IMPACT OF COPPER TOXICITY IN WHITELEG SHRIMP, *LITOPENAEUS VANNAMEI* (BOONE,1931)

Thesis submitted to

BHARATHIDASAN UNIVERSITY

TIRUCHIRAPPALLI

for the award of the Degree of

DOCTOR OF PHILOSOPHY

IN

ZOOLOGY

By

A.SHANMUGANATHAN, M.Sc.,

(Ref. No. 07236/Ph.D.K9/Zoology /FT/ October 2017)

Under the guidance of

Dr. A.MAHARAJAN,M.Sc.,M.Phil.,Ph.D.,

POST GRADUATE & RESEARCH DEPARTMENT OF ZOOLOGY

KHADIR MOHIDEEN COLLEGE

(M.K.N. Madarasa Trust - Waqf)

(Nationally Re - Accredited with B Grade by NAAC)

ADIRAMPATTINAM – 614 701, TAMILNADU, INDIA.

APRIL – 2022

P.G AND RESEARCH DEPARTMENT OF ZOOLOGY
KHADIR MOHIDEEN COLLEGE
(M.K.N. Madarasa Trust - Waqf)
(Nationally Reaccredited with B Grade by NAAC)
ADIRAMPATTINAM – 614701
THANJAVUR DIST, TAMIL NADU.
E,mail: drathimaha@gmail.com Mobile: 9443286900

Dr. A. MAHARAJAN, M.Sc., M.Phil., Ph.D.,
Assistant Professor & Research Advisor

CERTIFICATE

This is to certify that this thesis entitled **"IMPACT OF COPPER TOXICITY IN WHITELEG SHRIMP, *LITOPENAEUS VANNAMEI* (BOONE,1931)"** submitted to Bharathidasan University, Tiruchirappalli by **Mr.A.SHANMUGANATHAN** in partial fulfillment of the requirements for the degree of **DOCTOR OF PHILOSOPHY** in **ZOOLOGY** is a record of research work done by him during the period of study under my guidance and supervision. I further certify that the thesis has not previously formed the basis for the award of any degree, diploma, associate ship, fellowship or other similar title.

Place: Adirampattinam Research Guide and Supervisor
Date:

P.G AND RESEARCH DEPARTMENT OF ZOOLOGY
KHADIR MOHIDEEN COLLEGE
(M.K.N. Madarasa Trust - Waqf)
(Nationally Reaccredited with B Grade by NAAC)
ADIRAMPATTINAM – 614701
THANJAVUR DIST, TAMIL NADU

A.Shanmuganathan, M.Sc.,
Research Scholar

DECLARATION

I do hereby declare that this work has been originally carried out by me under the supervision of **Dr.A.MAHARAJAN,** Assistant Professor & Research Advisor, P.G. and Research Department of Zoology, Khadir Mohideen College, Adirampattinam and this work has not been submitted elsewhere for any other degree.

Station **:** Adirampattinam **(A.Shanmuganathan)**

Date **:** 16/04/2022

ACKNOWLEDGEMENT

I express my sincere gratitude to **Dr.A.Maharajan,** Assistant Professor & Research Advisor, P.G. and Research Department of Zoology, Khadir Mohideen College, Adirampattinam, whose guidance surpasses any word of command. The meticulous thinking, unceasing interest, critical analysis, scientific judgment and able guidance at every stage has made this research seek its due results. I feel indebted to him for his excellent guidance.

I have great pleasure in expressing my sincere thanks to **Haji.S.Mohamed Meerasahib**, **Secretary** and **Dr.A.Mohamed Nazer**, **Principal,** Khadir Mohideen College, Adirampattinam for the facilities provides to carry out this work.

I am extremely thankful to my Doctoral committee members **Dr.J.Sugumaran,** Assistant Professor, P.G. & Research Department of Zoology, Khadir Mohideen College, Adirampattinam and **Dr.M.Sukumaran,** Assistant Professor, P.G. & Research Department of Zoology, Rajah Serfoji Govt.College, Thanjavur for their steady support throughout the course of my work.

I am immensely grateful to **Dr,V.Ganapiriya** Assistant Professor of Zoology, Khadir Mohideen College, Adirampattinam, for her valuable suggestions in my research work.

I wish to express my gratitude to**, Dr. A. Amsath,** Associate Professor & Head, **Dr. K. Muthukumaravel, Dr. O. Sathick, Dr.N.Vasanthi, Mr. S. Malkar Oli and Dr.P.Maharajothi** Assistant Professors of Zoology, Khadir Mohideen College, Adirampattinam, for their valuable suggestions and encouragement.

I would like to express my gratitude to **Dr.S.Kandan, Director, Rajiv Gandhi Centre for Aquaculture,** Sirkali for permitting me to make use of the facilities at the Institute to carry out the Light Microscopic studies.

I thank **Lab Assistants** for our department for their moral support and encouragement during the course of this investigations.

I express my deep sense of gratitude to my **family members** for their boundless help in all possible ways and moral support at every stage provided me with inspiration during the course of research work.

A. SHANMUGANATHAN

CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

P.G AND RESEARCH DEPARTMENT OF ZOOLOGY
KHADIR MOHIDEEN COLLEGE
(M.K.N. Madarasa Trust - Waqf)
(Nationally Reaccredited with B Grade by NAAC)
ADIRAMPATTINAM – 614701
THANJAVUR DIST, TAMIL NADU.

PLAGIARISM FREE CERTIFICATE

Name of the Research Scholar	**A.SHANMUGANATHAN**
Course of study	**Ph.D**
Title of the thesis	**IMPACT OF COPPER TOXICITY IN WHITELEG SHRIMP, *LITOPENAEUS VANNAMEI* (BOONE,1931)**
Name of the Supervisor	**Dr.A.MAHARAJAN**
Department	PG & RESEARCH DEPARTMENT OF ZOOLOGY
Acceptable Maximum Limit	10%
% of Similarity content Identified	3%
Software used	URKUND
Date of Verification	09/04/2022

Report of the plagiarism check, items with % of similarity is attached

Signature of the Supervisor

1. INTRODUCTION

Aquaculture is the farming of fish, crustaceans, mollusks and aquatic plants in aquatic environment; sometimes it is referred to as aquatic agriculture, as an aquatic counterpart of terrestrial agriculture. The term farming implies the type of intervention in rearing process, such as regular stocking, feeding or protection from the predators. Aquaculture is the fastest growing food producing industry with a total global aquaculture production of 73.8 million tonnes. So far, a total of 582 species are farmed worldwide and of these 62 are crustaceans. It was estimated that the total global farmed crustacean was 6.9 million tonnes valued for 37 billion USD. Although many crustaceans attract lucrative markets, shrimp has become the single most successful crops, and mainstay of the brackish water coastal aquaculture in India and many Asian countries.

Globally, Aquaculture is one of the most fastest growing sectors of primary production in aquatic food for human consumption with rich protein in developing countries (Balarin, 1988; Pullin, 1993). The availability of the seafood is very limited. Therefore, the enhanced production of the aquatic food is an alternative method of aquaculture. Therefore, Shrimp aquaculture is one of the fastest growing economic activities in coastal areas of the Asia-Pacific region (FAO, 2016).

Aquaculture of shrimp is considered to be a success story of modern aquaculture. Shrimps had been raised as an incidental crops in coastal ponds or coastal low lying ecosystems including India. The advent of sophisticated refrigeration facilities aided traditional farmers to access international markets, thereby traditional coastal aquaculture shifted to an export oriented or industrialized aquaculture. During industrialization, the quality of the material exported or imported anywhere remains as major concern. Since, aquatic aquaculture is largely depending on numerous environmental factors, continuous studies and documentation of all possible factors associated with improving the quality of the material is in need. At this juncture, the influence of heavy metal pollution in the alteration of the quality of the reared subjects are major concern.

1.1 Indian aquaculture

The fact that the fisheries sector employs more than five million people in India, contributes to food and nutritional security and employment, supports livelihoods and improves the socioeconomic standing of underprivileged fishing communities demonstrates the relevance of the sector. Indian fish production has increased dramatically over the last half-century, from a modest 0.75 million tonnes in 1950 to 6.3 million tonnes in 2002. (Anon, 2000). The industry is worth roughly INR 200 trillion to the Indian economy, accounting for 1.4 percent of national GDP and 5.4 percent of agricultural GDP (Anon, 2000). The

industry is a significant contributor to overseas exports. Inland fisheries in India, which comprise both capture and culture fisheries, have experienced remarkable expansion and transformation during the last two decades. Capture fisheries were the primary source of inland fish output until the mid-1980s. However, fish output in natural waterways such as rivers and lakes has declined since then, owing to a proliferation of water management devices, indiscriminate fishing, and habitat destruction (Katiha, 2000). Due to diminishing resources, the energy crisis, and the high cost of fishing as a result, aquaculture's potential and versatility as a viable and cost-effective alternative to capture fisheries has become more widely recognised (Ayyappan, 2004; Jana and Jena, 2004; Pillai and Katiha, 2004). The current research examines features of India's inland aquacultural resources, production practises and trends, and future possibilities, keeping in mind the importance of inland aquaculture in India. Fresh and brackish waters are included in this sector. Based on the findings, recommendations are provided for how India's inland aquaculture sector might be further developed.

The northeast region of India, comprised of the states of Arunachal Pradesh, Assam, Manipur, Meghalaya, Mizoram, Nagaland, Sikkim, and Tripura, is blessed with rich biodiversity and fisheries resources. With more than 90% of population being fish eaters, there is heavy demand for

fish but a wide gap exists between supply and demand. The region produces over 0.214 million tons of fish annually, with almost 50% coming from aquaculture. Aquaculture development in the region is taking place at a rapid rate. However, efforts are necessary to increase the present level of production through both horizontal and vertical expansion. The region has rivers, cold water streams, floodplain wet lands, reservoirs, lakes, ponds, paddy fields, and mini-barrages to support large-scale aquaculture activities, which can not only produce fish to meet regional requirements, but also export the surplus.

1.2 Shrimp aquaculture

Several primary forces have driven the rapid expansion of shrimp aquaculture. They include potentially high profits, buoyant demand for high-value seafood products, increasing demand for farmed shrimp due to limitations and fluctuations in supplies from capture fisheries, and the industry's capacity to generate foreign exchange and employment in poor coastal areas of tropical and subtropical developing countries. Efforts are being incorporated into some projects to provide access to the benefits of shrimp farming to poor coastal communities, thereby reducing poverty and preventing the communities' interests being overtaken by external parties. Shrimp farming has become a major aquaculture activity and attractor of investment over the past two to three decades. Currently,

shrimp farming accounts for some 30% of total world shrimp production, and this share is growing. In the face of stagnating or declining catches from the wild, shrimp farming is expected to play an even more important role in the future.

There is a pressing need for the development and dissemination of a range of shrimp culture systems that are both environmentally and economically sustainable (Funge-smith and Briggs, 1998). Currently, nearly 70% of the shrimp consumed globally is farmed. India ranks as one of the largest producers of the black tiger shrimp, *Penaeus monodon* in the world. It contributed 21 and 44% by volume and value, respectively to Indian seafood exports in 2008-2009 (MPEDA - 2010, Newsletter). Shrimp farming plays an important but controversial role in the economic development of many countries in Asia because of the high economic returns and often catastrophic environmental impact of production in coastal areas (Senarath and Visvanathan, 2001; Huitric *et al.,* 2002; Hall, 2003; Islam, 2008). The shrimp aquaculture sector in India has witnessed several changes in the last two decades. Shrimp industry in the east coast of India was seriously affected by white spot virus disease since 1993, as in most countries in the Asia-Pacific region (Kongkeo and Davy, 2010). Farmed shrimp has become a significant factor in world shrimp markets over the past five to six years. Worldwide farmed shrimp production has risen significantly since 1985, from

213,000 metric tons (MT) to 931,788 MT in 1995, although it has since declined slightly. The market for shrimp has grown in most parts of the world, and demand is likely to remain high, assuming that major markets continue to have overall economic growth. However, the current downturn in the southeast Asian and Japanese economies has affected price, and prices may settle at a level somewhat lower than in recent years, at least in the short to medium term. Nonetheless, shrimp remains a high-value product with a very large international market. It is expected that any future growth in the market and shortfall from capture fisheries will be covered by increased aquaculture production. Crop failures due to disease outbreaks have occurred in several shrimp farming countries, and, along with other macroeconomic factors, have introduced an element of uncertainty in the market, with resulting price fluctuations. These fluctuations may be detrimental to the sustainability of shrimp farming.

Over the last three decades, shrimp farming has been one of the most rapidly growing aquaculture sectors throughout the world. It is documented that Shrimp as a largest single seafood commodity by value, accounting for 15% of all internationally traded among fishery products. Shrimp is one of the most traded seafood commodities, and aquaculture of shrimp is considered to be one of the success stories of modern aquaculture. The shrimp aquaculture activities generated millions of employments, and provides foreign exchange for various countries which rely hugely on

coastal economy. Farm raised shrimp is comprised of 55 % of global shrimp production and this is entirely dominated by two species - the black tiger shrimp (*Penaeus monodon*) and the white Pacific white shrimp, *Penaeus vannamei* (FAO, 2014).

1.3 Toxicological study in heavy metals

Toxicological studies are widely applied in the field of research, environmental monitoring, ecosystem management, and clinical medicine for determining the health status of an organism in the niche. Among various pollutants, the presence of heavy metals in the environment remains as major problem across the globe. Increased urbanization, expansion of industrial activities and exploitation of natural resources, the excess occurrence of heavy metals in natural loads has become a serious problem throughout the world (FAO,1992). The long term impact of heavy metals pollution in the environment is the relatively slower rate of elimination and no way of destruction from the environment (Mudal *et al*., 2010). Available evidences indicated that factors such as fly ash from power stations, burning of fossil fuels, smelting, refining of metals and leaded petrol from vehicles played a critical role in accumulation of metal pollution in the environment (La Colla *et al.*, 2019). The combination of metals deposition in the sediments and bioaccumulation by the living organisms residing in the niche greatly enhances the problems of heavy

metal pollution at large scale (Rajkowska and Protasowicki, 2013; Saha *et al.*, 2016). The occurrence and distribution pattern of metals in various ecosystems is often related with factors such as natural and anthropogenic activity (Krishnapriya and Ramesh, 2018).

1.4 Effect of copper in aquatic organisms

Copper, an essential trace metal, is required for a variety of physiological and biochemical processes in many organisms and usually exists in natural waters and sediments (Lorentzen *et al.,* 1998; Shao *et al.,* 2010). The natural role of copper as a cofactor for several enzymes is well documented over the years (Gaetke and Chow, 2003; Trevisan *et al*., 2010, Lorentzen *et al*., 1998; Meng *et al*., 2016). Copper, an essential trace element for plants and animals; it is a component of many metalloenzymes and respiratory pigments (Demayo and Taylor, 1981). The role of copper in various physiological and chemical processes in almost all organisms including plants and original is well documented. It is required in the synthesis of chlorophyll (a photosynthetic pigment in plants) and haemoglobin (a respiratory blood pigment). Copper also serves as the oxygen coupling site in haemocyanin, the respiratory blood pigment in many molluscs and certain other invertebrates (Birge *et al*.,1979).

The natural occurrence of copper in rocks, soil, water, sediments and even in air (approximately 50ppm in entire earth crust) is well

documented. Copper is a minor mineral component in natural waters that usually occurs at concentrations of 0.002-0.004 mg/l in sea water and 0.005-0.020 mg/l in fresh water. Accumulated evidences suggested that among the man made activities, sources derived from agriculture product such as fungicide, algaecide and nutritional supplements play a crucial role in accumulation of copper salts (Copper hydroxide, Copper carbonate and Copper sulphate) in the environment. Further, wide usage of copper and its derivatives in veterinary practices and industrial applications. The excessive amount of copper accumulation in the environment especially in water bodies and causes toxicity to aquatic fauna and flora and finally to human beings. The higher concentration of copper may affect the survival, growth, reproduction and other biological and physiological functions. It also affects the macro-molecules such as lipids, nucleic acids, carbohydrates, and proteins.

Due to its fast growth at high densities and ability to survive in a wide range of environments, *L. vannamei* is now the species farmed in the greatest quantities, accounting for 80% of total marine penaeid shrimp production (Bardera *et al.*, 2019). As a result of long term usage of copper by humans in various industrial aspects, accumulation of copper in the environment poses as serious threat to the shrimp in the natural habitat and also in reared ponds. Hence, the present study was performed to document the accumulation of copper and its impact on the shrimp.

2.OBJECTIVES OF THE STUDY

The present study aimed to elucidating the aquatic toxicity of copper in the whiteleg shrimp, *Litopenaeus vannamei*. Copper sulphate is a disinfecting agent used in most of the shrimp farms. It is effective against a wide range of organisms including blue-green algae, bacteria, fungi, protozoans, digeneans, leeches and monogeneans. Overdosing the chemical can easily kill the animals being treated, and thus caution is called for in its use. In the context of the local industry, copper sulphate is used at a rate of 0.5 - 1 ppm in marine shrimp ponds.

The present studies intended to evaluate the lethal and sublethal effect of copper in the white leg shrimp, *L. vannamei,* The plan of work included:

- Finding out the LC50 value of copper in *L.vannamei*
- Investigation of the effect of two sublethal concentrations of copper (10 and 20% of LC_{50} value for 96hrs) on the morphological colour changes including bioaccumulation of the metal in various tissues such as gills, muscle, hepatopancreas and uropod in *L.vannamei*
- Haematological parameters of haemolymph in *L.vannamei* exposed to sublethal concentrations of copper.

- Changes in proximate composition, fatty acids, vitamins, minerals, amino acids, in muscle of *L. vannamei* due to copper toxicity.
- Impact of copper on the histological changes in various tissues such as muscle, gills, hepatopancreas, lymphoid organs and nerve cord of *L. vannamei.*

Furthermore, it is aimed / hoped that the study will provide an useful blueprint for avoiding copper toxicity in *L.vannamei* in aquaculture farming system.

3. REVIEW OF LITERATURE

Ever since Bronze Age, Copper (Cu) is widely used by humans across the globe for several thousand years. The higher concentration of copper may affect the survival, growth, reproduction and other biological and physiological functions. It also affects the macro-molecules such as lipids, nucleic acids, carbohydrates, and proteins. The impact of pollution in the marine environment can be assessed by monitoring the changes in the exterior surfaces, in blood and histopathological examination of the tissues or internal parts of the organism (Lasheen *et al.,* 2012; Poopal *et al.,* 2017; Mierzejewski *et al.,*2014; Saleh, and Marie,2016). Hence, combined usage of aforementioned parameters as a biomarker may aid us to understand the impact of pollutants on living organisms thereby necessary counter measures may be developed to reduce the negative impacts of pollutants in the living beings and in the ecosystem (Cazenave *et al.*, 2009; Saleh, and Marie,2016).

3.1. Aquaculture practices in India

In India, shrimp aquaculture started as a traditional practice in natural water bodies such as bheries or pokkali fields and subsequently transformed to commercial industry during late 20^{th} century. The first recorded data for farmed shrimp production in India were 20 mt in 1970 and first major

change became obvious in 1991 when it reached 40000 MT. By 2016, it is documented that Indian shrimp farming sector showed a remarkable growth, from 50000 mt in 1990 to 600000 mt. Although growth of shrimp aquaculture is remarkable, the sector has been facing several issues such as infections, marketing, bio-accumulation of pollutants, and poor farm level performances

Rapid growth of shrimp aquaculture induced an increase in area of shrimp farming and production across the globe. This growth occurred in spite of the set-back caused by white spot syndrome virus (WSSV) in the late 1994. The disease impacted aquaculture industry severely, and it caused the exit of almost all corporate investors by 1997. A recovery and moderate growth happened in the post WSSV era, from 2000 to 2006, where shrimp farming gradually increased and peaked with a maximum production of about 1.4 lakh tonnes in 2006, but production reduced drastically in 2008.

Using locally available species such as *Penaeus monodon* and *Fenneropenaeus indicus* growing in brackish water culture ponds, Indian shrimp has emerged as a prominent culture industry in the international landscape. BMR industries Pvt. Ltd introduced foreign species like *L.vannamei* stock to India with authorization from the Indian government in 2002. In 2009, the species *L.vannamei* was approved for commercial cultivation in India, despite the dangers of introducing foreign species

and the potential for the spread of various endemic illnesses in *L.vannamei* culture operations. The main causes of disease outbreaks such as virus, bacteria, fungal, and other diseases might be attributed to pond management techniques, specifically the direct pumping of water from creeks and canals without any pathogen screening and elimination procedures. After the introduction of *L.vannamei*, the country has witnessed a remarkable upsurge of farmed shrimp production with production of 5,00,000 MT of farmed shrimp in 2015.

3.2. Background information on Copper Toxicity

In aquatic invertebrates, heavy metals such as copper, zinc, Mercury and cadmium change both morphological and physiological parameters such as growth, swimming movement, food intake, rate of breathing, productivity, survival and life cycles (La Breche *et al*., 2002). For copper, the main process leading to the mobilization into the ecosystem is extraction from man-made activities including mining, milling and smelting, and also from agriculture industry and waste disposal (Wright and Welbourn, 2001).

In aquatic ecosystems, copper generally enters from agriculture runoff, though the deliberate use of copper sulphate to control the growth of microscopic algae and also from direct discharges from industrial processes. Copper is widely used in antifouling paint, treatment for fish diseases and as an algaecide (Gutierrez *et al*., 2010) and is an essential

element in metabolic processes for both plants and animals, but high concentrations can be toxic to aquatic species such as fish, algae and crustaceans (Sullivan *et al*., 1983). In a small amounts copper is not toxic and is required by a living organism for its biological function including the metabolic process. Copper can be defined as a toxic to an organism only if the doses of copper concentration was higher than the volume of physiological detoxification processes.

Copper is the most toxic metal to marine organisms in general (Bryan,1976). The pathways of entry of copper into the body of crustaceans, the organs or organelles where accumulation takes place and the processes by which the metal gets sequestered and is possibly eliminated from the body were the subject of much investigation over the past 4 decades (Kerkut and Munday, 1962; Gibson, and Barker, 1979; Arumugam, and Ravindranath, 1983; White and Rainbow, 1986; Manisseri, and Menon, 1995). The shrimp, *Crangon crangon* (Portmann and Wilson,1971), the lobster, *Homarus americanus* (Mcleese,1974), the ghost shrimp, *Callanassa australiensis* (Ahsanullah *et al.*, 1981), the penaeid shrimp, *Penaeus merguiensis* (Denton, and Burdon-Jones, 1982), the shrimps, *Paratya australiensis* (Daly *et al.*, 1990) and *Penaeus japonicus* (Yuliantio Bambang *et al.*, 1995) are reported to be highly sensitive to copper.

3.3 Acute Toxicity

The Environmental Protection Agency of the United States has produced a priority list of contaminants that includes eight heavy metals: arsenic, cadmium, chromium, copper, lead, mercury, nickel, and zinc (Moore, and Ramamoorthy, 1984). When Minamata sickness struck Japan in 1953 as a result of eating contaminated fin fish and shellfish, heavy metal contamination drew a lot of attention (Nammalwar,1985).

Heavy metals are divided into two types: essential and non-essential. Eleven heavy metals have been identified as being necessary for the survival of living creatures (Schroeder *et al*., 1970). Essential metals are always found in complexes with organic molecules, most notably proteins, which are either tightly bound in metalloproteins or loosely bound in metal protein complexes (Vallee, and Wacker, 1970).

The sensitivity of aquatic invertebrates to copper varies. For example, crustaceans have an LC_{50} at 48h ranging from 5 to 86 ug/L but for annelids (Tubifex *sp*.), the 48h LC_{50} ranges from 10 to 890 ug/L/ (Hodson *et al*., 1979). This variation in sensitivity depends in part on the surface area and respiration rates of the animals, which can influence the copper uptake.

Bioassay is an important test for determining the effects of pollutants on both aquatic and terrestrial species. The acute toxicity test has been the 'workhouse' in monitoring pollution impacts since the 1950s (Buikema *et*

al., 1982). The toxicological dangers determined by bioassay procedures, according to Genjatulin, (1990), are more realistic than those anticipated by chemical analysis. According to Waldichuk (1978), a conventional bioassay for acute toxicity is usually performed for 48 or 96 hours. The LC_{50} (Lethal Concentration 50) values are determined in the acute toxicity bioassay.

3.4. Morphological Colour Changes

Color and visual appearance are important quality attributes of shrimp that can have a large impact on their market value. Consumers prefer cooked shrimp that have a bright orange color. In some countries, subjective color grading scores are used to provide market segmentation of high quality shrimp. In the shrimp, *L. vannamei*, Cu exposure affects the body color, survival, reproduction, structural damages in the gill and hepatopancreas, increased susceptibility to *V. alginolyticus* infection and depressed the immune ability, increased apoptosis. Nicoara *et al*., (1970) observed an elevated level of copper concentration among the crustaceans when compared to other groups, as crustaceans use copper in blood pigment for respiration. Higher levels of copper changed the colour of internal organs of crustaceans which is due to the oxidation of copper. Similar colour changes of gill and liver were also observed among fishes like the channel cat fish, rainbow trout and carps (George, *et al*.,1955).

The hepatopancreas of *Palaemon elegans* changed its colour from pale yellow to greenish blue when exposed to sublethal effect of copper (Gibson and Barker, 1979). Similarly, Kerkut and Munday, (1962) revealed that varied concentrations of copper had direct influence on the colour changes in *Carcinus maenas*. Nimmo *et al.*, (1977) first described pathological gill blackening in marine shrimps, *Penaeus duorarum, Palaemonetes pugio* and *Palaemonetes vulgaris* exposed to another heavy metal, cadmium.

3.5. Bioaccumulation

Heavy metals are found in various levels in all ground and surface waters and occur naturally in the environment (Martin and Coughtrey, 1982). Metal levels in many ecosystems have risen significantly as a result of anthropogenic activities such as agriculture, mining, and industry, notably in the last century (Guimaraes-Soares *et al*., 2006). Because of their toxicity, lengthy persistence, bioaccumulation, and biomagnification in the food chain, increased discharge of both necessary and non-essential metals into natural aquatic environments can expose aquatic animals to excessively high amounts of these metals, posing a major concern (Van Dyk *et al*., 2007).

Metalloids are serious pollutants as they are stable compounds, not readily removed by oxidation, precipitation or any other natural processes. Metal concentrations in the bodies of fish and shellfish are known to fluctuate based on species, ambient factors, and inhibitory processes. Because fish and shellfish are a common element of human diets, they can be an indirect source of metals entering the body, causing a variety of metabolic, physiological, behavioural, and ecological effects. The severity of such impacts is determined on the metals' inherent toxicity, their concentration and chemical form, as well as the species affected.

Concentrations as low as 5.3 to 31.9 mg/L in soft waters may be toxic to larval fish (depending on the pH and dissolved organic carbon and calcium concentrations). High concentrations of copper are toxic to aquatic organisms and may cause a significant decrease in populations of aquatic invertebrates, plants and fish. Waterborne copper exposure can exert a variety of physiological effects in fish, including the disruption of sensory system function, which has far-reaching implications on fish behavior. In developing fish larvae, copper is known to affect key parameters, such as survival, growth and more recently has been shown to interfere with the octavo lateral system (Johnson *et al.*, 2007). Copper intoxication increases mortality of fish offspring and weakens their condition, resistance and viability (Jezierska and Witeska, 2001). There

are several reports available on accumulation of copper in various tissues of different animals (Jaffer and Ashraf 1988; Harding and Goyette, 1989; Yong and Harvey, 1989). Cuadras *et al*., (1981) investigated the levels of copper and zinc in the tissues of the hermit crab, *Dardanus arrosor* from the Barcelona shore. Harris and Santos (2000) found heavy metal pollution and physiological heterogeneity in Brazilian mangrove crabs, *Ucides cardatus* and *Callinectes danae* .

In a study on accumulation of copper toxicity in *Penaeus orientalis*, Liu Fuyi *et al.,* (1988) reported maximum level accumulation of copper in the tissue of hepatopancreas. Similarly, in *Metapenaeus dobsoni*, the ultrastructural study revealed in hepatopancreas showed many number of electron dense granules when exposed to copper (Manisseri and Menon, 1995). Alliot and Frenet Piron (1990) established a link between metals in seawater and metal buildup in shrimps. Badsha and Goldspink (1982) presented studies on heavy metal concentrations in polluted environments, suggesting probable heavy metal concentration in different strata of aquatic ecosystems.

3.6 Haematology

High concentrations of copper can alter haematology (James *et al*., 1998), respiratory and cardiac physiology (Sorensen 1991), and may also lead to retarded growth and inhibition of spawning (Benoit, 1975).

Experiments evaluating the toxicity of copper in shrimps indicate that high concentrations of copper particularly affect osmoregulation, molting frequency and survival. In Decapoda crustaceans, there are generally three types of circulating haemocytes: hyaline cells, semigranular cells and large granular cells. They play an essential role in cellular immune function such ascoagulation, phagocytosis and encapsulation.The generation of superoxide anion by *L. vannamei* following 24 h exposure to20 mgl-1 Cu_2^+ was considered to be cytotoxic to the host.

Excess growth of blue-green algae releases geosmin in low saline ponds. Shrimp cultured in such waters will have unpleasant flavour. Farmers often apply excess amount of copper sulphate due to lack of information to eradicate the filamentous and bluegreen algae. Dose of copper sulphate application varies from 0.1 to 0.2 ppm and it depends mainly on the total alkalinity of pond water.

Copper based disinfectants are known to inhibit growth of plankton and induce shrimp moulting, may cause harmful impact if proper dosage is not used or residues are leftover. Environmental deterioration due to Cu accumulation in the pond sediments poses serious concern to shrimp health and growth. Further, copper may affect the incidence and nature of parasitism in many ways, for example, by altering host susceptibility through alteration of host defence mechanisms, by altering the availability of matching intermediate or final host populations, or by

reducing the performance or survival of infected hosts (Sures, 2004).

Shrimp feeds, with an estimated Cu content close to 50 μg kg−1, are an additional source of Cu for shrimp pond waters (Lacerda *et al*., 2006). This might explain the relatively higher levels of Cu in the tissues of wild-caught shrimp compared to those in cultured shrimp (Páez-Osuna and Tron-Mayen, 1996). These findings suggested that the persistent exposure to sublethal levels of copper might have affect some of the humoral and cellular (hemocytic) responses to stress.

The impact of pollution in the marine environment can be assessed by monitoring the changes in the exterior surfaces, in blood and histopathological examination of the tissues or internal parts of the organism (Lasheen *et al.,* 2012; Poopal *et al.,* 2017; Mierze jewski *et al.,* 2014; Saleh and Marie, 2016). Hence, combined usage of aforementioned parameters as a biomarker may aid us to understand the impact of pollutants on living organisms thereby necessary counter measures may be developed to reduce the negative impacts of pollutants in the living beings and in the ecosystem (Cazenave *et al*.,2009; Saleh and Marie, 2016).

3.7 Proximate composition

In general, blood samples obtained from the organism provides substantial information on possible major problems which develop in future due to the exposure to the toxicants thus functioning as a biomarker to diagnose the degree of toxicity (Zutshi *et al.,* 2010).

Likewise, assessment of biochemical parameters in the blood of aquatic organisms also aid to estimate the health of natural wild populations (Anderson *et al.,* 2010; Lewbart *et al.,* 2014; Poopal *et al.,* 2017). Further, these hematological assays can be used to study the water balance, nutritional status and the adverse effects of various xenobiotic present in our environment. Villalan *et al.,* (1988) observed that heavy metal altered protein, lipids and carbohydrate levels in the crab, *Thalamita crenata.* Impact of nickel and chromium on the protein content in the liver of the fresh water fish, *Cyprinus carp* during spawning and post spawning phases were observed by Virk and Kaur (1999). Depledge and Bjerregaard (1989) reviewed changes in haemolymph and tissue copper concentration in decapod crustaceans associated with starvation. Similarly, in starved benthic crustaceans maintained in hypoxic conditions, a depletion of glycogen reserves was observed by Hagerman and Szaniswaka, (1990). Baden *et al.,* (1994) also reported similar changes in the distribution of glycogen in the tissues of Norway lobster, *Nephrops norvegicus* exposed to copper. Findings from several studies suggested that copper induces several physiological (Ex, gill injury) and metabolical (Ex osmoregulatory disturbances) damages to the aquatic organism, especially fishes (Monteiro *et al*., 2005; Eyckmans *et al*., 2010; Abdel-Khalek *et al*., 2015; Crémazya *et al*., 2016, Lushchak, 2011; Sovová *et al*.,2014; Philippe *et al.,* 2017). As we know that, fishes are

directly linked to the aquatic environment, studying the variations in the haematological profile may provide information about internal body conditions, earlier than any noticeable disease indication (Fernandes and Mazon, 2003; Ullah *et al*., 2015). However, such alterations observed while hematological profiling is largely depend on the fish species, age, the cycle of sexual maturity and health condition (Blaxhall,1972; Wedemeyer *et al*., 1983; Hrubec *et al*., 2001). Hematological parameters are reliable biomarkers which are used to assess the general health condition of the fish and closely associated to the response of the animal to the environment (Oshode *et al*., 2008; Akinrotini *et al*., 2012; Sayed and Moneeb, 2015; Saravanan *et al*., 2017). Examination of haematological profile of the aquatic organisms is an effective and sensitive tool used in monitoring hepathophysiological changes (Kori-Siakpere *et al*., 2005; Motlagh *et al*.,2012).

Fatty acids are of great importance to humans for the prevention of coronary heart disease and also decreased incidence of breast cancer, rheumatoid arthritis, multiple scelerosis, psoriasis and inflammation (Conner, 2000; Kinsella, 1987; Mozaffarian *et al.*, 2005). Fatty acids are important for human and animal health and they are of interested because they are precursors in the eicosanoids biosynthesis, which are viewed as important bioregulators of many cellular processes (Khotimchenko, 2005). Seafood products have attracted considerable attention as

important sources of nutrients in the human diet. Apart from their delicacy, crustacean species such as shrimp, crab and lobster, consist of amino acids, peptides, protein and other useful nutrients (Sriket *et al.*, 2007). Shrimp meat is an excellent source of protein and minerals (Yanar and Celik, 2006). Furthermore, shrimp muscle consists of highly unsaturated fatty acids (HUFA) such as eicosapentaenoic (20:5n3, EPA) and docosahexaenoic (22:6n3, DHA) acids, considered as essential for the human diet (Abdullah *et al*., 2009).

3.8. Histopathology

Cellular assay techniques are employed to study pollutant induced injuries on the internal organ system of organisms. Such injuries serve as reliable biological indicators of pollution and are effectively used in assessing stress effects. In crustaceans, hepatopancreas, gills, muscle, lymphoid organ and nerve card display considerable cytological, cytochemical and structural alterations at chronic exposure to low levels of copper. The hepatopancreas followed by gills have been identified as the target organs of interest in toxicity investigations. Histological studies aid in locating the specific cell types in different organs which constitute the targets. The estimation of biochemical constituents helps to assess the nutritive value of an organisms. It has become imperative to study how the biochemical constituents vary the nutritive values of the crustaceans which are exposed to increased copper concentration.

Crustaceans are diversified in nature and easily accumulate pollutants present in the aquatic environment, as well as through biomagnification by the trophic chain, representing an important route of contamination to public health (Pinheiro *et al.*, 2013). These organisms present high sensitivity to the xenobiotics available in the environment (Carvalho-Neta *et al.,* 2019; Oliveira *et al*., 2019). The histological study of gills, hepatopancreas, and muscle of crustaceans has been documented as a appreciated tool for the assessment of the impacts of toxicants on crustaceans (Maharajan *et al.*, 2015; Carvalho-Neta *et al.,* 2019; De Freitas-Rebelo *et al.,* 2000). The gills are the first and prime organ to get in communication with environmental pollution and are highly sensitive to chemicals which can lead to severe problems for the organism. The hepatopancreas of crustaceans is a very dynamic organ mostly related to the digestive functions, being responsible for most of the detoxification activity responding with intensity to toxic substances (Maharajan *et al.,* 2015). In crustacea, the lymphoid organ (LO) is present only in penaeid prawns. In other crustaceans like crabs, lobsters and crayfish there is absence of lymphoid organs and many scientists believe that the lymphoid organ is involved in defence against invading pathogens. The nervous system of shrimps consists of supraoesphagial ganglion connected to a ventral nerve cord or nerve centres.

4. MATERIALS AND METHODS

4.1 Area of the present study

The present research was carried out at Post Graduate and Research Department of Zoology, Khadir Mohideen College, Adirampattinam.

4.2 Experimental animal White leg shrimp, *Litopenaeus vannamei*

Litopenaeus vannamei is a decapod crustacean native to the eastern Pacific coast of Central and South America. It has been widely introduced around the world since the 1970s, but notably since 2000, when it became Asia's primary cultivated shrimp species. The species isn't a serious danger to biodiversity, doesn't appear to have established reproductive colonies, and has generally had favorable economic consequences in non-indigenous locations.

4.3. Schematic Classification

Kingdom	: Metazoa
Phylum	: Arthropoda
Sub phylum	: Crustacea
Class	: Malacostraca
Sub class	: Eumalacostraca
Order	: Decapoda

Sub order : Dendrobranchiata

Family : Penaeidae

Genus : Litopenaeus

Species : vannamei

Binomial name : *Litopenaeus vannamei* (Boone, 1931) (Plate 1)

Plate 1: White leg shrimp, *Litopenaeus vannamei* (Boone, 1931)

4.4 Collection and Acclimatization of Experimental animal

Healthy hatchery produced experimental animal *Litopenaeus vannamei* with a mean total length of 8.25±0.65cm and a mean total weight of 8.70±0.50gm. Shrimps were acclimatized for two weeks in a stock tank to experimental FRP tank 500 l capacity supplied with 300 l of water with a salinity of 23±2 ppt and a natural photoperiod of 12 h:12 h

(light:dark). The tanks' water was filtered via a 1 m filter, UV-sterilized, and replaced 20% on a daily basis. Shrimps were given an artificial pellet feed (I Feed 3SP, 1.2mm Dia and 3-4mm length manufactured by Deepak Nexgen Feeds & Foods PVT. Ltd) for twice a day. Before and during the trial, they were famished for 24 hours.

4.5 Preparation of a copper toxicity test stock solution

The stock solution for preparing various concentrations of copper in raising water was 3.9 gram of Copper ll sulphate pentahydrate ($CuSo_4$ 5 H_2O) (Merck) diluted in one litre of double distilled water. It was kept at room temperature in a clean standard flask in the laboratory.

4.6 Procedure for Experimentation

4.6.1 Exploratory test for Experimental animal

Exploratory studies, also known as range finding tests, were conducted to determine the estimated effective concentration range of copper required for conducting short-term testing to examine the influence of copper on shrimp metaboloic function, as suggested by the American Public Health Association (1985). The test solutions were made with a variety of concentrations in mind. These tests were carried out by immersing 20 numbers of white leg shrimp, *L.vannamei* specimens in 200 litres of sea water containing various copper concentrations. The dead

animals were removed as soon as possible. Each animal's death was noted. For short-term toxicity studies, three replicates were created; the least lethal concentration was chosen when no mortality was observed in 24 hours, and the greatest lethal dose was chosen when 100 percent mortality was recorded in 24 hours.

4.6.2 Acute Toxicity test

Following the Standard Methods, a static bioassay test was done to determine the 96 h LC_{50} of copper to *L.vannamei* (APHA, 1995). The shrimp were transferred from the stock tank to the experimental tank after an acclimation time, with a total length of 9.25±0.55cm and a mean total weight of 10.50±0.50gm. Each trough was filled with 200 l of water and ten shrimp were inserted at random. Nominal copper concentrations were given to shrimp (0.8,1.0,1.2,1.4,1.6,1.8,2.0 and 2.2ppm). Three duplicates of each concentration were carried out. Control shrimps were kept in a comparable environment but were not exposed to copper. Dissolved oxygen (DO) 6.9±0.5 mg/l, temperature 27.5±0.4ºC, salinity 23±2 ppt, and pH 7.55±0.2 were all tested on a daily basis. Sprague (1973) approach was used to record shrimp mortality in each trough at intervals of 24, 48, 72, and 96 hours. The dead shrimps were removed as soon as possible.

The values were translated into the probit scale after calculating the percent fatality. Finney's probit analysis was carried out (1971). A probit

regression line was drawn against a logarithmic transformation of the calculations. The regression line with correlation coefficient's slope (S) function and confidential limit (upper confidential limit and lower confidential limit) were calculated as follows:

$$S = \frac{\frac{LC_{84}}{LC_{50}} + \frac{LC_{50}}{LC_{16}}}{2}$$

$$F = \text{Antilog}\frac{2.77 \log S}{\sqrt{N}} = S^{2.77/\sqrt{N}}$$

N = Total number of animals tested.

Upper Confidential Limit (UCL) = LC_{50} X F

Lower Confidential Limit (LCL) = LC_{50} / F

Based on acute toxicity, four fatal values were calculated for 24, 48, 72, and 96 hours of exposure, which were utilized as the copper toxicant experimental concentrations in following investigations.

4.6.3 Sub lethal toxicity test

For sublethal toxicity tests, the shrimps were grouped into three batches. Each batch had 10 animals and had 3 replicates.

Group : I

Shrimps maintained in normal sea water and served as control.

Group : II

Shrimps exposed to the sublethal concentration of 0.112ppm (10%of LC_{50} value for 96 hours) of copper in sea water.

Group : III

Shrimps exposed to the sublethal concentration of 0.224 ppm (20% of LC_{50} value for 96 hours) of copper in sea water. Shrimps were daily fed with artificial pellet feed (I Feed 3SP, 1.2mm Dia and 3-4mm length manufactured by Deepak Nexgen Feeds & Foods PVT. Ltd). The media were renewed every alternate day. Water quality (dissolved oxygen, temperature, pH and salinity) was measured daily and water chemistry (ammonia nitrogen, nitrite nitrogen, nitrate nitrogen) was measured twice weekly. The ammonia nitrogen and nitrite nitrogen levels were controlled not to exceed 0.1 mg/l. All chemical parameters were determined following the techniques of APHA (1995) using analytical grade

reagents. The actual concentration of copper was measured weekly before and after its addition to maintain concentrations at the designed level. The water characteristics and the actual copper concentrations are shown in Table 1. Mortality and behavior were observed daily in each concentration. One specimens each from the groups I, II and III were sacrificed after 0,7,14,21 and 28 days of the experiment.

Parameters	**Range**	**Mean ±S.D**
Dissolved oxygen (mg/l)	6.73–6.94	6.84±0.09
Temperature (ºC)	26.5–27.8	27.1±0.53
Salinity (%)	23.4–25.2	24.2±0.74
pH	7.21–8.14	7.73±0.39
Total alkalinity (ppm)	102-116	109.3±5.73
Total ammonia (ppm)	0.62–0.71	0.66±0.04
Nitrite (ppm)	0.84–0. 87	0.87±0.02
Actual copper concentration (mg/l)	0.009±0.012	0.010±0.001

Table 1. Water quality variables and actual copper concentration during sublethal exposure to *L.vannamei*

4.7 External and internal organ morphological observation

The shrimps were exposed to 0.112ppm and 0.224ppm concentrations of copper for 28 days. After 0,7,14,21 and 28 days, the shrimps were sacrificed and examined for external morphology. Internal organ colour changes were also recorded.

4.8 Bioaccumulation study

The shrimps were exposed to 0.112ppm and 0.224ppm concentrations of copper for 28 days. After 0,7,14,21 and 28 days, the shrimps were sacrificed and the tissues, muscle, gills, hepatopancreas and exoskeleton were excised out. The tissues were dried in an oven at 60ºC till constant weight was recorded. Dried samples of individual tissue were weighed and subjected to dry digestion with 20 ml of nitric acid and perchloric acid mixture (1:1) until a clear solution was obtained. The digested samples were then made upto 100 ml with double distilled water and analyzed in Atomic Absorption Spectrophotometer (Perkin- Elmor 2380) for copper concentration by the method described by Perkins,(1970).

4.9 Haematology

4.9.1 Haemolymph collection

The haemolymph of the shrimp, *L.vannamei* was drawn from haemocoel through a syringe. The haemolymph was thoroughly mixed

with 1% sodium citrate, used as an anticoagulant. Thin hemocyte films were prepared by carefully spreading a drop of unfixed hemolymph. They were then air-dried, fixed in 10% formalin-methanol (1:9) and flooded in Giemsa and haemotoxylin and counterstained with eosin (Cornick and Stewart, 1978). The identification of hemocyte types were based on cell size, shape, and staining properties (Wood and Visenten, 1967).

4.9.2 Haemocyte Counts

The counting of free hemocytes (THC) was done by using a hemocytometer with improved double Neubauer ruling and a diluting fluid suitable for crustaceans. Whenever cell rupture, agglutination and plasma clot formation appeared either in the dilution pipette or in the counting chamber, the count was not made. Differential haemocyte counts (DHC) were made on freshly stained smears following the method of Mix and Sparks (1980). The different types of hemocytes were counted at random under the microscope (×400) and the number of each cell type expressed in percentage, to which the following formula was applied.

$$\text{DHC (\%)} = \frac{\text{number of cells of a type}}{\text{Total number of cells counted}} \times 100$$

4.10 Evaluation of Proximate Composition

4.10.1 Estimate of Biochemical composition

The shrimps were exposed to 0.112ppm and 0.224ppm concentrations of copper for 28 days. After 0,7,14,21 and 28 days, the shrimps were sacrificed. Muscle tissue were excised out and analyzed for biochemical composition. Total protein was estimated in UV visible double beam spectrophotometer by Biuret method using bovine serum albumin as standard as suggested by Gornall *et al.,* (1949). Total carbohydrate was estimated by Phenol - Sulphuric acid Method of Dubois *et al.*, (1956). Total lipids were estimated by gravimetric methanol - chloroform extraction method suggested by Floch *et al.,* (1957) and modified by Linford,(1965).

4.10.2 Determination Fatty acid profile

The lipid compositions were determined by thin-layer chromatography/flame ionization detector (TLC-FID). Scanned quartz rods (silica gel powder- coated Chroma rod S III) were dipped in 3% boric acid solution for 5 min, dried and rescanned with the TLC-FID analyzer. The sample solution (1 ll) was spotted on the rod and the separation was carried out with the mixture of benzene: chloroform: acetic acid (52:20:0.7) for approximately 35 min. Then, the rods were dried in an oven (105°C) for 5 min before analyzing with the flame

ionization detector. The analytical conditions were H_2 flow rate of 160 ml. min^{-1}, air flow rate of 2000 $ml.min^{-1}$ and scanning speed of 30 s/scan. Retention time of lipid composition standards was used to identify chromatographic peaks. Peak area was quantitated and expressed as mg of total lipid. The fatty acid compositions were determined as fatty acid methyl esters using a gas chromatography, GC-14A (Shimadzu, Kyoto, Japan) equipped with fused silica capillary column Carbowax-30 M (30 m, 0.25 mm ID) and flame ionization detector (FID). Nitrogen was used as the carrier gas at a flow rate of 30 cm/s. The initial temperature of the column was set at 170 °C and was increased to 225 °C with a rate of 1 °C/min and then held at 220 °C for an additional 20 min. The detector temperature was set at 270 °C, while the temperature at the injection port was maintained at 250 °C. Retention time of standards was used to identify chromatographic peaks. Peak area was quantitated and expressed as mg of total lipid (AOAC, 1999).

4.10.3. Determination of Vitamins and Minerals

Shrimp muscle tissue were collected after the treatment of copper. After washing with tap water and sliced into 10 mm thick transverse slices using a fruit slicer. Subsequently, they were dried under direct sunlight. The muscle tissue were weighed and stored in polyethylene bags at room temperature. The vitamin B group was extracted and analyzed

according to a described method (AQAC International, 1990). Vitamin C was extracted according to a modification of a published method (Babarinde and Fabunmi, 2009). Vitamin A and D was extracted and analysed according to the spectrophometric method, (Aremu and Nweze, 2017).

Calcium, magnesium, manganese, iron, sodium, potassium and zinc contents were determined by the inductively coupled plasma optical emission spectrophotometer (ICP-OES) (Perkin–Elmer, Model 4300 DV, Norwalk, CT, USA) according to the method of AOAC (1999). Shrimp meat (4 g) was mixed well with 4 ml of nitric acid. The mixture was heated on the hot plate until digestion was completed. The digested samples were transferred to a volumetric flask and the volume was made up to 10 ml with deionised water. The solution was subjected to (ICP-OES) analysis. Flow rates of argon to plasma, auxiliary and nebulizer were kept at 15, 0.2, and 0.8 $l.min^{-1}$, respectively. Sample flow rate was set at 1.5 $ml.min^{-1}$. The concentration of mineral was calculated and expressed as mg/g sample.

4.10.4. Amino acid analysis

After the experiment the tail muscle samples were collected and dried at 60°C for 24 h in an oven and packed in airtight polyethylene covers and kept in desiccators. The oven-dried samples were finely grounded before estimating amino acid profile. Samples were hydrolyzed

under reduced pressure in 4.0 M methane sulfonic acid containing 0.2% (v/v) 3-2 (2- amino ethyl) indole at 115°C for 24 h. The hydrolysates were neutralized with 3.5 M NaOH and diluted with 0.2 M citrate buffer (pH 2.2). An aliquot (0.4 ml) was applied to an amino acid analyzer (D-7000 HPLC).

4.11. Evaluation of histopathology

At the interval of 0, 7 and 28 days one shrimp from each concentration of copper was picked out randomly. The animal was sacrificed and muscle, gills, hepatopancreas, lymphoid organ and nerve cord cut in small pieces of 4-5mm sizes were fixed immediately in Davidson's Fixative (Humason,1972) of the following composition.

i. 95% Ethyl alcohol - 330 ml
ii. 100% Formalin - 220 ml
iii. Glacial acetic acid - 115 ml
iv. Distilled water - 335 ml

After a period of 24 hours the fixed tissues were washed and transferred directly to 70% Ethanol and kept overnight. The next day the tissues were transferred to 90% solution of Ethanol and after one hour the tissues were given two changes of half an hour each in 100% Ethanol for complete dehydration.

The tissues were cleared by immersing in 1:1 ratio mixture of Ethanol and chloroform for one hour. After that, two changes were given in 100% chloroform for half an hour each. The tissues were kept in saturated chloroform - paraffin wax mixture overnight for cold impregnation.

Finally, the tissues were given three changes of 15 minutes each in molten wax for hot impregnation for block preparation. Paraffin with ceresin having melting point of 58-60°C was used. The blocks were prepared in paper boats using molten wax after hot impregnation. The prepared blocks were labelled, and kept in cool water for proper solidification. Tissue sections were taken at 7µ thickness using a Rotary microtome.

The sections were spread over clean glass micro-slides smeared with egg-albumin, warmed with a few drops of distilled water and kept at least for 24 hours before staining. The slides were marked appropriately. The slides carrying sections were deparaffined in absolute xylene, passed through a descending series of Ethanol solution (100%, 90%, 70%, 50% and 30%) and stained with Delafields haematoxylin for 1-2 minutes and in case of over staining, destaining was done using a solution of very dilute hydrochloric acid and then stained with 0.5% aqueous eosin for 3-5 minutes. After staining, the sections were taken through an ascending series of Ethanol solutions (30%, 50%, 70%, 90%, and 100%) and finally to pure

xylene. The cleared sections were mounted on DPX mountant and covered with No.1 cover slip. The stained sections were micro photographed using Nikon Bright field transmission microscope.

4.12 Statistical analysis

Concentration effect relationships were analyzed with probit analysis to calculate the LC_{50} point estimate and associated 95% confidence intervals (Finney,1971). All statistical tests such as mean, standard deviation and two way analysis of variance (ANOVA), linear regression were carried out by SPSS 11.5 software.

5. RESULTS

5.1. Acute toxicity test to determine the LC_{50} values of *L.vannamei*

In the present study, *L. vannamei* were exposed to copper for a maximum period of 96 h and the mortality of was observed after 24, 48, 72 and 96 h exposure. Acute toxicity test on *L. vannamei* was conducted using 0.8, 1.0, 1.2, 1.4, 1.6, 1.8, 2.0 and 2.2 ppm of copper. The 100% survival of *L. vannamei* was found in 0.08 ppm of copper up to 72 h and 80% of survival was observed after 96 h in the same concentration. Similarly, 100% of survival was observed in 1.0 ppm of copper after 24 h of exposure. In 2.2 ppm of copper 100% mortality was observed within 48 h. However, the 100% mortality of test animal was observed at 96 h in 1.8, 2.0 ppm. More than 50 % of test animal were killed when exposed to 1.4 ppm and 1.6 ppm copper after 72 h exposures respectively (Table 2 & Fig.1). No mortality was observed in control medium and solvent control at all exposure period. Concentration response curves was presented in Fig.2. The above results reveal that the rate of mortality was directly correlated with dose as well as exposure period. The mortality increased significantly when the concentration and the time of exposure to the metals increased.

