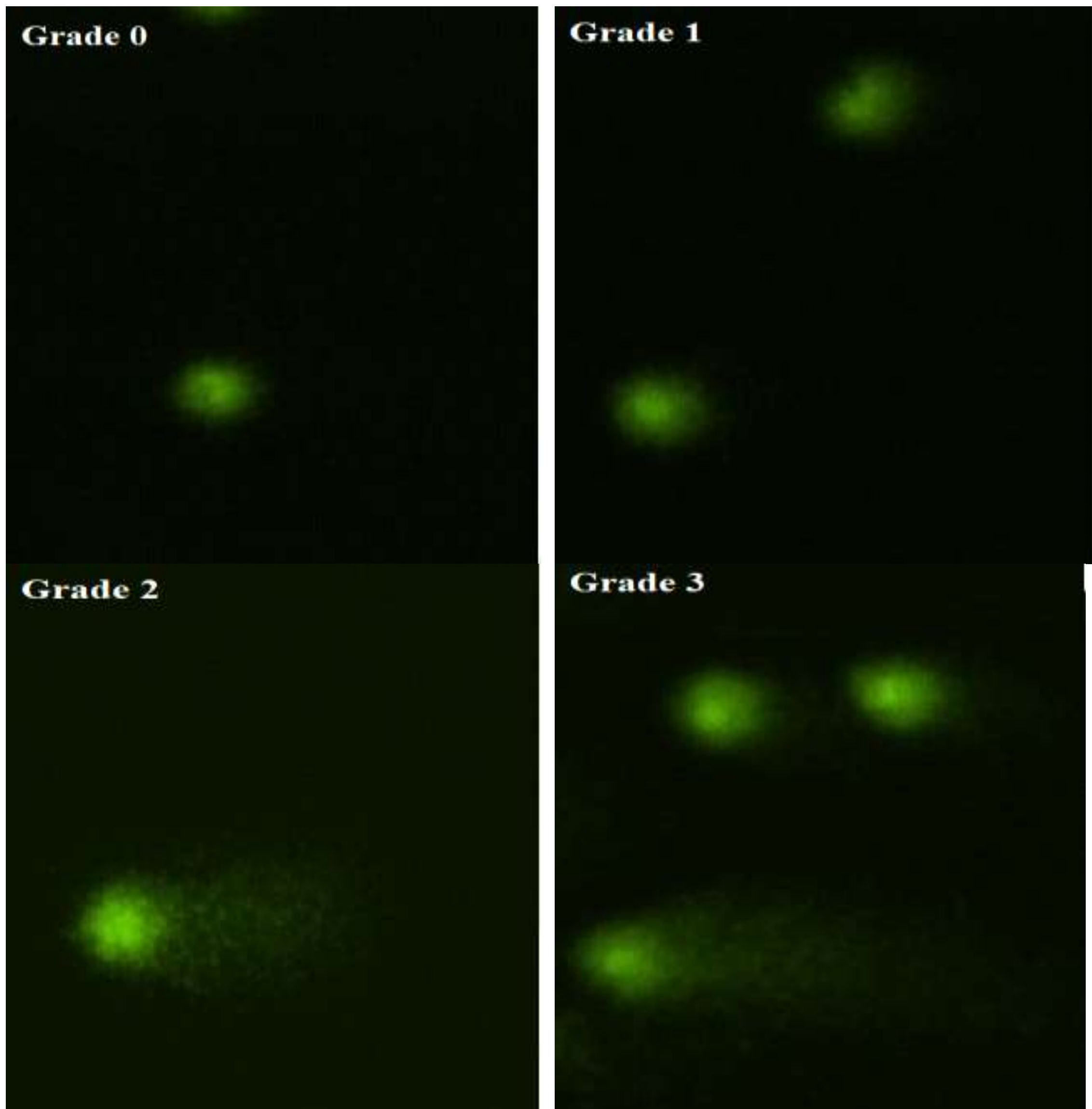
**(Mean ± SD; * denotes statistically significant at $p<0.05$ against the control group; $H_2O_2$ - hydrogen peroxide)**

