Pesticide induced genotoxicity and oxidative stress related studies in whiteleg shrimp, *Litopenaeus vannamei* (Boone, 1931)

A Thesis submitted to the

Goa University for the award of the Degree of

DOCTOR OF PHILOSOPHY

in

ZOOLOGY

By

Ms. ASHWINI P. PAWAR

(M. Sc.)

Goa University,

Taleigao Plateau, Goa

(2020)

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Under the Guidance

of

Dr. S. K. SHYAMA

Professor (Retd.)

Department of Zoology,

Goa University, Goa-403206, India

(2020)

STATEMENT

As required under the University Ordinance OA 19, I state that the present thesis entitled **"Pesticide induced genotoxicity and oxidative stress related studies in whiteleg shrimp, *Litopenaeus vannamei* (Boone, 1931)"** is my original contribution and the same has not been submitted on any previous occasion. To the best of my knowledge, the present study is the first comprehensive work of its kind from the area mentioned. The literature related to the problems investigated has been appropriately cited. Due acknowledgements have been made wherever facilities and suggestions have been availed of.

Ashwini P. Pawar

Date: 3rd February 2020

Place: Taleigao Plateau, Goa

CERTIFICATE

This is to certify that the thesis entitled "**Pesticide induced genotoxicity and oxidative stress related studies in whiteleg shrimp, *Litopenaeus vannamei* (Boone, 1931)**" submitted by Ms. Ashwini P. Pawar for the award of the degree of Doctor of Philosophy in Department of Zoology is based on original studies carried out by her under my supervision. The thesis or any part, therefore, has not been previously submitted for any degree or diploma in any universities or institutions.

Dr. S. K. Shyama
Research Guide
Professor (Retd.)
Goa University, Goa-403206

Date: 3rd February 2020
Place: Taleigao Plateau, Goa

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DEDICATED TO MY BELOVED

"FATHER AND MOTHER"

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Chapter 1

General Introduction with Review of Literature

1.1 Background information

As the global demand for seafood has increased due to population outburst, the advanced technologies made it possible to grow food in adjacent coastal marine waters and the open ocean. Aquaculture is a method used to produce food and other commercial products, restore habitat, replenish wild stocks and rebuild populations (NOAA, 2017). According to a report published by FAO (2018), about 567 aquatic species are currently farmed all over the world, representing a wealth of genetic diversity both within and among species. The global aquaculture production (including aquatic plants) in 2016 was estimated to be 110.2 million tonnes that includes farmed fish production of 54.1 million tonnes of finfish (USD 138.5 billion), 17.1 million tonnes molluscs (USD 29.2 billion), 7.9 million tonnes of crustaceans (USD 57.1 billion) and 938500 tonnes of other aquatic animals (USD 6.8 billion) such as turtles, sea cucumbers, sea urchins, frogs and edible jellyfish.

Shrimps being a popular food and profitable business for financial organizations, industries and governments are a path for developing countries to reach their targets and alleviate poverty. Thus, shrimp culture gained higher popularity among all kinds of aquaculture practices. The main leading countries in shrimp farming are Thailand, China, Indonesia, India, Vietnam, Philippines, Bangladesh, Mexico and Brazil. The area under shrimp production by brackish water aquaculture is 1,21,600 hectares which is high up compared to previous years and counts to 25% of total shrimp culture. Wild production of penaeid and non-penaeid shrimps were around 5,60,000 metric tonnes in the financial year of 2017-18 (Balakrishnan et al., 2011). The major marine wild species of shrimps are tiger shrimp (*Penaeus monodon*), white shrimp (*Fenneropenaeus indicus*), pink shrimp (*Metapenaeus dobsoni*), king prawn (*Metapenaeus affinis*) and marine shrimp (*Parapenaeopsis stylifera*) (Balakrishnan et al., 2011). However, native species such as *P. monodon and F. indicus* as well as an introduced species, *Litopenaeus vannamei* are the most favoured ones for farming in India. Cultured shrimps are favoured for growth over wild shrimps. Andhra Pradesh is the largest shrimp producer state in India followed by West Bengal, Tamil Nadu, Pondicherry, Gujarat and Odisha. The tiger shrimp (*P. monodon*) has been farmed in the states of West Bengal, Odisha and Kerala while the *L. vannamei* is preferred in the states of Andhra Pradesh, Gujarat, Tamil Nadu and Maharashtra.

India being a tropical country is blessed with highly diverse nature of marine fishery resources in its 2.02 million km^2 exclusive economic zone with an estimated annual harvestable potential of 4.414 million metric tonnes (FAO, 2018). The Coastal Aquaculture Authority of

India, an agency involved in the promotion of sustainable shrimp aquaculture has introduced whiteleg shrimp, *L. vannamei* in India (Balakrishnan et al., 2011) as the best alternative to *P. monodon*. Due to the introduction of *L. vannamei*, majority of shrimp farming industry shifted to the culture of *L. vannamei* and displaced the other shrimp species, especially tiger shrimp *P. monodon* which was considered of being more prone to white spot syndrome virus (WSSV). The preference of *L. vannamei* has increased over other shrimp species due to its superiority such as fast growth rate, higher stocking density, low feed requirement, disease resistance and higher survival rate (Bett and Vinatea, 2009). The compound annual growth rate for shrimp production has grown at a rate of 14 % from the financial year the 2014-15 to 2017-18. India's cultured shrimp production in 2017-18 was about 423,600 metric tonnes with *L. vannamei* representing 80 % of total shrimp production of India (MPEDA, 2019). These facts prompted us to consider this commercially important species for the present study.

1.2 Test animal

Uptake of contaminants by an organism in an aquatic ecosystem can take place from the surrounding water, sediments and also from another biota. This may lead to the accumulation of compound inside the organism's body and its quantum may vary depending upon various factors such as the rate of uptake, metabolism of the compound and its elimination rate (Livingstone, 1992). Further, it may also depend on other factors such as the bioavailability, lipophilicity, concentration of the chemical and the type of animal species (Valvanidis et al., 2006).