The LC_{50} values for *L. vannamei* exposed to copper were calculated using trimmed spearman- karber method. The calculated LC_{50} value for

L. vannamei 1.746 ppm after 24 h, while it was 1.515 ppm after 48 h, 1.407 ppmafter 72 h and 1.113 ppm after 96 h of exposures to copper. While increasing the exposure period, decrease in LC_{50} value was observed. The regression analysis showed r2 value of 0.9722 which is close to 1 indicated the linearity of the test data. LC_{50}, slope, intercept values of *L. vannamei* exposed to copper were determined and given in the Table 2. & Fig.3,4,5 & 6).

Exposure Period(h)	Total Number of animals exposed	Concentration of Copper in water (ppm)															
		0.8		1.0		1.2		1.4		1.6		1.8		2.0		2.2	
		Mortality rate (number of animals and percentage of mortality)															
		Nos	%	Nos	%	Nos	%	Nos	%	Nos	%	Nos	%	Nos	%	Nos	%
24	**10**	Nil	0	Nil	0	1	10	3	30	4	40	5	50	6	60	8	80
48	**10**	Nil	0	1	10	2	20	4	40	5	50	8	80	9	90	10	100
72	**10**	Nil	0	2	20	3	30	5	50	6	60	8	80	10	100	10	100
96	**10**	2	20	4	40	5	50	7	70	9	90	10	100	10	100	10	100

Table: 2 Average mortality rate of *L vannamei* in different concentrations of copper during acute toxicity study

Exposure Periods	LC_{50} (ppm)	95 % Confidence Limit (ppm)		Slope Function	Regression equation	Correlation coefficient (r)
		Lower Limit	Upper Limit			
24	1.746	1.551	1.966	7.113	y=3.340+6.587x	0.957*
48	1.515	1.163	1.183	6.976	y=3.578+8.074x	0.962*
72	1.407	1.232	1.606	6.443	y=4.063+6.442x	0.970*
96	1.113	0.947	1.308	6.585	y=4.704+6.585x	0.947*

Table: 3 Median lethal concentrations of Copper to *L. vannamei* under different exposure duration

5.2. Impact of copper on the morphological colour changes in internal organs and uropod of *L.vannamei*

Copper exposure caused changes in the appearance of external and internal organs in *L.vannamei*. There were no significant alterations in both exterior and internal organs after 7, 14, 21, and 28 days of exposure to 0.112ppm copper concentration. After 7, 14, and 21 days of exposure to a dose of 0.224ppm, similar results were seen. Eventually, the toxicity of copper was highlighted in the colour changes observed only after 28 days of exposure to 0.224ppm in muscle, hepatopancreas, gill tissues and uropod.

After 28 days of exposure to 0.224ppmconcentration of copper, the colour of muscle changed from normal creamy white to blackish white (Plate 2 A &B). Loosely packed muscle blocks and a deposition of thin film of copper (black spots) on the muscle tissue were also observed.

The gill is a major indicator of toxicity in general and heavy metal accumulation such as, copper in particular. The gill colour changed from pale green with black deposits of copper on the gill lamellae (Plate 2C &D). The primary and secondary gill lamellae were found bordered with black deposits or granules of copper in shrimps exposed to 0.224 concentration for 28 days.

The colour of hepatopancreas also turned from the normal yellowish brown to greenish black (Plate 3A &B) at the end of 28 days of exposure to 0.224concentration. The uropod region normal orange colour turned into black colour. Black deposits were also observed in uropod region (Plate 3C &D).

5.3. Investigation of the effect of copper on the bioaccumulation of copper in various tissues of *L.vannamei*

5.3.1. Muscle

The exposure of shrimps to copper through water medium provided several evidences for accumulation of copper in various tissues of shrimps. The relationship between the accumulation of copper in the muscle was directly related with exposure time and concentration of accumulation in a dose-dependent manner. After 28 days, both groups treated with low and high copper concentration had significantly accumulated higher copper in their muscle tissue compared to the control group, 2.68$\pm$0.42, 3.11$\pm$0.21, and 1.49 $\pm$0.19 respectively (Fig.7). The r2 values of the low, high and control values are 0.798, 0.867 and 0.0024, which indicated that the accumulation of copper is increased with increased exposure time and the copper is come from the exposure.

5.3.2. Gills

As observed earlier, accumulation of copper in the gills after exposed to both low and high concentrations are linear and increased with both time and concentration (Fig.8). The regression analysis showed r2 value of 0.9822 and 0.9711respectively, which indicated the linearity of the test data. Whereas the level of copper in the gills of control did not show any significant changes after 28 days with r2 value of 0.3745.

5.3.3 Hepatopancreas

Likewise, the level of copper in hepatopancreas in both low and high concentrations followed linear pattern and found to be increased with exposure time and concentration. The regression analysis showed r2 value of 0.9495 and 0.9582 respectively (Fig.9), which indicated the increase of copper in hepatopancreas is linear. Whereas the level of copper in the hepatopancreas of control did not show any significant changes after 28 days with r2 value of 0.133.

5.3.4. Exoskeleton

The copper level in the exoskeleton of the shrimp also measured and it was found that the copper accumulation of copper in exoskeleton is higher at the high concentration of copper exposed animals and also after the highest exposed period (Fig.10). The level of copper accumulation in exoskeleton after exposure to low and high concentration is significantly differed from control after 28 days of exposure. Whereas, there was no

significant difference was observed between the low and high level of exposed animals. The level of copper and increased with increased exposure time and the increase in copper level is also linear which is indicated by the r2 values such as 0.911 and 0.8656 respective to high and low concentration of copper.

It was observed that the highest accumulation of copper found in all the body parts hence it would be better to compare the accumulation at different parts of the body to find where the accumulation is high. The comparison of the test results were presented in Fig.11 and it is evident from the figure that the highest accumulation of copper is found in hepatopancreas followed by gills. There was a significant difference in the rate of accumulation between hepatopancreas and gills however there was no significant difference was observed in both low and high concentrations of copper and control. The lowest rate of copper concentration accumulation was found in muscle preceded by exoskeleton .

5.4. Sublethal effect of copper on the hematological changes in *L.vannamei*

5.4.1. Total Haemocyte Count (THC)

Figure 12. depicted the total haemocyte count of *L.vannamei* exposed to different concentrations of copper after 0, 7, 14, 21 and 28

days. After 28 days of copper exposure changes in THC values were observed, and the highest value was obtained in 0.224 ppm showed 8.1×10^6cells/ml followed by 0.112 ppm copper resulting THC value of 6.8×10^6cells/ml, and the lowest was observed from the control resulting in 4.7×10^6cells/ml. The total haemocyte count of the shrimp exposed to 0.112 ppm concentration in cells/ml was 4.6×10^6, 5.6×10^6, 5.9×10^6, 6.1×10^6, and $6.8\times10^6$and 0.224 ppm concentration was 4.8×10^6, 6.3×10^6, 7.4×10^6, $7.8\times10^6$and 8.1×10^6 after respective exposure duration 0, 7, 14, 21 and 28 days. Whereas the haemocyte count of the control shrimp was found to be 4.3×10^6, 4.6×10^6, 4.1×10^6, 4.9×10^6, and 4.7×10^6 after 0, 7, 14, 21 and 28 days of exposure.

Total haemocyte count was found to be increased in all the concentrations of copper treated animals as compared with the control and the count is increased with increasing exposure duration. At the end of exposure, the highest total haemocyte count was found in the high copper concentration and the level is significantly differed from control and it is insignificant from the lower concentration. Similarly, the low copper concentration showed significantly increased haemocyte count compared to control.

5.4.2. Differential Haemocyte Count (DHC)

Crustaceans differential haemocytes classified into three major types such as hyaline cells, small granule haemocyte (SGH) and large granule haemocyte (LGH) based on cytoplasmic granular size. In the present study differential haemocyte count (DHC) were carried out for hyaline, SGH and LGH.

5.4.2.1. Hyaline cells

On day 0 of exposure the highest hyaline cell count was observed from 0.224 ppm copper (75.6±0.16%) followed by 0.112 ppm copper (74.2±0.28%) and control (73.4±0.19%). Whereas, on 7thday both treatments and control displayed an increase in the number of hyaline cells and the highest value was obtained from 0.224 ppm (77.9±0.32%), followed by 0.112 ppm (77.2±0.26%) (Fig.13). However, there was no significant difference (P>0.05) observed between treatment on 7thday of exposure. Similarly, the level of hyaline cells was increased while increasing the exposure period in all the treatments however the level of hyaline cells between two copper concentrations is insignificant. Although, at the end of experiment the increase in hyaline cells were significantly higher in the treatments compared to control. Interestingly, the hyaline cell content decreased in control after 7th day of exposure and increased after 14th day of exposure and the increase is insignificant (P>0.05).

5.4.2.2. Large granular cells

After 28 days of copper exposure the lowest arge-granular cell count was observed from 0.224 ppm copper (11.5±0.18%) followed by 0.112 ppm copper (11.42±0.21%) and control (14.4±0.09%) (Fig.13). However, the change in LGC level after 28 day of experiment in both high and low copper exposure is insignificant ($P>0.05$) and the level of LGC is significant ($P<0.05$) when compared to control. After 7 days of exposure LGC level decreased insignificantly ($P>0.05$) in all the treatments including control. Similarly, the level of LGC level was decreased while increasing the exposure period in all the treatments however the level of LGC between two copper concentrations is insignificant ($P>0.05$). Although, at the end of experiment the decrease in LGC level were significantly ($P<0.05$) lower in the treatments compared to control.

5.4.2.3. Small-granular cells

After 28 days of copper exposure the lowest small-granular cell count was observed from 0.224 ppm copper (7.9±0.18%) followed by 0.112 ppm copper (8.42±0.31%) and control (13.8 ±0.12%) (Fig.13). After seven days of exposure SGC level decreased in all the treatments however no difference was found in control. However, there was no significant difference ($P>0.05$) observed between treatment on 7^{th}day. Similarly, the level of SGC level was decreased while increasing the

exposure period in all the treatments however the level of SGC between two copper concentrations is insignificant (P>0.05). Although, at the end of experiment the decrease in SGC level were significantly (P<0.05) lower in the treatments compared to control.

5.5. Changes in proximate composition, fatty acids, vitamins, minerals and amino acids in muscle of *L. vannamei* due to the effect of copper

5.5.1. Effect of the copper on proximate composition of muscle tissue

5.5.1.1. Total protein

When *L. vannamei* exposed to lower and higher concentration of copper, the protein content in the muscle decreased at all the exposure periods when compared to control. The maximum decrease was observed with *L. vannamei* exposed to higher concentration of copper at 28th day. In the control *L. vannamei*, the mean protein content in the muscle tissues was 36.4± 0.18%, 34.5± 0.21%, 33.9± 0.11%, 35.7± 0.23% and 34.5± 0.29% wet wt. of tissues for respective exposure periods of 0, 7, 14, 21 and 28 days. Whereas in the *L. vannamei* treated with lower concentration of copper, mean muscle protein content was 32.5± 0.09%, 31.6± 0.18%, 30.9± 0.21%, 29.5± 0.12%, and 27.6± 0.15% wet wt. of tissue, and in higher concentration, 35.3± 0.27%, 29.8± 0.19%, 29.4±

0.17%, 28.4± 0.23%, and 26.8± 0.14% wet wt of tissue for 0, 7, 14, 21 and 28 days exposure periods respectively (Fig.14). The percent decrease over the control was 10.71, 8.41, 8.85, 17.37 and 20.00 in lower concentration and 3.02, 13.62, 13.27, 20.45, and 22.32 in higher concentration for 0, 7, 14, 21 and 28 days of exposure period respectively (Table 4). The decreased level of protein in both high and lower exposure periods was statistically significant ($P<0.05$).

Copper Concentration (ppm)	**Percentage decrease of total protein**				
	0 day	**7th day**	**14th day**	**21st day**	**28th day**
0.112	10.71	8.41	8.85	17.37	20.00
0.224	3.02	13.62	13.27	20.45	22.32

Table:4. The percent decrease of protein *L. vannamei* exposed to lower and higher concentration of copper content over the control after different exposure period

5.5.1.2. Total Carbohydrate

The exposure of *L. vannamei* to copper cause distinct dose and time dependent decrease in the carbohydrate content of all exposure period. On day 28, carbohydrate content of *L. vannamei* was found to decrease respectively 37.29%, 42.12% in the muscle exposed to 0.112 and 0.224 concentration of copper (Table 5). In the present study, the highest decrease of carbohydrate content was observed after 28 days of exposure to copper at 0.224 ppm. Similarly, the percentage of decrease also found to be higher on *L. vannamei* exposed to the 0.224 ppm of copper than 0.112 ppm copper concentration. From the figure 15 it is evident that the observation of changes in carbohydrate was depend significantly ($P<0.05$) on the concentration and period of exposure. In the control *L. vannamei*, the carbohydrate content in the muscle tissues was 5.94±0.21%, 5.84 ±0.26%, 5.86±0.11%, 5.21±0.06%, and 5.39±0.15%, for 0, 7, 14, 21 and 28 days exposure periods respectively. Whereas in the *L. vannamei* treated with lower concentration of copper, carbohydrate content was 5.81±0.26%, 4.71±0.10%, 4.13±0.14%, 3.95±0.25% and 3.38±0.19%, and in higher concentration, 5.23±0.19%, 4.28±0.11%, 3.97±0.20%, 3.62±0.25% and 3.12±0.19% wet wt of tissue for 0, 7, 14, 21 and 28 days exposure periods respectively (Fig.15).

Copper Concentration Ippm)	Percentage decrease of Total carbohydrate				
	0 day	**7th day**	**14th day**	**21st day**	**28th day**
0.112	2.19	19.35	29.52	24.18	37.29
0.224	11.95	26.71	32.25	30.52	42.12

Table: 5. The percent decrease of carbohydrate *L. vannamei* exposed to lower and higher concentration of copper content over the control after different exposure period

5.5.1.3. Total lipid

Similar to carbohydrate, the exposure of *L. vannamei* to copper cause distinct dose and time dependent decrease in the lipid content of all exposure period. On day 28, lipid content of *L. vannamei* was found to decrease respectively 21.46% and 29.22% in the muscle exposed to 0.112 and 0.224 ppm concentration of copper (Table 6). In the present study, the highest decrease of lipid content was observed after 28 days of exposure to copper at 0.224 ppm. Similarly, the percentage of decrease also found to be higher on *L. vannamei* exposed to the 0.224 ppm of copper than 0.112 ppm copper concentration. From the figure it is evident

that the observation of changes in lipid was depend significantly ($P<0.05$) on the concentration and period of exposure. In the control *L. vannamei*, the lipid content in the muscle tissues was 12.49±0.25%, 12.38±0.12%, 13.32±0.08%, 13.12±0.21% and 12.49±0.14% for 0, 7, 14, 21 and 28 days exposure periods respectively. Whereas in the *L. vannamei* treated with lower concentration of copper, lipid content was 12.46±0.23%, 11.26±0.12%, 11.18±0.06%, 10.09±0.11% and 9.81±0.16%, and in higher concentration, 11.54±0.05%, 11.12±0.25%, 10.02±0.13%, 9.92±0.09% and 8.84±0.02% of tissue for 0, 7, 14, 21 and 28 days exposure periods respectively (Fig.16).

Copper Concentration (ppm)	**Percentage decrease of total lipid**				
	0	**7**	**14**	**21**	**28**
0.112	0.24	9.05	16.07	23.09	21.46
0.224	7.61	10.18	24.77	24.39	29.22

Table: 6. The percent decrease of lipid *L. vannamei* exposed to lower and higher concentration of copper content over the control after different exposure period

5.5.2. Effect of copper on Fatty acids composition of muscle tissue

Fatty acids are mainly two types. They are saturated fatty acids (SFA) and unsaturated fatty acids (UFA). Findings described that amount of Palmic acid 16:0 in the tissues of *L. vannamei* exposed to lower (0.112 ppm) copper concentration were 17.16%, 16.54%, 15.48%, 15.21% and 14.95% at 0, 7, 14, 21 and 28 days exposure days respectively (Table 7). Whereas *L. vannamei* exposed to higher (0.224 ppm) copper concentration showed 16.48%, 15.52%, 15.18%, 14.21%, and 13.92% Palmic acid 16:0 for the respective exposure periods of 0, 7, 14, 21 and 28 days (Table 8). Result indicated that at the end of exposure there was no significant difference ($p < 0.05$) found in the level of Palmic acid 16:0 between high and low concentrations of copper. In addition, the lowest values such as 13.92% and 14.95% were found in 0.224 and 0.112 ppm after 28 days of copper exposure (Table 7 & 8). The result showed that the decrease in the level of Palmic acid is time and dose dependent and the decrease is insignificant between high and low concentrations of copper exposure in all the exposure period.

Findings described that amount of Margaric acid 17:0 in the tissues of *L. vannamei* exposed to lower (0.112 ppm) copper concentration were 1.51%, 1.31%, 1.20%, 1.01%, and 0.95% at 0, 7, 14, 21 and 28 days exposure days respectively. Whereas *L. vannamei* exposed to higher

(0.224 ppm) copper concentration showed 1.68%, 1.41%, 1.15%, 1.03%, and 0.91% Margaric acid 17:0 in tissues for the respective exposure periods of 0, 7, 14, 21 and 28 days (Table 8). Result indicated that at the end of exposure there was no significant difference ($p < 0.05$) found in the level of Margaric acid 17:0 between high and low concentrations of copper. In addition, the lowest values such as 0.95% and 0.91% were found in 0.224 and 0.112 ppm after 28 days of copper exposure (Table 8). The result showed that the decrease in the level of Margaric acid is time and dose dependent and the decrease is insignificant between high and low concentrations of copper exposure in all the exposure period.

Findings described that amount of Stearic acid 18:0 in the tissues of *L. vannamei* exposed to lower (0.112 ppm) copper concentration were 10.85%, 10.12%, 9.84%, 9.12%, and 8.45% at 0, 7, 14, 21 and 28 days exposure days respectively. Whereas *L. vannamei* exposed to higher (0.224 ppm) copper concentration showed 9.85%, 9.12%, 8.74%, 8.56%, and 8.15% Stearic acid 18:0 in tissues for the respective exposure periods of 0, 7, 14, 21 and 28 days (Table 8). Result indicated that at the end of exposure there was no significant difference ($p < 0.05$) found in the level of Stearic acid 18:0 between high and low concentrations of copper. In addition, the lowest values such 8.15% and 8.45% were found in 0.224 and 0.112 ppm after 28 days of copper exposure (Table 7 & 8). The result

showed that the decrease in the level of Stearic acid 18:0 is time and dose dependent and the decrease is insignificant between high and low concentrations of copper exposure in all the exposure period.

Findings described that amount of Oleic acid 18:1 in the tissues of *L. vannamei* exposed to lower (0.112 ppm) copper concentration were 16.32%, 15.98%, 15.41%, 14.65% and 13.26% at 0, 7, 14, 21 and 28 days exposure days respectively. Whereas *L. vannamei* exposed to higher (0.224 ppm) copper concentration showed 15.42%, 15.18%, 14.57%, 14.12% and 13.07% Oleic acid 18:1 in tissues for the respective exposure periods of 0, 7, 14, 21 and 28 days (Table 8). Result indicated that at the end of exposure there was no significant difference ($p < 0.05$) found in the level of Oleic acid 18:1 between high and low concentrations of copper. In addition, the lowest values such 13.07% and 13.26% were found in 0.224 and 0.112 ppm after 28 days of copper exposure (Table 7 &8). The result showed that the decrease in the level of Oleic acid 18:1 is time and dose dependent and the decrease is insignificant between high and low concentrations of copper exposure in all the exposure period.

Findings described that amount of Linolenic acid in the tissues of *L. vannamei* exposed to lower (0.112 ppm) copper concentration were 8.58%, 7.94%, 7.36%, 7.16% and 6.92% at 0, 7, 14, 21 and 28 days exposure days respectively. Whereas *L. vannamei* exposed to higher

(0.224 ppm) copper concentration showed 8.98%, 7.54%, 7.12%, 6.96%, and 6.12% Linolenic acid in tissues for the respective exposure periods of 0, 7, 14, 21 and 28 days (Table 8). Result indicated that at the end of exposure there was no significant difference ($p < 0.05$) found in the level of Linolenic acidbetween high and low concentrations of copper. In addition, the lowest values such 6.12% and 6.92% were found in 0.224 and 0.112 ppm after 28 days of copper exposure (Table 7 &8). The result showed that the decrease in the level of Linolenic acid is time and dose dependent and the decrease is insignificant between high and low concentrations of copper exposure in all the exposure period.

Findings described that amount of Alpha linolenic acid 18:13 in the tissues of *L. vannamei* exposed to lower (0.112 ppm) copper concentration were 1.72%, 1.51%, 1.26%, 0.95% and 0.84% at 0, 7, 14, 21 and 28 days exposure days respectively. Whereas *L. vannamei* exposed to higher (0.224 ppm) copper concentration showed 1.62%, 1.57%, 1.46%, 1.35%, and 1.19% Alpha linolenic acid 18:13 in tissues for the respective exposure periods of 0, 7, 14, 21 and 28 days (Table 8). Result indicated that at the end of exposure there was no significant difference ($p < 0.05$) found in the level of Alpha linolenic acid 18:13 between high and low concentrations of copper. In addition, we found 2 fold decrease in the values of Alpha linolenic acid at the end of exposure.

Further, the lowest values such 0.84% and 1.19% were found in 0.112 and 0.224 ppm after 28 days of copper exposure (Table 7&8) and the observed difference is significant. The result showed that the decrease in the level of Alpha linolenic acid 18:3 is time and dose dependent and the decrease is insignificant between high and low concentrations of copper exposure in all the exposure period.

Findings described that amount of Morotic acid 18:4 in the tissues of *L. vannamei* exposed to lower (0.112 ppm) copper concentration were 0.59%, 0.52%, 0.49%, 0.42%, and 0.39% at 0, 7, 14, 21 and 28 days exposure days respectively. Whereas *L. vannamei* exposed to higher (0.224 ppm) copper concentration showed 0.62%, 0.58%, 0.41%, 0.36%, and 0.31% Morotic acid 18:4 in tissues for the respective exposure periods of 0, 7, 14, 21 and 28 days (Table 8). Result indicated that at the end of exposure there was no significant difference ($p < 0.05$) found in the level of Morotic acid 18:4 between high and low concentrations of copper. In addition, the lowest values such 0.31% and 0.39% were found in 0.224 and 0.112 ppm after 28 days of copper exposure (Table 7 &8) and the observed difference is insignificant. The result showed that the decrease in the level of Morotic acid 18:4 is time and dose dependent and the decrease is insignificant between high and low concentrations of copper exposure in all the exposure period.

The fatty acid composition of whiteleg shrimp muscle were presented in Table 7 &8. The major fatty acids in shrimp muscle were, in palmic acid, margaric acid, stearic acid, oleic acid, linolenic acid, alpha linolenic acid, morotic acid. The exposure of *L. vannamei* to low and high copper concentrations has affected in the fatty acid composition of the exposed shrimp muscle tissue. For instance, palmic, oleic stearic, and linolenic acid were in higher level in muscle tissue of shrimps exposed with both concentrations of copper. At the day 28, among the fatty acids measured, level of monounsaturated fatty acids (MUFA), particularly Palmic acid 16:0 was found to be highest (13.92% for 0.224 ppm and 14.95% for 0.112 ppm) in muscle tissue of shrimps exposed with both concentrations of copper followed by oleic acid 18:1 (13.07% for 0.224 ppm and 13.26% for 0.112 ppm). The saturated fatty acid Stearic acid 18:0 also found to be in high levels (8.15% for 0.224 ppm and 8.45% for 0.112 ppm) next to MUFA acids at the 28 day of exposure (Fig.17). Whereas, the Polyunsaturated Fatty Acid (PUFAs) linoleic acid (C18:2) level also found to be higher in the exposed animals at the end of exposure period. The levels of other fatty acids such as margaric acid, alpha linolenic acid, morotic acid are found to be significantly lower when compared with other fatty acids. At the end of exposure the order of the levels of fatty acids are palmic acid > oleic acid > stearic acid > linolenic acid > alpha linolenic acid > margaric acid > morotic acid.

Copper concentration (0.112 ppm)							
Exposure duration (days)	**Palmic acid 16:0**	**Margaric acid 17:0**	**Stearic acid 18:0**	**Oleic acid 18:1**	**Linolenic acid 18:2**	**Alpha linolenic acid 18:3**	**Morotic acid 18:4**
0	17.16	1.51	10.85	16.32	8.58	1.72	0.59
7	16.54	1.31	10.12	15.98	7.94	1.51	0.52
14	15.48	1.20	9.84	15.41	7.36	1.26	0.49
21	15.21	1.01	9.12	14.65	7.16	0.95	0.42
28	14.95	0.95	8.45	13.26	6.92	0.84	0.39

Table.7 Effect of copper on fatty acids (%) in shrimp, *L. vannamei* exposed to lower concentration of copper after 0,7,14,21 and 28 days of exposure

Copper concentration (0.224 ppm)							
Exposure duration (Days)	**Palmic acid 16:0**	**Margaric acid 17:0**	**Stearic acid 18:0**	**Oleic acid 18:1**	**Linolenic acid 18:2**	**Alpha linolenic acid 18:3**	**Morotic acid 18:4**
0	16.48	1.68	9.85	15.42	8.98	1.62	0.62
7	15.52	1.41	9.12	15.18	7.54	1.57	0.58
14	15.18	1.15	8.74	14.57	7.12	1.46	0.41
21	14.21	1.03	8.56	14.12	6.96	1.35	0.36
28	13.92	0.91	8.15	13.07	6.12	1.19	0.31

Table 8. Effect of copper on fatty acids (%) in shrimp, *L. vannamei* exposed to higher concentration of copper after 0,7,14,21 and 28 days of exposure

5.5.3 Effect of copper on Vitamins and Minerals contents in muscle tissue

The vitamin composition of whiteleg shrimp muscle were presented in Figure 18 &19. The major vitamins A, D, E, B1, B2, B3, B5, B6 and C were analyzed. The exposure to *L.vannamei* to low and high copper concentration does not show much difference among the vitamins in lower (0.112ppm) and higher 0.224ppm) concentration in 7,14 and 21 days of exposure. The significant changes were observed in 28 days of exposure in lower (Vitamin A 0.121 – 0.085%, Vitamin D 0.108 – 0.089%, Vitamin E 0.138-0.106%, Vitamin C 0.192 – 0.172%) and higher concentrations (Vitamin A 0.126 – 0.081%, Vitamin D 0.102 – 0.087%, Vitamin E 0.128-0.102%, Vitamin C 0.186 – 0.168% Vitamins B1, B2, B3, B5 and B6 also not showing any significant differences in both concentrations.

Minerals such as calcium, magnesium, manganese, phosphorus, iron, sodium, potassium and zinc were analyzed in *L.vannamei* when exposed to lower and higher concentration of copper in different exposure durations. At the end of 7,14 and 21 days of copper exposure minerals contents did not show any significant changes of muscle tissue in both concentrations. At the end of exposure in 28 days of showing significant changes in calcium and magnesium in both lower and higher concentrations (145.2–140.9 mg/g; 13.2-12.4 mg/g in 0.112ppm

concentration of copper, 147.4-139.5mg/g; 12.8-11.2mg/g in 0.224 ppm concentration of copper). Minerals Manganese, phosphorus, iron, sodium, potassium and zinc not showing any significant differences in both concentrations in 28 days of exposure (Fig.20 &21).

5.5.4. Effect of copper on Amino acids composition of muscle tissue

Amino acids profiles of *L. vannamei* after exposure to low and high concentration of copper was analyzed to assess effect of copper on the nutritional quality. The resulted depicted that the amount of amino acid in protein changes according to the concentration of copper and exposure period. The level of essential such as histidine, valine, methionine, isoleucine, phenylalnine, leucine, lysine, arginine, tryptophan and non essential amino acid such as aspartic acid, glutamic acid, serine, glycine, tyrosine, proline, alanine and cysteine level were measured at 0, 7, 14, 21 and 28 days.

5.5.4.1 Non Essential Amino acids

5.5.4.1.1 Aspartic acid

The present study observed that the amount of aspartic acid was found to 1.26%, 1.24%, 1.18%, 1.12% and 1.01% *L. vannamei* after exposed to 0.112 ppm of copper at 0, 7, 14, 21 and 28 days respectively. After the end of exposure, the lowest value of 0.87% was found in 0.224

ppm and for 0.112 ppm it was 1.01% (Fig.22). Result illustrated that no significant difference was observed between 0.224 ppm and 0.112 ppm concentration in all the exposure period. Whereas *L. vannamei* exposed to higher (0.224 ppm) copper concentration showed 1.32%, 1.21%, 1.05%, 0.96%, and 0.87% aspartic acid in tissues for the respective exposure periods of 0, 7, 14, 21 and 28 days (Fig.30). Further, it is obvious that the level of aspartic acid is decreased with increasing exposure period and concentration of copper.

5.5.4.1.2 Glutamic acid

The level of glutamic acid content in *L. vannamei* varied based on exposure concentration and time. Findings described that the amount of glutamic acid was found to 2.45%, 2.37%, 2.19%, 1.95%, and 1.87% *L. vannamei* after exposed to 0.112 ppm of copper at 0, 7, 14, 21 and 28 days respectively. After the end of exposure the lowest value of 1.63% was found in 0.224 ppm and for 0.112 ppm it was 1.87% (Fig.23). Result illustrated that no significant difference was observed between 0.224 ppm and 0.112 ppm concentration in all the exposure period. Whereas *L. vannamei* exposed to higher (0.224 ppm) copper concentration showed 2.62%, 2.32%, 2.09%, 1.91% and 1.63% glutamic acid in tissues for the respective exposure periods of 0, 7, 14, 21 and 28 days (Fig.30). Further, it is obvious that the level of glutamic acid is decreased with increasing

exposure period and concentration of copper.

5.5.4.1.3. Serine

The level of Serine content in *L. vannamei* varied based on exposure concentration and time. Findings described that the amount of Serine was found to 2.76%, 2.59%, 2.24%, 2.13% and 2.01% *L. vannamei* after exposed to 0.112 ppm of copper at 0, 7, 14, 21 and 28 days respectively. After the end of exposure the lowest value of 1.43% was found in 0.224 ppm and for 0.112 ppm it was 2.01% (Fig.24). Result illustrated that no significant difference was observed between 0.224 ppm and 0.112 ppm concentration in all the exposure period. Whereas *L. vannamei* exposed to higher (0.224 ppm) copper concentration showed 2.64%, 2.29%, 1.97%, 1.82%, and 1.43% Serine in tissues for the respective exposure periods of 0, 7, 14, 21 and 28 days (Fig.30). Further, it is obvious that the level of Serine is decreased with increasing exposure period and concentration of copper.

5.5.4.1.4. Glycine

The level of Glycine content in *L. vannamei* varied based on exposure concentration and time. Findings described that the amount of Glycine was found to 9.81%, 9.14%, 8.69%, 7.96%, and 7.19% *L. vannamei* after exposed to 0.112 ppm of copper at 0, 7, 14, 21 and 28

days respectively. After the end of exposure the lowest value of 7.19% was found in 0.224 ppm and for 0.112 ppm it was 6.48% (Fig.25). Result illustrated that no significant difference was observed between 0.224 ppm and 0.112 ppm concentration in all the exposure period. Whereas *L. vannamei* exposed to higher (0.224 ppm) copper concentration showed 9.74%, 9.09%, 8.21%, 7.49% and 6.48% Glycine in tissues for the respective exposure periods of 0, 7, 14, 21 and 28 days (Fig.30). Further, it is obvious that the level of Glycine is decreased with increasing exposure period and concentration of copper.

5.5.4.1.5. Tyrosine

The level of Tyrosine content in *L. vannamei* varied based on exposure concentration and time. Findings described that the amount of Tyrosine was found to 2.65%, 2.43%, 2.17%, 2.09%, and 1.98% *L. vannamei* after exposed to 0.112 ppm of copper at 0, 7, 14, 21 and 28 days respectively. After the end of exposure the lowest value of 1.64% was found in 0.224 ppm and for 0.112 ppm it was 1.98% (Fig. 26). Result illustrated that no significant difference was observed between 0.224 ppm and 0.112 ppm concentration in all the exposure period. Whereas *L. vannamei* exposed to higher (0.224 ppm) copper concentration showed 2.47%, 2.15%, 2.03%, 1.87%, and 1.64% Tyrosine in tissues for the respective exposure periods of 0, 7, 14, 21 and 28 days (Fig.30). Further,

it is obvious that the level of Tyrosine is decreased with increasing exposure period and concentration of copper.

5.5.4.1.6. Proline

The level of Proline content in *L. vannamei* varied based on exposure concentration and time. Findings described that the amount of Proline was found to 4.28%, 4.12%, 3.89%, 3.64%, and 3.21% *L. vannamei* afterexposed to 0.112 ppm of copper at 0, 7, 14, 21 and 28 days respectively. After the end of exposure the lowest value of 2.89% was found in 0.224 ppm and for 0.112 ppm it was 3.21% (Fig.27). Result illustrated that no significant difference was observed between 0.224 ppm and 0.112 ppm concentration in all the exposure period. Whereas *L. vannamei* exposed to higher (0.224 ppm) copper concentration showed 4.58%, 4.05%, 3.74%, 3.12%, and 2.89% Proline in tissues for the respective exposure periods of 0, 7, 14, 21 and 28 days (Fig.30). Further, it is obvious that the level of Proline is decreased with increasing exposure period and concentration of copper.

5.5.4.1.7. Alanine

The level of Alanine content in *L. vannamei* varied based on exposure concentration and time. Findings described that the amount of Alanine was found to 1.02%, 0.99%, 0.87%, 0.84%, and 0.76% *L.*

vannamei after exposed to 0.112 ppm of copper at 0, 7, 14, 21 and 28 days respectively. After the end of exposure the lowest value of 0.69%was found in 0.224 ppm and for 0.112 ppm it was 0.76% (Fig.28). Result illustrated that no significant difference was observed between 0.224 ppm and 0.112 ppm concentration in all the exposure period. Whereas *L. vannamei* exposed to higher (0.224 ppm) copper concentration showed 1.18%, 0.91%, 0.84%, 0.78%, and 0.69% Alanine in tissues for the respective exposure periods of 0, 7, 14, 21 and 28 days (Fig.30). Further, it is obvious that the level of Alanine is decreased with increasing exposure period and concentration of copper.

5.5.4.1.8. Cysteine

The level of Cysteine content in *L. vannamei* varied based on exposure concentration and time. Findings described that the amount of Cysteine was found to 5.46%, 5.16%, 4.89%, 4.62%, and 3.87% *L. vannamei* after exposed to 0.112 ppm of copper at 0, 7, 14, 21 and 28 days respectively. After the end of exposure the lowest value of 3.62% was found in 0.224 ppm and for 0.112 ppm it was 3.87% (Fig.29). Result illustrated that no significant difference was observed between 0.224 ppm and 0.112 ppm concentration in all the exposure period. Whereas *L. vannamei* exposed to higher (0.224 ppm) copper concentration showed 5.51%, 5.25%, 4.75%, 4.13%, and 3.62% Cysteine in tissues for the

respective exposure periods of 0, 7, 14, 21 and 28 days (Fig.30). Further, it is obvious that the level of Cysteine is decreased with increasing exposure period and concentration of copper.

The amino acid level at the end of exposure period from protein of Indian white shrimp were presented in Figure.30. The non-essential amino acids in shrimp protein were aspartic acid, glutamic acid, serine, glycine, tyrosine, proline, alanine, and cysteine. The exposure of *L. vannamei* to low and high copper concentrations has affected the amino acid composition of the exposed shrimp protein. For instance, glycine, cysteine and proline were in higher level in protein of shrimps exposed with both concentrations of copper. At the day 28, among the amino acids measured, level of non essential amino acids, particularly glycine was found to be highest (6.48% for 0.224 ppm and 7.19% for 0.112 ppm) in shrimps exposed with both concentrations of copper followed by cysteine (3.62% for 0.224 ppm and 3.87% for 0.112 ppm). Whereas, alanine was found to be the lowest among amino acids at the end of 28 day exposure to copper. The amino acid proline also found to be in high levels (2.89% for 0.224 ppm and 3.21% for 0.112 ppm) at the 28 day of exposure. Whereas, amino acids such as aspartic acid, glutamic acid, tyrosine, alanine, and cysteine were found to be significantly lower when compared with other amino acids. At the end of exposure, the order of the

levels of amino acids were glycine > cysteine > proline > tyrosine > serine > glutamic acid > aspartic acid > alanine.

5.5.4.2. Essential amino acids

5.5.4.2.1. Histidine

Findings described that the amount of Histidine was found to 1.09%, 1.05%, 0.98%, 0.87%, and 0.82% *L. vannamei* after exposed to 0.112 ppm of copper at 0, 7, 14, 21 and 28 days respectively. After the end of exposure the lowest value of 0.76% was found in 0.224 ppm and for 0.112 ppm it was 0.82% (Fig.31). Result illustrated that no significant difference was observed between 0.224 ppm and 0.112 ppm concentration in all the exposure period. Whereas *L. vannamei* exposed to higher (0.224 ppm) copper concentration showed 1.18%, 1.02%, 0.95%, 0.81%, and 0.76% Histidine in tissues for the respective exposure periods of 0, 7, 14, 21 and 28 days (Fig.40). Further, it is obvious that the level of Histidine is decreased with increasing exposure period and concentration of copper.

5.5.4.2.2 Valine

The level of Valine content in *L. vannamei* varied based on exposure concentration and time. The present study observed that the amount of Valine was 23.78%, 22.87%, 22.64%, 21.42%, and 20.34%

L. vannamei after exposed to 0.112 ppm of copper at 0, 7, 14, 21 and 28 days respectively. After the end of exposure the lowest value of 20.16% was found in 0.224 ppm and for 0.112 ppm it was 20.34% (Fig.32). Result illustrated that no significant difference was observed between 0.224 ppm and 0.112 ppm concentration in all the exposure period. Whereas *L. vannamei* exposed to higher (0.224 ppm) copper concentration showed 23.41%, 22.49%, 22.12%, 21.05%, and 20.16% Valine in tissues for the respective exposure periods of 0, 7, 14, 21 and 28 days (Fig.40). Further, it is obvious that the level of Valine is decreased with increasing exposure period and concentration of copper.

5.5.4.2.3 Methionine

The level of Methionine content in *L. vannamei* varied based on exposure concentration and time. Our findings suggested that the amount of Methionine was 13.87%, 13.41%, 12.89%, 12.76%, and 11.97% *L. vannamei* after exposed to 0.112 ppm of copper at 0, 7, 14, 21 and 28 days respectively. After the end of exposure the lowest value of 11.42% was found in 0.224 ppm and for 0.112 ppm it was 11.97% (Fig.33). Result illustrated that no significant difference was observed between 0.224 ppm and 0.112 ppm concentration in all the exposure period. Whereas *L. vannamei* exposed to higher (0.224 ppm) copper concentration showed 13.17%, 13.02%, 12.74%, 12.63%, and 11.42%

Methionine in tissues for the respective exposure periods of 0, 7, 14, 21 and 28 days (Fig.40). Further, it is obvious that the level of Methionine is decreased with increasing exposure period and concentration of copper.

5.5.4.2.4 Iso leucine

The level of Iso leucine content in *L. vannamei* varied based on exposure concentration and time. The present study observed that the amount of Iso leucine was 12.82%, 12.41%, 12.14%, 11.89%, and 11.68% *L. vannamei* after exposed to 0.112 ppm of copper at 0, 7, 14, 21 and 28 days respectively. After the end of exposure the lowest value of 10.42% was found in 0.224 ppm and for 0.112 ppm it was 11.68% (Fig.34). Result illustrated that no significant difference was observed between 0.224 ppm and 0.112 ppm concentration in all the exposure period. Whereas *L. vannamei* exposed to higher (0.224 ppm) copper concentration showed 11.89%, 11.42%, 11.01%, 10.96%, and 10.42% Iso leucine in tissues for the respective exposure periods of 0, 7, 14, 21 and 28 days (Fig.40). Further, it is obvious that the level of Iso leucine is decreased with increasing exposure period and concentration of copper.

5.5.4.2.5. Phenylalanine

The level of Phenylalanine content in *L. vannamei* varied based on exposure concentration and time. Findings described that the amount of

Phenylalanine was found to 1.37%, 1.28%, 1.12%, 1.01%, and 0.98% *L. vannamei* afterexposed to 0.112 ppm of copper at 0, 7, 14, 21 and 28 days respectively. After the end of exposure the lowest value of 0.85% was found in 0.224 ppm and for 0.112 ppm it was 0.98% (Fig.35). Result illustrated that no significant difference was observed between 0.224 ppm and 0.112 ppm concentration in all the exposure period. Whereas *L. vannamei* exposed to higher (0.224 ppm) copper concentration showed 1.24%, 1.12%, 1.02%, 0.97%, and 0.85% Phenylalanine in tissues for the respective exposure periods of 0, 7, 14, 21 and 28 days (Fig.40). Further, it is obvious that the level of Phenylalanine is decreased with increasing exposure period and concentration of copper.

5.5.4.2.6. Leucine

The level of Leucine content in *L. vannamei* varied based on exposure concentration and time. Our assay, reported the amount of Leucine was 5.74%, 5.24%, 4.98%, 4.72%, and 4.21% *L. vannamei* afterexposed to 0.112 ppm of copper at 0, 7, 14, 21 and 28 days respectively. After the end of exposure the lowest value of 3.56% was found in 0.224 ppm and for 0.112 ppm it was 4.21% (Fig.36). Result illustrated that no significant difference was observed between 0.224 ppm and 0.112 ppm concentration in all the exposure period. Whereas *L. vannamei* exposed to higher (0.224 ppm) copper concentration showed

5.89%, 5.13%, 4.26%, 3.95%, and 3.56% Leucine in tissues for the respective exposure periods of 0, 7, 14, 21 and 28 days (Fig.40). Further, it is obvious that the level of Leucine is decreased with increasing exposure period and concentration of copper.

5.5.4.2.7. Lysine

The level of Lysine content in *L. vannamei* varied based on exposure concentration and time. Findings described that the amount of Lysine was found to 13.67%, 13.12%, 12.87%, 12.51%, and 12.01% *L. vannamei* after exposed to 0.112 ppm of copper at 0, 7, 14, 21 and 28 days respectively. After the end of exposure the lowest value of 11.85% was found in 0.224 ppm and for 0.112 ppm it was 12.01% (Fig.37). Result illustrated that no significant difference was observed between 0.224 ppm and 0.112 ppm concentration in all the exposure period. Whereas *L. vannamei* exposed to higher (0.224 ppm) copper concentration showed 13.82%, 13.01%, 12.31%, 12.12%, and 11.85% Lysine in tissues for the respective exposure periods of 0, 7, 14, 21 and 28 days (Fig.40). Further, it is obvious that the level of Lysine is decreased with increasing exposure period and concentration of copper.

5.5.4.2.8. Arginine

The level of Arginine content in *L. vannamei* varied based on

exposure concentration and time. Findings described that the amount of Arginine was found to 1.20%, 1.12%, 1.09%, 0.95%, and 0.87% *L. vannamei* after exposed to 0.112 ppm of copper at 0, 7, 14, 21 and 28 days respectively. After the end of exposure the lowest value of 0.71% was found in 0.224 ppm and for 0.112 ppm it was 0.87% (Fig. 38). Result illustrated that no significant difference was observed between 0.224 ppm and 0.112 ppm concentration in all the exposure period. Whereas *L. vannamei* exposed to higher (0.224 ppm) copper concentration showed 1.41%, 1.06%, 0.95%, 0.86%, and 0.71% Arginine in tissues for the respective exposure periods of 0, 7, 14, 21 and 28 days (Fig.40). Further, it is obvious that the level of Arginine is decreased with increasing exposure period and concentration of copper.

5.5.4.2.9. Tryptophan

Findings described that the amount of Tryptophan was found to be 1.41%, 1.35%, 1.28%, 1.05%, 0.96% *L. vannamei* after exposed to 0.112 ppm of copper at 0, 7, 14, 21 and 28 days respectively. After the end of exposure the lowest value of 0.87% was found in 0.224 ppm and for 0.112 ppm it was 0.96% (Fig.39). Result illustrated that no significant difference was observed between 0.224 ppm and 0.112 ppm concentration in all the exposure period. Whereas *L. vannamei* exposed to higher (0.224 ppm) copper concentration showed 1.62%, 1.24%, 1.13%,

0.98%, and 0.87% Tryptophan in tissues for the respective exposure periods of 0, 7, 14, 21 and 28 days (Fig.40). Further, it is obvious that the level of Tryptophan is decreased with increasing exposure period and concentration of copper.

The essential amino acid level at the end of exposure period from protein of Indian white shrimp were presented in Figure.40. The essential amino acids in shrimp protein were histidine, valine, methionine, iso leucine, phenylalanine, leucine, lysine, arginine and. tryptophan. The exposure of *L. vannamei* to low and high copper concentrations has affected the essential amino acid composition of the exposed shrimp protein. For instance, valine, Lysine, methionine, and Iso leucine were in higher level in protein of shrimps exposed with both concentrations of copper. At the day 28, among the amino acids measured, level of essential amino acids, particularly valine was found to be highest (20.16% for 0.224 ppm and 20.34% for 0.112 ppm) in shrimps exposed with both concentrations of copper followed by lysine (11.85% for 0.224 ppm and 12.01% for 0.112 ppm). Whereas, Histidine was found to be the lowest (0.76% for 0.224 ppm and 0.82% for 0.112 ppm) at 28^{th} day. The amino acid methionine (11.42% for 0.224 ppm and 11.97% for 0.112 ppm) and Iso leucine (10.42%for 0.224 ppm and 11.68% for 0.112 ppm) also found to be in high levels at the 28 day of exposure. Whereas, amino acids such as Histidine, Phenylalanine, leucine, Arginine and tryptophan

were found to be significantly lower when compared with other amino acids. At the end of exposure, the order of the levels of amino acids were valine>lysine > methionine > iso leucine > leucine > phenylalanine > tryptophan >arginine > histidine.

5.6 Effcets of copper toxicity on the histopathological changes in *L.vannamei*

5.6.1 Histology of Muscle

Muscle is the tissue of motion and is widely distributed in various organs of the body. Muscle tissue of *L.vannamei* reared at without copper treatment revealed normal histoarchitecture. It is composed of elongated muscle fibers, each an individual muscle cell, held together by connective tissues. The photomicrograph of the muscle (Plate3.A and B.) depicted the presence of normal myotomes (MT) with equally spaced muscle bundles the fascicular arrangement of myofilaments (MF) with emarginated epimysium, binding to connective tissue and tendon at the extremities of the smooth muscles. The striated muscle fibres (SM) were tightly packed. The nuclei were arranged along the margins of the muscle bundles (Plate 4 A &B).

5.6.2. Histopathology of muscle

After 7 days of exposure to 0.112ppm concentration of copper the muscle histoarchitecture displayed severe alterations in the muscle tissue showed highly disintegrated epidermis (DE) with vacuolation, gap

formation (GF) in between the muscle bundles, necrosis (NE), marked thickening and separation of muscle bundle and pronounced intramuscular oedema with minor dystrophic changes (Plate 4.C) In lower concentration at 28 days of exposure the muscle bundles are completely disrupted with discontinuity of striations and complete disappearance of nuclei. In some regions of muscle tissue there is sloughing of epidermal layer (SEL) observed (Plate 4.D) Lesions (LN) and mild haemocyte infiltrations (HI) are the marked changes in fusion of muscle bundles (FMB) were observed at 0.224ppm concentration of copper after 7 days of exposure (Plate 4.E). At the end of 28^{th} day the muscle tissue expressed significant changes like broken myofibrils (BMF), coagulative necrosis (CNE) congestion of muscle bundles followed by rupture of muscle bundles. Severe haemocyte infiltration (HI) and accumulation of granular materials in between the muscle fibers (GMF) are also noted (Plate 4.F).

5.6.3 Histology of Hepatopancreas

It is the most important organ of store and detoxification of xenobiotics in crustaceans. This organ is highly responsive to physiological and environmental variations. The hepatopancreas tenanted the major part of the cepahalothorax and projects into abdomen into some extent with orange colouration. It is made up of many tubules. These tubules are internally lined with columnar epithelium and are supported

by meager connective tissue (CT). The epithelial luminal surface has fluctuating shape due to varied height of cells. The epithelial cells comprised of four different cell types, namely, E- embryonic, F- Fibrillar, B- vesicular and R- reabsorption cells. The columnar epithelium is present with many small vacuoles (V) assembled in cytoplasm with few basophilic granules. The nuclei and nucleolus is highly evident (Plate 4 A). The hepatopancretic tubule lumen (Lu) displays various shapes due to different heights of epithelial cells (Plate.5 A & B).

5.6.4 Histopathology of Hepatopancreas

Under the light microscope in lower concentration after 7 days, there are no conspicuous variations in the hepatopancreatic tubule in the experimental shrimp. In lower concentrations the connective tissue becomes very thin and meager looks like spongy (SCT). When the concentration increases, there is a proliferation of hepatopancreatic tubular cells (PHT). There is extensive vaculoations (EV) in different cell types and the tubules did not remain intact in most of the regions (Plate 5 C). Due to the proliferation of different cell types the columnar epithelial region burst in some parts of the tubule and basophilic granules moves in to lumen is evident in higher concentrations. In higher concentration, the star shaped lumen of the hepatopancreatic tubule pressed in many parts and it appears that the lumen is squeezed into the

epithelial cells (SLu) (Plate 5 D). The different cell types are inflamed and swollen in many areas with the secretions. There is a general degeneration of the tubule with intertubular spaces and complete disorientation with necrosis.

In the 28th day in lower concentration the picture of hepatopancreatic tubule depicts the expanded vacuolation, more secretion into the lumen, rupture of epithelial cell lining here and there and sloughing of tubular epithelium. The light microscopic picture depicts the enlargement of nuclei and nucleolus (Plate 5E). In higher concentrations, there is more vacuolation (EV) in various cell types. The epithelial cells highly disfigured (DCE) and clumped with poor intercellular spaces. Infiltration of haemocytes become highly evident after 28 days of exposure in experimental animals. The columnar epithelial cells of hepatopancreatic tubules becomes highly disintegrated (DT) and denuded leaving basal lamina alone in major part of the tissue (Plate 5F).

5.6.5 Histology of Gill

The control animal consists of eight pairs of gills on lateral sides typical to phyllobranch type. The gill tissue exhibits a gill base in which two rows of gill plates are present (Plate 6A) and gills are attached to the body through gill roots. The gills of control shrimp appeared normal with spaced gill plates arranged serially in pairs along a gill stem. The central

axis of the gill tissue is the primary gill lamellae and it divides further into secondary gill lamellae or filaments. In the control shrimp, the gill tissue shows a thin layer of cuticle which covers the entire outer surface and underlying the cuticle is a continuous layer of epithelial cells. At irregular intervals, there are pillar cells, which join the lamellae. The epithelial cells of the lamellae are continued as the lining of the gill stem and large connective tissue cells compose the chief support of the gill stem (Plate 6 B).

5.6.6 Histopathology of Gill

The light microscopic pictures in lower concentration after 7 days of exposure depict some architectural changes in the gill tissue of experimental specimens. The epidermal covering around the lamellae showed degenerative nature (Plate 6 C). Gill epithelial cells in the lamella exhibits flattened appearance with necrotic cells. The gill lamellar cells collapses in experimental animal due to disruption of pillar cells. At higher concentration after 7 days of exposure, there is thickening of gill lamellae and infiltration of haemocytes. In later stages, there is detachment of cuticle and bursting of capillaries in the secondary lamellae. Necrosis of epithelial cells (NEC) and hyperplasia (HP) are highly evident in higher concentrations (Plate 5 D).

After 28 days of exposure in higher concentrations, there is swelling of secondary gill lamellae (SSF) and poor arrangement of secondary gill lamellae with necrotic lesions. In the experimental gills there is edema and rupture of epithelial cells (EREC) and pyknotic nuclei are clearly evident in the pictures (Plate 6 E). Infiltration of haemocytes (IH) appears in some filaments and results in the secondary lamellar swelling. In higher concentration, the gills exhibited lamellar fusion (LF) in some regions because of filamentary epithelium proliferation (Plate 6 F).

5.6.7 Histology of Lymphoid organ

The Lymphoid organ (LO) consists of two distinctly clear lobes, located ventro lateral to the junction of the stomach. The lymphatic folded tubules are with a central haemal lumen and a wall, layered with cells. Under the light microscope, the lymphatic tubules consist of a lumen surrounding stromal matrix of cells with interstitial sinuses and fibrous connective tissue. The tubular lumen consists of circulating haemocytes (HC) The lumen is lined with endothelial layer consists of deeply stained cytoplasm. Haemal sinuses and a delicate fibrous material occupy spaces between the lymphatic tubules (LT). There are number of cells observed in controls with a clear cytoplasm and deeply stained clear nuclei (Plate 7 A & B).

5.6.8. Histopathology of Lymphoid organ

After 7 days in experimental shrimp, the lymphatic cells undergo inflammation and degradation of nucleus (IDNu) observed (Plate 7 C). The light microscopic pictures clearly depict the degranulation of haemocytes (DHC) in the lymphatic tubular lumen. In higher concentrations, the haemocytes burst out and disorientation occurs. There is clumping of lymphatic cells occurs and necrosis of cells (Plate 7 D). After 28th day of the experiment it is highly evident that from the lumen, more number of haemocytes enters the endothelial cells, move into the stromal matrix. Appearance of highly scattered pyknotic nuclei (PKNu) and lesions in the lymphatic tubular cells. There is many deeply stained spheroid cells (LOS) appear along with normal lymphatic cells (Plate 7 E). The lymphoid spheroid cells (LOS) present in the haemal sinuses with basophilic cytoplasm and there is absence of lumen. These LOS are irregularly arranged with few vacuoles in between. Necrotic and apoptotic cells are frequent in higher concentrations (Plate 7 F).

