Assessement of the toxicity of cobalt ferrite ($cofe_2o_4$) nanoparticle in short neck clam *Paphia malabarica, in vivo*

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PRIYANKA SHASHIKANT SATARKAR

Seat Number: 22P0440025

ABC ID: 426-096-620-965

PR Number: 201905713

Under the Supervision of

DR. AVELYNO H. D'COSTA

School of Biological Sciences and Biotechnology

Discipline: Zoology

GOA UNIVERSITY

Date: May 2024

SBSB, M.Sc., Zoology ★ Taleigao Plateau, Goa 403206 ★ GOA UNIVERSITY

Seal of the School

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DECLARATION BY STUDENT

I hereby declare that the data presented in this Dissertation report entitled, "**Assessment of the toxicity of cobalt ferrite ($CoFe_2O_4$) nanoparticle in short neck clam *Paphia malabarica*, in vivo**" is based on the results of investigations carried out by me in the Zoology at School of Biological Sciences and Biotechnology, Goa University under the Supervision of Dr. Avelyno H. D'Costa and the same has not been submitted elsewhere for any award of a degree or diploma by me. Further, I understand that Goa University or its authorities will not be responsible for the correctness of observations/experimental or other findings given in the dissertation.

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Priyanka S. Satarkar

Date: **22P0440025**

Place: Goa University. **Discipline: Zoology**

School of Biological Sciences and Biotechnology.

COMPLETION CERTIFICATE

This is to certify that the dissertation report entitled "**Assessment of the toxicity of cobalt ferrite ($CoFe_2O_4$) nanoparticle in short neck clam *Paphia malabarica,* in vivo**" is a bonafide work carried out by Miss. Priyanka Shashikant Satarkar under my supervision in partial fulfilment of the requirements for the award of the degree of Masters in Zoology in the Discipline Zoology at the School of Biological Sciences and Biotechnology, Goa University.

Date:

DR. AVELYNO D'COSTA

Assistant Professor

Discipline Zoology

Dr. Bernard F. Rodrigues
Dean
School of Biological Science and Biotechnology
Date: 8-4-24
Place: Goa University

Dean of School of Biological Sciences
& Biotechnology
Goa University, Goa-403206

School Stamp

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PREFACE

This thesis is presented to fulfill the requirements for the degree of Master's in Zoology, based on research conducted by the author under the Supervision of Dr. Avelyno D'Costa, Assistant Professor of Zoology at Goa University from 2023 to 2024.

Cobalt Ferrite Nanoparticles ($CoFe_2O_4$ NPs) are increasingly versatile in their applications. These nanoparticles have found wide usage in imaging (MRI) and cancer treatment. However, their large-scale utilization results in their disposal into the environment, which poses a significant threat to the global ecosystem (Novak et al., 2013). Natural organic matter can facilitate the degradation of $CoFe_2O_4$ NPs, leading to the potential release of Co2+ and Fe3+ ions into the environment. These ions can trigger ROS-mediated oxidative stress (Novak et al., 2013; Sun et al., 2013), cytotoxicity, genotoxicity, inflammations (Papis et al., 2007; Ponti et al., 2009), as well as protein oxidation and membrane disruptions (Azaria et al., 2011; Luo et al., 2014). Therefore, there is an urgent need to study the potential toxic effects resulting from the release of $CoFe_2O_4$ NPs and the subsequent dissolution of ions from these nanoparticles in the environment.

The aim of this study was to gain insight into the fate and interactions of medically significant NPs (such as $CoFe_2O_4$) within aquatic environments and their interactions with organisms (Ahmad et al., 2015).Therefore, studying the effect of Cobalt ferrite on *Paphia malbarica* will provide valuable data on its toxicity, which can be used to develop preventive measures to reduce $CoFe_2O_4$ NPs exposure to aquatic organisms. There are limited studies analyzing the toxicity of $CoFe_2O_4$ NPs in aquatic ecosystems, making this thesis a significant contribution to the field by investigating their impact on the anatomy and physiology of *Paphiamalbarica.*

The thesis is structured into four main chapters. The first chapter provides an introduction to Cobalt ferrite Nanoparticles, including their applications and toxicity. The second chapter includes a literature review and outlines the aims and objectives of the study. Chapter 3 details the materials and methods used for physiological and biochemical estimations. Chapter 4 presents the analysis and conclusions, including observations from histopathology and micronucleus genotoxicity studies, biochemical analysis graphs, and in-depth discussions on the effects of $CoFe_2O_4$ toxicity on bivalves. Finally, the thesis concludes with a summary of findings and recommendations.

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TABLES

FIGURES

ABBREVIATIONS USED

Entity	Abbreviations
Nanoparticles	NPs
Magnetic Nanoparticles	MNPs
Cobalt Ferrite	$COFe_2O_4$
Curie Temperature	Tc
Deoxyribonucleic Acid	DNA
Magnetic Resonance Imaging	MRI
Nanocrystals	NCs
Reactive Oxygen Species	ROS
Tetraiodotryonine And Triiodothryonine	T4 and T3
Nanobeads	NBs
Alternating Magnetic Field	AMF
Cobalt Ferrite Nanoparticles	CFNPs
Lethal Concentration 50	LC50
Biochemical Oxygen Demand	BOD
Bovine Serum Albumin	BSA
Rotations Per Minutes	RPM
Sodium Hydroxide	NaOH
Catalase	CAT
Superoxide Dismutase	SOD
Reduced Glutathione	GSH
Acid Phosphatase	AP
Alkaline Phosphatase	ALP
Alanine Amino Transferase	ALT
Malondialdehyde	MDA
5,5'-Dithiobis, 2-Nitrobenzoic Acid	DTNB
Tri Chloric Acid	TCA
Dichromate/ Acetic Acid	DCA
ThiobarbituricAcid	TBA
Dipotassium Phosphate	K_2HPO_4
Potassium Dihydrogen Phosphate	KH_2PO_4
Titanium Dioxide	TiO_2
Gold–Titania Catalyst	$AuTiO_2$
Dopamine	DA
Acetylcholine	ACh
Gamma-Aminobutyric	GABA
Copper Oxide Nanoparticles	CuO NPs
Multi-Walled Carbon Nanotube	MWCNT
Hydrogen Peroxide	H_2O_2
Glutathione S-Trasferase	GST
Tributyltin Chloride	TBTCL
Guanosine Triphosphate	GTP
Micronucleus Test	MN

ABSTACT

The extensive use of Cobalt Ferrite Nanoparticles ($CoFe_2O_4$ NPs) in various industries, medical fields, and environmental applications has raised considerable interest, leading to widespread exposure of organisms. To assess the associated risks, it is crucial to analyze their health effects and environmental impacts. In a study on acute toxicity, *Paphia malabarica* were exposed to three concentrations (100 mg/L, 200 mg/L, and 400 mg/L) for 96 hours, with subsequent exposure of bivalves to two concentrations (10 mg/L and 50 mg/L) based on the LC50 value. Biochemical changes were assessed using biomarkers such as Protein, Glycogen, and Reduced Glutathione (GSH), as well as enzymes including Catalase (CAT), Superoxide Dismutase (SOD), Alkaline Phosphatase (ALP), Acid Phosphatase (AP), Alanine Aminotransaminase (ALT), and Malondialdehyde (MDA). The agglomeration of nanoparticles and ion release induced oxidative stress in bivalves. Histopathological studies confirmed the toxicity of $CoFe_2O_4$ NPs and the presence of micronucleus indicated genotoxic effects.

Key words: Cobalt frerrite nanoparticles, *Paphia malabarica*, Oxidative stress, Toxicity, Genotoxicity, In vivo.

Introduction

CHAPTER 1: INTRODUCTION

1.1 BACKGROUND

1.1.1 Nanotechnology and Nanoparticles

Nanoparticles (NPs) are solid particles of nano-sized dimensions with a size ranging from 10-100 nm (Mohanraj & Chen 2006). A majority of synthesized NPs form the building blocks of nanotechnology which can be defined as a development and research at the macromolecular, molecular and atomic scales.These particles have been utilized by human societies and various industries for millennia due to their capacity for manipulation and synthesis. Due to their unique properties like particle size, surface reactivity, surface area, shape and charge relative to their dissolved counterparts, NPs find a broad range of applications in electronic, optoelectronic and magnetic, pharmaceutical, biomedical, cosmetic, catalytic and environmental utilization (Biswas et al, 2012.,Bundschuh et al, 2018).

1.1.2 Magnetic Nanoparticles

Magnetic Nanoparticles (MNPs) are the nanoparticles which undergo certain changes in the presence of an external magnetic field. They are composed of magnetic elements such as iron, cobalt, nickel and their oxides (Shubayev et al,2009). Magnetic Nanoparticles are of particular interest because their size ranges from few nanometers up to tens of nanometers which is smaller than the normal cells and comparable with genes, proteins and viruses. This allows them to interact with biomolecules by crossing the biological membranes (Duguet et al., 2006).

In a wide range of disciplines MNPs are of great interest in magnetic resonance imaging, catalysis, magnetic fluids, biotechnology, data storage, biosensing, environmental remediation, and in biomedicine importance tools for separating and purifying cell populations in cell biology, repair of the tissue, for drug delivery, hyperthermia, for cancer treatment etc. (Lu et al, 2007.,Tran et al, 2010).

1.1.3 Characteristics of Cobalt Ferrite Nanoparticle

In recent years, metal oxide nanoparticles have been found to be a subject of great interest because of their electronic, optical and magnetic properties (Sanpo et al., 2012). Similarly, cobalt ferrite ($COFe_2O_4$) has special mechanical and physical properties which enables its use in nanomedicine. It has a high Curie temperature (Tc) of 520°C, making it a high magnetic material. For a single domain of 40 nm size at room temperature, it exhibits a high coercivity of 4.3 kOe. The anisotropy constant ranges from 2.65×106 to 5.1×106 erg cm-3. It shows a moderate saturation magnetization at room temperature, approximately 80 emu/g, and a high magnetostrictive value of -225×10-6 (Amiri, 2012).

1.1.4 Applications of cobalt ferrite Nanoparticles

Cobalt Ferrite shows excellent properties of mechanical hardness, chemical stability, wear resistance, electric insulation and ease of synthesis which enables its wide applications in medical science including drug delivery in biomedicine, for cancer treatment as biocompatible magnetic nanoparticles, magnetic resonance imaging, magnetic-optical devices, magnetic hyperthermia, ferrofluids, technology and excellent material for high density magnetic recording (Sanpo et al., 2012). Cobalt ferrite's utility extends to the isolation of Deoxyribonucleic acid

(DNA), its self-heating property for hyperthermia applications, and its capacity to enhance the sensitivity ofmagnetic resonance imaging (MRI) through the use of spinel ferrite nanoparticles, which offer tunable and high magnetizations.(Amiri & Shokrollahi, 2013).

Pita (2008), synthesized a cobalt ferrite NPs for the development of biosensor specifically for (PNA)/DNA nucleic acid peptide with. Christopher S. Brazil studied heating agents for hyperthermia and for drug delivery which can be magnetically activated by aqueously dispersed $CoFe_2O_4$ NPs through heat generation. Shokrollahi et al., (2012) reported the effect of particle size and cation distribution on the structural and magnetic properties of $COFe_2O_4$ nanoparticles which were synthesized by chemical method (Amiri., 2012).

1.1.5 Disposal of Nanoparticles

In the aquatic environment Nanoparticles enters through their life cycle, the three scenarios of emission are very common; those are (i)-during raw material production and nano products. (ii)-during its actual use and (iii)- while disposing of products containing NPs. They undergo aging processes like chemical transformation, disaggregation and aggregation in the environment (Bundschuh et al, 2018).

Large scale disposal of cobalt ferrite nanoparticles in the environment poses alarming situations in the global ecosystem (Novak et al., 2013). The degradation of cobalt ferrite NPs, which is promoted by the natural organic matter, results in a release of CO_2+ and Fe_3+ ions in environment which includes oxidative stress mediated by reactive oxygen species (Novak et al., 2013; Xu et al., 2013). The released ions induce genotoxicity, cytotoxicity, and inflammations (Papis et al., 2007; Ponti et al., 2009), membrane disruptions and oxidation of proteins (Ahmad

et al., 2016). Therefore there is a need to study the toxic effects of $CoFe_2O_4$ nanoparticles in the environment (Ahmad et al., 2015).

1.1.6 Toxicity of cobalt ferrite Nanoparticle

Nanoparticles (NPs) and nanocrystals (NCs) are increasingly used for their beneficial properties, but their potential toxicity is a concern. Their composition and size play crucial roles in their toxicity. Despite their popularity in water treatment applications, NPs can be toxic to plants, aquatic life, and humans. Continued use of NPs in water treatment raises concerns about their environmental biocompatibility. Toxicity is not only linked to their chemical composition but also to their small grain size (Mmelesi et al., 2020). Cobalt ferrite nanoparticles have been observed to induce toxic effects, including cytotoxicity and genotoxicity, across multiple trophic levels. These effects have been documented in organisms ranging from primary producers such as photosynthetic algae to consumers, including humans and pigs (Barhoumi and Dewez, 2013; Azaria et al., 2013; Kapilevich et al., 2010). Even at lower concentrations, cobalt ferrite (CoFe2O4) nanoparticles (NPs) caused significant sensitivity in the microalgae *Chlorella vulgaris*. This sensitivity was attributed to the adsorption of $CoFe_2O_4$ NPs, leading to oxidative damage through the production of reactive oxygen species (ROS).

1.1.7 Toxicity of $COFe_2O_4$ NPs in aquatic organisms

$CoFe_2O_4$ nanoparticles induce acute developmental toxicity in zebrafish embryos, affecting cell cycle, apoptosis, metabolism, and causing hatching delay, heart issues, and behavioral changes. They also induce physical stress by enveloping the embryo chorion and damaging membranes through ROS-induced apoptosis (Ahmad et al., 2015). Cobalt ferrite nanoparticles ($CoFe_2O_4$

NPs) affect the thyroid endocrine system in zebrafish larvae, leading to developmental and metabolic issues. This disruption promotes the production of reactive oxygen species (ROS), causing oxidative membrane damage, apoptosis, and DNA aberrations. These DNA changes may transfer mutated genetic material to future generations, increasing cancer risks. The findings highlight the potential endocrine-disrupting effects of $CoFe_2O_4$ NPs on aquatic organisms and humans, potentially disrupting food webs.(Ahmad et al., 2016). Ecotoxicity and risk assessment studies revealed that *Chlorella vulgaris* is highly sensitive to low concentrations of $CoFe_2O_4$ Nanoparticles (NPs). The adsorption of $CoFe_2O_4$ NPs leads to severe oxidative damage due to reactive oxygen species (ROS) production. This excess ROS accelerates lipid peroxidation and denatures antioxidant enzymes, destroying the antioxidant defense system (Ahmad et al., 2015).

The scientific community faces a dilemma due to the lack of prior characterization of nanoparticles (NPs) for toxicity studies. This limitation not only diminishes the significance of such studies but can also render them entirely inconclusive (Krug, 2014). The objective of this study was to understand the biochemical mechanisms responsible for the acute toxic effects of Nano-$CoFe_2O_4$, focusing on their physicochemical properties. No research has yet examined the impact of $CoFe_2O_4$ Nanoparticles (NPs) in bivalves. Thus, the current study focuses on assessing the toxicity of $CoFe_2O_4$ NPs on bivalves in vivo.

1.2 OBJECTIVES

- To determine the LC50 of Cobalt Ferrite ($CoFe_2O_4$) Nanoparticle in *Paphiamalabarica*.
- To determine the toxicity of Cobalt Ferrite ($CoFe_2O_4$) Nanoparticle in *Paphiamalabarica* through biochemical assays.
- To evaluate the Genotoxicity and Histopathological alterations of viscera and gills in Cobalt Ferrite Nanoparticle exposed *Paphiamalabarica*.

1.3 HYPOTHESIS

cobalt ferrite ($CoFe_2O_4$) nanoparticles (NPs) induce adverse toxicity i*n Paphiamalabarica.*

1.4 SIGNIFICANCE OF THE WORK

Paphiamalabarica forms an inexpensive and delicious seafood among the locals therefore information on the presence or toxicity of cobalt ferrite nanoparticles in bivalves is of great importance since it reflects the magnitude of environmental contamination. Through the trophic levels, it may finally reach human beings and higher animals posing health hazards within the food chain. Therefore, the toxicity assessment of cobalt ferrite Nanoparticles becomes of utmost importance.