Several species of earthworms, plants and mammals are also used to evaluate the genotoxic potentials of xenobiotics in diverse terrestrial ecosystems. However, not any species are employed for toxicology studies of aquatic ecosystems. Amphibians, being the most sensitive organisms to the environmental changes due to their permeable skin and having two water-dependent life stages are often used as favourable species for toxicity study. Among the vertebrates, fishes are used more frequently to carry out the aquatic toxicology studies. Among invertebrates, mysid shrimp, *Americamysis bahia* was often used in recent years, as a test organism to biomonitor the estuarine and marine water (Cripe, 1994). However, Clark et al. (1986) reported that penaeids are more sensitive to toxic materials than mysids. Further, they also have found that penaeids are 36 times more sensitive to organochlorines and 5 times more sensitive to organophosphates than mysids (Clark et al., 1986). However, the toxic effect of xenobiotics on any moulting faunal species is reported to vary according to the age and stage

of moulting of that organism (Cripe, 1994). Hence, more studies are needed to understand the nature and the quantum of the toxicity of organophosphate pesticides on penaeid shrimps. Due to the commercial importance of *L. vannamei* amongst the penaeid shrimps it is selected as the test animal for the present study.

Water quality being one of the key regulatory factors for shrimp culture, variation in the salinity of the surrounding water not only affects the growth and survival rate of shrimps, but also affects the accuracy of the experimental result (Chim et al., 2008). Scientists have reported *L. vannamei* as a euryhaline species. According to Bray et al. (1994) its optimum growth occurs in 5–15 ppt salinity, whereas, Huang (1983) reported it as 20 ppt. Further, Zhu et al. (2004) reported that the optimum growth rate of *L. vannamei* occurs at higher salinities of 50 ppt and more. These reports showed the greater possibility of its selection as an ideal species for commercial shrimp production.

Protein requirement of a species has been defined by Guillaume (1997) as the amount of protein needed (minimum to maximum) for its optimum growth per animal per day. Protein requirements change with respect to changes in biotic factors (e.g., species, physiological state, size) and dietary characteristics (e.g., protein quality, energy: protein ratio). Further, abiotic factors such as temperature and salinity may also affect the protein requirement of a species (Guillaume, 1997).

Hence, in order to know the optimum salinity and temperature which may induce a higher resistance to pesticides and consequently a better growth and survival rate in shrimps, above commercial euryhaline shrimp, *L. vannamei* was selected as an ideal experimental animal for the present investigation.

1.3 Biology and life cycle of *L. vannamei*

Kingdom: Animalia
Phylum: Arthropoda
Subphylum: Crustacea
Class: Malacostraca
Order: Decapoda
Family: Penaeidae
Genus: *Litopenaeus*
Species: *vannamei*

Plate 1.1: Image of *L. vannamei* shrimp

L. vannamei is native to the Eastern Pacific coast. It lives in tropical marine waters. Adults live and spawn in the open ocean while post larvae migrate inshore to spend their juvenile to sub-adult stages in brackish water areas. *L. vannamei* grows to a maximum length of 230 mm. The rostrum contains 7–10 teeth on the dorsal side and two to four teeth on the ventral side.

At the age of 6-7 months, males weighing 20 g and females of 28 g onwards get matured. Matured female *L. vannamei* weighing around 30-45 g, spawn and release ~1 to 2.5 lakhs eggs and adult males subsequently fertilise these eggs. Hatching occurs at about 16 h after spawning and fertilization. Their embryonic developmental stages include nauplii (six stages), zoea (three stages) and mysis (three stages). They feed mainly on phytoplanktons and zooplanktons (FAO, 2006).