5.6.9 Histology of Nerve cord

The nervous system of control animal consists of supraoesphagial ganglion connected to a ventral nerve cord or nerve centres. The nerve cords originate from the postero-lateral part of supraoesopharyngeal ganglion or brain, run downwards around the oesophagus to unite with

ventral nerve cord. In the control animal the ventral nerve cord is formed by a pair of longitudinal nerve strands and features a fused subesophageal ganglion comprising the mandibular, maxillular, and maxillar neuromeres; nine segmental thoracic ganglia; and eight segmental abdominal ganglia, while the abdominal segments 9 and 10 lack ganglia. Many nerve cells are seen with deeply stained basophilic cytoplasm and nuclei (Plate 8 A &B).

5.6.10. Histopathology of nerve cord

After 7 days of copper exposure the experimental animal the nerve cord shows distinctive changes. In lower concentrations, the nerve cells with abnormal nuclei or misshapen nuclei. This leads to decrease in the normal nuclear density of the nerve cells (Plate 8 C). The interstitial spaces between the nerve cells show the presence of vacuolization. The light microscopic picture depicts the thickening of fibrous tissue capsule (Plate 8 D). In the experimental animal at higher concentrations after 28 days, there is infiltration of haemocytes and inflammation of nerve cells (Plate 8 E). There is edema, rupture of epithelial cells and degeneration of nerve cells appears. In the experimental animal, the nerve cells undergo necrosis and nuclear degeneration (Plate 8 F).

6. DISCUSSION

6.1 Acute toxicity test

The ability to detoxify toxic substances is of substantial importance to the organisms in an environment contaminated with heavy metals for its survival. The production of low molecular weight proteins that can bind with the heavy metals provides a way to detoxify the targeted heavy metal. For example, copper binding protein probably functions as a detoxifying feedback mechanism in which copper is bound up in an inert metabolic complex, preventing copper from binding with other sensitive sites (David, 1979).

Acute toxicity test is the measurement of the short-term lethality of a pollutant, by which, the concentrations of the substance proves to be lethal to the animals (Sparague,1969). Brongs and Mount (1978) stated that the LC_{50} value application is the significant related test for assessing the potential adverse effects of chemical contaminants of aquatic life. Bryan (1976) listed a series of factors, which influence the toxicity of heavy metals in solution are the dissolved form of metals, the presence of other metals and factors influencing the physiology and behavior of the organisms. Copper is highly toxic to crustaceans and in the present investigation, the median lethal concentration (LC_{50} for 96 hrs) of copper, in *L. vannamei* was determined to be 1.113ppm/l.

The information suggested that lobsters are highly sensitive to copper compared other animals (Mc Leese, 1974). The observation of higher toxicity of copper to the spiny lobsters, *P.homarus* and more or less similar LC_{50}, values for copper (22-33hrs = 1.0mg/1 and 105hrs = 0.8mg/1) in the American lobster *Homarus americanus*. A current finding indicated that the lethal toxicity of *L. vannamei* in shrimp farming the copper residues was analyzed in the pond water. It was observed that the concentration (2mg/1) of copper concentration in shrimp farming within few days after resulted in killing all shrimps without no external symptoms and any other diseases. As a result, it is clear that shrimps are highly sensitive to copper, and even exposure to sublethal concentrations might seriously impair its survival during the culture.

Species	Element	LC_{50}	Source
Penaeus indicus	Cu	48hrs=1.72mg	Govindrajan *et al*., 1993
Penaeus monodon	Cu	96hrs=1.20mg	Joseph *et al*., 2002
Paratelphusa hydrodroma	Cu	96hrs=114mg	Vardhanan and Radakrishnan. 2002
Balanus balanoides	Cu	6hrs = 0.23mg	Pyefinch and Mott, 1948
Balanus crenatus	Cu	6hrs = 1.2mg	Pyefinch and Mott, 1948
Crangon clangor'	Cu	96hrs = 1900pg	Portmann and Wilson, 1971
Homarus americanus	Cu	22-33hrs = 1.0mg	Mcleese,1974
Homarus americanus	Cu	105hrs =0.08mg	Mcleese, 1974
Paragrapsus quadridentatus	Cu	96hrs = 0.17ppm	Ahsanullah and Arnott, 1978
Homarus americanus (LS1)	Cu	96hrs = 0.048mg	Johnson and Gentile, 1979
Callianassa australiensis	*Cu*	96hrs =1030pg 168hrs = 340pg 240hrs = 220pg	Ahsanullah *et al.,* 1981
Penaeus merguiensis	*Cu*	96hrs = 350pg	Denton and Burdon-Jones, 1982
Metapenaeus dobsoni	Cu	96hrs = 2.25ppm	Sivadasan *et al.,*1986
Paratya australiensis	Cu	48hrs =43pg 96hrs =34pg 144hrs =30pg	Daly *et al.,* 1990
Macrobrachium lamarrei	Cu	96hrs = 0.247ppm	Murti and Sukla, 1984
Penaeus japonicas	Cu	48hrs =2500pg 96hrs =2050pg	Yulianto Bambang *et al.,* 1995
Panulirus Homarus	Cu	12hrs =190.5pg 24hrs =158.5pg 36hrs =141.2pg 48hrs =125.9pg 72hrs =112.2pg 96hrs =95.5pg	Maharajan, 2002
Litopenaeus vannamei	Cu	24hrs = 1.746ppm 48hrs =1.575 ppm 72hrs =1.407 ppm 96hrs =1.113ppm	**Present study**

Table: 9. LC_{50} values of copper toxicity in shell fishes

6.2 Morphological colour changes

The muscle tissue of *L. vannamei* exposed to 28 days of 0.224 concentration of copper reveals considerable colour changes. The normal muscle is creamy white, it changes to yellowish black, and this corroborates with *Jasus lalandii* (Paterson, 1968). Hence, changes in morphology and colouration of internal organs in *L. vannamei* exposed to sublethal levels of copper indicate that changes can be used as imperative biological indicators of heavy metal toxicity.

Due to copper toxicity the colour changes of the gill tissue in *L. vanname* is *clearly* evident. The observation of normal white colour changed to greenish black was related with concentration of copper toxicity. During the long term exposure of sublethality, the gill tissue changed to black colour with many copper deposits (as granules) on the gill rackers. A thick black deposits bordered on either sides of the lamellae. The pathological observation such as gill blackening and black deposits in cell cytoplasm in marine shrimps exposed to copper suggested that the discolouration of gills is common due to heavy metal pollution. Viarengo (1985) also reported similar effects due to copper deposits on gills of various species of marine penaeid shrimps. In other crustaceans blackening of gills and various other lesions have described due to heavy metal toxicity (Cough,1977).

Generally, the colour of the hepatopancreas is yellowish brown in *L. vannamei* and upon exposure to copper the colour changed to greenish black thus reveals the toxicity of copper. In the European lobster *H. gammarus* copper exposure changes the hepatopancreas to yellowish green (Barker and Gibson, 1977), in the spiny lobster *Panulirus polyphagus* orange colour hepatopancreas changed in to greenish black due to intervention of copper (George Reuben and Muthe 1955), in *P malcomsonii* orange red colour hepatopancreas changes to bluish black due to heavy metal toxicity. In *Metapenaeus bennettae* (Dall, 1957) and *Scylla serrata* long term exposure to copper resulted in yellowish brown colouration in hepatopancreas (Barker and Gibson,1978).

Similar observations on colour changes were reported in other crustaceans like *Cancer pagurus* (Pearson, 1908) and *Paratelphusa hydrodromus* (Reddy,1938). The change of colour in hepatopancreas is the effect of copper on 13 carotene which form the principal colouration in hepatopancreas and accounts for 40-70% of the total carotenoid content (Nicoara *et al.*, 1970). The early findings in the *P.monodon* showed that the colour changes of the cuticles occurs in response to the absorption and deposition of the copper in the cuticles (Vogt and Quinitio, 1994). The present study also observed that subtle changes in colouration of internal organs can be used as indicator of stress due to copper toxicity in spiny lobster, *L. vannamei* .

6.3 Bioaccumulation

This study is to investigate the bioaccumulation of copper in the body of shrimp *L. vannamei*, as well as its impact on fundamental biochemical markers that are frequently used as indicators to monitor shrimp health condition. Heavy metal accumulation varies between different tissues in various crustaceans and between various metals at different concentrations, which has been thoroughly studied (Vijayakumaran,1990). The rate of accumulation and the regulation of copper has been well established for various species of fin and shell fishes (Bryan and Ward, 1965). Several studies on crustaceans concluded that copper is deposited in the hepatopancreas, which is the major storage organ in decapods followed by gills (Vogt and Quinitio, 1994; Rainbow, 1998). No granular formation is showed in the muscle tissue of *L. vannamei*, which seems to be a general feature in decapods (Martin, 1973; Roldan and Shivers, 1987) and is explained by the differences in the susceptibility of the tissues to a certain metal and by the confinement of detoxification pathways to particular tissues (Nott,1991). The accumulation of copper in the muscle, hepatopancreas, gills and exoskeleton of *L. vannamei* showed increasing trend as the duration of exposure to two different concentrations of copper were increased.

Among the crustaceans, it was observed that *Panulirus homarus* accumulate plenty of copper as granules in the hepatopancreas

(Maharajan, 2002). The hepatopancreas of mature *P.homarus* appeared blue due to high copper deposits (Vijayakumaran,1990). The present study in *L. vannamei* supports the observation of copper accumulation in the hepatopancreas. The least interspecific variation is known for copper in muscle tissue than the gills (present study), since all decapods have a capacity to deposits more copper in the hepatopancreas.

The copper level often varies within the tissues tending to be concentrated in areas of mobilization and transformation or excretion of the organism. The hepatopancreas of crustaceans is often rich in copper, as a result of metal storage and mobilization (Hilmy *et al.,*1988) The capacity to accumulate and tolerate higher concentration of copper implies that *L. vannamei* possesses mechanism to prevent interaction of the toxic metal with essential enzymes. Previous findings suggested that crustacean muscle shows a very low degree of heavy metal uptake relative to other tissues (White, and Rainbow, 1986). Likewise, the present study also suggested that the accumulation of copper in muscle is very low.

The marine amphipods and penaeid prawn, *P.indicus* exhibited slow buildup of metal in different tissues (Ahsanullah and Williams, 1991). In *P. elegans* different copper concentrations exposure, the net accumulation in muscle tissue was lower than the gills and hepatopancreas (White and Rainbow,1982). The results in *L. vannamei* is

in accordance with the barnacle *Elminus modestus* and in the mud crab, *Scylla serrata* where a maximum level of copper was reported in the hepatopancreas. The concentration of copper in *L. vannamei* found doubled in the hepatopancreas than the other tissues as in the case of *Palaemon elegans* (White and Rainbow,1982). Similar findings in gills of sunfish exposed to a sublethal concentration of copper, the gills exhibited significant increase of the metal exposure (Felts, and Heath,1984).

From the present study it is apparent that copper accumulation in *L. vannamei* follows a pattern such as hepatopancreas > gills > Exoskeleton> muscle. It is suggested that the low level of metal accumulation in muscle is due to isolation of the muscle tissue from the surrounding tissues by the branchial epithelium protects against further invasion of the toxicant (Mallatt, 1985). The gills act as a barrier accumulating a major part of the heavy metal. The inhibition of copper excretion and detoxification results in anoxia where energy metabolism decreased and contrastingly accumulation increases (Depledge, 1987).

6.4. Haematology

The circulating haemocyte number is a stress indicator, and haemocyte counts may be an expensive tool for evaluating the health state of crustacean species (Le Moullac and Haffner, 2000). The study of haemocytes in crustacean contributes to the accumulation of basic knowledge about haemocytes, particularly the physiological status. When

exposed to nurocombi, the freshwater crab *P.jacquemontii* revealed haematological abnormalities (Maharajan *et al*., 2017).

Copper is toxic to aquatic organisms at lower and higher concentrations (Baldwin *et al*., 2003). Copper is being a transitional metal usually participates in Fenton and Habere Weiss reactions and help the generation of ROS which results in oxidative stress (Regoli and Principato,1995). There is a link between the external environment and circulatory system of fish, analysis of blood assays helps to identify the physiochemical changes and health status of fish exposed to different toxicants (Lavanya *et al*., 2011; Sampaio *et al*., 2012; Poopal *et al*., 2017). Haematological variables may be used as a possible biomarker in the clinical diagnosis of fish health and also helps to assess the toxic effects of xenobiotic substances (Tellez- Banuelos *et al*., 2009; Krishnapriya *et al*., 2015, 2017). Moreover, these parameters will give the health status of fishes (Adriana *et al*., 2007; Harikrishnan *et al*., 2011; Saravanan *et al*., 2017).

In the present investigation, a significant alteration has noted in haematological profiles of the shrimp exposed to two different sublethal concentrations of copper. Hamoglobin is a protein responsible for carrying oxygen in the body, and its concentration is closely related to red blood cell counts (Clark *et al*., 1989). Hct is the ratio of blood volume that is occupied by red blood cells, expressed as a percentage of total

blood volume. The decrease in the haemoglobin and hematocrit value of Copper treated fish during acute and chronic toxicity studies may be due to lysing of erythrocytes due to toxicant stress whereas increased Hb and Hct values of copper treated fish due to toxicant exposure (Lavanya *et al*., 2011).

Red blood cells are the most common cells seen in the fish blood (Bastami *et al*., 2009). In the present investigation, RBC count was increased significantly copper exposed shrimp during acute study when compared with control shrimp due to the reduction in the oxygen level in the blood resulted from the histological alterations in the gill lamellae due to accumulation of copper. A fall in RBC count in *Oreochromis mossambicus* (Nussey *et al*., 1995), *Heteropneustes fossilis* (Singh and Reddy, 1990) and in *Dicentrarchus labrax* (Gwoździński *et al*., 1992), when exposed to copper, may be due to hemolysis caused by copper toxicity. In general, hemolysis causes a fall in RBC count (Hedayati *et al*., 2016). Similar results in fresh water fish treated with other metals such as arsenic (Cockell *et al*., 1992; Lavanya *et al*., 2011), arsenate (Kavitha *et al*., 2010) cadmium (Remyla *et al*., 2008) and bisphenol (Krishnapriya *et al*., 2017).

White blood cells are responsible for the immune function of an organism (Jurd, 1985). Davis, (2008) reported that variations in leucocytes counts are useful in the field of conservation physiology as

they directly altered by stress and their role in the regulation of immunological functions. In the present investigation leucocyte count was significantly increased in all the two groups of copper treated fish when compared to control shrimp which may be due to the direct effect of the copper in the shrimp.

Nanoparticles based studies reported that CuNPs have similar types of toxic effects on haematology and biochemistry of rainbow trout (*Oncorhynchus mykiss*) as compared to $CuSo_4$ which can occur at lower tissue Cu concentrations than expected for the dissolved metal (Shaw *et al*., 2012). Likewise, Wang *et al*. (2014) reported the dissolved Cu was more toxic than Cu-NPs to juvenile *E. coioides*.

A frequent pathogenic reaction in lobsters and crabs experimentally infused with endotoxins was a decrease in the number of circulating haemocytes (Newman and Feng 1982). The decrease in the quantity of circulating haemocytes alter the development of haemocyte aggregates in the blood sinuses (Smith, *et al*., 1984). In crustaceans the total heamocytes count is a measure of the individual's defence, as the number of circulating haemocytes might indicate the defence system of the host (Brehelin *et al*., 1978). In the present investigation the total haemocyte count (THC) of the shrimps exposed to copper dramatically decreased. Haemocytes were found in four different types. After exposure the proportion of granule haemocytes in shrimps decreased.

6.5 Proximate composition

The observation and documentation of stress specific biological, physiological and behavioral aspects of any organisms in aquaculture farms aid us to take corrective and preventive measures in large scale to avoid substantial economic loss. In general, crustaceans mobilize maximum energy from muscle which might possibly indicate the first phase of detoxification. The increment in weight-age of the gills when exposed to copper in *L. vannamei,* was related with the increment of water holding capacity which correlated with catabolism of large molecules of proteins and fats into smaller molecules, subsequently increase the osmotic pressure resulting in tissue hydration (Nuwayhid and Young, 1985).

Protein are the major energy reserves in crustaceans. The sequence of utilization of these reserves and the relative importance of the muscle tissue as storage organs during adverse conditions however vary among species (Armitage *et al.,* 1972; Barclay *et al.,* 1983; White and Rainbow,1986). Contrastingly, in the spiny lobster *P.ornatus*, the hepatopancreas is reported to be a more sensitive indicator of physiological stress than the muscle tissue (Trendal and Prescott, 1989).

In the present study, the total protein in the muscle, hepatopancreas and gills of *L. vannamei* showed decreasing trend as the duration of

exposure to copper increased (muscle: 19.39% to 16.38%, hepatopancreas: 16.19% to 11.33% and gills: 4.60% to 2.77%). It is likely that the observed reduction in total protein of *L. vannamei* is due to a direct consequence of the stress imposed by copper. The depletion of total protein suggests an increased proteolysis and possible utilization of the products of their degradation for metabolic purpose. The decreased protein level during exposure to pollutants may be due to increased catabolism and decreased anabolism of proteins as reported in freshwater bivalve *Parrysia corrugata* (Deshmukh and Lomte, 1998).

A decrease in protein content in shrimps in mangrove crab *Sesarma brockii* increases the amino acid pool which may be attributed towards its conversion to the detoxification mechanism of the heavy metal, cadmium (Kannupandi *et al.*, 2001). Copper causes adverse effects on the lysosomal membrane as stabilizers and release nucleases and proteases thereby affecting the RNA and protein metabolism. Subsequently, the lower rate of protein synthesis depends on the reduced availability of ATP or an increased activity of proteolytic lysosomal enzymes (Jana and Choudhari, 1984).

Cellular and biochemical changes have also been found in the oyster *Crassostrea viriginica* (Cheng, 1988; Farley, 1988) on exposure to heavy metal. Similar reduction in total protein is evidenced by Moore

(1988) in *Cyclope neritea* and *Littorina littorea* exposed to high levels of copper. It was suggested that the effects of copper on the oyster to a reduced ability to phagocyte bacteria and thus a reduced capability of defence mechanism.

Concentration of copper in the medium as well as the exposure duration influenced the protein profile in *L. vannamei.* Similar alterations due to copper was highlighted in the dog fish (Tort *et al.,* 1987), the crab *Carcinus muenas* (Wright and Brewer, 1979) and the penaeid prawn *Penaeus indicus* (Seenivasan, 1988). Similarly, Krishnamoorthy and Subramanian (1995) reported a decrease in the protein content during the process of copper accumulation in freshwater prawn *Macrobrachiurn lamerrei lamerrei.* All these findings collectively suggested that the accumulation of copper alters the levels of protein.

The initial declining levels of protein may be attributed to the higher rate of energy production at the onset of various enzymatic blockages. It is known that copper blocks the mRNA synthesis and thereby the protein synthesis at the level of transcription. However, copper inhibits the action of enzyme protease reflecting a steady decline in the total percentage of protein.

Carbohydrates are very important biological compounds as they are the chief source of energy and also structural constituents of

protoplasm. In the present study the total carbohydrate in the muscle, hepatopancreas and gills of *L. vannamei* showed decreasing trend with increasing concentration of copper (muscle: 0.51% to 0.32%, hepatopancreas: 1.32% to 0.78% and gills: 0.43% to 0.27 %). But a narrow increase is observed during 7 days of copper exposure in gills (0.43% to 0.45%). It may be due to the breakdown of glycogen to cope with the high energy demand for the detoxification process, since carbohydrate forms major source of energy under toxicity (Hochachka and Somero, 1973).

Changes in the percentage of carbohydrate in response to differential concentrations as an indirect response to internal hypoxia is well explained in cat fish *Heteropneustes fossilis* (Misshra and Srivastava, 1983). In *L. vannamei* also, a decrease in percentage of total carbohydrate in muscle and hepatopancreas has been induced by copper. Excess in dietary copper has been shown to cause lower percentage of carbohydrate in *Homarus americanus.* Similar result is evidenced by Jana and Sahana (1988) in fresh water fish *Claxias batrachus.*

The association between carbohydrate and copper metabolism is complex and reduces its bioavailability in *Arnphora coffeaeformis* (Brown *et al.*, 1988). However, on exposure to heavy metal, glycogen content was found to be reduced in the hepatopancreas and muscle tissues

of brook trout, (Mckim and Benoit,1971). Glycogen plays an important role as a readily mobilized storage form of carbohydrate in muscle (Stryer, 1988), which decreases during toxicity as evidenced in *L. vannamei* also. Similarly, the carbohydrate declines in the gills and it deposit as granules on the lamellae interrupting the uptake of oxygen. This is consistent with the earlier studies in Norway lobster, *Nephrops noruegicus* resulting in hypoxic condition (Baden *et al.*, 1994), where the binding of oxygen with haemoglobin decreases with the increase in concentration of copper, thereby resulting in hypoxia (Depledge and Bjerregaard, 1989). The carbohydrate, may have been used to fuel detoxification mechanism operating within the animal as reported in *P.longipes* and *Jasus lalandii* (Cockcroft, 1997).

In aquatic organisms, the carbohydrate reserves may be rapidly utilized under unfavorable conditions and the great variations found in the tissues indicate that the level of mobilizable carbohydrate reserves may fluctuate widely and rapidly in response to fluctuations in the nutritional state of the animal. In the present study the carbohydrate content decreased in the gill tissues of *L.vannamei* exposed to sublethal concentrations of copper (Table 1). The fenvalerate exposed *Ctenopharyngodon idellus* showed a decrease in the carbohydrate content in the various tissues (Tilak and Yacobu, 2002). The decrease in total carbohydrate level signifies it utility to meet the higher energy demands

of the fish reeling under metal toxicity. Therefore, the synthesis and utilization of carbohydrate are altered in the organism subjected to copper stress.

Carbohydrates supply the major portion of the metabolities for the energy requirements in a normal individual is oxidized for the energy requisites. Carbohydrates are converted to glycogen or shunted in the metabolic pathway to supply the carbon chain for aminoacids or converted in to fat. At sublethal concentration, when the liver carbohydrate content decreased and the haemolyph sugar level increased suggests the breakdown of hepatopancreas glycogen (glycogenolysis). The glucose movement from the hepatopancreas to the haemolymph and the availability for utilization by the needy tissues for ensuring normal metabolic processes in the body appears inevitable when the prawn exposed to toxic medium. Many authors claimed that decreased carbohydrate level in various tissues of aquatic organisms during the toxicity study. Due to pesticide exposure in *C.punctatus*, there is quantitate variations in the sugar content of liver and muscle tissues (Visvanathan *et al.*, 2009).

In the present study the muscle carbohydrate content of *L. vannamei* showed a decrease when exposed to sublethal concentrations of copper. There is a fall in muscle carbohydrate level in *L. rohita* when

exposed to tannery, electroplating and textile effluents (Muley *et al*., 2007). The high concentration of Nuvacron caused a decline in muscle carbohydrate level in *C.punctatus* (Sastry and Dasgupta, 1991). These observations were in conformity with the reports on the fall in muscle glycogen level in *C.punctatus*, when exposed to organophosphate pesticide, Dimethoate (Tripathi *et al.*, 2003). Studies in general have suggested that exposure to metal treatment interferences with the carbohydrate metabolism. A greater decrease of carbohydrate content indicates greater utilization of carbohydrate to cope with enhanced metabolism under stressful situations. Despite a continuous and rapid release of glucose by glycogenolysis in the hepatopancreas, there is a fall in the overall carbohydrate content in prawns subjected to heavy metal treatment is imminent. Proteins are mainly involved in the architecture of the cell and during chronic period of stress, they are also a source of energy (Umminger, 1970).

Lipid is an important constituent of animal tissue, which plays a prime role in energy metabolism. It is well established that lipids are important in cellular and sub-cellular membranes. A gradual decrease in the lipid content in various tissues of *L. vannamei* after chronic treatments of copper. Earlier researchers suggested that the decrease in lipid content in *C. carpio* is either due to the uptake of lipid by the tissue for utilization at cellular levels or due to increased lipolysis or mitochondrial injury,

which affect the fatty acid oxidation (Ware, 1980; Anusha *et al*., 1996). The considerable decrease in total lipid in tissues might be due to drastic decrease in glycogen content in the same tissue which is an intermediate source of energy during toxic stress conditions. After glycogen, lipid content may be used for energy production to over come toxic stress. Some workers support these results in which lipid content decreased in animals after exposure to pollutants. A preliminary study on the effect of dairy effluent on *O. mossambicus* reported that lipid content was decreased (Amutha *et al*., 2002). Similar decrease in lipid content level has also been observed in *C.punctatus* when exposed to mercurial fungicide (Raj Narayan Ram and Sathyanesan, 1987). Reduction of lipid content of *L. vannamei* in this study may have been due to the utilization of lipids for energy demand under stress condition (Harpert *et al*., 1977).

Lipids in hepatopancreas of *L. vannamei* declined significantly with increase in the concentration of copper. This trend was reported in many crustaceans concluded that, in crustaceans with well-defined hepatopancreas, energy reserves are stored in it and are apparently utilized for detoxification. In crustaceans lipids are considered as the major energy reserves. The role of hepatopancreas and muscle as storage organ during adverse conditions, like stress and detoxification are very well documented (Vijayakumaran, 1990). In *L. vannamei,* the effect of

copper toxicity results in the reduction of total lipids as reported in crab *Thalamita crenata* (Villalan *et al.,* 1988).

The total lipids in the muscle, hepatopancreas and gills *of P.hornarus* showed decreasing trend as the duration of exposure in each concentration of copper increased (muscle: 2.96% to 1.90%, hepatopancreas: 10.15% to 7.23% and gills: 0.47% to 0.35%). A significant decrease in muscle and hepatopancreas is due to the utilization for energy. In the spiny lobster *P.ornatus,* the hepatopancreas is the most sensitive indicator of physiological stress than the muscle tissue (Trendal and Prescott, 1989). This results in accordance with *L. vannamei,* although muscle and hepatopancreas are the major energy stores.

Lipids were the primary source of energy under stress condition in *Penaeus duorarum* (Schafer, 1968). An increase in metal concentration and exposure resulted in lipid reduction in *Oreocheonis mossambicus* (Overstreet, 1988) and similar results are reported in bivalve *Sunetta scripta* (Katticaran *et al.,* 1995). As the lipid reserves are ultimately transferred to the detoxification process in *L. vannamei,* the total percentage of lipids decline sternly in hepatopancreas followed by muscle and gills. This suggests the important role of hepatopancreas in storage and mobilization of energy during detoxification of copper in *L. vannamei.*

The concentration of the essential and nonessential amino acids in most of the crustaceans is higher than that in vertebrate tissues (Clay brook, 1983), possibly for osmoregulatory functions (Awapara, 1962). The amino acid profile for *L. vannamei* is similar to that of *P. kerathurus* (Torres, 1973). The amino acid pattern in *L. vannamei* is also broadly similar to those in other crustaceans and its concentration is comparable with those in *Carcinus maenas* and *Palaemon xiphias* (Clay brook, 1983; Dall and Smith, 1987). In the present study, some notable differences between the copper concentration in different exposure periods in amino acid tail-muscle tissue. Lyndon *et al*., (1993) found a significant increase in tryptophan levels in white muscle 12 h after a meal in cod, and correlated this increase with the protein synthesis rates at around this time. Tryptophan is a candidate amino acid that limits the rate of protein synthesis. Despite considerable variation in individual amino acid concentrations, the total amount of essential and non-essential amino acids in the tail-muscle remained steady after 28 days of copper exposure, according to the current study. This shows that the intracellular amino acid pools are regulated by active transmembrane transport rather than passive amino acid motions.

However, the proximate composition, fatty acid, vitamins, amino acid and minerals in muscle of *L. vannamei* was significantly varied due to the effect of copper. The copper toxicity was shows the significant

positive correlation between the Palmic acid 16:0, Linolenic acid, Vitamin A, Vitamin B2, Vitamin C, essential amino acids asparagine, glycine, threonine, histidine, histidine, iso leucine, phenylanin and chloride, whereas it also showed the negative correlations between the Vitamin B3, B6, C, gultamic acid, valine, zinc, sodium. The results of the correlation analysis revealed that the concentration of the essential amino acids was decreased in the muscle of the shrimps while increasing the copper concentrations.

6.6 Histopathology

Understanding the prawn immunity is highly significant in reducing the effects of copper toxicity. The study of lymphoid organs in copper toxicity leads to the development of immune intervention strategies. Crustaceans for eg., crabs, lobsters, crayfish, and prawns do not possess antibody-based immunity. These animals depend on an innate immunity consisting of cellular and humoral components that are extremely effective in understanding and sequestering invading pollutants (Roch, 1999; Bachere, 2003; Loker *et al.*,2004). Haemocytes are important for immune activity in which the immune factors are stored in stationary state and are released during the entry of pollutants (Rodriguez and Le Moullac, 2000; Smith *et al.*, 2003).

In crustacea, the lymphoid organ has identified only in penaeid species. In other crustacea such as crabs, lobsters, and crayfish there is no

lymphoid organs. The presence of the lymphoid organ in penaeids was first noticed in koroi prawn (*Penaeus orientalis*) and then noticed in the ginger prawn (*Penaeus japonicus*), (*Penaeus carinatus*, the white prawn (*Penaeus indicus*) and the banana prawn (*Penaeus merguiensis*) (Oka, 1969).

The lymphoid organ lies in the midst of hepatopancreas and the stomach in mal shrimps. On the contrary in the females this organ is placed in between the ovary and the hepatopancreas (Oka, 1969). The position of the LO possibly changes with the life cycle of the prawns (Nakamura, 1987). Structurally, the penaeid lymphoid organ composes of a pair of lobes. These two lobes are similar, surrounded by connective tissue capsules and connected directly to the heart via subgastric artery (Bell and Lightner, 1988; Van de Braak *et al*., 2002; Duanguwan *et al*., 2008). In penaeid species the lymphoid organ is involved in immune defence against alien materials (Kondo *et al.*, 1994; Hasson *et al.*, 1999; Van de Braak *et al.*, 2002; Anggraeni and Owens, 2008) and functions as a major phagocytic organ in penaeid prawns (Martin *et al.*, 1996 ;Van de Braak *et al.*,2002). The formation of spheroid cells in the lymphoid organ has been explained in many experimental penaeid prawns (Alday-santz *et al.*, 2002; Pongsomboon *et al.*,2008) The reticular connective tissue and haemal sinuses of lymphoid organ widely expand during spheroid formation and these abnormal cells are formed in the haemal sinuses in

our study (Anggraeni and Owens, 2008). In the experimental animal in higher concentrations, gapping between tubules appears and abnormal interstitial space or gapping between tubules has been observed.

The present study observed the destruction of hepatic tissue due to the copper toxicity in experimental shrimps. There is complete degeneration of epithelial cells and collapsing of epithelial tubules observed in higher concentration at 28 days. There is dysfunction of central hepatopancreas and its cells, massive sloughing hepatopancreatic tubule and epithelial cells and massive intertubular hemocytic aggregation in experimental animals compared to control. Prachumwat *et al.* (2012) obtained comparable histopathological result in which there were dysfunction of the tubule epithelial cells that progress from proximal to distal ends of HP tubules.

The gross morphological changes noticed in the p resent study were rather interesting and helps in the identification process of the affected animals. The control prawn consists of eight pairs of gills on lateral sides and typically phyllobranch type and almost comparable to Palaemon (Patwardhan,1937). The gills of control shrimps appear typical to other prawns with spaced gill plates and cuticle appeared smooth and regular circulating haemocytes. Gills are the primary site directly comes in contact of the external environment and are affected by the toxicants. After 7 days of exposure in higher concentrations, there was branchial

inflammation; vacuolization and distention of gill plates were noted. After 28 days of exposure in lower concentrations cuticular disruption and bulging of gills plate were frequent. Hyperplasia was seen at most of the gill plates. The most recurrent alterations in gills of *L.vannemi* observed in this study, such as rupture of pilaster cells, marginal canal deformation and necrosis, were also found in gills of crustaceans analyzed by De Freitas Rebelo *et al.,* 2000).

Metal and toxic chemical caused haemocyte infiltration in branchial gills and distension of gill plates as observed in present study. Various workers in other crustaceans (Anderson and Baatrup, 1988; Victor *et al*., 1990; Abraham and Radhakrishna, 2002) have reported this type of haemocyte infiltration. Gills play major in role in metal elimination in crustaceans. Haemocyte collects metal from circulation, transport to gills, accumulate within gill tissues and eliminated by sloughing of affected portion of gills. These types of accumulation and elimination by gills, hepatopancreas and haemocytes have been studied of clearance of carmine particles in shrimps (Fontine and Lightener, 1974).

Heavy metals are well known to significantly decrease the oxygen consumption as well as disruption of osmoregulation in crustaceans (Jadhav, 1993; Sen *et al*., 2008; Asih *et al*., 2013; Putranto *et al*., 2014; Soegianto *et al*., 2016). In our study, histopathological alterations like inflammation, vacuolization, distention of gill plates, cell necrosis,

bulging in gill plate tip, hyperplasia, heavy influx of haemocytes, thickening of gill plates and reduction in interlamellar space, hypertrophy and cuticular irruption collection of granular material below cuticle and leakage of haemocytes in interlamellar spaces, mucous coating after 28 days of exposure. At higher concentrations there was clumping of gill plates, necrotic and degenerative changes showing pycnosis and karyolysis, increased influx of haemocytes, fragmentation of nuclear material in haemocyte and aggregate of haemocyte noticed in haemolymph channel in gill base.

Gills showed complete disorganization after acute and sub-acute exposure of copper in *L.vannamei*. Other workers also reported similar histopathological alterations in gills of fishes and crustaceans after exposure of Lead and other heavy metals (Gill *et al.,* 1988; Kumari and Kumar, 1995; Dutta *et al.,* 1996; Parashar and Banerjee, 2002; Olojo *et al.,* 2005; Asih *et al.,* 2013; Putranto *et al.,* 2014; Soegianto *et al.,* 2016;).

In this study, the abnormal hepatopancreatic lumen changes were the most recurrent alteration in the shrimps exposed to copper. It is characterized by morphological alterations of the hepatopancreatic tubular epithelial cells, as the cell decreases in height, from normal columnar cells to cuboidal cells (Maharajan *et al.,* 2015). There is an increase of Vacuolization of more cells that there was a proliferation of these cells. That may be related to the high rate of excretion of

hepatopancreas, which is a storage and detoxification organ, so that it is possible to eliminate pesticides and pollutantsby increasing the number of F cells and converting them to B cells (Maharajan *et al.*, 2015). These alterations directly affect the tissue and cause damage to the cells which included is hemocytic infiltration in the interstitial sinuses, thickening of basal laminae and necrosis of the tubules of the hepatopancreas: accumulation of hemocytes in the hemocoelic space. Swelling and fusion of lamellae, abnormal gill tips, hyperplastic, necrotic, and clavate-globate lamellae of the gills. The results obtained suggest that the hepatopancreas and gills of prawns exposed to copper were structurally altered.

Tissue atrophy in experimental animal is a narrowing of the tissue accompanied by a widening of the hepatopancreatic lumen (Jayadi, 2016). The thickened lumen of the hepatopancreas indicates that the hepatopancreas lumen is bare because the shrimp do not eat for days. In the present study, the hepatopancreas showed changes in the F and B cells in low concentration of copper, and cells were found clumped, and intercellular spaces invisible and a general degeneration, loss of tubules structures, vacuolation, star shape of lumen and necrosis of cells in the high concentrations of copper in experimental animal. The star shape of the lumen was partially lost due to morphological changes of the tubular epithelial cells, because some cells decreased in height from a normal columnar height to a low cuboidal form. Previous studies on the

hepatopancreas concluded that this digestive organ possesses several functions, including absorption, digestion, storage, and secretion (Dall and Moriarty, 1983; Caceci *et al*., 1988). Krishnamoorthy and Subramonian (1996) also reported changes such as elongation of hepatopancreatic cells, and shrunken cells in *Macrobrachium lamarrg* exposed to copper.

` 7. SUMMARY AND CONCLUSION

Aquaculture of shrimp is considered to be a success story of modern aquaculture. The advent of sophisticated refrigeration facilities aided traditional farmers to access international markets, thereby traditional coastal aquaculture shifted to an export oriented or industrialized aquaculture. During industrialization, the quality of the material exported or imported anywhere remains as major concern. Among various pollutants, the presence of heavy metals in the environment remains as major problem across the globe. Increased urbanization, expansion of industrial activities and exploitation of natural resources, the excess occurrence of heavy metals in natural loads has become a serious problem throughout the world. The excessive amount of copper accumulation in the environment especially in water bodies and causes toxicity to aquatic fauna and flora and finally to human beings

7.1 The LC_{50} values for *L. vannamei* exposed to copper were calculated using trimmed spearman- karber method. The calculated LC_{50} value for *L. vannamei* 1.746 ppm after 24 h, while it was 1.515 ppm after 48 h, 1.407 ppm after 72 h and 1.113 ppm after 96 h of exposures to copper.

7.2 The toxicity of copper was highlighted in the colour changes observed only after 28 days of exposure to 0.224ppm in muscle, hepatopancreas, gill tissues and uropod.

7.3 The exposure of shrimps to copper through water medium provided several evidences for accumulation of copper in various tissues of shrimps. After 28 days, both groups treated with low and high copper concentration had significantly accumulated higher copper in their muscle tissue compared to the control group, 2.68$\pm$0.42, 3.11$\pm$0.21, and 1.49 $\pm$0.19 respectively.

7.4 There was a significant difference in the rate of accumulation between hepatopancreas and gills however there was no significant difference was observed in both low and high concentrations of copper and control. The lowest rate of copper concentration accumulation was found in muscle preceded by exoskeleton.

7.5 Total haemocyte count was found to be increased in all the concentrations of copper treated animals as compared with the control and the count is increased with increasing exposure duration.

7.6 Shrimp differential haemocytes classified into three major types such as hyaline cells, small granule haemocyte (SGH) and large granule

haemocyte (LGH) based on cytoplasmic granular size. The level of hyaline cells was increased while increasing the exposure period in all the treatments however the level of hyaline cells between two copper concentrations is insignificant. The level of LGC and SGC level was decreased while increasing the exposure period in all the treatments however the level of LGC and SGC between two copper concentrations is insignificant

7.7 The percent decrease over the control was 10.71, 8.41, 8.85, 17.37 and 20.00 in lower concentration and 3.02, 13.62, 13.27, 20.45, and 22.32 in higher concentration for 0, 7, 14, 21 and 28 days of exposure period respectively.

7.8 The exposure of *L. vannamei* to copper cause distinct dose and time dependent decrease in the carbohydrate content of all exposure period.

7.9 Similar to carbohydrate, the exposure of *L. vannamei* to copper cause distinct dose and time dependent decrease in the lipid content of all exposure period.

7.10 The major fatty acids in shrimp muscle were, in palmic acid, margaric acid, stearic acid, oleic acid, linolenic acid, alpha linolenic acid, morotic acid. The exposure of *L. vannamei* to low and high copper concentrations has affected in the fatty acid composition of the exposed

shrimp muscle tissue. For instance, palmic, oleic stearic, and linolenic acid were in higher level in muscle tissue of shrimps exposed with both concentrations of copper. At the end of exposure the order of the levels of fatty acids are palmic acid > oleic acid > stearic acid > linolenic acid > alpha linolenic acid > margaric acid > morotic acid.

7.11 The major vitamins A, D, E, B1, B2, B3, B5, B6 and C were analyzed. The exposure to *L.vannamei* to low and high copper concentration does not show much difference among the vitamins in lower (0.112ppm) and higher 0.224ppm) concentration in 7,14 and 21 days of exposure.

7.12 Minerals such as calcium, magnesium, manganese, phosphorus, iron, sodium, potassium and zinc were analyzed in *L.vannamei* when exposed to lower and higher concentration of copper in different exposure durations. At the end of 7,14 and 21 days of copper exposure minerals contents did not show any significant changes of muscle tissue in both concentrations. At the end of exposure in 28 days of showing significant changes in calcium and magnesium in both lower and higher concentrations (145.2–140.9 mg/g; 13.2-12.4 mg/g in 0.112ppm concentration of copper, 147.4-139.5mg/g; 12.8-11.2mg/g in 0.224 ppm concentration of copper).

7.13 The exposure of *L. vannamei* to low and high copper concentrations has affected the amino acid composition of the exposed shrimp protein. For instance, glycine, cysteine and proline were in higher level in protein of shrimps exposed with both concentrations of copper. At the day 28, among the amino acids measured, level of non essential amino acids, particularly glycine was found to be highest (6.48% for 0.224 ppm and 7.19% for 0.112 ppm) in shrimps exposed with both concentrations of copper followed by cysteine (3.62% for 0.224 ppm and 3.87% for 0.112 ppm). Whereas, alanine was found to be the lowest among amino acids at the end of 28 day exposure to copper. At the end of exposure, the order of the levels of amino acids were glycine > cysteine > proline > tyrosine > serine > glutamic acid > aspartic acid > alanine.

7.14 The essential amino acids in shrimp protein were histidine, valine, methionine, iso leucine, phenylalanine, leucine, lysine, arginine and. tryptophan. The exposure of *L. vannamei* to low and high copper concentrations has affected the essential amino acid composition of the exposed shrimp protein. For instance, valine, Lysine, methionine, and Iso leucine were in higher level in protein of shrimps exposed with both concentrations of copper. At the day 28, among the amino acids measured, level of essential amino acids, particularly valine was found to be highest (20.16% for 0.224 ppm and 20.34% for 0.112 ppm) in shrimps

exposed with both concentrations of copper followed by lysine (11.85% for 0.224 ppm and 12.01% for 0.112 ppm). Whereas, Histidine was found to be the lowest (0.76% for 0.224 ppm and 0.82% for 0.112 ppm) at 28th day. The amino acid methionine (11.42% for 0.224 ppm and 11.97% for 0.112 ppm) and Iso leucine (10.42%for 0.224 ppm and 11.68% for 0.112 ppm) also found to be in high levels at the 28 day of exposure. At the end of exposure, the order of the levels of amino acids were valine>lysine > methionine > iso leucine > leucine > phenylalanine > tryptophan >arginine > histidine.

7.15 Muscle histoarchitecture displayed severe alterations in the muscle tissue showed highly disintegrated epidermis (DE) with vacuolation, gap formation (GF) in between the muscle bundles, necrosis (NE), broken myofibrils (BMF), coagulative necrosis (CNE) congestion of muscle bundles marked thickening and separation of muscle bundle and pronounced intramuscular oedema with minor dystrophic changes.

7.16 Connective tissue of hepatopancreas becomes very thin and meager looks like spongy (SCT). When the concentration increases, there is a proliferation of hepatopancreatic tubular cells (PHT). There is extensive vaculoations (EV) in different cell types and the tubules did not remain intact in most of the regions. The epithelial cells highly disfigured (DCE) and clumped with poor intercellular spaces. Infiltration of haemocytes.

7.17 The epidermal covering around the lamellae showed degenerative nature in gill tissue. Gill epithelial cells in the lamella exhibits flattened appearance with necrotic cells, swelling of secondary gill lamellae (SSF) and poor arrangement of secondary gill lamellae with necrotic lesions. The gill lamellar cells collapses in experimental animal due to disruption of pillar cells.

7.18 The lymphatic cells undergo inflammation and degradation of nucleus (IDNu) observed. The light microscopic pictures clearly depict the degranulation of haemocytes (DHC) in the lymphatic tubular lumen. In higher concentrations, the haemocytes burst out and disorientation, highly scattered pyknotic nuclei (PKNu) and lesions in the lymphatic tubular cells occurs. There is clumping of lymphatic cells occurs and necrosis of cells.

7.19 The interstitial spaces between the nerve cells show the presence of vacuolization. The light microscopic picture depicts the thickening of fibrous tissue capsule, edema, rupture of epithelial cells and degeneration of nerve cells appears. In the experimental animal, the nerve cells undergo necrosis and nuclear degeneration.

7.20. CONCLUSION

Copper has been utilized in freshwater farm ponds and aquaculture operations for many years as a chemical tool. It works as an algicide as well as a parasite therapy. Copper, as well as filamentous and higher algae, can be used to manage algae in ponds. The chemistry of copper in marine systems is more difficult than in freshwater systems due to the higher ion content of saltwater. Many additional factors also have an impact on the final concentration of free copper in water.

In the present study concluded that the increase in accumulation of copper interferes with the changes in colouration of external and internal organs can be used as an indicator of stress due to copper toxicity in *L. vannamei.* It is apparent that copper accumulation in *L. vannamei* follows a pattern such as hepatopancreas > gills > muscle> Exoskeleton.

The declining levels of proteins may be attributed to the higher rate of energy production at the onset of various enzymatic blockages to contain toxic effect of copper. Copper at sublethal level affect the structure and functioning of cellular component, leading to impairment of vital functions of *L. vannamei.* To avoid toxicity, copper utilisation in aquaculture farming systems should be restricted, according to the current study.

8. REFERENCES

Abdel-Khalek, A.A., Kadry, M., Hamed, A., Marie, M.A.S., 2015. Ecotoxicological impacts of zinc metal in comparison to its nanoparticles in Nile tilapia (*Oreochromis niloticus*). *Jour. of Basic and Appl. Zoo.,* 72, 113–125.

Abdullah, O., Ayse, O., Mevlut, A., Gozde, G. and Jelena, M.2009. A comparative study on proximate, mineral and fatty acid compositions of deep seawater rose shrimp (*Parapenaeus longirostris*, Lucas, 1846) and red shrimp (Plesionika martia, A. Milne-Edwards, 1883). *J.Anim. Vet. Adv.* 8(1):183-189.

Abraham, K. M. and Radha Krishna, T. 2002. Study on the gill of field crab, *Paratelphusa hydrodromous* (Herbst.) exposed to nickel. *J. Environ. Biol.,* 23 (2): 151-155.

Adriana, B., Almodóvar, A.N.M., Pereira, C.T., Mariângela, T.A., 2007. Antimicrobial efficacy of *Curcuma zedoaria* extract as assessed by linear regression compared with commercial mouth rinses. *Brazil. Jour.of Microbiol*., 38, 440-445.

Ahsanullah, M. and Arnott, G.H. 1978. Acute toxicity of copper, cadmium and zinc to larvae of the crab, *Paragrapsus quadridentatus* (H.Milne Edwards) and implications for water quality criteria. *Aus. J. Mar. Freshwat. Res.,* 29 : 1 - 8.

Ahsanullah, M., Negilski, D.S. and Mobley, M.C. 1981. Toxicity of zinc, cadmium, and copper to the shrimp, *Callianassa australiensis* 1. Effect of individual metals. *Mar. Biol.,* 64 : 299 - 305.

Ahsanullah, M. and Williams, A.R. 1991. Sublethal effects and bio accumulation of cadmium, chromium, copper and zinc in the marine amphipod, *Allorchstes compressa. Mar. Biol.,* 108 : 59 - 65.

Akinrotini, O.A., Agokei, E.O., Aranyo, A.A., 2012. Changes in blood parameters of *Tilapia guineensis* exposed to different salinity levels. *Jour. of Environ. Engineer. and Tech*., 1, 4–12.

Alday-Sanz, V., Roque, A. and Turnbull, J.F. 2002.Clearing mechanisms of *Vibrio vulnificus* biotype I in the black tiger shrimp *Penaeus monodon*. *Dis. Aquat. Org*.,48:91-9.

Alliot, A. and Frenet. Piron, M. 1990. Relationship between metals in seawater and metal accumulation in shrimps. *Mar. Pollut. Bull.,* 21 : 30-33.

Amutha, P., Sangeetha, G. and Mahalingam, S. 2002.Diary effluent induced alterations in the protein, carbohydrate and lipid metabolism of a freshwater teleost fish *Oreochromis mossambicus. Poll. Res.,* 21(1): 51-53.

Anderson, J. T. and Baatrup, E.1988. Ultrastructural localization of mercury accumulation in the gills hepatopancreas, midgut and antennal glands of the brown shrimp, *Crangon crangon. Aquat. Toxicol*., 13: 309-324.

Anderson, B.S., Phillips, B.M., Hunt, J.W., Clark, S.L., Voorhees, J.P., Tjeerdema, R.S., Casteline, J., Stewart, M., Crane, D., Mekebri, A., 2010. Evaluation of methods to determine causes of sediment toxicity in San Diego Bay, California, USA. *Ecotoxic. and Environ. Safe*., 73, 534-540.

Anggraeni, M.S. and Owens, L. 2000. The haemocytic origin of lymphoid organ spheroid cells in the penaeid prawn, *Penaeus monodon. Dis. Aquat. Org.,* 40:85-92.

***Anon. 2000** Handbook on fisheries statistics. Ministry of Agriculture (Department of Agriculture and Co-operation, Fisheries Division), Government of India. 153 p.

Anusha, A, Cyril Arunkumar, I., Elizabeth Jayanthi,F. and Selvanayagam, M. 1996. Quinolphos induced biochemical anomalies in *Cirrhinus mrigala* (Ham.). *J. Environ. Biol.,* 17(2): 121-124.

AOAC. 1990. Official methods of analysis 15th Edition. Washington DC. pp. 222-245

AOAC. 1999. Official methods of analysis, 16th Edn. Association of Official Analytical Chemists, Washington.

APHA, 1995. Standard methods for the estimation of water and waste water. American Public Health Association, New York. pp. 4-127.

APHA, 1985. Standard methods for the examination of water and waste water APHA, AWWA and WPCF, New York.

Aremu, S.O. and Nweze, C.C. 2017. Determination of Vitamin A Content from Selected Nigerian Fruits Using Spectrophotometric Method. *Bangla. Jour.of Scien. and Indus. Rese.,* 52, 153-158.

Armitage, K.B., Buikema, A.L. and Willems, Jr. N.J. 1972. Organic constitutents in the annual cycle of the cray fish, *Orconectes nais (Faxon). Corn. Bio and Phy.,* 41 : 825 - 92.

Arumugam, M. and Ravindranath, M.H. 1983. Nature and distribution of copper in green lagoon crab, *Scylla serrata* (Forskal). *J. Exp. Mar. Biol. Ecol.,* 70 : 271 - 280.

Asih, A. Y. P., Irawan, B. and Soegianto, A. 2013. Effect of copper on survival, osmoregulation, and gill structures of freshwater prawn (*Macrobrachium rosenbergii*, de Man) at different development stages. *Mar. and Fresh. Behav. and Physiol*. 46(2):75-88.

Awapara, J. 1962. Free amino acids in invertebrates: a comparative study of their distribution. In Amino Acid Pools (ed. J. T. Holden), pp. 158-175. Amsterdam: Elsevier.

Ayyappan, S. 2004. Enhancing global competition. *Survey of Indian Agriculture* . The Hindu, 97–100.

Babarinde, G. O. and O. A. Fabunmi. 2009. Effects of packaging materials and storage temperature on quality of fresh okra (*Abelmoschus esculentus*) fruit. *Agric. Trop. Subtrop*., 42: 151–156.

Bachere, E. 2003. Anti-infectious immune effectors in marine invertebrate: potential tools for disease control in larviculture. *Aquaculture*.237:427-38.

Baden, S.P., Depledge, M.H. and Hagerman, L. 1994. Glycogen depletion and altered copper and manganese handling in *Nephrops norvegicus* following starvation and exposure to hypoxia. *Mar. Research,Vol.,* 103: 65 - 72.

Badsha, K.S. and Goldspink, C.R. 1982. Preliminary observations on the heavy metal content of four species of fresh water fish in NW England. *J. Fish. Biol.,* 21 : 251 - 267.

Balarin, J.D,, 1988. Development planning for Tilapia farming in Africa. In: R.S.V. PuUin, T., Bhukaswan, K. Tonguthai and J. Maclean (Editors), *The Second International Symposium on Tilapia in Aquaculture.* ICLARM, Bangkok, 15: 531-538.

Baldwin, D.H., Sandahl, J.F., Labenia, J.S. and Scholz, N.L. 2003. Sublethal effects of copper on coho salmon: Impacts on non overlapping receptor pathways in the peripheral olfactory nervous system. *Environ.Toxicol. and Chemis.,* 22, 2266-2274.

Barclay, C.J., Dan, W. and Smith, D.M. 1983. Changes in lipid and protein during starvation and the moulting cycle in the tiger prawn. *J. Expt. Mar. Biol. Eco.,* 68 : 229-49.

Bardera,G., Owen, M.A.G., Pountney, D., Alexander, M.E. and Sloman, K.A. 2019. The effect of short-term feed-deprivation and moult status on feeding behaviour of the Pacific white shrimp (*Litopenaeus vannamei*) *Aquaculture.* 511. 10.1016/j.aquaculture.2019.734222

Barker, P.L. and Gibson, R. 1977. Observations on the feeding mechanisms structure of the gut and digestive physiology of the European lobster, *Homarus gammarus* (L) (Decapoda : Nephropidae). *J. Expt. Mar. Biol. Eco.,* 26 : 297-324.