CHAPTER 2: LITERATURE REVIEW

Ahmad et al. (2015) exposed zebrafish embryos to $CoFe_2O_4$ nanoparticles and found dose- and time-dependent toxicity, including developmental issues and biochemical changes. The nanoparticles caused severe oxidative stress and apoptosis, highlighting the need for careful risk assessment in their use. Ahmad et al. (2016) investigated the potential endocrine-disrupting effects of cobalt ferrite ($CoFe_2O_4$) nanoparticles (NPs) on zebrafish larvae. The NPs caused malformation of the hypothalamus-pituitary axis, leading to elevated levels of thyroid hormones (T4 and T3), delayed hatching, and developmental abnormalities.

In another study, Ahmad et al. (2015) examined the toxicity of Cobalt Ferrite nanobeads (NBs) on *Chlorella vulgaris*. The NPs caused damage to cell morphology and membrane integrity, releasing ions and inducing oxidative stress. This led to reduced enzyme activity, genetic aberrations, and metabolic dysfunction. Gokce et al. (2020) addressed the impact of three types of magnetic nanoparticles (CuFe2O4, $CoFe_2O_4$, and $NiFe_2O_4$) on *Daphnia magna* over a 96-hour period. $CuFe_2O_4$ nanoparticles were found to be the most toxic, followed by $NiFe_2O_4$ and $CoFe_2O_4$. The research underscores the potential ecological risks of magnetic nanoparticles on freshwater ecosystems.

Mariani et al. (2011) investigated the use of a microphysiometer to assess real-time changes in cell metabolism caused by Cobalt Ferrite Nanoparticles (NPs) on fibroblast cultures. The microphysiometer detected a cytotoxic effect of the NPs, which was confirmed by conventional in vitro assays. Cobalt Ferrite Nanoparticles (Co–Fe NCs) act as magnetic hyperthermia and cytotoxic agents, forming chains under an alternating magnetic field (AMF). This unique

property, along with their mild hyperthermia and cobalt ion toxicity, leads to complete tumor regression and improved survival rates in animal models, suggesting their potential for enhancing cancer therapy (Balakrishnan et al.,2020).

Akhtar et al. (2020) examined the fate of cobalt ferrite NPs coated with bare, polyethylene glycol, and citrate in the biological environment. Structural and morphological analyses confirm their properties. In vivo toxicity studies in rats reveal degradation effects and transfer of degraded ions into apoferritin, indicating potential for safe therapeutic design. Abudayyak et al. (2016) investigated the toxic effects of cobalt ferrite nanoparticles (CoFe2O4-NPs) on various cell types in vitro and reported dose-dependent cytotoxicity, genotoxicity, oxidative damage, and apoptosis induction in liver, colon, lung, and neuron cells.

Abudayyak et al. (2017) investigated the toxic effects of cobalt ferrite nanoparticles ($CoFe_2O_4$-NPs) on kidney cells in vitro. $CoFe_2O_4$-NPs did not affect cell viability at concentrations ranging from 100 to 1000 µg/mL after 24 hours of exposure. However, they significantly induced DNA damage at concentrations ≤100 µg/mL, with no observed apoptotic or necrotic effects. Iron oxide and Cobalt Ferrite magnetic nanoparticles (MNPs) were synthesized and functionalized for potential biomedical applications. They exhibited superior superparamagnetic properties and low toxicity, indicating their suitability for safe use in biomedicine.(Peeples et al., 2014).

Cobalt-Ferrite Nanoparticles (Co-Fe NPs) show promise for nanotechnology-based therapies. Evaluating their effect on the viability of seven different cell lines representing various human organs is crucial. The observed correlation between oxidative stress induced by Co-Fe NPs and

the sensitivity hierarchy of different cell types suggests oxidative stress as a possible mechanism for Co-Fe NP toxicity (Azaria et al., 2012).

Hassanenet al.,(2023) investigated the pulmonary toxicity of Cobalt Ferrite Nanoparticles (CFN) in rats following repeated oral intake Results showed dose-dependent oxidative stress and pulmonary inflammation, suggesting potential health risks associated with CFN exposure. Cobalt Ferrite Nanoparticles (CFNPs) were synthesized and characterized for cancer treatment using hyperthermia. They showed promising properties, including monodispersity, cubic structure, and enhanced cytotoxicity against triple-negative breast cancer cells when exposed to microwaves (Medina et al.,2020).

Khan et al. (2023) investigated the toxicity of synthetic Cobalt Iron Oxide Nanoparticles (CIONPs) in rabbits. The CIONPs were synthesized and characterized before being administered intravenously to the rabbits. Blood analysis and histological examinations were conducted post-exposure. The results showed that CIONPs induced toxicity in the rabbits, affecting various tissues and organs.

2.1 LACUNAE

The toxicity of the Cobalt Ferrite ($CoFe_2O_4$) Nanoparticle has not been fully explored in aquatic organisms such as bivalves leaving a scope for the area to be unexplored. Although bivalves are a good environmental indicator of pollution, no studies have been reported with respect to toxicity of $CoFe_2O_4$ nanoparticles in vivo. The widespread utilization of CoFe2O4 nanoparticles (NPs) in various applications leads to significant environmental disposal, posing a critical concern for the global ecosystem. Upon disposal, these CoFe2O4 NPs are subject to biodegradation, potentially releasing Co2+ and Fe3+ ions into aquatic environments.

Methodology

CHAPTER 3: METHODOLOGY

3.1 CHEMICALS

The commonly used organic solvents like Ethanol, Methanol, Chloroform, Diethyl ether etc and acids like Sulphuric acid, Hydrochloric acid, Glacial acetic acid, Citric acid were obtained from M/S. SDFine-Chem.Ltd, Mumbai India. Standard chemical like Cholesterol, Palmitic acid which are used for the biochemical analysis along with enzymatic analysis which include Glycine, P-nitrophenyl phosphate, α-ketoglutaric acid, Glutathione, 5.5- Dithiobis nitrobenzoic acid etc were procured from M/S, sigma- Aidrich chemical Co.USA. Various other chemical powders like Manganous sulphate, Potassium hydroxide, Sodium nitroprusside, Sodium carbonate, Copper sulphate, Sodium molybdate, Sodium citrate, Sodium hydroxide, Potassium di chloride, Magnesium chloride, Gallium chloride , Potassium chloride, Potassium bromide, Sodium bicarbonate, Sodium sulphate, Boric acid etc along with liquid chemicals like Hydrogen peroxide, Nitrophenol, Formalin were obtained from the M/S, S.dfine.Chem.Ltd., Mumbai, India and or M/S Hi.Media lab. Pvt. Ltd, Mumbai, India.

3.2 GLASSWARES

The Glassware's which are required for the present study like beakers, test-tubes, conical flask, measuring cylinders, glass rods, watch glasses, glass funnel, pipettes etc along with the lab wares like centrifuge tubes, microtips, microfuge tubes, pipette stand, test tube stand, plastic reagent bottles, plastic coupling jars, mortar and pestle of high quality were used. Before making use, all glassware's were soaked in chromic acid overnight and then cleaned with detergent and rinsed with distilled water and dried for sterilization and were kept in the oven for about three hours.

3.3 INSTRUMENTATION

Instruments used in the present steady were UV-visible spectrophotometer (Bio Erass Elite), light microscope, pH meter (µp pH meter TMP3), centrifugation machine (REMI CM101), Analytical machine (WENSAR PGB 200), water bath (i-therm AI-7981), and hot air oven (MIC-MIC-165) were used.

3.4 STUDY ANIMAL

For the conduct of research work *Paphiamalabarica* which is a bivalve species were selected as an experimental animal for the study. Necessary approval was obtained for carrying out experimentation on model organisms from institutional Animal Ethics Committee of Goa University, with approval reference no:. GUZ/IAEC/23-24/N22 dated 12/09/2023. The bivalves were collected from a Zuari river of Madkai village, Ponda Goa.

Paphiamalabarica

Venerid clams of the family Veneridae display diverse morphological adaptations based on their habitat, including mud, sand, gravel, and coral reefs (Chen et al., 2013). The short-neck bivalve *Paphiamalabarica* is highly prized as seafood in coastal India. (Chemnitz, 1782). *Paphiamalabarica is* widely distributed along the Southwest and Southeast coasts of India representing a significant bivalve resource in numerous estuaries, backwaters, and coastal waters. This species is harvested for local consumption and export purposes (Kavitha et al., 2021)

CLASSIFICATION

Kingdom:	Animalia
Phylum:	Mollusca
Class:	Bivalvia
Order:	Venerida
Family:	Veneridae
Genus:	Paphia
Species:	*P. malabarica*

Paphiamalabarica, commonly known as the short neck clam, is a highly prized species found in the estuarine systems of the southwest coast of India. This clam is sought after for its delectable meat and its shell, which is used in various applications(Chemnitz 1782). Bivalves like mussels, oysters, and clams are widely consumed worldwide, with an annual consumption exceeding 90,000 tons. They are valued for their nutritional benefits, including high protein content, essential minerals, and omega-3 fatty acids.*Paphiamalabarica*, locally known as "tisreo" in Goa, is selected as a model organism for toxicological studies due to its filter-feeding nature. Morphologically, it has a triangular to oval shell with rounded margins and concentric ridges. The hinge area is short with narrow diverging teeth, and the 'U' shaped pallial sinus is not deeply pronounced (Gawas ,2023).

Goa's plentiful edible bivalve resources are a crucial fishery and income source for local fishermen. However, metal pollution and bioaccumulation in these bivalves, though significant, are inadequately addressed. Regular monitoring of metal concentrations in these bivalves is essential due to their popularity as seafood and their ability to accumulate toxins (Usmani., 2015). Bivalves can accumulate heavy metals and pollutants in their tissues, and some can

transform xenobiotics into mutagens, making them important for environmental monitoring and pollutant studies.due to the accumulation of nanoparticles in bivalves, have the potential to act as a bioindicator of nanoparticle contamination.

3.5 MAINTENANCE OF STUDY ANIMAL

Bivalves were maintained in the animal house of Zoology Discipline, School of Biological Science and Biotechnology, Goa University. They were brought to the Aqua room of the animal house and were immediately transferred to a tank containing artificial sea water. The acclimatization period of 12 hrs dark and 12 hrs light and temperature of 28^0C was maintained for 15 days from the day of collection under the laboratory conditions. The continuous supply of oxygen was maintained by providing proper aeration by aerator pumps. A group of 10 bivalves were maintained in each aquarium tank. The tank water was changed every alternate day.

3.6 EXPERIMENTAL SET UP

To evaluate the toxin effect of Cobalt Ferrite ($CoFe_2O_{4)}$ Nanoparticles, the bivalves were exposed to two different concentrations along with the control. 10 bivalves were maintained in each three Aquarium tanks. The bivalves were exposed to concentrations like 50 mg, 100mg and 200mg of Cobalt Ferrite Nanoparticles for 98 hours in order to determine the LC50 values by Probit analysis (Finney 1952). The Nanoparticles were introduced in the tank by suspending in 5 ml of artificial sea water and then added in tanks. (Canesi et al., 2008). The LC50 values were determined based on the behaviour of bivalves and the mortality rate.

For the assessment of toxicity of $CoFe_2O_4$ nanoparticles,Two different concentrations (10 mg/L and 50 mg/L) were selected based on the LC50 values. In this setup, three tanks were maintained: one control tank, one experimental tank with 10 mg of CoFe2O4 nanoparticles, and a second experimental tank with 50 mg of CoFe2O4 nanoparticles. Each tank housed 10 bivalves per liter of water. The control tank was not exposed to the nanoparticles. The exposure study was carried out for 15 days.

Maintenance of bivalves

Fig. 1 Maintenance of the bivalves

3.7 EUTHANASIA

After the exposure of 15 days, bivalves were sacrificed by placing them in chilled water, dissected, rinsed with artificial sea water and freezed at -80^{0}C in order to determine biological and enzyme activities. Gills were processed for the histopathological study and also used for the genotoxicity study.

3.8 DETERMINATION OF PHYSIOLOGICAL PARAMETERS

3.8.1 Determination of oxygen consumption rate

Principle:

The Manganous sulphate becomes the manganous hydroxide in water. The oxygen in the water sample oxidizes manganous hydroxide to manganic hydroxide. The acidic manganic hydroxide liberates iodine from potassium iodide. The iodine that has been freed is titrated using sodium thiosulphate. Thus, one molecule of oxygen can liberate 4 atoms of iodine which is titrated with 4 molecules of thiosulphate.

Chemical preparation

I. Winkler's solution A- In a 100 ml pre-boiled distilled water dissolve 20g of manganous sulphate.

II. Winkler's solution B – 20g of potassium iodine and 100 g of potassium hydroxide was added in pre-boiled 200 ml of distilled water.

Sample collection:

Before determining the oxygen consumption rate, cover the tanks with lid for 2 hours so that no air space is left between lid and the water surface. After 2 hrs, collect the water from the tank without introducing air bubbles in the BOD bottle (welsh and smith.,1960).

Procedure

The water sample was fixed immediately by adding 1 ml of Winkler's solution A and 1 ml of Winkler's solution B. After a 30-minute incubation period, 1 ml of concentrated H_2SO_4 was added and mixed well until the solution turned dark yellow. The solution was mixed repeatedly by inverting the bottle several times. In a conical flask, 20 ml of the resulting solution was added and titrated against a 0.025N sodium thiosulfate solution in the burette until the color changed to light yellow. Subsequently, 1 to 2 drops of starch indicator were added to the flask, resulting in the formation of a dark blue color. The titration was continued against the same solution until the color changed to colorless. The endpoint for blank and experimental was noted down.

Calculations:

Dissolved oxygen in mg/ml= 8 x 100 x N / V x V

Where

V= Volume of sample taken (ml)

N= Normality of sodium thiosulphate (0.025M)

v= Volume of titreart used (ml)

Calculation

Oxygen consumption in mg/ml=dissolved oxygen in blank – dissolved oxygen in experimental

3.8.2 Determination of excretory rate

Principle:.

The anime or imine in the presence of sodium hypochlorite reacts with phenol to give p-nitrophenol which gives green coloured substance. The intensity of colour depends upon the ammonia present, which can be measured at 650 nm.

Chemical preparation

I. Phenol Colour Reagent:. 125 ml of sodium Nitroprusside and 27 mg of phenol was dissolved in 100 ml of distilled water

Sample Collection:

5ml of water sample was collected from each tank separated and filtered for the estimation of ammonia.

Procedure:

To the 2 ml of the obtained sample, 1 ml of sodium hypochlorite solution and 1 ml of phenol-colored reagent were added. The sample aliquot was incubated for 15 minutes at 40°C and then cooled to room temperature. The intensity of the colored complex was measured against a suitable blank at 650 nm. The amount of ammonia present in the sample was calculated using a standard curve of ammonia (mg/ml).

3.9 EXTRACTION AND ESTIMATION OF BIOMOLECULES

3.9.1 Total proteins

Principle:

The carbonyl groups of protein molecules react with copper and potassium present in the reagent to give a blue copper-potassium complex. This complex together with the tyrosine and phenolic compounds present in the protein, reduces the phosphomolybdate of the folin's reagent to give a blue colour complex after 20 minutes.

Chemical preparations

BSA stock solution :- 5mg (0.05g) of BSA was dissolved in 20 ml of 1N NaOH solution.

lowry's reagent: In 98 ml of 4% Na_2CO_3 , 1ml of 2% $CuSO_4$ was added along with 1 ml of 4% Sodium potassium tartrate to make a volume up to 100 ml.µ

Folin's reagent10 ml of folin-giogalter was added in 10 ml of distilled water for little dilution

Extraction

Tissue homogenate was prepared by using 10 ml of ice cold phosphate buffered saline (PBS). The homogenate was cold centrifuged at 6000 RPM for 5 minutes. The supernatant was used as a sample source and the residue was discarded.