Life cycle of *L. vannamei*

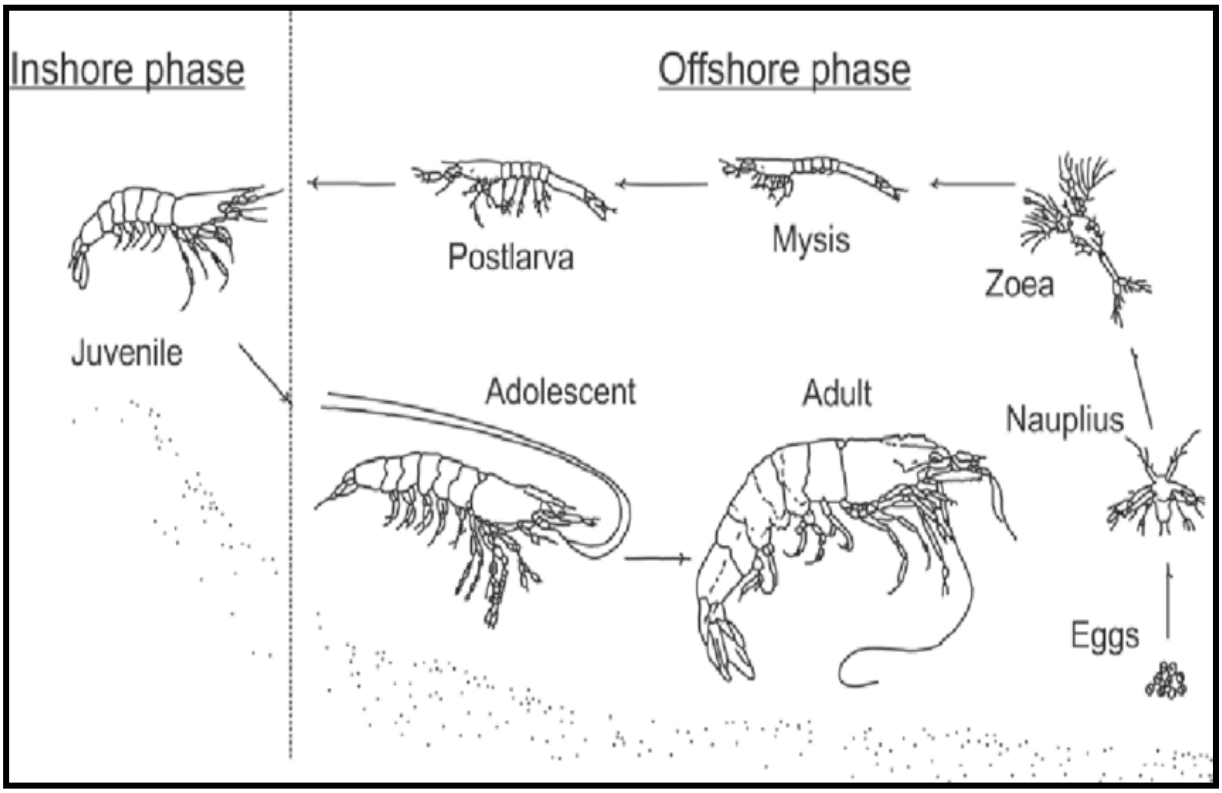


Fig. 1.1 Life cycle of *L. vannamei* (Martin et al., 2012)

1.3.1 Selection of two life stages for the present study

Larvae (10-11 days): The commercial production of shrimp larvae is done in a hatchery. After hatching, penaeid shrimp go through nauplii (six stages), zoea (three stages) and mysis (three stages). The first larval stage is known as nauplius. The unsegmented body which is pyriform in shape possesses three pairs of appendages. The nauplius undergoes six moultings within 50 hours into a protozoea (Fig.1.2). The body becomes elongated with a distinct cephalothorax. The early zoea stage has a pair of protruded compound eye, the next stage is characterized by the presence of a rostrum and the late zoea stage has a pair of uropods (Fig. 1.3). After 4–6 days, the zoea finally metamorphoses into a mysis (Fig. 1.4 a, b and c). At this stage, the larvae assume the form of a juvenile shrimp at which the pleopods are to develop. At this stage, tiny protrusion known as pleobases are seen on the ventral side of the abdominal segments. The next stage is marked by the development of first segment of the pleopods development. The mysis remain drifting in the water column until they metamorphose into post larvae within 10–12 days (FAO, 2018). During these stages the shrimps go through physical and ontogenic changes (mainly on digestive physiology level) and have specific nutritional requirements (Samocha, 2019).

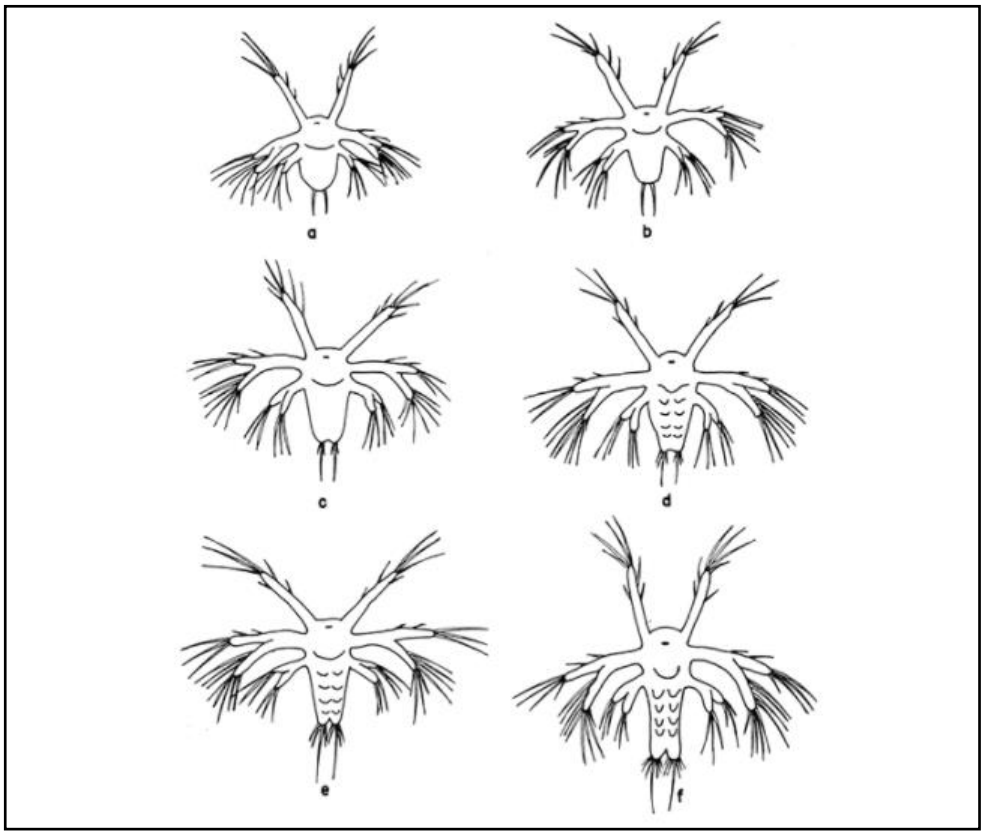


Fig. 1.2 Nauplius Stages of *L. vannamei* (FAO, 2018)