Barker, C.J. and Gibson, R. 1978. Observations on the structure of the mouth jarrs, histology of the alimentary tract and digestive physiology of the mud crab, *Scylla serrata* (Forskal) (Decapoda : Portunidae). *J. Expt. Mar. Biol. Eco.,* 32 : 177 - 196.

Bastami, D.K., Moradlou, H.A., Zaragabadi, M.A., Mir, S.S.V., Shakiba, M.M., 2009. Measurement of some haematological characteristics of the wild carp. *Compar. Clini. Pathol.,* 18, 321-323.

Bell, T.A. and Lightner, D.V.1988. A handbook of normal penaeid shrimp histology. *Baton Rauge, La World Aquacult Soc.*, 52-73.

Benoit, D.A. 1975. Chronic effects of copper on survival, growth, and reproduction of the bluegill (*Lepomis macrochirus*). *Trans. Am. Fish. Soc.* **2***: 353-358.*

Birge, W.J., Black, J.A. and Westerman, A.G. 1979. Evaluation of aquatic pollutants using fish and amphibian eggs as bioassay organisms. *AGRIS.*, 108-118.

Blaxhall, P.C., 1972. The haematological assessment of the health of freshwater fish. *Jour. of Fish. Biol.*,4 (4): 593-604.

Brehelin, M., Zachary, D. and Hoffmann, J.A. 1978. A comparative ultra structural study of blood cells from nine insect orders. *Cell Tissue Res.,* 195: 45-57.

Brongs, W.A. and Mount, D.I. 1978. Introduction to a discussion of the use of aquatic toxicity tests for evaluation of the effects to toxic substances. In : *Estimating the hazard of chemical substances to aquatic life* (ANTMATP 657). Cairns, J., K.L. Dickson and A.W. Maki (eds), American Society for testing and materials, *Philadelphia,* pp.15 - 26.

Brown, L.N. Robinson, M.G. and Hall, B.D. 1988. Mechanisms for copper tolerance in *Amphora coffeaeformis* internal and external binding. *Mar. Biol.,* (Berlin), 97 : 581 - 586.

Bryan, G.W. 1976. Some aspects of heavy metal tolerance in aquatic organisms. In : A.P.M. Lockwood, ed., *Effects of pollutants on aquatic organisms.* pp.7-34. Cambridge University Press, Cambridge, England.

Bryan, G.W. and Ward, E. 1965. The absorption and loss of radioactive and non radioactive manganese by the lobster, *Homarus vulgaris. J. Mar. Biol. Aus.,* 45 : 65 - 95.

Buikema, Jr. A.L., Nider-Lehner, B.R. and Cairns, Jr. J. 1982. Biological monitoring. Part IV - Toxicity testing. *Wat. Res.,* 16 : 239 - 262.

Caceci, T., Neck, K.F., Lewis, D.H. and Sis, R.F. 1988. Ultrastructure of the hepatopancreas of the pacific white shrimp, *Penaeus vannamei* (Crustacea: Decapoda). *J. Mar. Biol. Assoc*. *UK.*, 68, 323-337

Carvalho-Neta, R.N.F., Mota Andrade, T.S.O., Oliveira, S.R.S., Torres, A.R., Cardoso, W.S., Santos, D.M.S., Batista, W.S., Serra, I.M.R. and Brito, N.M. 2019. Biochemical and morphological responses in *Ucides cordatus* (Crustacea, Decapoda) as indicators of contamination status in mangroves and port areas from northern Brazil. *Environ. Sci. and Poll. Res.,* 26(16): 15884-15893.

Cazenave, J., Bacchetta, C., Parma, M.J., Scarabotti, P.A. and Wunderlin, D.A., 2009. Multiple biomarkers responses in *Prochilodus lineatus* allowed assessing changes in the water quality of Salado River basin (Santa Fe, Argentina). *Environ. Pollut., 157,* 3025–3033.

Cheng, T.C. 1988. In vivo effects of heavy metals on cellular defence mechanisms of *Crassostrea virginica :* Total and differential cell counts. *J. Invertebr. Pathol.,* 51 : 207 - 214.

Clark, I.A., Chaudhri, G. and Cowden, W., 1989. Some roles of free radicals in malaria. *Free Radical Biology and Medicine.,* 6, 315-321.

Clay brook, D. L. 1983. Nitrogen metabolism. In The Biology of Crustacea: *Internal Anatomy and Physiological Regulation*, vol. 5 (ed. L. H. Mantel), pp. 163-213. New York: Academic Press Inc.

Cockcroft, A.C. 1997. Biochemical composition as a growth predictor in male west-coast rock lobster *(Jasus lalandii). Mar. Fresh Water Res.,* 48 : 845 - 856.

Cockell, K.A., Hilton, J.W. and Bettger, W.J., 1992. Hepatobiliary and hematological effects of dietary di sodium arsenate heptahydrate in juvenile rainbow trout (*Oncorhynchus mykiss*). Comparative Biochemistry and Physiology Part C 103, 453–458.

Conner, W. E. 2000. Importance of n- 3 fatty acids in health and disease. *The Amer. Jour. of Clini.Nutr*. 17(1), 171S–175S.

Cornick, J.W. and Stewart, J.E. 1978. Lobster (*Homarus americanus*) hemocytes: Classification, differential counts, and associated agglutinin activity. *J. Invertebr. Pathol.,* **31:** 194-203.

Cough, J.A. 1977. Ultra structural study of lesions in gills of a marine shrimp exposed to cadmium. *J. Invertebr. Pathol.,* 29 : 267 - 288.

Crémazya, A., Wood, C.M., Smith, D.S., Ferreirac, M.S., Johannsson, O.E., Giacomina, M. and Val, A.L. 2016. Investigating copper toxicity in the tropical fish cardinal tetra (*Paracheirodon axelrodi*) in natural Amazonian waters: Measurements, modeling, and reality. *Aquatic Toxicol.,* 180, 353–363

Cuadras, J., Gimeno, A., Flos, R. and Crespo, S. 1981. Levels of copper and Zinc in tissues of the hermit crab, *Dardanus arrosor* (Herbst) from the Barcelona coast (Decapoda, Anomura). *Crustaceana,* 40(1) : 79 - 87.

Da11, W. 1957. The functional anatomy of the digestive tract of a shrimp, *Metapenaeus bennettae.* Racek and Da11 (Crustacea : Decapoda : Peneidae). *Aus. J. Zool.,* 15 : 699-714.

Dall, W. and Moriarty, D.J.W. 1983. Functional aspects of nutrition and digestion. In:Mantel, L.H. (Ed.), *The Biology of Crustacea. Internal Anatomy and Physiological Regulation,* vol. 5. Academic Press, New York, pp. 215-261

Dall, W. and Smith, D. M. 1987. Changes in protein-bound and free amino acids in the muscle of the tiger prawn, *Penaeus esculentus* during starvation. *Mar. Biol.,* 95, 509-520.

Daly, H.R., Camphell, I.C. and Hart, B.T. 1990. Copper toxicity to *Paratya australiensis* 1. Influence of nitrilo acetic acid and glycine. *Environ. Toxicol. Chem.*, 9: 997 - 1006.

David, W. E. 1979. Copper and Cadmium induced changes in the metabolism and structure of molluscan gill tissue. In *Marine Pollution Functional Response*, pp. 241 - 256.

Davis, A.K., 2008. Ontogenetic changes in erythrocyte morphology in larval mole salamanders, *Ambystoma talpoideum*, measured with image analysis. *Compar. Clini. Pathol.*,17, 23–28.

De Freitas-Rebelo, M., Rodriguez, E.M., Santos, E.A. and Ansaldo, M. 2000. Histopathological changes in gills of the estuarine crab *Chasmagnathus granulata* (Crustacea-Decapoda) following acute exposure to ammonia. Comparative Biochemistry and Physiology Part C: *Pharmacology, Toxicology and Endocrinology*, 125: 157-164. doi: 10.1016/S0742-8413(99)00093-6.

***Demayo, A., and Taylor M. 1981.** Guidance on the Site-Specific Application of Water Quality Guidelines in Canada: Procedures for Deriving Numerical Water Quality Objectives "Site-Specfic guidness", Volume 1Inorganic (1981).

Denton, G.R.W. and Burdon Jones, C. 1982. The influence of temperature and salinity upon acute toxicity of heavy metals to banana prawn *(Penaeus merguiensis). Chem. Ecol., 1 :* 131 - 143.

Depledge, M.H. 1987. Enhanced copper toxicity resulting from environmental stress factor synergies. *Comp. Biochem. Physiol.,* **87** : pp. 15 - 19.

Depledge, M.H. Bjerregaard, P. 1989. Haemolymph protein composition and copper levels in decapod crustaceans. *Helgolander meeresunters*, 43 : 207 - 23.

Deshmukh, R. and Lomte, A. 1998. Effect of heavy metal (CuSo1) on protein activity of fresh water bivalve, *Parlysia corrugata. J. Ecotoxicol. Monist.,* 16(3) 704 - 708.

Duangsuwan, P., Phoungpetchara, I., Tinikul, Y., Poljaroen, .J, Wanichanon, C. and Sobhon, P. 2008. Histological and three dimensional organizations of lymphoid tubules in normal lymphoid organ of *Penaeus monodon*. *Fish Shell fish Immunol.,* 24:426-35.

Dubois, M., Gilles, K.A., Hamilton, J.K., Rebees, P.A. and Smith, F. 1956. Calorimetric method for determination of sugars and related substances. *Anal. Chem.,* 28 : 350-356.

Dutta, H. M., Munshi, J. S. D., Roy, P. K., Singh, N. K., Adhikari; S. and Killius, J. 1996. Ultrastructural changes in the respiratory lamellae of the catfish, *Heteropneustes fossilis* after sublethal exposure to malathion. *Environ. Poll*., 92: 329-341.

Eyckmans, M., Tudorache, C., Darras, V.M., Blust, R. and De Boeck, G., 2010. Hormonal and ion regulatory response in three freshwater fish species following waterborne copper exposure. *Comparative Biochemistry and Physiology Part C: Toxicology and Pharmacology* 152, 270–78.

FAO, 1992. Committee for inland fisheries of Africa: Report of the third session of the working party on pollution and fisheries, *Accra. Ghana*, 25-29.

FAO, 2014. FAOSTAT. Food and Agriculture Organization of the United Nations, Rome, Italy. *FAO Fisheries Report* No. 471, FAO, Rome, Italy, 1-24.

FAO. 2016 Global aquaculture production dataset 1950-2014 (Fishstat).Available at:www.fao.org/fishery/statistics/software/fishstatj/en

Farley, C.A. 1988. Histochemistry as a tool for examining possible pathologic cause - and - effect relationship between heavy metal and inflammatory lesions in oyster, *Crassostrea vitginica. Mar. Environ. Res.*, 24 : 271 - 275.

Felts, P.A. and Heath, A.G. 1984. Interactions of tempeature and sublethal environmental copper exposure on the energy metabolilsm of blue gill, *Lepomis rnacrochirus. J. Fish. Biol., 25: 445.*

Fernandes, M.N., and Mazon, A.F., 2003. Environmental pollution and fish gill morphology. In: Val, A.L., & Kapoor, B.G., (Eds.). Fish adaptations. Enfield, Science Publishers, 203-231.

Finney, D.J. 1971. *Probit analysis.* Cambridge Univ. Press. London and New York, *3rd Ed.*

Floch, J., Lees, M. and Sloane - Stanley, G.H. 1957. A simple method for the isolation and purification of total lipids from animal tissues. *J.biol.chem.,* Vol. 226: 497 - 509.

Fontine, C. T. and Lightener, D. V. 1974. Observation on the phagocytosis and elimination of carmine particles injected in to the abdominal musculature of the white shrimp *Penaeus setiferus J. Invert. Pathol.,* 24(2): 141-8.

Funge-Smith, S. J. and Briggs, M. R. P. 1998. Nutrient budgets in intensive shrimp ponds: implications for sustainability. *Aquaculture* 164(1-4): 117-133.

Gaetke, L.M., and Chow, C.K., 2003. Copper toxicity, oxidative stress, and antioxidant nutrients. *Toxicology.* 189, 147-163.

Genjatulin, K.V. 1990. Controlling chemical and biological water pollution by quantitative bioassaying. *Water Res.,* 24(5) 539-542.

George, S.G. 1982. Subcellular accumulation and detoxification of metals in aquatic animals. *Physiological mechanism,* 3 - 53.

***George, C.J., Reuben, N. and Muthe, P.T. 1955.** J. *Anim. Morph. Physiol.,* 2 : 14-22.

Gibson, R. and Barker, P.L. 1979. The decaped hepatopancreas. *Oceanogr. Mar. Biol. Ann. Rev.,* 17 : 285 - 346.

Gill, T. S.,Pant, J. C. and Tewari, H. 1988. Bronchial pathogenesis in a fresh water fish, *Puntius conchonius* (Ham), chronically exposed to sublethal concentration of cadmium. *Ecotoxicol. Environ. Saf.,* 15 (2): 153 – 161.

Gornall, A.G., Bardawill, C.J. and David, M.M. 1949. Determination of total serum proteins by means of biuret reaction. *J.Biol.Chem.,* 177: 751- 766.

Govindarajan, S., Valsaraj, C.P., Mohan, R., Hariprasad, V. and Ramasubramanian, **R.** 1993. Toxicity of heavy metals in aquaculture organisms : *Penaeus indicus, Perna viridis, Artemia salina* and *Skeletonema costatum*. *Poll. Res.,* 12(3): 187-189.

Guimaraes-Soares, L., Felicia, H., Bebianno, M.J. and Cassio, F. 2006. Metal-binding proteins and peptides in the aquatic fungi *Fontanospora fusiramosa* and *Flagellospora curta* exposed to severe metal stress. *Sci. Total Environ.,* 372, 148-156.

Gutierrez, M.F., Gagneten, A.M. and Paggi, J.C. 2010. Copper and Chromium Alter Life Cycle Variables and the Equiproportional Development of the Freshwater Copepod, *Notodiaptomus conifer* (SARS). *Water Air and Soil Pollution.* 213(1):275-286

Gwoździński, K., Roche, H. and Pérès, G. 1992. The comparison of the effects of heavy metal ions on antioxidant enzyme activities in human and fish *Dicentrarchus labrax* erythrocytes. *Comp. Bioche. and Physiol.*, 102, 57–60.

Hagerman, L. and Szaniawaka, A. 1990. Anaerobic metabolic strategic of the glacial relief isopod *Saduria* (Mesidotea) *entomon. Mar.Ecol.Prog. Ser.*, 59: 91 - 96.

Hall, D. 2003. The International Political Ecology of Industrial Shrimp Aquaculture and Industrial Plantation Forestry in Southeast *Asia. Jour. of Southeast Asian Stud*., 34(02): 251-264.

Harding, L. and Goyette, D. 1989. Metals in Northeast Pacific coastal sediments and fish, shrimp and prawn tissues. *Mar. Pollut. Bull.*, 20(4) : 187 -189.

Harikrishnan, R., Balasundaram, C. and Heo, M.S., 2011. Impact of plant products on innate and adaptive immune system of cultured finfish and shellfish. *Aquaculture*. 317: 1–15.

Harris, R.R. and Santos, M.C.F. 2000. Heavy metal contamination and physiological variability in the Brazilian mangrove crabs, *Ucides cardatus* and *Callinectes danae* (Crustacea : Decapoda). *Marine Biology*, 137 : 691 - 703.

***Harpert, A., Rodwell, N.M. and Mayer, A. 1977.** A review of physiological chemistry.16, Edition, California Lange, Medical Publication, 269.

Hasson, K.W., Lightner, D.V., Mohney, L.L., Redman, R.M. and White, B.M. 1999. Role of lymphoid organ spheroids in chronic Taura syndrome virus (TSV) infections in *Penaeus vannamei*. *Dis. Aquat. Org*., 38:93-105.

Hedayati, A., Hoseini, S.M. and Hoseinifar, S.H. 2016. Response of plasma copper, ceruloplasmin, iron and ions in carp, *Cyprinus carpio* to waterborne copper ion and nanoparticle exposure. *Com. Biochemist. and Physiol - Part C*., 179: 87–93.

Hilmy, A.M., El - Hamid, N.F.A. and Ghazaly, K.S. 1988. Biochemical and physiological changes in the tissues and serum of both sexes in *Portunus Pelagicus* (1) following acute exposures to zinc and copper. Folia. *Morphol.* (Prague)., 36 : 79-94.

Hochachka, P.W. and Somero, G.N. 1973. Strategies of biochemical adaptations W.B. Saunders Company. *Philadelphia.* 24 : 145.

Hodson, P.V. Borgmann, U. and Shear, H. 1979. Toxicity of Copper to Aquatic Biota: *Copper in the Environment Health Effects.* Vol. 2, Wiley-Interscience, New York, pp: 307-372.

Hrubec, T.C., Smith, S.A. and Robertson, J.L. 2001. Age related in haematology and chemistry values of hybrid striped bass chrysops *Morone saxatilis. Vet. Cli.Pathol*., 30, 8–15.

Huitric, M., Folke,C. and Kautsky, N. 2002. Analysing development and government policies of the shrimp farming industry in Thailand in relation to mangrove ecosystems. *Ecological Economic.,* 40: 441-445.

***Humason, G.L. 1972.** Animal tissue Techniques, 3rd ed., W.H. Freeman and Company, San Francisco.

Islam, M.S. 2008. From pond to plate: Towards a twin-driven commodity chain in Bangladesh shrimp aquaculture. *Food Policy*. 33(3): 209-223.

Jadhav, S. M. 1993. Impact of pollutions on some physiological aspects of the fresh water bivalve, *Corbicula seriatella*. *Ph.D. Thesis, Marathwada Universit*y, Aurangabad (M.S).

Jaffer, M. and Ashraf. 1988. Selected trace metal concentration in different tissues of fish from coastal waters of Pakistan (Arabian Sea). *Indian. J. Mar. Sci.,* 17(3) : 231 - 234.

James, R., Sampath, K. and Selvamani, P. 1998. Effect of EDTA on reduction of copper toxicity in *Oreochromis mossambicus* (Peters). *Bull. Environ. Contam. Toxicol.* **60** 487-493.

Jana, S.S. and Choudhari, M.A. 1984. Synergistic effect of heavy metal pollutants on senescence in submerged aquatic plants. *Water Air Soil Poll.,* 21 : 351 - 357.

Jana, R.K. and Jena, J.K. 2004. Overwhelming growth. Enhancing global competition. Survey of Indian Agriculture 2004. The Hindu, 101–103.

Jana, S. and Sahana, S.S. 1988. Effects of Copper, Cadmium and Chromium cations on the freshwater *fish, Clarias batrachus L. Physiol. Bohomoslove.,* 37 : 79 - 82.

***Jayadi, M. 2016.** Peningkatan Bahan Organik dan Vibrio spp. Budidaya Udang Vaname Dicurigai sebagai Penyebab Infeksi White Feces Syndrome.*Tesis*. Fakultas Perikanan dan Imlu Kelautan Universitas Brawijaya, Malang.

Jezierska, B. and Witeska, M. 2001. Metal Toxicity to Fish. Wydawnictwo Akademii Podlaskiej, Siedlce, 318 p.

Johnson,A.F., Carew, E. and Sloman,K.A.2007. The effects of copper on the morphological and functional development of zebrafish embryos. *Aquatic toxicology*. 84(4):431-38.

Johnson, M.W. and Gentile, J.H. 1979. Acute toxicity of cadmium, copper and mercury to larval American lobster, *Homarus americanus. Bull. Envir. Contarn. Toxicol.,* 22: 258 - 264.

Joseph, K.O., Krishnani K.K., Gupta, B.P. and Muralidhar, M. 2002. Acute toxicity of some heavy metals to the shrimp *Penaeus monodon. Aquacult*., 3 (2): 143-148.

Jurd, R.D. 1985. Specialization in teleost and anuran immune response: a comparative critique. In: Manning M. J., & Tatner M. F. (Eds) *Fish Immunology*. Academic Press, London, 9-28.

Kannupandi, T., Vijayakumar, G. and Soundarapandian, P. 2001. Impact of cadmium on the total protein, carbohydrate and lipid contents of the mangrove crab, *Sesarma brockii*, De man. *J. Mar. Biol. Ass. India,* 48 (1 & 2): 193-196.

Katticaran, C.M., Mohammed Salib, K.Y. and Joseph, P.S. 1995. Copper induced alterations in total carbohydrate and protein levels in bivalve, *Sunetta scripta (Bivalvia). Indian. J. Mar. Sci.,* 24 : 171 - 174.

Katiha, P.K. 2000. Freshwater aquaculture in India: Status, potential and constraints. In: Proceedings of the Aquaculture Development in India: Problems and Prospects Workshop (eds M. Krishnan & P.S. Birthal).

Kavitha, C., Malarvizhi, A., Senthil Kumaran, S., Ramesh, M., 2010. Toxicological effects of arsenate exposure on hematological, biochemical and liver transaminases activity in an Indian major carp, *Catla catla*. *Food and Chemi.Toxicol*., 48: 2848–54.

Kerkut, G.A. and Munday, K.A. 1962. The effect of copper on the tissue respiration of the crab, *Carcinus maenas. Cah. Biol. Mar.*, 3: 27 - 35.

Khotimchenko, S.V.2005. Lipids from the Marine Alga *Gracilaria verrucosa*. *Chemistry of Natural Compounds.* 41(3):285-288.

Kinsella, J. E. 1987. Sea foods and fish oils in human health and disease. New York: Marcel Dekker. Inc. New York, 307p.

Kondo, M., Itami, T., Takahashi, Y., Fujii, R. and Tomonaga, S. 1994. Structure and function of the lymphoid organ in the kuruma prawn. *Dev. Comp. Immunol*.,18 (Suppl. 1): S109.

Kongkeo, H. and Davy, F.B. 2010. Backyard hatcheries and small scale shrimp farming in Thailand. In: De Silva SS, Davy FB (eds) *Success stories in Asian aquaculture*. Springer, Dordrecht, pp 67–84.

Kori-Siakpere, O., Ake, J.E.G. and Idoge, E. 2005. Haematological characteristics of the African snakehead, *Parachacnna obscura*. *Afri. Jour. of Biotech.*, 4: 527–530.

Krishnamoorthy, P. and Subramanian, P. 1995. Biochemical variation during accumulation and deporation of copper in *Macrobrachium lamerrei lamerrei* (H.M. Edwards). *Bull. Pure. Applied. Sci.*, 19A(1) : 27 - 33.

Krishnamoorthy, P. and Subramanian, P., l996. Effect of Schwaiger, J., Wanke, R., Adam, S., Pawert, M., Honnen, sub lethal doses of copper on the hepatopancreas of the freshwater prawn, *Macrobrachium lamarrei lamarrei. Geo bio.*, 23(1), 16-18.

Krishnapriya, K., and Ramesh, M., 2018. Comparative risk assessment of copper nanoparticle with its bulk counterpart in an Indian major carp *Labeo rohita*. Chapter 7 In: Environmental Toxicity of Nanomaterials, Taylor & Francis Group, Articles in press.

Krishna Priya, K., Ramesh, M., Saravanan, M. and Ponpandian, N. 2015. Ecological risk assessment of silicon dioxide nanoparticles in a fresh-water fish *Labeo rohita*: Hematology, ionoregulation and gill Na^+/K^+-ATPase activity. *Ecotoxi. and Environ. Safe*., 120: 295–302.

Krishnapriya, K., Shobana, G., Narmadha, S., Ramesh, M. and Maruthappan, V. 2017. Sublethal concentration of bisphenol A induces haematological and biochemical responses in an Indian major carp *Labeo rohita*. *Ecohydro. and Hydrobiol*., 17:306–313.

Kumari, S. A. and Kumar, N. S. R. 1995. Effects of water pollution on the histology of fish, *Channa striatus* in Hussaina sagar, Hyderabad. India. *Environ. Ecol.* 13 (4): 932-934.

Labreche, T., Dietrich, A.M., Gallagher, D.L and Shepherd, N. 2002. Copper toxicity to larval *Mercenaria mercenaria* (Hard clam). *Environ. Toxicol. and Chemistry.*, 21(4):760-66.

Lacerda, L ,D., Santos, J.A. and Madrid, R. M.2006. Copper emission factors from intensive shrimp aquaculture. *Mar. Pollut. Bulletin.*, 52(12):1823-1826.

La Colla. N.S, Botté, S.E and Marcovecchio, J.E. 2019. Mercury cycling and bioaccumulation in a changing coastal system: From water to aquatic organisms. *Mar. Pollu.Bulletin*., 140: 40-50.

Lasheen, M.R., AbdelGawad, F.K., Alaneny, A.A. and Abd Elbary, H.M.H. 2012. Fish as Bio indicators in aquatic environmental pollution assessment: a case study in Abu-Rawash Area, Egypt. *Wor. Appl. Sci. Jour*., 19, 265–275.

Lavanya, S., Ramesh, M., Kavitha, C. and Malarvizhi, A., 2011. Hematological, biochemical and ionoregulatory responses of Indian major carp *Catla catla* during chronic sublethal exposure to inorganic arsenic. *Chemosphere.* 82, 977–985.

Le Moullac, G. and Haffner, P.2000. Environmental factors affecting immune responses in Crustacea. *Aquaculture* 191(1-3):121-131.

Lewbart, G.A., Hirschfeld, M., Denkinger, J., Vasco, K., Guevara, N., García, J., Muñoz, J. and Lohmann, K.J., 2014. Blood gases, biochemistry, and hematology of Galápagos green turtles (*Chelonia mydas*). *PLoS One* 9: 964-87.

Linford, E. 1965. Biochemical studies on marine zooplankton. II. Variations in the lipid content of some mysidacea. *J. Cons. Perm. Int. Explor. Mer.,* 30 : 16-27.

Liu, F., Yulin, W., Hongru, Z., Lanying,H. and Fend, S. 1988. Accumulation and toxicity of copper in *prawn, Penaeus orientalis. Oceanol Limnol sin* (Haiyaog Yu - Huzhao)., 19 : 133 - 139.

Loker, E.S., Adema, C.M., Zhang, S.M. and Kepler, T.B. 2004. Invertebrate immune system -not homogeneous, not simple, not well understood. *Immunol. Rev.,* 198:10-24.

Lorentzen, M., Maage, A., Julshamn, K., 1998. Supplementing copper to a fishmeal- based diet fed to *Atlantic salmon* parr affects liver copper and selenium concentrations. *Aquacul. Nut.*, 4: 67–72.

Lushchak, V.I., 2011. Environmentally induced oxidative stress in aquatic animals. *Aqua. Toxicol.*, 101(1):13-30.

Lyndon, A. R., Davidson, I. and Houlihan, D. F. 1993. Changes in tissue and plasma free amino acid concentrations after feeding in Atlantic cod. *Fish Physiol. Biochem*. 10, 365-375.

Maharajan. A.2002. Evaluation of copper toxicity in the spiny lobster, *Panulirus homarus,* (Linnaeues,1758). Ph.D Thesis, *University of Madras.* P:1-85

Maharajan, A., Narayanasamy, Y., Ganapiriya, V. and Shanmugavel, K. 2015. Histological alterations of a combination of chlorpyrifos and cypermethrin (Nurocombi) insecticide freshwater water crab, *Paratelphusa jacquemontii* (Rathbun). *Jour. of Basic and Appl. Zool.,* 72: 104-112. doi:10.1016/j.jobaz.2015.08.002.

Maharajan, A., Narayanaswamy, Y. and Ganapiriya, V. 2017. Haematological Changes of Fresh Water Crab, *Paratelphusa jacquemontii* in Response to the Combination of Chlorpyrifos and Cypermethrin (Nurocombi) Insecticide. *Ann. Aquac. Res., **4(3):** 1041: 1-6.*

Mallatt, J., 1985. Fish gill structural changes induced by toxicants and other irritants: a statistical review. Canadian Journal of Fisheries and Aqua. Sci., 42, 630-648.

Manisseri, M.K. and Menon, N.R. 1995. Copper induced damage to the hepatopancreas of the penaeid shrimp, *Metapenaeus dobsoni* an ultrastructural study. *Dis. Aquat. Org.,* 22 : p.51-57.

Martin, J.L.M. 1973. Iron metabolism in Cancer irroratus (Crustacea. Decapoda) during the intermoult cycle with special reference to iron in the gills. *Comp. Biochem. Physiol.,* 46A : 123 - 129.

Martin, M.H., Coughtrey, P.J., 1982. Biological monitoring of heavy metal pollution. Land and Air Applied Science, London 475pp.

Martin, G.G., Hose, J.E., Minka, G. and Rosenberg, S. 1996. Clearance of bacteria injected into the hemolymph of the ridgeback prawn, *Sicyonia ingentis* (Crustacea: Decapoda): role of hematopoietic tissue. *J. Morphol.,* 227:227-33.

Mckim,J.M. and Benott, D.A. 1971. Effects of long term exposures to copper on survival, growth and reproduction of brooktrout, *Salvelinus jontinalis. J. Fish. Res. Board. Canada.,* 28 : 655 - 662.

Mcleese, D.W. 1974. Toxicity of copper at two temperatures and three salinities to the American lobster *(Homarus americanus). J. Fish. Res. Board Canada.,* 31 : 1949 - 1952.

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

Mierzejewski, J., Haney, D.C. and Van den Hurk, P. 2014. Biomarker responses in sunfish and bass from the Saluda river, SC. *Ecotox. and Environ. Safety*., 110:8–15.

Misshra, J. and Srivastava, A.K. 1983. Malathion induced hematological biochemical changes in the Indian catfish, *Heteropneustes fossilis. Environ Res.,* 30 : 393.

Monteiro, S.M., Mancera, J.M., Fontainhas, F.A. and Sousa, M. 2005. Copper induced alterations of biochemical parameters in the gill and plasma of *Oreochromis niloticus. Comp. Biochem. and Physiol*., 141, 375-383.

Moore, M.N. 1988. Cytochemical responses of the lysosomal system and NADPH. Ferrihemoprotein reductase in molluscan digestive cells to environmental and experimental exposure to Xenobiotics. *Mar. Ecol. Prog. Ser.,* 46 : 81 - 89.

Moore, J.W. and Ramamoorthy, S. 1984. *Heavy metals in natural waters -applied monitoring and impact assessment.* Spring series on Environmental Management, Springer - Verald, New York.

Motlagh, S.P., Zarejabad, A.M., Nasrabadi, R.G., Ahmadifar, E. and Molaee, M. 2012. Haematology, morphology and blood cells characteristics of male and female Siamese fighting fish (*Betta splendens*). *Comp. Clin. Patho*., 21: 15–21.

Mozaffarian, D., Bryson, C. L., Lemaitre, R. N., Burke, G. L.and Siscovick, D. S. 2005. Fish intake and risk of incident heart failure. *Jour. of the Amer. College of Cardiol*., 45(12): 2015–2021.

Mudgal, V., Madaan, N., Mudgal, A., Singh, R.B. and Mishra, S. 2010. Effect of toxic metals on human health. *Open Nutraceuticals J.* 3:94–99.

Muley, D.V., Karanjakar, D.M. and Maske, S.V. 2007. Impact of industrial effluents on the biochemical composition of fresh water fish *Labeo rohita*. *J. Environ Biol.,* 28(2):245-249.

Murti, R. and Shukla, **G.S. 1984.** Toxicity of copper sulphate and zinc sulphate to *Macrobrachium lamarrei* (H. Milne Edwards) (Decapoda, Palaemonidae). *Crustaceana,* 47(2):168-173.

Nakamura, K. 1987. Lymphoid organ and its developmental property of larval prawn *Penaeus japonicus*. Mem Fac Fish, Kagoshima University 36:215-20.

Nammalwar, P. 1985. Heavy metal pollution in Adyar Estuary, Madras, India. *Proc. Symp. Assess. Environ. Pollut.,* pp. 235 - 238.

Newman, M.C. and Feng, S.Y. 1982. Susceptibility and resistance of the rock crab, *Cancer irroratus* to natural and experimental bacterial infection. *J. Inve.r Pathol.,* 40: 75-88.

***Nicoara, E., Bilaus, C., Bodea, C. and Petrescu, I. 1970**. *StudiiCere.Biochim.,* **13** : 65 - 68.

Nimmo, D.W.R., Lightner, D.V. and Bahner, L.H. 1977. Effects of cadmium on shrimps. *Penaeus duorarum, Palaemonetes pugio* and *P.uul,garis* In : *Physiological Response of marine biota to pollutants.* F.J. Verenberg, A. Calabrese, F.P. Thurberg and W.B. Vernberg (Eds). Academic Press, New York, 131 - 183.

Nott, J.A. 1991. Cytology of pollutant metals in marine invertebrates. A review of microanalytical application. *Scann. microsc.,* 5 : 191-204.

Nussey, G., Van Vuren, J.H. and Du Preez, H.H. 1995. Effect of copper on blood coagulation of *Oreochromis mossambicus* (Cichlidae). *Com. Biochemist. and Physiol*., Part C: Toxicology and Pharmacology 111: 359-367.

Nuwayhid, M.B. and Young, L.G.L. 1985. Biochemical composition and starvation effects of the shrimp, *Penaeus semisulcatus* Dettaan 1844, found off the Labanese Coast. *Lebanese science Bullettin, 1(1) :* 33 - 41.

Oka, M. 1969. Studies on *Penaeus orientalis* KISHINOUYE-VIII structure of the newly found lymphoid organ. *Bull. Jap. Soc. Sci. Fish*.,35:245-50.

Oliveira, S.R.S., Batista, W.S., Sousa, J.B.M., Noleto, K.S., Lima, I.M.A., Andrade, T.S.O., Cardoso, W.S. & Carvalho-Neta, R.N.F. 2019. Enzymatic and histological biomarkers in *Ucides cordatus* (Crustacea, Decapoda) in an industrial on the north coast of Brazil. *Bullet. of Environ. Contamin. and Toxicol*., 102(6): 802-810. doi: 10s00128-019-02594-1

Olojo, E. A. A., Olurin, K. B., Mbaka, G. and Oluwemimo, A. D. 2005. Histopathology of gill and liver tissues of the African catfish Clarias garipinus exposed to lead. *African J. Biotech*., 4(1): 117-122.

Oshode, O.A., Bakare, A.A., Adeogun, A.O., Efuntoye, M.O. and Sowunmi, A.A. 2008. Ecotoxicological assessment using *Clarias gariepius* and microbial characterization of leachate from municipal solid waste landfill. *Inter. Jour. of Environ. Res.*, 2: 391-400.

Overstreet, R.M. 1988. Aquatic Pollution Problems, South eastern U.S. Coarts: Histopathological indicators. *Aquat. Toxicol.*, 11 : 213 - 239.

Paez - Osuna, F. and Tron -Mayen, L. 1996. Concentration and distribution of heave metals in tissue of wild and farmed shrimp *Penaeus vannamei* from the north-west coast of Mexico. *Arch. of Environ.Contamin. and Toxicol*., 22 : 443-450.

Parashar, R. S. and Banerjee, T. K. 2002. Toxic impact of lethal concentration of lead nitrate on the gills of air breathing cat fish *Heteropueustes fossilii* (Bloch) *Veteran. Arch*., 72 (3), 176-83.

***Paterson, N.F. 1968**. *Ann. S. Afr. Mus.*, 51 : 1-232.

***Patwardhan, S.S. 1937.** *Indian Zool. Mem. Ind Anim. Types*, 6 : 1-100

***Pearson, J. 1908.** L.M.B.C. *Mein. Typ. Br. Mar. Pl. Anim. Types*, 16 : 1-209.

***Perkins, H.F., 1970**. *Soil Science and Plant Analysis,1 :* 35.

Philippe, C., Gregoir, A.F., Janssens, L., Pinceel, T., DeBoeck, G. and Brendonck, L. 2017. Acute and chronic sensitivity to copper of a promising ecotoxicological model species, the annual killifish *Nothobranchius furzeri*. *Ecotoxicol. and Environ. Safety*., 144 :26– 35.

Pillai, N.G.K. and Katiha, P.K. 2004. Evolution of Fisheries and Aquaculture in India. Central Marine Fisheries Research Institute, Kochi, p. 34.

Pinheiro, M.A.A., Duarte, L.F.A., Toledo, T.R., Adam, M.L. and Torres, R.A. 2013. Habitat monitoring and genotoxicity in *Ucides cordatus* (Crustacea: Ucididae), as tools to manage a mangrove reserve in southeastern Brazil. *Environ. Monitor. and Assess*., 185: 8273-8285. doi: 10.1007/s10661-013-3172-9.

Pongsomboon, S., Wongpanya, R., Tang, S., Chalorsrikul, A. and Tassanakajon, A.2008. Abundantly expressed transcripts in the lymphoid organ of the black tiger shrimp, *Penaeus monodon*, and their implication in immune function. *Fish Shellfish Immunol*., 25:485-93.

Poopal, R.K., Ramesh, M., Maruthappan, V. and Babu Rajendran, R. 2017. Potential effects of low molecular weight phthalate esters (C16H22O4 and C12H14O4) in a Freshwater Fish, *Cyprinus carpio. Toxicol. Res*., 6: 505–520.

Portmann, J.E. and Wilson, K.W. 1971. The toxicity of 140 substances to the brown shrimp and other marine animals. *Shell fish information Leaflet,* No.22 pp.1-38. Ministry of Agriculture, Fisheries and Food, Fisheries laboratory, Burnham on Crouch, UK.

Prachumwat, A., Thitamadee, S., Sriurairatana, S., Chuchird, N. and Limsuwan,C. et al., 2012. Shotgun sequencing of bacteria from AHPNS, a new shrimp disease threat for Thailand. Poster, National Institute for Aquaculture Biotechnology, Mahidol University, Bangkok, Thailand.

Pullin, R.S.V.1993. An overview of environmental issues in developing-country aquaculture. In: R.S.V. Pullin, H. Rosenthal and J.L. Maclean (Editors), Environment and Aquaculture in Developing Countries. ICLARM Conf. Proc., 31: 1-19.

Putranto, T. W. C., Andriani, R., Munawwaroh, A., Irawan, B. and Soegianto, A. 2014. Effect of cadmium on survival, osmoregulation and gill structure of the Sunda prawn, *Macrobrachium sintangense* (de Man), at different salinities. *Mar. and Fresh. Behav. and Physiol*., 47(5):349-360.

Pyefinch, K.A. and Mott, J.C. 1948. The sensitivity of barnacles and their larvae to copper and mercury. *J. Exp. Biol.*, 25 : 276 - 298.

Rainbow, P.S. 1998. Phylogeny of trace metal accumulation in crustacean. In: Langston WJ, Bebianno MJ (eds) *Metal metabolism in aquatic environments.* Chapman and Hall, London, pp. 285 - 319.

Raj Narayan, R. and Sathyanesan, A.G. 1987. Histopathological and biochemical changes of a teleost fish, *Channa punctatus* (Bloch) induced by a mercurial fungicide. *Environ. Poll.,* 47: 135-145.

Rajkowska, M. and Protasowicki, M. 2013. Distribution of metals (Fe, Mn, Zn, Cu) in fish tissues in two lakes of different trophy in Northwestern Poland. *Environ. Monitor. and Assess*., 185: 3493–3502.

***Reddy, A.R. 1938**. *Proc. Indian. Acad. Sci.,* 6 : 172-193.

Regoli, F. and Principato, G. 1995. Glutathione, glutathione-dependent and antioxidant enzymes in mussel, *Mytilus galloprovincialis*, exposed to metals under field and laboratory conditions: implications for the use of biochemical biomarkers. *Aqua. Toxicol*., 31: 143-164.

Remyla, S.R., Ramesh, M., Sajwan, K.S. and Senthil Kumar, K. 2008. Influence of zinc on cadmium induced haematological and biochemical responses in a freshwater teleost fish *Catla catla*. *Fish Physiol. and Biochem.*, 34: 169–174.

Roch. P. 1999. Defense mechanism and disease prevention in farmed marine invertebrate. *Aquaculture.* 172:125-45.

Rodriguez, J. and Le Moullac, G.2000. State of the art of immunological tools and health control of penaeid shrimp. *Aquaculture*.191:109-19.

Roldan, B.M. and Shivers, R.R. 1987. The uptake and storage of iron and lead in cells of the tray fish *(Roconectes propinguus)* hepatopancreas and antennal gland. *Corn. Biochern. Physiol.,* 86C : 201 - 214.

Saha, N., Mollah, M.Z.I., Alam, M.F. and Safiur Rahman, M. 2016. Seasonal investigation of heavy metals in marine fishes captured from the Bay of Bengal and the implications for human health risk assessment. *Food Control*, 70:110-118.

Saleh, Y.S., and Marie, M.A.S., 2016. Use of *Arius thalassinus* fish in a pollution biomonitoring study, applying combined oxidative stress, hematology, biochemical and histopathological biomarkers: A baseline field study. *Mar. Pollu. Bullet*., 106:308–322.

Sampaio, F.G., Boijink, C.L., Bichara dos Santos, L.R., Tie Oba, E., Kalinin, A.L., Barreto Luiz, A.J. and Rantin, F.T. 2012. Antioxidant defenses and biochemical changes in the neotropical fish pacu, *Piaractus mesopotamicus:* Responses to single and combined copper and hypercarbia exposure. **Compar. Biochemist. and Physiol**., Part C 156: 178–186.

Saravanan, M., Kim, J.Y., Hur, K.J., Ramesh, M. and Hur J.H. 2017. Responses of the freshwater fish *Cyprinus carpio* exposed to different concentrations of butachlor and oxadiazon. *Biocatal. and Agricul. Biotech*.,11: 275-281.

Sastry, K.V. and Das gupta, A.1991. Effect of Nuvacron on the nutritive value of fresh water teleost fish *Channa punctatus*. *J. Environ. Biol.*,12(3): 243-248.

Sayed, A.E.H. and Moneeb R.H. 2015. Hematological and biochemical characters of monosex tilapia (*Oreochromis niloticus,* Linnaeus, 1758) cultivated using methyltestosterone. *The Jour. of Basic and Appl. Zool*., 72: 36-42.

Schafer, H.J. 1968. Storage materials utilized by starved pink shrimp, *Penaeus duorarum.* Burken. road FAO *Fish. Rep. Ser.*, 57 (2) : 393 - 403.

***Schroeder, H.A., Frost, D.V., and Balasse, J.J. 1970**. *J. Chron. Dis.,* **22**: 227 -243.

Seenivasan, R. 1988. Individual and combined effect of heavy metals (Ca, Zn, and Cd) on prawn, *Penaeus indices. M.Phil. Dissertation,* Annamalai Uni.42 Pp.

Sen, P., Tiwari, K. J., Shukla, S., Shukla, R. and Sharma, U. D. 2008. Effects of cadmium on ventilation and oxygen consumption of freshwater prawn, Macrobrachium dayanum (Crustacea - Decapoda). *Aquaculture*. 9(1): 95-100.

Senarath, U. and Visvanathan,C. 2001. Environmental Issues in Brackish Water Shrimp Aquaculture in Sri Lanka. *Environ. Manag*e., 27(3): 335-348.

Shao, L., Kang, S.H.L., Li, J., Hixson, P., Taylor, J., Yatsenko, S.A., Shaw, C.A., Milosavljevic, A., Chang, C.C., Cheung, S.W. and Patel, A. 2010. Array comparative genomic hybridization detects chromosomal abnormalities in hematological cancers that are not detected by conventional cytogenetics. *The Jour. of Mole. Diagnostics.*,12: 670-679.

Shaw,B.,Al-bairuty, G. and Handy.R.D. 2012. Effects of waterborne copper nanoparticles and copper sulphate on rainbow trout, (*Oncorhynchus mykiss*): Physiology and accumulation. *Aqua. Toxicol.*, 116: (117): 90-101.

Singh, H.S., and Reddy, T.V. 1990. Effect of copper sulphate on heamatology, blood chemistry and hepato-somatic index of an Indian catfish, *Heteropneustes fossils* (Bloch), and its recovery. *Ecotoxicol. and. Environ. Safe*., 20: 30-35.

Sivadasan, C.R. Nambian, P.N. and Domodaran, R. 1986. Toxicity of mercury, copper and zinc to the prawn *Metapenaeus dobsoni* (Miers), *Current Sci.*, 55(7) : 337 - 340.

Smith, V.J., Brown, J.H. and Hauton, C.2003. Immunostimulation in crustaceans: does it really protect against infection? *Fish Shellfish Immunol*., 15:71-90.

Smith, V.J., Soderhall, K. and Hamilton, M.B.1984. 1, 3-Glucon induced cellular defence reactions in the shore crab, *Carcinus maenas*. *Comp. Biochem.Physiol* . 77A: 635-639.

Soegianto, A.; Asih, A. Y. P. and Irawan, B. 2016. Lead toxicity at different life stages of the giant prawn (*Macrobrachium rosenbergii*, de Man). Considerations of osmoregulatory capacity and histological changes in adult gills. *Mar. and Fresh. Behav. and Physiol*., 49(3):187-20.

***Sorensen, E.M. 1991**. *Metal Poisoning in Fish*. CRC Press, Florida.

Sovová, T., Boyle, D., Sloman, K.A., Pérez, C.V. and Handy, R.D. 2014. Impaired behavioural response to alarm substance in rainbow trout exposed to copper nanoparticles. *Aqua. Toxicol*., 152:195–204.

Sparague, J.B. 1969. Measurements of Pollutant toxicity to fish. I. Bioanay methods for acute toxicity. *Wat. Res.*, 3 : 793 - 821.

***Stryer, L. 1988.** *Biochemistry,* 3rd Edn. Freeman and Co. New York.

Sriket, S., Benjakul, P., Visessanguan, W. and Kijroongrojana, K. 2007. Comparative studies on chemical composition and thermal propertiesof black tiger shrimp (*Penaeus monodon*) and white shrimp (*Penaeus vannamei)* meats. *Food Chem.*, 103:1199-1207

Sullivan, B. K., Buskey, E., Miller, D. C. and Ritacco, P. J. 1983. Effects of copper and cadmium on growth, swimming and predator avoidance in *Eurytemora affinis* (Copepoda). *Mari. Biol.*, 77: 299–306.

Tellez-Banuelos, M.C., Santerre, A., Casas-Solis, J., Bravo-Cuellar, A. and Zaitseva, G. 2009. Oxidative stress in macrophages from spleen of Nile tilapia (*Oreochromis niloticus*) exposed to sublethal concentration of endosulfan. *Fish and Shellfish Immunol*., 27:105–111.

Tilak, K.S. and Yacobu, K. 2002. Toxicity and effect of fenvalerate on fish *Ctenopharyngodon idellus.* J. Ecotoxicol. Environ. Monit., 12(1): 09-15.

Torres, C. 1973. Variations Du Pool Des Amines Libres Du Muscle Abdominal De *Penaeus kerathurus* Au Cours Du Cycle d'intermue, Etau Cours Du Jeune. *Comp. Biochem. Physiol*., 45A, 1-12.

Tort, L., Torres, P. and Flos, R. 1987. Effects on dogfish haematology and liver compostion after acute copper exposure. *Comp. Biochem. Physiol.,* 87C : 349.

Trendall, J.T. and Prescott, J. 1989. Servere physiological stress associated with the annual breeding emigration of *Panulirus ornatus* in the Torres strait. *Marine Ecology program series.*, 58 : 29 - 39.

Trevisan, J., Angelov, P.P., Patel, I.I., Najand, G.M., Cheung, K.T., Llabjani, V., Pollock, H.M., Bruce, S.W., Pant, K., Carmichael, P.L., Scott, A.D. and Martin, F.L. 2010. Syrian hamster embryo (SHE) assay (pH 6.7) coupled with infrared spectroscopy and chemometrics towards toxicological assessment. *Analyst.,* 135: 3266- 3272.

Tripathi, P.K., Srivastava, V.K. and Singh, A. 2003. Toxic effects of di methoate (organophosphate) on metabolism and enzyme system of fresh water teleost fish *Channa punctatus*. *Asian Fish. Sci*., 16: 349-359.

Ullah, R., Zuberi, A., Naeem, M. and Ullah, S. 2015. Toxicity to hematology of liver, brain and gills during acute exposure of Mahseer (*Tor putitora*) to cypermethrin. *Pak. Jour. of Agri. and Biol*., 12-18.

Umminger, B.L. 1970. Physiological studies on super cooled Hilli fish *Fundulus heteroditus*, carbohydrate metabolism and survival at sub zero temperature. *J. Exp. Zool.,* 173: 159- 174.

***Vallee, B.L. and Wacker, W.E.L. 1970.** The proteins. In: metalloproteins H. Nourath (Ed.) Vol.5 Academic Press, London, New York.

Van de Braak, C.B.T., Botterblom, M.H.A., Taverne, N, Van Muiswinkel,W.B., Rombout, J.H.W.M., Van der Knaap, W.P.W.2002. The roles of haemocytes and the lymphoid organ in the clearance of injected Vibrio bacteria in *Penaeus monodon* shrimp. *Fish Shellfish Immunol*., 13:293-309.

Van Dyk, J.C., Pieterse, G.M. and Van Vuren, J.H.J. 2007. Histological changes in the liver of Oreochromis mossambicus (Cichlidae) after exposure to cadmium and zinc. *Ecotoxicol. Environ. Saf.,* 66: 432-440.

Vardhanan, Y.S. and Radhakrishanan, T. 2002. Acute toxicity evaluation of copper, arsenic and HCH to paddy field crab, *Paratelphusa hydrodomus* (Herb.). *J. Environ. Biol.,* 23(4): 387-392.

Viarengo, A. 1985. Biochemical effects of trace metals. *Mar. Poliut. Bull.,* 16: 153 -158.

Victor, B., Mahalingam,S. and Sarojini, R. 1990. Gill Pathology and haemocyte response in mercury exposed *Macrobrachium idea* (Heller). J. *Environ. Biol*., 11(1): 61-65.

Vijayakumaran, M. 1990. Energetics of a few marine crusteceans. *Ph.D. Thesis,* Cochin University of Science and Technology, Cochin.

Villalan, P., Narayanan, K.R., Ajmal Khan, S. and Natarajan, R. 1988. Proximate composition of muscle, hepatopancreas and gill in the copper exposed estuarine *crab,Thalamita crenata* (Latreille). *Proc. 11. Natl. Symp. Ecotoxicol.,* 55 - 59.

Virk, S.Q. and Kaur, K. 1999. Impact of mixture of nickel and chromium on the protein content of flesh and liver of *Cyprinus carpio* during spawing and post-spawning phases. *Bull. Env. Cont. and Toxi.,* 63 : 499 - 502.

Visvanathan, P., Maruthanayagam, C. and Govindaraju, M.2009. Effect of malathion and endosulfan on biochemical changes in *Channa punctatus. J. Ecotoxicol. Environ. Monit.,* 19(3): 251- 257.

Vogt, G. and Quinitio, E.T. 1994. Accumulation and excretion of metal granules in the *prawn,Penaeus monodon,* exposed to water borne copper, lead, iron and calcium. *Aqua. Toxicol.,* 28 : 233 - 241.

Waldichuk, M. 1978. Disposal of mine wastes into the sea. *Mar.Pollut. Bull. N.S.,* 9, 141 - 143.

Wang, T., Long, X., Cheng, Y., Liu, Z. and Yan, S.2014. The potential toxicity of copper nanoparticles and copper sulphate on juvenile *Epinephelus coioides*. *Aqua. Toxicol.,* 152: 96–104.

Ware, G.M. 1980. Effects of pesticides on non-target organisms. *Residue Rev*., 1980; 76: 173-301

Wedemeyer, G.A., Gould, R.W. and Yasutake, W.T. 1983. Some potential and limits of the leucocrit test as a fish health assessment method *Jour. of Fish Biol.,* 23: 711-716.

White, S.L. and Rainbow, P.S. 1982. Regulation and accumulation of copper, zinc and cadmium by the shrimp, *Palaemon elegans. Mar. Ecol. Prog. Ser.,* 8: 95 - 101.

White, S.L. and Rainbow, P.S. 1986. Accumulation of Cadmium by *Palaemon elegans* (crustacea) : Decapeda). *Mar. Ecol. Prog. Ser.,* 32 : 17-25.

Wright, D.A. and Brewer, C.C. 1979. Cadmium Mar. turnover in the shore *crab,Carcinus maenas. Marine Biol.,* 50 : 151 - 156.

Wood, P.J. and Visenten, L.P. 1967. Histology and Histochemicl observation of the hemolymph cellsw in the crayfish, *Orconectes virilis*. *J. Morphol.,* **123:** 559-568.

Wright, D.A and Welbourn, P.2001. Environmental Toxicology. Cambridge University Press. P:1-22.

Yanar, Y. and Celik, M. 2006. Seasonal amino acid profiles and mineral content of green tiger shrimp (*Penaus semisulcatus*, De Haan, 1844) and speckled shrimp (*Metapenaus monoceros*, Fabricus 1789) from the Eastern Mediterranean sea. *Food Chem*., 94:33-36.

Yong, L.B. and Harvey, H.H. 1989. Concentrations and distribution of Fe, Zn and Cu in tissues of the Whitesucker *(Catostomus commersoni)* in relation to elevated levels of metals and low pH. *Hydrobiologia,* 176/177 : 349 - 354.