Procedure

To the 0.1 ml of obtained sample 0.4 ml of distilled water was added to make the volume to 0.5ml.5 ml of lowry's reagent was added and incubated at room temperature for 10 minutes.

After incubation 0.5 ml of folin'sCiocalteu reagent was added and again incubated at room temperature for another 10 minutes. After 10 minutes, the intensity of the blue coloured complex was measured against a blank at 660nm.Protein quantification of sample was done with the help of a standard curve of BSA (µg/ml in 1N NaOH) (Lowry et at.,1951).

3.9.2 Estimation of Glycogen

Principle:. Hydrolyzing glycogen in the presence of strong sulphuric acid causes it to break down into glucose units that make up glycogen. In acidic medium the hydrolyzed glucose unit reacts with tricyclic aromatic chemical anthrone that reacts with keto groups of glucose resulting in the formation of blue coloured complexes.

Chemicals required

1. Anthrone reagent
2. Glycogen

Reagent preparation

I. Glycogen stock solution: 0.005g of Glycogen was dissolved in 50 ml of distilled water.

II. Anthrone reagent: 0.2g of Anthrone was added in 100ml of concentrated sulphuric acid.

Extraction

500mg of bivalve tissue was homogenised in 10 ml of 5M KOH solution. Homogenate was transferred to the test tube and kept in a boiling water bath for 30 minutes. After boiling, cool the test tube and add 10 ml of 10% PCA solution. Homogenate was centrifuged at 3000 rpm for 10

minutes. Residue was discarded and to the collected supernatant, the same amount of 96% ethanol was added to double the volume. The sample was again kept in a boiling water bath for heating till bubbles came out and then it was cooled. After cooling, again centrifuge at 3000 rpm for 10 minutes and dissolve the precipitate in 3 ml of distilled water. Add 3 ml of ice cold 30% ethanol and 3 ml of ice cold diethyl ether and again centrifuge at 3000 rpm for 10 minutes. Resulting precipitate was dissolved in 1 ml of distilled water.

Procedure

To the 1 ml of sample 4 ml of Anthrone reagent was added and incubated at room temperature for 25 minutes. After incubating, the intercity of the green coloured complex was measured against a suitable blank at 620 nm.Quantification of the glycogen was done with the help of standard curve of glycogen (100µg/ml) (Bavalekaret al.,1951).

3.9.3 Estimation of reduced glutathione (GSH)

Principle:

. GSH reacts with 5,5'- Dithiobis, 2-nitrobenzoic acid producing yellow coloured compound of TNB and oxidized glutathione 2GSH + DTNB□ GSSG + 2TNB

Chemical required

1. DTNB reagent
2. Reduced glutathione
3. Disodium hydrogen phosphate

Preparation of reagents

I. Phosphate solution: 5.34g of Disodium hydrogen phosphate was dissolved in 100ml of distilled water.

II. DTNB reagent: 40 mg (0.04g) of DTNB was dissolved in 100ml of distilled water

III. Reduced glutathione: 5mg (0.005g) of Reduced Glutathione was dissolved in 50 ml of distilled water.

Extraction

The tissue homogenate was prepared by using 1ml of 5% TCA and then cold centrifuged at 6000 rpm for 5 minutes. Residue was discarded and supernatant was used as a sample.

Procedure

To 0.5 ml of tissue sample, 2 ml of phosphate solution was added, after which 2 ml of 5,5'-Dithiobis, 2-nitrobenzoic acid (DTNB) reagent was added.The intensity of the coloured complex was measured against a suitable blank at 412 nm (Moron et al.,1979).Quantification of reduced glutathione was done with the help of standard curve of Reduced Glutathione (0.2µmole/ml in 5% of TCA).

3.10 EXTRACTION AND ESTIMATION OF ENZYMES

3.10.1 Catalase (CAT)

Principle:.

Catalase activity was measured by using a method reported by Sinha. The reaction of catalase is the decomposition of hydrogen peroxide into water and molecular oxygen.

$2H_2O_2 \rightarrow 2H_2O + O_2$

Dichromate in acetic acid is reduced to chromic acetate, with the creation of perchromic acid as an unstable intermediate. The concentration of hydrogen peroxide is proportional to the concentration of chromic acetate produced by the reaction , which gives an orange coloured complex, the intensity of which is measured at 620 nm.

Chemical required

1. Potassium dichromate
2. Glacial acetic acid
3. Disodium hydrogen phosphate
4. Sodium dihydrogen phosphate
5. Hydrogen peroxide

Preparation of reagents

Phosphate buffer: Dissolve 3.56g of Disodium hydrogen phosphate in 100 ml of distilled water label it as solution A. 3.12g of Sodium dihydrogen phosphate was dissolved in 100ml of distilled water and label it as Solution B. Mix 80 ml of solution A and 20 ml of solution B and adjust the pH of solution to 7.80.2M 30% Hydrogen peroxide :2.26ml of hydrogen peroxide was added in 97.74 ml of distilled water

I. Dichromate/ acetic acid (DCA): 50 ml of potassium dichromate mixed in 150ml of glacial acetic acid with 1:3 distribution.

Extraction

Tissue homogenate was prepared by using phosphate buffer saline of pH 7.2 . Homogenate was cold centrifuged at 3000 rpm for 15 minutes. The residue was discarded and supernatant was used as a sample source.

Procedure

2 ml of homogenate was added and thoroughly mixed into an assay mixture containing 0.5ml of 0.2M H_2O_2 solution. After 1 minute 2 ml of dichromate acetic acid solution was added and kept in a boiling water bath for 10 minutes. Mixed well and intensity of the Orange colour was measured at reference blank at 620 nm (sinha.,1962).The enzyme activity was measured with the help of reference curve of hydrogen peroxide (2µ molecule/ml) and expressed in mole of H_2O_2 consumed / min/mg protein

3.10.2 Estimation of Alkaline Phosphates

principle:.

The enzyme catalyses the hydrolysis of p-nitrophenyl phosphate (pNPP), under alkaline conditions. The reaction produces p-nitrophenol, which is yellow in color and can be quantified spectrophotometrically at 405 nm The amount of p-nitrophenol produced is directly proportional to the ALP activity in the sample.

Chemical required

1. Glycine
2. HCL
3. P-nitrophenol
4. P-nitrophenyl phosphate

5. Sodium hydroxide
6. Magnesium chloride

Preparation of reagents

I. ALP substrate (9.2): 0.375g of glycine along with 0.166g of NaOH, 0.01 g of magnesium chloride, 0.165g of P-nitrophenyl phosphate were dissolved in 100ml of distilled water and 9.2 pH was maintained.

II. Glycine buffer: 0.375g of glycine and 0,035ml of HCL was dissolved in 40 ml of distilled water. pH was adjusted to 3 by making the volume to 50 ml

Extraction

Tissue homogenate was prepared by using tris-hcl buffer centrifuge at 300 rpm for 15 minutes. The residue was discarded and supernatant was used as a sample.

Procedure

To 0.1 ml of diluted sample add 1 ml of substrate buffer and incubate for 30 minutes at 37^0C. The reaction is stopped by the addition of 0.02M NaOH. The intensity of the colour change was measured against suitable blank at 405 nm (Bavalekar et al.,1951). The enzyme activity was measured with the help of reference curve of P-Nitrophenol (1µm P-Nitrophenol /Min)

3.10.3 Estimation of Acid Phosphates (AP)

Principle:.

Acid phosphatase catalyzes the hydrolysis of pNPP into p-nitrophenol and phosphate p-nitrophenyl phosphate+H2O→p-nitrophenyl+phosphate. The formation of p-nitrophenol is directly proportional to the acid phosphatase activity in the sample.

Chemical required

1. citric acid
2. Sodium citrate
3. P-nitrophenyl phosphate
4. Sodium hydroxide
5. P-nitrophenol

Preparation of reagents

I. Citrate buffer (0.1M, pH 4.8): 410mg citric acid along with 1.1g of sodium citrate were dissolved in 100 ml of distilled water.

II. Substrate solution: Dissolve 165 mg of P-nitrophenyl phosphate in 100 ml of citrate buffer.

I. NaOH (0.1M): 4gm of NaOH dissolved in 1000 ml of distilled water.

Extraction

A tissue homogenate was prepared by using a potassium phosphate buffer which was centrifuged at 3000 rpm for 15 minutes. Residue was discarded and supernatant was used as a sample.

Procedure

1 ml of citrate buffer was added to the tubes and were equilibrated for 10 minutes at 37^0C. The enzyme activity was arrested by the addition of 4 ml of NaOH in all tubes. The intensity of the colour change was measured at 405 nm (Bavalekar et al.,1951). The enzyme activity was measured with a reference curve of P-nitrophenol (1µm of P-nitrophenol / min).

3.10.4 Estimation of Malondialdehyde (MDA)

Principle:.

The byproduct of lipid oxidative damage arethiobarbituric acid reactive substance (TBARS) which can be identified by using thiobarbituric acid (TBA) as reagent.TBA reacts with Monodialdelyde (MDA) to give a dark pink colour absorbing light maximally at 535 nm.

Chemical required

1. Trichloroacetic acid
2. Thiobarbituric acid
3. Hydrochloric acid

Preparation of reagents

I. 0.37% Thiobarbituricacid(TCA) -0.37g of TBA was dissolved in 100mL of distilled water.

II. 0.24 N HCL- 1.6 mL of concentrated HCL was pipette out in a 100 ml volumetric flask and diluted up to the mark with distilled water.

III. 15% Trichloroacetic acid (TCA)-15g of TCA was dissolved in 100 ml of distilled water.

Extraction

A tissue was removed and rinsed with ice cold media (5mM tris hydrochloric acid (HCL) . The sample was homogenised in 100% methanol and centrifuged at 10,000xg for 15 minutes at 4^0C

Procedure

Supernatant was used as a sample in which 2 ml of Trichloroacetic acid ,Triobarbitanic acid-HCL was added. After that the mixture was placed in a boiling water bath for 15 minutes. The protein precipitate was removed after cooling by centrifuge at 10,000xg for 5 minutes. The resulting solution was measured at 535 nm against reagent blank (Buege et al.,1978)

3.10.5 Estimation of Alanine Aminotransferase (ALT)

Principle: -

Alanine reacts with α-Ketoglutaric acid and undergoes a transamination reaction. alanine donates its amino group to α-Ketoglutaric acid because of which α-Ketoglutaric acid becomes glutamic acid. Alanine becomes pyruvate which reduces 2,4 dinitrophenyl hydrazine to dinitrophenyl hydrazone. In alkaline medium, hydrazone produces a coloured complex

Alanine transaminase

Alanine + Alpha.Ketoglutaric aci ⟶ Pyruvate + glutaric acid

Chemical required

1. Dipotassium hydrogen phosphate
2. Potassium hydrogen phosphate
3. Sodium hydroxide
4. α-Ketoglutarate

Preparation of reagents

1. ALT substrate

   0.087g (K_2HPO_4) along with a 0.068g (KH_2PO_4) was dissolved in 50 ml distilled water, from which 20ml is taken and 0.1ml NaOH is added into it along with 0.532g of alanine and 0.006g of α-Ketoglutarate.

Extraction

The tissue homogenate was prepared by using a potassium phosphate buffer and cold centrifuged at 3000 rpm Supernatant was used as a sample by discarding tissue debris.

Procedure

ALT substrate was prepared in a phosphate buffer and was incubated at 37^0C for 5 minutes. To this tissue homogenate was added to assay the enzyme activity. Enzyme blank was also prepared by taking 0.5ml of substrate and 0.1 ml of distilled water. They were further incubated for another 15 minutes. The reaction was stopped by addition of 0.5ml of dinitrophenylhydrazine reagent kept for another 20 minutes at room temperature. Reaction was stopped by addition of 5 ml of 0.4N NaOH. The coloured intensity was read against a blank at 540 nm (Reitman and Frankel., 1957). Quantification with a standard curve of Pyruvate (220µg/ml).

3.11 GENOTOXICITY STUDY

3.11.1 The Micronucleus Assay

Bivalve Gills were dissected and chopped into small pieces to separate the cells and dropped onto a microscope slide and allowed to air dry at room temperature. The cells were then fixed with absolute methanol and again air dry. The slides were subsequently stained with 5% Giemsa in phosphate buffer saline. The slides were observed under the microscope for intact cells (Siu et al. ,2004).

3.12 HISTOLOGY

The bivalves were sacrificed and the gills were isolated and removed. Tissues were washed in physiological saline. The tissues were weighed and stored in 10% phosphate buffered formalin for 24 hours and then transferred in 70% ethanol until processed. The slides were prepared in the Ashwini pathology lab. They were further analysed under a light microscope at 20x and light micrographs were captured.

Analysis and Conclusion

CHAPTER 4: ANALYSIS AND CONCLUSION

4.1 RESULTS

4.1.1 Lc50 of $CoFe_2O_4$

The Lc50 value of Cobalt Ferrite Nanoparticle ($CoFe_2O_4$) for the Acute study of 96 hrs was found to be 100.93 mg/L in *Paphiamalabarica.*

4.1.2 Laboratory Exposure Study

The study animal *was Paphiamalabarica* of which gills and visceral tissue were used for biochemical estimations.

4.1.3 Effect of $CoFe_2O_4$ Nanoparticle on Physiological Parameters

A. Effect of $CoFe_2O_4$ Nanoparticle on excretion of Ammonia

The rate of ammonia Excretion show significance difference with the concentration of doses ($F=28.26$, $P=0.0013$) but there was no significant difference with respect to days ($F=3.336$, $P=0.1061$). The interection between the concentration of doses with the days show significant difference ($F=8.514$, $P<0.0001$).

B. Effect of CoFe2O4 NPs on Oxygen Consumption

The oxygen consumption show significance difference with the concentration of doses ($F=16.66$, $P=0.0001$), also there is a significance difference with the days ($F=10.83$, $P=0.0040$) and also no difference with the concentration of doses with the days ($F=1.796$, $P=0.0890$).

4.1.4 Effect of CoFe2O4 NPs on Biomolecules of Gills and Viscera

The graph for protein estimation in Paphiamalabarica Visceral tissues indicates that a higher concentration of protein was present in the Experimental 2 group and the lower quantity was found in the Control group. Anova analysis showed significant difference (F=40.54, P=0.0003) with respect to protein estimation in tissues. Further the protein estimation in gills indicates higher concentration in Experimental 1 and equal quantity of protein was found in Control and Experimental group. Anova analysis does not show a significance difference (F= 0.8937, P=0.4574) between the control and experimental groups.

The Glycogen content of visceral tissues found to be high in experimental 2 group exposed to higher concentration of doses with a significance difference (F= 92.12, P<0.0001) and in the gills tissues higher quantity was found in control and experimental 2 group and lower was found in experimental 1. Anova analysis does not show any significance difference between the groups (F= 0.1208, p=0.8784).

The Reduced Glutathione concentration found to be high in Experimental 2 group of visceral tissues and lower in control group with a significance difference (F=72.43, P<0.0001) revealed by Anova analysis. In the gill tissue the results show higher concentration in Experimental 1 group followed bt Experimental 2 and then in Control group with a sifnificance difference of (F=3.881, P=0.0438) revealed by one wayAnova.

4.1.5 Effect of $CoFe_2O_4$ NPs on Enzymes of Gills and Vicera

The result of Catalase estimation enzymes showa elevated level in Experimental 2 Group of visceral tissues with a significance difference (F=12.8, P=0.0006) but there was no significant difference (F=2.503, P=0.1153) in terms of groups in Gills tissues. The outcome of Alanine

Amino Transferase estimation revealed that the higher concentration found in Experimental 2 group of visceral tissue but no significance difference of (F=1.062, P=0.3703) among groups. In Gills higher concentration found in Experimental 1 Group with a significance difference of (F=3.795, P=0.0464)among groups.

Estimation of Alkaline Phosphatase (ALP) Enzyme shows higher activity in Experimental 2 and Lower in Control group of visceral tissue with a significance difference (F=19.42, P=0.0005), but there was no significance difference in Gills tissues of Control and Experimental groups.Although higher concentration found in Experimental 2 group. The graph of Acid Phosphatase (AP) Enzyme show higher concentration in Control group but does not show significance difference (F=2.259, P=0.1262) in visceral tissues, Also there was no significance difference in the gill tissue (F=1.43, P=0.2702), although Experimental 2 group show slight elevation.

