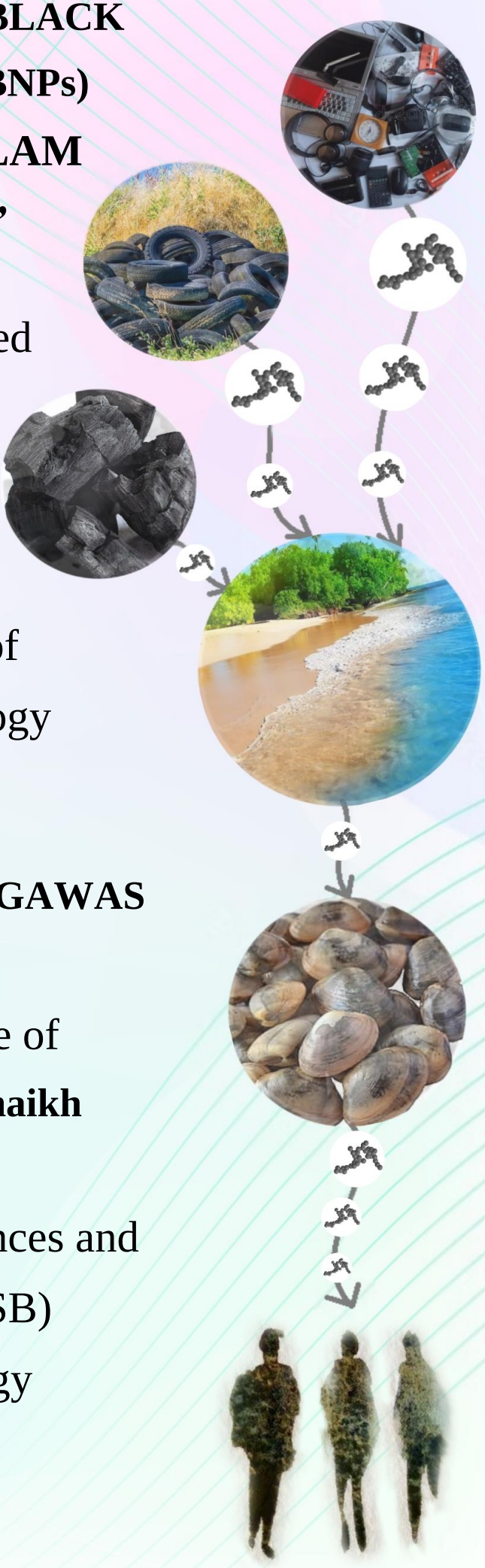
EFFECT OF CARBON BLACK NANOPARTICLES (CBNPs) ON SHORT NECK CLAM *"Paphia malbarica"*



Dissertation submitted

To

Goa University

In partial fulfillment of

Masters Degree in Zoology

By

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MSc. Zoology

Under the guidance of

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Effect Of Carbon Black Nanoparticles (CBNPs) on Short Neck Clam "*Paphia malbarica*"

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Examined by: Seal of the School

DECLARATION BY STUDENT

I hereby declare that the data presented in this Dissertation report entitled, "Effect of Carbon Black Nanoparticles (CBNPs) on Short Neck Clam *Paphia malbarica*" is based on the results of investigations carried out by me in the Zoology Discipline at the School of Biological Sciences and Biotechnology (SBSB), Goa University under the supervision of Dr. Shamshad Bi M. Shaikh and the same has not been submitted elsewhere for the 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 the dissertation.

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COMPLETION CERTIFICATE

This is to certify that the dissertation report "Effect of Carbon Black Nanoparticles (CBNPs) on Short Neck Clam *Paphia malbarica*" is a bonafide work carried out by Ms. Riya Ramchandra Gawas, under my supervision in partial fulfillment of the requirements for the award of the degree of Master of Science in Zoology in the Discipline Zoology at the School of Biological Sciences and Biotechnology (SBSB), Goa University.

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Dr. Savita S. Kerkar
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Dedicated

To My Loving

Parents

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Riya Gawas

8th May 2023

LIST OF FIGURES

ABBREVITIONS

µm- micrometer

µmole- micromole

ANOVA- Analysis of Variance

CAT- Catalase activity

CB-Carbon Black

CBNP- Carbon Black Nanoparticles

CNP- Carbon Nanoparticles

CNT- Carbon Nanotubes

COOH-MWCNT- Carboxyl Multi-Walled Carbon Black Nanoparticles

DNA- Deoxyribonucleic Acid

DTNB- 5, 5'- dithiobis, 2-nitrobenzoic acid

DWCNT- Double-Walled Carbon Black Nanoparticles

Exp.- Experiment

Fig.- Figure

GSH- Reduced Glutathione

H_{20}- Water

H_2SO_4- Sulphuric Acid

HCl- Hydrochloric Acid

IU- Unit

Kg- Kilogram

M- Molarity

mg- milligram

mg- Milligram

$MgCl_2$- Magnesium chloride min- Minutes

mL-Millilitres

MWCNT- Multi-Walled Carbon Black Nanoparticles

N- Normality

NaOH- Sodium Hydroxide

nm- Nanometer

NP- Nanoparticles

NS- Non-significant

OH-MWCNT- Hydroxyl Multi-Walled Carbon Black Nanoparticles

pH- Potential of hydrogen

ROS- Reactive Oxygen Species

SWCNT- Single-Walled Carbon Black Nanoparticles

TCA- Trichloroacetic Acid

UCB- Ultrafine Carbon Black

ZnO- Zinc oxide

PREFACE

This thesis is submitted in fulfillment of the requirement for the degree of Masters in Zoology and comprises research work carried out by the author under the guidance of Dr. Shamshad Bi M. Shaikh Assistant Professor of Zoology, Goa University from 2022 to 2023.

Carbon Black Nanoparticles (CBNPs) are growing in their applicative dynamism. Simultaneously, their exposure to aquatic organisms and the environment is also increasing rapidly by the direct or indirect disposal of the various industrial byprodutcts. The smaller size, high surface area and ability to form the agglomerates allow them to induce toxicity via differential effects and causes toxicity in various cell types (Catalan et al., (2011). Thus, investigations of the CBNPs effect on *Paphia malbarica* will provide data about its toxicity that can be further used to generate preventive measures to reduce CBNPs exposure to aquatic organisms. There are very few studies, undertaken to analyze the toxicity of CBNPs in the aquatic ecosystem. This thesis is contributing to the knowledge investigating their toxicity in anatomy and physiology of *Paphia malbarica*. The thesis is divided into four main chapters. The first chapter's introduction gives the information on toxicity caused by different types of Carbon Nanoparticles (CNPs), Carbon Black Nanoparticles (CBNPs), and applications of CBNPs. The 2nd chapter includes a survey of literature and the aims and objectives of the work. Chapter 3 gives the material and methods used for the study different physiological and biochemical estimations. Chapter 4 represents the results embodying observations of anatomy, and physiology of *Paphia malbarica*. Chapter 5 gives elaborate discussions. The reasons and the effects of changes

occurring in the bivalves as a result of CBNPs toxicity are discussed. Conclusion with a summary, future work, references, and contributions from the thesis follows chapter 5.

CHAPTER 1:

INTRODUCTION

1. INTRODUCTION

"The impact of Nanotechnology is expected to exceed the impact that the Electronics Revolution has had on our lives."

By Richard Schwartz

In recent years, due to rapid globalization the expansion of nanoparticles (NPs) in different sectors has tremendously increased because of their extensive uses and consequent production and exploitation (De Marchi et al., 2018). The Nanoparticles (NPs) are being defined as the tiny materials which ranges in size from 1 to 100 nm and are differentiated based on their sizes, shapes and unique properties (Khan et al., 2017). Based on the composition of NPs Joudeh and Linke, (2022); and Ealia and Saravanakumar, (2017) classified the NPs into three classes: organic, carbon-based, and inorganic. According to Khan et al. (2017) the nanoscale size and the high surface area of these NPs makes them to possess unique physical and chemical properties. These physicochemical properties such as chemical stability, optical properties, electrical conductivity, density, melting point, magnetic properties, etc. make them suitable candidates to be used in several fields and are employed in daily consumable products, industrial production, biotechnology, medicine, electronics and to other numerous and important commercial areas (Singh et al., 2020; Cid et al., 2015). According to De Felice and Parolini, (2020); and Murr and Garza, (2009) the NPs can originate from both natural (volcanic eruptions, photochemical reactions in the atmosphere, forests fires, oxidation and degradation of minerals and organic matter) and anthropogenic processes which includes unintentionally created (i.e.,

ashes and combustion by-products and diesel engines exhaust) and intentionally designed nanoparticles by humans (which are used in industry, medicine, and day to day life i.e., engineered NPs).

There has bene a rapid growth in the Nanotechnology industries due to the increase use of NPs in domestic and industrial applications which have led to the increase production and discharge into the environment giving rise to diverse risks to the environment (Lekamge et al., 2020; Gusev et al., 2021). The emissions of these NPs in the soil, atmosphere and water harm the respective ecosystems but in particular marine ecosystems have been identified as a major sink of diverse anthropogenic contaminants and are prone to receive notable amounts of NPs (De Felice and Parolini, 2020; Matysiak et al., 2016; Corsi et al., 2014; Klaine et al., 2008). In the aquatic system, 70% of industrial waste effluents are discharged without being treated, which contaminates the supply of potable water. Additionally, these components may influence human health by entering the food chain (Kaul and Sharma, 2022). Due to the smaller size of the NPs the gravitational settling of these particles is inhibited and therefore they remain suspended in the water, NPs only gets settled down when they increase their size through agglomeration with other particles (Turan et al., 2019). The increasing amounts of NPs contaminates waterbodies and thereby causes adverse effects on the aquatic organisms (Lekamge et al., 2020).

1.1. CARBON NANOPARTICLES (CNPs)

In the field of Nanotechnology, Carbon Nanoparticles (CNPs) are some of the most used tools because of their unique properties, such as high chemical versatility, attractive optical properties, high conductivity, high mechanical strength, light weight etc. (Crevillen et al., 2018; Peng et al., 2020; Jiang et al., 2018). The majority of the novel nanoparticles, such as fullerene (C_{60}), graphene (GRA), and carbon nanotubes (CNTs), have at least one dimension and are made of carbon components (Peng et al 2020). The different types of CNPs includes, Carbon Black Nanoparticles (CBNPs), Fullerenes, Carbon nanotubes (CNTs), Fibres, Graphene oxide, Nanodiamonds, Carbon Quantum Dots (CDs), Diamond-like Carbon (DLC), Mesoporous carbon biomaterials (MCNs), Carbon nanofibers, Carbon nanocones-disks, Nanohorns (Yuan et al., 2019; Bhattacharya et al., 2016; Rahmati and Mozafari, 2019; Zhang et al., 2013). Due to the possession of above-mentioned unique properties by these CNPs, a myriad of new electronic devices, batteries, sensors and composites have been invented covering the majority of scientific areas, example, engineering, biology, physics, medicine and chemistry (Crevillen et al., 2018). Besides these, CNPs also play an important role in controlling environmental pollution by absorbing heavy metals, antibiotics and harmful gases (Peng, et al., 2020).

From the past few decades, due to the rapid development in the Nanotechnology the CNPs are widely used and with the increasing research and application, more and more attention has been obtained on their effects on the environment and their behavior in the environment (Peng et al 2020; Chen et al 2018). Peng et al. (2020) have listed various applications of CNPs which includes, Pollutant removal (Sewage treatment, Heavy metal adsorption, Removal of organic pollutants), Nanocomposite (Metal-based nanocomposite, Ceramic-based

nanocomposite, Polymer-based nanocomposite, CNPs/rubber composite, Carbon nanocomposite electrode), Biomedicine (Tissue Engineering, Gene/ drug carrier, Biological imaging, Tumor medication, Anti-bacterial activity, Biosensor), Catalyst (Electrocatalysis, Photoelectrocatalysis, Heterogeneous catalytic) and Electrochemistry (Biosensor, Supercapacitor, Fuel cell) etc. However, CNPs not only bring benefits to us, but also cause a series of environmental problems. Their increase in the aquatic environment may produce detrimental effects to aquatic organisms (Mueller and Nowack 2008). Because CNPs have small particle size, they have the ability to penetrate cell walls, cell membranes in a living body, triggering reactions such as lung tumors and cellular inflammation, which would produce direct damage to animals and humans and thus are likely to spread and accumulate in the food chain (Oberdorster et al., 2006; Pangule et al., 2009; Lam et al., 2004; Wu et al., 2006). Among the various CNPs which are mentioned above, the Carbon Black Nanoparticles (CBNPs) play important roles in various sectors which includes electronics, plastics, green technology, medicine, inks, coatings, cosmetics, rubber industries etc. and for this reason the Carbon Black Nanoparticles (CBNPs) have been selected as a candidate for the present study.

1.2. CARBON BLACK NANOPARTICLES (CBNPs)

Carbon black Nanoparticles (CBNPs) are defined as a powdered form of elemental carbon with a morphology consisting of grape-like aggregates of highly fused spherical particles and diameter between 10 to 100 nm with three nanometric dimensions (McCunney et al., 2001; Yuan et al., 2019). The composition of CBNPs is mainly carbon, with a little quantity of other elements which includes oxygen and hydrogen (Lindner et al., 2017). One of the

characteristics of Carbon Black (CB) is the formation of aggregates nanostructures, by presenting semispherical groupings and these aggregate groups, with a distinctly long dimension, can form agglomerates (Silva et al., 2017). The possession of various exceptional properties by the CBNPs such as conducting nature, high specific surface area, strong electric forces, and varied particle size are responsible for the bounding of the aggregates (Rani et al., 2020). The common subtypes of CB are thermal black, acetylene black, channel black, lamp black and furnace black and is also known by different trade names such as Printex-140, Printex-G, Printex-90 and Lampblack-101 (Niranjan et al., 2017).

The production of CB is by the partial combustion of gaseous or liquid hydrocarbons and by the controlled vapour-phase pyrolysis (IARC 1996; US EPA 2005). From the different available processes for the production of CB, the oil-furnace black process and the thermal black process are dominantly used (Wang, 2003; Baan, 2007). Besides the manufactured CBNPs, nanoscale carbon particles exist naturally in the environment and this includes diesel exhaust and carbonaceous nanoparticles which are an important part of diesel exhaust (Murr et al., 2004 and Oberdorster et al., 2006). According to BeruBe et al., (2007), in daily life the combustion of fuel and waste is responsible for the generation of large amounts of CBNPs. In the environment the presence of ultrafine ambient nanoparticles and consumable products are the major concern for daily exposure to these nanoparticles (Yuan et al., 2020).