Yulianto, B., Pierre,T., Mireille C.D., Jean Paul, T. and Guy, C. 1995. Effect of copper on survival and osmoregulation of various developmental stages of the shrimp, *Penaeus japonicus* Bate (Crustacea, Decapoda). *Aqu.Biol.,* 33 : 125 - 139.

Zutshi, B., Raghu Prasad, S.G. and Nagaraja, R., 2010. Alteration in hematology of *Labeo rohita* under stress of pollution from lakes of Bangalore, Karnataka, India. Environ. Monitor. and Assess. 168: 11–19.

**Originals not referred*

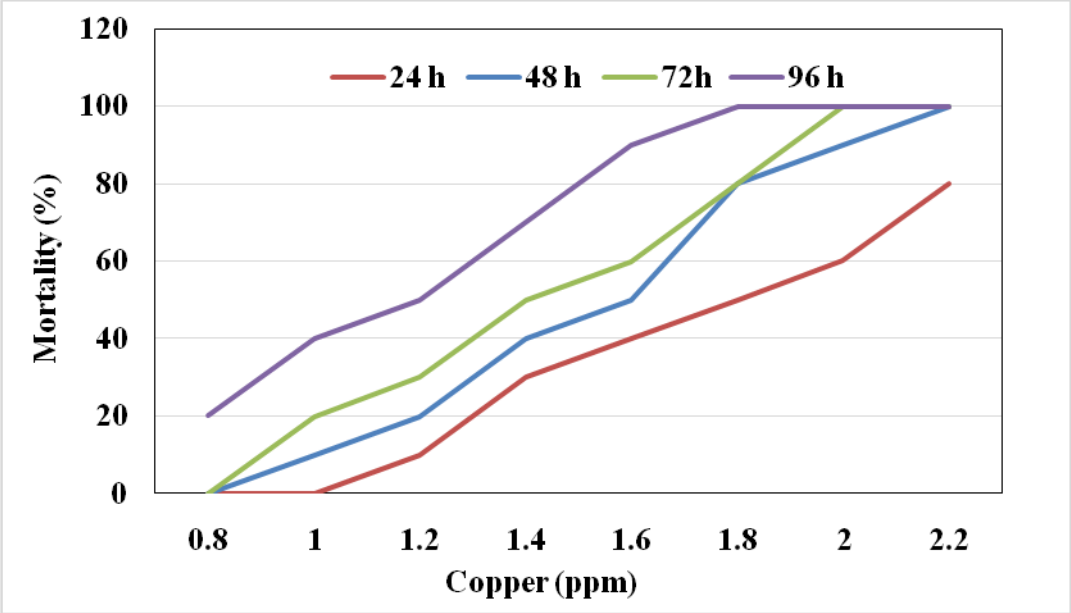


Fig. 1. Percentage mortality of *L.vannamei* exposed to different concentrations of copper after 24, 48, 72 and 96 h under acute bioassay test

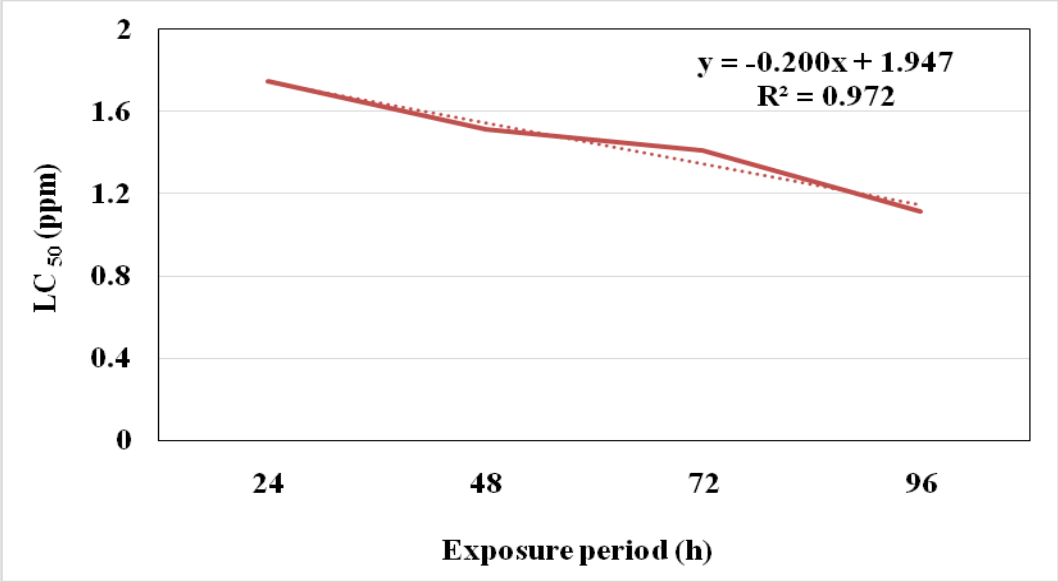


Fig.2. Toxicity curve for *L. vannamei* exposed to copper based on LC_{50} values

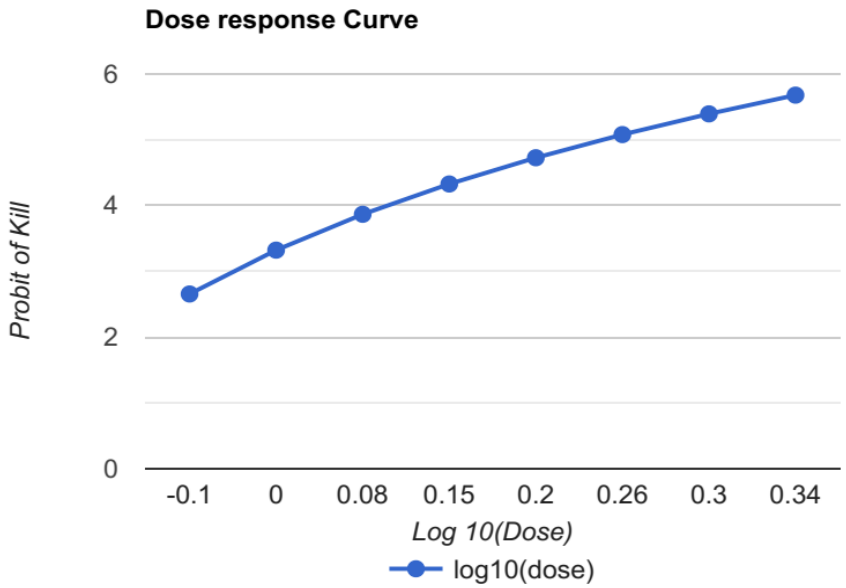


Fig.3. Dose response relationship (Probit graph) *L. vannamei* exposed to copper under acute static renewal bioassay of at 24 hrs

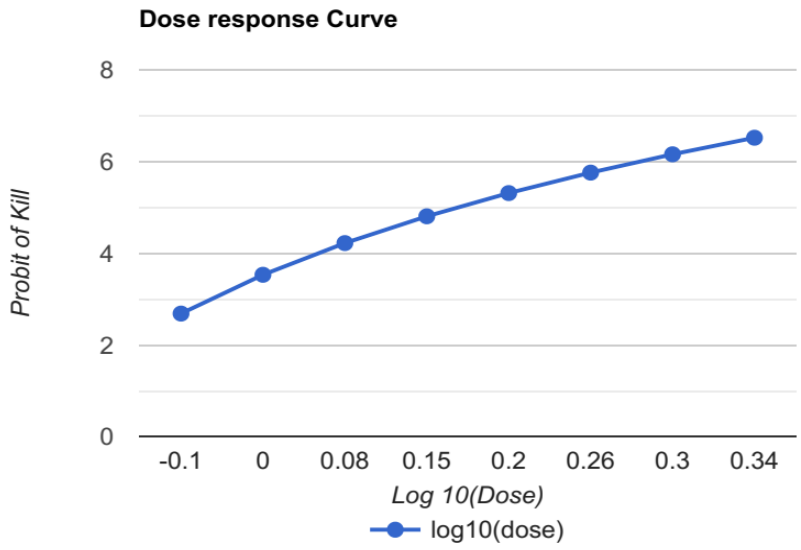


Fig. 4. Dose response relationship (Probit graph) – *L. vannamei* exposed to copper under acute static renewal bioassay of at 48 hrs

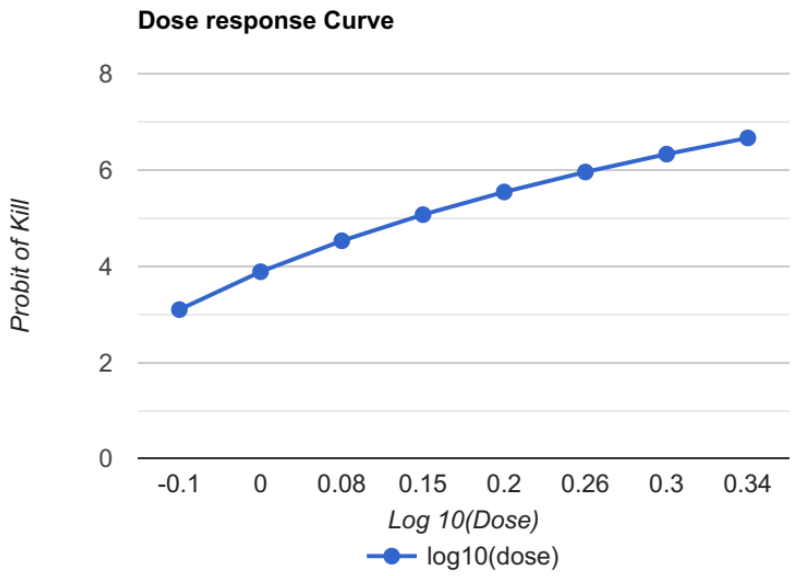


Fig. 5. Dose response relationship (Probit graph) – *L. vannamei* exposed to copper under acute static renewal bioassay of at 72 hrs

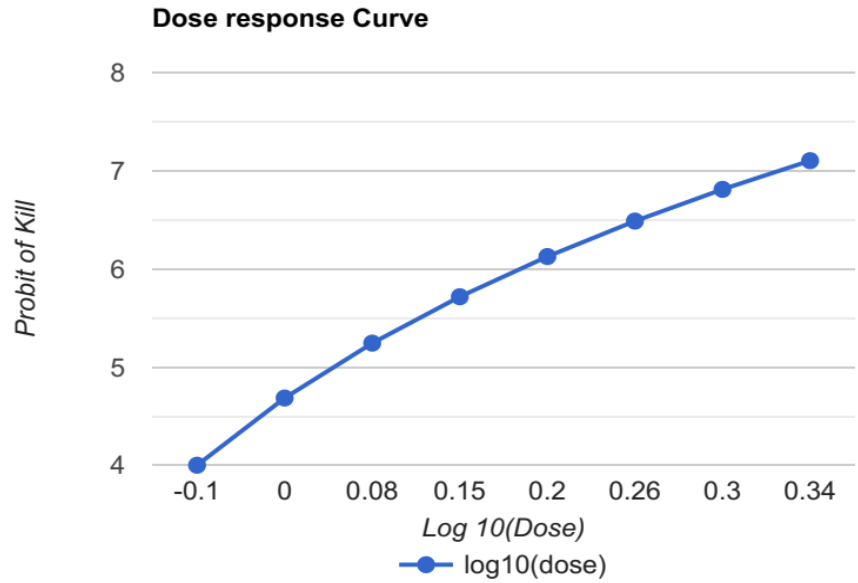


Fig.6. Dose response relationship (Probit graph) – *L.vannamei* exposed to copper under acute static renewal bioassay of at 96 hrs

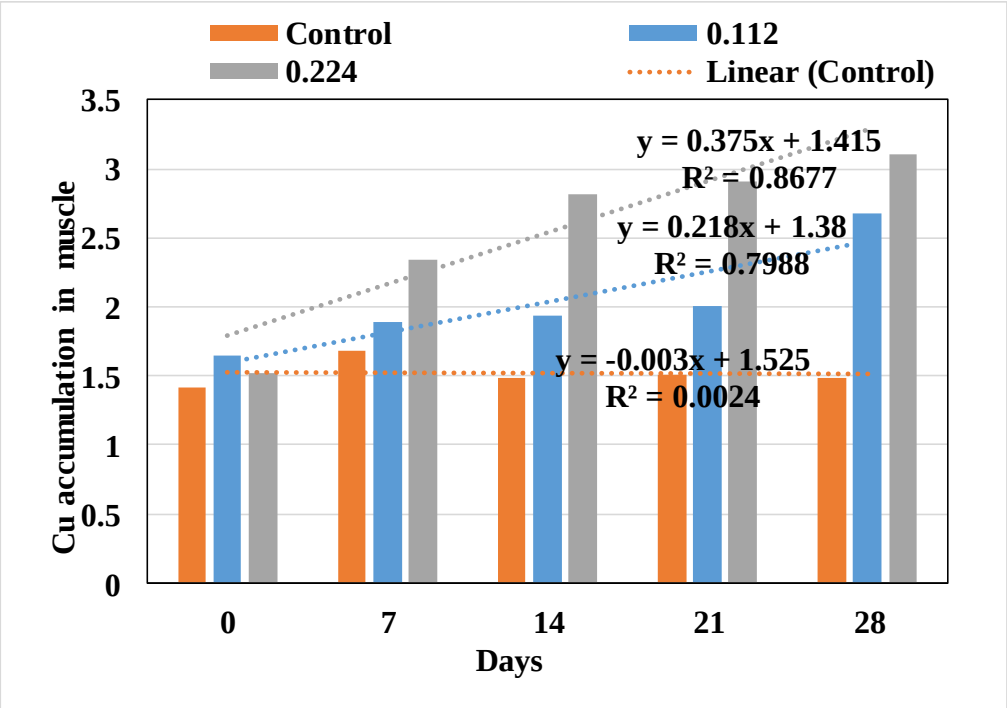


Fig.7. Accumulation of copper in muscle of *L. vannamei* exposed to different concentrations of copper after 0, 7, 14, 21 and 28 days of exposure

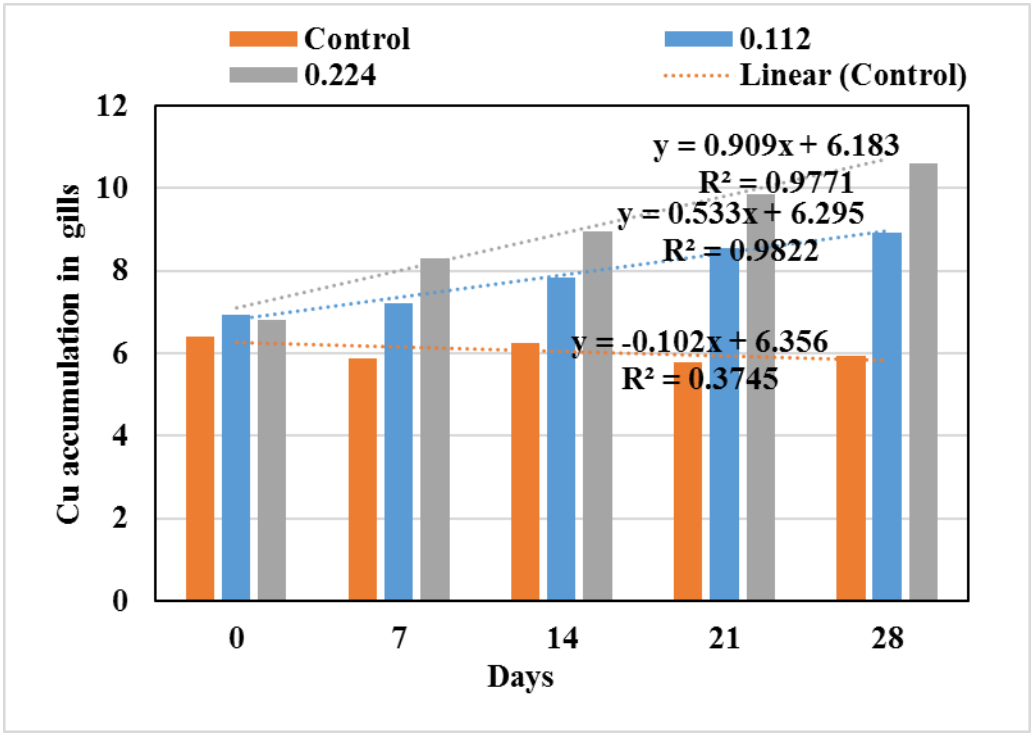


Fig.8. Accumulation of copper in gills of *L. vannamei* exposed to different concentrations of copper after 0, 7, 14, 21 and 28 days of exposure

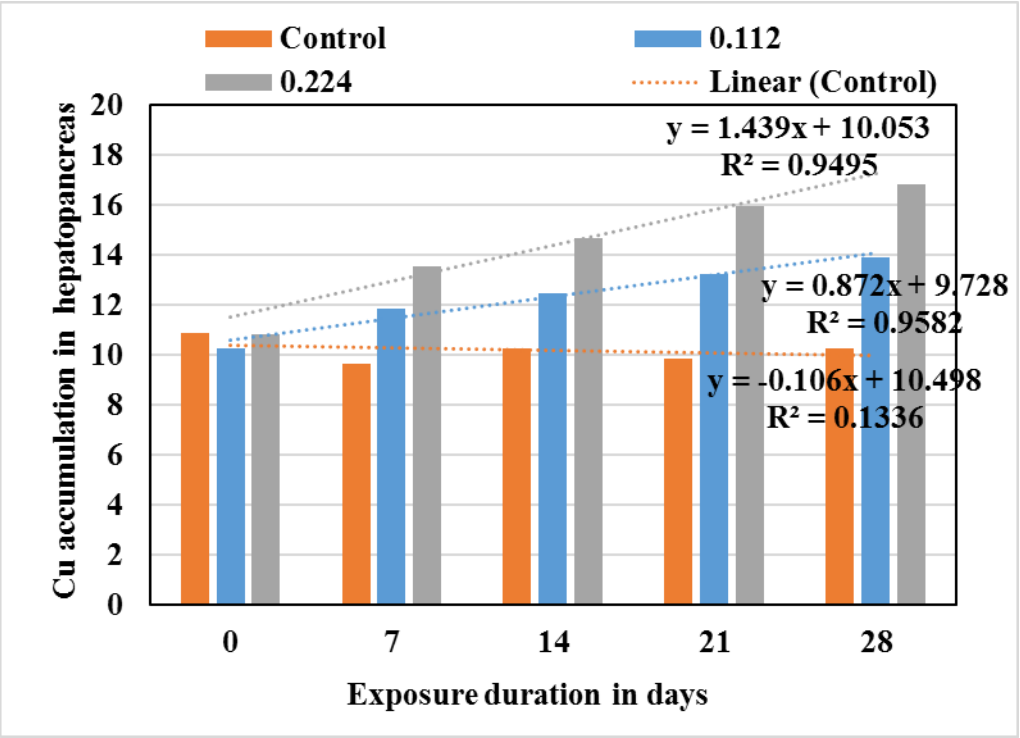


Fig.9. Accumulation of copper in hepatopancreas of *L. vannamei* exposed to different concentrations of copper after 0, 7, 14, 21 and 28 days of exposure

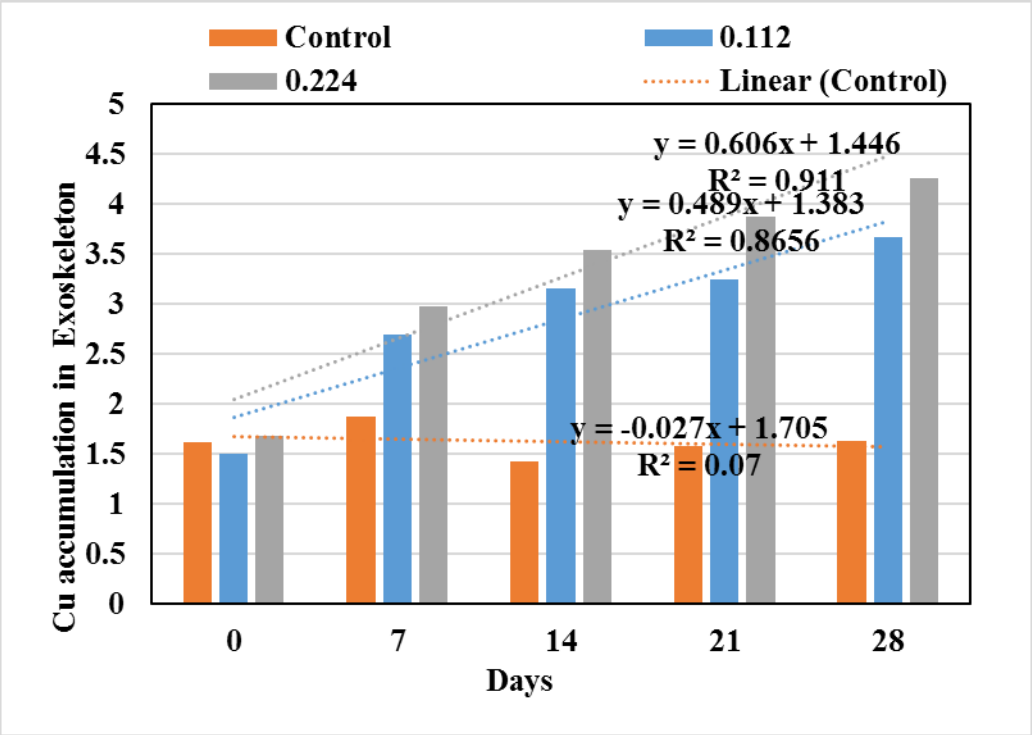


Fig.10.Accumulation of copper in exoskeleton of *L. vannamei* exposed to different concentrations of copper after 0, 7, 14, 21 and 28 days of exposure

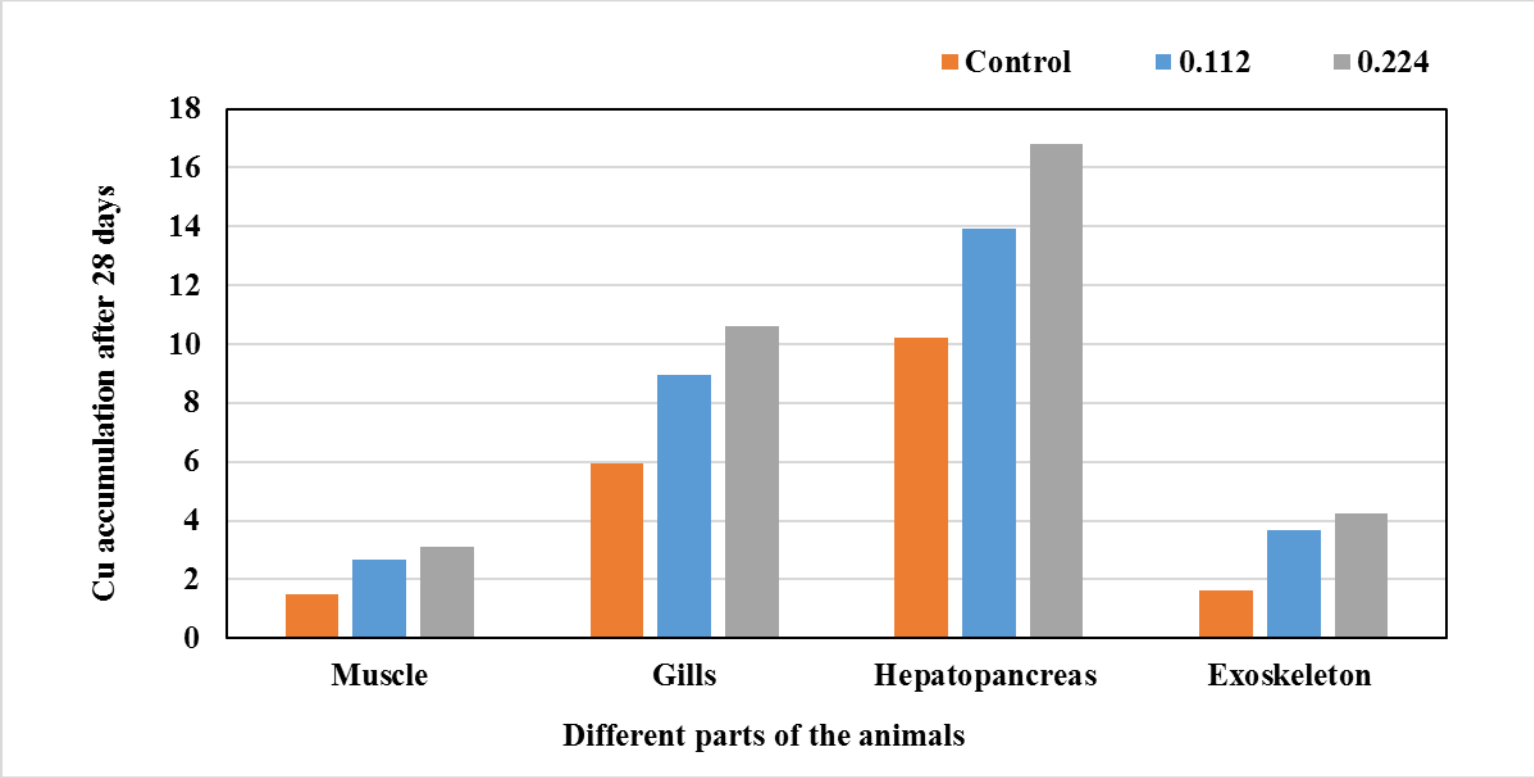


Fig.11. Accumulation of copper in muscle, gill, hepatopancreas and exoskeleton of *L.vannamei* exposed to different concentrations of copper after 28 days

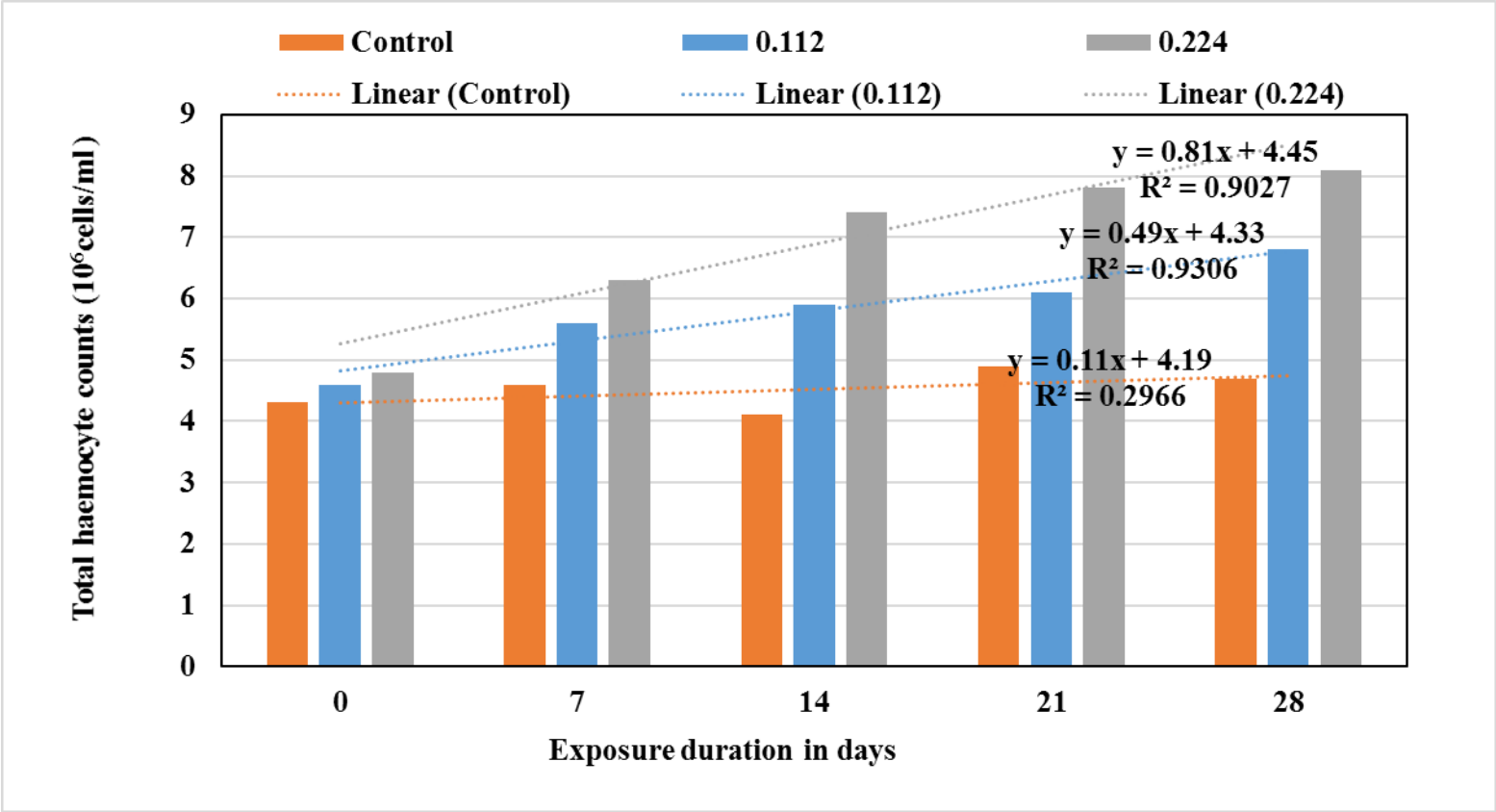


Fig.12.Changes in the total haemocyte count of *L. vannamei* exposed to different concentrations of copper after 0, 7, 14, 21 and 28 days

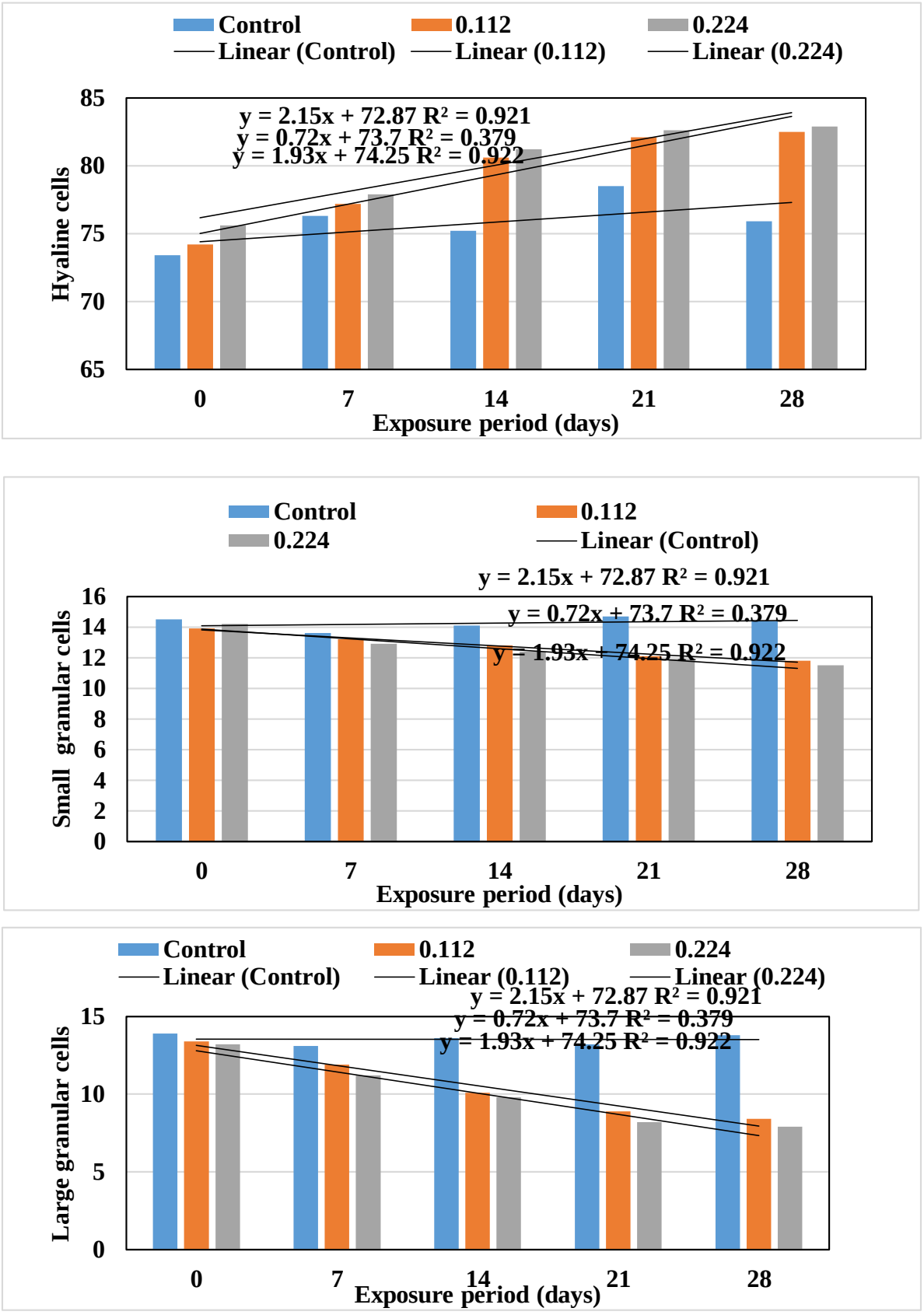


Fig.13.Changes in the Differential Haemocyte Count (DHC) such as hyaline, small granule haemocyte (SGH) and large granule haemocyte (LGH) of *L.vannamei* exposed to different concentrations of copper after 0, 7, 14, 21 and 28 days

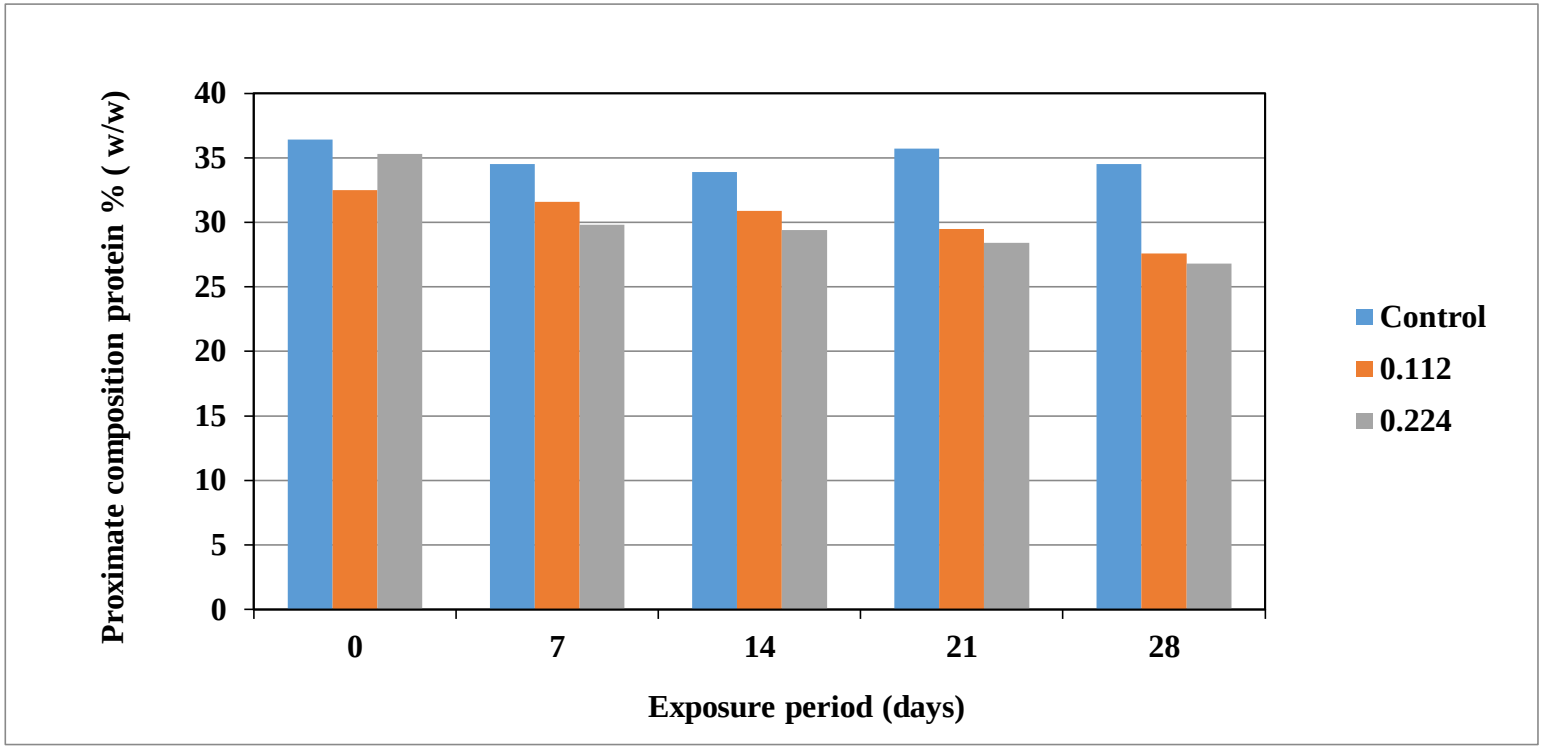


Fig. 14. Level of protein content of muscle tissue in shrimp, *L. vannamei* exposed to lower and higher concentration of copper over the control after different exposure period

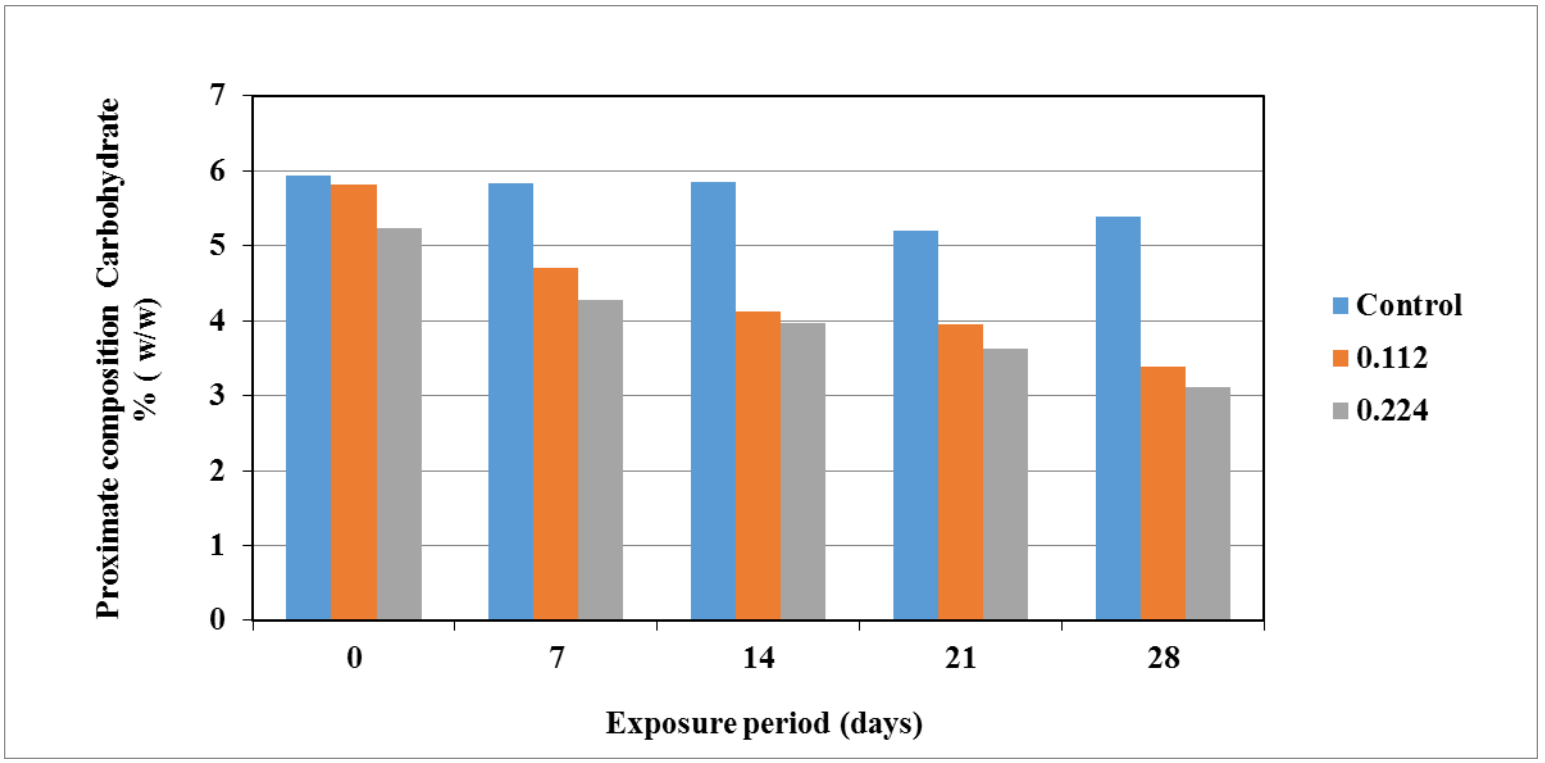


Fig.15.Level of carbohydrate content of muscle tissue in shrimp, *L. vannamei* exposed to lower and higher concentration of copper over the control after different exposure period

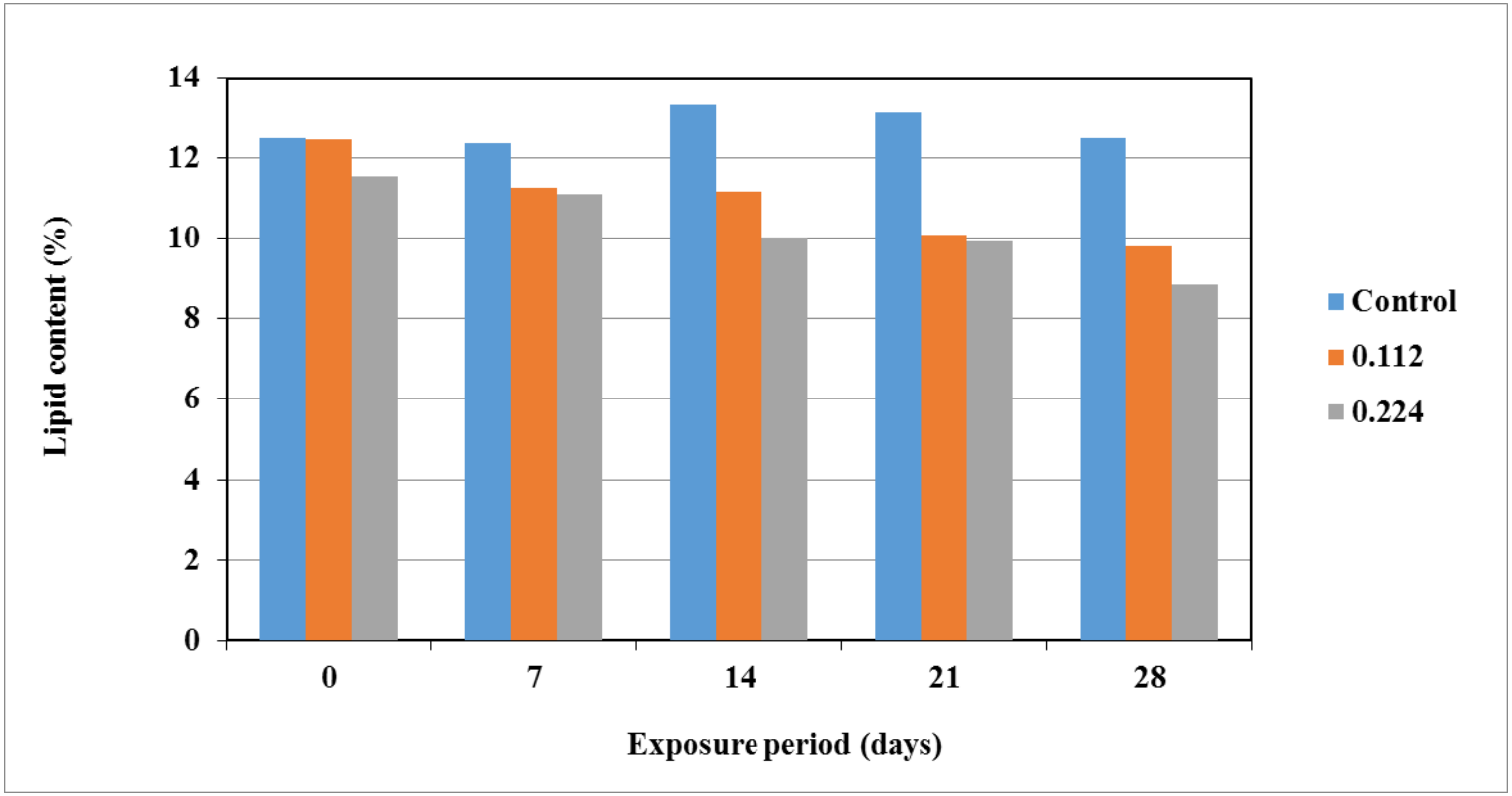


Fig.16. Level of lipid content of muscle tissue in shrimp, *L. vannamei* exposed to lower and higher concentration of copper over the control after different exposure period

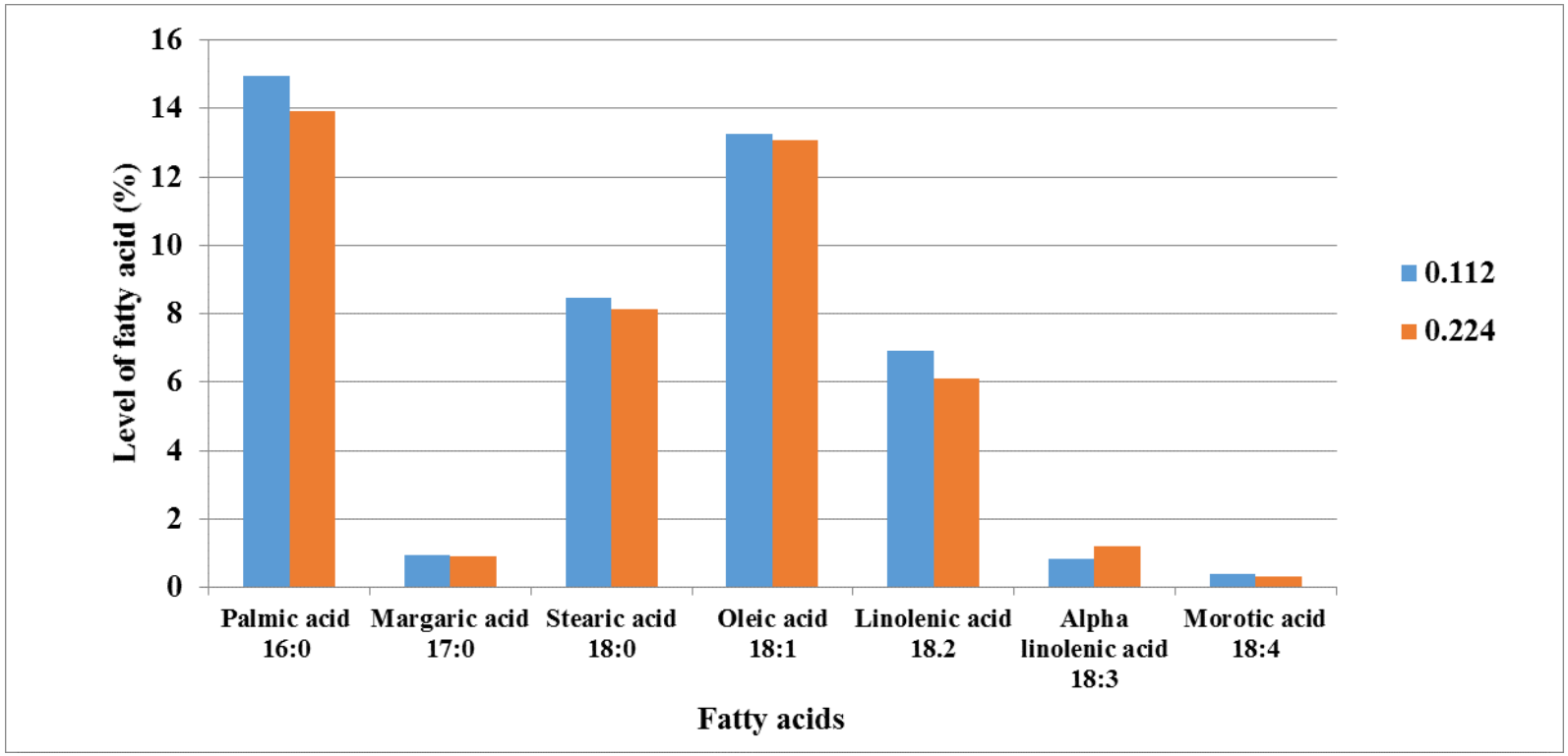


Fig.17. The levels of fatty acids in *L. vannamei* exposed to lower and higher concentration of copper after 28 day of exposure period

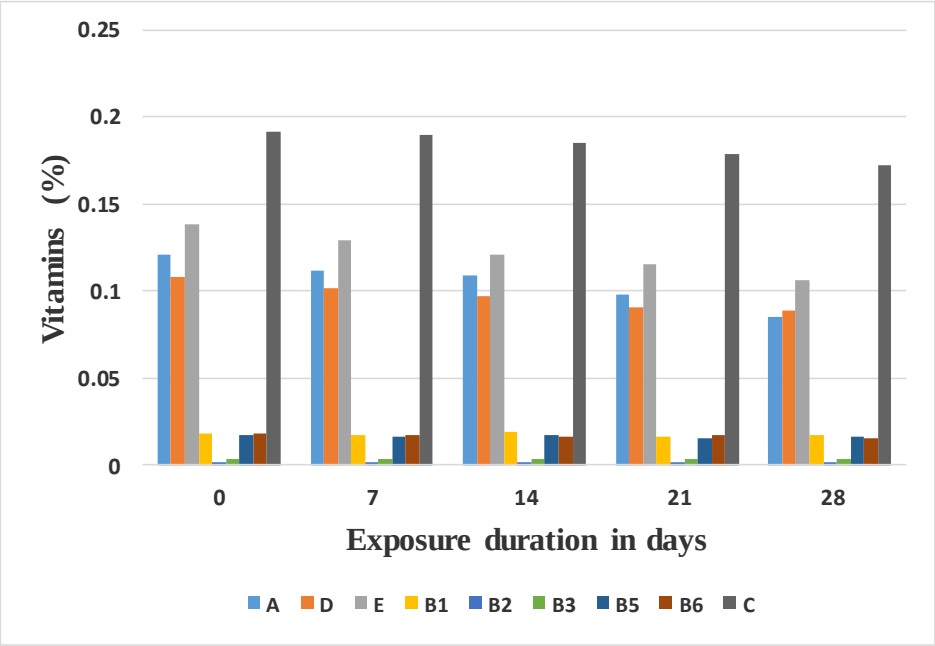


Fig.18. Level of vitamins in muscle of shrimp, *L. vannamei* exposed to lower concentration of copper over the control after different exposure period

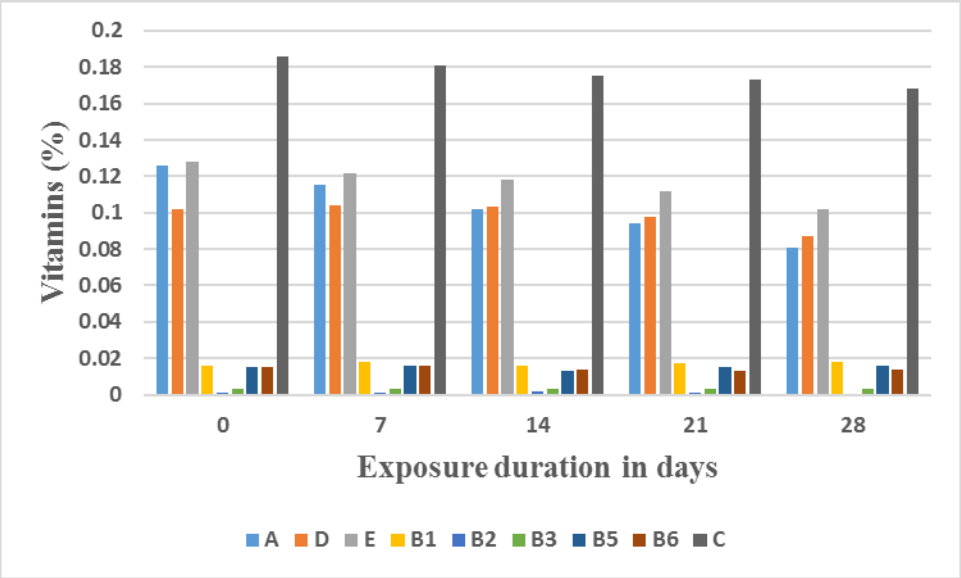


Fig.19. Level of vitamins in muscle of shrimp, *L. vannamei* exposed to higher concentration of copper over the control after different exposure period