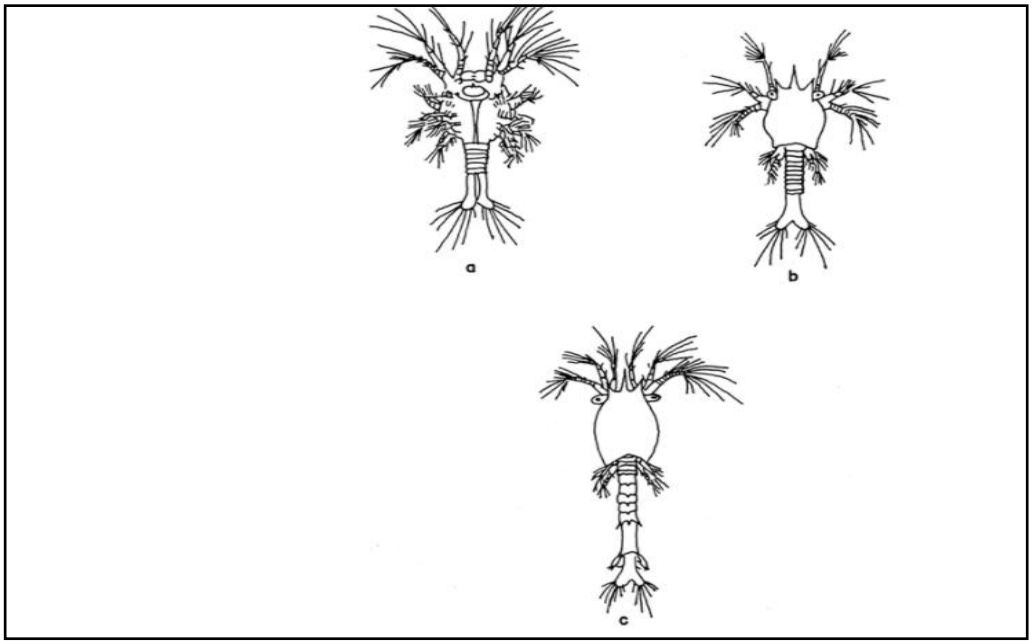


Fig. 1.3 Zoea Stages of *L. vannamei* (FAO, 2018)

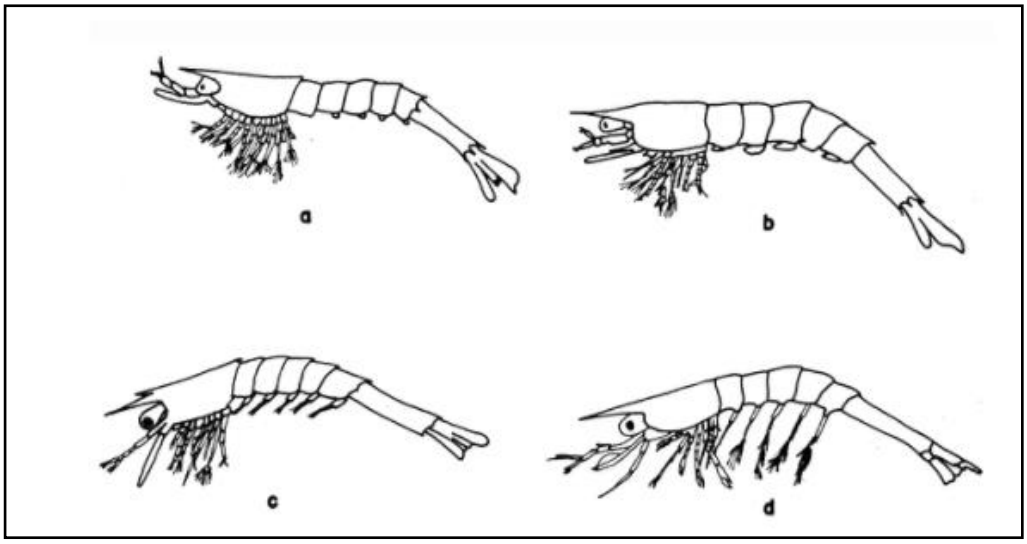


Fig. 1.4 a, b and c (Mysis stages) and d (Post larval stage) of *L. vannamei* (FAO, 2018))

Post Larvae: The stocking of post larvae for prawn culture is usually done in nursery ponds or directly in grow-out ponds. Feeding as well as quality and handling of post larvae are critical for successful grow out (Samocha, 2019). At post larval stages (Fig. 1.4 d), the pleopods become fully developed and functional. The animals grow very fast in terms of size and are able to swim freely although early post larvae are still planktonic in offshore waters. At a body size of 0.8–1 cm in body length, they enter into estuaries and inshore waters where they first adopt to a benthic existence. Shrimps spend their juvenile, adolescent and sub-adult stages in

estuarine waters and then gradually move towards deeper water as they grow and eventually return to offshore water when they attain sexual maturity (FAO, 2018).

The post larvae are reared at hatcheries or culture ponds for stocking purpose till the cropping. Once they attain the post larval stage they will be named as PL1 (One day old), PL2, PL3 and so on, depending on the age in days of the post larva. We have selected PL14 which means it's a 14 day old post larva. This age of post larvae specifically selected because PL14-PL20 being stocked in the ponds for culture. At this stage of development, they get stressed out due to the transportation and also exposure to different stress environments in ponds during stocking. During further development, these post larvae undergo additional two consequential moultings and attain bigger sizes (Laramore et al., 2007). The process of moulting of post larvae makes them more sensitive and susceptible to any toxicant. Hence, post larval stage was selected in the present research to carry out various toxicological studies.

Plate 1.2 Image of *L. vannamei* post larvae

Juvenile and adult stages: Final moulting of post larvae yield juveniles which further moult and become adults. During these grow out phases, nutrition and feeding needs are very crucial and are to be tailor made according to the specificity of the rearing systems to optimise the growth rate and support the health of the animals. They are laterally compressed, elongate decapods, with a well-developed abdomen adapted for swimming. Each somite (segment) is enclosed by a dorsal tergum and ventral sternum. It is usual to call the side plates (and any extensions thereof) of each somite the pleura (Dall et al., 1990).