According to Yuan et al. (2020), CBNPs make up a significant portion of ambient particle pollution and can be hazardous to the lungs mostly through inflammation. Inhalation is the predominant route of exposure to CBNPs (Lindner et al., 2017). The main causes of nanoparticles potential toxicity in comparison to most bulk-

sized particles of the same makeup are their nanoscale size, large surface area/mass ratio, higher translocation rate, high chemical or catalytic reactivity, and slow clearance (Samak et al., 2018). Because of these characteristics, some NPs can easily cross cell membranes and translocate to the organism from the surrounding environment.

1.3. APPLICATIONS OF CARBON BLACK NANOPARTICLES (CBNPs)

Carbon black is primarily used in the manufacture of rubber goods, especially tyres, although it is also used in several non-automotive and automotive rubber applications. Moreover, carbon black is utilized in paint, ceramics, inks, polymers, and other minor industrial processes. Carbon black is used in the reinforcement of rubber in order to increase the resistance of rubber to abrasion, fatigue, flexing and tear. It is used to improve the processing characteristics and tensile strength of many natural and synthetic elastomers. The rubber industry accounts for 89– 91% of total consumption of carbon black worldwide (Auchter, 2005).

The production of tyres (for automobiles, trucks, buses, agricultural equipment, and industrial use), retread rubber, and inner tubes are the main uses for carbon black in elastomers. Typically, the weight of the carbon black makes up 20-40% of the tyre. In the automotive industry, carbon black is also utilized in elastomers for wire and cable, belts, hoses, O-rings, insulation stripping, shock and motor mounts, and other comparable products. Coated fabrics, gaskets, packaging,

gloves, shoes, floor mats, tape, hard rubber goods, pontoons, and toys are all made with carbon black (Auchter, 2005).

The largest non-elastomer use for carbon black is in making of plastics. Carbon black is often employed as a colourant, as well as an additive for regulating electrical conductivity, a filter that adds strength, and as an excellent stabilizer of UV light (Auchter, 2005). The printing ink industry consumes almost one-third of the special industrial (non-rubber) carbon blacks produced in the USA. The grade and concentration are chosen for characteristics like the desired level of colour, gloss, tone, viscosity, tack, and rheological qualities, depending on the kind and quality of the ink. The range of carbon black inks is between 5% and 22% (Auchter, 2005).

All kinds of paints and varnishes employ carbon black as a colourant for tinting and coloring. To improve electrical conductivity, relatively modest amounts are added to several industrial formulas (such primers and floor coatings) (IARC 1996). The main application for carbon black in the paper industry is the creation of carbon paper. The backing paper for photographic film, leatherboard, wrapping and bag papers, highly conductive and electrosensitive sheets, and picture albums and further uses (Lyon and Burgess, 1985).

Other sporadic uses for carbon black include magnetic tapes, xerox toners, and dry-cell batteries (Auchter, 2005). With the rise in the production by nanotechnological industries, for the use of nanoparticles in different applications, the different nanoscale products and byproducts generated enter the aquatic and terrestrial ecosystem causing the accumulation of these nanoparticles

over the course of time which persistently threaten the entire environment and faunal biodiversity (Canesi et al., 2008; Xu et al., 2021).

1.4. *Paphia malbarica* AS A MODEL ORGANISM

Scientific Classification

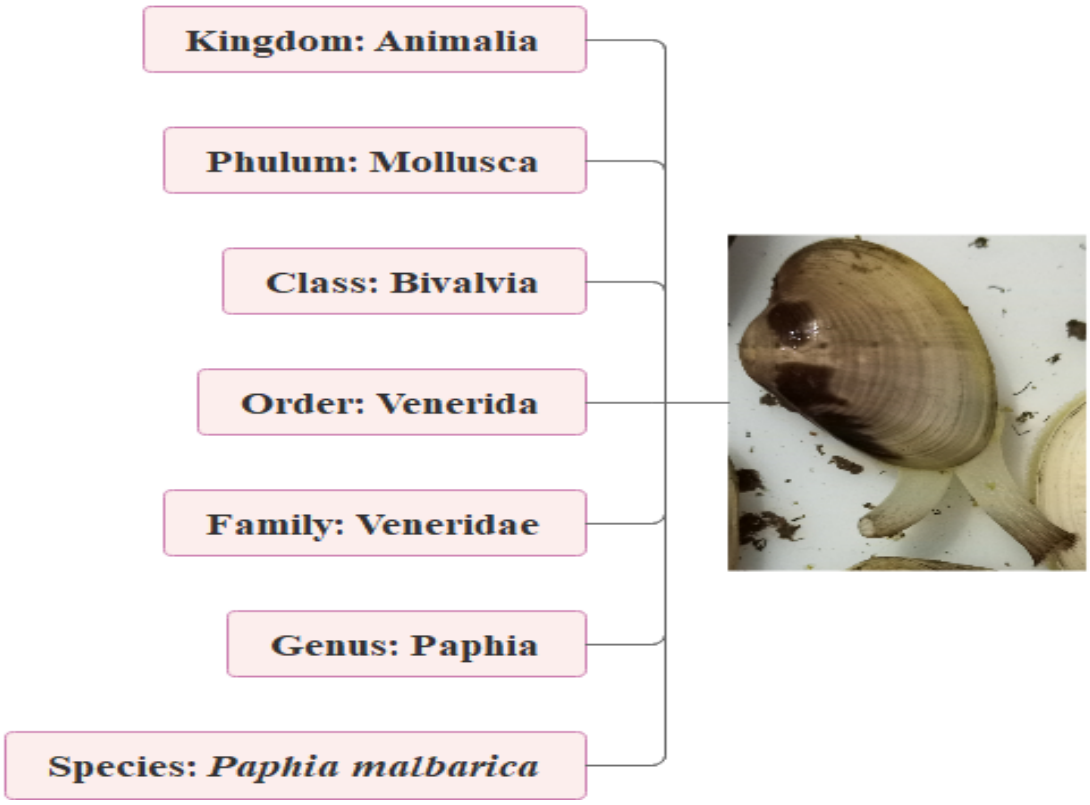

Figure No. 1: Scientific classification of *Paphia malbarica*

Being a filter-feeder, bivalves are widely used as a model organism among invertebrate group in most of the toxicological studies. The present study has selected *Paphia malbarica* as a model organism which is locally referred to as "tisreo" in Goa.

Morphologically the Short Neck Clam has a triangular to oval shape shell with rounded anterior and posterior margins. The outer surface of shell has concentric ridges and is more or less long and smooth. It has a short hinge area possessing narrow diverging teeth. The pallial sinus is 'U' shaped and is not too deep.

In India it is found in many estuaries and coastal waters along the east and west coast. It is distributed in the sandy mud flats up to a depth of 4m. Being a filter feeder, it feeds on phytoplankton viz., *Nannochloropsis salina, Isochrysis galbana, Dicrateria inornate, Chaetoceros calcitrans, Tetraselmis gracilis and Dunaliella salina.* In India *Paphia malbarica* is a predominant and commercially important bivalve which is persistently challenged by wavering salinity in a monsson influenced estuary (Gajbhiye & Khandeparker 2017). It is an important economical species in Indian waters. For the local population, it is a traditional staple food due to its nutritional values especially high protein content.

1.5. THE SIPHON AND GILLS; VITAL ORGANS IN MAINTAINING PHYSIOLOGICAL HOMEOSTASIS

The infaunal bivalves in order to escape from extreme environmental changes and biological predators, burrow at various depths into the substrate for the

survival (Stanley, 1968). For the evolutionary study of the species, the bivalves siphon is considered as an important organ (Shin et al., 2022). During the process of burrowing, the bivalves expand the siphon to absorb and discharge the water and also it has functions in food intake, pseudofeces and excretion, gas exchange,

photoreception, germ cell ejection and mechanosensation (Vitonis et al., 2012). Adult siphons consisted of a pair of incurrent and excurrent siphon which are developed posteriorly to the shell and are connected to the edge of the mantle. The excurrent siphon is narrower than the incurrent siphon. Toward the distal ends of the lumen of the incurrent and excurrent canal, irregularly placed tentacles are present (Shin et al., 2022). The water is drawn inside through the incurrent siphon and is returned from the body to the surrounding through excurrent siphon.

The gills of bivalves are referred to as ctenidia. The gills are leaf-like organs that function in both respiratory and feeding (Carroll and Catapane, 2007). On each side of the body there are two pairs of gills. Two pairs of flaps, called labial palps, are present at the anterior end which surrounds the mouth and help in directing food in the mouth. On the gill filaments there are presence of cilia in the lateral, laterofrontal and frontal cells and each type have specific arrangements and functions. The lateral cilia help in the generation of water currents which allows the exchange of gases and also help in the regulation of food intake and waste removal (Crroll and Catapane, 2007).

The present study has selected gills and siphon to evaluate toxicological effects as these two organs play primary function in filtration and in the uptake of suspended particles from the water respectively. And also, when it comes to exposure of internal organs of the bivalve to external environment, siphon and gills will be the first line of exposure to nanoparticles from the water in the present study.

1.6. PHYSIOLOGY OF OSMOREGULATION, EXCRETION AND RESPIRATION IN BIVALVES

The bivalve mollusc is subject to several abiotic variables that may exert significant effects on their biology and therefore the disturbances caused to hemolymph osmolality, tissue water content and cellular volume may affect the homeostasis maintenance (Medeiros et al., 2020). Most of the marine molluscs are osmoconformers and following changes in the surrounding environment, osmoconformation would result in osmotic stress to tissues of bivalves (Lin et al., 2016). The inorganic ions such as Na^{+}, K^{+}, Ca^{++}, Mg^{++}, Cl^{-}, SO_4^{2-} chiefly determines the osmotic concentration of hemolymph and found to be equal to, or marginally greater than, the osmotic concentration of seawater (Bayne et al., 1976). In the process of cellular ion-regulation, the transmembrane ion countertransporter, such as Na^{+}-K^{+}-ATPases, Mg^{++}-ATPases and Ca^{++}-ATPases have found to play a major role in converting its metabolic energy of ATP to transport the ions like Na^{+}, K^{+}, Ca^{++}, Mg^{++} across the cell membranes (Lin et al., 2016).

In bivalves two types of excretory glands are present for excretion, the pericardial glands (also called as Keber's organs) and the paired kidneys. Pericardial glands develop from the epithelial lining of the pericardium and lie over the auricular walls of the heart. In the certain cells of the pericardial glands, waste accumulates and is periodically discharged in to the pericardial cavity and from there it is eliminated via the kidneys. In bivalves the first stage of urine formation is the filtration of hemolymph by the cells of pericardial glands. The filtrate is received by the glandular part of the kidney where the process of secretion and re-

absorption of ions occurs. The end result of excretion is formation of urine containing high concentration of ammonia, and smaller amounts of creatine and amino acids. Though ammonia is highly toxic to organisms, due to its small molecular size and high solubility in water it diffuses rapidly in water ensuring its exposure to organisms. Besides these major excretory organs, the excretory products are also probably lost across the general body surface and particularly across the gills.

In bivalves, the gills, as well as the mantle, play an important role in respiration because they have large surface area and rich supply of hemolymph. The hemolymph circulates through the hollow tubes of gill filaments. From the afferent gill vein, the hemolymph flows from kidney to the gill. As water passes through the gills, oxygen from the water diffuses into the hemolymph.

CHAPTER 2:

LITERATUE REVIEW

2. LITERATURE REVIEW

With the advent of nanotechnological industries, the CNPs have attracted increased attention due to their applications in the various fields. However there have been widespread concern about the inherent cytotoxicity caused by these CNPs which have remained controversial with the different studies demonstrating conflicting results (Garriga et al., 2020). In order to evaluate the toxicity of such nanoparticles, a multitude of studies have been carried out on the invertebrate and vertebrate organisms to study the mechanism of their toxicity and effects caused by these CNPs.

Also, a multitude of research has been done on the toxicity of CBNPs in the Aquatic and Terrestrial organisms as there has been a growing potential for human and ecological toxicity with the nanoparticles (Canesi et al., 2008). The toxicity of CBNPs have been investigated in different types of Cells such as hepatocytes, renal cells, neurons, lung epithelial cells, lymphocytes, hemocytes, embryonic cells, larval cells, sperm cells in order to understand the mechanisms of toxicity and its physiological effects (Jennifer and Maciej 2013).

2.1. CNPs TOXICITY STUDIES IN INVERTEBRATES

Freitas et al., (2018) assessed the impacts of nanoparticles, Multi-Walled Carbon Nanotubes (MWCNTs) when acting in combination with classical pollutants such as meta(loid)s (Arsenic) in the clam "*Ruditapes philippinarum"* when both contaminants were acting individually (As, MWCNTs) and as a mixture (As+

MWCNTs) for a period of 28 days and found that clams exposed to MWCNTs showed higher injuries with higher oxidative stress and metabolic depression, regardless of the presence of As, also the neurotoxicity was found to be higher in clams exposed to combination of both contaminants as compared to the effects of As and MWCNTs individually. De Marchi et al., (2018) conducted a research on the toxicity of the unfunctionalized MWCNTs (Nf-MWCNTs) and functionalized MWCNTs (f-MWCNTs) by chronic exposure (28 days) to "*R. philippinarum*" and found that exposure to both MWCNT materials altered energy-related responses, with higher metabolic capacity and lowered glycogen, protein and lipid concentrations and higher oxidative stress in lipid peroxidation and lower ratio between reduced and oxidised glutathione, also the neurotoxicity was observed by inhibition of Cholinesterases activity.

Moore et al., (2009) investigated the *In vitro* cellular toxicity of C_{60}-fullerene and carbon nanotubes in the marine mussel "*Mytilus galloprovincialis*" by measuring the intra-lysosomal retention of neutral red and found that the aqueous suspensions of C_{60}-fullerene induced cytotoxicity in circulating phagocytic hemocytes whereas the hemocytes which were exposed to the same concentration range of carbon nanotubes were found to be unaffected. In the year 2013, Manno et al., (2013) explored the effects of silver (Ag) and carbon (C) NPs on fertilization and development in the Sea Urchin "*Paracentrotus lividus*" (*P. lividus*) and found that both AgNPs and CNPs are embryotoxic because they caused embryo malformations and changes in the normal progression through the development stages wherein AgNPs-induced malformations leading embryos to die in a concentration-dependent manner and slowed down embryonic

development; while embryos bearing CNPs-induced malformations survived for a longer time and speed up embryonic development.