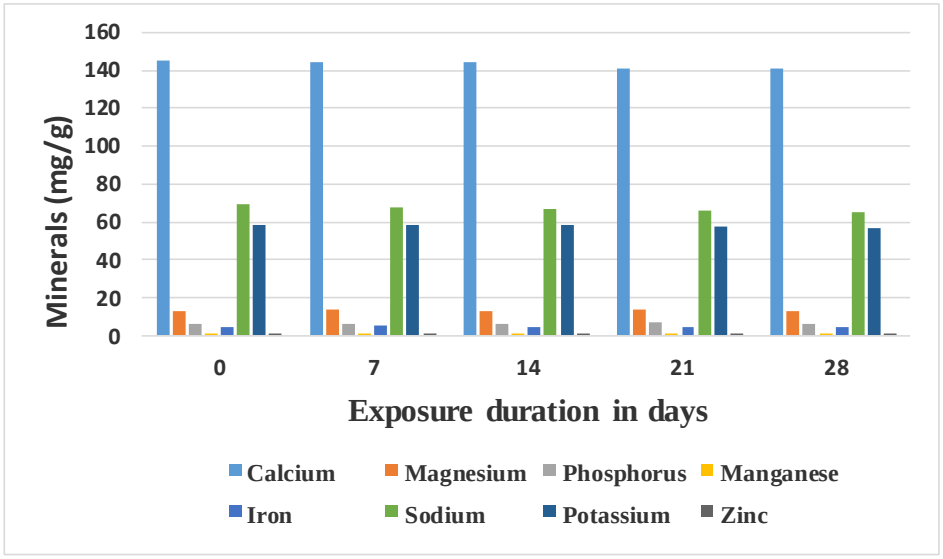


Fig.20. Level of minerals in muscle of shrimp, *L. vannamei* exposed to lower concentration of copper over the control after different exposure period

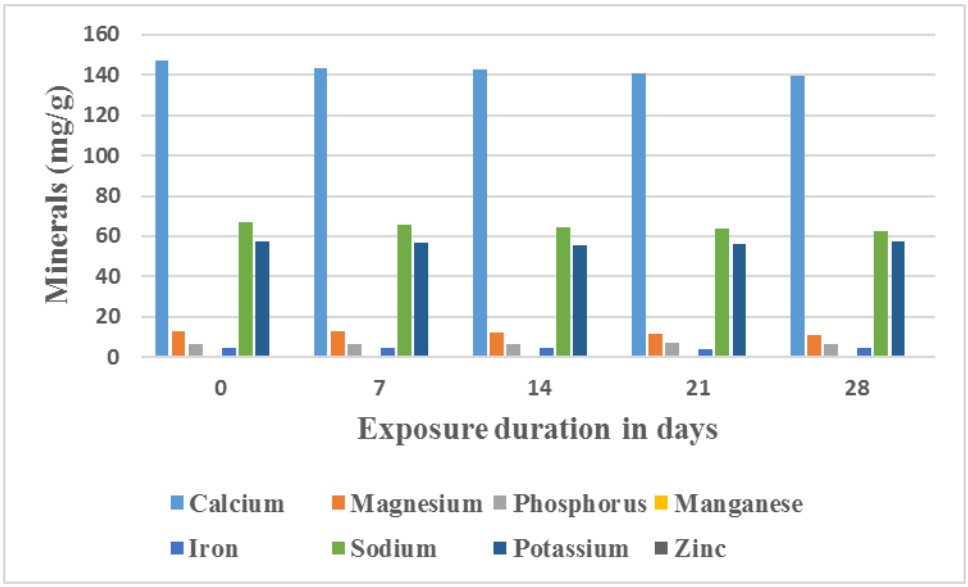


Fig.21. Level of minerals in muscle of shrimp, *L. vannamei* exposed to higher concentration of copper over the control after different exposure period

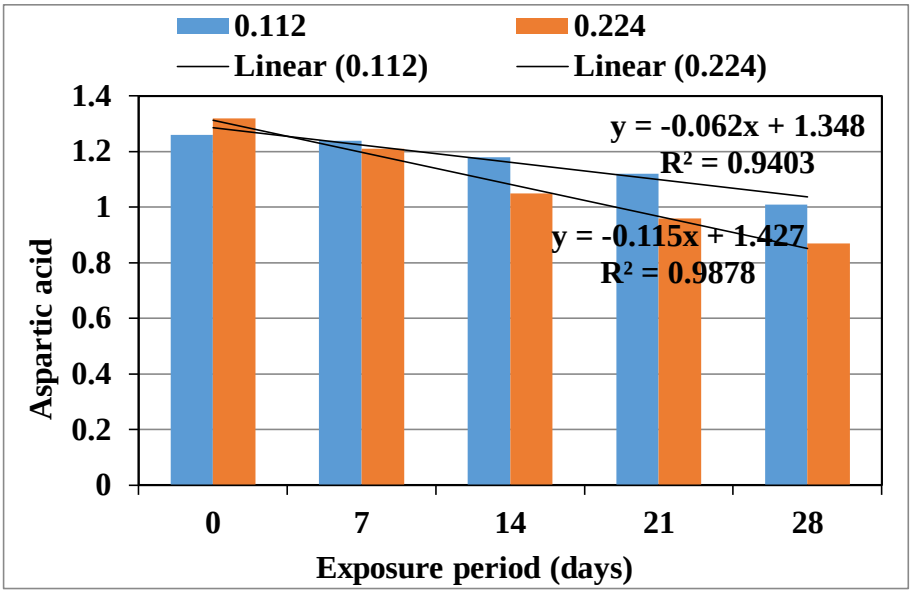


Fig.22. Variation of aspartic acid (%) of *L. vannamei* after exposure to low and high concentration of copper at 0, 7, 14, 21 and 28 days

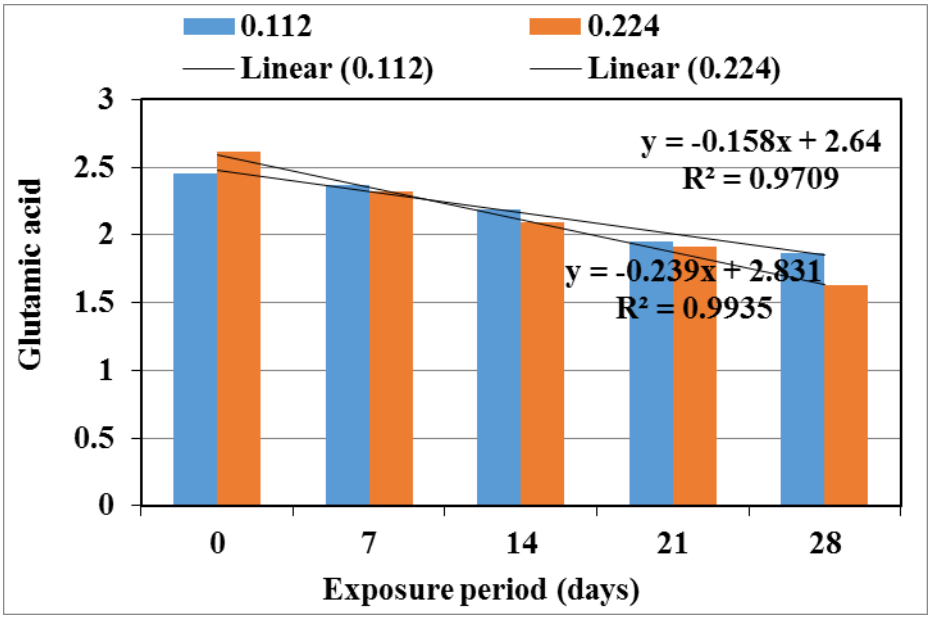


Fig.23. Variation of glutamic acid (%) of *L. vannamei* after exposure to low and high concentration of copper at 0, 7, 14, 21 and 28 days

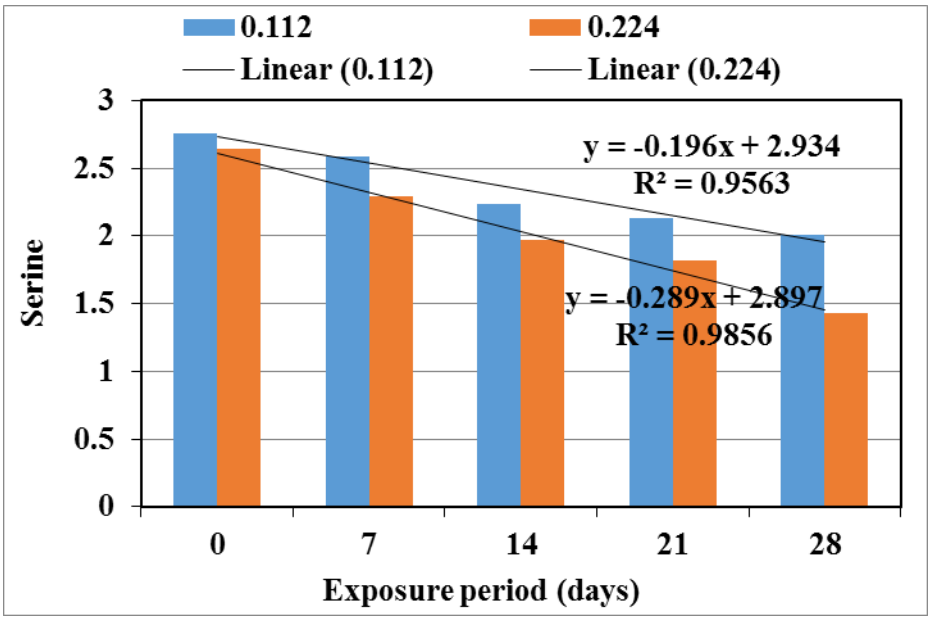


Fig.24.Variation of Serine (%) of *L. vannamei* after exposure to low and high concentration of copper at 0, 7, 14, 21 and 28 days

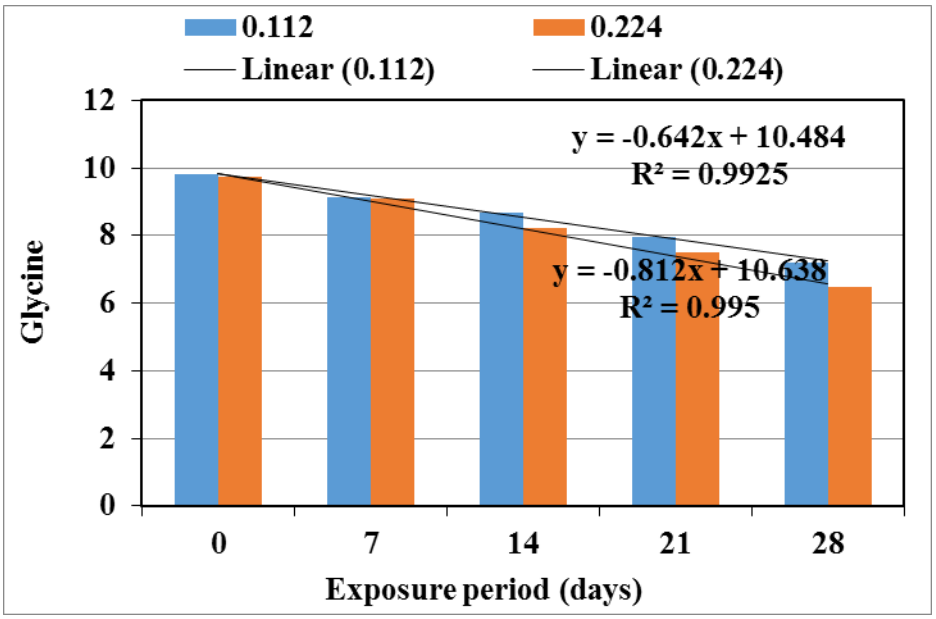


Fig.25. Variation of Glycine (%) of *L. vannamei* after exposure to low and high concentration of copper at 0, 7, 14, 21 and 28 days

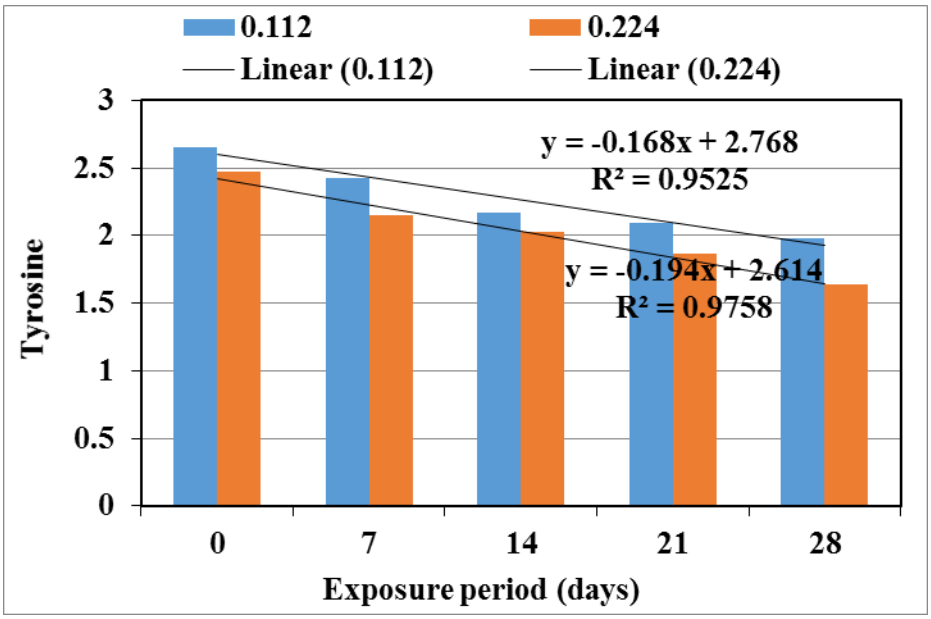


Fig.26. Variation of Tyrosine (%) of *L. vannamei* after exposure to low and high concentration of copper at 0, 7, 14, 21 and 28 days

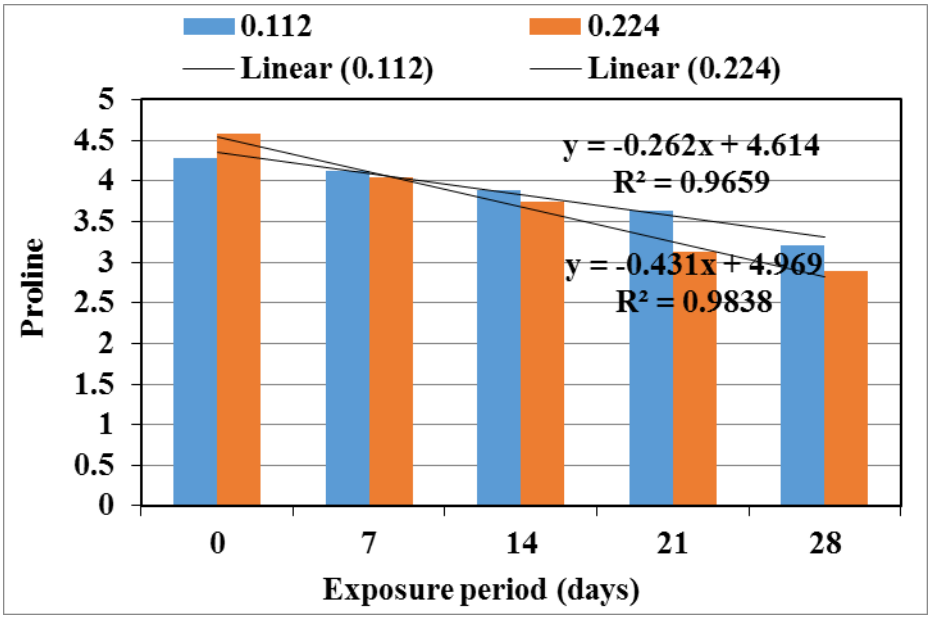


Fig.27. Variation of Proline (%) of *L. vannamei* after exposure to low and high concentration of copper at 0, 7, 14, 21 and 28 days

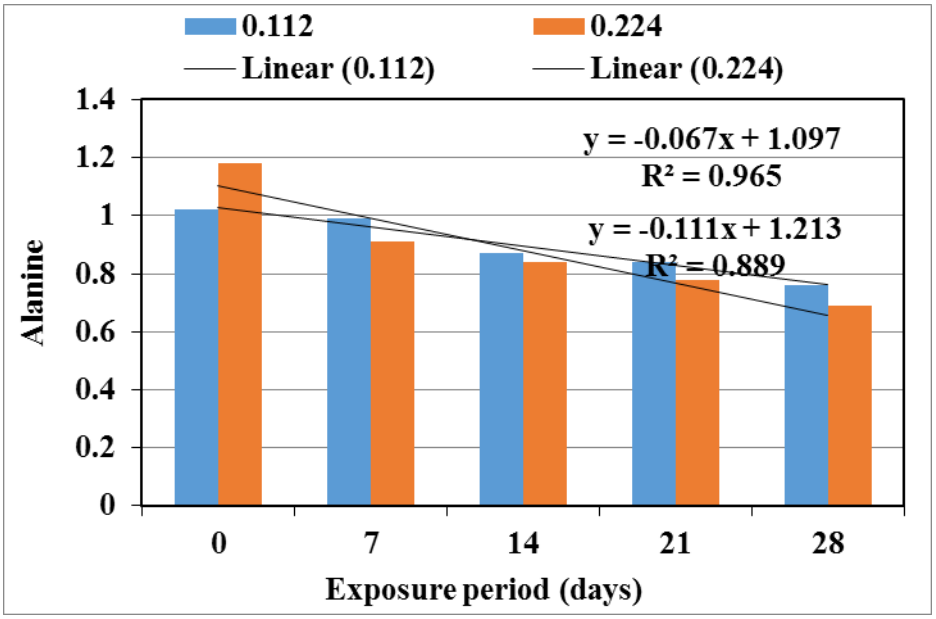


Fig.28. Variation of Alanine (%) of *L. vannamei* after exposure to low and high concentration of copper at 0, 7, 14, 21 and 28 days

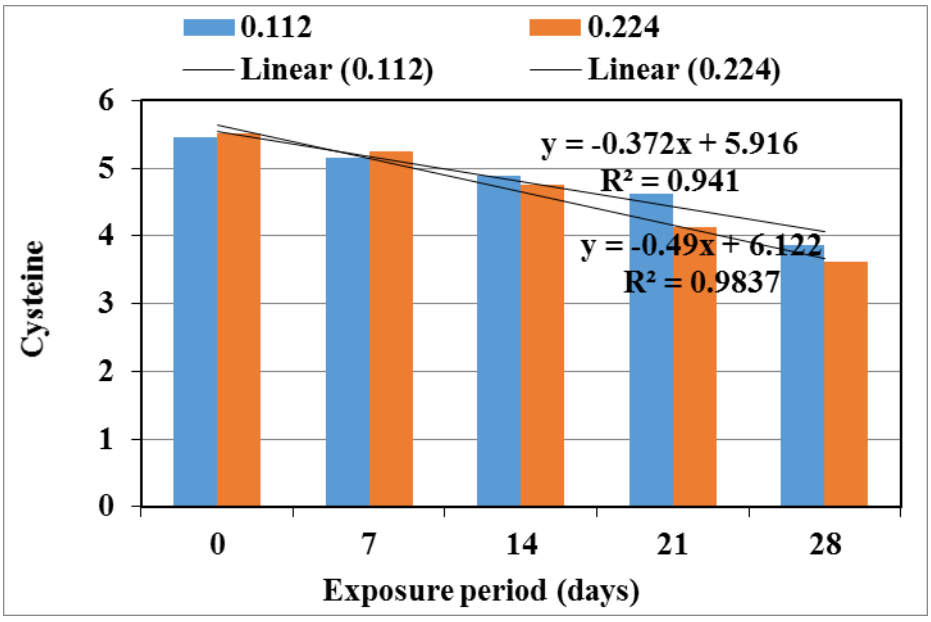


Fig.29. Variation of Cysteine (%) of *L. vannamei* after exposure to low and high concentration of copper at 0, 7, 14, 21 and 28 days

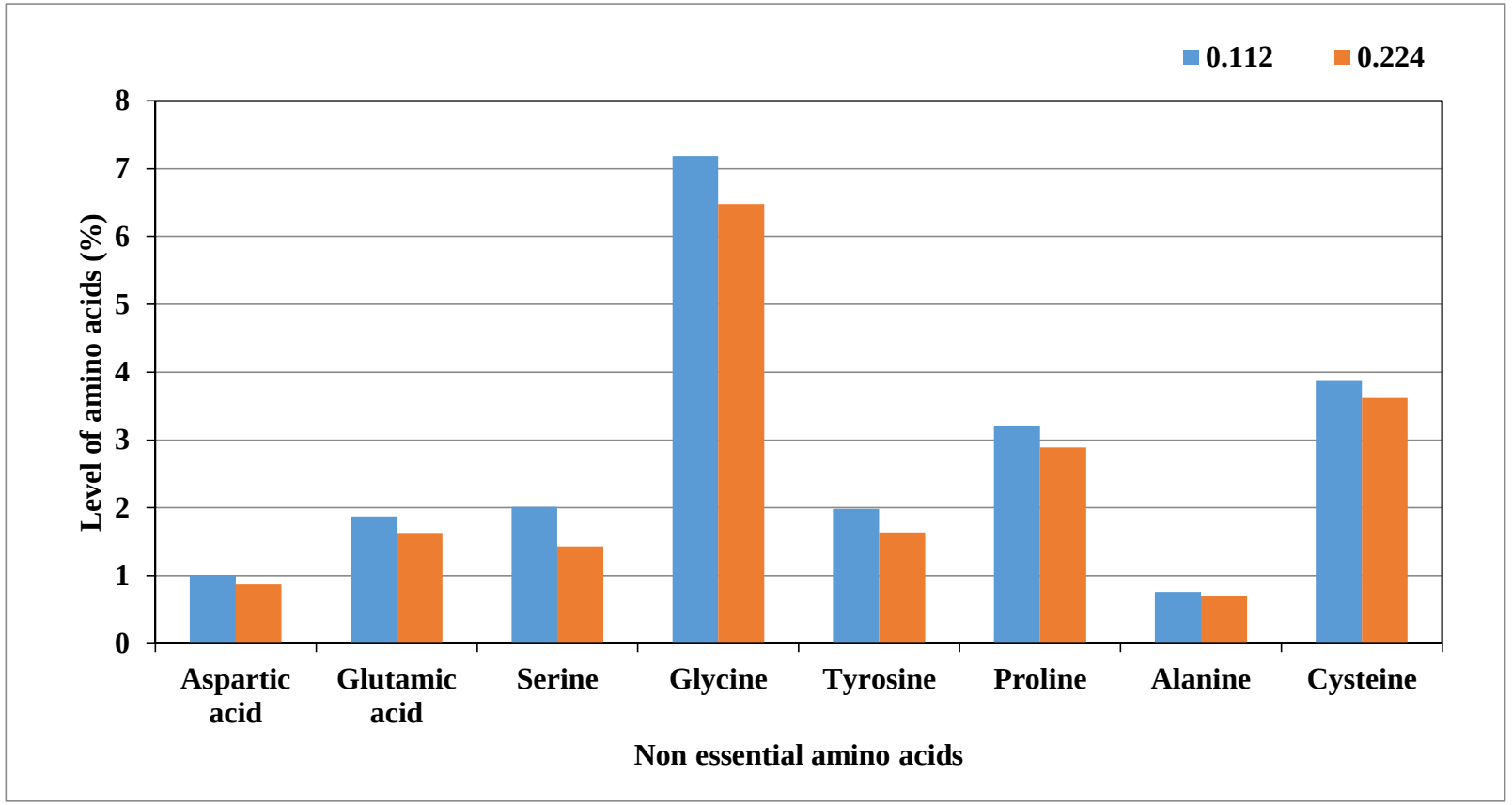


Fig.30. The levels of non essential acids in *L. vannamei* exposed to lower and higher concentration of copper content after 28 day of exposure period

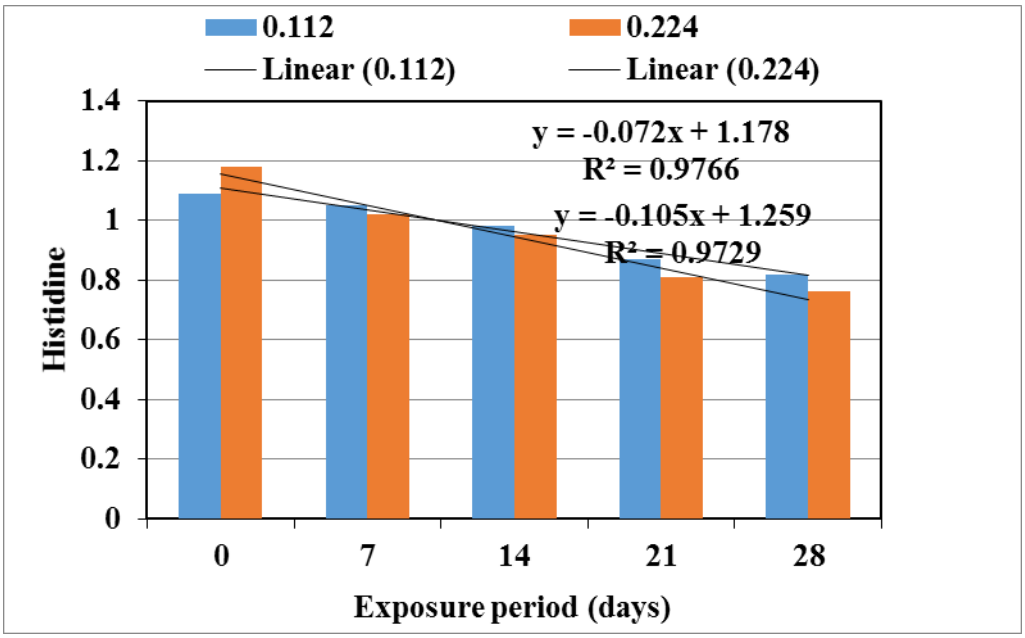


Fig.31. Variation of Histidine (%) of *L. vannamei* after exposure to low and high concentration of copper at 0, 7, 14, 21 and 28 days

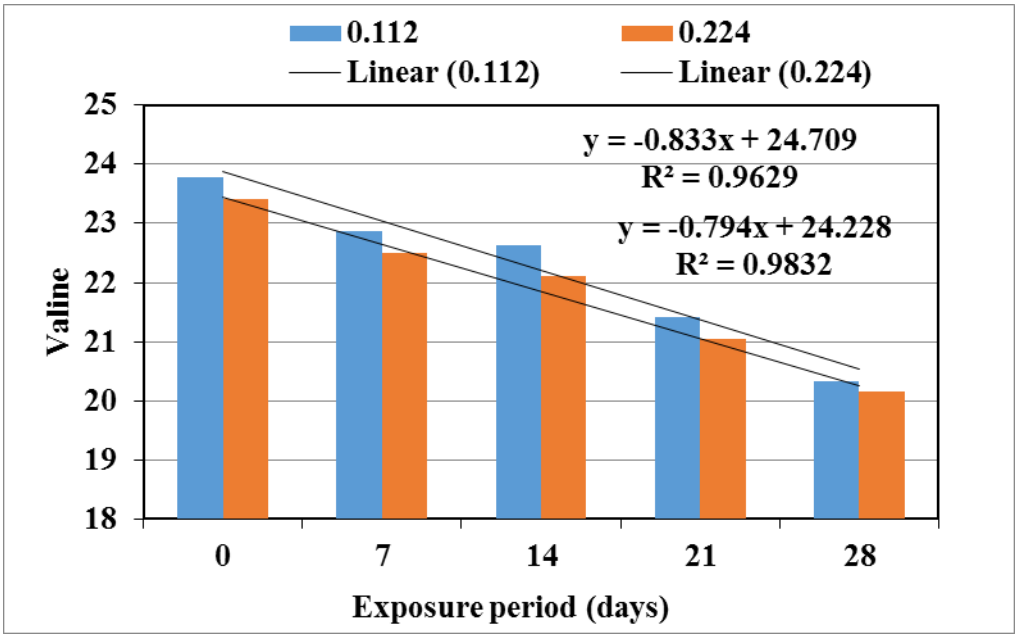


Fig.32.Variation of Valine (%) of *L. vannamei* after exposure to low and high concentration of copper at 0, 7, 14, 21 and 28 days

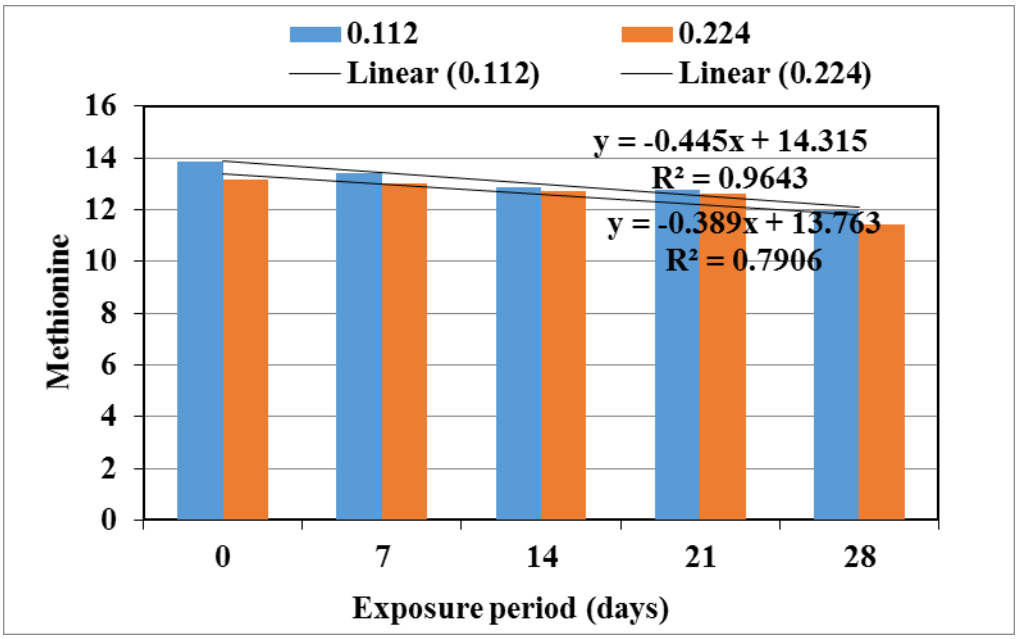


Fig.33. Variation of Methionine (%) of *L. vannamei* after exposure to low and high concentration of copper at 0, 7, 14, 21 and 28 days

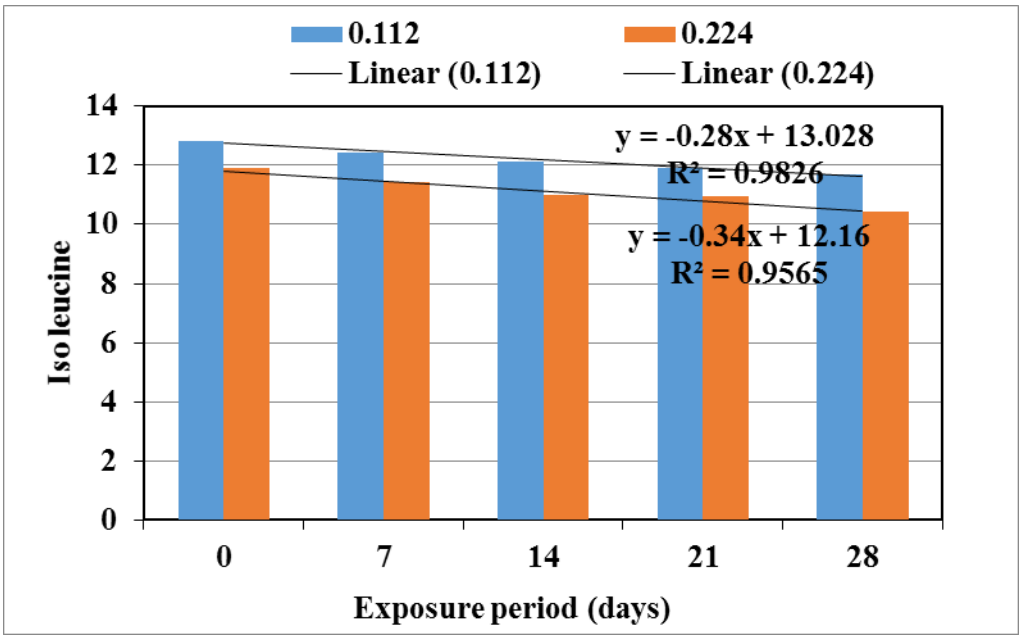


Fig.34. Variation of Iso leucine (%) of *L. vannamei* after exposure to low and high concentration of copper at 0, 7, 14, 21 and 28 days

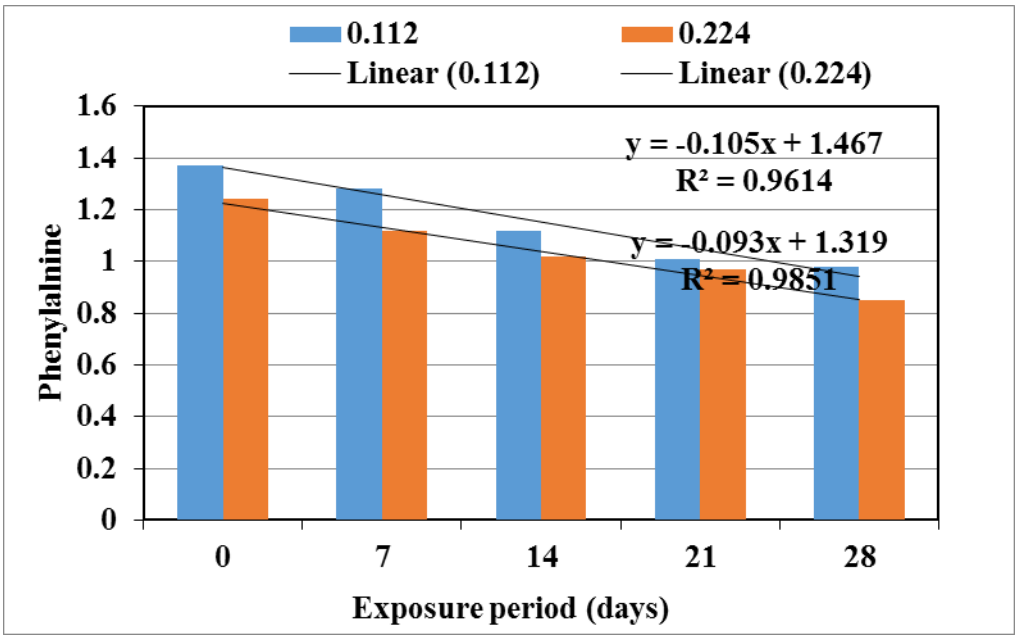


Fig.35. Variation of Phenylalanine (%) of *L. vannamei* after exposure to low and high concentration of copper at 0, 7, 14, 21 and 28 days

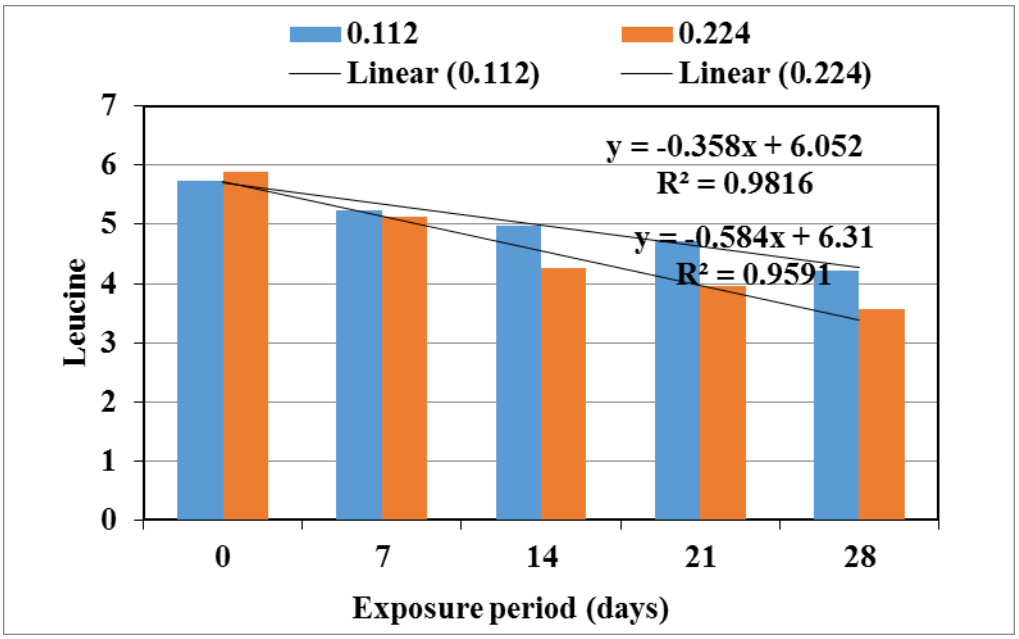


Fig.36. Variation of Leucine (%) of *L. vannamei* after exposure to low and high concentration of copper at 0, 7, 14, 21 and 28 days

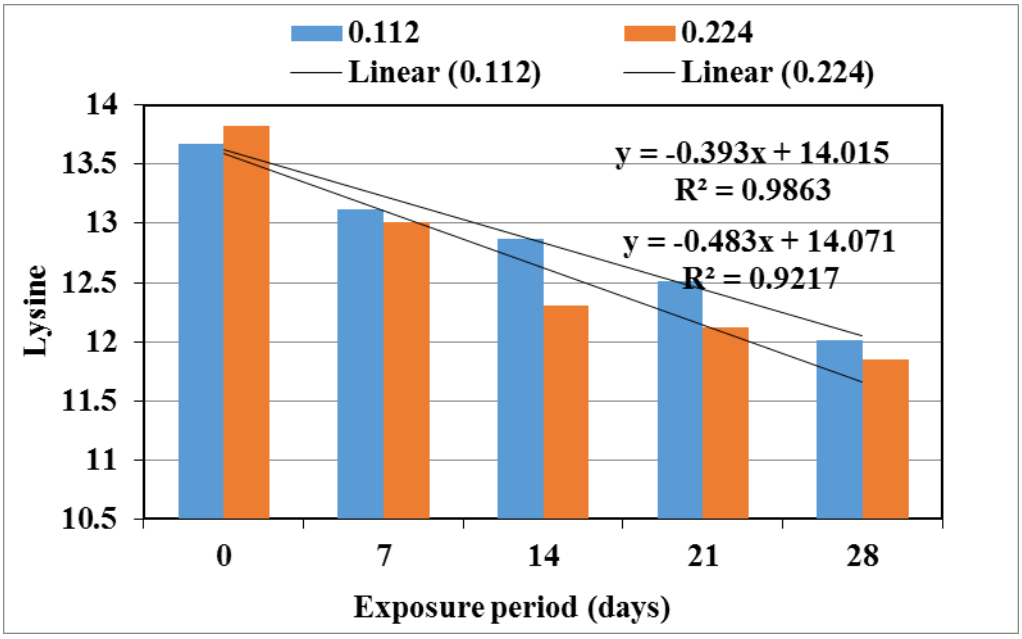


Fig.37.Variation of Lysine (%) of *L. vannamei* after exposure to low and high concentration of copper at 0, 7, 14, 21 and 28 days

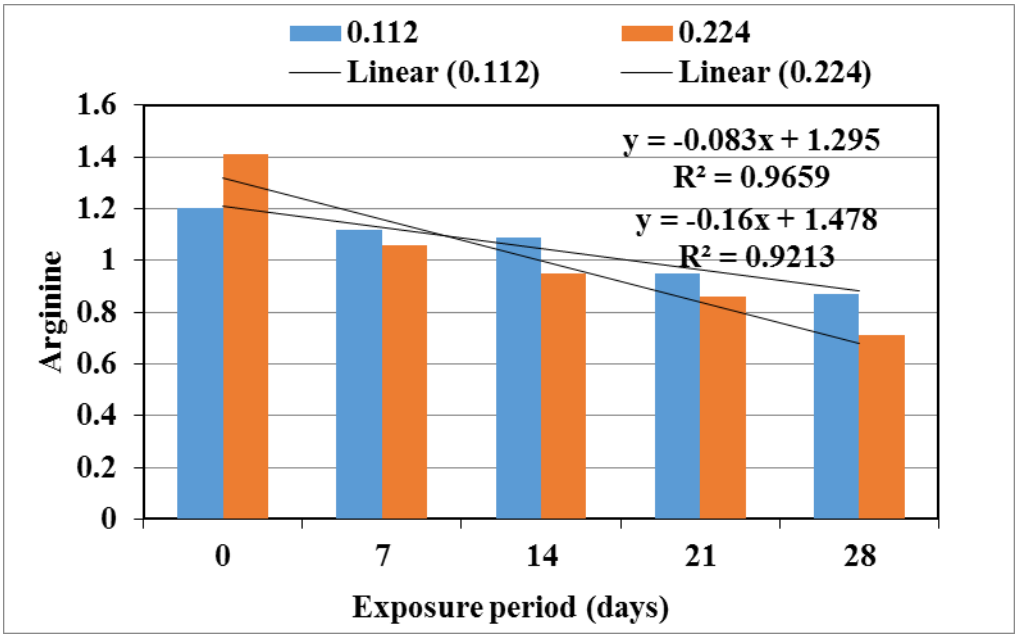


Fig.38. Variation of Arginine (%) of *L. vannamei* after exposure to low and high concentration of copper at 0, 7, 14, 21 and 28 days

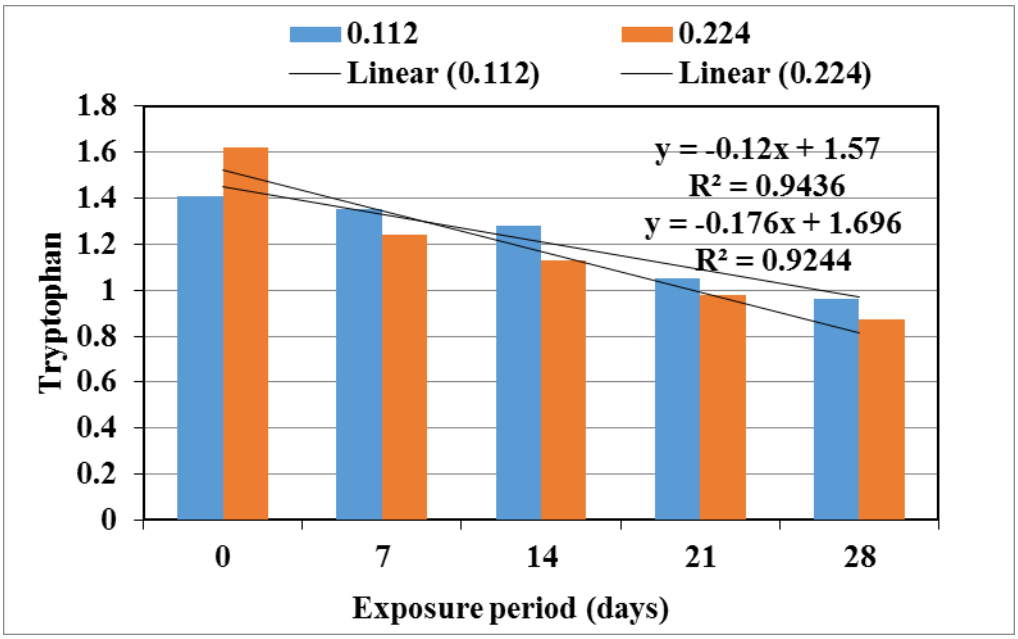


Fig.39. Variation of Tryptophan (%) of *L. vannamei* after exposure to low and high concentration of copper at 0, 7, 14, 21 and 28 days

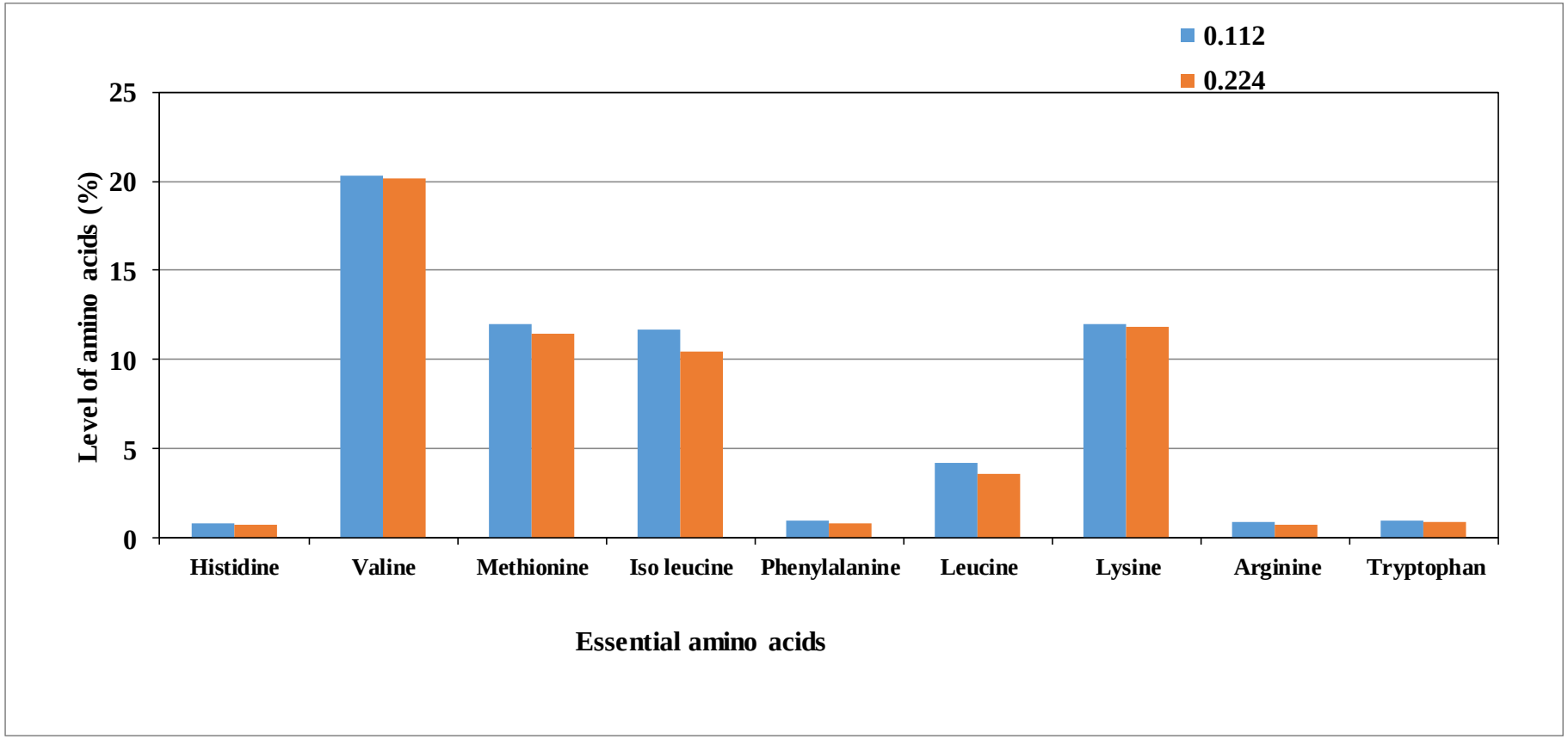
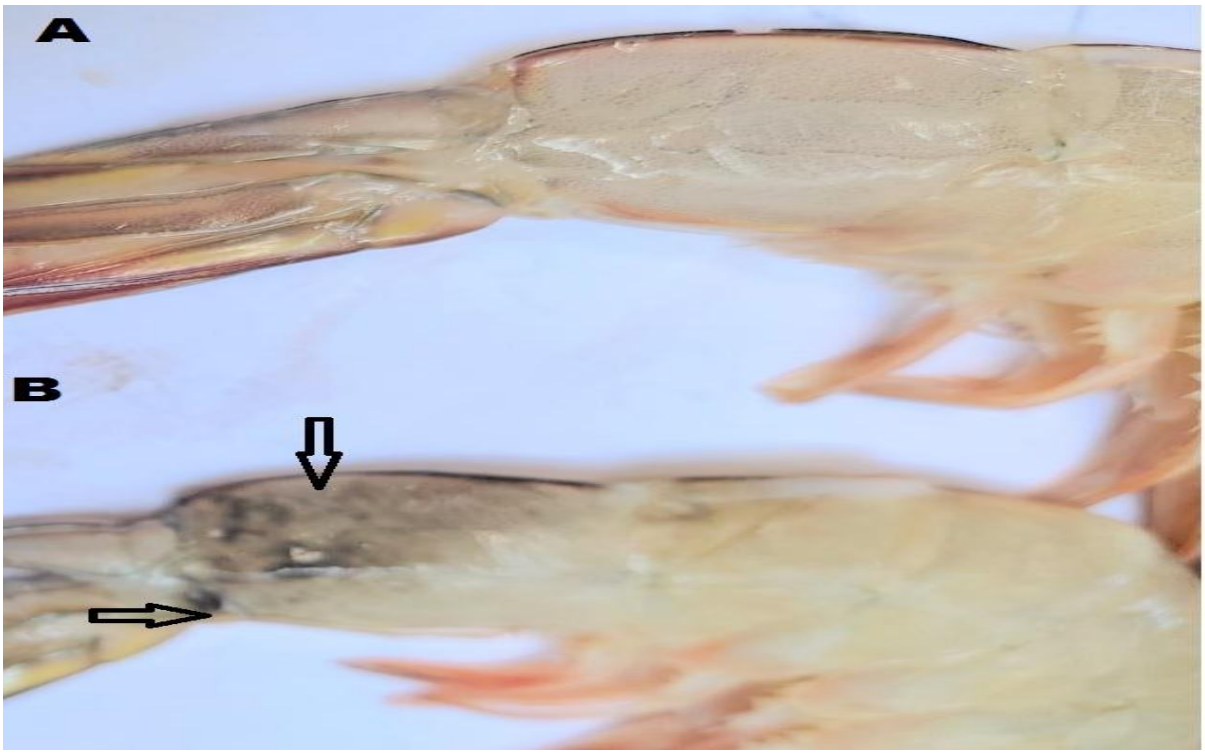


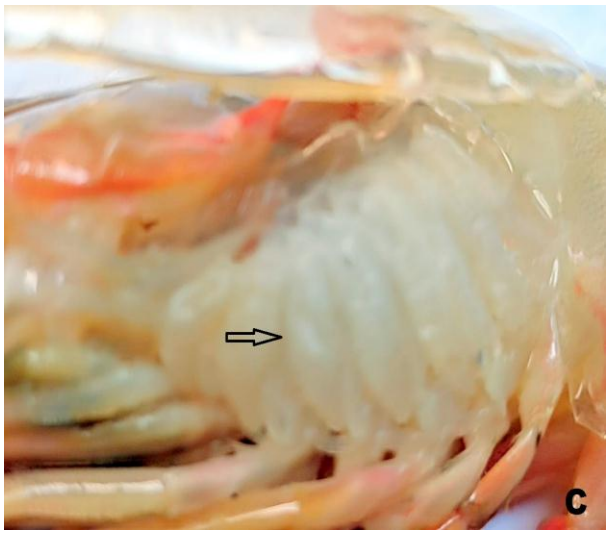
Fig.40. The levels of non essential acids in *L. vannamei* exposed to lower and higher concentration of copper content after 28 days of exposure period

PLATE: 2

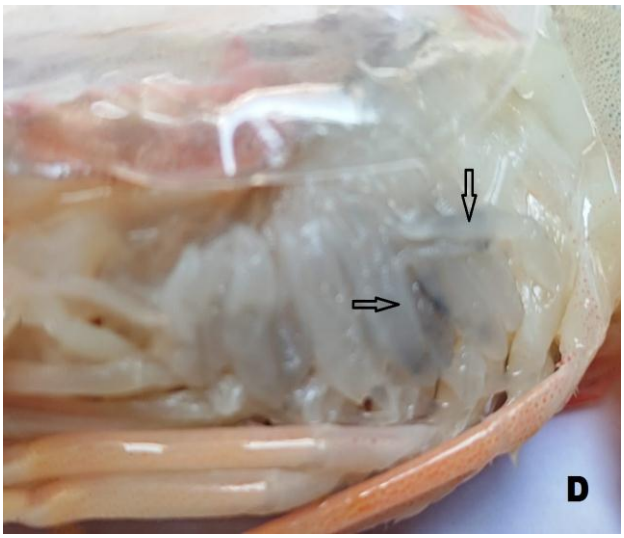


A: Control tail muscle showing creamy white in colour

B: Arrow indicates black spots were seen in tail muscle after 28 days of the copper treatment in higher concentration (0.224ppm)