Malondiadehyde (MDA) concentration seen higher in Control group for visceral tissue with a significance difference (F=10.87, P=0.002), but found higher in Experimental 2 group for gill tissues with a significance difference (F=4.605, P=0.0328) among groups. The graph of Superoxide Dismutase (SOD) showed that the Enzyme activity was highest in experimental 2 group and lowest in control group for visceral tissues with a significance difference (F=10.55, P=0.0439), but there was no significance difference (F=2.839, P=0.2033) with and without exposure for gill tissues although a slight higher concentration found in Experimental 1 group.

4.1.6 Correlation Analysis

A. In visceral tissue

According to Pearsons correlation test, In the visceral tissue Protein concentration was significantly correlated with Reduced Glutathione (P value=0.0002, r value=0.9358) and catalase (P value=0.0003, r value=0.9317) but not significantly correlated with glycogen (P=0.3325, r=0.3661), Superoxide dismutase (P=0.1463, r=0.5254), Alkaline Phosphatase (P=0.2554, r=0.424) and Alanine Amino Transferase (P=0.1352, r=0.5379), while protein concentration was not significantly negatively correlated to Acid Phosphatase (P=0.7501, r= -0.1243) and Malondialdehyde (P=0.105 , r= -0.5755) concentration.

Glycogen in the visceral tissue was significantly correlated with Superoxide Dismutase (P=0.0032, r=0.8571) and Alkaline Phosphatase (P=0.0005, r=0.9162) but significantly negatively correlated with Alkaline phosphatase (P=0.0291,r= -0.7188).Glycogen concentration was not significantly correlated with Reduced Glutathione (P=0.1311, r=0.5427),and catalase (P=0.0753, r=0.6194) while not significantly negatively correlated with MDA (P=0.0528, r= -0.6605) and ALT (P=0.4903,r= -0.2652).

Concentration of Reduced Glutathione was significantly correlated with Catalase (P=<0.0001, r=0.9856) and SOD (P=0.0200.37378, r=0.7466) and not significantly correlated with ALP (P=0.0588, r=0.6486) and ALT (P=0.6486, r=0.3737) and also not significantly negatively correlated with AP (P=0.5533, r= -0.229 and MDA (P=0.0128, r= -0.7816).

Enzyme catalase Show significantly Positive correlation with SOD (P=0.0161, r=0.766) and not significantly positive correlation with ALP (P=0.051, r=0.6643) and ALT (P=0.4356, r=0.2983). Catalase enzyme is significantly negatively correlated with MDA (P=0.0168, r=-0.7631) and non significantly negatively correlated with AP (P=0.5096, r= -0.254).

Alkaline Phosphatase (ALP) concentration was significantly positively correlated with SOD (P=0.0008, r=0.9062) and significantly negatively correlated with AP (P=0.0228, r= -0.7395) and MDA (P=0.0294, r= -0.7179), but the enzyme was non significantly negatively correlated with ALT (P=0.6902, r=-0.1552).

Acid Phosphatase (AP) enzyme activity was not significantly positively correlated with MDA (P=0.448, r=0.2906) and negatively correated with ALT (P=0.9796, r= -0.009996) and SOD (P=0.2188, r= -0.4547). Similarly enzyme Alanine Amino Transferase (ALT) was not significantly negatively correlated with SOD (P=0.7901, r= -0.1039) and MDA (P=0.7545, r= -0.122). Concentration of enzyme Malondialdehyde (MDA) was significantly negatively correlated with Superoxide Dismutase (SOD) (P=0.008, r=-0.8111).

B. In Gill tissue

In the Gill tissue Protein concentration was significantly correlated with SOD (P=0.0041, r=0.8449) and not significantly correlated with ALT (P=0.3188 , r=0.3759) and Catalase (P=0.2175, r=0.4558) while non significantly negatively correlated with Glycogen (P=0.4209, r= -0.3075), ALP (P=0.5297, r= -0.2424), AP (P=0.4268, r= -0.3037), MDA (P=0.2875, r= -0.3989), and Reduced Glutathione (P=0.3177, r= -0.3767).

Concentration of Glycogen was significantly correlated with MDA (P=0.0053, R=0.8331) and non significantly correlated with AP (P=0.54, r=0.2366) and Reduced Glutathione (P=0.5324, r= 0.2409). While the concentration was not significantly negatively correlated with ALP (0.6664, r= -0.1676) and significantly negatively correlated with ALT (P=0.0015, r= -0.8864) and Catalase (P=0.0029, r=-0.8604).

Reduced Glutathione was not significantly correlated with ALP (P=0.8497, r=0.07414), AP (P=0.3543, r=0.3511), ALT (P=0.9463 , r=0.02637) and MDA (P=0.1437, r=0.5283). But negatively correlated with Catalase (P=0.2714 , r= -0.4113) and SOD (P=0.545, r= -0.2337). Enzyme Catalase was Significantly correlated with SOD (P=0.0216, r=0.7439) and ALT (P=0.0129, r=0.7813), non significantly correlated with ALP (P=0.8949, r=0.05172), and it was significantly negatively correlated with MDA (P=0.0206, r= -0.7475).

Concentration of Alkaline Phosphatase was not significantly correlated with ALT (P=0.6408, r=0.1812)and SOD (P=0.8474, r=0.07528) and negatively correlated with AP (P=0.1466, r=-0.5251) and MDA (P=0.7269, r=-0.1361).

Acid Phosphatase was not significantly Positively correlated with MDA (P=0.5686, r=0.2205) and negatively with ALT (P=0.4848, r= -0.2685) and SOD (P=0.1234, r= -0.552).

Alanine Amino Transferase activity was not significantly positively correlated with SOD (P=0.0647, r=0.6376)and negatively correlated with MDA (P=0.1257,r=-0.5491). Malondialdehye was significantly negatively correlated with SOD (P=0.008, r= -0.8111).

4.1.7 Genotoxicity study

Results show a prominent micronucleus which were seen in the exposed bivalve cells, which confirms that a higher percentage of micronucleus are found in a exposed groups than control. The maximum number of micronucleus are found in a Experimental 2 group, exposed to 50mg/L concentration. Anova analysis show a significant difference (F=64.65, P<0.0001) in the micronucleus of exposed and unexposed groups of bivalve gill cells.

4.1.8 Histopathological Study

Gill

The histopathology of control bivalve gills show uniform interlamellar space and uniform arrangement of gill lamellae which are divided into three zones upper Frontal zone middle Intermediate Zone and the lower Abfrontal Zone. Inside each lamellaeheamolymph pass through the vessel. The outer surface of the gill lamellae lined by the Epithelial cells and the inner surface lined by the endothelial cells. In the experimental 1 bivalve gills show narrower gill lamellae with a obstructed gill heamolymph vessels. Whereas experimental 2 bivalve gill shows Narcotic gill lamellae which are fused and clubbed with the other gill lamellae along with narrower heamolymph vessels.

Graph of Physiological Parameters

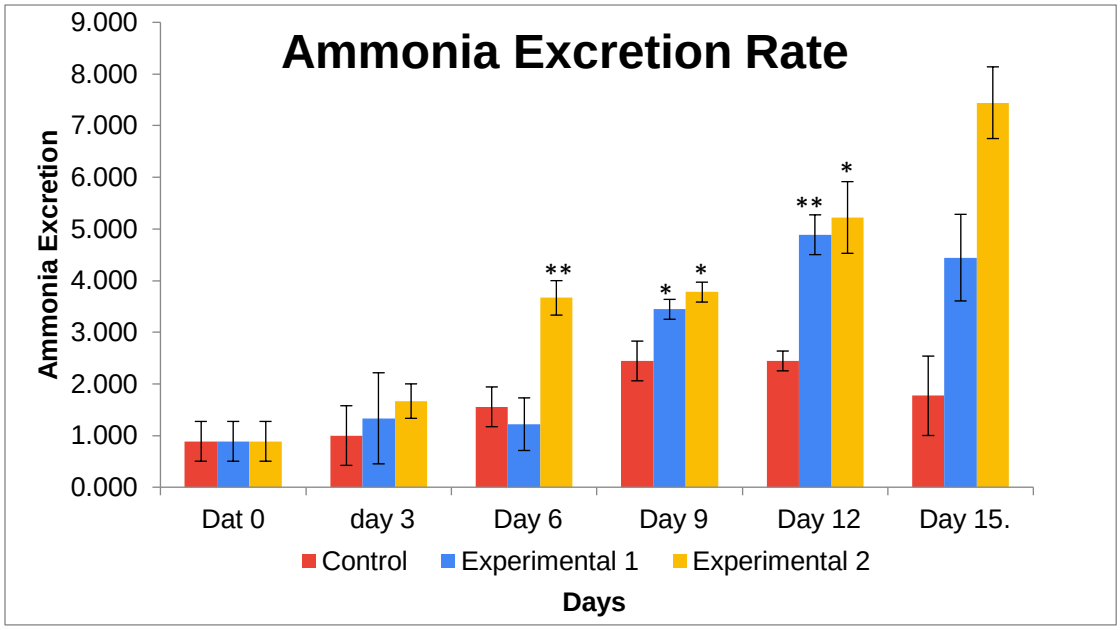

Figure 2. Effect of $CoFe_2O_4$ Nanoparticle on the Ammonia Excretion Rate of bivalve. ($p<0.05^*$, $P<0.01^{**}$)

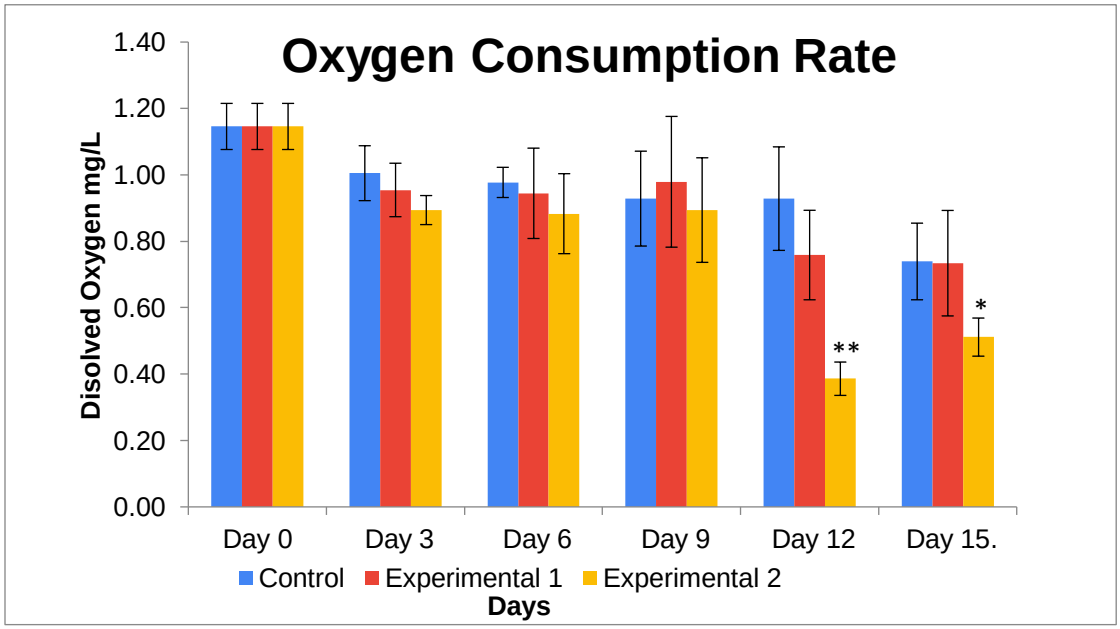

Figure 3. Effect of $CoFe_2O_4$ Nanoparticle on the Oxygen Consumption Rate of bivalve. ($p<0.05^*$, $P<0.01^{**}$)

Graph of Biomolecules Estimation

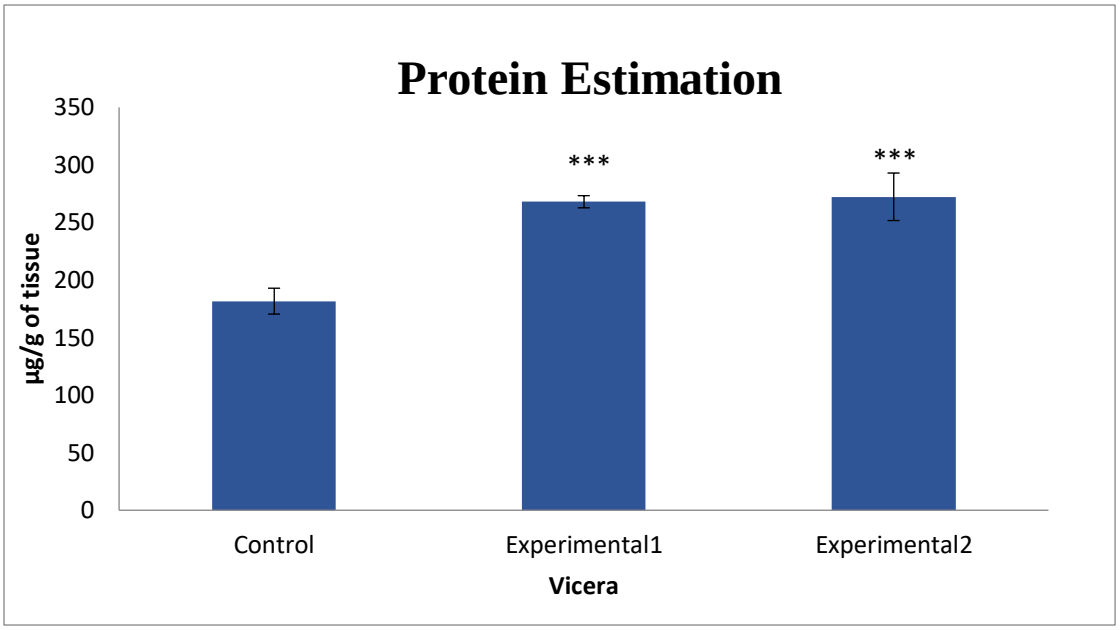

Figure 4. Effect of $CoFe_2O_4$ Nanoparticle on the total protein content of bivalve visceral tissue . ($p<0.001$***).

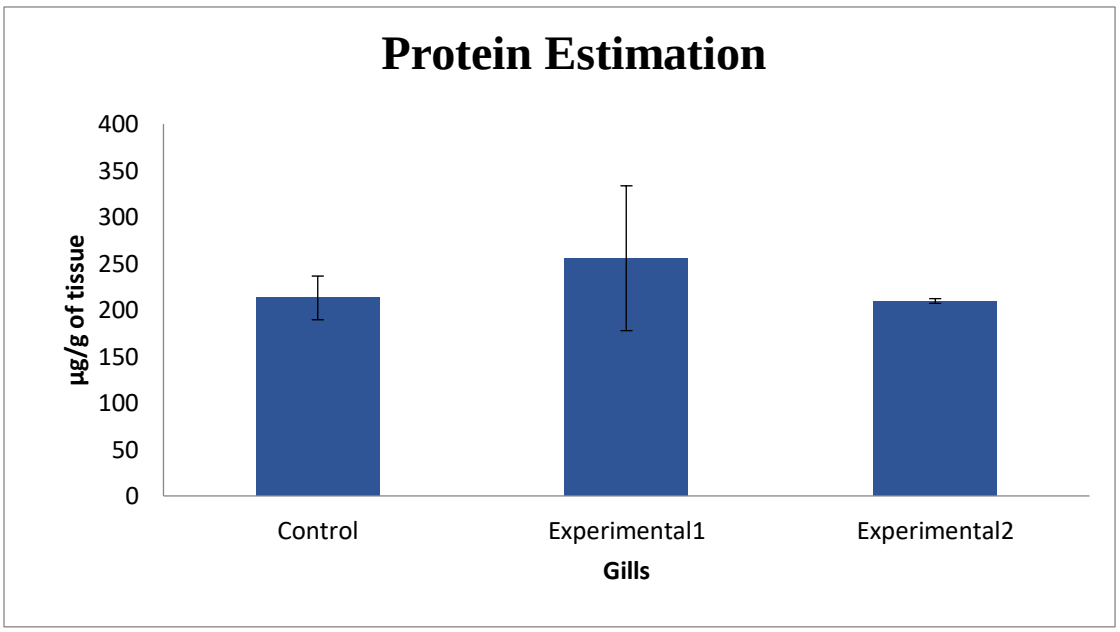

Figure 5. Effect of $CoFe_2O_4$ Nanoparticle on the total protein content of bivalve Gill tissue.