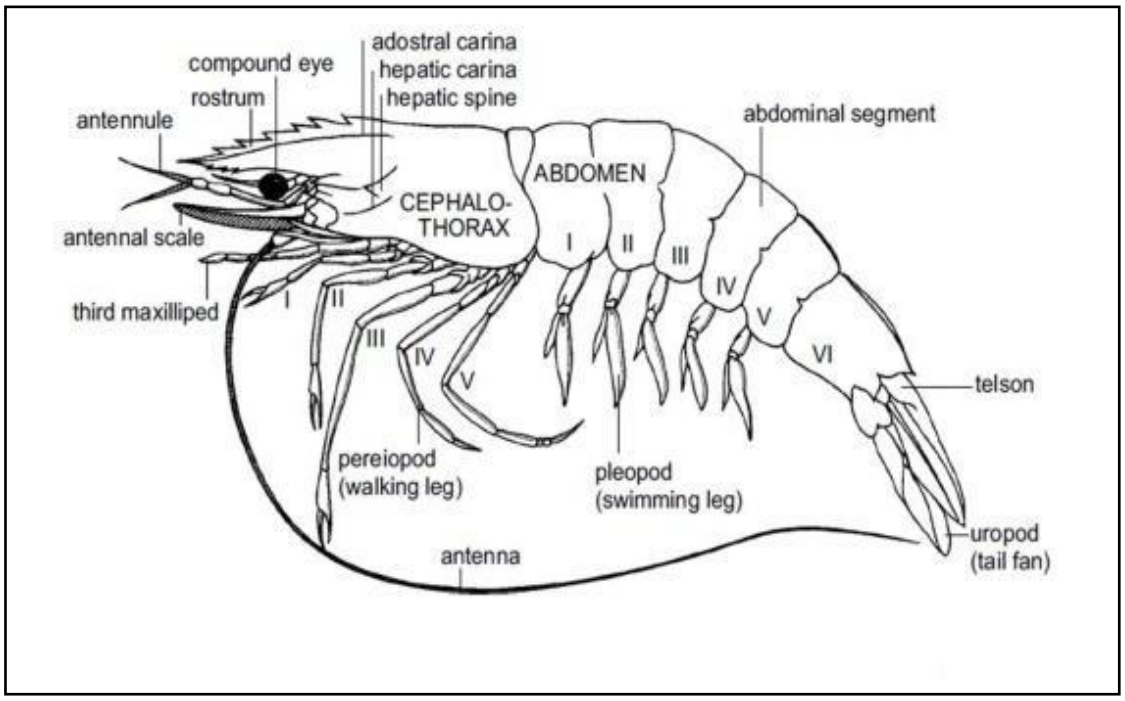


Fig. 1.5 Schematic diagram of Juvenile stage of *L. vannamei* (Sucharita and Jyoti, 2013)

In the members of Penaeidae, their head (five somites) and thorax (eight somites) are fused into a cephalothorax, which is completely covered by the carapace. The pleura of the cephalothorax forms the branchiostegite or gill cover. The carapace has characteristic ridges (carinae) and grooves (sulci). The rostrum is always prominent, with a high median blade bearing dorsal teeth and, in some genera, ventral teeth as well. The compound eyes are stalked and laterally mobile and the somites of the head bear, in order, pairs of antennules, antennae, mandibles, maxillules (maxillae 1) and maxillae (maxillae 2). The thorax has three pairs of maxillipeds and five pairs of pereiopods (legs), the first three being chelate and used for feeding, and last two simple (non-chelate) and used for walking. The abdomen consists of six somites, the first five with paired pleopods and the last one with the pair of uropods and a telson. The mouth is situated ventrally and the cephalic appendages surrounding it, plus the first and second maxillipeds and sometimes the third as well, may be referred to collectively as the mouth parts. The anus is on the ventral surface of the telson, towards its base (Dall et al., 1990).

In addition, constant attention needs to be given to the rearing system itself (Samocha, 2019). Similar to the post larvae, juveniles too undergo repeated moultings during development (Laramore et al., 2007). These moultings make them easy prone for the toxicants due to their

soft shell. Hence, the juvenile stage being another sensitive stage is also selected for the present study.

Plate 1.3 Image of Juvenile of *L. vannamei*

1.4 Pesticide

Pesticide is a chemical or biological substance that is intended to prevent or repel or destroy the pests that may damage or disturb the growth or health of living organisms which may be plants or animals causing harm during or otherwise interfering with the production, processing, storage, transport or marketing of food, agricultural commodities, wood and wood products or animal feed stuffs or substances that may be administered to animals for the control of insects, arachnids pests in or on their bodies (Pandey et al., 2003; Mhadhbi and Beiras, 2012; Mellish, 2013). These pests include insects, rodents, fungi, weeds, nematodes, algae, plant pathogens, molluscs, birds, mammals, fish, nematodes and microbes.

1.4.1 Types of pesticides

Pesticides are of different types and are classified into the following different categories as per National Pesticide Centre (NPIC, 2018):

1.4.1.1 Classification based on the types of pests they kill

- Insecticides – insects eg. DDT, Malathion, Aldicarb
- Herbicides – plants eg. Aminopyralid, Atrazine
- Rodenticides – rodents (rats & mice) eg. Bromadiolone, Chlorophacinone, Difethialone
- Bactericides – bacteria eg. Bronopol, Cresol, Dodicin
- Fungicides – fungi eg. Captan, Benomyl, Zineb
- Larvicides – larvae eg. Temephos, Methoprene

1.4.1.2 Based on their biodegradability

Pesticides can be

1.4.1.2.1 Biodegradable:

Capable of being broken down (decomposed) rapidly by the action of microorganisms and other living beings into less harmful/ harmless compounds.Eg. Neem, Mineral oil, salt spray, Citrus oil.

1.4.1.2.2 Persistent:

Persistent pesticides are those that do not breakdown/ which may take months or years to break down in the environment but remain toxic to fish, animals and humans for many years. They can bioaccumulate their toxic effects multiplying as they move up the food chain from plants to animals and finally to a human being. Eg. Aldrin, Chlordane, Endosulphan, DDT.