| **Concentration (µg/ml)** | **Tail length** | **Tail DNA (%)** |
|---|---|---|
| **$Al_2O_3$-NPs** | | |
| Untreated control | 11.13 ± 3.04 | 6.00 ± 1.86 |
| 5 | 14.87 ± 3.87* | 9.34 ± 1.71* |
| 10 | 28.13 ± 3.61* | 13.38 ± 1.89* |
| 15 | 45.74 ± 4.76* | 20.18 ± 2.79* |
| $H_2O_2$ | 92.32 ± 4.62* | 72.03 ± 4.98* |
| **$Fe_2O_3$-NPs** | | |
| Untreated control | 11.13 ± 3.04 | 6.00 ± 1.86 |
| 18.75 | 16.64 ± 3.92* | 15.2 ± 3.18* |
| 37.50 | 33.78 ± 4.36* | 27.9 ± 2.58* |
| 56.25 | 41.70 ± 4.67* | 38.3 ± 4.05* |
| $H_2O_2$ | 92.32 ± 4.62* | 72.03 ± 4.98* |
| **$SiO_2$-NPs** | | |
| Untreated control | 11.13 ± 3.04 | 6.00 ± 1.86 |
| 15 | 22.34 ± 3.94* | 10.57 ± 3.27* |
| 30 | 35.41 ± 4.09* | 19.15 ± 1.27* |
| 45 | 52.33 ± 4.86* | 24.22 ± 1.90* |
| $H_2O_2$ | 92.32 ± 4.62* | 72.03 ± 4.98* |
| **$TiO_2$-NPs** | | |
| Untreated control | 11.13 ± 3.04 | 6.00 ± 1.86 |
| 20 | 33.50 ± 3.97* | 17.69 ± 2.59* |
| 40 | 54.74 ± 3.52* | 30.13 ± 2.73* |
| 60 | 75.60 ± 4.84* | 49.12 ± 1.61* |
| $H_2O_2$ | 92.32 ± 4.62* | 72.03 ± 4.98* |

**Table 6** **Grades of DNA damage in human peripheral lymphocytes exposed to the selected nanoparticles**

| Treatment groups | | Grades of DNA damage | | | | | Score |
|---|---|---|---|---|---|---|---|
| | | Grade 0 | Grade 1 | Grade 2 | Grade 3 | Grade 4 | |
| Untreated control | | 29 | 21 | 0 | 0 | 0 | 42 |
| Hydrogen peroxide | | 0 | 0 | 0 | 50 | 0 | 300 |
| $Al_2O_3$-NPs (µg/ ml) | 5 | 7 | 43 | 0 | 0 | 0 | 86 |
| | 10 | 2 | 40 | 8 | 0 | 0 | 112 |
| | 15 | 2 | 24 | 24 | 0 | 0 | 144 |
| $Fe_3O_4$-NPs (µg/ ml) | 18.75 | 2 | 38 | 10 | 0 | 0 | 116 |
| | 37.50 | 0 | 10 | 32 | 8 | 0 | 196 |
| | 56.25 | 0 | 1 | 22 | 27 | 0 | 252 |
| $SiO_2$-NPs (µg/ ml) | 15 | 4 | 46 | 0 | 0 | 0 | 92 |
| | 30 | 3 | 24 | 23 | 0 | 0 | 140 |
| | 45 | 2 | 12 | 36 | 0 | 0 | 168 |
| $TiO_2$-NPs (µg/ ml) | 20 | 3 | 33 | 14 | 0 | 0 | 122 |
| | 40 | 0 | 4 | 46 | 0 | 0 | 192 |
| | 60 | 0 | 0 | 2 | 48 | 0 | 296 |

**Figure 6** **Comet morphology showing different grades of DNA damage after nanoparticles cultured in human peripheral lymphocytes *in vitro***



# Discussion

## 5.1 Significance of the study

Nanoparticles have potential applications in several industrial sectors, medical and other consumer products. Inappropriate usage, overproduction and hazard identification of nanoparticles have potentially damaged the growth of nanotechnology. Regulated new technologies and innovations are required to maintain proper balance between nanotechnology and nanotoxicology. Recently nanotoxicology is focused much than nanotechnology, and this is because of risk assessment and increased genotoxic potential of nanoparticles identified both *in vivo* and *in vitro*. However, the effects and mechanisms behind the genotoxicity of nanoparticles remain controversy. The literatures reviewed found inconsistency regarding the mechanisms; therefore, drawing a conclusion behind the genotoxic potential of nanoparticles is difficult. Research on the cytotoxic and genotoxic potential of engineered nanoparticles are fairly increasing in recent years as the exposure promote a range of adverse health effects in micro-organisms, animals and humans.