Pikula et al., (2020) evaluated the biochemical effects caused by different types of carbon nanotubes, carbon nanofibers, and silica nanotubes on four marine microalgae species (two types of diatom species "*Attheya ussuriensis*" and "*Chaetoceros muelleri*" a raphidophyte "*Heterosigma akashiwo*", and a red algae "*Porphyridium purpureum*" and found that these nanoparticles cause change in growth rate, membrane polarization, esterase activity and changes in microalgae cells, also it was found that toxic effects caused by the carbon nanotubes strongly correlated with the content of heavy metal impurities in the NPs, and the red microalga *P. purpureum* was more affected by hydrophobic carbon NPs. Additionally, Silica nanoparticle significantly slowed the growth rate of microalgae. In the year 2014, Pretti et al., (2014) investigated the ecotoxicity of pristine graphene nanoparticles (graphene nanopowder grade C1 (GNC1) and pristine graphene monolayer flakes (PGMF) in the marine organism's bioluminescent bacterium "*Vibrio fischeri*", unicellular alga "*Dunaliella tertiolecta*" and crustacean "*Artemia salina*" and found that PGMF is more toxic than GNC1 to the bioluminescent bacterium and unicellular alga on the basis of EC_{50} values and 48-h exposure to *A. salina* revealed increase in activities of biomarker enzymes Catalase (in both PGMF and GNC1), glutathione peroxidase (in PGMF) and lipid peroxidation of membranes (in PGMF).

Lovern et al., (2006) exposed "*Daphnia magna*" to the four solutions (filtered C_{60} nanoparticles, sonicated unfiltered C_{60}, filtered TiO_2 and sonicated unfiltered TiO_2) using U.S. Environmental Protection Agency 48-h acute toxicity tests and

found that exposure to filtered C_{60} and filtered TiO_2 caused an increase in mortality with an increase in concentration wherein exposure to fullerenes show higher levels of toxicity at lower concentrations and varied mortality was observed for the exposure to sonicated solutions.

2.2. CNPs TOXICITY STUDIES IN VERTEBRATES

Smith et al., (2007) reported the toxicity of single walled carbon nanotubes (SWCNT) to Rainbow Trout stating that SWCNT exposure for a period of 10 days caused a dose-dependent rise in ventilation rate, gill pathologies and mucus secretion with SWCNT precipitation. Similar study was carried the year 2021on the vertebrate frog. Zhao et al., (2021) studied the toxicity of MWCNTs to the growth and reproduction of "*Xenopus* tropicalis" and their results showed that toxicity of MWCNTs inhibits the growth of body, including the gonadal organs (testis, ovaries) and fat of *X. tropicalis*, also from the histopathological sections it was noted that MWCNTs affects the formation of spermatogonia and oocytes also it induced the formation of lung cannons in the *X. tropicalis* lungs and in addition to this, MWCNTs were found to changed diversity of gut microbiota and the microbial community structure.

Tong et al., (2014) investigated the toxic effects of three nanomaterials i.e., multi-walled carbon nanotubes (MWCNTs), graphene oxide (GO), and reduced graphene oxide (RGO), on zebrafish embryos and found that RGO significantly inhibited the hatching of Zebra fish embryo and later RGO and MWCNTs

decreased the length of the hatched larvae at 96 hours post-fertilization (hpf) but there were no morphological malformation or mortalities in the exposed embryos.

2.3. TOXICITY OF CBNPs TO VARIOUS CELL TYPES

The CBNPs have found to induce toxicity via differential effects. Catalan et al. (2012) observed the Chromosomal aberrations in the lymphocyte cells. L'Azou et al. (2008) and Belade et al. (2012) observed the cytotoxicity in the renal and lung epithelial cells respectively. The CBNPs have found to produce neurotoxic effect by disturbing the electrical activity of neuronal networks and inflammatory and genotoxic effects (Gramowski et al., 2010; Bourdon et al., 2012 and Canesi et al., 2008). Mesaric et al. (2015) observed that the CBNPs inhibits swimming behaviour, alters enzymatic activities and various signaling pathways involved in embryonic development. Also, the CBNPs have found to reduce the success rate of fertilization (Nielsen et al., 2008).

2.4. CBNPs TOXICITY STUDIES IN INVERTEBRATES

Canesi et al., (2008) found that CBNPs induces inflammatory responses in hemocytes (immune cells) of a marine mussel "*Mytilus galloprovincialis*" by releasing oxyradical production, extracellular Lysozyme, nitric oxide (NO) and changes in mitochondrial production. In another study by Mesaric et al. (2013) it was found that very high concentration of nano Carbon Black (nCB) inhibits swimming behavior of II stage nauplii larvae in "*Amphibalanus amphitrite*". A

similar study was carried out by Mesaric et al. (2015) in the larvae of "*Artemia salina*" and found that CBNPs concentrates in the gut and attach onto the body surface and thereby inhibiting larval swimming behavior and partly alters the enzyme activities. Mesaric et al. (2015) found that the exposure of CBNPs to sperm cell of purple sea urchin "*Paracentrotus lividus*" affects the fertilization and the various signaling pathways of early stages of development and also reduces skeletogenesis. Nielsen et al. (2008) found that in the brown algae "*Fucus serratus*" CBNPs forms agglomerates of CB in the sperm, embryo and zygote of the algae and reduces the success rate of fertilization and correct alignment of the polar axis.

Zhao et al., (2017) investigated the impact of three types of modified nano-carbon black on the earthworm "*Eisenia fetida*" by exposing them to artificial soil supplemented with 5% $H2SO_4$-, HNO_3- and $KMnO_4$-modified nano-CB (SCB, NCB and KCB, respectively) under turfgrass growing conditions and found that SCB and NCB were found to be more toxic and ecologically dangerous to *E. fetida* as there was a notable decline in biomass and survival following exposures of 35- and 60 days, and the survival rate tended to drop with exposure time. The activities of SOD, CAT and POD were found to be inhibited in all treatments with modified nano-CBs at 35- and 60-d, indicating that oxidative stress was induced by modified nano-CBs. A similar study was carried out by Xu et al., (2021) on the earthworm "*Eisenia fetida*" by exposing the earthworm to three types Carbon Nanomaterials (CNs), carbon black (CB), Single-wall carbon nanotubes (SWCNT) and reduced graphene oxide (RGO) are examples. The multivariate regression analyses showed that Carbon Black with a higher aspect ratio

(SWCNT followed by RGO) exerted more stress on the metabolic processes of earthworms. Membrane-associated metabolite choline changes were found to be indicative for potential harm of the sharp CNs due to membrane disruption.

2.5. CBNPs TOXICITY STUDIES IN VERTEBRATES

Liu et al. (2021) exposed the CBNPs to Zebrafish "*Danio rerio*" and found that CBNPs reduces the intestinal floral diversity, changes structure of core microbial populations and also causes vacuolar degeneration and lipid accumulation in liver of zebrafish. Inhalation of Printex 90 carbon black nanoparticles by Nulliparous Female Mice induce denaturation of PVM and astrocytes in dose dependent manner (Umezawa et al., 2018)

Carbon black nanomaterial are recognized as a risk factor for prolonged inflammatory response and diffused alveolar injury, it induces acute cell necrosis through lysosomal rupture and release of mtDNA play as a key role in mediating pulmonary neutrophilic inflammation in the invitro studies performed on the cell of *Danio rario* (Yuan et al., 2020). Parental exposures to carbon nanoparticles affect entire life of the fetus. Pregnant women are more susceptible to nanoparticles toxicity. A nanoparticle cross placental barrier and can generate oxidative stress, inflammation and alters gene expression and also leads to abnormal fetal development (Wang and Wang 2020).

In vivo exposure studies of carbon Nanoparticles on Rainbow trout indicated oxidative stress in the brain and change in gene expression. The survival of the exposed fishes was reduced with increase in concentration of nanoparticles (Scown et al., 2010).

2.6. *Paphia malbarica* AS A MODEL ORGANISM IN RESEARCH STUDIES

The bivalve mollusc exhibits a suspension feeding habit and thus filter large volumes of water containing bacteria, microalgae, sediments and other particulates thus accumulating different chemicals in their tissues and this makes them valuable indicators of environmental monitoring (Desai, 2012 and Luiz et al., 2010). The bivalve mollusc "*Paphia malbarica*" is widely distributed along the cost of Goa (India) and it forms an inexpensive and delicious seafood among the locals (Desai, 2012 and Krishna Kumari et al., 2006). Modassir and Ansari (2000), reported this species as a useful bioindicator. Various studies have been carried out using *"Paphia malbarica"* as a model organism. Gajbhiye and Khandeparker (2017), studied the immune response of the Short Neck Clam *Paphia malbarica* to salinity stress using flow cytometry in a two-way experimental approach (*in vitro* and *in vivo*) by providing with different salinities (0, 5, 15, 25 and 35) and found that at lower salinities (0 and 5) hemocyte parameters were significantly compromised with an evident immune-salinity tolerance range of 15-35 also with the longer exposure period to lower (0 and 5) salinities the damaging impact on hemocyte function was found to be intensified.

Iqbal and Navalgund (2021), evaluated the *in vivo* immunotoxic effects in Bivalves "*Perna virdis"* and "*Paphia malbarica*" by treating mussels and clams with the five-sublethal doses of atrazine for 14 days and found that atrazine caused immunotoxicity by reducing the viability of bivalve hemocytes.

2.7. LACUNAE

The above literature shows that in aquatic environment the toxicity of CBNPs have been studied only in few organisms leaving much of scope in this area unexplored. Exposure of bivalve "*Mytilus galloprovincialis*" to CBNPs showed inflammatory responses, and in Sea Urchin, "*Paracentrotus lividus*" and crustacean, "*Artemia salina*" these NPs inhibits growth. In brown algae and sperm of Sea urchin, CBNPs have found to affect the fertilization rate. Its exposure to nauplius larvae "*Amphibalanus amphitrite*" and "*Artemia salina*" has found to inhibit swimming behavior. In Zebra fish it reduces intestinal floral diversity. Based on the above referencing it seems that there is lack of work done of the effects of CBNPs with respect to physiology and biochemical effects in the organisms which leaves the lacunae that needs to be filled.

2.8. HYPOTHESIS

The study hypotheses that CBNPs can induce adverse toxicity on "*Paphia malbarica*" by affecting its anatomy, morphology and physiology in turn affecting its normal functioning.

2.9. OBJECTIVES OF THE STUDY

Based on the Lacunae stated, objectives of the current study include:

- To determine the LC50 of the CBNPs in *"Paphia malbarica"*.
- To analyze effect of CBNPs on various physiological parameters such as Respiratory rate, excretory rate and osmoregulation in "*Paphia malbarica*".
- To evaluate the effects of CBNPs on the anatomy of Gill and Siphon in "*Paphia malbarica*".
- To determine the effect of CBNPs on the enzymes and major biomolecules.

CHAPTER 3:

MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1. MATERIALS USED

CHEMICALS

All the chemicals used for the study were of Analytical Grade from Hi-media and Sigma-Aldrich.

GLASSWARES

Glasswares and labware used were beakers, conical flasks, test tubes, test tube stands, pipettes, pipette canisters, measuring cylinders, eppendorf tubes, and funnels of high quality were used. All glassware were first soaked in 4% chromic acid and kept overnight and cleansed thoroughly with detergent and water. These clean glasswares were rinsed with distilled water and dried in the oven before use.

INSTRUMENTATION

UV-Visible Spectrophotometer (BioEra's Elite), centrifugation machine (REMI CM 101), water bath (*i-therm*, AI- 7981) analytical balance (WENSAR PGB 200), vortex (RECM 101), incinerator, hot air oven (MIC-MIC-165), light microscope, Ph meter (µP pH meter TMP3) was used.

BIOLOGICAL MATERIALS

The animal model chosen for the conduct of research work was a bivalve species *Paphia malbarica*. The bivalves were collected from river in Madkai village, Ponda Goa. Necessary approval was taken for carrying out experimentation on animals from the Institutional Animal's ethics committee of Goa University (IAEC Ref. No. GUZ/IAEC/22-23/N5).

3.2. MAINTAINANCE OF BIVALVE *Paphia malbarica*

The bivalves were brought to the Animal House of Goa University and were immediately transferred to static tank containing sea water which was directly bought from the site. The acclimatization period of 12Hr light and 12Hr dark and temperature 18°C was maintained for 7 days from the day of collection under the laboratory conditions. The tank was provided with proper aeration by inserting aerators in order to maintain continuous supply of oxygen. No feed was provided since the water was used from the site itself. The tank water was changed daily i.e., once a day to maintain good quality conditions of water.

3.3. EXPERIMENTAL SETUP

In order to evaluate the toxic effects of the CBNPs the bivalves were exposed to three different concentrations of CBNPs weighing 100mg/L, 200mg/L and 400mg/L for 28 days in order to determine LC50 value of CBNPs by Probit Analysis (Finney, 1952). With these doses the LC50 value was determined for 28 days as per the mortality rate and behaviour. The CBNPs addition to the tanks

was done by suspending in 5mL of sea water and was then added in the aquarium (Canesi L, et al., 2008).

Based on LC50 value of CBNPs three concentrations were decided for the experimentation purpose to carry out the analysis of the effect of CBNPs on bivalves. During the experimental period four different test conditions were maintained i.e., (1) Control, (2) Experimental A, (3) Experimental B, and (4) Experimental C, containing 10 bivalves in each tank and CBNPs concentration weighing 3mg/L, 6mg/L and 12mg/L in Experimental A, Experimental B and Experimental C respectively whereas control tank was set without any exposure to NPs. The exposure period considered for the study was 28 days.

3.4. EUTHANASIA

After the exposure period of 28 days the bivalves were removed from the aquarium and placed in cold/chilled water to make them unconscious and then tissues (Siphon and Gills) were quickly dissected, pooled washed/rinsed in seawater and stored at -80° C in order to determine the biochemical and enzyme activities as well as the tissues were processed for histological analyses (Canesi et al., 2010).

3.5. HISTOLOGY

After sacrificing the bivalves, the siphon and gill were isolated, removed aseptically, and washed in physiological saline (Phosphate buffered saline, Ph=7.2). They were weighed and immediately stored in 10% formalin. They were transferred to 70% ethyl alcohol and stored until processed. The tissue specimens

(siphon and gill) were further processed, embedded in paraffin, sectioned at 0.1 µm, and stained with hematoxylin and eosin at the Ashwini Pathology Lab. They were further analyzed under a light microscope at 20x and light micrographs were captured.

3.6. DETERMINATION OF LC_{50} OF CBNPs

The LC_{50} was determined by Probit Analysis (Finney, 1952) in Microsoft Excel 2011. Initially, the three different concentrations (100mg/L, 200mg/L, 400mg/L) used to determine the LC_{50} value were conveterd to their log concentrations. Then, the Probit of Kill value was taken from the Finney's table based on the mortalities values observed. The obtained data was then used to calculate the LC_{50} value by using Regression Analysis.