1.4.1.3 Based on their chemical nature:

Based on their chemical nature they are of the following types:

1.4.1.3.1 Organophosphates:

Also known as phosphate esters are a class of organophosphorus compounds with the general structure $O=P(OR)_3$. They can be considered as esters of phosphoric acid. Most organophosphates are insecticides and they affect the nervous system of the pests by disrupting the enzyme acetylcholinesterase (AchE), which is a neurotransmitter. It acts as irreversible

covalent inhibition. Hence, the potential of the poisoning varies in degree. Eg. Chlorpyrifos, Dimethoate, Monocrotophos, Diazinon.

1.4.1.3.2 Carbamates:

A carbamate is an organic compound derived from carbamic acid (NH_2COOH).Similar to the organophosphorus pesticides, the carbamate pesticides also reversibly affect the nervous system by disrupting an enzyme that regulates the neurotransmitter. Eg. Carbofuran, Oxamyl, Fenobucarb.

1.4.1.3.3 Organochlorines:

An organochlorine is a chlorinated hydrocarbon containing an organic compound with at least one covalently bonded atom of chlorine that changes the chemical behaviour of the molecule. Most of them widely used as insecticides to control the wide range of insects and they have a long-term residual effect in the environment. These insecticides may disrupt the nervous system of the insects leading to convulsions and paralysis followed by eventual death. However, many countries have removed organochlorine insecticides from their market due to their persistence and badly effects on health and environment. Eg. DDT, Chlordane, Endosulphan, Toxaphene.

1.4.1.3.4 Pyrethroids:

A pyrethroid is an organic compound similar to the natural pyrethrins produced by the flowers of pyrethrums (*Chrysanthemum cinerariaefolium* and *C. coccineum*). Pyrethroids constitute the majority of commercial household insecticides. They may also have insect repellent properties. Moreover, they are developed in such a way as to maximize their stability in the environment and are generally harmless to humans. Eg. Allethrin, Resmethrin, Permethrin.

1.5 Selection of pesticides for the present study

The pattern of agrochemical-application in India is not similar to that for the world in general. India is a tropical country and to control pests the usage of chemical compounds has more skewed towards the insecticides (Indian Pesticides Industry, 2011). About 76% of the

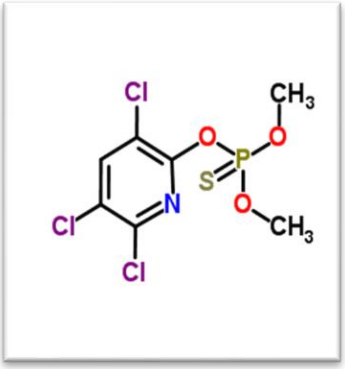
pesticides used are insecticides compared to 44% global usage (Mathur, 1999) while the herbicides and fungicides applications are correspondingly less. If we consider the crop-wise distribution, cotton accounts for the maximum share of pesticide consumption i.e around 37% followed by paddy field (20%). Together they account for 57% of the total pesticide consumptions. Moreover, the wheat and pulses contribute about 4%, vegetable 9% and other plantation crops 7% (Ministry of Agriculture, 2009). According to state-wise consumption, Andhra Pradesh is the highest pesticide consumer state (23%) followed by Punjab & Maharashtra (Bharedwaj and Sharma, 2013).

Among 135 registered pesticide products in India, 33 are banned due to their non-target toxicity and accumulation in the environment whereas, 35 are extensively available in the market (Rao et al., 2007). Hence, the production and usage of these permitted pesticides have increased abruptly in recent years. It has also been reported that 70% of these chemical formulations employed in agriculture are reaching the aquatic water bodies through agricultural runoff, leaching, spray drift, seepage etc., which are affecting non target organisms inhabiting adjacent aqua cultural ponds, rivers, estuaries and ocean (Selvakumar et al., 2005; Suryavanshi et al., 2009; Agrawal et al., 2010).

The major classes of pesticide which are getting used exclusively and likely to pose a serious environmental problem are organochlorine, organophosphate and carbamates (Dyk and Pletschke, 2011). The introduction of new generation pesticides i.e organophosphates (OP) on one hand benefiting to man replacing problematic organochlorine pesticides (OC), as non-persistent and degradable. But on the other hand, they are posing differential threats to the environment being highly toxic to aquatic organisms and cause long term ecological implications (Tu et al., 2012). The primary effect of OP is to inhibit the acetylcholinesterase enzyme (AChE) which regulate the functioning of nervous system (Jokanovic, 2009). Hence, causes hyperactivity, paralysis in organisms and finally leading to death (Key and Fulten, 1993). Last few decades have witnessed the effect of OP pesticides on biological and physiological parameters in fresh and marine aquatic organisms (Krishnapriya and Padmaja, 2014; Shoaib and Sidiqqui, 2015). Despite being low-persistent in nature and higher biodegradability in the aquatic environment, the major concern is inclined toward its effect on non-target wildlife populations (Frasco et al., 2006, Kumar et al., 2010, Kumar et al., 2017). Such entry into natural water bodies was observed to cause deleterious effects on non-target organisms (Chandrasekara and Pathiratne, 2007).