The available literatures provided valued information that some nanoparticles induced DNA strand breaks and chromosomal aberrations in the exposed organisms. The reasons for genetic damage depends not only on the nano-size, surface modification and physico-chemical properties, but also known to depend on the dose, duration and selection of experimental model. Nanoparticles are known to interact with biological molecules such as proteins, lipids and nucleic acids in several ways. Interestingly, most of the experiments conducted on the genotoxic effects of nanoparticles have been documented only in *in vitro* studies. Damage to genetic material also leads to the initiation of carcinogenesis and genetic instability. The cellular genotoxicity *in vivo* and *in vitro* can be quantified by several test batteries established by OECD guidelines. The present study was aimed to evaluate the genotoxic potential of selected nanoparticles both *in vivo* and *in vitro* considering the current test batteries of OECD. Further, an attempt was made to assess if the genetic damage induced by nanoparticles followed direct interaction between DNA by primary mechanism, or could be due to the induction of oxidative stress through the secondary mechanism. Quantification using *in vivo* and *in vitro*

experiments helps to identify the mode of genetic damage induced by the selected nanoparticles.

## 5.2 Effects of selected nanoparticles on genotoxicity tests in erythrocytes of the fish, *Oreochromis mossambicus*

There is an increasing attention paid by the toxicologists on the use of genotoxicity as a biomarker for nanoparticles induced genetic damages, especially in aquatic organisms. Fish serves as aquatic genotoxicological model as they could bioaccumulate and biomagnify the pollutants, and provide early warning signals for toxicant induced genetic abnormalities. Most promising, economical and sensitive techniques widely used to detect cytogenetic damages in fish include micronucleus test and comet assay. Fish micronucleus test along with other nuclear abnormalities presented an incredibly important assay due to its proven suitability in diverse fish species as well as detect toxicant stress and pollution load in aquatic ecosystem (De Flora *et al*., 1993; Al-Sabti and Metcalfe, 1995). Micronucleus test help to detect both aneugenic and clastogenic effects of the toxicants on the exposed cells or organisms thereby identify the genotoxicity of toxic compounds (Heddle *et al*., 1991). Besides micronuclei formation, other nuclear abnormalities such as blebbed, lobed, notched, fragmented, binucleated and budded nuclei are considered as diagnostic indicators of cytotoxicity (Kirschbaum *et al*., 2009).

The micronucleus assay can be performed in various tissues like kidney, gill, fin, hepatocytes and so on however, cell isolation procedures from the different cell types remain complex and the output is limited. However, fish erythrocyte was used as a perfect alternative since the simple protocol make advantageous over the other complex procedures and the peripheral blood as target cells prevent from killing of animals at each sampling time (Pfuhler *et al*., 2009). Exposure of toxicants is known to cause micronuclei formation in the interphase cells, which are visualized as DNA fragmentation. Micronuclei appear as a result of the failure of whole chromosome or a chromosome fragment migration with one of the two daughter nuclei formed during mitosis (Bolognesi and Fenech, 2012). Thus the present study selected

micronucleus test as one of the biomarkers to detect the nanoparticles induced genotoxicity.

### 5.2.1 Effects of selected nanoparticles on micronucleus test

The present study evaluated the frequencies of micronuclei formation and other nuclear anomalies such as binucleated, budded, irregular nuclei, and cellular anomalies such as sticky cells and vacuolated cytoplasm in the erythrocytes of selected nanoparticles exposed groups. The results observed showed exposure to selected nanoparticles namely $Al_2O_3$-NPs at 4 mg/ L, $Fe_3O_4$-NPs at 15 mg/ L, $SiO_2$-NPs at 12 mg/ L and $TiO_2$-NPs at 16.4 mg/ L concentrations for 24, 72 and 96 h (short-term exposures) and 15, 30 and 60 days (long-term) exposures resulted in significant ($P<0.05$) increase in nuclear and cytoplasmic abnormalities in the erythrocytes of the fish, *Oreochromis mossambicus* in time-dependent manner when compared to the negative control group (Table 1; Figure 1).