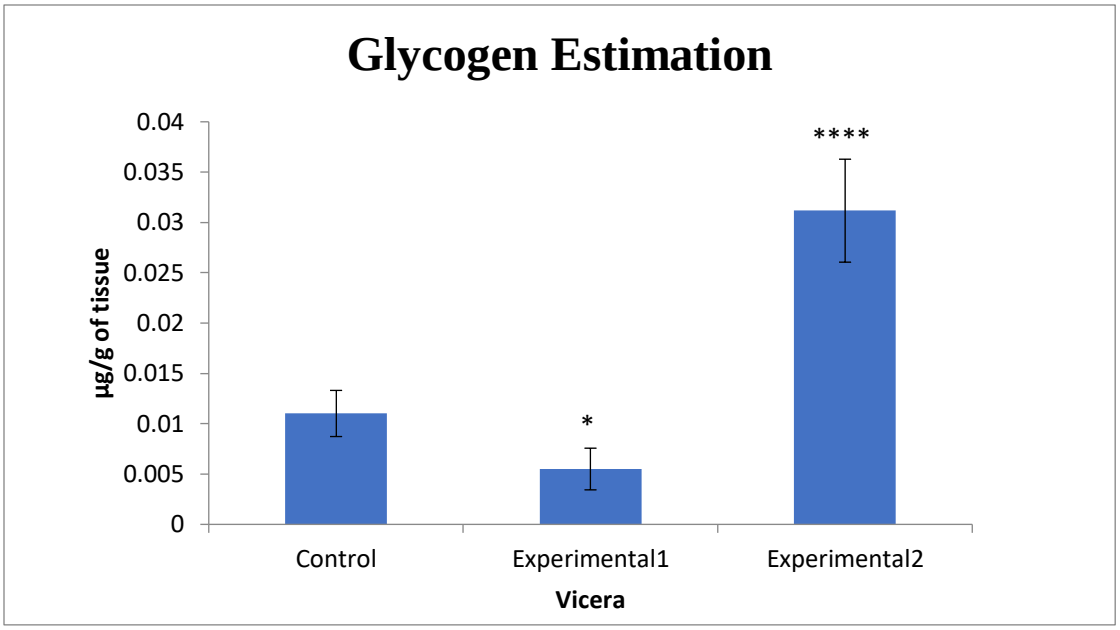

Figure 6. Effect of $CoFe_2O_4$ Nanoparticle on the Glycogen content of bivalve visceral tissue. (p<0.05* , P<0.0001 ****)

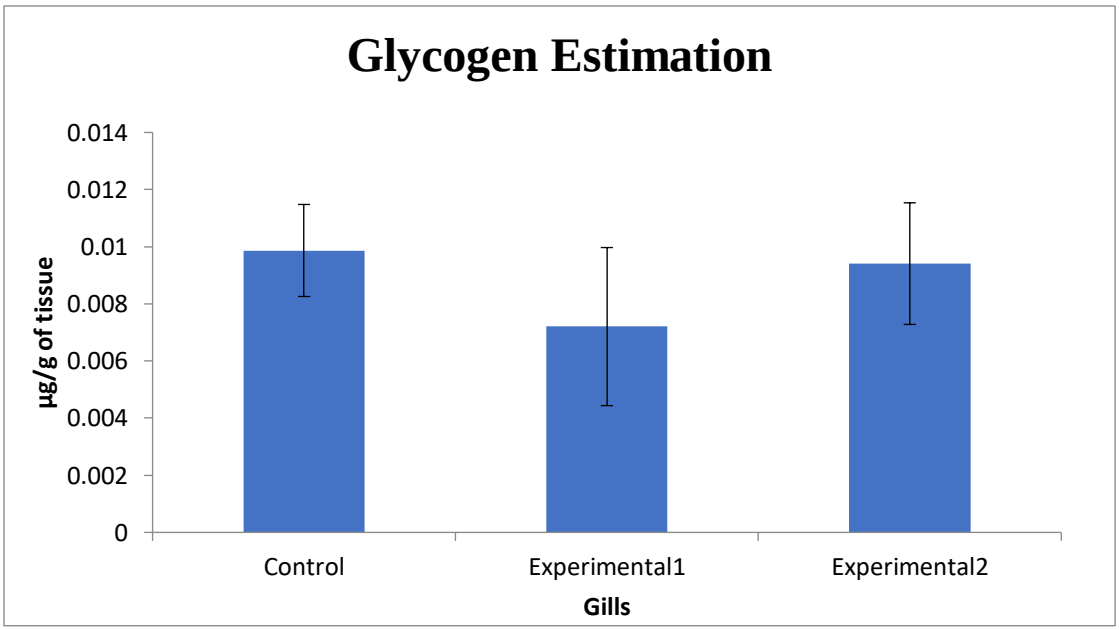

Figure 7. Effect of $CoFe_2O_4$ Nanoparticle on the Glycogen content of bivalve Gill tissue.

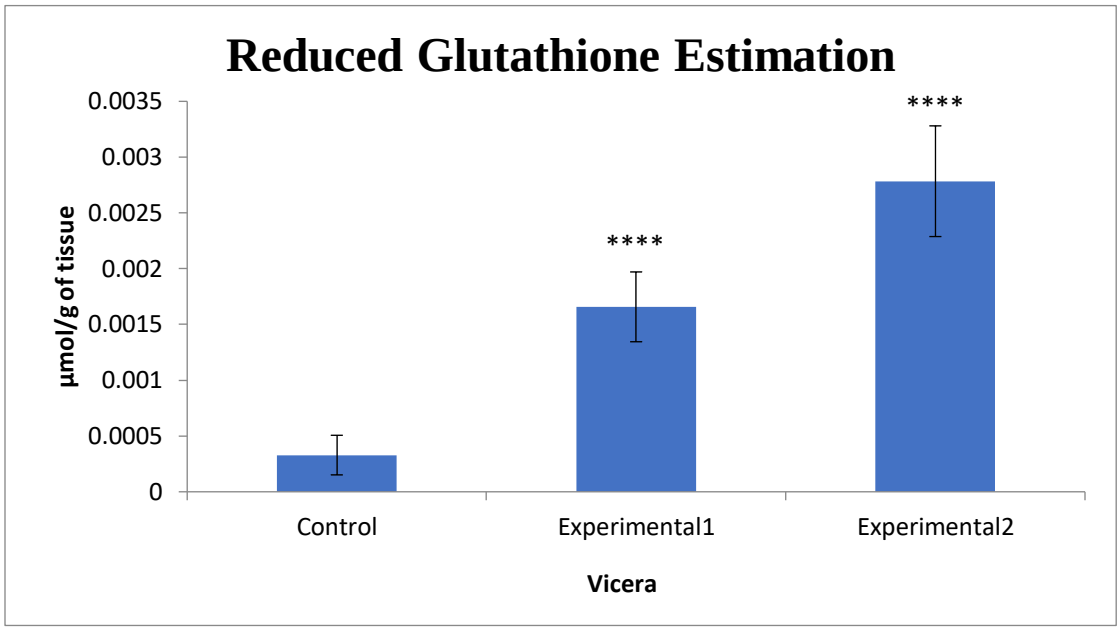

Figure 8. Effect of $CoFe_2O_4$ Nanoparticle on the Reduced Glutathione content of bivalve visceral tissue. (P<0.0001 ****)

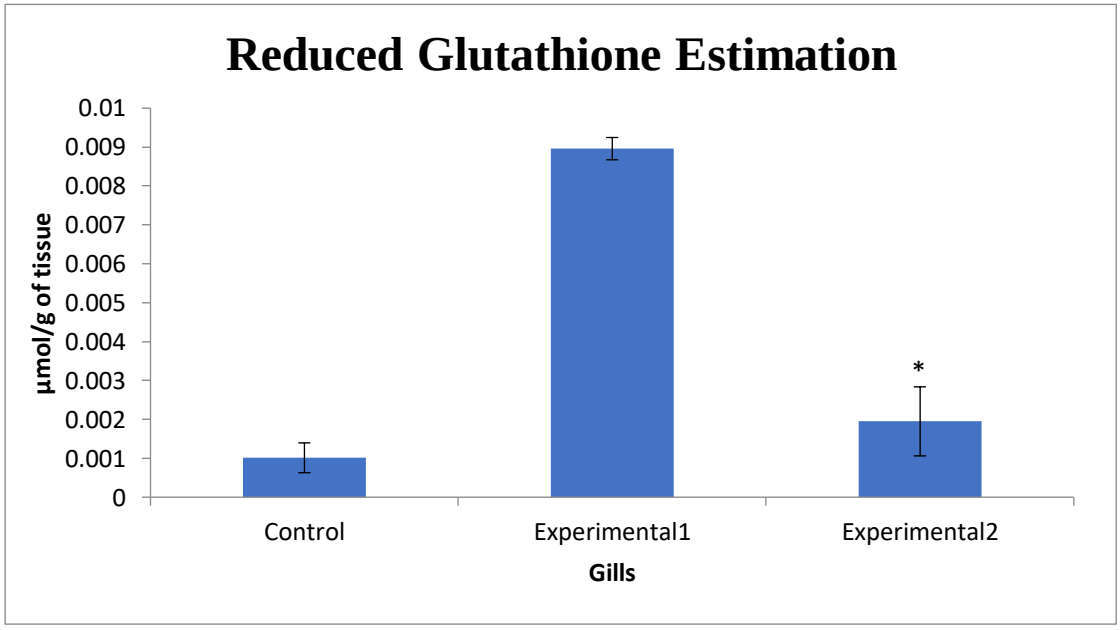

Figure 9. Effect of $CoFe_2O_4$ Nanoparticle on the Reduced Glutathione content of bivalve Gill tissue. (P<0.05*)

Graph of Enzyme Estimation

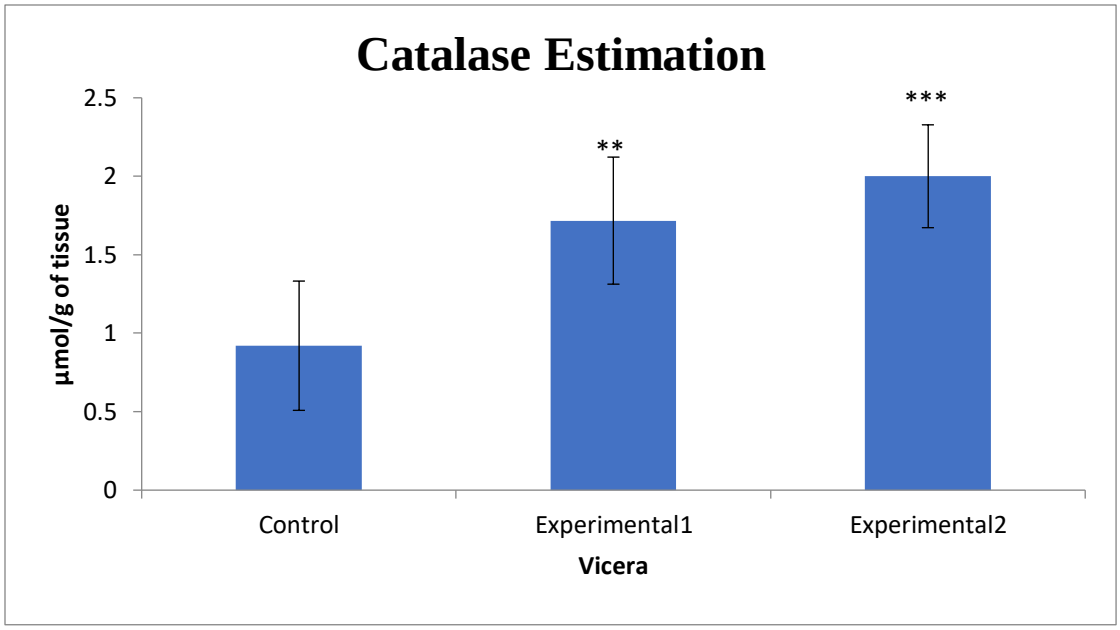

Figure 10. Effect of $CoFe_2O_4$ Nanoparticle on the Catalase activity of bivalve visceral tissue. (P<0.01**, P<0.001 ***)

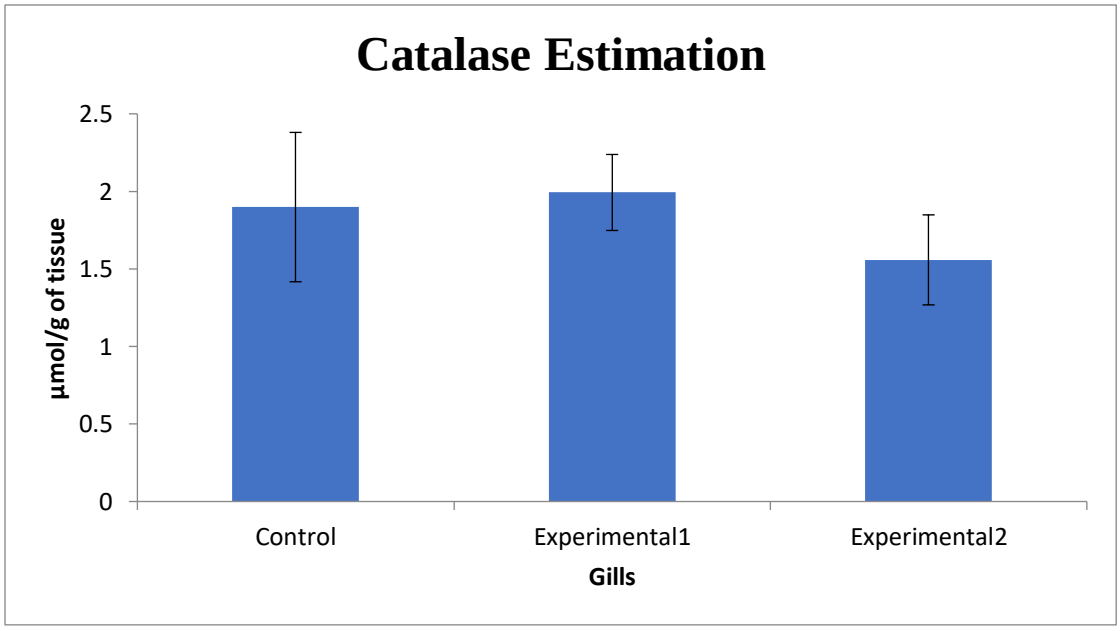

Figure 11. Effect of $CoFe_2O_4$ Nanoparticle on the Catalase activity of bivalve Gill tissue.