Shrimps inhabit naturally in the estuary during a noteworthy section of their life cycle. Hence, for the culturing of shrimps, people mostly avail water from these natural resources. Consequently, shrimps possibly get exposed to diverse pesticide pollutants through these water sources (Roque et al., 2005). Moreover, the extension of farming areas to inland areas often adjacent to the agriculture field (Roque et al., 2005) makes them highly prone to get exposed to pesticides. Exposure may lead to differential stress production in aquatic animals which may further leads to the several damages in organisms. Many studies have been reported on harmful effects of pesticide in various crustaceans but very less attention was given to the penaeid shrimp industry although it contributes to the economic growth of India. Many studies have reported the effect of pesticides in aquatic marine organisms which include, the chlorpyrifos and endosulfan effect on crab embryo, toxicity of nine pesticides (Carbofuran, Chlordane, Malathion, Methoxychlor, 2,4-D, DEF, Propanil, Trifluralin and Captan) on larval, juvenile and adult stages of dungeness crab (*Cancer magister*), acute toxicity of acephate, aldicarb, chloro-nicotinylimidacloprid, lambda-cyhalothrin and roundup in post larval and juvenile stages of blue crab (*Callinectes sapidus*) and the effect of methoprene on bioaccumulation and metabolism in lobster *Homarus americanaus* (EPA, 2005; Walker et al., 2005; Osterberg et al., 2012; Negro et al., 2015). Further, the effect of pesticide on freshwater prawn, protein and DNA of *L. stylerotris*, behavioural and biomarker response in penaeid shrimps have also been reported (Reyes et al., 2002; Tu et al., 2010; Mensah et al., 2014). According to the Environmental Protection Agency, it has been proved that the penaeid shrimps are very sensitive to the pesticides than fishes and molluscs. Moreover, their distribution throughout the world which includes the temperate, subtropical and tropical regions makes them the indicators of estuarine health (Couch, 1978). Based on the severity of the toxic effects of OP pesticides on penaeid shrimps, the following two pesticides have been selected for the present study.

1.5.1 Chlorpyrifos



Structural formula

Chlorpyrifos (CPF) with chemical name 0, 0-diethyl 0-(3, 5, 6-trichloro-2-pyridinyl-phosphorothioate).

It is a broad-spectrum chlorinated organophosphate insecticide, acaricide and nematicide. It is a colourless to white crystalline solid (EPA, 2005; Tomlin, 2006) with mild mercaptan odour. It smells like sulphur compounds found in rotten eggs, onions, garlic and skunks (Lewis, 1998; Tomlin, 2006).

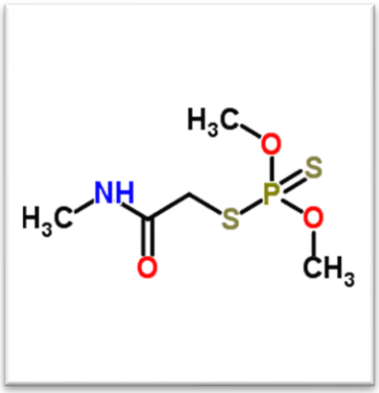
Table 1.1: The physicochemical properties of Chlorpyrifos

Molecular weight	350.6 $gmol^{-1}$
Solubility in water:	0.0020 gL^{-1} (2 mgL^{-1}) at 25°C
Vapor pressure	1.85 x 10^{-5} mmHg at 25°C
Partition Coefficient (Log $K_{OW})_2$	4.70

It affects mainly the nervous system of an organism by inhibiting acetylcholinesterase enzyme (AChE) which is neurotransmitter (Smegal, 2000).

Chlorpyrifos is the second largest selling OP agro chemical in India. Chlorpyrifos is the most toxic OP compound in aquatic animals compared to the OC compounds (Ramesh and Sarvanan, 2008). Few reports are available on the toxicity of CPF in tilapia fish *Oreochromis mossambicus* and fresh water shrimps *Paratya australiensis* and *Palaemonetes argentines* (Olima et al.*;* Rao et al., 2003; Montagna and Collins, 2007). However, no information is available on the toxicity of CPF on commercially important penaeid shrimps. Therefore, in the present investigation an attempt has been made to study the effect of Chlorpyrifos on acute toxicity, DNA damage, antioxidant enzyme activity and their gene regulation on *L. vannamei*.

1.5.2 Dimethoate



Structural formula

Dimethoate (DMT) ($C_5H_{12}NO_3PS_2$) with chemical name O, O-dimethyl S-methylcarbamoylmethyl phosphorodithioate.

It is also an organophosphate insecticide and acaricide. It is a grey-white crystalline solid at room temperature with a camphor-like (mercaptan) odour (Hudson et al., 1984).

Table 1.2 The physicochemical properties of Dimethoate

Molecular weight	229.26 $gmol^{-1}$
Solubility in water	2.5 g100 ml^{-1}
Vapor pressure:	8.5 x 10^{-6} mm Hg at 25°C
Partition Coefficient (Log $K_{OW})_2$	0.6990

Dimethoate is also an acetylcholinesterase enzyme inhibitor due to its Oxon metabolite *viz.* dimethoxon (Dogan et al., 2011). Dimethoate first introduced into the market in 1956 and described by the Hoegberg and Cassaday in 1951. It is absorbed by the organisms following ingestion, inhalation and cutaneous contact (Pandey et al., 2009). Dimethoate is an inhibitor of enzyme cholinesterase and causes accumulation of acetylcholine in nerve tissue (synapses of the central and peripheral nervous system) and effectors organs with the principal site of action being the peripheral nervous system (Cope, 2004). The accumulation of acetylcholine results in a prolonged stimulation of the cholinergic receptors downstream leading to intense activation of autonomic nervous system, which depending upon the severity of acetyl cholinesterase inhibition results in tremors, convulsion, respiratory arrest and death (Breckenridge and Stevens, 2008).

The exposure of fish to different concentrations of dimethoate shows altered behavioural responses, such as restlessness, hyperactivity, abrupt erratic and jerky swimming, decline in opercular movement, frequent surfacing and gulping, avoidance behaviour, increased mucus secretion, discolouration of skin, drooping of fins, loss of balance and finally the death (Pandey et. al., 2009). The restlessness and hyperactivity in fish and prawns are also reported on exposure to Dimethoate due to the inactivation of acetylcholinesterase (Dogan et al., 2011; Krishnapriya and Padmaja, 2014). Lack of reports on Dimethoate toxicity in penaeid shrimps prompted us to undertake this study to generate the baseline data on toxicity of Dimethoate and other associated parameters in one of the commercially important shrimp species.