In fish, micronucleus formed during cell division occurs due to misrepair of DNA double strand breaks leading to formation of acentric chromosomes or chromosomal fragments, which are not incorporated into the daughter nuclei at mitosis (Bolognesi and Hayashi, 2011). Nanoparticles exposure also resulted in other nuclear and cytoplasmic abnormalities in the erythrocytes of the fish. The major nuclear anomalies observed are deformed nuclei, binucleated cells and nuclear buds and cytoplasmic anomalies include sticky cells and vacuolated cytoplasm (Figure 1). Nuclear anomalies usually occur in association with micronuclei formation as a consequence of improper cell division. Binucleated cells formed as a result of nanoparticles exposure could be due to the failure of tubulin polymerization during the formation of mitotic spindle in cell cycle (de Campos Ventura *et al.*, 2008). Nanoparticles induced DNA amplification and breaking or shrinking of nucleoplasmic bridge probably lead to the formation of nuclear buds during the exposure period (Anbumani and Mohankumar, 2012). Thus all possible deformities in the morphology of nucleus observed in time-dependent manner during the experiment could be due to inappropriate chromatin condensation (Braham *et al.*, 2017).

The cytoplasmic anomalies like sticky cells and vacuolated cytoplasm were also evident in all treatment groups where fish exposed to $SiO_2$-NPs for 60 days showed highest number of vacuolated cytoplasm (Figure 1). The exact mechanism of vacuole formation in the cytoplasm is not well understood, but similar results were identified in cytoplasm of erythrocytes on exposure to gamma radiation in the fish, *Catla catla* (Anbumani and Mohankumar, 2012). Erythrocytes are associated with immune response, and the formation of sticky cells after nanoparticles exposure could be due to the functional response of erythrocytes against the nanoparticles.

The possible reason behind the nanoparticles induced DNA damages observed using micronucleus test could be the induction of oxidative stress through Fenton reaction. Oxidative stress is a state of redox imbalance within the cells, resulted due to the increased production of reactive oxygen species and decreased antioxidant activity, which could disturb the oxidative homeostasis within the cells (Moller *et al*., 2010). The effects of selected nanoparticles on the induction of oxidative stress were discussed earlier in chapter 2. The metal or metal oxides of the selected nanoparticles such as aluminium, iron, silicon and titanium oxides could have interacted with the mitochondrial membrane for free radical production. The transition metals ions released from the selected nanoparticles has been shown to have the potential ability for the conversion of cellular oxygen metabolic products, such as hydrogen peroxide and superoxide anions, to hydroxyl radicals, which could have diffused through cellular and nuclear membranes to react with DNA thereby resulted in genetic damage (Song *et al*., 2012).

Micronucleus test thus served as a marker to detect and quantify the damages in genetic material after the selected nanoparticles exposure. The present findings are in agreement with other studies where exposure of petroleum refinery effluent and the insecticide, lambda-cyhalothrin has been shown to spontaneously induce the formation of micronuclei and other nuclear anomalies in *Oreochromis niloticus* and *Gambusia affinis*, respectively (Hoshina *et al*., 2008; Muranli and Guner, 2011). There are report suggesting DNA oxidation as a result of reactive oxygen species generation and oxidative stress as one of the major reasons behind of DNA damage in fishes (Dusinska *et al*., 2011).

### 5.2.2 Effects of selected nanoparticles on comet assay in erythrocytes of the fish

Comet assay is another sensitive quantitative technique used to measure DNA damages, which detects single and double stranded breaks, alkali labile sites, protein-DNA and DNA-DNA cross-links and minute nuclear damages in single cell suspensions (Singh *et al.*, 1988). Under high alkaline condition and in the presence of lysing solution, DNA unwinds, relaxes and makes supercoiling of DNA, where DNA move towards the anode thereby giving comet like appearance (Karlsson, 2010). In most of the aquatic organisms, comet assay was performed in erythrocytes as target cells because only small volumes of samples are required that are obtained through a non-damaging technique where cell dissociation is not required (Belpaeme *et al.*, 1998). There are various comet parameters used to detect the DNA damages namely comet head and tail area, intensity, length and DNA, tail moment and Olive moment, which provides the intensity of DNA damage. The most commonly used comet parameters are tail length, tail percent DNA, tail moment, and olive moment. The present study evaluated only the relevant parameters such as tail length and percent tail DNA because the increase in DNA damage are reflected mainly on the tail length and percent tail DNA, thus it is considered as the direct measurement of DNA damage (Karlsson, 2010).