3.7. DETERMINATION OF PHYSIOLOGICAL PARAMETERS

3.7.1. Determination of Oxygen consumption rate

Principle: *The manganous sulphate becomes manganous hydroxide in water. The oxygen in the water sample oxidizes manganous hydroxide to manganic hydroxide. The acidified 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.*

- ***Reagents:***

Winkler's Solution A- 20g of Manganous sulphate was dissolved in 100ml of pre-boiled distilled water.

Winkler's Solution B- l00g of Potassium hydroxide and 20g of Potassium iodide was dissolved in 200 ml of pre-boiled distilled water.

Sample Collection: Prior to determination of dissolved oxygen, the tanks were covered with a lid for 2 hours so that there was no air space left between water surface and the lid. After 2Hrs, 60 mL of water sample was siphoned out into an amber-coloured bottle without introducing water bubbles to estimate the dissolved oxygen (*Welsh and Smith, 1993*).

Protocol:

The collected water was immediately fixed by adding 1ml Winkler's Solution A and 1ml of Winkler's Solution B and then mixed the sample by inverting several times which resulted in the formation of brown colour precipitate. To this, immediately, 1ml of concentration sulphuric acid was added by inserting the calibrated pipette just below the surface of the liquid. The sample was mixed by repeatedly inverting it. In a conical flask, 50 ml of resulting solution was titrated against 0.025 (N) Sodium thiosulphate solution to a pale straw colour. After these two drops of 1% starch indicator was added resulting in the formation of dark blue colour. The titration was continued until the water sample turns colorless. At this point the burette reading was noted. A 'blank' was estimated parallelly by taking 60 ml distilled water.

Calculations:

Dissolved oxygen in mg/ml= 8×100×N/V ×v

where,

V= volume of sample taken (mL)

v= volume of titrant used (mL)

N= normality of sodium thiosulphate (0.025 M)

8= equivalent weight of oxygen (8)

FORMULA

Oxygen consumption in mg/mL= Dissolved oxygen in blank – Dissolved oxygen in experimental

3.7.2. Determination of Excretory rate

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

Reagents:

Phenol colour reagent- 27 mg of Phenol and 125 mg of Sodium nitroprusside were dissolved in 100 ml distilled water.

Sample Collection: The water samples were collected from the four experimental tanks separately and filtered for estimation of Ammonia. A total of 5 samples of water from each tank were taken for the estimation of ammonia.

Procedure:

Samples aliquot was made up to 4ml with distilled water. To this 1 ml of sodium hypochlorite solution and 1ml of Phenol reagent was added. It was incubated in water bath at 40°C for 15 minutes and then cooled to room temperature. The intensity of colour was measured at 650 nm against a suitable blank *(Chaney and Marbach, 1962)*. The amount of ammonia present in samples was calculated by using standard curve of ammonia (mg/ml). The rates of excretion were expressed as mg/L.

3.8. EXTRACTION AND ESTIMATIONS OF BIOMOLECULES AND ENZYMES

Siphon and Gill tissue samples were used for biochemical estimations.

3.8.1. Estimation of Biomolecules

1. Total Proteins

Principle: *The carbamyl 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.*

- ***Reagents***

Lowry's reagent: To 98 ml of 4% Sodium carbonate, 1ml of each 1% Copper sulphate and 4% Sodium-potassium tartarate were added to make the volume upto 100 ml.

Folin-Ciocalteau reagent (1:1 dilution)

Extraction

Tissue homogenate was prepared by using 1ml of ice-cold distilled water. The homogenate was cold centrifuges at 6000 RPM for 5 minutes. The residue was discarded and supernatant was used as a sample source.

Procedure:

To 1ml of the obtained sample 5ml of Lowry's reagent was added and incubated at room temperature for 15 minutes. To this 0.5 ml of Folin- Ciocalteau reagent was added and incubated at room temperature for 30 minutes. The intensity of the blue coloured complex was measured against a suitable blank at 720 nm. Quantification of the protein content of the sample was done with the help of standard curve of BSA (250 µg/ml in 1N NaOH). (Lowry et al., 1951).

Total Carbohydrate and Free sugars

Extraction

The tissue was homogenised in 1ml of ice-cold distilled water. The prepared homogenate was then deproteinized with equal volume of $ZnSO_4$ and BaOH. The homogenate was then cold centrifuged to 3000 RPM for 15 minutes. The residue was discarded and the supernatant was used to estimate the total carbohydrate and free sugars.

3. Total carbohydrate

Principle: *Carbohydrates are dehydrated by conc. H2SO4 to form furfural which condenses with anthrone to form a blue-colored complex, which is measured calorimetrically at 620nm.*

- ***Reagents***

Anthrone reagent: 0.2gm of anthrone was dissolved in 100m1 of concentrated sulphuric acid.

Procedure:

0.1ml of the deproteinized aliquot was diluted with distilled water to make 1.0ml, to which 4ml of the anthrone reagent was added, and the mixture was then incubated for 10 minutes in a boiling water bath. The intensity of the color developed was measured at 620nm against a suitable blank (Carroll, 1956). Total carbohydrate content was quantified using a standard curve of total carbohydrate (100ug of glucose/ml).

3. Free sugars

Principle: *Sugars when heated with alkaline copper reagent it forms a cuprous oxide, giving a blue-colored complex with arsenomolybdate reagent, the intensity of which can be measured at 540 nm.*

- ***Reagents***

A) Alkaline copper reagent- 12.0 g anhydrous sodium carbonate and 6.0 g sodium potassium tartrate were dissolved in 125 ml of distilled water (Solution a). 2.0g of copper sulfate was dissolved in 25 ml distilled water (Solution b). Both the

solutions a and b were mixed and to this 8.0 g of sodium bicarbonate was added by stirring to prepare solution A.

B) 90.0g of anhydrous sodium sulfate was dissolved in 250 ml of distilled water. Boiled to expel air and then cooled to room temperature to prepare solution B. Now both the solutions A and B were mixed and the volume was made up to 500ml with distilled water.

C) Arsenomolybdate color reagent – 450ml of distilled water were used to dissolve 25.0g of ammonium molybdate, and while the mixture was being stirred, 21 ml of concentrated sulfuric acid was added. To this, 3.0 g disodium hydrogen arsenate (previously dissolved in 25 mL of water) was added mixed, and stored in the amber-colored bottle at 37°C for 48 hours.

Procedure:

To 1.0 ml deproteinized sample, 1.0 ml alkaline copper reagent was added and incubated in the boiling water bath for 20 minutes. After cooling at room temperature 1 ml of arsenic-molybdate color reagent was added furthermore the mixture was diluted with 7 ml distilled water. The intensity of the color was evaluated at 540 nm against a suitable blank (Nelson, 1944). Quantification of tissue-free sugar concentration was assessed with the help of a glucose standard curve, prepared by using 100 µg/ml glucose as a standard solution.

4. Reduced glutathione (GSH)

Principle: *GSH reacts with 5, 5'- dithiobis, 2-nitrobenzoic acid producing a yellow-colored compound. The intensity of the color can be measured spectrophotometrically at 412nm.*

- ***Reagents***

  5, 5'- dithiobis, 2-nitrobenzoic acid (DTNB) reagent- In 100ml of 0.1% sodium nitrate, 19.8mg of 5,5-dithiobis, 2-nitrobenzoic acid was dissolved.

  Extraction

  The tissue was homogenized by using 1ml of 5% TCA and cold centrifuged at 6000 RPM for 5 minutes. The supernatant was used aa a sample source and residue were discarded.

  Procedure:

  To 1.0 ml of diluted tissue extract, 2 ml of 5, 5'- dithiobis, 2-nitrobenzoic acid (DTNB) reagent was added making the final volume of 3.0 ml (Moron et. al., 1979). Absorbance was read at 412 nm against a suitable blank. The samples were quantified for Reduced Glutathione content with the help of a standard curve of reduced glutathione (0.2µmole/m1 in 5% TCA).

3.8.2. Estimation of Enzymes

1. Catalase

Principle: *The reaction of catalase is the decomposition of hydrogen peroxide into water and molecular oxygen.*

$2H_2O_2$ ----------> $2\ H_2O + O_2$

The remaining H_2O_2 in the samples was not decomposed by the enzyme. reacts with dichromate to give a blue precipitate of perchromic acid. This unstable

precipitate is then decomposed by heating to give a green-colored stable compound. The intensity of the green color can be measured at 620nm.

- ***Reagents***

  Dichromate acetic acid reagent- 5% potassium dichromate and glacial acetic acid were taken in a 1: 3 ratios to prepare this reagent.

Extraction

The tissue homogenate was prepared by using 1ml of Phosphate buffered saline (PBS) of pH 7.2. The homogenate was then cold centrifuged to 6000 RPM for 5 minutes. The supernatant was used as a sample source and residue was then discarded.

Protocol:

1.5 ml of phosphate buffer (0.01 M, pH 7.0) and 0.4 ml of the substrate (0.2 M H202) were taken and incubated at 37°C for 5 minutes. 0.1 ml phosphate buffer was added to prepare the enzyme blank and 0.1 ml serum or tissue homogenate was added to assay the enzyme activity. This reaction mixture was incubated at 37°C for 15 minutes. The reaction was stopped by the addition of 2.0 m1 of dichromate acetic acid reagent. The solution was then heated in a boiling water bath for 10 minutes, mixed well and the intensity of the color was measured against a reference blank at 620 nm (Sinha, 1972). As previously mentioned, an estimate of the enzyme's protein content was made. The enzyme activity was quantified with the help of a reference curve of hydrogen peroxide (2 µmole/ ml) and expressed as moles of H202 consumed/min/mg protein.

Estimation of Ion transporting enzymes (ATPases):

Principle: *The ATPases (Na^+-K^+/-ATPase, M g^{++}-ATPase, Ca^{++}-ATPases) play a major role in converting its metabolic energy of ATP to transport the ions like Na^+, K^+, Ca^{++}, Mg^{++} across the cell membranes. Therefore, the activities of ATPases are directly linked with the hydrolysis of ATP. The released inorganic phosphate, upon the hydrolysis of ATP, is measured with ammonium molybdate reagent to form phosphomolybdate acid which was reduced by ascorbic acid to give blue colour.*

Reagents:

- **Ammonium molybdate reagent**- 0.42% of ammonium molybdate solution in 1(N) sulphuric acid and 10% aqueous ascorbic acid solution were taken in the ratio of 6 :1 in order to prepare this reagent (to be freshly prepared).
- ***Reagent A:*** Copper acetate buffer (pH-4)

  Dissolve 2.5g of copper sulphate and 46g of sodium acetate in 1 L of 2mol/L acetic acid.
- ***Reagent B:*** 5% ammonium molybdate solution
- ***Reagent C:*** 2% metol prepared in 5% NaCl (Filter the solution as it does not dissolve completely).
- ***Reagent mixture:*** In a 500ml of distilled water dissolve 2.364g of Tris HCl, 0.014g of $MgCl_2$, 0.0074g of KCl and 0.292g of NaCl.

Extraction

The tissue was homogenised in 1 ml of 0.32M sucrose solution. The homogenate was cold centrifuged at 10, 000 RPM for 10 minutes. The residue was discarded

and the supernatant was used as a sample source for the estimation of Na^{+}-K^{+}/-ATPases, M g^{++}-ATPases.

Protocol for estimation of Na^{+}-K^{+}/-ATPases

For the estimation of Na^{+}-K^{+}/-ATPases the 0.1 ml of sample was taken and to this 0.2ml of reagent mixture was added and incubated for 10 minutes at 100°C in a boiling water bath. After cooling the contents of the test tubes 0.2ml of 0.5mM ATP was added and incubated at 37°C for 10 minutes. To this 0.2ml of 10% TCA was added and centrifuged for 3000 RPM for 5 minutes. After centrifugation, to the above reaction mixture, 3ml of Reagent A, 0.5ml of Reagent B and 0.5ml of Reagent C was added and incubated at room temperature for 7 minutes. The blue colour developed was measured against a suitable blank at 820 nm.

Protocol for estimation of Mg^{++}-ATPases

For the estimation of Mg^{++}-ATPases, 0.1ml of sample was taken and to this 0.2ml of Reagent mixture, 0.1ml of 0.1mM Oubain was added and incubated at 100°C for 10 minutes in a boiling water bath. To this 0.2ml of 0.5mM ATP was added and incubated at 37°C for 10 minutes. To this 0.2ml of 10% TCA was added and centrifuged at 3000 RPM for 5 minutes. After centrifugation, to the above mixture 3ml of Reagent A, 0.5ml of Reagent B, 0.5ml of Reagent C wad added and incubated at room temperature for 7 minutes. The blue colour developed was measured against a suitable blank at 820 nm.

The released inorganic phosphate was estimated following the modified methods of Fiske and Subbarow, (1925). In brief, to 1 ml of the obtained supernatant 3.5 ml of freshly prepared ammonium molybdate reagent was added and kept it on

water bath at 60°C for 30 minutes. After 30 minutes the intensity of colour developed was measured at 820nm. The quantification of released phosphate was done with the help of standard curve of phosphate (1µmole of Phosphate/ml).

CHAPTER 4:

RESULTS

4. RESULTS

4.1. LC_{50} of CBNPs

The LC_{50} value of CBNPs for chronic period of 28 days exposure to bivalve *Paphia malbarica* was found to be 64.77mg/L.

4.2. CBNPs effects on the anatomy of major organs of *Paphia malbarica*

4.2.1. Gill

The gills of the control bivalve showed uniform arrangement of the gill lamellae with uniform interlamellar space. The surface of each gill lamellae is lined by a monolayer epithelium and also contains the pillar cells which extends itself into the lamellar sinus. Each gill lamellae are organized into three different regions, terminal of the gill lamellae the frontal, middle intermediate and inner Abfrontal. The surface of the gill filaments is covered by basophilic cells (Figure 2A). The experimental group 1 showed narrower or obstructed gill lamellae (Figure 2B). The experimental group 2 showed narrower gill lamellae with necrosis and the basic cells are clubbed at discrete locations (Figure 2C). The experimental group 3 showed necrotic gill lamellae fused with the adjacent gill lamellae containing clubbed basophilic cells. Also, the gill lamellae showed hyperplasia resulting in clavate-globate lamellae (Figure 2D).

4.2.2. Siphon

The control grouped showed normal structure containing hemolymph space, erect outer epithelium and glandular layer containing AB-positive cells (Figure 3A). The experimental group 1 showed distorted outer epithelium and AB-positive cells separating from a glandular layer (Figure 3B). The experimental group 2 showed distorted outer epithelium and clubbed AB- positive cells (Figure 3C). The experimental group 3 showed distorted outer epithelium and fused glandular layer with AB- positive cells. Also, in the hemolymph space there was a vacuole bubble (Figure 3D).

