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

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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.

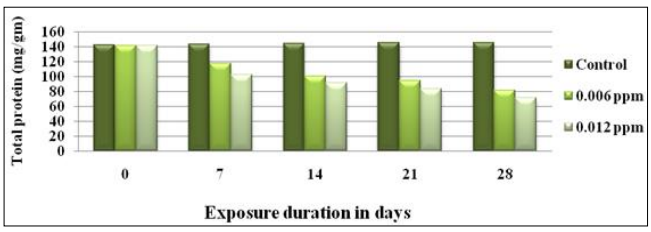

Fig 1: Changes of total protein (mg/gm) in muscle of *L.vannamei* exposed to sublethal concentration of chlorpyrifos

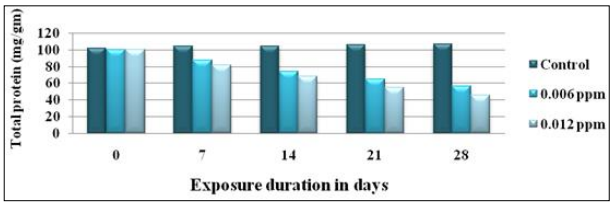

Fig 2: Changes of total protein (mg/gm) in muscle of *L.vannamei* exposed to sublethal concentration of chlorpyrifos

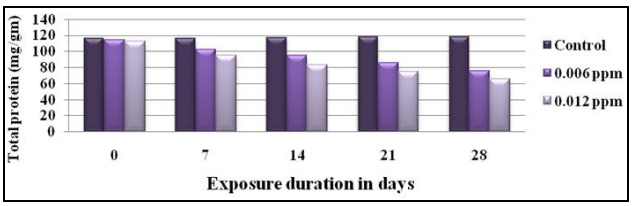

Fig 3: Changes in total protein (mg/gm) in hepatopancteas of *L. vannamei* exposed to sublethal concentration of chlorpyrifos

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).

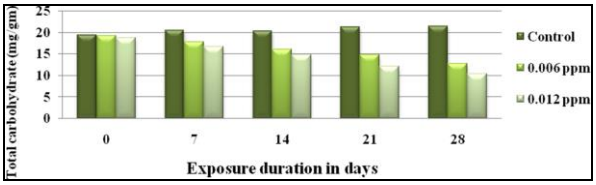

Fig 4: Changes of total carbohydrate (mg/gm) in muscle of *L. vannamei* exposed to sublethal concentration of chlorpyrifos

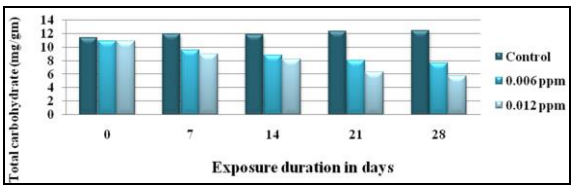

Fig 5: Changes of total carbohydrate (mg/gm) in gill of *L.vannamei* exposed to sublethal concentration of chlorpyrifos

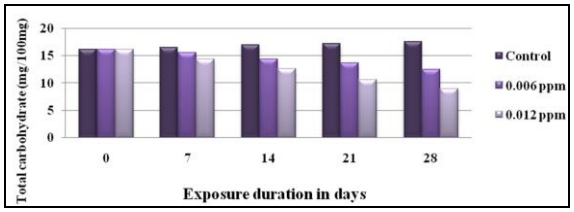

Fig 6: Changes of total carbohydrate (mg/gm) in hepatopancreas of *L. vannamei* exposed to sublethal concentration of chlorpyrifos

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.

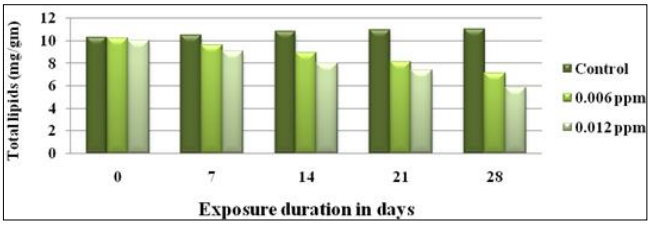

Fig 7: Changes of total lipids (mg/gm) in muscle of *L.vannamei* exposed to sublethal concentration of chlorpyrifos

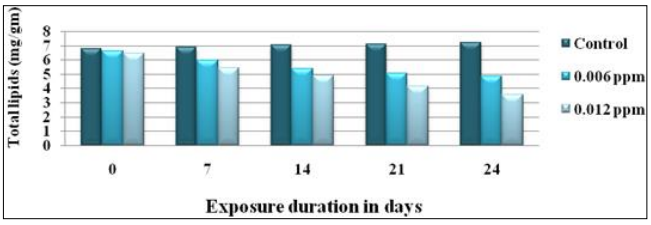

Fig 8: Changes of total lipids (mg/gm) in gill of *L. vannamei* exposed to sublethal concentration of chlorpyrifos

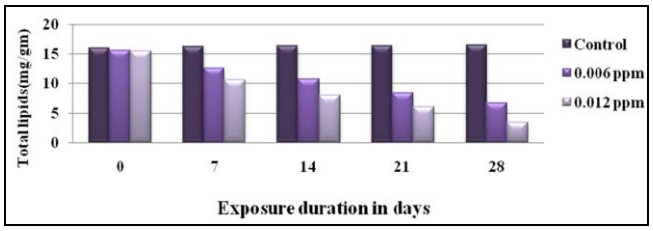

Fig 9: Changes of total lipids (mg/gm) in hepatopancreas of *L. vannamei* exposed to sublethal concentration of chlorpyrifos

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).