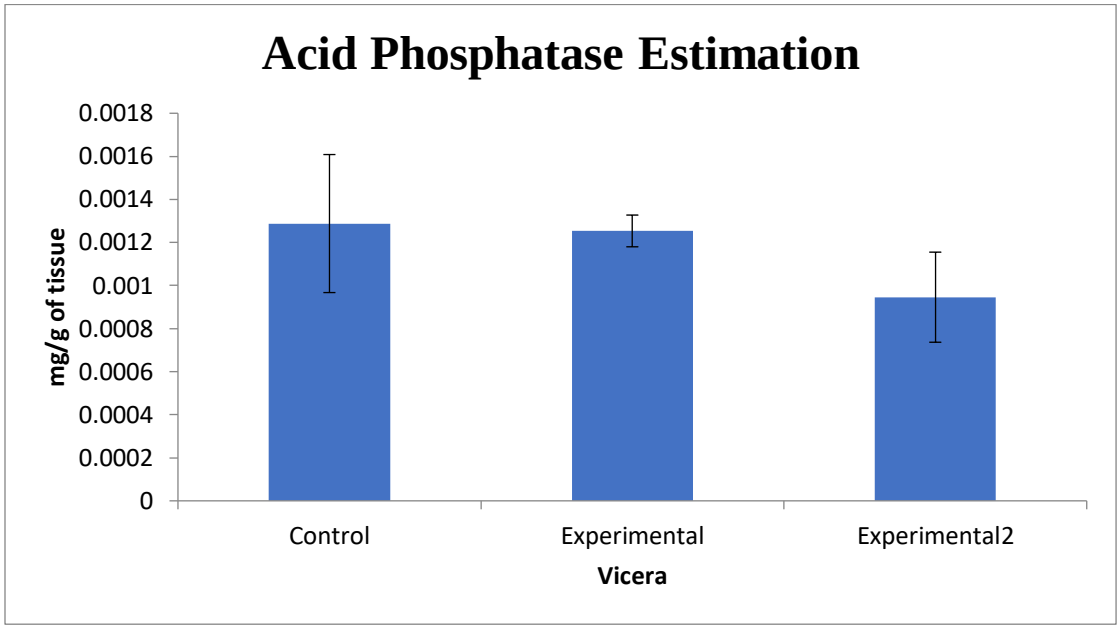

Figure 12. Effect of $CoFe_2O_4$ Nanoparticle on the Acid Phosphatase Activity of bivalve Gill tissue**.**

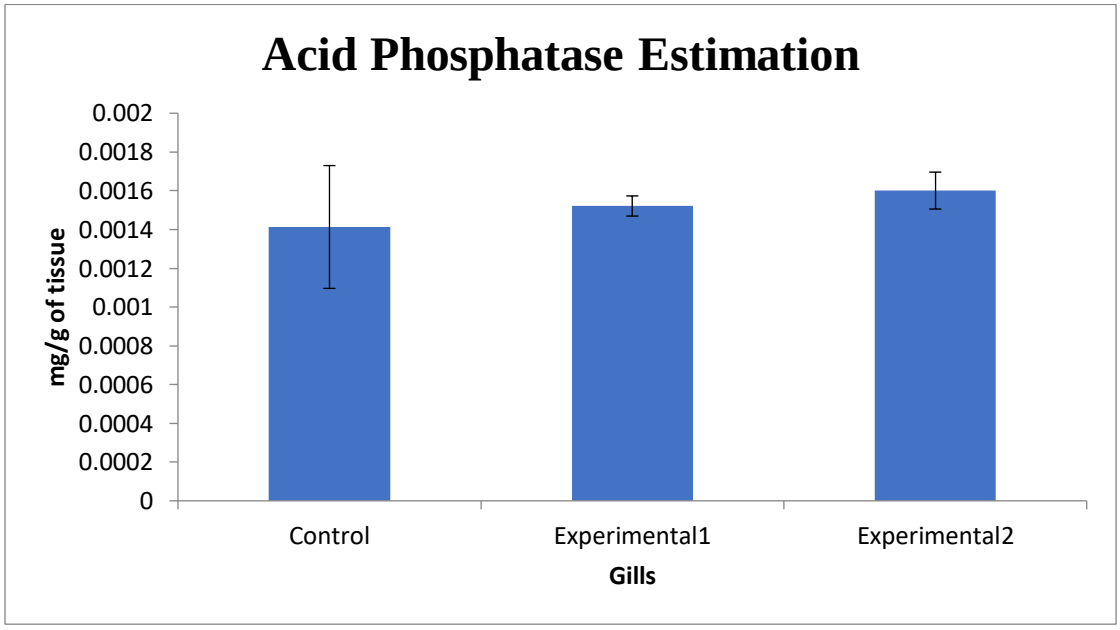

Figure 13. Effect of $CoFe_2O_4$ Nanoparticle on the Acid Phosphatase Activity of bivalve Gill tissue.

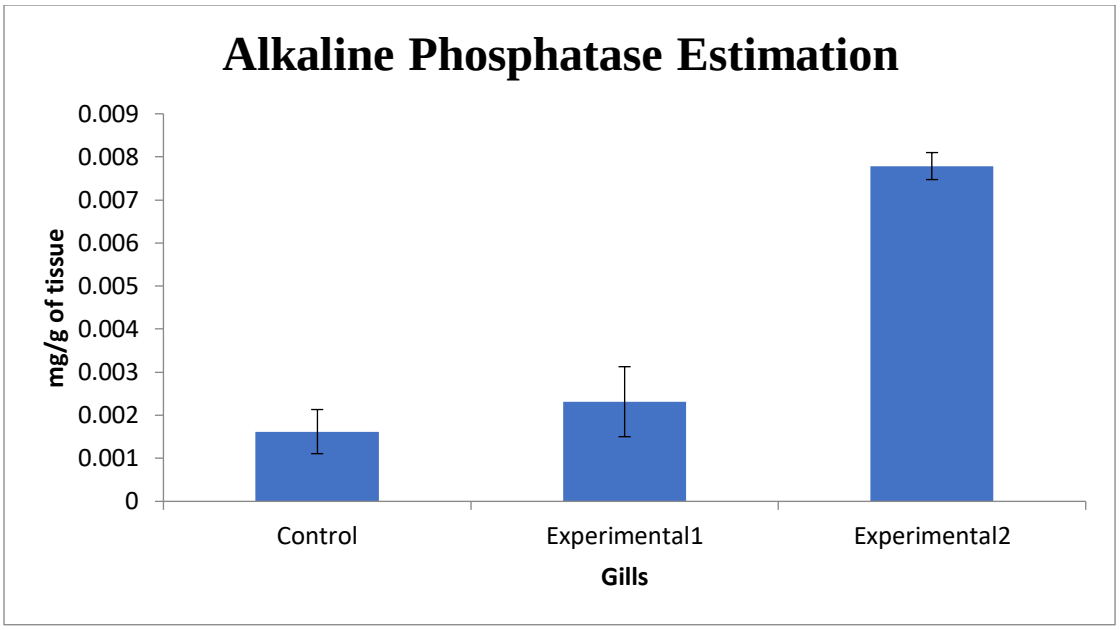

Figure 14. Effect of $CoFe_2O_4$ Nanoparticle on the Alkaline Phosphatase Activity of bivalve Viceral tissue. (P<0.001***)

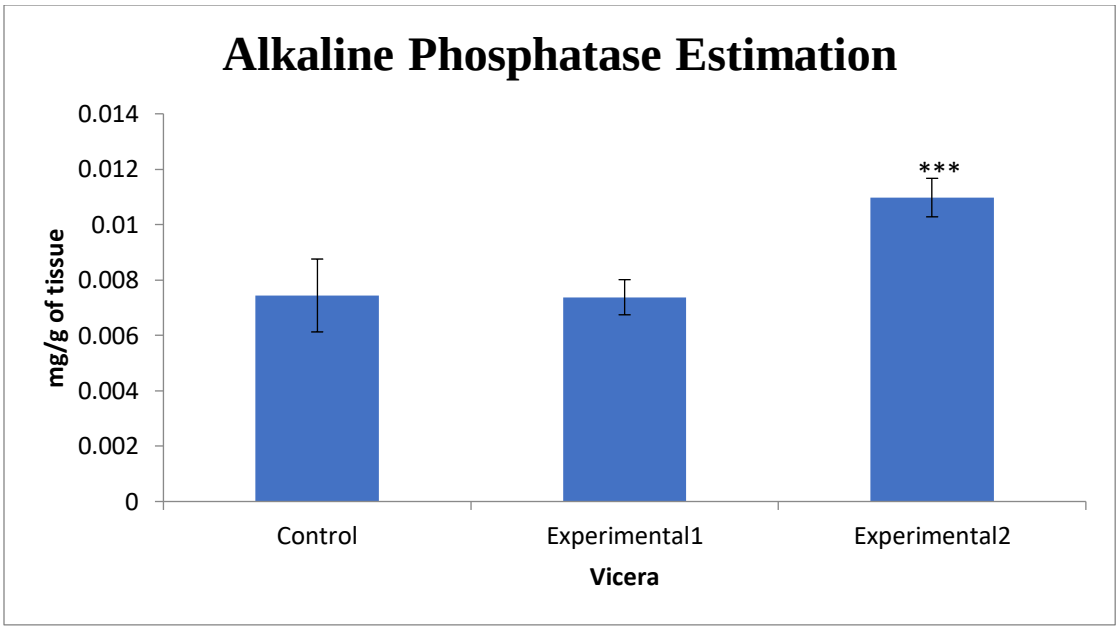

Figure 15. Effect of CoFe2O4 Nanoparticle on the Alkaline Phosphatase Activity of bivalve Gill tissue.

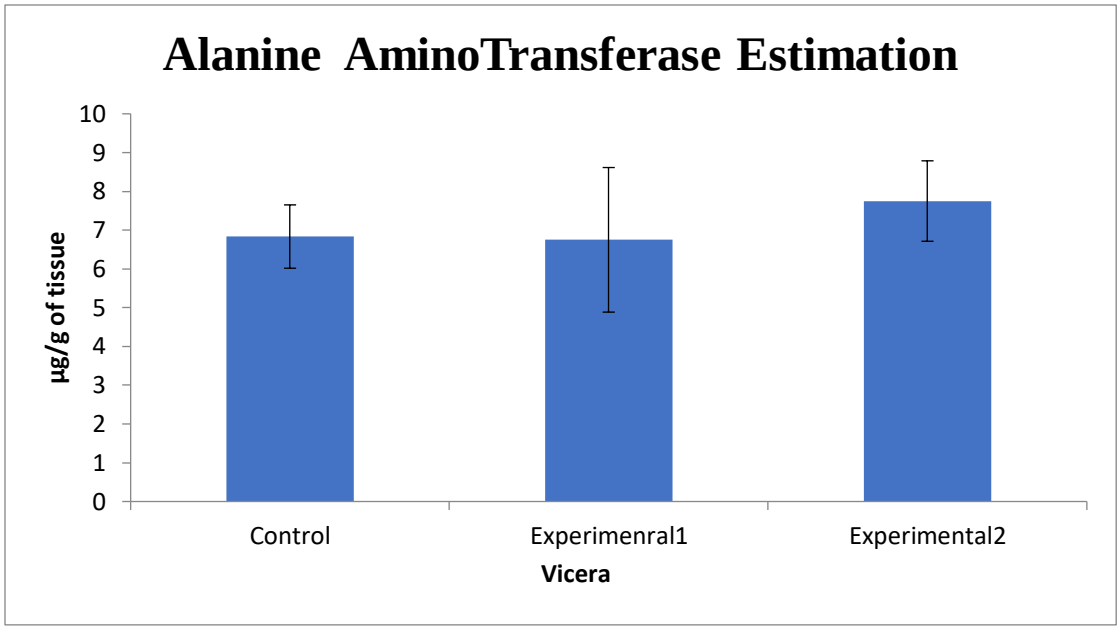

Figure 16. Effect of $CoFe_2O_4$ Nanoparticle on the Alanine Amino Transferase Activity of bivalve viceral tissue. (P<0.05*).

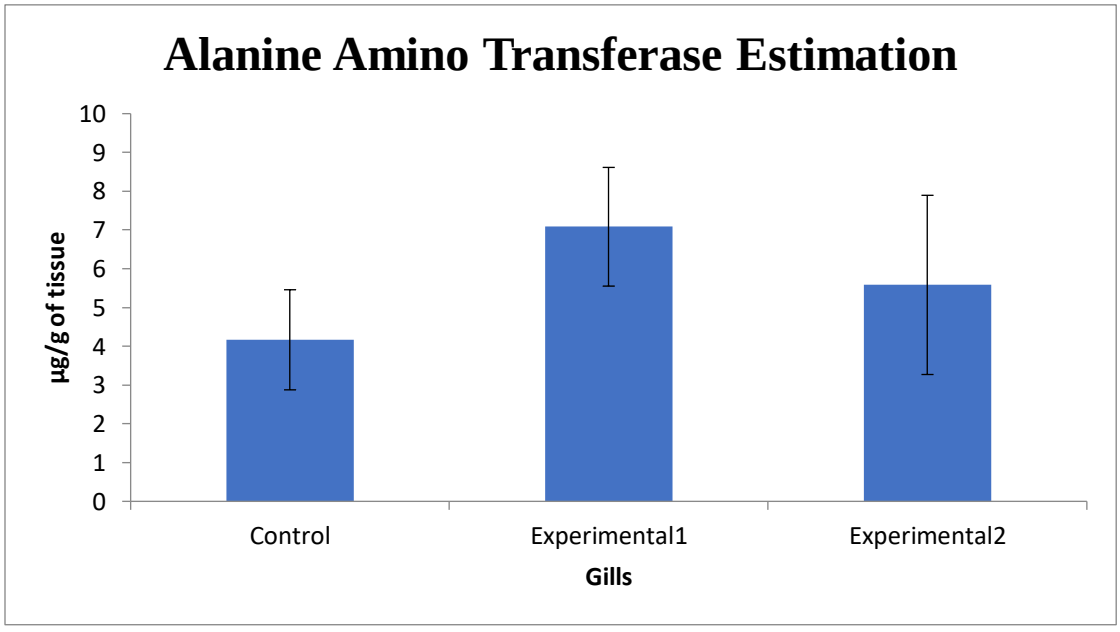

Figure 17. Effect of $CoFe_2O_4$ Nanoparticle on the Alanine Amino Transferase Activity of bivalve Gill tissue.

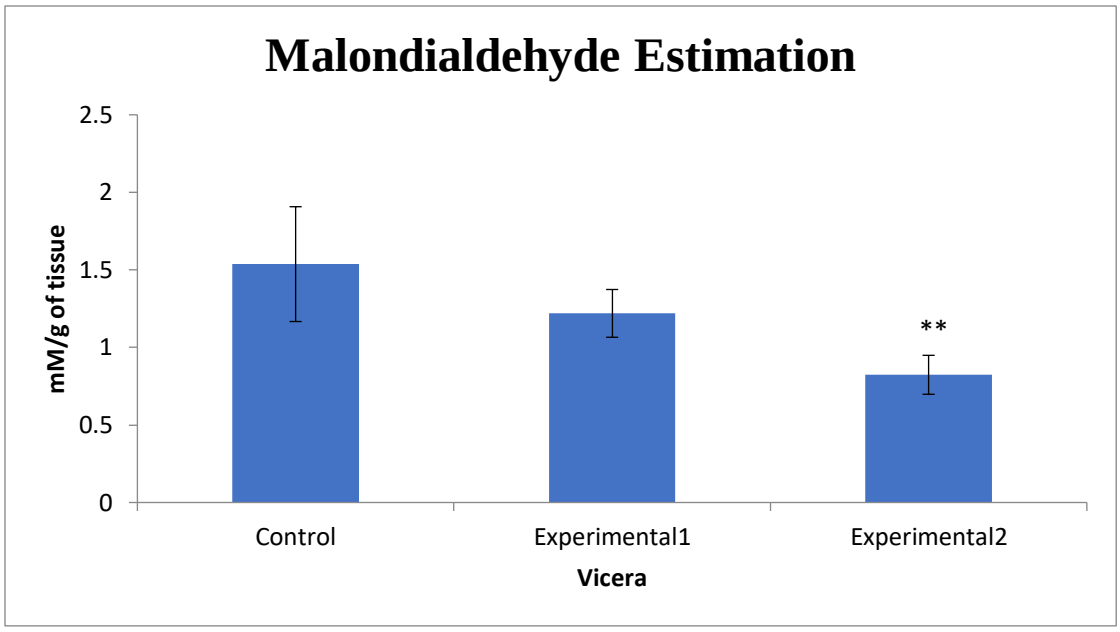

Figure 18. Effect of $CoFe_2O_4$ Nanoparticle on the Malondialdehyde Activity of bivalve Viceral tissue. (P<0.01**)

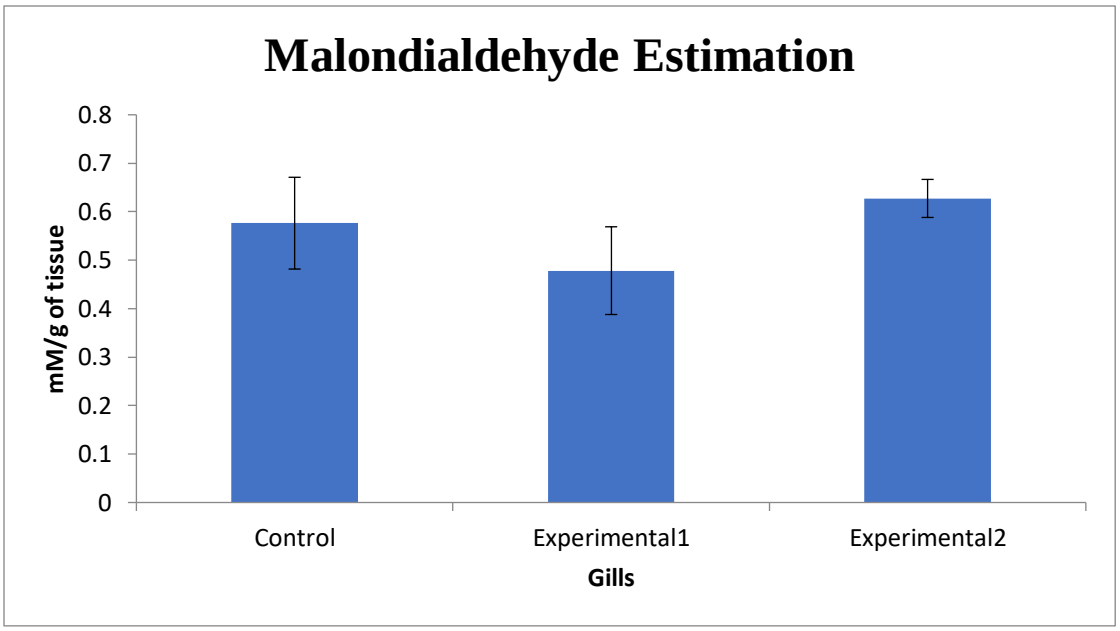

Figure 19. Effect of $CoFe_2O_4$ Nanoparticle on theMalondialehyde Activity of bivalve Gill tissue.