4.3. Effect of CBNPs on Physiological parameters

4.3.1. Effect of CBNPs on Oxygen Consumption

The oxygen consumption rate decreased significantly with days ($F=239.6$, $P\leq0.001$) and also declined significantly with respect to doses ($F=617.4$, $P\leq0.001$). The interaction between the days and the doses was also showing significant decrease ($F=3.752$, $P\leq 0.001$) (Figure No.4).

4.3.2. Effect of CBNPs on Excretion of Ammonia

The rate of ammonia excretion increases significantly with days ($F=13.38$, $P\leq 0.001$) and also showed significant increase with respect to doses ($F=111.8$, $P\leq 0.001$). The interaction between the days and the doses was also showing significant decrease ($F=3.314$, $P\leq 0.001$) (Figure No.5).

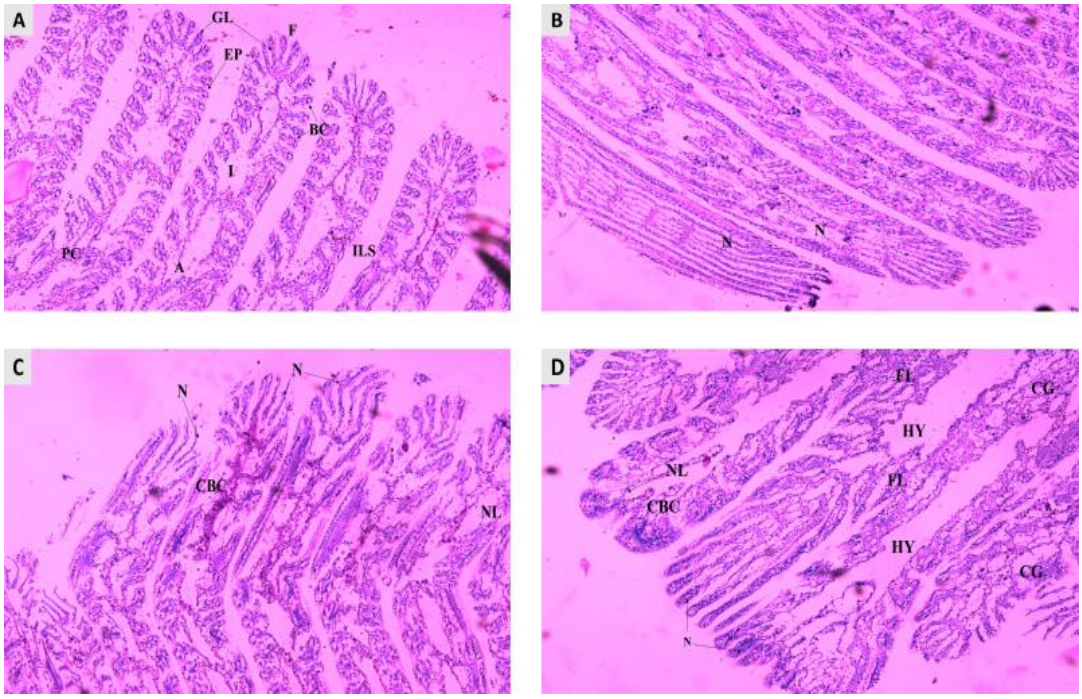

Figure No. 2: CBNPs effect on the histopathology of the Gill. **A**- Control bivalve gill section showing Gill lamellae with uniform interlamellar space (ILS), Gill lamellae (GL), Epithelium (EP), Basophilic cells (BC), Distinguished three zones of gill lamellae 1. Frontal (F), 2. Intermediate (I), 3. Abfrontal (A) and Pillar cells (PC).). **B**- Experimental 1 bivalve gill shows Narrower (N) or obstructed hemolymph vessels in gill lamellae. **C**- Experimental 2 bivalve gill shows Narrower (N) or obstructed hemolymph vessels in gill lamellae, Clubbed basophilic cells (CBC) and Necrotic lamellae (NL). **D**- Experimental 3 bivalve gill shows Necrotic lamellae (NL), Clubbed basophilic cells (CBC), Fusion of lamellae (FL) and Hyperplasia (HY) resulting in clavate-globate (clubbing) lamellae (CG) and completely disorganized mass of ruptured gill lamellae.

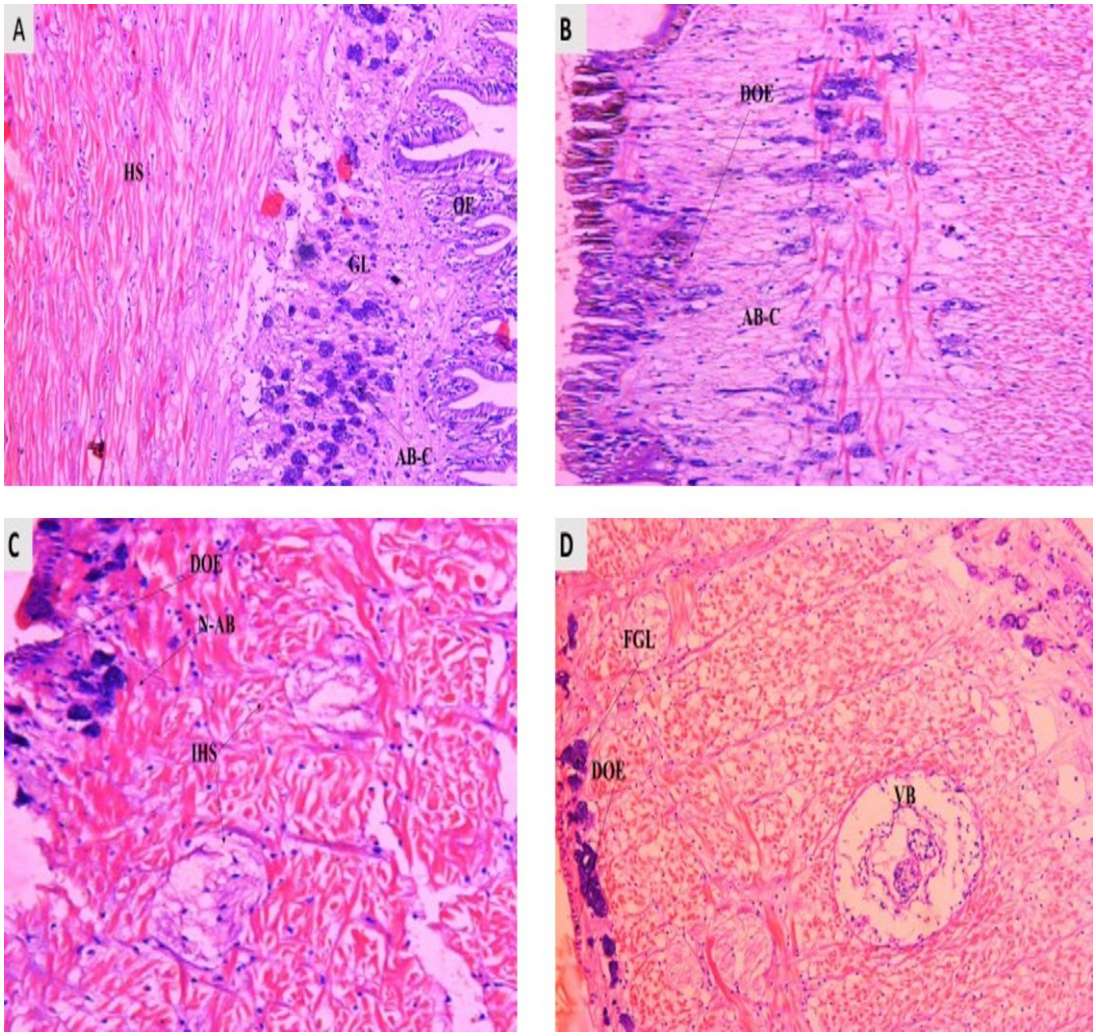

Figure No. 3: CBNPs effect on the histopathology of the Siphon. **A**-Control bivalve siphon section showing normal siphon architecture with normal hemolymph space (HS), outer epithelium (OE), glandular layer (GL) containing many AB-positive cells (AB-C). **B**-Experimental 1 bivalve siphon section shows distorted outer epithelium (DOE), AB-positive cells in the hemolymph space separating from glandular layer. **C-** Experimental 2 bivalve siphon section showing distorted outer epithelium (DOE), Necrotic AB-positive cells (N-AB). **D**-Experimental 3 bivalve siphon section shows fused glandular layer (FGL), Distorted outer epithelium (DOE), Vacuole bubble (VB).

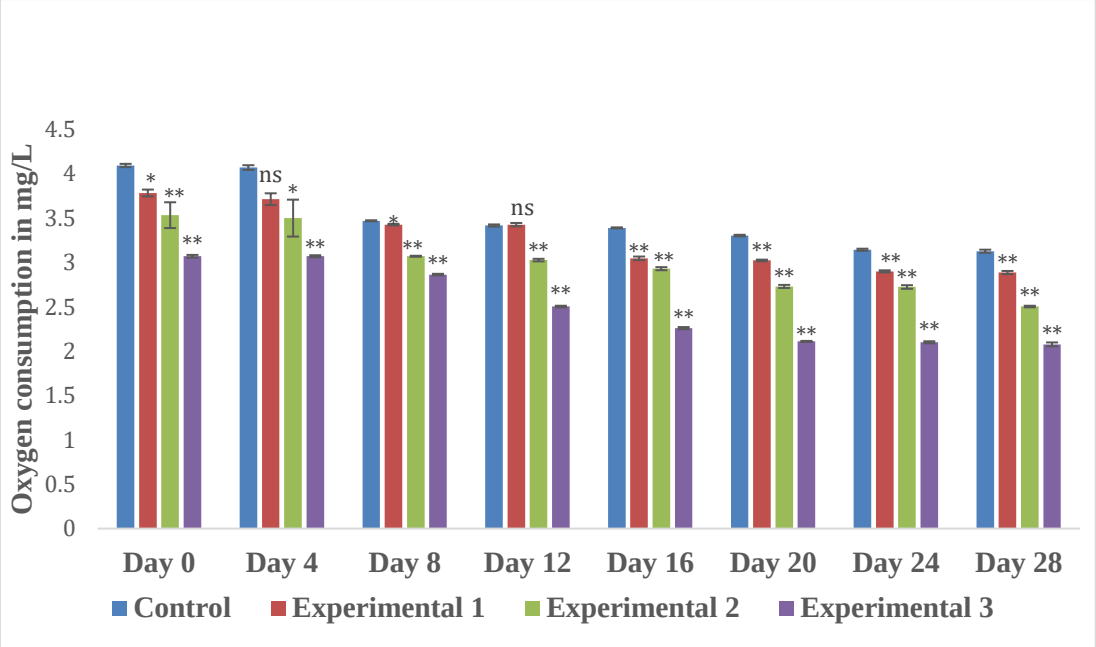

Figure No. 4: Effect of CBNPs on Oxygen consumption ns (non-significant), *(P≤ 0.05, significant), **(P≤ 0.01, very significant).

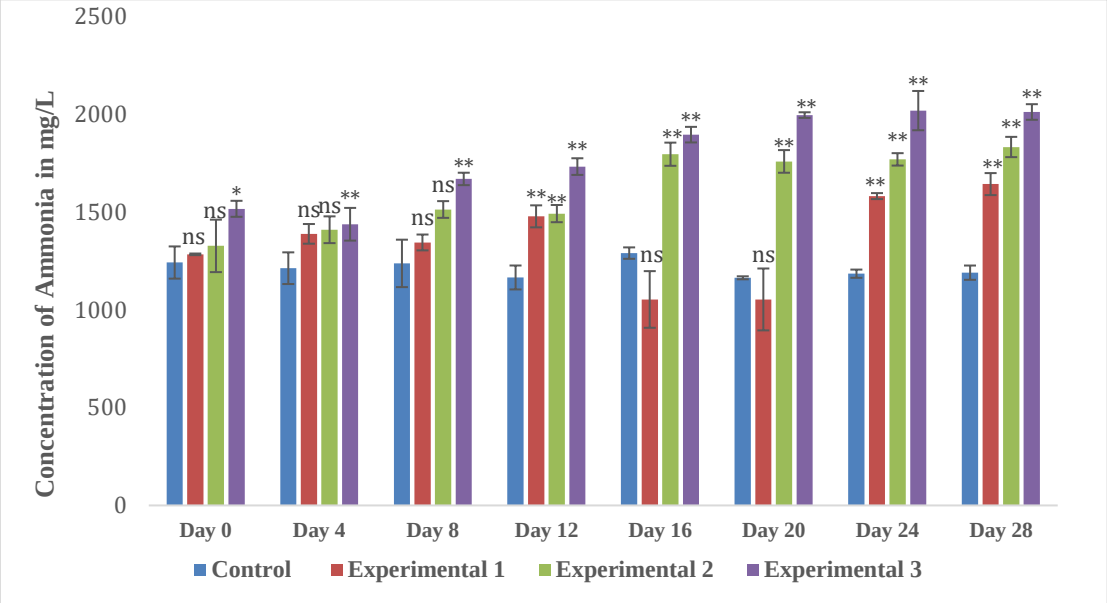

Figure No. 5: Effect of CBNPs on the Ammonia excretion ns (non-significant), *(P≤ 0.05, significant) ** (P≤ 0.01, very significant).

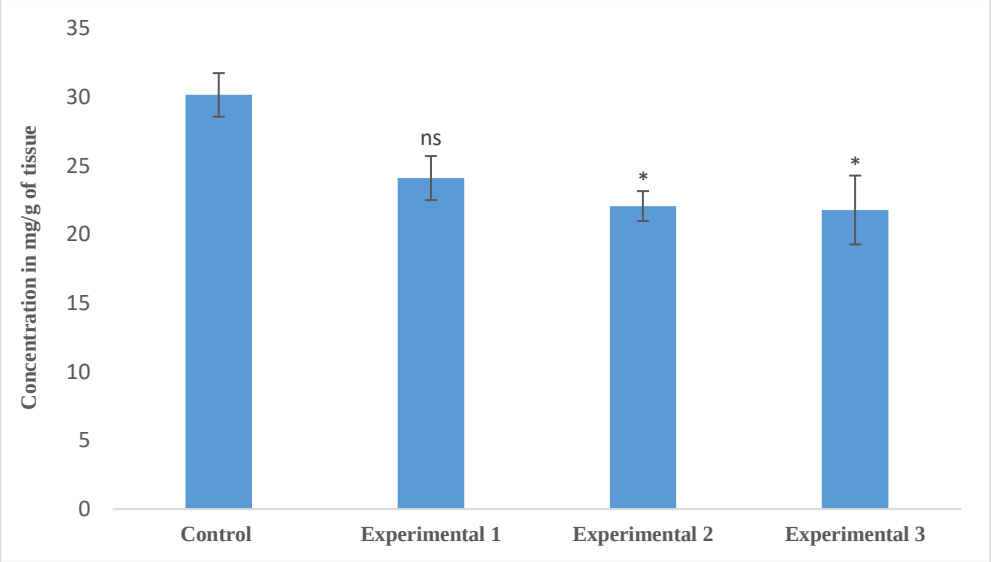

Figure No. 6: Effect of CBNPs on the Total Protein content of bivalve Gill tissue. Ns (non-significant), *($P \leq 0.05$, significant).