In the present study, exposure to $Al_2O_3$-NPs, $Fe_3O_4$-NPs, $SiO_2$-NPs and $TiO_2$-NPs for short-term (24, 72 and 96 h) and long-term (15, 30 and 60 days) durations showed significant ($P<0.05$) and time-dependant increase in the tail length and percent tail DNA in the erythrocytes of *Oreochromis mossambicus* (Table 2). Comets scored using the percent tail DNA was expressed in different grades of damages, and the total scores showed in Table 3 and Figure 2 indicated equal contribution of selected nanoparticles in the induction of DNA damage. Grade 0 represents no damage; untreated control group draw closer to the least grade with total score of 14 out of 400 scores (Table 3). Short-term exposure groups showed the score between 86 and 178 out of total 400 scores thereby represent grade 1 (Figure 2). The severity of damage was found to be increased with exposure durations, thus long-term treatment groups resulted with a damage score between 142 and 278.

Therefore, the long-term exposure groups achieved grade 2 and 3, respectively based on the intensity of damages (Table 3 and Figure 2). The results coincided with increase in the comet tail as reported in the rat model on exposure to aluminium oxide nanoparticles (Balasubramanyam *et al.*, 2009). The present findings suggest that induction of oxidative stress could be the possible reason for the genotoxicity observed after selected nanoparticles exposure.

## 5.3 Effects of nanoparticles on genotoxicity tests in human peripheral blood lymphocytes *in vitro*

The interaction of nanoparticles with human peripheral blood lymphocytes is critical in toxicological studies. Nanoparticles are known to induce hemolysis thus create pores in the membrane of blood cells thereby disrupt the balance of intra- and extracellular ions, proteins and other macromolecules leading to toxicity. The formation of cellular interactions between nanoparticles and cellular macromolecules such as protein, lipid and DNA forms nano-biocomplex that could adversely affect the cell functions (Ghosh *et al.*, 2013). Cytotoxicity of nanoparticles in human lymphocytes was evaluated by cell viability test using trypan blue dye exclusion method.

Trypan blue is a dye capable of selective penetration into the cytoplasm of dead cells because of the loss of membrane integrity whereas the live cells do not stain the dye. Changes induced by nanoparticles exposure included the uptake of dye that resulted to distinguish between the control viable cell and damaged or dead experimentally treated cells. Non-viability of lymphocytes cells observed after 3 and 24 h could be due to loss of membrane integrity as a result of nanoparticles exposure. Thus the half-maximal inhibitory concentration or $IC_{50}$ values of the selected nanoparticles observed based on cell viability of dye exclusion test was $Al_2O_3$-NPs - 20 µg/ ml, $Fe_3O_4$-NPs - 75 µg/ ml, $SiO_2$-NPs - 60 µg/ ml and $TiO_2$-NPs - 80 µg/ ml. Three sublethal concentrations namely three-fourth, one-half and one-fourth of $IC_{50}$ values of respective nanoparticles were selected according to OECD guidelines for further genotoxicity tests in human peripheral lymphocytes.

### 5.3.1 Effects of nanoparticles on nuclear anomalies in human peripheral blood lymphocytes using cytokinesis block micronucleus (CBMN) assay

The genotoxic responses of sublethal concentrations of selected nanoparticles was analysed *in vitro* in human peripheral blood using cytokinesis block micronucleus (CBMN) assay and comet assay. CBMN assay is the most reliable and preferred assay used to measure the frequency of micronuclei in human cultured cells, which are also more suitable for the interpretation of chromosome breakage or chromosome loss, DNA misrepair, non-disjunction, apoptosis, necrosis and cytostasis in cells (Fenech and Morley, 1985). CBMN assay are usually performed in once-divided cells that are recognized by binucleated appearance by blocking the cytokinesis with cytochalasin-B so that it prevents the confounding effects caused by the altered cell cycle kinetics (Fenech, 2000).