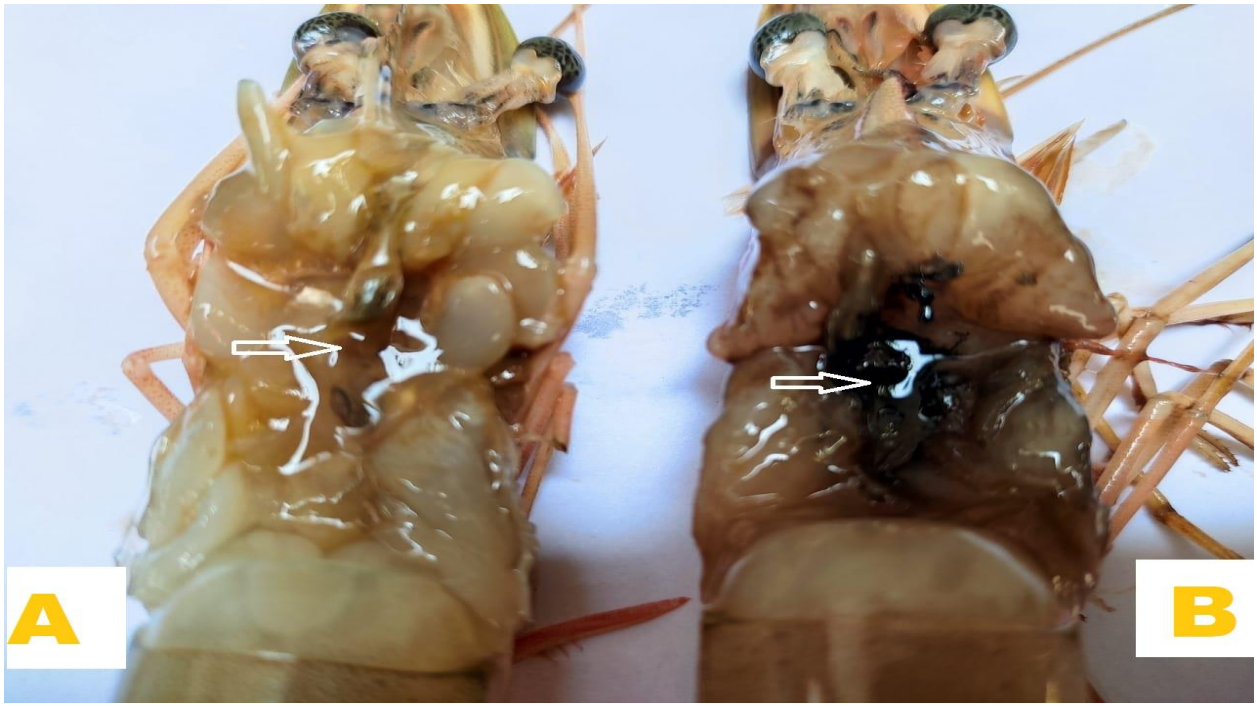


C: Control Gills showing pale green colour



D: Arrow indicateds black deposits in gill lamellae after 28 days of copper treatment in higher concentration (0.224ppm)

PLATE : 3



E: Arrow indicates control hepatopancreas showing yellowish brown in colour

F: Arrow indicates Greenish black colour after 28 days of copper treatment in higher concentration (0.224ppm)

C: Control uropad showing yellowish orange in colour

D: Arrow indicates balack deposits in uropod region after 28 days of copper treatment in higher concentration (0.224ppm)

PLATE : 4

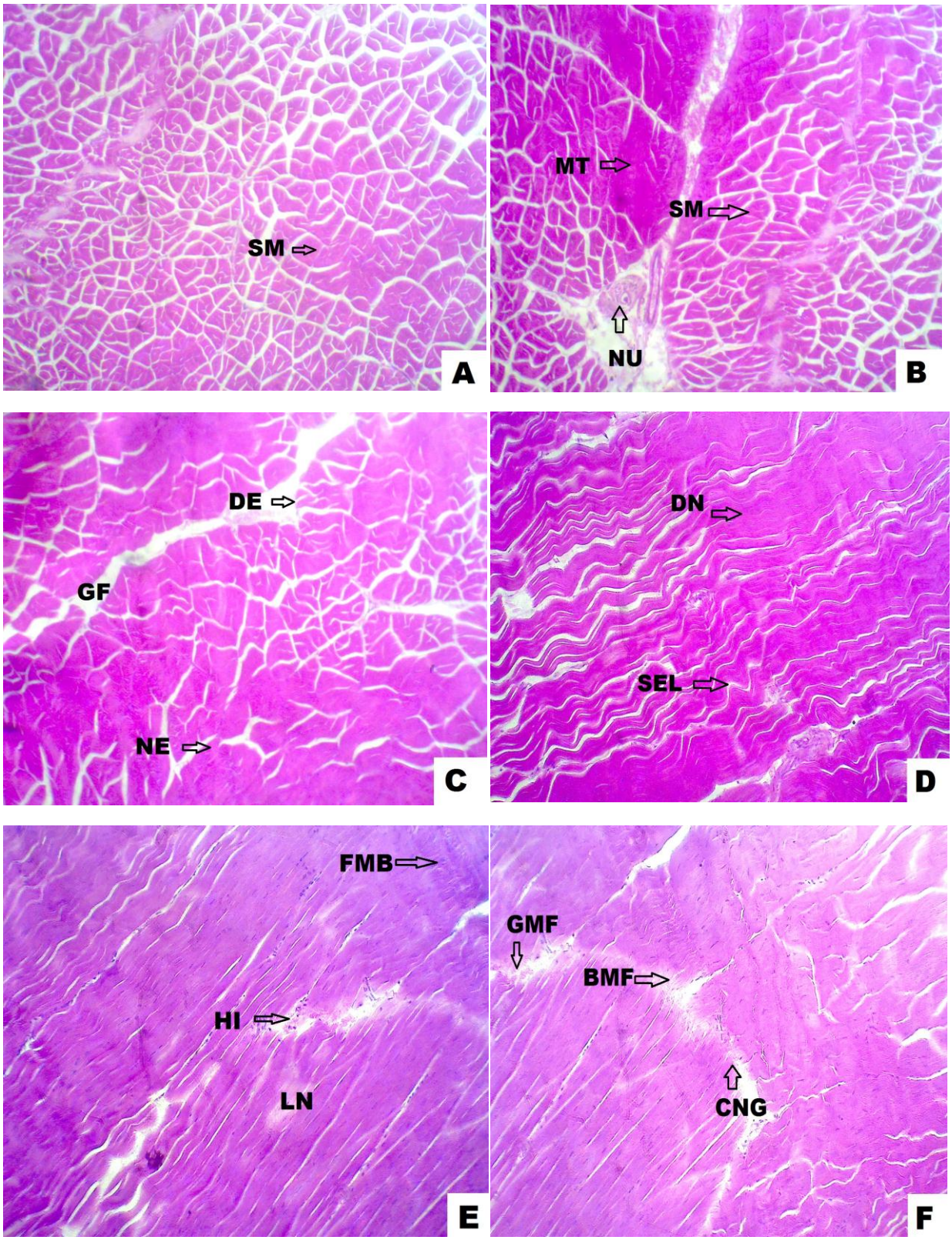


PLATE : 5

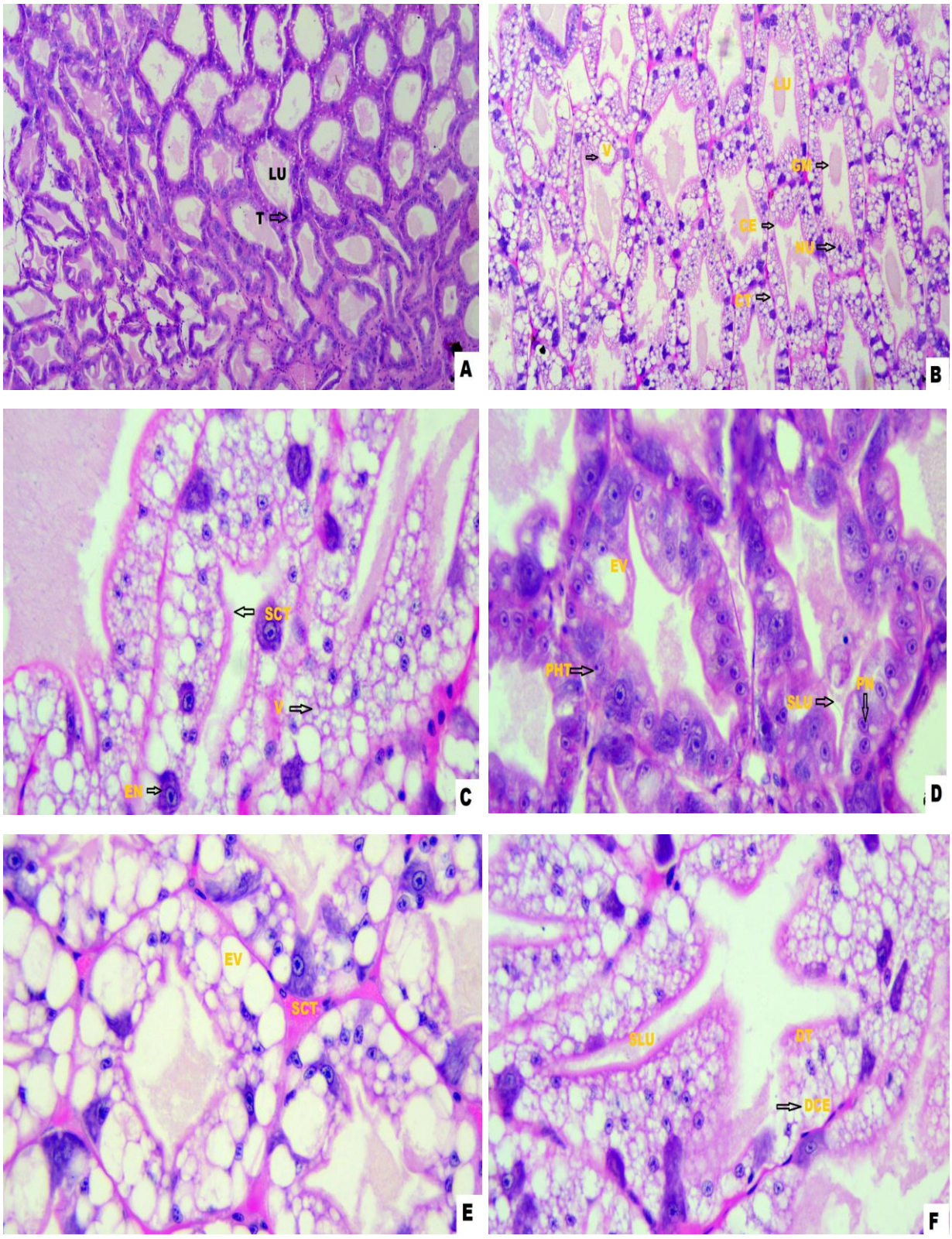


PLATE : 6

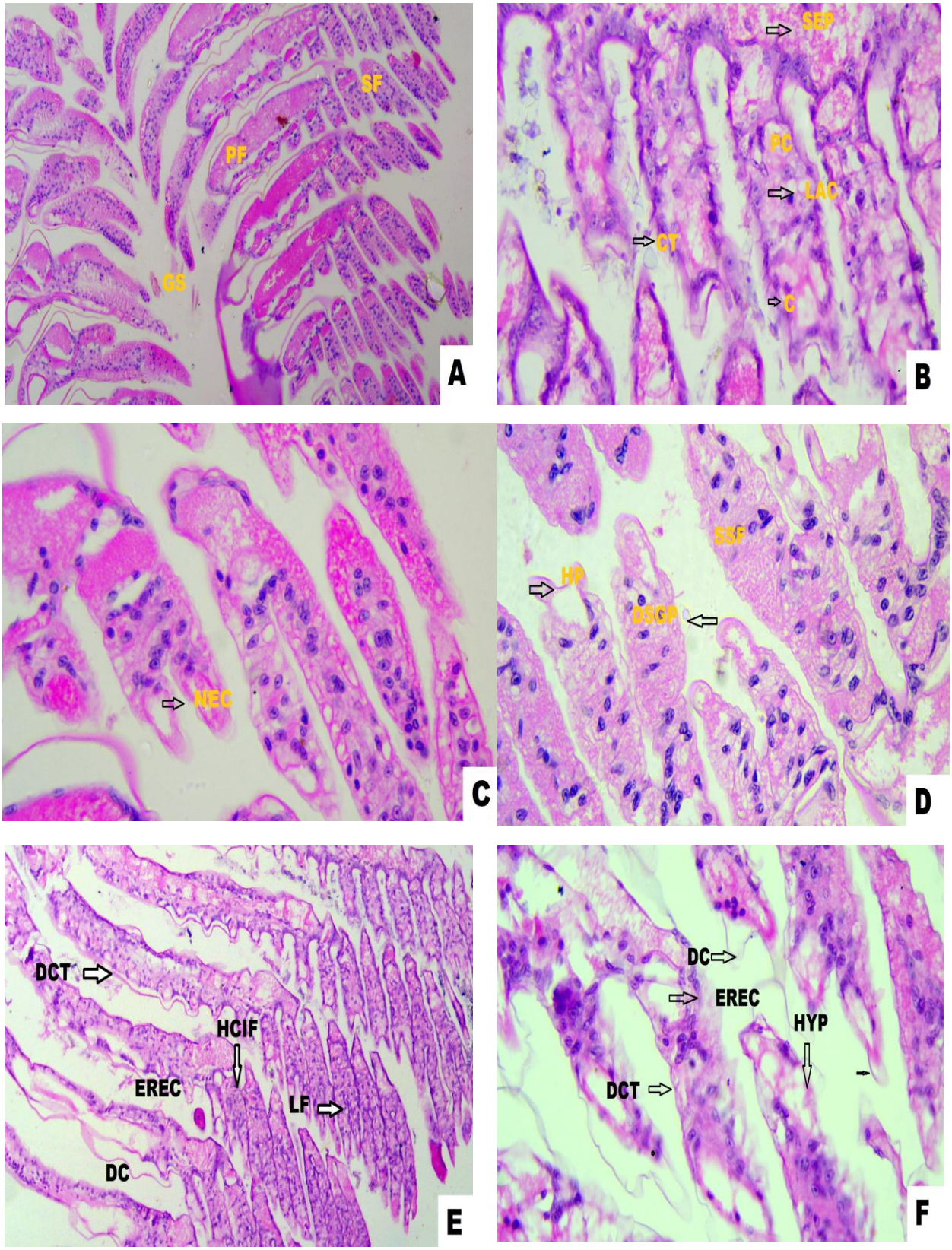

SF
PF
GS
A
SEP
PC
LAC
CT
C
B
NEC
C
SSF
HP
DSGP
D
DCT
HCIF
EREC
LF
DC
E
DC
EREC
HYP
DCT
F

PLATE : 7

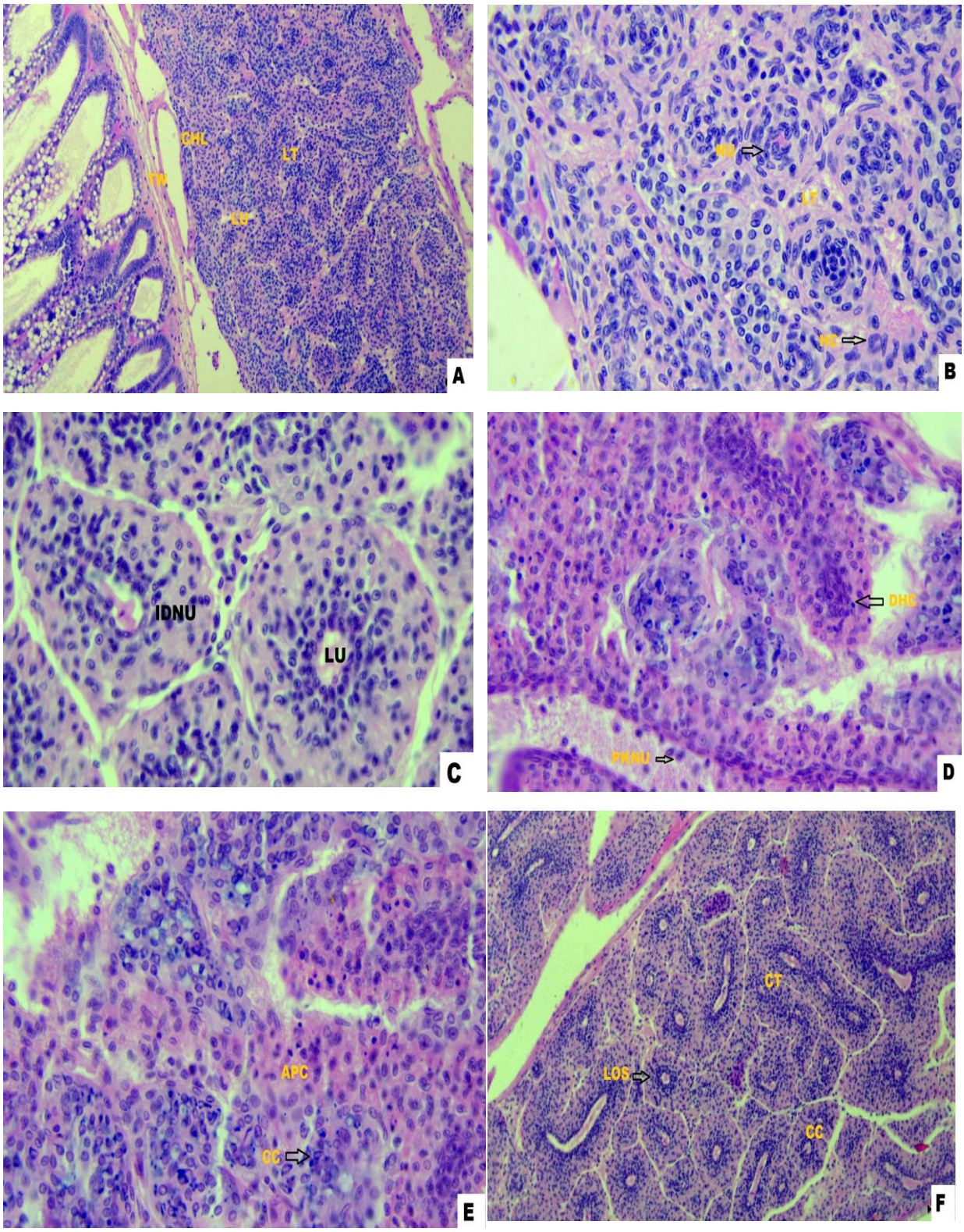


PLATE : 8

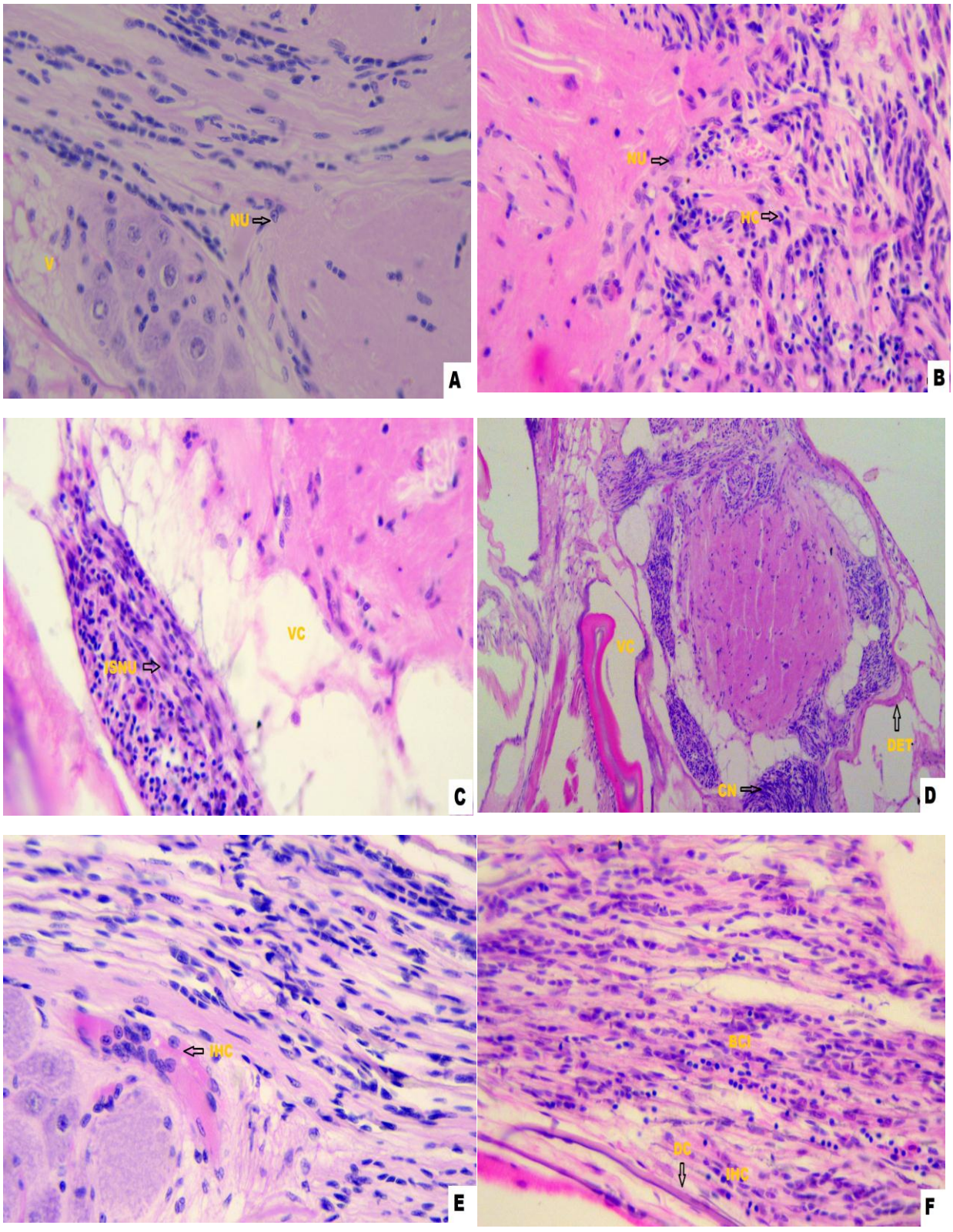


Plate: 4 Histological changes of muscle in *L.vannamei*

Light micrographs of a paraffin section stained with Hematoxylin and Eosin (40x)

A & B- Control

C- After 7 days of exposure to 0.112 ppm concentration of copper

D- After 28 days of exposure to 0.112 ppm concentration of copper

E- After 7 days of exposure to 0.224 ppm concentration of copper

F - After 28 days of exposure to 0.224 ppm concentration of copper

Abbreviations used:

SM	- Striated muscle
MT	- Myoton
NU	- Nuclei
DE	- Disintegrated epidermis
GF	- Gap formation
LN	- Lesions
HI	- Haemocyte infiltration
FMB	- Fusion of Muscle Bundle
SEL	- Sloughing of epidermal layer
DN	- Disappearance of nuclei
BMF	- Broken myofibrils
CNG	- Coagulative necrosis
GMF	- Granular materials in muscle fibers

Plate: 5 Histological changes of hepatopancreas in *L.vannamei*

Light micrographs of a paraffin section stained with Hematoxylin and Eosin (20x & 40x)

A & B- Control

C- After 7 days of exposure to 0.112 ppm concentration of copper

D- After 28 days of exposure to 0.112 ppm concentration of copper

E- After 7 days of exposure to 0.224 ppm concentration of copper

F - After 28 days of exposure to 0.224 ppm concentration of copper

Abbreviations used:

LU	- Lumen
GM	- Granular material
NU	- Nuclei
T	- Tubules
V	- Vacuole
CT	- Connective tissue
CE	- Columnar epithelium
SCT	- Spongy connective tissue
EN	- Enlarged nuclei
PHT	- Proliferated hepato pancreatic tubule
EV	- Extensive vacuolation
SLU	- Squeezed lume
PN	- Pyknotic nuclei
EV	- Extensive vacuolation
DT	- Degeneration of tubule
DCE	- Disintegration of columnar epithelium

Plate: 6 Histological changes of gills in *L.vannamei*

Light micrographs of a paraffin section stained with Hematoxylin and Eosin (40x)

A & B- Control

C- After 7 days of exposure to 0.112 ppm concentration of copper

D- After 28 days of exposure to 0.112 ppm concentration of copper

E- After 7 days of exposure to 0.224 ppm concentration of copper

F - After 28 days of exposure to 0.224 ppm concentration of copper

Abbreviations used:

GS	- Gill slem
PF	- Primary filament
SF	- Secondary filament
PC	- Pillar cell
C	- Cuticle
CT	- Connective tissue
LAC	- Lacunae
SEP	- Thin septum
DSGF	- Degeneration of secondary gill filament
SSF	- Swelling of secondary gill filament
NEC	- Necrotic cell
HP	- Hyperplasia
HYP	- Hypertrophy
EREC	- Edema and rupture of epithelial cell
HCIF	- Haemocyte infiltration
DC	- Detached cuticle
DCT	- Disintegrated connective tissue
LF	- Lamellar fusion

Plate: 7 Histological changes of lymphoid organ in *L.vannamei*

Light micrographs of a paraffin section stained with Hematoxylin and Eosin (40x)

A & B- Control

C- After 7 days of exposure to 0.112 ppm concentration of copper

D- After 28 days of exposure to 0.112 ppm concentration of copper

E- After 7 days of exposure to 0.224 ppm concentration of copper

F - After 28 days of exposure to 0.224 ppm concentration of copper

Abbreviations used:

CHL	- Central haemal lumen
TW	- Tissue wall
HC	- Haemocyte
LT	- Lymphatic tubules
NU	- Nuclei
LU	- Lumen
IDNU	- Inflammation and degration of nuclei
DHC	- Degranulation of haemocytes
PHNU	- Pyknotic nuclei
LOS	- Lymphoid spherical cells
APC	- Apoptotic cell
LT	- Lymphatic tubule
CC	- Clumping of cells

Plate: 8 Histological changes of nerve cord in *L.vannamei*

Light micrographs of a paraffin section stained with Hematoxylin and Eosin (40x)

A & B- Control

C- After 7 days of exposure to 0.112 ppm concentration of copper

D- After 28 days of exposure to 0.112 ppm concentration of copper

E- After 7 days of exposure to 0.224 ppm concentration of copper

F - After 28 days of exposure to 0.224 ppm concentration of copper

Abbreviations used:

NU	- Nuclei
HC	- Haemocytes
V	- Vacuoles
VC	- Vacuolated cytoplasm
ISNU	- Irregularly shaped nuclei
DET	- Disintegrated epithelial tissue
CN	- Clumping of nuclei
IHC	- Infiltration of haemocytes
DC	- Disintegration of cuticle
BCI	- Basophilic cytoplasmic inclusion

Received: 15 September 2021 | Revised: 30 September 2021 | Accepted: 10 November 2021

DOI: 10.1002/jemt.24000

RESEARCH ARTICLE

MICROSCOPY RESEARCH & TECHNIQUE WILEY

Cytopathological and ultrastructural changes in the male reproductive organs of freshwater crab *Paratelphusa jacquemontii* (Rathbun) exposed to nurocombi

Shanmuganathan Archunan[1] | Maharajan Athisuyambulingam[1] | Kumara Perumal Pradhoshini[2] | Narayanaswamy Yelappu[1] | Ganapiriya viswambaran[1] | Muthukumaravel Kannayiram[1] | Marckasagayam Priyadharshini[2] | Mohamed Saiyad Musthafa[2] | Caterina Faggio[3]

[1]PG & Research Department of Zoology, Khadir Mohideen College, Bharathidasan University, Thanjavur, India

[2]Unit of Research in Radiation Biology & Environmental Radioactivity (URRBER), PG & Research Department of Zoology, The New College, University of Madras, Chennai, India

[3]Department of Chemical, Biological, Pharmaceutical and Environmental Sciences, University of Messina-Viale Ferdinando Stagno d'Alcontres, Messina, Italy

Correspondence
Caterina Faggio, Department of Chemical, Biological, Pharmaceutical and Environmental Sciences, University of Messina-Viale Ferdinando Stagno d'Alcontres, 31, 98166 Messina, Italy.
Email: cfaggio@unime.it
Mohamed Saiyad Musthafa, Unit of Research in Radiation Biology & Environmental Radioactivity (URRBER), PG & Research Department of Zoology, The New College, University of Madras, Chennai 600014, Tamil Nadu, India.
Email: saiyad_musthafa@rediffmail.com

Funding information
Science and Engineering Research Board, Department of Science and Technology, Grant/Award Number: SB/YS/LS/254/2013

Review Editor: Alberto Diaspro

Abstract

Accumulation of pollutants in the aquatic system has a high impact on the reproductive physiology of crustaceans. The objective of the present study was to assess the possible histopathological effects of combined chlorpyrifos and cypermethrin (nurocombi) exposure on reproductive tissue in male freshwater crab *Paratelphusa jacquemontii* using light and electron microscopy. The testis of experimental crabs showed disorganization of testicular lobules, increased inters cellular space, necrosis, and cellular damage in both germinal cells and Sertoli cells. The treated vas deferens exhibited epithelial degeneration, misshaped spermatophores, decline in the number of spermatophores, and dehiscence of spermatophore wall. These clinical manifestations expressed in crabs following the exposure of nurocombi significantly reduce the testicular activity and substantially inhibits the seminal secretions, which ultimately lead to impairment of reproduction.

KEYWORDS
nurocombi, *Paratelphusa jacquemontii*, testis, vas deferens

1 | INTRODUCTION

Usage of pesticides in agriculture not only impacts the public health but also affects the natural environment and nontarget aquatic organisms through surface runoff from the treated area (Blahova, Cocilovo, Plhalova, Svobodova, & Faggio, 2020; Chromcova et al., 2015; Faria et al., 2021; Fiorino et al., 2018; Plhalova et al., 2018; Stara, Kubec, et al., 2019; Stara, Bellinvia, et al., 2019; Stara et al., 2021; Strungaru et al., 2021; Radovanović et al, 2021; Vajargah et al., 2021; Vajargah, Mohsenpour, Yalsuyi, Galangash, & Faggio, 2021). Chlorpyrifos (*O*,*O*-diethyl-O-(3,5,6-trichloro-2-pyridil) phosphorothioate) is an organophosphate (OP), predominantly used in the world in controlling cutworms, corn rootworms, cockroaches, flea beetles, flies, termites, fire ants, and lice. It is the second largest OP insecticide used in India

(Mathur & Tannan, 1999). Cypermethrin is one of the light stable synthetic pyrethroids registered to control cockroaches and other indoor pests (Sharma, Jindal, & Faggio, 2021). However, when they are applied, the nontarget organisms in addition to the environment they are in and the users are affected (Banaee et al., 2020; Dhara, Saha, Panigrahi, Saha, & Faggio, 2021; Kennedy, ; Petrovici et al., 2020; Sharma, Dar, Singh, Kaur, & Faggio, 2021).

Reproduction, an essential biological need of animals, dominates all other physiological processes and plays an important role in replacing population losses due to death and migration (Petrovici et al., 2020; Sattari et al., 2020; Vajargah, Imanpoor, Shabani, Hedayati, & Faggio, 2019). Accumulation of pollutants in the aquatic ecosystem decelerates the reproductive cycle of animals, thereby causing considerable damage to reproductive tissues and restriction in the egg development upon long-term exposure. Several studies illustrated that the testis is extremely sensitive to toxicity (Ji, Wang, Liu, Wang, & Zhao, 2010). Damage caused to such vital organs affects the metabolic process such as growth and development (Hodkovicova et al., 2020; Pagano, Stara, Aliko, & Faggio, 2020; Sula, Aliko, Barceló, & Faggio, 2020; Sula, Aliko, Marku, Nuro, & Faggio, 2020).

There are numerous examples of male related reproductive abnormalities observed in vertebrates. Extensive studies were conducted with chloropyrifos and cypermethrin in albino rats regarding pulmonary expression of TNF-α in response to toxicity, on respiratory parameters and sleep apnea by Darwiche et al. (2018), on liver and lung toxicity by Yazdinezhad et al. (2017), and with respect to reproductive perturbation by Humadi (2011). Some studies highlighted that chloropyrifos and cypermethrin alone or in combination induced developmental abnormalities in exposed vertebrate model (Shaikh & Sethi, 2020). Contrastingly, on the invertebrates there have been noticeably less examples from both laboratory and field investigations. The organophosphate insecticides are known to affect the crustaceans, as evidenced in *Barytelphusa guerini* (Patil, Paul, & Malkanna.,), *Palaemonetes argentines*, *Trichodactylus borellianus* (Montagna & Collins, 2008), *Paratelphusa jacquemontii* (Maharajan, Narayanasamy, Ganapiriya, & Shanmugavel, 2015), and *Pomacea canaliculata* (Arrighetti, Ambrosio, Astiz, Capítulo, & Lavarías, 2018).

Concerned with histopathological studies, they are useful in evaluating the pollution potential (Mohsenpour et al., 2020; Sinha, Jindal, & Faggio, 2021; Stara et al., 2020a; Stara et al., 2020b; Sula, Aliko, Pagano, & Faggio, 2020). Moreover, ultrastructural study, documented as an important tool to assess the effects of contaminants on vital organs, serves to be helpful in detecting early effects of toxicants on cells (Gharaei, Karimi, Mirdar, Miri, & Faggio, 2020; Hinton et al., 1992; Qyli, Aliko, & Faggio, 2020). On the other hand, the use of resident species as sentry organisms is proposed as a more suitable way to obtain information about a specific site. Study by Neta et al. (2019)) employed *Ucides cordatus* (crab) to evaluate the environmental contamination of the mangrove in Northern Brazil. Also, application of crab species as best bioindicator of environmental contamination was suggested. With respect to *P. jacquemontii*, histological alterations in gills were evaluated succeeding chronic exposure to silver nanoparticles by Kadam & Raut (2019). Accordingly, *P. jacquemontii*, a local crab that is widely distributed in Thiruvarur district, Tamil Nadu, India, was chosen for the current study accounting for the advantages including territorial, easy to collect, and resistant to pollutants. Since this species serves as an efficient bioindicator for assessing the presence of pollutants in aquatic systems, the present study has been designed to exploit the nurocombi bioaccumulative capacity of this species, although more studies will be needed. The present study envisages the pathological effects of nurocombi on testis and vas deferens of freshwater crab *P. jacquemontii*.

2 | MATERIALS AND METHODS

2.1 | Animal collection and acclimatization

The experiments were performed in accordance with the local or national guidelines for experimentation in animals and enough care was exercised to prevent cruelty of any kind. Freshwater crab, *P. jacquemontii* with carapace size ranging from 5.6 to 6.1 mm and weight from 45 to 55 g was collected from the paddy field of Muthupettai, Thiruvarur District, Tamil Nadu, India. They were transported and reared in a 100-L tank containing well aerated, filtered, freshwater that was maintained at ambient temperature (27 ± 2°C) for a period of 1 week. Before stocking, the tank was disinfected with 0.1% $KMnO_4$.

2.2 | Chemicals

For preparation of stock solution, 1 mL of insecticide nurocombi (chlorpyriphos (CPF) 50% and cypermethrin (CPM) 5% EC) was purchased from Cheminova, FMC Corporation, Mumbai and were diluted with 1 L of Milli-Q deionized water (Banaee et al., 2020).

2.3 | Test concentration

Crabs were exposed to 0.0187 and 0.0374 ppm sublethal concentration of combined insecticide doses at 10 and 20%, respectively, of the maximum acceptable toxicant concentration (MATC), which was 0.187 ppm.

2.4 | Test procedure

After 2 weeks of acclimatization in a holding tank, 10 healthy crabs of carapace size ranging from 5.9 to 6.2 mm and weight from 50 to 60 g were transferred to each aquarium. Three replicates were performed for test concentration and control. Crabs were fed twice daily with commercially available trash fish tilapia at 10:00 a.m. and 04:00 p.m. Uneaten food was quickly removed from the system. The media were renewed every alternate day. Water quality (dissolved oxygen [6.5 ± 1.0 mg/L], temperature [25.5 ± 1.5°C], and pH [6.8 ± 1.2]) was

measured every day and water chemistry (ammonia nitrogen [0.56 ± 0.45 mg/L], nitrite nitrogen [0.52 ± 0.28 mg/L], nitrate nitrogen [0.75 ± 0.18 mg/L]) was measured twice weekly. All chemical parameters were determined following the techniques of APHA (1995) using analytical grade reagents. Mortality and behavior of the crayfish were observed every day in each concentration. Two crabs from each aquarium were sampled at 0, 7, and 28 days post-exposure.

2.5 | Light and transmission electron microscopy study

The reproductive tissues testis and vas deferens were collected from the treated as well as control crab after 0, 7, and 28 days post-exposure. The dissection was performed under a dissection microscope in a medium of 0.9% physiological saline. For histological examinations, minimum of seven control and experimental specimen reproductive tissues testis and vas deferens were taken. The method of Bernet, Schmidt, Meier, Burkhardtholm, and Wahli (1999) was adopted for processing of tissues for histological studies. The tissues were fixed in Davidson's fixative and dehydrated in a graded ethyl alcohol series and cleared in xylene for 15 min. The tissues were then embedded in Paraffin wax, sectioned at 5–7 μm thickness, and the thin sections of the tissues were stained by haematoxylin and eosin for observation by the Nikon bright field transmission microscope with Koehler illumination and automatic exposure unit and photomicrographed.

Testis and vas deferens were fixed in 4% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) and post-fixed in buffered 1% osmium tetroxide for 1 hr at 4°C. Post-fixed tissues dehydrated through a graded series of ethanol and embedded in resin. Ultrathin sections (250–500 Å) were cut using glass knives, collected on naked copper-meshed grids, and stained with uranyl acetate and lead citrate. The sections were examined and viewed using a Hitachi H-300 electron microscope operated at 50 kV.

3 | RESULTS AND DISCUSSION

3.1 | Histological assessment of testis

Light microscopic observation of the testis in control crabs displayed normal architecture as evidenced by well-organized distribution of cells in the seminiferous epithelium (Figure 1). Similar to that of other crabs, the testis of *P. jacquemontii* consists of compactly arranged testicular lobules, lined by an outer layer of germinal epithelium which gives rise to the germinal cell lineage consequently. The spermatogonial cells occur in clusters around the germinal ridge along with accessory cells. The ensuing stages consist of a rapid proliferation of spermatogenic elements by the division of spermatogonia into primary and secondary spermatocyte and spermatids and finally crescent shaped spermatozoa. After 7 days of exposure, the seminiferous tubules enlarged and formation of more spaces between the germinal cells was witnessed. The seminiferous epithelium compared to the control was atrophied. Increased size of testicular lumen, disorganization of tubules, and less chromatin material in the germinal cells clearly indicated testicular atrophy. Condensation of spermatogenic cells in testis at higher concentrations was observed by prominent clumping of germinal cells. On Day 28, the testis exhibited extensive necrosis associated with impaired spermatogenesis as well as edema in the interstitial space. Histological observations of testis in the higher concentration (0.0374 ppm) displayed loosely arranged proliferating zone and testicular tubules (LTF) as presented in Figure 2. These results thereby prove negative effects of nurocombi on spermatogenesis in male crabs.

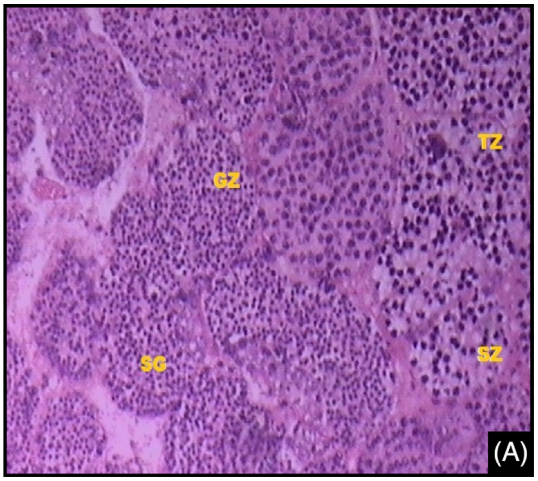


FIGURE 1 Histological changes of testis in *P. jacquemontii*. Light microscope of a paraffin section stained with haematoxylin and eosin (40×). Control testicular tubules showing germinal zone (GZ) and transformation zone (TZ) with spermatonia (SG) and Spermatozoa (SZ)

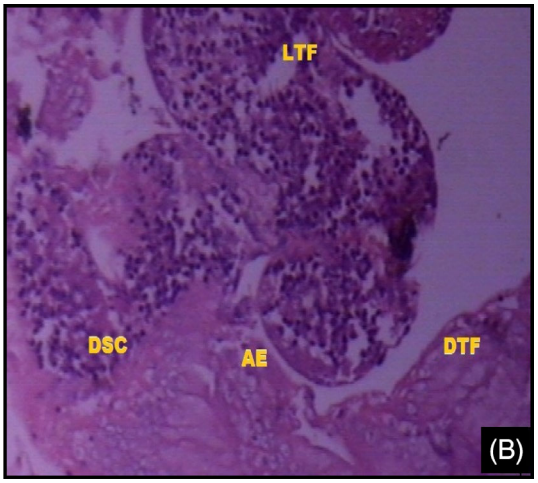


FIGURE 2 Histological changes of testis in *Paratelphusa jacquemontii*. Light microscope of a paraffin section stained with haematoxylin and eosin (40×). After 7 days of exposure to 0.0374 ppm concentration of nurocombi. The picture depicts loosely arranged spermatogenic tubules (LTF), disintegrated spermatocytes (DSC), atrophied epithelium (AE), and damage of testicular follicle (DTF)

Gangshettiwar (1986) showed thickening and rupturing of testicular tubules, deformation of tubules affecting proliferation zone, reduced spermatogenic mass, vacuolization, and tissue degeneration of prawn *Macrobrachium lamerri* after exposure to phenol. In congruence to this, disintegration and deformation of spermatocyte, spermatids, and spermatozoa were observed in *P. jacquemontii* following nurocombi exposure. The treated testis of *P. jacquemontii* harbored distinct changes upon different exposure periods to nurocombi pesticide. Testicular inflammation was documented as one of the common responses in aquatic animals exposed to environmental toxicants (Sokal, Madding, & Swerdloff, 1985). Extensive cytotoxic damage, inflammation, and other histological abnormalities were quite evident in the treated reproductive tissue. Spermatogenesis progression, especially involving the spermatocyte transformation to spermatozoa, was inhibited succeeding nurocombi administration. Observations made through light microscopic studies were further supported by the ultrastructural observations in the treated testis.

3.2 | Ultrastructural profile of testis

Ultrastructural studies, performed in the control group, reveal the cytoplasm, nucleus, and other organelles of spermatogonia and primary spermatocytes to be normal, with mitochondria exhibiting prominent cristae as presented in Figure 3. The spermatozoa comprised globular concentric acrosome, bounded by a cup shaped nucleus in a thin layer of cytoplasm as in other crabs. However, after 7 days of nurocombi exposure, the nuclear materials dispersed within the nucleus in spermatogonia and spermatocytes. The germinal cells which were wrapped by accessory cells (AC) separated with vacuolation appearance in between both cell types.

Inter and intravacuolation worsened with increasing concentration and duration of pesticide exposure (Figure 4). Nuclei of accessory cells were dislocated from the basal portion with vacuoles, dense material, and whorled structures in their nucleoplasm. Large number of phagocytic vesicles (PV) appeared in the AC, thereby suggesting the destruction of abnormal germinal cells. There was extensive coalescence of accessory cell (CA) and spermatogonia with signs of germ cell depletion and exfoliation (Figure 4). Numerous vesicles and electron dense materials were seen in their germ cell cytoplasm. After 28 days of exposure to nurocombi, condensation of nuclear chromatin and degeneration of cytoplasmic organelles, leading to apoptosis was visualized as presented in Figure 5. The acrosome and nuclear structures of spermatozoa highly disorganized (Figure 6).

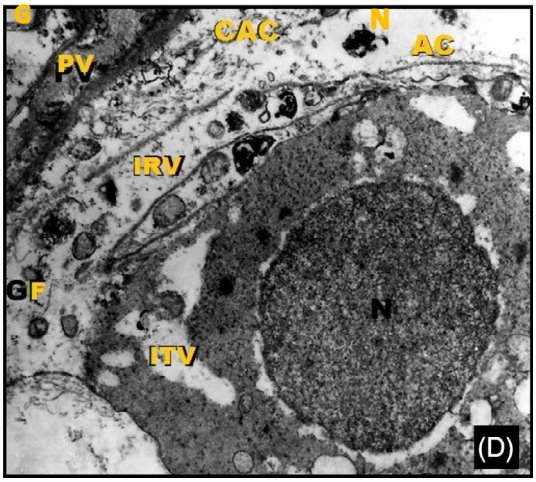


FIGURE 4 Transmission electron microscopic structures of *Paratelphusa jacquemontii* testis. After 7 days of exposure to 0.0374 ppm concentration nurocombi exhibits a disorganized spermatogonium with inter (ITV) and intra vacuolation (IRV) and surrounded by phagocytic vesicles (PV) 10,000×. AC, accessory cell; CAC, coalescence of accessory cell; N, nucleus; GF, gap formation

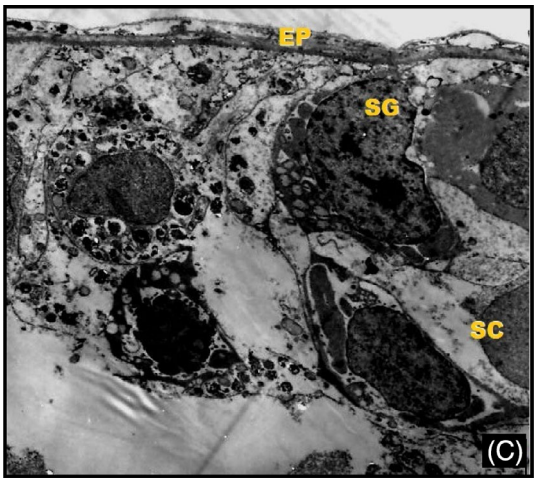


FIGURE 3 Transmission electron microscopic structures of *Paratelphusa jacquemontii* testis. Transmission electron microscopic structures of control testis of *P. jacquemontii* showing germinal epithelium (EP) with spermatogonia (SG) and spermatocyte (SC) 3,000×

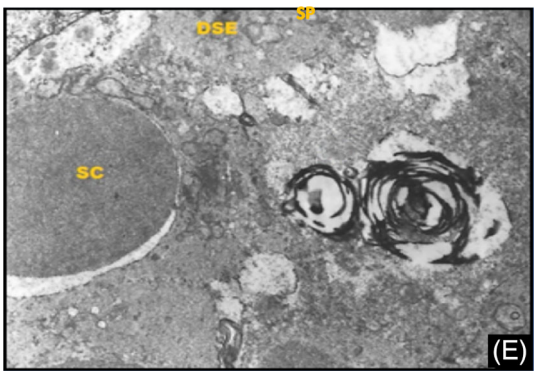


FIGURE 5 Transmission electron microscopic structures of *Paratelphusa jacquemontii* testes. After 28 days of exposure to 0.0374 ppm concentration nurocombi depicts the distortion of seminiferous epithelium (DSE) with damages spermatocytes (SC) and spermatids (SP) 4,000×

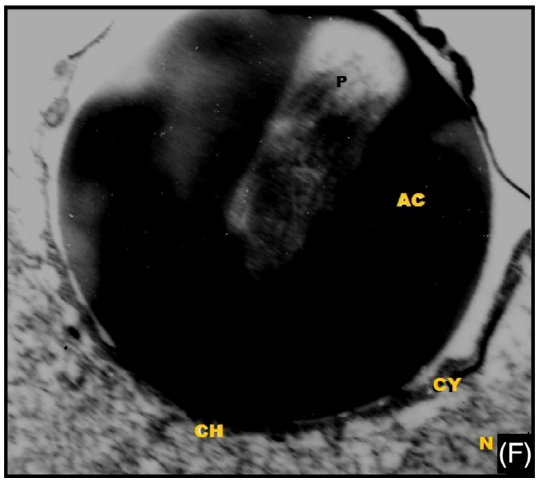


FIGURE 6 Transmission electron microscopic structures of *Paratelphusa jacquemontii* testis. The disorganized spermatozoa (SZ) with improper acrosome (AC) 5,000×. CY, cytoplasm; CH, chromatin; N, nucleus

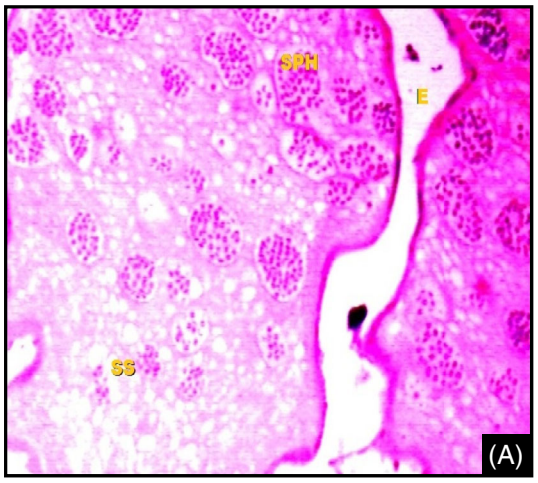


FIGURE 7 Histological changes of vas deferens in *Paratelphusa jacquemontii*. Light microscope of a paraffin section stained with haematoxylin and eosin (40×). Control crab (unexposed) showing fully formed spermatophores (SPH) and seminal secretion (SS) with normal epithelium in the vas deferens

3.3 | Histological assessment of vas deferens

Histological examination of the vas deferens from control crabs displayed normal structure as evidenced by the presence of well-organized epithelium and spermatophores (Figure 7). It was found to be lined internally with squamous epithelium, possessing a definite long nucleus and nucleolus. The lumen was filled with eosinophilic homogenous secretions in which spermatozoa were seen surrounded by a wall as spermatophores (Figure 7). Within the spermatophores, the spermatozoa remained embedded in a homogenous spermatophore matrix. Size of the spermatophore ranged from 10 to 70 μm. Nurocombi treatment, however, resulted in cellular disorganization of the epithelium and highly irregular appearance of the spermatophore

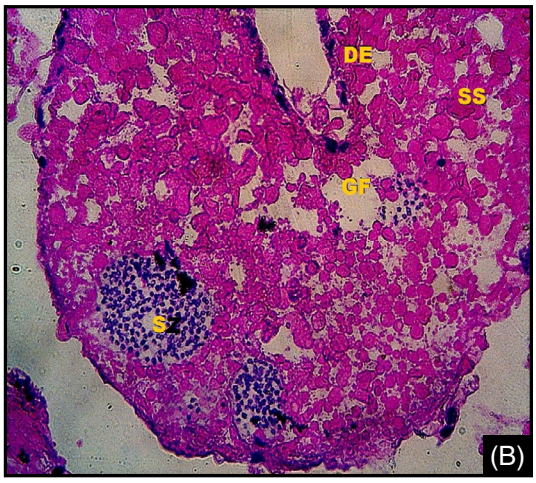


FIGURE 8 Histological changes of vas deferens in *Paratelphusa jacquemontii*. Light microscope of a paraffin section stained with haematoxylin and eosin (40×). After 7 days of exposure to 0.0374 ppm concentration of nurocombi showing damaged epithelium (DE) and gap formation (GF) in the lumen of vas deferens. S, Sertoli cells; SS, seminal secretions

matrix after 7 days for a lower concentration of (10%LC_{50} 0.0187 ppm) (Figure 8). Following progression, the destruction was highly evident in the seminal plasma and spermatophore matrix. In accordance to our results, Bodkhe (1983) observed an irregular arrangement of spermatozoa in the testicular tubules of the crab *Barytelphusa cunicularis* as a result of exposure to sevimol.

Our findings are consistent with the results of El-Ashmawy and Youssef (1999), which reported the disordered arrangement of germ cells, sloughing, and decreased spermatogenic cell layer in the seminiferous tubules, destruction of basement membranes, disintegration of spermatocytes, and complete absence of sperms following Cd toxicity. Moreover, number of spermatophores declined with prominent dehiscence of most of the spermatophores in the lumen. The vas deferens exhibited severe necrosis and atrophy against the control (Figure 8). At a higher concentration (20%LC_{50} 0.0374 ppm), after 28 days of exposure, the spermatophore matrix collapsed and the spermatozoa exposed in most of the spermatophores. The spermatophore wall dissolved and dehisced in some regions as illustrated in Figure 9.

3.4 | Ultrastructural profile of vas deferens

Ultrastructurally the vas deferens encompasses an outer connective tissue, middle muscular layer, and inner cuboidal epithelium. The inner lumen was filled with seminal secretion and spermatozoa. Within the seminal secretion the spermatophores were witnessed with double layers as presented in Figure 10. Upon exposure to 7 days of nurocombi pesticide, the epithelium was visualized with less number of multilobated nuclei, thereby suggesting a gradual decline in the secretory activity (Figure 11). The structural organization of nuclei and ER were sternly altered. nurocombi exposure resulted in vacuolar degeneration of the vasal epithelium and muscular disruption.