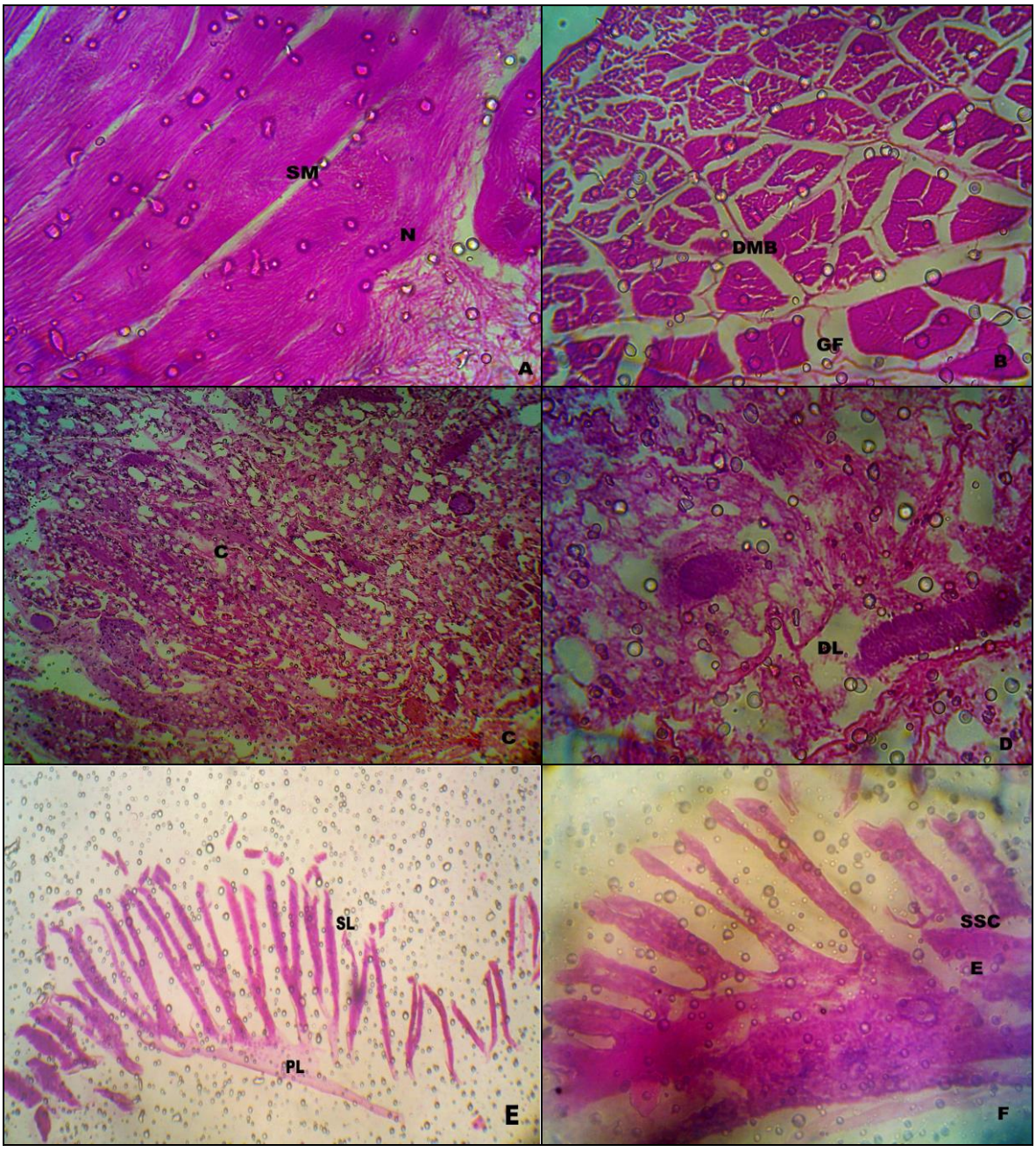

Plate 1: Histological changes of various tissues in *L. vannamei*. A & B: Muscle*:* SM - Striated muscle, N - Nuclei, DMB - Distruption of muscle bundle, GP- Gap formation C&D: Hepatopancreas: L - Lumen, DL -Distended lumen, E &F: Gills: PL - Primary lamellae, SL - Secondary lamellae, E- Edema, SSL- 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

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