Sublethal exposure of selected nanoparticles such as $Al_2O_3$-NPs, $Fe_3O_4$-NPs, $SiO_2$-NPs and $TiO_2$-NPs in human peripheral blood lymphocytes showed significant ($P<0.05$) induction of nuclear anomalies such as micronuclei, nucleoplasmic bridge and nuclear buds in concentration-dependent manner (Table 4; Figure 3). There are several possible reasons for the formation of micronuclei in human blood cells that are mainly due to unrepaired chromosome breakage, DNA misrepair, acentric chromososome fragments, asymmetrical chromosome rearrangement, malaggregation of chromosomes or defective cell cycle checkpoints, chromosomal instability and so on (Fenech, 2007). The expression of micronuclei is also considered as the marker of DNA hypomethylation that could have occurred due to nanoparticles exposure. The present results also showed the formation of nuclear bud but only in $Al_2O_3$-NPs exposure group at three-fourth of $IC_{50}$ concentration (Table 4). Nuclear bud is widely used as biomarker of gene amplification, and the results observed suggested that $Al_2O_3$-NPs exposure could have induced gene amplification followed by selectively localizing the amplified DNA to the periphery of nucleus and eliminated through nuclear budding. Nuclear buds are peculiar in having the same morphology like micronucleus with an exception of small narrow or wide stalk of nucleoplasmic material to which it link to the nucleus depending on the stage of the budding process (Shimizu *et al*., 1998).

Nucleoplasmic bridge is considered as the biomarker of dicentric chromosomes resulting from telomere end-fusions or DNA misrepair (Fenech, 2007). Exposure to $Fe_3O_4$-NPs at 56.25 µg/ ml concentration showed the formation of nucleoplasmic bridge (Table 4). Anaphase nucleoplasmic bridge formed by pulling of centromeres of dicentric chromosomes to opposite poles could have occurred due to the inhibition of cytokinesis after $Fe_3O_4$-NPs treatment in the human peripheral lymphocytes (Fenech, 2011). Maximum induction of micronuclei formation was observed at highest concentration of $SiO_2$-NPs treated cultures than the positive and negative controls (Table 4) and this could have formed from the broken anaphase bridges or derived from either acentric chromosome fragments or chromosome loss (Fenech, 2007). In positive control, mitomycin C treated group, all cytogenotoxic abnormalities were found at highest frequency when compared with all three sublethal concentrations of nanoparticles and untreated control group (Table 4).

Cytokinesis block micronucleus assay performed in peripheral blood lymphocytes after exposure to the selected nanoparticles showed a concentration-dependant increase in total nuclear anomalies. Thus the assay provided better approach to evaluate cytotoxicity and genotoxicity *in vitro* in human cells. The study also identified that among the tested nanoparticles, the effects of $SiO_2$-NPs in the induction of micronuclei and other anomalies was more prominent, and this could be due to internalization of nano-silica of 1 nm size, the smallest among the other selected nanoparticles. Moreover, the genotoxicity of nanoparticles revealed by CBMN assay indicated the direct primary mechanism of toxicity exerted on the nucleus of human lymphocyte cells.

### 5.3.2 Cytokinesis block proliferation index (CBPI)

The cytotoxicity of CBMN assay can be expressed by cytokinesis block proliferation index (CBPI), which is actually a ratio between the three subpopulations of cells with different numbers of nuclei (OECD guideline 487). Thus in the present study a sound evaluation of cell kinetics and micronuclei frequencies was quantified by determining rate of cell proliferation after sublethal

exposure of selected nanoparticles by a standard proliferation index protocol. Concurrent positive and negative controls were also maintained along with the treatment groups. The results showed high proliferation index in $Al_2O_3$-NPs at high sublethal concentration whereas the other nanoparticles such as $Fe_3O_4$-NPs, $SiO_2$-NPs and $TiO_2$-NPs showed concentration-dependent increase in the proliferation index (Figure 4). The results obtained by the proliferation index rate thereby confirmed the cytotoxicity of selected nanoparticles.

### 5.3.3 Effects of nanoparticles on comet assay in human peripheral blood lymphocytes

Comet assay, the standard method for assessing DNA damage was also assessed *in vitro* in human peripheral blood lymphocytes. Tail length and percent tail DNA are the parameters selected for measuring DNA damage. Among all the parameters, percentage of DNA in the tail provides an appropriate measure of genetic damage which is calculated by the ratio between the total intensity of tail and total intensity of comet that measure the exact amount of DNA that migrated out of nuclei (Tice *et al.*, 2000). The untreated control culture did not showed comet tail and the grade of DNA damage was observed as grade 0 whereas in mitomycin C – positive control exposed culture group, the DNA damage was observed as grade 3 (Table 6 and Figure 5).