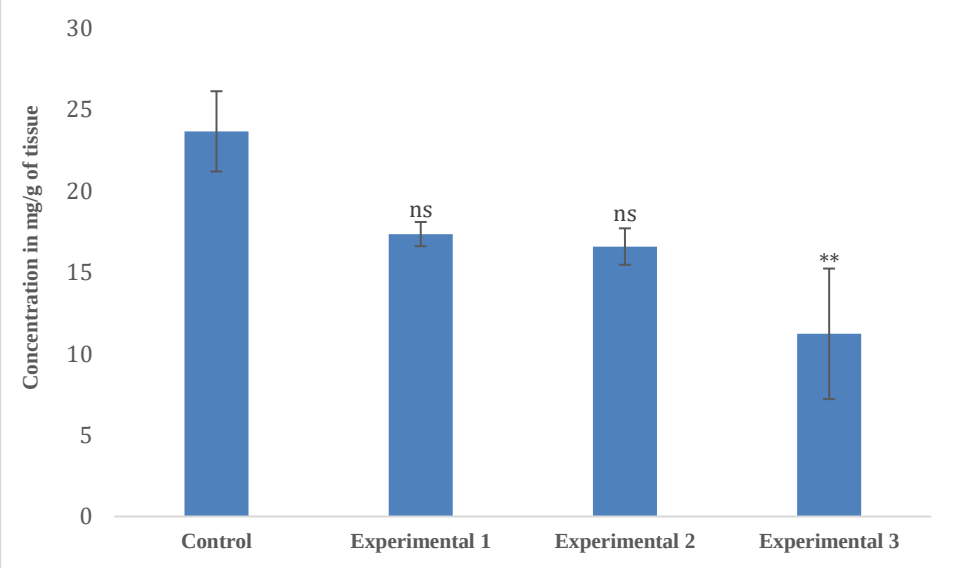

Figure No. 7: Effect of CBNPs on the Total Protein content of bivalve Siphon tissue. Ns (non-significant), **($P \leq 0.01$, highly significant).

4.4. Effect of CBNPs on the Biomolecules of gill and siphon

The total protein concentration significantly and dose-dependently decreased in the CBNPs exposed bivalves in the Experimental group 2 and experimental group 3 ($F= 4.836$, $P< 0.05$), whereas showed non-significant decrease in the experimental 1 group compared to the control (Figure No.6).

Further, the total protein concentration also showed a significant and dose dependent decrease in the Experimental 3 set of CBNPs exposed bivalves ($F= 4.333$, $P< 0.05$), whereas showed a non-significant decrease in the experimental 1 and experimental 2 set (Figure No. 7).

The Total carbohydrate concentration significantly and dose-dependently decreased in the CBNPs exposed bivalves in the experimental 2 and experimental 3 set ($F= 65.52$, $P<0.001$), whereas showed a non-significant decrease in the experimental 1 set compared to the control (Figure No.8).

The Total carbohydrate concentration significantly and dose-dependently decreased in the CBNPs exposed bivalves in the experimental 2 and experimental 3 set ($F= 13.64$, $P< 0.001$), whereas showed non-significant decrease in the experimental 1 set compared to the control (Figure No. 9).

The Free sugar concentration significantly and dose-dependently decreased in the CBNPs exposed bivalves in the experimental 3 set ($F= 4.849$, $P<0.05$), whereas showed non-significant decrease in the experimental 1 and experimental 2 set compared to the control (Figure No.10).

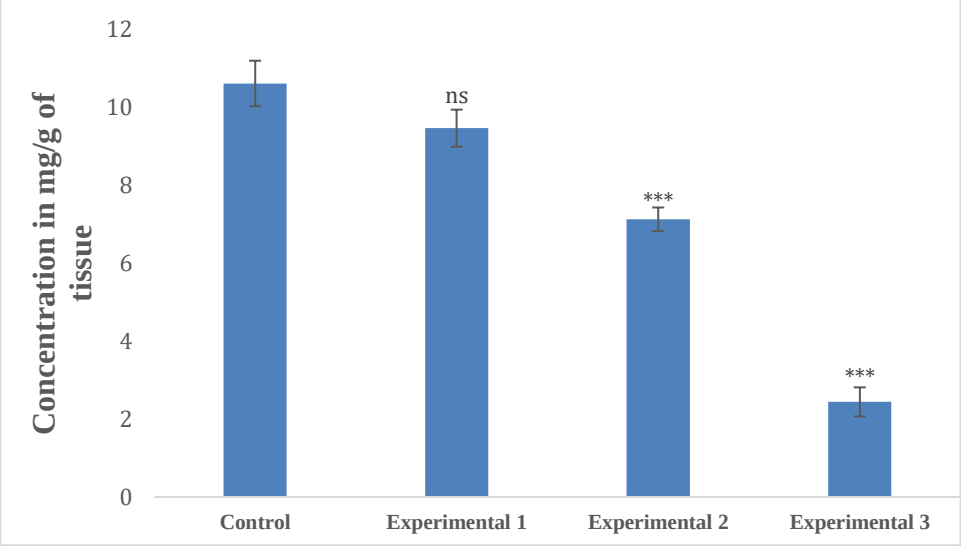

Figure No. 8: Effect of CBNPs on the Total carbohydrate content of bivalve Gill tissue. ns (non-significant), ***(P≤ 0.001, very highly significant).

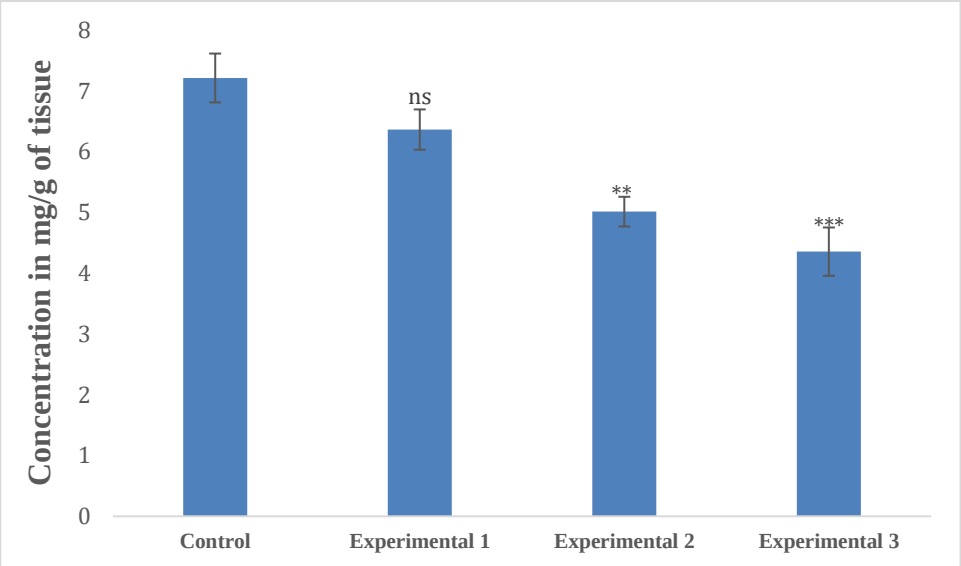

Figure No. 9: Effect of CBNPs on the Total carbohydrate content of bivalve Siphon tissue. ns (non-significant), **(P≤0.01, highly significant), ***(P≤ 0.001, very highly significant).

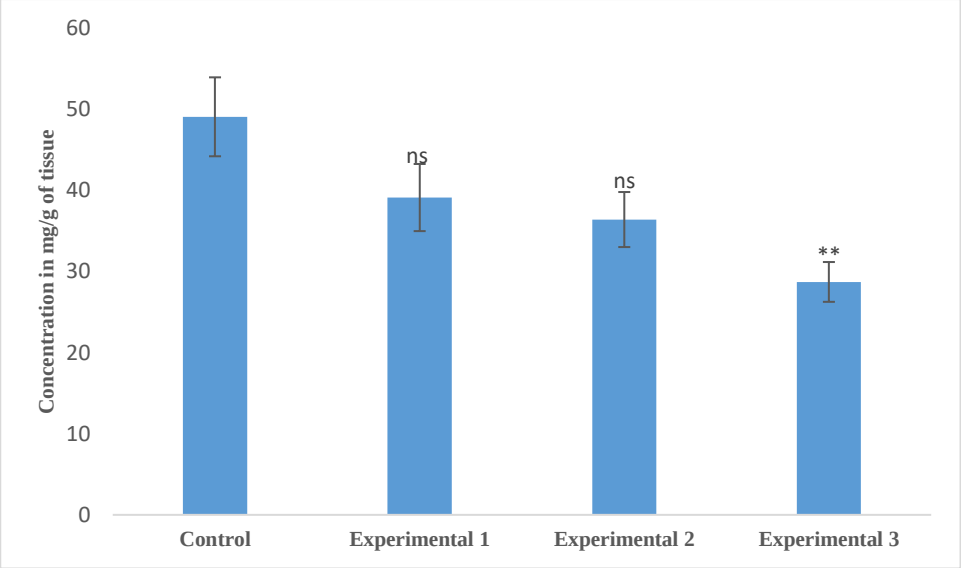

Figure No. 10: Effect of CBNPs on the Free sugar content of Bivalve Gill tissue

ns (non-significant), **(P≤ 0.01, highly significant).

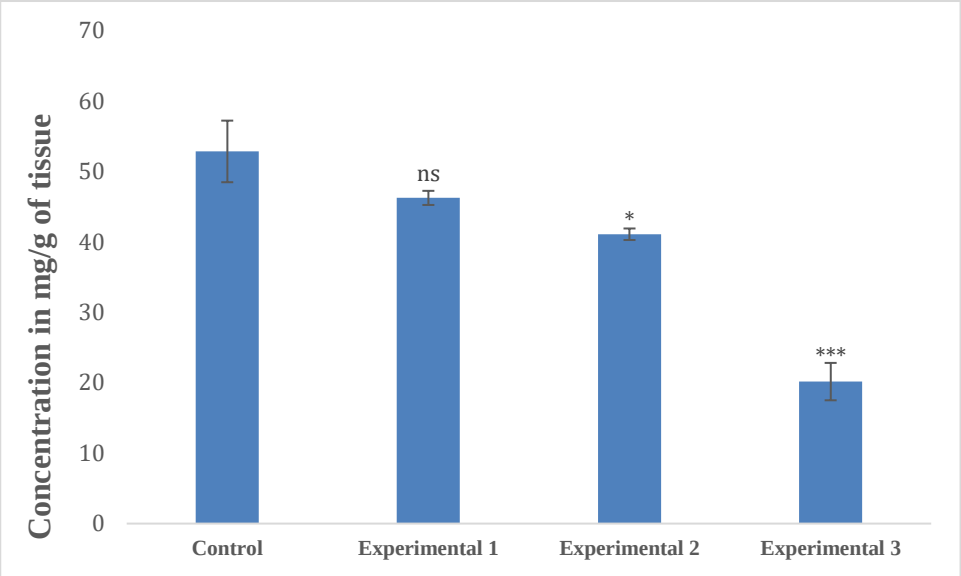

Figure No.11: Effect of CBNPs on the Free sugar content of bivalve Siphon tissue

ns (non-significant), ***(P≤ 0.001, very highly significant).

Further the free sugar concentration significantly and dose-dependently decreased in the CBNPs exposed bivalves in the experimental 2 and experimental 3 set ($F= 28.67$, $P<0.001$), whereas showed a non-significant decrease in the experimental 1 set compared to the control (Figure No.11).

The Reduced glutathione concentration significantly and dose-dependently decreased in the CBNPs exposed bivalves in the experimental 2 and experimental 3 set ($F= 9.710$, $P<0.001$), whereas showed non-significant decrease in experimental 2 set (Figure No. 12).

The Reduced glutathione concentration significantly and dose-dependently elevated in the CBNPs exposed bivalves in the experimental 2 and experimental 3 set ($F= 8.810$, $P<0.001$), whereas showed a non-significant decrease in the experimental 1 set (Figure No.13).

4.3. Effect of CBNPs on the Enzymes of gill and siphon

The activity of catalase enzyme was significantly and dose-dependently elevated in the CBNPs exposed bivalves in the experimental 3 set ($F=7.762$, $P< 0.01$), whereas showed non-significant decrease in experimental 1 and experimental 2 (Figure No. 14).

The activity of catalase enzyme showed very high significance dose-dependently in the CBNPs exposed bivalves in the experimental 3 set ($F=7.604$, $P\leq0.01$), whereas showed a non-significant decrease in experimental 1 and experimental 2 set (Figure No. 15).

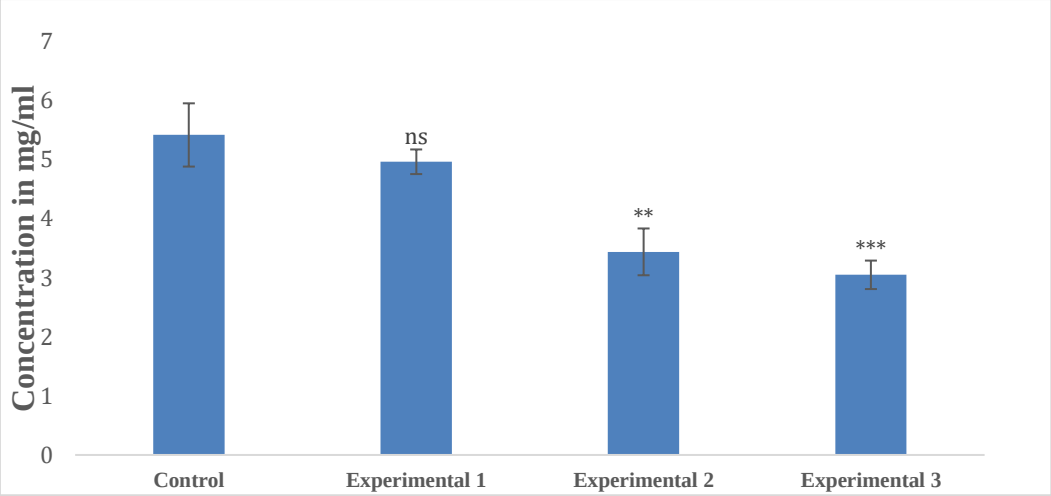

Figure No.12: Effect of CBNPs on the Reduced glutathione content of bivalve Gill ns (non-significant), **(P≤0.01, highly significant) ***(P≤ 0.001, very highly significant).