FIGURE 9 Histological changes of vas deferens in *Paratelphusa jacquemontii*. Light microscope of a paraffin section stained with haematoxylin and eosin (40×). After 28 days of exposure to 0.0374 ppm of nurocombi showing disintegrated spermatophores (DSPH) with dissolution of spermatophore wall. SZ, spermatozoa; SS, seminal secretions

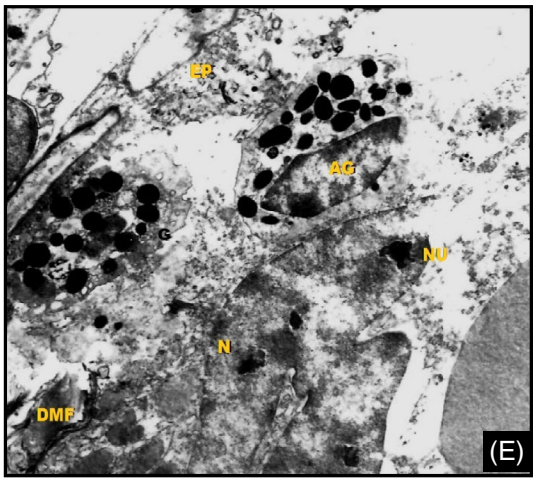


FIGURE 11 Transmission electron microscopic structures of *Paratelphusa jacquemontii* testis. After 28 days of exposure to 0.0374 ppm concentration nurocombi exhibiting damaged epithelium (EP) with the entry of haemocytes 4,500×. AC, accessory cell; G, granuocytes; N, nucleus; Nu, nucleolus; DMF, disintegrated muscle fibers

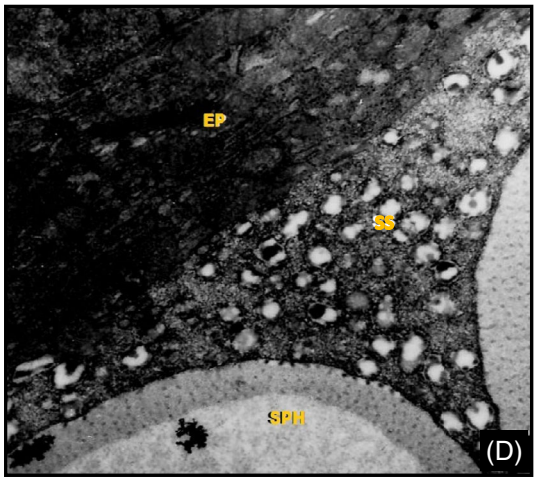


FIGURE 10 Transmission electron microscopic structures of *Paratelphusa jacquemontii* vas deferens. Transmission electron microscopic structure of control vas deferens of *P. jacquemontii* showing highly secretory epithelium (EP) with seminal secretion (SS) and spermatophores (SPH) 3,000×

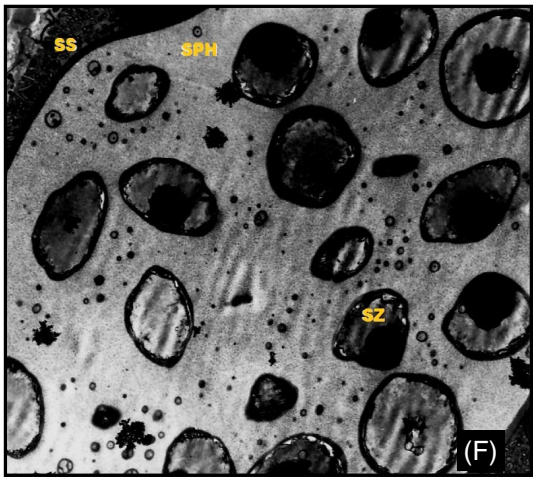


FIGURE 12 Transmission electron microscopic structures of *Paratelphusa jacquemontii* vas deferens. The micrograph showing collapsed spermatophores with severely damaged spermatozoa (SZ) 3,000×. SS, seminal secretions; SPH, spermatophores

After 28 days, hemocytes were visualized in vas deferens due to the hemolymph damage and consequent entry of the hemocytes, as shown in Figure 11. At a higher concentration (20% LC_{50} 0.0374 ppm) of nurocombi exposure, the spermatophore matrix appeared to be highly irregular followed by scattering and collapse of spermatozoa. The structure of such spermatozoa was highly disorganized as well, with decondensed and scattered nucleus (Figure 12). Moreover, irregularity in the arrangement of spermatophores and epithelial cells were discerned with reduction in tubule membrane thickness. On the other hand, at relatively low concentrations, treated crabs exhibited disruption in the tubular architecture, arrangement of epithelial cells, reduction in number of spermatophores, and aggregation of the granular substances in the vas deferens. It is to annotate that TBT (tributyltin) has similar effect on sperm count and male reproductive system of aquatic organisms (Fent & Hunn, 1995; Zhang et al., 2007). Results of our study are in accordance with the findings of Eman et al. (2016), which reported similar manifestations such as deterioration in spermatozoa motility, viability, quality, histopathological changes, and significant decline in hormone production of male albino rats, following the exposure of chloropyrifos and cypermethrin. It is also in accordance with the results of Prashanthi et al. (2006), having been visualized the impairment of spermatozoa, severe impact on vas deferens, cellular necrosis, and nuclear pyknosis, upon exposure to organophosphate

pesticide in rats. Vas deferens in such organophosphate treated rats were highly altered and resulted in the depletion of testosterone production (Humadi, 2011).

4 | CONCLUSION

In the present study, nurocombi treatment in freshwater crab *P. jacquemontii* for a post-exposure period of 28 days caused distinct changes in gonadal histology. Ultrastructural and cytological deformations produced in the reproductive tissues, perturbated the reproductive physiology and over all metabolic performance of the crab. Hence, it can conclude that utilization of pesticides should be minimized in the paddy field area of Muthupettai mangrove ecosystem. Enhancement and designing of alternative or remedial measures are recommended to protect the crustacean biodiversity.

ACKNOWLEDGMENTS

Authors would like to acknowledge their gratitude to Science and Engineering Research Board, Department of Science and Technology, New Delhi, India (SB/YS/LS/254/2013) for the financial assistance and Head of the Institution, Khadir Mohideen College, Adirampattinam for the facilities provided.

ORCID

Caterina Faggio https://orcid.org/0000-0002-0066-2421

REFERENCES

APHA. (1995). *Standard methods for the examination of water and waste water* (19th ed.). Washington, DC: American Public Health Association, American Water Works Association, and Water Pollution Control Federation.

Arrighetti, F., Ambrosio, E., Astiz, M., Capítulo, A. R., & Lavarías, S. (2018). Differential response between histological and biochemical biomarkers in the apple snail *Pomacea canaliculata* (Gasteropoda: Amullariidae) exposed to cypermethrin. *Aquatic Toxicology*, *194*, 140–151.

Banaee, M., Akhlaghi, M., Soltanian, S., Sureda, A., Gholamhosseini, A., & Rakhshaninejad, M. (2020). Combined effects of exposure to sublethal concentration of the insecticide chlorpyrifos and the herbicide glyphosate on the biochemical changes in the freshwater crayfish Pontastacus leptodactylus. *Ecotoxicology*, *29*(9), 1500–1515.

Bernet, D., Schmidt, H., Meier, W., Burkhardtholm, P., & Wahli, T. (1999). Histopathology in fish: Proposal for a protocol to assess aquatic pollution. *Journal of Fish Diseases*, *22*, 25–34.

Blahova, J., Cocilovo, C., Plhalova, L., Svobodova, Z., & Faggio, C. (2020). Embryotoxicity of atrazine and its degradation products to early life stages of zebrafish (*Danio rerio*). *Environmental Toxicology and Pharmacology*, *77*, 103370.

Bodkhe MK. 1983. *Effect of some pesticidal pollutant on the physiology of Barytelphusa cunicularis* (PhD thesis). Dr. Babasaheb Ambedkar Marathwada University, Aurangabad, India.

Chromcova, L., Blahova, J., Zivna, D., Plhalova, L., Casuscelli, F., Di Tocco, L., … Svobodova, Z. (2015). NeemAzal T/S–toxicity to early-life stages of common carp (*Cyprinus carpio* L.). *Veterinární Medicína*, *60*(1), 23–30.

Darwiche, W., Gay-Quéheillard, J., Delanaud, S., El Khayat El Sabbouri, H., Khachfe, H., Joumaa, W., … Ramadan, W. (2018). Impact of chronic exposure to the pesticide chlorpyrifos on respiratory parameters and sleep apnea in juvenile and adult rats. *PLoS One*, *13*(1), e0191237.

Dhara, K., Saha, S., Panigrahi, A. K., Saha, N. C., & Faggio, C. (2021). Biochemical, physiological (haematological, oxygen-consumption rate) and behavioural effects of mercury exposures on the freshwater snail, *Bellamya bengalensis*. *Comparative Biochemistry and Physiology, Part C*, *251*, 109195.

El-Ashmawy, I. M., & Youssef, S. A. (1999). The antagonistic effect of chlorpromazine on cadmium toxicity. *Toxicology and Applied Pharmacology*, *161*, 34–39.

Faria, M., Prats, E., Ramírez, J. R. R., Bellot, M., Bedrossiantz, J., Pagano, M., … Raldua, D. (2021). Androgenic activation, impairment of the monoaminergic system and altered behavior in zebrafish larvae exposed to environmental concentrations of fenitrothion. *Science of the Total Environment*, *775*, 145671.

Fent, K., & Hunn, J. (1995). Organ tins in freshwater harbours and river temporal distribution, annual trends and fate. *Journal of Environmental Toxicology and Chemistry*, *14*, 1123–1132.

Fiorino, E., Sehonova, P., Plhalova, L., Blahova, J., Svobodova, Z., & Faggio, C. (2018). Effects of glyphosate on early life stages: Comparison between *Cyprinus carpio* and *Danio rerio*. *Environmental Science and Pollution Research*, *25*(9), 8542–8549.

Gangshettiwar, V. B. (1986). *Effect of phenol poisoning on the physiology of the prawn, Macrobrachium lamerrii* (PhD thesis). Dr. Babasaheb Ambedkar Marathwada University, Aurangabad, India.

Gharaei, A., Karimi, M., Mirdar, J., Miri, M., & Faggio, C. (2020). Population growth of *Brachionus calyciflorus* affected by deltamethrin and imidacloprid insecticides. *Iranian Journal of Fisheries Sciences*, *19*(2), 588–601.

Hinton, D. E., Baumann, P. C., Gardner, G. R., Hawkins, W. E., Hendricks, J. D., Murchelan, R. A., & Okihiro, M. S. (1992). *Biomarkers: Biochemical, physiological and histopathological markers of anthropogenic stress* (pp. 155–208). Boca Ration, FL: Lewis.

Hodkovicova, N., Enevova, V., Cahova, J., Blahova, J., Siroka, Z., Plhalova, L., … Svobodova, Z. (2020). Could the musk compound tonalide affect physiological functions and act as an endocrine disruptor in rainbow trout? *Physiological Research*, *69*, S595–S606.

Humadi, A. L. (2011). The toxic effects following gestational and lactational exposure of rats to 4-tert-octylphenol on the subsequent development of the vas deferens tissue. *American-Eurasian Journal of Toxicological Sciences*, *3*(3), 161–171.

Ji, Y. L., Wang, H., Liu, P., Wang, Q., & Zhao, X. F. (2010). Pubertal cadmium exposure impairs testicular development and spermatogenesis via disrupting testicular testosterone synthesis in adult mice. *Reproductive Toxicology*, *29*, 176–183.

Kadam, G. H., & Raut, P. D. (2019). Histological alterations in the gills of crab, *Paratelphusa jacquemontii* after chronic exposure to silver nanoparticles (AgNP). *Indian Journal of Ecology*, *46*(2), 422–426.

Maharajan, A., Narayanasamy, Y., Ganapiriya, V., & Shanmugavel, K. (2015). Histological alterations of a combination of chlorpyrifos and cypermethrin (nurocombi) insecticide in the fresh water crab, *Paratelphusa jacquemontii* (Rathbun). *Journal of Basic and Applied Zoology*, *72*, 104–112.

Mathur, S. C., & Tannan, S. K. (1999). Future of Indian pesticides industry in next millennium. *Pesticide Information*, *24*(4), 9–23.

Mohsenpour, R., Mousavi-Sabet, H., Hedayati, A., Rezaei, A., Yalsuyi, A. M., & Faggio, C. (2020). In vitro effects of silver nanoparticles on gills morphology of female Guppy (*Poecilia reticulate*) after a short-term exposure. *Microscopy Research and Technique*, *83*(12), 1552–1557.

Montagna, M. C., & Collins, P. A. (2008). Oxygen consumption and ammonia excretion of the fresh water crab *Trichodactylus borellianus* exposed to chlorpyrifos and endosulfan insecticides. *Pesticide Biochemistry and Physiology*, *92*, 150–155.

Neta, R. N. F. C., de Oliveira Mota, T. D. S., de Oliveira, S. R. S., Junior, A. R. T., da Silva Cardoso, W., Santos, D. M. S., … Brito, N. M. (2019). Biochemical and morphological responses in *Ucides cordatus* (Crustacea, Decapoda) as indicators of contamination status in mangroves and port areas from northern Brazil. *Environmental Science and Pollution Research*, *26*(16), 15884–15893.

Pagano, M., Stara, A., Aliko, V., & Faggio, C. (2020). Impact of neonicotinoids to aquatic invertebrates—In vitro studies on *Mytilus galloprovincialis*: A review. *Journal of Marine Science and Engineering*, *8*(10), 801.

Petrovici, A., Strungaru, S. A., Nicoara, M., Robea, M. A., Solcan, C., & Faggio, C. (2020). Toxicity of deltamethrin to zebrafish gonads revealed by cellular biomarkers. *Journal of Marine Science and Engineering*, *8*(2), 73.

Plhalova, L., Blahova, J., Divisova, L., Enevova, V., Casuscelli di Tocco, F., Faggio, C., … Svobodova, Z. (2018). The effects of subchronic exposure to NeemAzal T/S on zebrafish (*Danio rerio*). *Chemistry and Ecology*, *34*(3), 199–210.

Prashanthi, N., Narayana, K., Nayanatara, A., Chandra Kumar, H. H., Bairy, K. L., & D'Souza, U. J. (2006). The reproductive toxicity of the organophosphate pesticide 0, 0-dimethyl 0-4-nitrophenyl phosphorothioate (methyl parathion) in the male rat. *Folia Morphology*, *65*(4), 309–321.

Qyli, M., Aliko, V., & Faggio, C. (2020). Physiological and biochemical responses of Mediterranean green crab, *Carcinus aestuarii*, to different environmental stressors: Evaluation of hemocyte toxicity and its possible effects on immune response. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, *231*, 108739.

Radovanović, T. B., Gavrilović, B. R., Petrović, T. G., Despotović, S. G., Gavrić, J. P., Kijanović, A., … Prokić, M. D. (2021). Impact of desiccation pre-exposure on deltamethrin-induced oxidative stress in *Bombina variegata* juveniles. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, *250*, 109191.

Sattari, M., Imanpour Namin, J., Bibak, M., Forouhar Vajargah, M., Bakhshalizadeh, S., & Faggio, C. (2020). Determination of trace element accumulation in gonads of *Rutilus kutum* (Kamensky, 1901) from the south Caspian Sea trace element contaminations in gonads. *Proceedings of the National Academy of Sciences, India Section B: Biological Sciences*, *90*(4), 777–784.

Shaikh, N. I., & Sethi, R. S. (2020). Exposure to chlorpyrifos and cypermethrin alone or in combination induces developmental abnormalities and lung damage in animal models: A review. *Journal of Entomology and Zoology Studies*, *8*(5), 1923–1928.

Sharma, R., Jindal, R., & Faggio, C. (2021). Cassia fistula ameliorates chronic toxicity of cypermethrin in *Catla catla*. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, *248*, 109113.

Sharma, S., Dar, O. I., Singh, K., Kaur, A., & Faggio, C. (2021). Triclosan elicited biochemical and transcriptomic alterations in *Labeo rohita* larvae. *Environmental Toxicology and Pharmacology*, *88*, 103748.

Sinha, R., Jindal, R., & Faggio, C. (2021). Nephroprotective effect of *Emblica officinalis* fruit extract against malachite green toxicity in piscine model: Ultrastructure and oxidative stress study. *Microscopy Research and Technique*, *84*, 1911–1919.

Sokal, R. Z., Madding, C. E., & Swerdloff, R. S. (1985). Lead toxicity and the hypothalamicpitutary testicular axis. *Biology of Reprodution*, *33*, 722–728.

Stara, A., Bellinvia, R., Velisek, J., Strouhova, A., Kouba, A., & Faggio, C. (2019). Acute exposure of neonicotinoid pesticide on common yabby (*Cherax destructor*). *Science of the Total Environmental*, *665*, 718–723.

Stara, A., Kubec, J., Zuskova, E., Buric, M., Faggio, C., Kouba, A., & Velisek, J. (2019). Effects of S-metolachlor and its degradation product metolachlor OA on marbled crayfish (*Procambarus virginalis*). *Chemosphere*, *224*, 616–625.

Stara, A., Pagano, M., Albano, M., Savoca, S., Di Bella, G., Albergamo, A., … Faggio, C. (2021). Effects of long-term exposure of *Mytilus galloprovincialis* to thiacloprid: A multibiomarker approach. *Environmental Pollution*, *289*, 117892.

Stara, A., Pagano, M., Capillo, G., Fabrello, J., Sandova, M., Albano, M., … Faggio, C. (2020a). Acute effects of neonicotinoid insecticides on *Mytilus galloprovincialis*: A case study with the active compound thiacloprid and the commercial formulation calypso 480 SC. *Ecotoxicology and Environmental Safety*, *203*, 110980.

Stara, A., Pagano, M., Capillo, G., Fabrello, J., Sandova, M., Vazzana, I., … Faggio, C. (2020b). Assessing the effects of neonicotinoid insecticide on the bivalve mollusc *Mytilus galloprovincialis*. *Science of the Total Environment*, *700*, 134914.

Strungaru, S. A., Pohontiu, C. M., Nicoara, M., Teodosiu, C., Baltag, E. S., Jijie, R., … Faggio, C. (2021). Response of aquatic macroinvertebrates communities to multiple anthropogenic stressors in a lowland tributary river. *Environmental Toxicology and Pharmacology*, *87*, 103687.

Sula, E., Aliko, V., Barceló, D., & Faggio, C. (2020). Combined effects of moderate hypoxia, pesticides and PCBs upon crucian carp fish, *Carassius carassius*, from a freshwater lake-in situ ecophysiological approach. *Aquatic Toxicology*, *228*, 105644.

Sula, E., Aliko, V., Marku, E., Nuro, A., & Faggio, C. (2020). Evaluation of kidney histopathological alterations in Crucian Carp, *Carassius carassius*, from a pesticide and PCB-contaminated freshwater ecosystem, using light microscopy and organ index mathematical model. *International Journal of Aquatic Biology*, *8*(3), 154–165.

Sula, E., Aliko, V., Pagano, M., & Faggio, C. (2020). Digital light microscopy as a tool in toxicological evaluation of fish erythrocyte morphological abnormalities. *Microscopy Research and Technique*, *83*(4), 362–369.

Vajargah, M. F., Imanpoor, M. R., Shabani, A., Hedayati, A., & Faggio, C. (2019). Effect of long-term exposure of silver nanoparticles on growth indices, hematological and biochemical parameters and gonad histology of male goldfish (*Carassius auratus* gibelio). *Microscopy Research and Technique*, *82*(7), 1224–1230.

Vajargah, M. F., Mohsenpour, R., Yalsuyi, A. M., Galangash, M. M., & Faggio, C. (2021). Evaluation of histopathological effect of roach (*Rutilus rutilus caspicus*) in exposure to sub-lethal concentrations of Abamectin. *Water, Air, & Soil Pollution*, *232*(5), 1–8.

Vajargah, M. F., Namin, J. I., Mohsenpour, R., Yalsuyi, A. M., Prokić, M. D., & Faggio, C. (2021). Histological effects of sublethal concentrations of insecticide Lindane on intestinal tissue of grass carp (*Ctenopharyngodon idella*). *Veterinary Research Communications*, 1–8. https://doi.org/10.1007/s11259-021-09818-y

Yazdinezhad, A., Abbasian, M., Hojjat Hosseini, S., Naserzadeh, P., Agh-Atabay, A. H., & Hosseini, M. J. (2017). Protective effects of *Ziziphora tenuior* extract against chlorpyrifos induced liver and lung toxicity in rat: Mechanistic approaches in subchronic study. *Environmental Toxicology*, *32*(9), 2191–2202.

Zhang, J., Zhenghong, Z., Yixin, C., Yang, Z., Shuai, H., & Chonggang, W. (2007). Effect of tributyltin on the development of ovary in female cuvier (*Sebasticus marmoratus*). *Aquatic Toxicology*, *83*, 174–179.

How to cite this article: Archunan, S., Athisuyambulingam, M., Pradhoshini, K. P., Yelappu, N., viswambaran, G., Kannayiram, M., Priyadharshini, M., Musthafa, M. S., & Faggio, C. (2021). Cytopathological and ultrastructural changes in the male reproductive organs of freshwater crab *Paratelphusa jacquemontii* (Rathbun) exposed to nurocombi. *Microscopy Research and Technique*, 1–8. https://doi.org/10.1002/jemt.24000

International Journal of Entomology Research
www.entomologyjournals.com
ISSN: 2455-4758
Received: 08-11-2021, Accepted: 24-11-2021, Published: 09-12-2021
Volume 6, Issue 6, 2021, Page No. 174-179

Biochemical and cytological changes of Whiteleg shrimp, *Litopenaeus vannamei* exposed to chlorpyrifos

A Shanmuganathan, A Maharajan*, V Ganapiriya, D Nancy
PG and Research, Department of Zoology, Khadir Mohideen College (Affiliated to Bharathidasan University, Tiruchirappalli), Adirampattinam, Thanjavur, Tamil Nadu, India.

Abstract
Chlorpyrifos is used extensively as a pesticide in consumer products for commercial agricultural applications as well as for household purposes. The aim of the present research is to evaluate the effect of a sub-lethal concentration of chlorpyrifos in the biochemical and cytological parameters of the Indian white shrimp, *Litopenaeus vannamei*, after 0,7,14,21 and 28 days of exposure.TP, TC and TL levels were significantly lower in test *L. vannamei* than in controls for all days of the experiment. Experimental gill tissue exhibited epithelial lifting, edema, necrosis, fusion of secondary lamellae, and bleeding. Infiltration, large lumen formation and disappearance of hemocytes were detected in the dead hepatopancreas. Muscle tissue exhibited pathological signs such as atrophy, necrosis, wavy appearance, accumulation of granular material between muscle fibers, fragmentation, loss of muscle structure, presence of basophilic deposits.

Keywords: Chlorpyrifos, *Litopenaeus vannamei*, Biochemistry, Cytology

Introduction
Numerous studies have been conducted on the toxicity of various insecticides on aquatic and terrestrial organisms. Most studies concerning the effects of insecticides on shrimp mainly focus on short-term investigations that include behavioral changes in overall animal responses such as gross abnormalities, growth rates, and mortality. Recently, more research is being done on the physiological and biochemical reactions of agricultural insecticides on shrimp. The crustacean hepatopancreas or digestive gland involves in food absorption, synthesis and secretion of digestive enzymes, storage of lipids, glycogen and minerals during intermoult period. It is the main organ of reserve and detoxification of xenobiotics and is highly sensitive to physiological and environmental changes [1]. Reddy *et al.* [2] observed that the sumithion affects the ovarian growth of crab, *Oziotelphusa sensex sensex*. Victor [3] observed structural changes in ovary of freshwater prawn, *Caridina rajadhari* exposed to malathion and DDT.
Histopathological examination has been increasingly recognizes as a valuable tool for the assessment of the impact of environmental pollutants on aquatic animals [4, 5, 6]. Gills apart from being the primary respiratory organ in crabs, are also responsible for other vital physiological functions like excretion, acid base balance and ion regulation. So when crabs are exposed to environmental pollutants, these vital functions are deleteriously affected and the functional impairment of gills can significantly damage their health. The gills are efficient tools for biomonitoring potential impacts because of their large area in contact with water and high permeability [7]
The present study was undertaken to evaluate the toxicity of the largest market-selling and multipurpose insecticide chlorpyrifos, on the commonly available and edible aquatic organism shrimp. The study was carried out with special emphasis on biochemical and cytological effects of the insecticide, chlorpyrifos in the Indian white shrimp, *Litopenaeus vannamei.*

Materials and Methods
Animal collection and acclimatization
The experiments were performed in accordance with local/national guidelines for experimentation in animals and all care was taken to prevent cruelty of any kind. Whiteleg shrimp, *Litopenaeus vannamei*) size ranging from 4- 5cm and weight 2-3g were collected from the culture pond Mallipattinam, Thanjavur Dist, Tamil Nadu. They were transported and kept in 100 L tank containing well aerated filtered sea water maintained at ambient temperature (27± 2 °C) for a period of one week. Before stocking, the tank was washed with 0.1% KMnO4 for disinfection.

Chemicals
For preparation of stock solution 1 ml of insecticide Chlorpyrifos, Jeyban, Sabari Crop Care Sciences (P) Ltd. Chennai, diluted with 1 L of Milli-Q deionized water.

Test concentration
Shrimps were exposed to 0.006 and 0.012 ppm sublethal concentration of chlorpyrifos insecticide doses at 10% and 20% respectively.

Test procedure
After 2 weeks of acclimatization in a holding tank, ten healthy shrimps with size ranging from 4.5- 5cm and weight 2.5-3.5g were transferred to each aquarium. Three replicates were performed for test concentration and control. Shrimps were fed twice daily with commercially prepared pellet feed at 10:00 and 16:00 h. Uneaten food was quickly removed from the system. The media were renewed every alternate day. The actual concentration of chlorpyrifos was measured weekly before and after its addition to maintain chlorpyrifos

concentrations at the designed level. Mortality and behavior were observed every day in each concentration. Two shrimps from each aquarium were sampled at 0, 7, 14, 21and 28 days post-exposure.

Biochemical Composition

The shrimps were exposed to 0.006 and 0.012ppmconcentrations of chlorpyrifos for 28 days. After 0, 7, 14, 21 and 28 days of chlorpyrifos exposed shrimps were sacrificed and tissues, were taken out and analyzed for biochemical composition. Total protein was estimated in UV visible double beam spectrophotometer by Biuret method using bovine serum albumin as standard as suggested by [8]. Total carbohydrate was estimated by Phenol - Sulphuric acid Method of Roe [9]. Total lipid was estimated by gravimetric methanol - chloroform extraction method suggested by Floch *et al.,* [10] and modified by Linford [11].

Cytological study

The muscle, hepatopancreas and were collected from the treated as well as control shrimp after 0 & 28 days post-exposure and preserved in Davidson's fixatives for 24 h, dehydrated through a graded ethanol series and embedded in paraffin.

Tissue sections (5 mm thick) were stained with haematoxylin–eosin. The thin sections of the tissues were stained by haematoxylin and eosin for observation by the Nikon bright field transmission microscope with Koehler illumination and automatic exposure unit was used.

Results

Chlorpyrifos induced changes in proximate composition

Changes in the Total Protein (TP) Levels

TP levels in the various tissues of the control and *L. vannamei* exposed during the exposure period are depicted in Figure 1, 2, and 3, TP concentrations tested were significantly lower in *L. vannamei* than in controls at all days of exposure (DOE) ($P<0.05$). The rate of reduction was found to be highly time and tissue dependent. At the end of 28 DoE the sequence of decrease in percentage of TP concentration in different tissues was observed as gill>hepatopancreas>muscle.

A progressive decrease in the tested TP levels was recorded in the GL and HP tissues during the exposure period. A significant variation in TP content was observed ($p > 0.05$) between exposure concentrations of 0.006 ppm and 0.012 ppm. Hepatic protein levels of test *L.vannamei* were found to be approximately similar to those of control *L.vannamei* at 0 and 7 DoE, but the reduction was more prominent at 14, 21 and 28 DoE. The magnitude of the reduction in liver protein *L. vannamei* was directly proportional to the concentration.

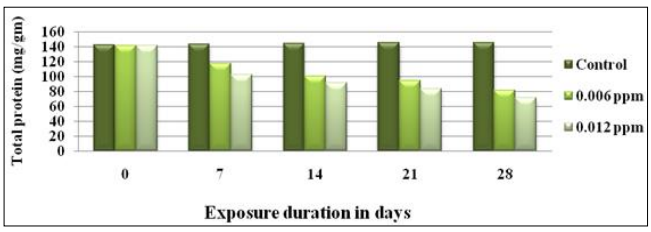


Fig 1: Changes of total protein (mg/gm) in muscle of *L.vannamei* exposed to sublethal concentration of chlorpyrifos

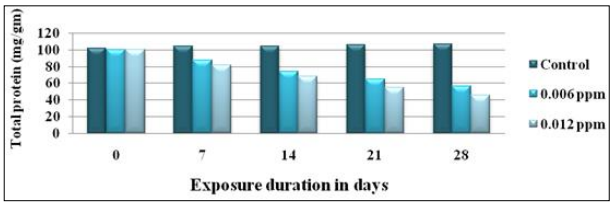


Fig 2: Changes of total protein (mg/gm) in muscle of *L.vannamei* exposed to sublethal concentration of chlorpyrifos

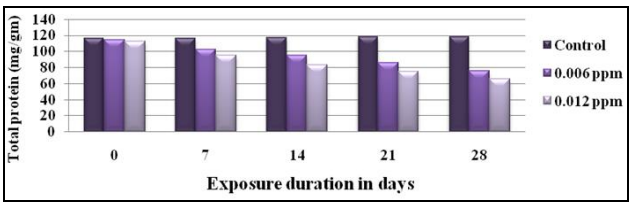


Fig 3: Changes in total protein (mg/gm) in hepatopancteas of *L. vannamei* exposed to sublethal concentration of chlorpyrifos

Changes in the Total Carbohydrate (TC) Levels

The levels of TC in the different tissues tested for *L. vannamei* and controls during the exposure period are shown in Figures 4, 5 and 6. TC concentrations were significantly lower in the test. *L.vannamei* compared to controls at all DoEs. The reduction in TC levels in the GL and HP of trial *L.vannamei* was significant with progression in the duration of exposure. The TC levels in the GL of the test *L.vannamei* displayed a biphasic pattern: high concentrations at 0 DOE and 7 DOE and low at 14 DOE and 21 DOE and 28 DOE. The order of percent reduction in TC levels in the studied tissues was found to be GL>HP>MU on the last day of exposure (28 DoE).

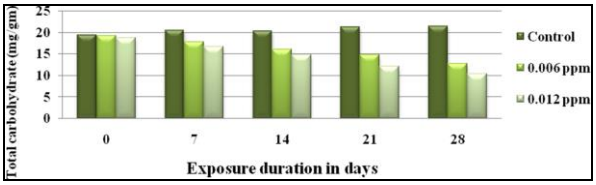


Fig 4: Changes of total carbohydrate (mg/gm) in muscle of *L. vannamei* exposed to sublethal concentration of chlorpyrifos

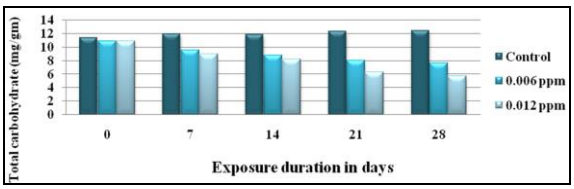


Fig 5: Changes of total carbohydrate (mg/gm) in gill of *L.vannamei* exposed to sublethal concentration of chlorpyrifos

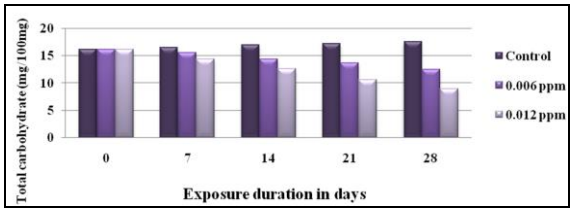


Fig 6: Changes of total carbohydrate (mg/gm) in hepatopancreas of *L. vannamei* exposed to sublethal concentration of chlorpyrifos

Changes in the Total Lipid (TL) Levels

The levels of TL in different tissues of the test *L.vannamei*

and controls during the exposure period are shown in Figure 7, 8 and 9. In general, TL concentrations in all studied tissues of *L.vannamei* exposed to sub-lethal doses. The amount of chlorpyrifos was significantly lower than that of the control ($P<0.05$). As the duration of exposure progressed, the concentrations of total lipids decreased significantly in all tissues regardless of exposure concentrations.

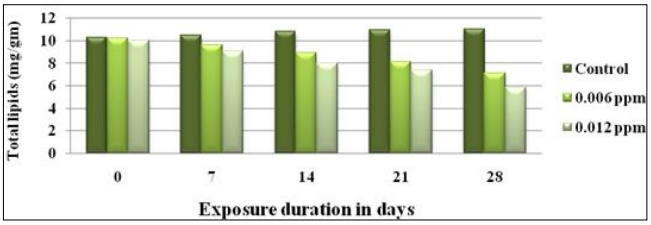


Fig 7: Changes of total lipids (mg/gm) in muscle of *L.vannamei* exposed to sublethal concentration of chlorpyrifos

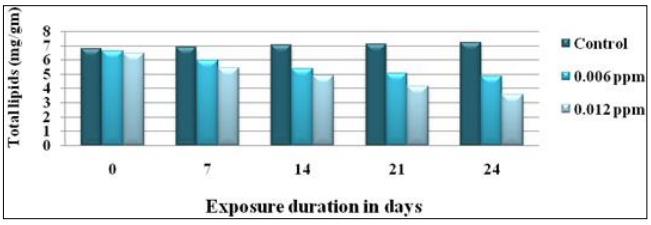


Fig 8: Changes of total lipids (mg/gm) in gill of *L. vannamei* exposed to sublethal concentration of chlorpyrifos

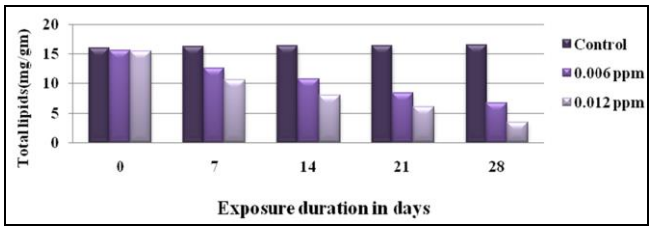


Fig 9: Changes of total lipids (mg/gm) in hepatopancreas of *L. vannamei* exposed to sublethal concentration of chlorpyrifos

Cytological studies

Muscle

The muscle tissue of the control shrimp was made up of muscle cells containing contractile filaments that move each other and change the size of the cell. Muscle tissue derived from mesoderm contains protein, and myosin filament (thread-like) form multi nucleate cells that assemble into fibers called myofibrils (Plate 1A). The striated muscle fibres were tightly packed. Muscle is the tissue of motion and is widely distributed in various organs of the body. The photomicrograph of the muscle depicted the presence of normal myotomes (MT) with equally spaced muscle bundles the fascicular arrangement of myofilaments (MF) with emarginated epimysium, binding to connective tissue and tendon at the extremities of the smooth muscles. The striated muscle fibres (SM) were tightly packed. The nuclei were arranged alongthe margins of the muscle bundles. After the pesticide exposure, the muscle tissue showed disintegrated epidermis (DE) with vacuolation, gap formation (GF) in between the muscle bundles, necrosis (NE), marked thickening and separation of muscle bundle and pronounced intramuscular oedema with minor dystrophic change. In the higher concentration, the muscle bundles are completely disrupted with discontinuity of striations and complete disappearance of nuclei. In some regions of muscle tissue shows the sloughing of epidermal layer (SEL) Lesions (LN) and mild haemocyte infiltrations (HI) are the marked changes after 28 days low concentration followed by fusion of muscle bundles (FMB). In higher concentration the muscle tissue expressed significant changes like broken myofibrils (BMF), coagulative necrosis (CNE) congestion of muscle bundles followed by rupture of muscle bundles. Severe haemocyte infiltration (HI) and accumulation of granular materials in between the muscle fibers (GMF) are also noted. Congregation of nucleus occurs in the vacuolated region and banding patterns were completely altered in higher concentration (Plate 1 B).

Hepatopancreas

The cross section of hepatopancreas showed the presence of large number of elongated tubules collectively brought together by a connective tissue and stained pink with haematoxylin and eosin. Each tubule has an outer thin cuticle and inner epithelial lining. Each tubule contained lumen of different shape and size. Each tubule is covered with a thin epidermal layer enclosing a central cavity, the lumen. Three types of cells are noticed in each tubule beneath the epidermal layer. Absorptive cells (A-cells): These cells are of columnar type having nucleus towards the base. The cells appear vacuolated due to the presence of fat globules. Secretory cells (S-cells): These cells are larger in size, having large globular mass and small vacuoles. The globular masses of cells discharge their secretions into lumen of the tubule. Embryonic cells (E-cells) (Plate 1C): These cells are small as compared to absorptive and secretory cells. The nucleus is present in the centre and cells are located beneath the secretory cells towards the lumen of the tubule and very few in numbers. The experimental shrimps treated with lethal concentration of pesticides showed many histological changes in hepatopancreas through the period of exposure. The hepatopancreas shows the damage of tubules and distortion of connective layers. Vacuolation of the epithelial cells are observed. Epithelial layer is ruptured and the central cavity [the lumen] is observed decreased in size. The absorptive cells are increased in size; whereas in secretory cells the globular mass is reduced, sometimes disappeared and number of vacuoles are increased (Plate 1D).

Gills

The gills of *L. vannamei* are formed of a number of lamellae or broad flattened plates arranged serially in pairs along a control gill stem. The central axis of gill tissue is the primary gill lamellae and it further divides into secondary gill lamellae or filaments. The control gill exhibit a thin layer of cuticle covers the entire outer surface. Underlying the cuticle is a continuous layer of epithelial cells. At irregular intervals pillar cells join the lamellae. The distal part of the lamella is expanded. The epithelial cells of the lamellae are continued as the lining of the gill stem and large connective tissue cells compose the chief support of the gill stem (Plate 3E). In lower concentration, the changes were perceptible enlargement of intralamellar space densely packed with granular material, and loss of gill structure. The gill lamellae get collapsed in exposed crab gill due to the disruption of the pillar cells. In the case of higher concentration after 7 days of exposure the following changes were seen: haemocoel with coarse amorphous to

fibrous materials, thickened gill lamellae, and massive haemocytic infiltration. Detached cuticle (DC) and rupture of capillaries (RC) at tip of the secondary lamellae releasing haemocytes are evident in later stages. In low concentration after 28 days of exposure the cytoplasm of phagocytes were found to be free from any engulfed material, and gills developed bulbular swelling at the tip. Epithelial necrosis and hyperplasia were also observed in later stages. Enlargement of secondary gill lamellae (ESGL) and disarrangement of secondary gill lamellae (DSGL) are seen in the exposed crabs at higher concentrations after 28 days of exposure. Edema and rupture of epithelial cells (EREC) and pyknotic nuclei (PN) are distinctly seen in experimental gills. These pathologies with the absence of the pillar cells collapse the entire lamellae. In some regions infiltration of haemocytes (IH) are also noted and this resulted in the swelling of secondary lamellae (SSL). In higher concentration, the gills exhibited lamellar fusion in some regions because of filamentary epithelium proliferation (Plate 3F).

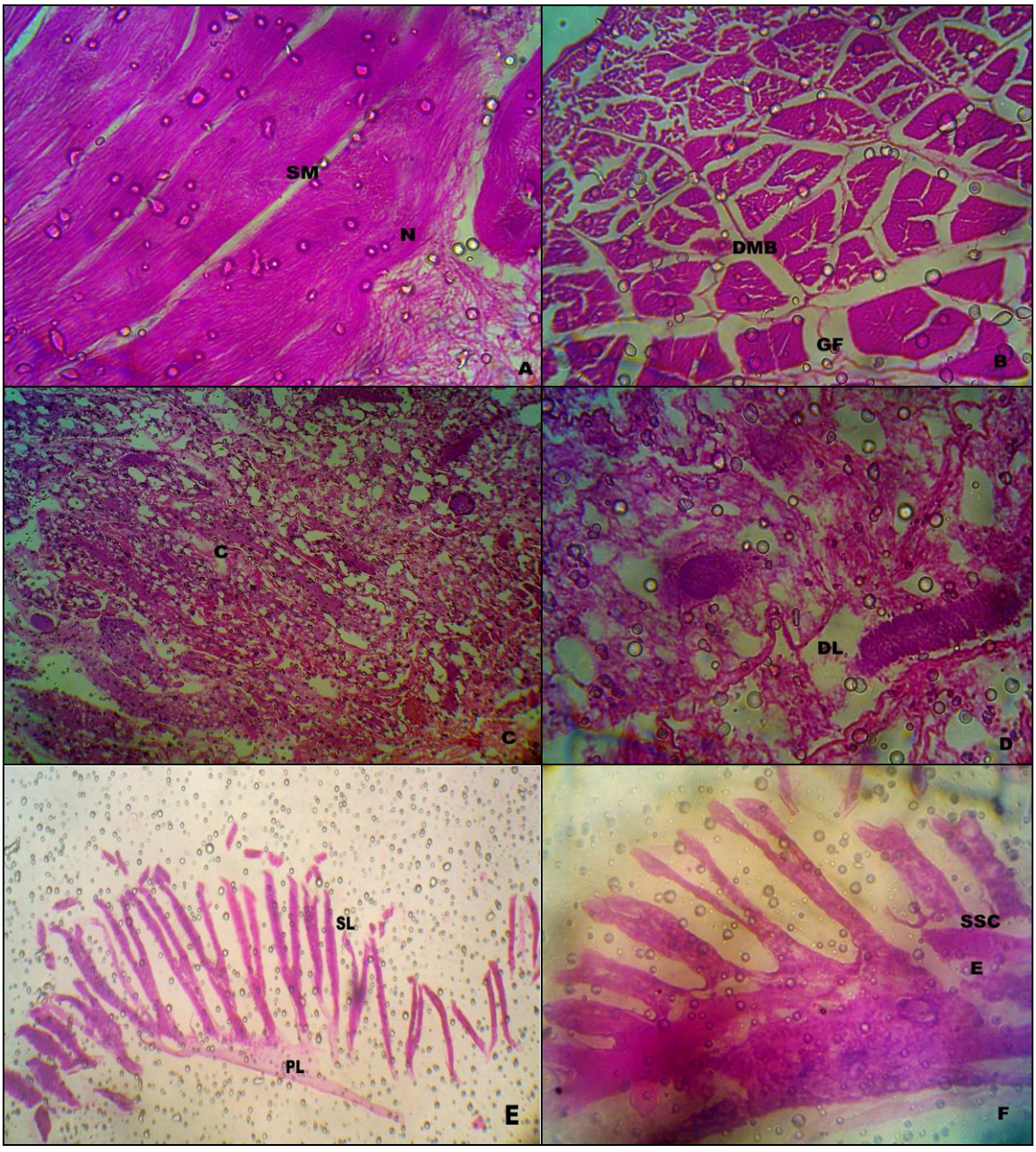


Plate 1: Histological changes of various tissues in *L. vannamei*. A & B: Muscle*:* SM - Striated muscle, N - Nuclei, DMB - Distruption of muscle bundle, GP- Gap formation C&D: Hepatopancreas: L - Lumen, DL -Distended lumen, E &F: Gills: PL - Primary lamellae, SL - Secondary lamellae, E- Edema, SSL- Swelling of secondary lamellae.

Discussion

Assessment of biochemical components helps in estimating the nutritive value of an organism. It has become imperative to study how nutritive value changes with changes in the biochemical components of crustaceans, which are exposed to increasing environmental pollution. The nutritional value of different species of fish and shellfish depend on their biochemical components such as protein, carbohydrate and lipids. These proximate components could serve as sensitive indicators for detecting potential adverse effects, particularly the early events of pollutant damage because their alterations appear before the clinical symptoms

produced by the toxicant [12]. It is therefore important that potential effects of acute and chronic concentrations of pollutant on proximate composition are determined and interpreted to delineate mechanisms of pollutant action and possibly ways to mitigate adverse effects [13]. Histopathological, biochemical, and physiological changes in different species of crustaceans after exposure to endosulfan have been widely reported [14].

Biochemical changes induced by pesticidal stress is due to disturbed metabolism manifested by inhibition of enzymes, retardation of growth and reduction in the fecundity and longevity of the organism. Most of the pesticides act as metabolic depressors and generally affect the activity of biologically active molecules such as proteins, carbohydrates and lipids. The exposure of aquatic organisms to even very low levels of pesticides causing alterations in the nutritional value of finfish and shellfish as well as their biochemical constituents, physiological and histological functions has been widely documented [4].

Depletion of the total protein content in the tissues of may constitute a physiological mechanism under pesticidal stress, to provide intermediates to the Kreb's cycle or to enhance osmalality, by retaining free amino acid content in the haemolymph, to compensate osmoregulatory problems encountered due to the leakage of ions and other essential molecule, during the pesticide stress. Carbohydrate metabolism is broadly divided into the anaerobic segment or glycolysis in which the breakdown of glucose or glycogen through Embden–Meyerhaf pathway occurs and the aerobic segment that consists of oxidation of pyruvate to acetyl co-A to be utilized through citric acid cycle [15]. The lipids provide energy for almost all enderogonic processes and are of atmost importance in maintaining the structural and physiological integrity of cellular and subcellular structure. Lipids are important energy resources in crustaceans and are required during reproductive cycles. The total lipid concentrations in different tissues chlorpyrifos treated shrimps in the present study were found to be significantly lower than the concentrations in the same organs of controls (P<0.05). Similar observations have been made in the freshwater prawn, *M. kistensis* on exposure to pesticides [16]. The concentrations of the total lipid decreased in all the tissues significantly with the progress of exposure period irrespective of exposure concentrations. The hepatopancreas of crustaceans is analogous to the liver of vertebrates and is the centre of lipid metabolism [17]; higher levels of the lipid could be expected in the hepatopancreas compared to other tissues.

The histopathological changes were more evident in specimens exposed to chlorpyrifos and were not observed in the control shrimp. As muscle tissue is the primary site of exposure, pollutants affected the muscle epidermis abruptly. Pigmented cells are prominent feature of chronic inflammatory response. The present investigation closely agreed with a similar report by Tehrani *et al.* [18] in the muscle tissues of *Artemia urmaiana* in response to carbamates pesticide resulting in degeneration, Zenkers necrosis of muscle fiber with haemorrhages and RBC like cells. Gangshettiwar[19] showed thickening and rupturing of testicular tubules, deformation of tubules affecting proliferation zone, reduced spermatogenic mass, vacuolization and degeneration of tissue of prawn, *M. lamerri* after exposure to phenol. Jaiswal *et al.* [20] observed the effect of naphthalene on the testis of *M. kistnensis* and noticed changes like degeneration, necrosis and rupture of testicular wall, reduction in spermatogenic mass and damage to tissue. These observations coincide with the results obtained in the present investigation on *L.vannamei.*

Hepatopancreas is not only a digestive organ possesses abilities of absorption, digestion, storage and secretion but also a major site where biotransformations and detoxification undergo crustaceans. In the present study, the hepatopancreas showed changes in the F and B cells in low concentration of chlorpyrifos, and cells were found clumped, and intercellular spaces invisible in the medium concentration, and a general deterioration, loss of tubules structures, vacuolation, star shape of lumen and necrosis of cells in the high concentrations of chlorpyrifos exposed to *L. vannamei.* Krishnamoorthy and Subramanian [21] also reported changes such as elongation of hepatopancreatic cells, and shrunken cells in *M. lamarrg* exposed to low (0.0065ppm), and high (0.02l5ppm) concentrations of copper. Destructive and deteriorative changes in the hepatopancreas and gills were observed in *Penaeus indicus* exposed to Zn at a low concentration of 100 ppb [22]. The noted histopathological changes in the hepatopancreas may be due to accumulation of the pesticide since this organ is the centre of storage, metabolism and detoxification.

After exposure an excessive amount of mucus was observed over the gills of live specimens. It has been reported that the stress caused by the variations in the environment and pathologic agents induced the proliferation of mucus cells and increased secretion. The lifting of the epithelium, oedema, epithelial necrosis, fusion of adjacent secondary lamellae and haemorrhage at primary lamellae were observed in the gills of the crab examined after 30 days of exposure to 0.5µg/l. Epithelial necrosis and rupture of gill epithelium are direct deleterious effect of the irritants. The animal's defense responses are excessive mucus secretion. Lifting of the epithelium, lamellar fusion and club shaped lamellae could be protective in that it diminishes the amount of vulnerable gill surface area [23]. Our results propose that the lethal effect of chlorpyrifos is a result of damage to gas exchange mechanisms as consequence of the gill pathologies observed. The present histopathological study on various tissues showed progressive damage and degeneration and it is clearly evident with the progress of exposure period i.e. extent of tissue damage increases with the increase of chlorpyrifos exposure of *L.vannamei.*

Acknowledgements

Authors would like to acknowledge their gratitude to Science and Engineering Research Board, Department of Science and Technology, New Delhi, India (SB/YS/LS/254/2013) for the financial assistance and Head of the Institution, Khadir Mohideen College, Adirampattinam for the facilities provided.

References

1. Johnson DJ, Alexander CG, Yellowlees D. Epithelial cytology and function in the digestive gland of *Thenus orientalis* (Decapoda, Scyllaridae). J.Crust. Biol,1998:18(12):271-278.
2. Reddy PS, Bhagyalakshmi A, Ramamurthi R.Effect of sumithion on ovarian growth of freshwater rice field crab, *Oziotelphusa senex* (Fab). Toxicol. Lett,1983:18:273-276.
3. Victor B. Reproductive biology of freshwater prawn,

Caridina rajadhari (Ph.D. thesis). Marathwada University, Aurangabad, 1984.
4. Saravana Bhavan P, Geraldine P. Manifestation of carbaryl toxicity on soluble protein and histopathology in the hepatopancreas and gills of the prawn, *Macrobrachium malcolmsonii.* J. Environ. Biol, 2009:30(4):533-538.
5. Maharajan A, Neelakandamoorthy N, Kumarasamy P. Impact of Profenofos on oxygen consumption and gill histopathology of the Fresh water crab, *Paratelphusa jacquemontii* (Rathbun). Res. Rev.: J. Toxicol,2012:2(2):46-55.
6. Chourpagar AR, Kulkarani GK. Effect of mercuric chloride on gill structure of a freshwater female crab, *Barytelphusa cunicularis* (Westwood). J. Glob. Biosci,2014:3(2):423-427.
7. Oliveira Ribeiro CA, Vollaire Y, Sanchez-Chardi A, Roche H. Bioaccumulation and the effects of organochlorine pesticides, PAH and heavy metals in the eel (*Anguilla anguilla*) at the Camargue Nature Reserve, France. Aquat. Toxicol, 2005:74:53-69.
8. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with folin phenol reagent. J. Biol. Chem, 1951:193:265-275.
9. Roe JH. The determination of sugar in blood and spinal fluid with anthrone reagent. J. Biol. Chem, 1955:212:335-343.
10. Folch J, Lees M, Sloare- Stavely GH. A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem, 1957:226:497-509.
11. Linford E. Biochemical studies on marine zooplankton variations in the lipid content of some Mysidacea. J. Cons. perm. int. Explor. Mer,1965:30:16-27.
12. Almeida JA, Diniz YS, Marques SFG, Faine IA, Ribas BO, Burneiko RC *et al*. The use of the oxidative stress responses as biomarkers in Nile tilapia (*Oreochromis niloticus*) exposed to *in vivo* cadmium contamination. Environ. Int, 2002:27:673-679.
13. Matos P, Fontaınhas-Fernandes A, Peixoto F, Carrola J, Rocha E. Biochemical and histological hepatic changes in Nile tilapia, *Oreochromis niloticus* exposed to carbaryl. Pes. Biochem. Physiol, 2007:89:73-80.
14. Omkar VB, Upadhyay R, Shukla GS. Endosulfan induced changes in the carbohydrate metabolism of a freshwater prawn, *Macrobrachium lamarrei*. Curr. Sci, 1984:53:280-281.
15. Nelson D, Cox M WH Lehninger Principles of Biochemistry Freeman and Company, New York, 2005, 1216.
16. Jaiswal K, Nagabhushanam R, Sarojini R. Influence of naphthalene on tissues of *Macrobrachium kistnensis*. Geobios, 1989:16(5):197-202.
17. Chang ES, O'Connor JD. Metabolism and transport of carbohydrates and lipids. In: Mantel, L.H (Ed.), The Biology of Crustacea, Vol. 5. Internal anatomy and physiological regulation. Academic Press, New York, 1983:263-287.
18. Tehrani AAG, Sadeghi Z, Badamchi NH, Sanjou Mansoub A, Azhari. Effect of Carbamates pesticides on Instar I-II larvae and Adult *Artemia urumiana.* Anna. of Bio. Rese ,2011:2(3):515-525.
19. Gangshettiwar VB. Effect of phenol poisoning on the physiology of the prawn, *Macrobrachium lamerrii*. Ph.D. Thesis, Dr. Babasaheb Ambedkar Marathwada University, Aurangabad. India, 1986.
20. Jaiswal K, Nagabhushanam R, Sarojini R. Influence of naphthalene on tissues of *Macrobrachium kistnensis*. Geobios, 1989:16(5):197-202.
21. Krishnamoorthy P, Subramanian P. Effect of sub lethal doses of copper on the hepatopancreas of the freshwater prawn, *Macrobrachium lamarrei lamarrei*. Geobios, l996:23(1).16-18.
22. Viswanathan S, Manisseri MK. Histopathological studies on Zinc toxicity in *Penaeus indicus* H. Milne Edwards. Mariculture Research under the postgraduate programme in Mariculture Part-6. Rengarajan, K.eds. Cochin India, l995:61:25-29.
23. Richmonds C, Dutta HM. Histopathological changes induced by malathion in the gills of bluegill *Lepomis macrochirus.* Bull Envir. Con. Toxicol, 1989:43:123-130.