Low grade of DNA damage was observed after one-fourth of $IC_{50}$ concentration of the selected nanoparticles, but the grade was found above the untreated controls (Figure 5). At one-half of $IC_{50}$ concentration of all selected nanoparticles, the grade of DNA damage observed was grade 1 whereas the same grade was observed for $TiO_2$-NPs exposed culture group at one-fourth of $IC_{50}$ concentration, and when the concentration was increased to one-half showed grade 2 (Table 5; Figure 5). Nanoparticles treated at three-fourth of $IC_{50}$ concentration showed grade 2 DNA damage while $TiO_2$-NPs exposure group showed grade 3 DNA damage (Table 6; Figure 5) thereby indicated concentration-dependent genetic damage. The present results are in agreement with another study where cereium dioxide nanoparticles exposure in human peripheral blood lymphocyte cells showed

genotoxic potential which has been demonstrated by CBMN test, comet assay gamma H2AX test (Könen-Adıgüzel and Ergene, 2018).

The chapter therefore concludes that both *in vivo* and *in vitro* exposure of nanoparticles induced genetic damage in time-dependent and concentration-dependent manner irrespective of its unique physico-chemical properties. There are several studies reporting the nanoparticles-induced genotoxicity by different mechanisms. The present observations conclude that the genotoxic potential of selected nanoparticles could have mediated through the induction of oxidative stress, which is the secondary mechanism of nanoparticles toxicity. It was proved by the generation of reactive oxygen species in vital tissues as gill, liver and brain as discussed in chapter 2. However, on pursuing the results of *in vitro* studies using human peripheral lymphocytes showed genotoxicity in the cultured cells thereby stating the direct interaction of nanoparticles with biomolecules such as nucleic acids in human lymphocyte cells. Therefore, genotoxic potential of selected nanoparticles by the induction of DNA damage could have occurred either directly interacting with the genetic material or indirectly by generation of reactive oxygen species.

# Conclusions

1. Sublethal exposure of nanoparticles such as $Al_2O_3$-NPs, $Fe_3O_4$-NPs, $SiO_2$-NPs and $TiO_2$-NPs demonstrated induction of micronuclei and other nuclear anomalies such as binucleated cells, deformed nucleus and nuclear bud along with cytoplasmic anomalies such as sticky cells and vacuolated cytoplasm in the erythrocytes of the fish, *Oreochromis mossambicus* thereby indicated genotoxicity of the selected nanoparticles.
2. Comet assay performed in erythrocytes of fish detected the DNA damages in the form of increased tail length, tail percent DNA thus confirmed the genotoxic potential of selected nanoparticles.
3. Effects of nanoparticles evaluated by micronucleus test and comet assay as biomarkers of genotoxicity in erythrocytes of the fish were found time-dependent, and suggested DNA oxidation as a result of reactive oxygen species generation and oxidative stress could be the possible mechanism behind the DNA damage in fish.
4. Viability of human peripheral blood lymphocytes evaluated by trypan blue dye exclusion method showed half-maximal inhibitory concentration or $IC_{50}$ values of the selected nanoparticles as $Al_2O_3$-NPs - 20 µg/ ml, $Fe_3O_4$-NPs - 75 µg/ ml, $SiO_2$-NPs - 60 µg/ ml and $TiO_2$-NPs - 80 µg/ ml concentrations.
5. Culture of human peripheral lymphocytes at sublethal concentrations of $IC_{50}$ values of selected nanoparticles demonstrated genetic damage as evidenced by cytokinesis block micronucleus assay, cytokinesis block proliferation index and comet assay.
6. The genotoxic potential of selected nanoparticles evaluated *in vitro* were found concentration-dependent, thus indicated the direct primary mechanism of toxicity exerted on the nucleus of human lymphocyte cells.
7. To brief, positive genotoxicity of nanoparticles was observed both *in vivo* and *in vitro*, and also further assumed that induction of DNA damage could have occurred either directly interacting with the genetic material or indirectly by the generation of reactive oxygen species.

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