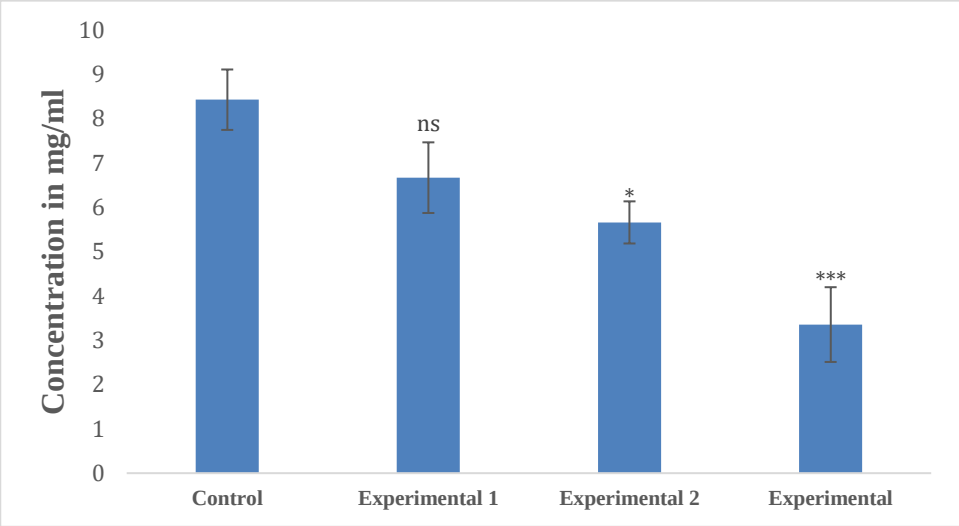

Figure No.13: Effect of CBNPs on the Reduced glutathione content of bivalve Siphon tissue ns (non-significant), *(P≤ 0.05, significant), *** (P≤ 0.001, very highly significant).

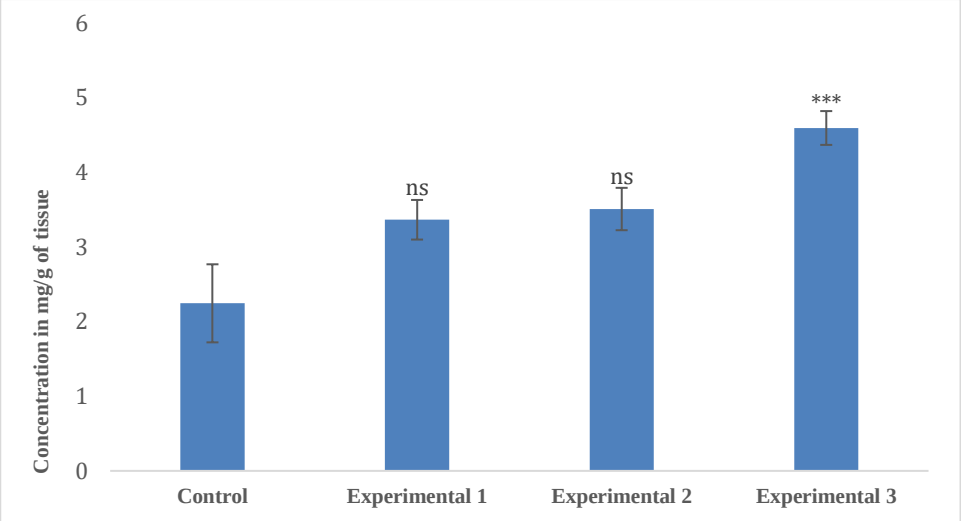

Figure: No. 14: Effect of CBNPs on the Catalase activity of bivalve Gill tissue ns (non-significant), ***(P≤ 0.001, very highly significant).

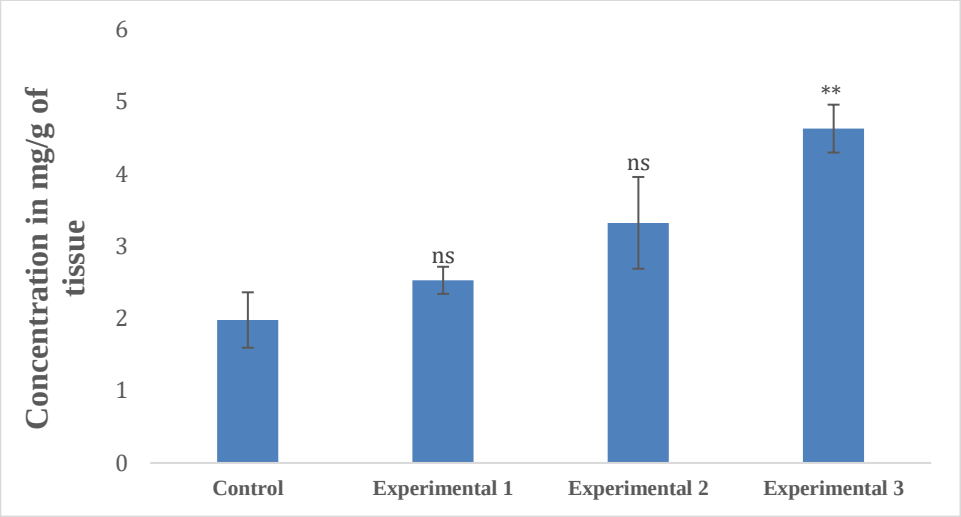

Figure No. 15: Effect of CBNPs on the Catalase activity of bivalve Siphon tissue ns (non-significant), **(P≤ 0.01, highly significant).

The concentration of Na^+/K^+ ATPases significantly and dose-dependently decreased in the CBNPs exposed bivalves in the experimental 3 set ($F=3.981$, $P<0.05$), whereas showed non-significant decrease in experimental 1 and experimental 2 set compared to the control (Figure No.16).

Further the concentration of Na^+/K^+ ATPases significantly and dose-dependently decreased in the CBNPs exposed bivalves in the experimental 3 set ($F=10.08$, $P\leq0.001$), whereas showed a non-significant decrease in experimental 1 and experimental 2 set (Figure No. 17).

The concentration of Mg^{++}- ATPases significantly and dose-dependently decreased in the CBNPs exposed bivalves in the experimental 3 set ($F= 4.735$, $P<0.05$), whereas showed a non-significant decrease in experimental 1 and experimental 3 set compared to control (Figure No. 18).

Further the concentration of Mg^{++}- ATPases significantly and dose-dependently decreased in the CBNPs exposed bivalves in the experimental 2 and experimental 3 set ($F= 9.804$, $P\leq0.01$), whereas showed a non-significant decrease in the experimental 1 set compared to control (Figure No. 19).

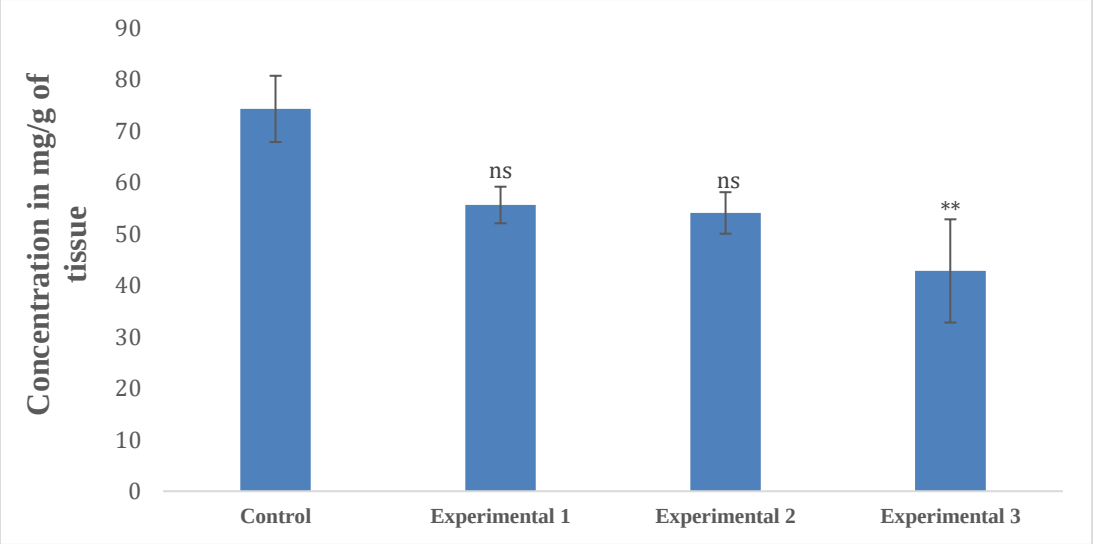

Figure No. 16: Effect of CBNPs on the concentration of Na^+/K^+ ATPases of bivalve Gill tissue ns (non-significant), **(P≤0.01, highly significant).

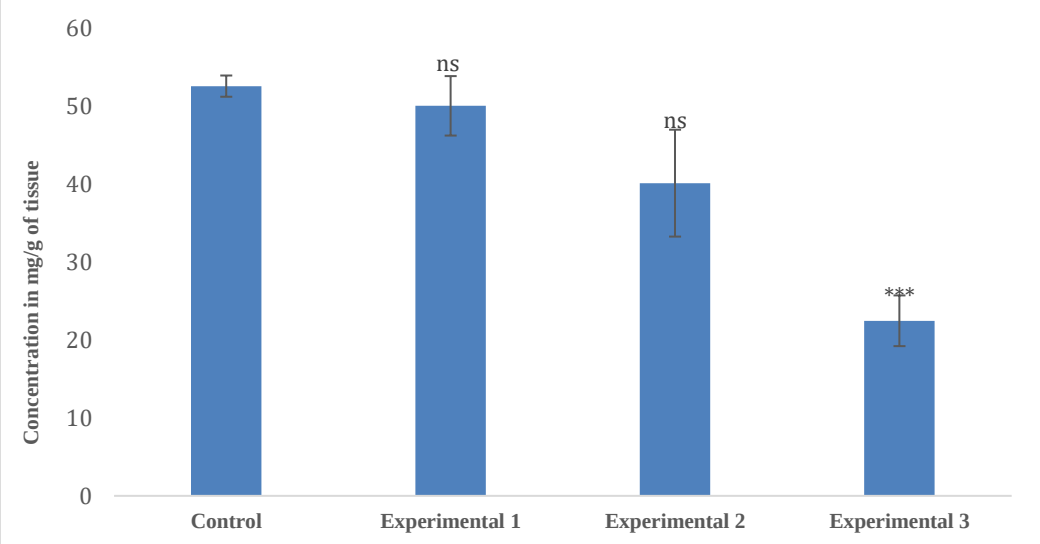

Figure No.17: Effect of CBNPs on the concentration Na^+/K^+ ATPases of bivalve Siphon tissue ns (non-significant), ***(P≤ 0.001, very highly significant).

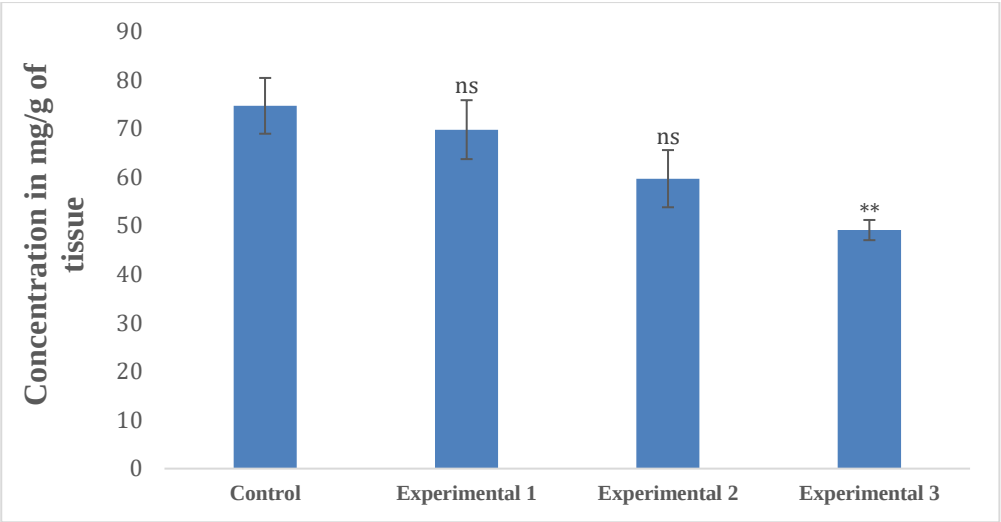

Figure No. 18: Effect of CBNPs on the concentration of Mg^{++}- ATPases of bivalve Gill tissue ns (non-significant), **(P≤ 0.01, highly significant).

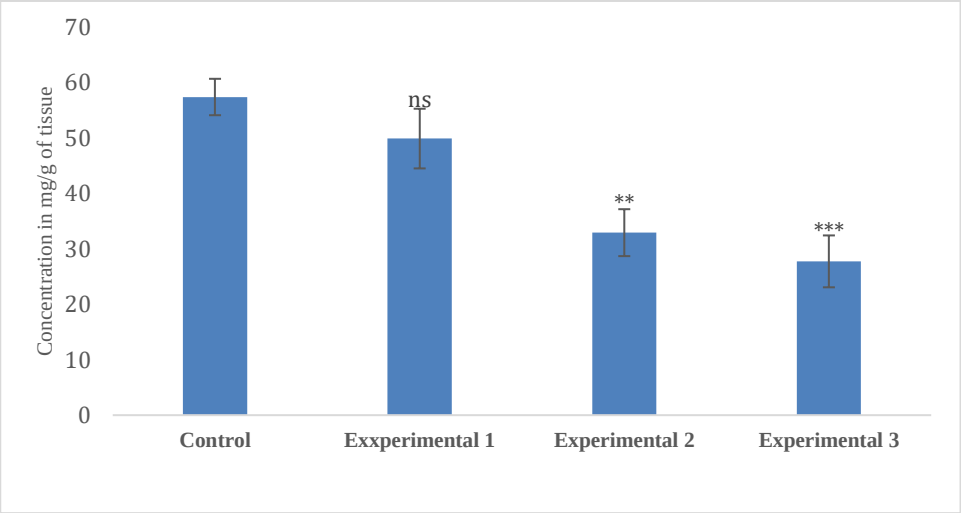

Figure No. 19: Effect of CBNPs on the concentration of Mg^{++}- ATPases of bivalve Siphon tissue ns (non-significant), **(P≤ 0.01, highly significant), ***(P≤0.001, very highly significant).