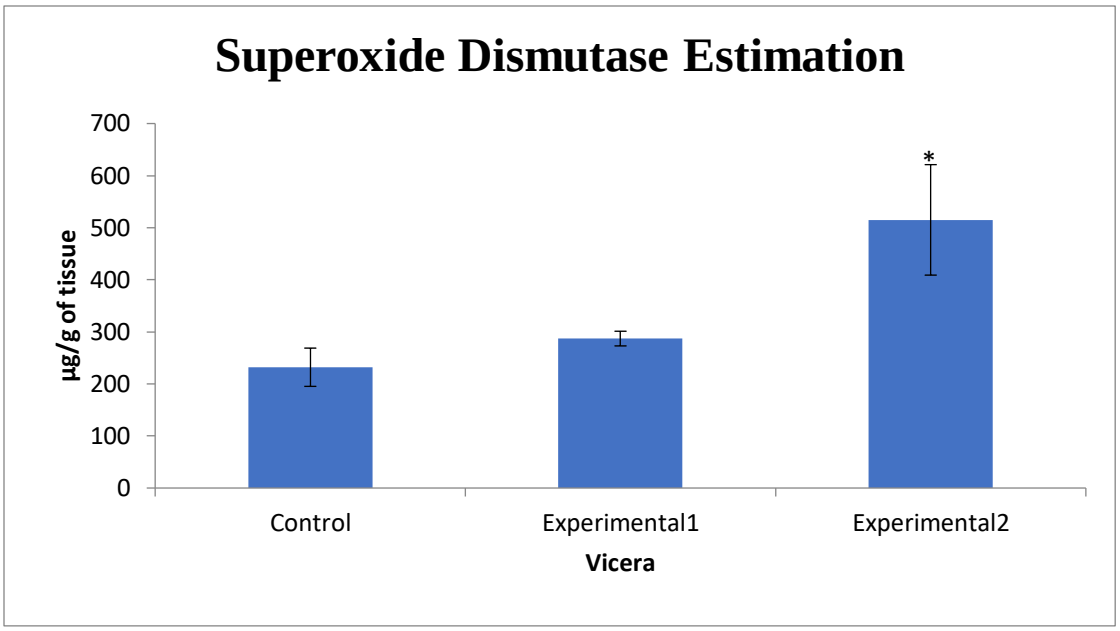

Figure 20. Effect of $CoFe_2O_4$ Nanoparticle on the Superoxide Dismutase Activity of bivalve Viceral tissue. ($P<0.05$*).

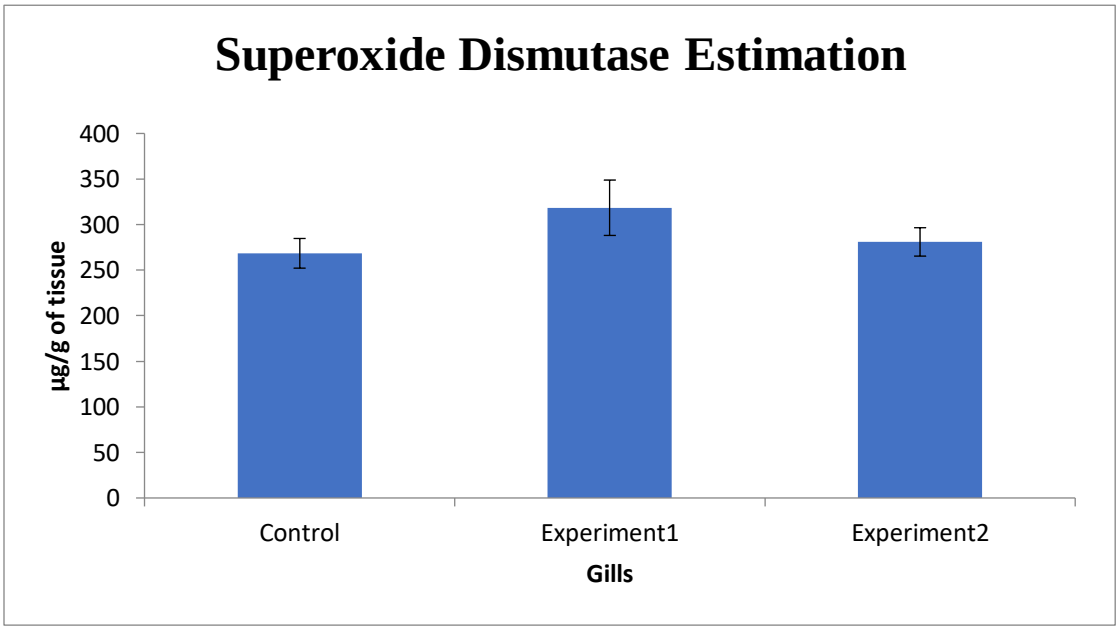

Figure 21. Effect of $CoFe_2O_4$ Nanoparticle on the Superoxide Dismutase Activity of bivalve Gill tissue.

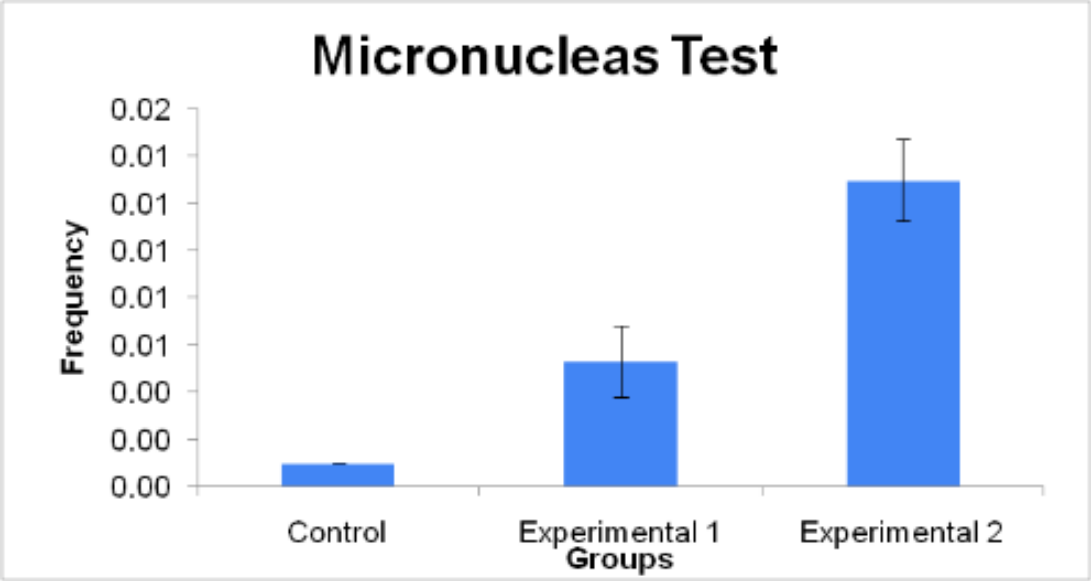

Figure 22. Micronucleas Test of Exposed cells(P<0.01**, P<0.0001****).

Table 3.1 Viscera correlation

Pearson r value	Protein	Glycogen	SOD	Catalase	ALP	AP	MDA	ALT
Protein								
Glycogen	0.37							
SOD	0.53	0.86						
Catalase	0.93	0.62	0.77					
ALP	0.42	0.92	0.91	0.66				
AP	-0.12	-0.72	-0.45	-0.25	-0.74			
MDA	-0.58	-0.66	-0.81	-0.76	-0.72	0.29		
ALT	0.54	-0.27	-0.10	0.30	-0.16	-0.01	-0.12	
Reduced Glutathione	0.94	0.54	0.75	0.99	0.65	-0.23	-0.78	0.37

Table 3.2 Gills correlation

Pearson r value	Protein	Glycogen	Reduced Glutathione	AP	Catalase	ALP	MDA	SOD
Protein								
Glycogen	-0.31							
R Glutathione	-0.38	0.24						
AP	-0.30	0.24	0.35					
Catalase	0.46	-0.86	-0.41	-0.44				
ALP	-0.24	-0.17	0.07	-0.53	0.05			
MDA	-0.40	0.83	0.53	0.22	-0.75	-0.14		
SOD	0.84	-0.57	-0.23	-0.55	0.74	0.08	-0.54	
ALT	0.38	-0.89	0.03	-0.27	0.78	0.18	-0.55	0.64

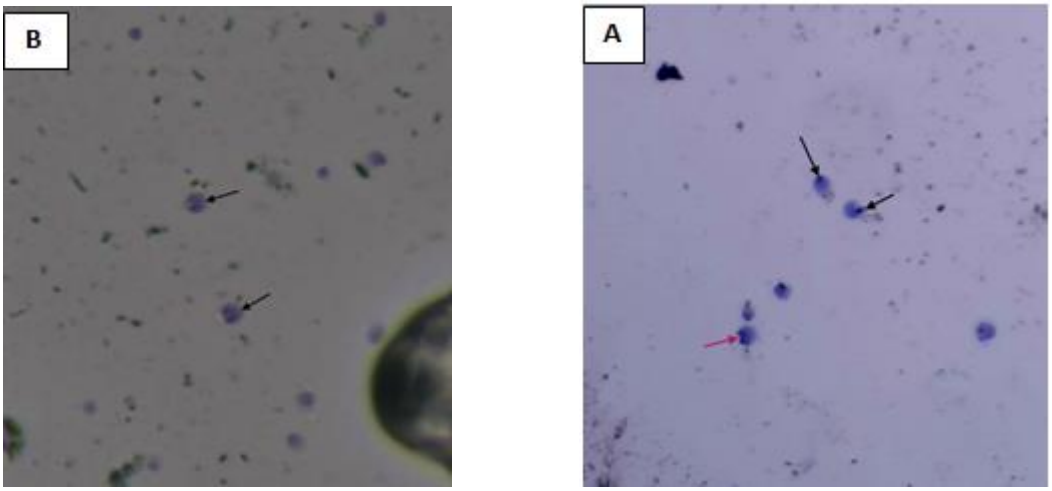

Figure No. 23; Genotoxicity study in bivalves gill cells. A-Experimental 1 bivalve gill cells showing normal cells as well as micronucleus-Experimental 2 bivalve gill cells showing micronucleus

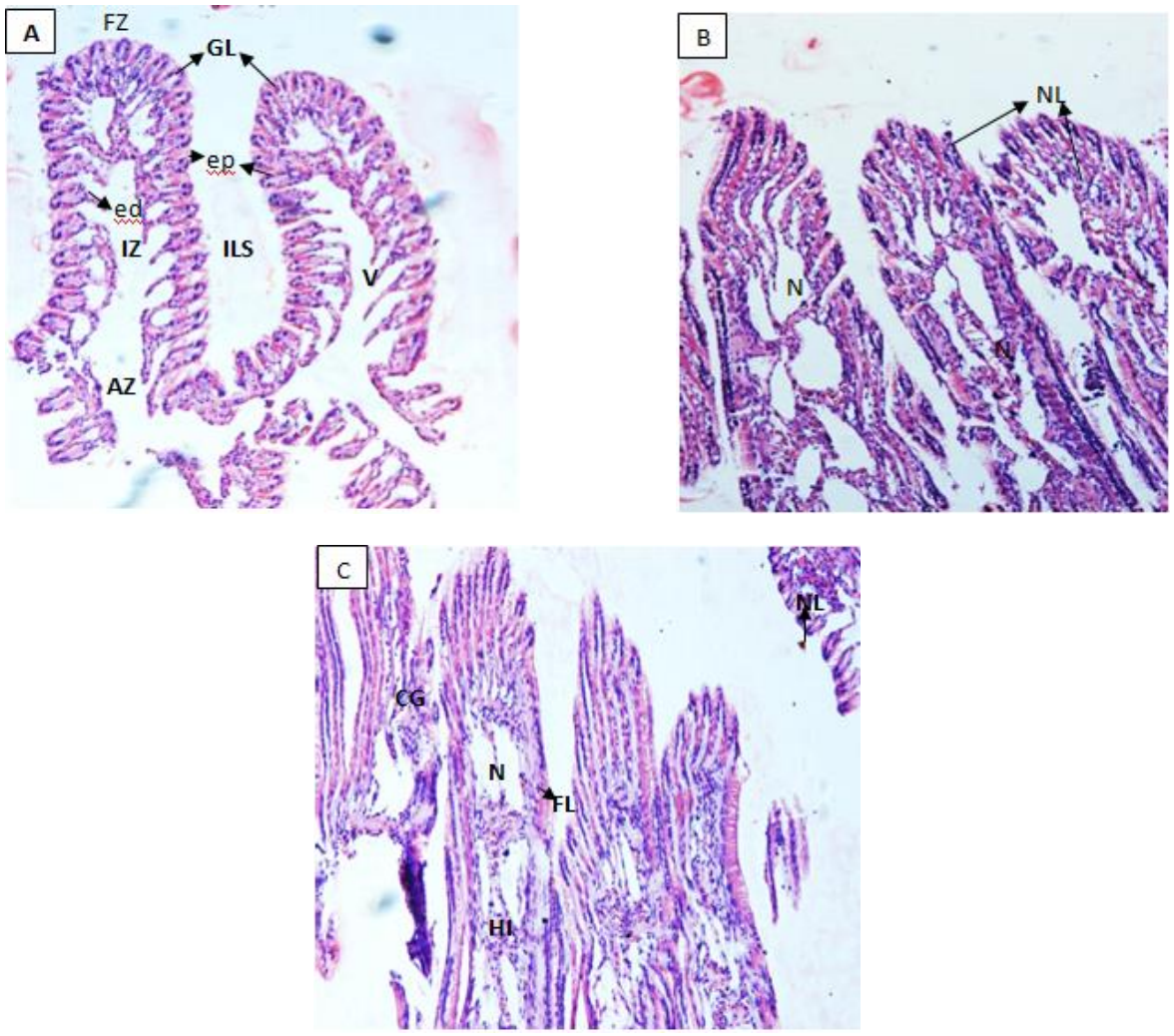

Figure No. 24 Effect of $CoFe_2O_4$ on the Histopathology of Gills. A-Bivalve Control Gill section showing Gill Lamellae (GL), Uniform Interlamellar Space (ILS), Epithelium (ep), Endothelium (ed), Three zones of Gill Lamellae 1-Frontal Zone (FZ), 3-Intermediate Zone(IZ), 3-Abfrontal Zone (AZ),Heamolymph Vessel (V). B- Exprimental 1 Bivalve Gill showing Narcotic Lamellae (NL), and obstructed or Narrower heamolymph vessel of gill lamellae (N). C- Experimental 2 Bivalve gill section showing Narrower heamolymph vessel (N), Narcotic Lamellae (NL), Fused Lamellae (FL), Clubbed Lamillae (CG), HeamolymphInfiltration(HI).