CHAPTER 5:

DISCUSSION

5. Discussion

The LC_{50} value determination is of utmost significance because it offers key information for the creation of a more complicated disposal models. The results are very helping in determining a pollutant's safe amount or tolerance level (Sherkhane., et al., 2013). Edgington et al. (2010), found that Natural organic matter (NOM) increases the stability of the suspensions of MWNT. Using chronic bioassays with *Ceriodaphnia dubia* and *Daphnia magna* they evaluated the acute and chronic bioassays and found that 96Hr LC_{50} for *D. magna* exposed to MWNT suspended in Suwannee River (USA) NOM is approximately 2.0mg/L. Hull et al., (2009), determined the (48Hr) LC50 by exposing the *P. promelas* and *C. dubia* to metallofullerene waste solids leachates and found that 54 and 5% of metallofullerene causes the mortality. In another study, Lukhele et al., (2015) exposed Doubled- walled Carbon nanotube (DWCNY) to *Pseudokichneriella subcapitata*, *Daphnia pulex*, and *Poecilia reticulata* and found the acute toxicity for *D. pulex* which was 2.81mg/L and 4.45mg/L, for pristine and oxidised DWCNT respectively. For *P. reticula* the LC_{50} values were 113.64 mg/L and 214.0 mg/L pristine and DWCNT respectively. The *P. subcapitata* showed the mortality of EC_{50} of 17.95mg/L and 10.93 mg/L for pristine and oxidised DWCNT respectively.

In the present study the LC_{50} for CBNPs was found to be 64.77/L. From the above studies it seems that in comparison to pristine and DWCNT the CBNPs is more toxic to aquatic organisms in comparison to other Carbon nanoparticles. But when compared with MWNT and metallofullerene, CBNPs are less toxic to

aquatic organisms. As per the literature review the toxicity of nanoparticles depends on its size and surface area. Also, it seems that the lower concentrations of CBNPs does not results in toxicity. The lower concentrations of CBNPs might not lead to mortality but higher concentration of CBNPs is responsible for the cause of mortality in the aquatic organism. The present study forms a baseline study for studying the chronic toxicity of CBNPs in relation to other forms of carbon nanoparticles.

The CBNPs have the ability to induce the toxic effects in the organisms because of its smaller particle size and higher surface area which helps it to easily form the aggregates. The results of the present study investigated the toxicity of CBNPs on different parameters associated with anatomy and physiology. The following results from the experiment shows that CBNPs can cause pathogenicity. Thus, the experiment provides new insight into the relation between CBNPs and their toxicity on *Paphia malbarica*.

Histological examinations serve as an identifier of the cytoarchitecture of any tissue or organ and also help in determining the changes caused by any toxicant. In the present study different cytoarchitectural changes were observed in the Gill and Siphon of bivalves exposed to CBNPs. The observations from histopathology of the gill tissue demonstrated structural changes in the form of narrower or obstructed hemolymph vessels in gill lamellae, clubbed basophilic cells, necrotic lamellae, hyperplasia, fusion of lamellae, clavate-globate (clubbing) lamellae and disorganized mass of disrupted gill lamellae. Similar histopathological findings were demonstrated in bivalve gills exposed to organophosphorus insecticide, chlorpyrifos and mercury exposure thus justifying our present results where

damage in the gill tissue is a consequence of CBNPs toxicity (Arumugam et al., 2011; Pandey et al., 2016).

According to Lignot et al. (1997), deadly levels of fernitrothion, cause histological alterations in the epipodites and gill lamellae, our study also showed morphological alterations in the gills of CBNPs exposed *P. malbarica* revealing alterations in the lesions with increase in levels of CBNPs. Higher doses of CBNPs resulted in massive destruction in normal architecture of gill tissue as compared to the lower doses.

The observations from histopathology of the siphon tissue demonstrated structural changes in the form of distorted outer epithelium (DOE), separation of AB-positive cells from glandular layer, necrosis of AB-positive cells, fusion of glandular layer with outer epithelium, and vacuole bubble. From the histopathological changes observed in the section of siphon tissue, it seems that the level of pathogenicity caused is concentration dependent and at highest concentration the physiological role might be affected. According to Costa et al. (2012), the AB-positive cells present in the glandular epithelium of the siphon tissue help in the secretion of mucous. Due to the toxicity of CBNPs in the exposed siphon tissue, the secretion of mucous by AB-positive cells might be inhibited which will going to cause the destruction of the tissue. The essential physiological roles of bivalves such as filtration and excretion might be affected due to the pathogenicity caused by CBNPs.

The amount of oxygen consumption was also found to decrease significantly with increase in CBNPs concentration. The amount of oxygen consumed by aquatic creatures has been determined in proportion to their physiological activities. In

the present study the decrease in the oxygen consumption may be due to, respiratory epithelium damage, suppression of ATPase function, and respiratory enzymes (Suresh et al. 1995; Vijayavel and Balasubramanian, 2006). Additionally, the bivalves may have consumed less oxygen due to a valve closure mechanism to prevent metal stress, severe epithelial surface destruction that may have prevented gaseous exchange, inhibition of enzyme systems and a compensatory lowering of respiration rate associated with gill perfusion in an effort to slow the rate of CBNPs distribution throughout the body (Doherty et al., 1987).

In crustaceans and bivalves, the principal end product of excretion is ammonia, which results from the catabolism of protein. In the present study the exposure of CBNPs to *P. malbarica*, significantly and dose dependently increased the excretion of ammonia from day 0 to day 28 may be due to higher catabolism of amino acids or other nitrogenous substances (Zhen et al., 2010). The freshwater crab *Trichodactylus borellians* treated to cypermethrin solutions was also found to excrete more ammonia in a study carried out by Williner and Collins (2003).

Pollutants cause a certain type of stress on organisms, and in response, those organisms develop the capacity to withstand that stress. An organism needs enough energy during stressful times, and this energy is provided by reserve elements including proteins, carbs and lipids. When organisms are under little or less stress, glycogen reserves are used as a source of energy; however, when under severe stress, proteins and lipids are used to provide energy. According to Palanichamy et al. (1986), when the principle and immediate energy sources are exhausted, the other sources also show a proportional depletion since their metabolism is connected to the Tricarboxylic cycle, a shared metabolic route. The

total protein content of the cell is considered as an important biomolecule for building and repairing. In the current study, due to the CBNPs stress in exposed *P. malbarica* there was a decrease in total protein content levels. It is widely acknowledged that the protein is a crucial component of animal cells and that it facilitates interactions between intracellular and extracellular media. The protein participates in delicately balanced subcellular processes since it is a component of an enzyme. As a result of CBNPs exposure, the bivalves tend to utilize protein at very high amount to overcome toxic effects of CBNPs as a result the Protein level may have decreased (Reddy et al., 1987; Raman Rao and Ramamurthy;1980).

At the transcriptional level, the contaminants are known to prevent the synthesis of m-RNA, which in turn prevents the production of proteins. According to reports, pollutants speed up the action of the enzyme protease, which causes the protein content to steadily decrease (Vijayaram et al., 1989). The decrease in the total protein content in *P. malbarica* exposed to CBNPs may be because of the increased proteolysis as observed by Rani and Balusubramanian (1999). Devi (1981), on the other hand, claimed that there may be a connection between the decrease in total protein and the way that chemicals affect nucleic acids. Further she claims that any chemical can impact the protein content of a tissue by preventing the synthesis of RNA, preventing the synthesis of proteins owing to anaerobiosis brought on by chemical toxicity, which depletes the ATP molecules, or speed up the pace at which proteins are degraded in cells which may also be a reason for depletion in protein levels in case of CBNPs exposed bivalve tissues.

According to recent research on invertebrates, the changes in the total carbohydrate brought on by pollution stress are similar to those seen in larger

vertebrates (Dange and Masurekan 1982). Therefore, the present investigation was aimed to investigate the impact of CBNPs on the free sugar and total carbohydrate in a saline water bivalve *Paphia* malbarica. According to Clarke (1975), among the organic nutrients, carbohydrates are thought to be the first to deteriorate when animals are under stress. Despite the fact that protein serves as the majority of an animal's energy supply, stress from exercise or acute hypoxia causes the body's reserves of carbohydrates to quickly deplete, with the hepatopancreas and muscles being the main sites (Health, 1987).

In the present study the total carbohydrate concentration and free sugar were found to decrease in gill and siphon tissue of bivalves exposed to CBNPs which might also be due to the stress created by CBNPs. Similar results were obtained in animals such as *Anguilla anguilla* exposed to pentachlorophenol, *Salmo gairdneri* exposed to endrin, *Salmo gairdeneri* exposed to zinc, *Sarotherodon mossambicus* exposed to malathion and mercury (Ramalingam, 1988; Holmberg et al., 1972; Grant and Mehrle, 1973; Watson and Mckeown, 1976). The decrease in the carbohydrate levels indicated that in the gill and siphon tissue carbohydrates was readily utilized to meet the energy requirements during the stress condition.

In the present study the activity of catalase increased gradually with increase in concentrations of CBNPs. According to multiple investigations, fish exposed to various toxicants that modulate the antioxidant defense systems experienced increase in the catalase activity (Jemec et al. 2010; Oost et al. 2003). An earlier work (Barbin et al. 2008) demonstrating the production of ROS by chlorhexidine aqueous solutions lends weight to this notion. In the present study the significant increase in the activity of catalase enzyme may be due to the production of

reactive oxygen species (ROS) in order to reduce the toxic stress caused by the CBNPs.

In bivalve molluscs, the Na+/K+-ATPases plays a critical function in controlling intracellular Na^+ content in response to varied environmental variables (Parisi et al., 2019). In the present study the gill and siphon ATPase activity of *P. malbarica* have undergone diverse changes as a result of chronic exposure to CBNPs. According to Balasundaram et al., (1995), inhibitions in ATPase activities may be the result of bivalves entering the energy economy while they are hypometabolic. The osmoregulatory and acid base regulatory systems in the gills, however, may be damaged by environmental pollutants, and these changes in ATPase activities may serve as early warning signs of this harm (Atli and Canli, 2011).

In the present study the Na+/K+-ATPase activity was significantly inhibited compared to control which may be due to the damage caused by CBNPs. An ionic pump called Na+/K+-ATPase is primarily responsible for maintaining the potential and osmotic equilibrium of cell membranes (Begum, 2011). Changes in the lipid content and structure of the membrane may result in a decrease in Na+/K+-ATPase activity because membrane fluidity permits membrane-bound proteins like Na+/K+-ATPase to function (Palecz et al., 2005). These findings also suggest that the CBNPs are able to damage the membranes of the gill and siphon tissue which were also evident through histological examination which may also be a reason for decline in Na+/K+-ATPases.

The effect of CBNPs also showed significant decrease in the Mg++ ATPases in both the tissues of bivalves exposed to CBNPs. The homeostasis of Mg2+, is

essential in many toxicological processes, and is provided by Mg2+- ATPase (David et al., 2014). The most significant component of energy metabolism is Mg2+ ATPase, and blockage of this enzyme causes oxidative phosphorylation to degrade (Dogan et al., 2015). The decrease in the Mg2+- ATPase in the present study may be due to the increase use of Mg2+- ATPase in the oxidative phosphorylation to generate more amount of ATP to overcome the depletion of energy demands caused by CBNPs stress.

In the present study the decrease in the ATPases activity may be due to the partitioning in the enzyme complex and or strong attraction of CBNPs for the -SH groups (Pham et al., 2017; Atli and Canli, 2011). According to Sunila and Lindstrom (1985), the gills of aquatic creatures are critical organs that are crucial in the transfer of gases, as well as the control of osmotic and ionic equilibrium. In the present study the overall decrease in the Na^{+}/K^{+}-ATPases and Mg^{2+}-ATPases may be due to disruption in the osmoregulatory function and oxygen intake of bivalve by as a consequence of CBNPs (Ghate and Mulherkar, 1979).

GSH level in the gill and siphon tissue dropped in the *P. malbarica exposed* to CBNPs. NPs accumulation exceeded exposure concentration by a factor of a thousand, and its accumulation in the cells to bind with the GSH and oxidation both contribute to lower GSH's decreased availability (Canesia et al., 1999). In the present study the decrease in the GSH level may be due to the direct interaction of nanoparticles with the enzyme (GST) or indirectly via the creation of ROS that directly interact with the enzyme leading to the depletion of its substrate (GSH) or increased GSH utilization in toxic conditions to counteract the prooxidants (Roling and Baldwin, 2006). By catalysing the addition of GSH to xenobiotic substrates, the biotransformation enzyme GST plays a crucial part

in the detoxification of xenobiotic substances (Elia et al., 2007). The present findings are similar with the references made and it seems that the endogenous antioxidant defense mechanism of *P. malbarica* may have been activated by CBNPs. This suggests that a detoxifying process versus pro-oxidation forces, mediated by this enzyme, was induced (Elia et al., 2007).

CHAPTER 6:

SUMMARY

6. SUMMARY

The rapid progress in the field of nanotechnology for its various applications in the different research fields have made this industry as a sunrise industry in the world. However, giving attention only to the benefits of these nanoparticles while ignoring its toxic effects will be detrimental to the organisms as well as to the environment. The CBNPs nanoparticles are being utilized in the field of electricity, rubber industries, cosmetics and automobiles but are also used in the field of medicine. Due to their wide application in the industries, the various byproducts generated by these nanoparticles enter the aquatic ecosystem because of the direct release in the water body thereby causing harm to its faunal diversity and changes the water parameters.

The follow up work was to understand the toxicity of CBNPs on *Paphia malbarica*. The bivalves were divided into 1 control group and 3 experimental group. In order to carry out the experiment, initially the LC_{50} was determined for a period of 28 days and based on the LC_{50} the bivalves were exposed to three different concentrations of CBNPs. The effects of CBNPs were evaluated on the different parameters of physiology and anatomy.

The histological alterations in the gill and siphon tissue from the normal tissues were found to be more with increasing CBNPs concentrations. The different metabolites studied such as total protein, total carbohydrate, free sugars and reduced glutathione were found altered with significant differences affecting the metabolic activity. The activity of the antioxidant enzyme (catalase) and non-antioxidant (reduced glutathione) play a significant role in overcoming the toxic

effects. Thus, the current study reveals that CBNPs act as toxicant to *Paphia malbarica*.

CONCLUSION

CBNPs have adverse effects on the physiology and anatomy of *Paphia malbarica*. The direct or indirect release of these CBNPs in the aquatic ecosystem can induce toxic effects in the organisms at genetic and cellular level. The root cause of CBNPs-induced toxicity is its smaller particle size, high surface area and ability to form the agglomerates in the cells.

FUTURE PROSPECTS

- Genotoxicity studies concerning CBNPs in bivalves
- Detoxification studies concerning CBNPs in bivalves
- Ameliorative effects of CBNPs toxicity in bivalves

CHAPTER 7: REFERENCES

7. REFERENCES

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