4.2 DISCUSSION

Nanoparticles can accumulate in various tissues of aquatic species when present in the aqueous phase. In bivalves, these particles are ingested from the water and tend to accumulate primarily in the gills and then the viscera. Our study demonstrated significant accumulation of cobalt ferrite nanoparticles in both the gills and viscera of bivalves after 15 days of exposure. Similar accumulation patterns were observed in Mediterranean clams (*Ruditapesdecussatus*) exposed to two photocatalyst nanocomposites (TiO2 NPs and AuTiO2 NPs), with accumulation primarily in the gills followed by the digestive gland. (saidani et al.,2019)

Cobalt ferrite has attracted significant scientific interest due to its distinct properties and highly promising applications.(Jauhar et al.,2016). But the few studies highlight toxic effects of NPs. In the present study Acute toxicity and the toxicological impacts of NPs *on Paphiamalabarica* were evaluated.

Bivalve mollusks are prevalent in both freshwater and marine environments and are extensively used in monitoring environmental changes. They are suspension feeders with advanced mechanisms for internalizing nano- and micro-scale particles through endo- and phagocytosis. These processes are vital for functions like intra-cellular digestion and cellular immunity. In bivalve mollusks, nanoparticles (NPs) taken up through feeding are directed to the digestive gland, where they can cause lysosomal disruptions and oxidative stress. This makes bivalves a valuable model for studying the toxicity of NPs in marine invertebrates. (Canesi et al.,2012).

The LC50 (lethal concentration, 50%) of nanoparticles is important because it helps determine the concentration at which 50% of a test population is expected to die. This value is critical in

assessing the toxicity of nanoparticles and establishing safe exposure limits. (Oberdörster et al., 2005).

Ramakritinan, et al.,(2012) aimed to compare the acute static renewal toxicity of copper (Cu), cadmium (Cd), lead (Pb), zinc (Zn), and mercury (Hg) on the bivalve Modiolus philippinarum. The 96-hour LC50 values for M. philippinarum, were 0.023 mg/L (Cu), 0.221 mg/L (Cd), 2.876 mg/L (Pb), 2.337 mg/L (Zn), and 0.007 mg/L (Hg).

The neurotoxic effects of titanium dioxide nanoparticles (TiO_2 NPs) on the blood clam Tegillarcagranosa. Exposure to high doses of TiO_2 NPs led to increased concentrations of dopamine (DA), gamma-aminobutyric acid (GABA), and acetylcholine (ACh) in the clams. This suggests that TiO_2 NPs have a neurotoxic impact on T. granosa, affecting its physiological processes. (Guan et al.,2018).

Hietanen, et al.,(1988)The study assessed the toxicity of zinc on the common mussel, Mytilus edulis L., in brackish water conditions. Mussels were exposed to elevated zinc concentrations for 24 hours at 12°C, followed by a six-week observation period. The LC50 (lethal concentration) was determined to be 20.8 mg Zn/L.

In the present study the LC50 value of the cobalt ferrite nanoparticle was found to be 100.98mg/L. By comparing the above studies, it seems that $CoFe_2O_4$ NPs is less toxic as compare to mercury,TiO_2 and Zinc.

Freshwater mussels, such as Lampsilismarginalis, possess highly developed gill lamellae that serve for both respiration and filter feeding. Their efficient and continuous filter feeding allows them to process a large volume of water quickly. (Ward and Shumway, 2004). The amount of oxygen consumption was found to be decreased with increased in concentration, similar

conditions was observed in the study examined by Saidani et al. (2019) the effects of nanotoxins on gill filtration and respiration in the clam Ruditapesdecussatus. Experimental exposure to TiO2 nanoparticles and AuTiO2 nanoparticle composites led to a dose-dependent decrease in filtration and respiration rates, suggesting that these nanoparticles altered the clam's behavior.

The decrease in respiratory rate observed in clams exposed to nanoparticles could be attributed to a potential reduction in energy consumption, as suggested by Basti et al. (2016). The reduction in oxygen consumption, which is also linked to water filtration by the gills, may be attributed to nanoparticle-induced closure of the valves and damage to the gills.(Zha,et al.,2022). Exposure to copper oxide nanoparticles (CuO NPs) led to a reduction in the filtration rate of Lampsilismarginalis, resulting in decreased oxygen consumption and filter feeding efficiency in mussels inhabiting contaminated habitats. These findings suggest impaired gill function and metabolic stress in the experimental mussels.(Ray,et al.,2020).

Ammonia is the predominant nitrogenous end product resulting from protein catabolism in crustaceans. (Zhen et al.,2010).In the present study exposure to Cobalt Ferrite Nanoparticle result in a increased in the excretion rate. Similar studies observed in Blood clams exposed to the lower dose of MWCNT and CuO Resulted in notable increases in the excretion of ammonia. (Zha,et al.,2022) Given that ammonia waste primarily originates from protein metabolism through nitrogen transfer and oxidation reactions, the observed increase in ammonia excretion following nanoparticle exposure suggests an elevated utilization of proteins for energy generation. (Anestis et al., 2010, Hu et al.,2017).

During the process of metabolism, macronutrients such as carbohydrates, proteins, and fats undergo enzymatic breakdown into their respective basic components, such as glucose, amino

acids, and lipids. These constituents are subsequently oxidized through biochemical reactions, leading to the production of carbon dioxide and water. This oxidation process releases the chemical energy stored within these macronutrients, which is utilized to power various physiological processes within the organism (Shang et al., 2018; Zha et al., 2019). The metabolic response to oxidative stress induced by nanoparticles (NPs) imposes an energetic burden on the organism, potentially affecting its energy reserves such as protein and glycogen stores. Therefore, changes in energy reserves can serve as an indirect indicator of NP toxicity (Zhang et al.2018).The presence of adequate stores of protein and glycogen enables organisms to cope with environmental stressors and ensure sufficient energy allocation for maintaining homeostasis, supporting growth, and facilitating reproduction. In the present study after 15 days of exposure glycogen and protein content was significantly increase in visceral tissues and decrease in gill tissues similar study was found in a Ag NP exposure decreased clam glycogen content indicating an energetic cost associated with Ag NP exposure. by 30%, lowest with 40mg/L HA. Protein concentration in clam stayed mostly unaffected, except with 20mg/L HA, decreasing by 22%. (Zhang et al.2018). During the exposure period, there was a significant decrease in glycogen concentration, particularly pronounced in the presence of HA, despite lower tissue silver burdens and reduced oxidative stress with added HA. However, protein concentrations remained similar to the NP-free treatment throughout the study.

One distinctive feature of these enzymes is their ability to be induced as an adaptive response to environmental changes, particularly when marine invertebrates experience oxidative stress. Variations in habitat environmental conditions can trigger a functional response in antioxidant enzymes. (Rahman et al.2019).Catalase (CAT) is the primary antioxidant enzyme responsible

for detoxifying hydrogen peroxide (H_2O_2) by converting it into water and oxygen (Woo et al. 2013).The bivalves were exposed to the increasing concentration of the nanoparticles show significant increase in catalase and SOD activity in bivalves visceral tissues.our results are par with the Zha et al. (2019) in which CAT activity increased in the digestive gland of clams exposed to all concentrations of TiO2 and AuTiO2 NPs for 2 weeks, likely due to the generation of H2O2 necessary for cell protection against oxidative damage. In another study, SOD activity in abalone tissue significantly increased in the 1.0 mg/L group but returned to a level not different from the control in the 10 mg/L group was found to decrease significantly (Zhu et al.2011).

Our results show decrease in CAT and SOD activity in unequal trend in bivalve gill tissueIn a similar study found that antioxidant enzyme levels were initially higher but decreased over time, especially SOD and GPx in the gills, and SOD, CAT, GPx, and GST in the liver of mussels. These variations could be due to natural fluctuations or stress from experimental manipulation. The low SOD activity suggests that dismutation may not be the primary factor driving the high catalase activity detected in this organ (Nogueira,et al.2017). Catalase typically operates alongside superoxide dismutase (SOD), which converts superoxide radicals to hydrogen peroxide. The observation of low SOD activity suggests that the high catalase activity detected in this organ is not primarily influenced by dismutation processes. (Soldatov et al.2007).

A significant decrease in the MDA activity was observed in the visceral tissues which show similarity with The inverse relationship between MDA level and CAT activity suggests that alterations in CAT activity could lead to increased lipid damage, affecting physiological processes like respiration and filtration behavior. This indicates reduced individual fitness due to the negative impact of nanoparticles (NPs) (Zha,et al.2019). A varied pattern of MDA activity

increase was observed in gill tissues, which can be attributed to MDA serving as an indicator of membrane damage caused by reactive oxygen species (ROS) (Liu et al. 2018). A significant increase in the GSH activity was found in the visceral tissues in a similar study observed a significant increase in glutathione (GSH) levels in both gills and digestive glands of mussels Perna perna exposed to hypoxia, starting as early as 6 hours and lasting up to 48 hours. This elevation in GSH levels is a typical adaptive response to combat oxidative stress, as any depletion of GSH due to various processes can be balanced by increased GSH synthesis (Nogueira, et al. 2017). An uneual trend in the increase in GSH in gill tissues was found in the present study which finds similarity with The increase in GSH levels in mussels' gills and digestive glands under hypoxia indicates an early protective response against oxidative damage during oxygen reperfusion, as well as potential detoxification of accumulated pro-oxidants (Nogueira, et al. 2017).

Acid phosphatase activity was increased in the gill tissue and decreased in the visceral tissues similar condition was found in Jing et al.(2006)Exposure of Pinctada fucata to copper resulted in higher AcPase activity in gills at 0.05 μM, likely due to stimulated cell defense mechanisms, while 0.5 μM depressed activity, possibly due to excessive copper interfering with defenses. In the digestive gland, 0.5 μM initially inhibited AcPase but increased as oysters adapted, while 0.05 μM did not increase activity due to rapid copper accumulation, disrupting defenses, aligning with mussel studies. In another study increased acid phosphatase activity in the digestive gland of intoxicated clams suggests lysosomal membrane damage, releasing the enzyme. This response aligns with previous findings of inducible enzyme activity in animal tissues exposed to various

chemicals. The activity in A. rhombea increased with both dose and exposure time (Jing et al.2006).

ALP activity was found to be increased in both the gill and visceral tissues similar conditions found in the study observed increased alkaline phosphatase enzyme activity in the digestive gland of A. rhombea exposed to three sublethal doses of TBTCL,where tissue alkaline phosphatase levels increased in response to various stressors. The significant difference in phosphatase activities between control and experimental groups suggests damage to the digestive gland tissue, leading to disrupted digestive function (Ranilalitha,et al.2014).

ALT shows significant increase in the activity in the visceral tissues and also in gill tissue first increase and then decrease The suppression of alanine aminotransferase (ALT) activity by cadmium and lead in soft body and gill tissues, and by copper in the gills, indicates the potential of this enzyme as a biomarker for detecting sublethal stress induced by heavy metals(Blasco,et6 al.1999). During the succinate thiokinase reaction, alanine aminotransferase (ALT) catalyzes the conjugation of glutamate with pyruvate, leading to the formation of alanine and α-ketoglutarate. This process helps reduce the accumulation of toxic lactate. Subsequently, α-ketoglutarate is converted to succinate, generating energy equivalents in the form of GTP and NADH2.

The micronucleus test (MN) is a widely used method in environmental genotoxicity studies, serving as an indicator of cytogenetic damage for over 30 years. This test relies on the detection of micronuclei, which are small chromatin fragments separated from the main cell nucleus. The presence of micronuclei indicates chromosome breakage or mitotic spindle dysfunction, often caused by clastogenic and aneugenic agents. These micronuclei form when a chromosome

fragment or whole chromosome is not properly segregated during cell division, resulting in a small secondary nucleus in the interphase. (Baršienė et al.2008).The present study show significant increase in micronucleus. Our results are par with the genotoxic effects of copper (Cu) on mussel gill cells are potentially linked to the excessive generation of reactive oxygen species (ROS), which can induce oxidative harm, including single and double strand DNA breaks (SSBs and DSBs), as well as modifications or oxidation of DNA bases [8]. Additionally, Cu2+ has the ability to interact with DNA, leading to the formation of DNA adducts (Vernon,et al.2019).

To comprehensively understand the toxicity of nanoparticles in bivalves, it is crucial to investigate their rate of accumulation, routes of uptake, and distribution within the organisms.Histological changes in the gill tissues of bivalves are known to be vital for both food collection and respiration (Morad,et al.2023). The gills serve as the primary respiratory system in bivalves, responsible for filtering particles and suspended matter from the water. As a result, they are directly exposed to crucial xenobiotics present in the water (Mohamed et al.2021).

The present study shows the normal control gill cells, our results par with the Katalayet al.(2016) the gill tissue of bivalves is composed of various cell types serving multiple functions, including gas exchange, excretion, osmoregulation, and food material transport. Gill filaments are divided into frontal, intermediate, and abfrontal zones, with the frontal zone responsible for nutrient and contaminant transport and uptake. Observed cilial erosion, degeneration of endothelial cells, cell loss, necrosis, and vessel lumen enlargement in the gill samples. The abfrontal zone showed compromised tissue integrity, with thinning of the vessel lumen and adhesion in some areas.

Exposed bivalve gill cells show narcotic gill lamellae, narrower and fused lamellae, burst of epithelial cells, our results shows similarity with the increased doses and longer exposures to pollutants lead to histological changes in mussel gill tissues, including hemosit infiltrations, disruption of structure, filament thinning, and hyperplasia. Due to their direct contact with water, mussel gills are the first to show prominent histopathological findings from environmental pollutants. Lamellar fusion is a defense mechanism against pollutant-induced damage. The effects of ZnO nanoparticles on mussel gills are similar to those reported in other studies, likely due to similar exposure mechanisms (GÜNEŞ et al.2023).

Gills are the primary target tissues for xenobiotic toxicity. Histopathological abnormalities such as inflammation, necrosis, and tubule thinning were the most prominent symptoms observed in the gill filaments. Another study shows similarity with exposure to heavy metals and pesticides has significant effects on the morphology of mussel tissues. Heavy metal exposure in M. galloprovincialis leads to fusion of residual bodies in digestive cells and gill filament fusion, while M. edulis shows increased vacuolization. Pesticide exposure in M. edulis results in moderate vacuolization, cilia loss, and conjunctive alteration with Lindane, and moderate cilia loss, infiltration, dilation of hemolymphatic space, and increased mucocytes with atrazine. Gill tissue analysis indicates cilia loss, cell loss, endothelial cell degeneration, necrosis, lumen thinning, and adjoined lumens (Katalay et al.2016).

4.3 CONCLUSION

The concentration-dependent adverse effects of Cobalt Ferrite nanoparticles ($CoFe_2O_4$ NPs) were observed in *Paphiamalabarica*. Biochemical analysis revealed toxicity in the gills and visceral tissues of bivalves, attributed to oxidative stress induced by reactive oxygen species (ROS) production. The release of $CoFe_2O_4$ NPs into aquatic ecosystems can lead to genetic-level toxicity and tissue alterations, as confirmed by micronucleus genotoxicity study and histopathological studies. These findings highlight the potential environmental risks of $CoFe_2O_4$ NPs and underscore the need for comprehensive risk assessments before their biological applications.Based on these findings and existing research, it may be prudent to assess the potential safe use of low but efficacious concentrations of $CoFe_2O_4$ nanoparticles (NPs) in various applications such as biomedicine, electronics, magneto-optics, sensors, data storage, catalysis, and microwaves. However, further in vivo studies are essential to comprehensively understand the mechanisms underlying the toxicity of $CoFe_2O_4$ NPs.

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and three by nature painted and illuminated by vivid colors copper plates.

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