1.6 Genotoxicity

The chemical and physical pollutants induced DNA alterations leads to single and double strand breaks, DNA-DNA cross-links and DNA-protein cross-links which is caused directly or indirectly by an interaction with oxygen radicals (Canaan and Pederson, 2016; Barnes et al., 2018). The chemical effects on DNA involve the release of free bases and destruction of deoxyribose residues. This further leads to interference with DNA integrity. Different methods have been established to study the DNA alteration. ^{32}P post-labelling assay used during initial periods but due to its complexity have not found suitable for a further period. The alkaline elution assay in which the DNA samples passes through the alkaline membrane filter which is directly related to the length of DNA strand itself (Kohn et al., 1976). This methodology was used to study the single and double strand breaks, DNA-DNA and DNA-protein cross linkage. It has been extensively applied for the fish and invertebrates on the exposure of chemical compounds (Bolognesi and Cirillo, 2014). The DNA alkaline unwinding assay has also been largely employed to aquatic organisms to detect the DNA strand breaks (Sarkar et al., 2008; Oliveira et al., 2010). In this methodology, the crude DNA extracts or whole cells are subjected to the alkaline assay for unwinding under controlled conditions with the usage of fluorescent dyes.

1.6.1 Comet assay

Recently comet assay (single cell gel electrophoresis) has been used more frequently to detect DNA damage at the individual cell level. It proved as one of the promising tools in environmental monitoring and ecotoxicology study due to the less labour work, less complexity, low time consuming and rapid results (Jha, 2008; Frenzilli and Lyons, 2013). The Comet assay

was launched by Singh et al. (1988) and after that it has itself proved one of the promising tools in the detection of DNA damage. In this technique, the cells are immobilized in gel and mounted on the glass slides which further lysed with a lysing solution and then finally subjected to the electrophores. During electrophoresis, the damaged DNA migrates away from the nucleus and length of migration indicates the number of DNA fragments (Fairbairn et al., 1995). It has been extensively used in genetic toxicology study *in vitro* and *in vivo*.

Many studies have reported on comet assay to study the DNA damage in a group of flora including fishes, mollusc and crustaceans. Based on their observation it has been proved that the comet assay is a fast and reliable biomarker tool to study DNA damage on exposure to environmental pollutant in short duration of time. The DNA damage by comet assay were reported in fishes *Therapon jarbua*, *Danio rerio*, *Cyprinus carpio* (Moradi et al., 2012; Nagarani et al., 2012; Bhuvaneshwari et al., 2013) as well as two bivalve species, *Macoma balthica* and *Mytilus edulis* (Barsienė et al., 2008). Moreover, few studies have also been reported on DNA damage in penaeid shrimp *P. monodon* (Jose et al., 2011; Salam, 2014) and *L. vannamei* (Frias-Espericueta, 2011).

Evaluation of pesticide exposure on DNA in post larvae and juveniles of *L. vannamei* would help us to understand the potential of pesticides to induce DNA damage in post larvae and different cells (gill, haemolymph and muscle) of juveniles. This study also reveals the sensitivity of both the stages towards the pesticide exposure. The DNA damage study could be used as biomonitoring tool to assess the genotoxic effect of pesticides in shrimp culture industry. Hence, comet assay was selected as a parameter to investigate the genotoxic potential of selected pesticides on the post larvae and juveniles of *L. vannamei* in the present study.

1.7 Oxidative stress and defence system

The oxidative stress arises in the body when there is an imbalance between the production of reactive oxygen species (ROS) and the ability of body to detoxify it. ROS includes superoxide radicals, hydroxyl radicals and hydrogen peroxide. These generated ROS interact with the cellular membrane and leads to the injury of cells. It mainly affects the protein, lipid, DNA and carbohydrates (Vuillaume et al., 1987; Heikal et al., 2012). It also causes the peroxidation of lipid bio-membrane (Ogutcu et al., 2006; Rai and Sharma, 2007; Mehta et al., 2009). This includes the process in which the free radicals attack the lipid to take away an electron from it which eventually leads to cell damage (Heikal et al., 2012). Thus, pesticides

are known to produce ROS in organisms which eventually leads to the damage of vital organs, reduce the reproduction ability and alteration in biochemical composition (Sharma et al., 2012).

1.7.1 Antioxidant enzymes

The antioxidant enzyme systems protect organisms from these harmful ROS to maintain the proper balance inside the cells (Schafer and Buettner, 2001; Singh et al., 2016). These enzymes include superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, etc. For their effective catalytic functioning, these enzymes require the co-factors such as manganese, selenium, zinc, copper and iron. If these minerals are provided in an insufficient amount it may leads to the dysfunction of these enzyme systems (Mandal, 2019).

Superoxide dismutase (SOD): It catalyses the breakdown of the superoxide anion into oxygen and hydrogen peroxide. It is present in almost all aerobic cells and in extracellular fluids and needs co-factors copper, zinc, manganese or iron for catalytic activity. The Mn-SOD is present in mitochondria while Cu-SoD, Zn-SOD are present in cytosol of the cell.

Catalase (CAT): Convert hydrogen peroxide into water and oxygen. It depends on the co-factor iron or manganese.

Glutathione enzymes: Includes glutathione reductase, glutathione peroxidases (GPx) and glutathione S-transferases (GST). Among them glutathione peroxidases is an enzyme containing four selenium-cofactors that catalyses the breakdown of hydrogen peroxide and organic hydroperoxides (Ajitha and Jayaprakash, 2016).

Antioxidant enzymes are considered as sensitive biomarkers to evaluate the environmental stress in organisms (Geoffroyet al., 2004). Studies have reported on antioxidant enzymes activities in various crustaceans. Exposure effect of the water-soluble fraction of petroleum on CAT, GST, GPx and SOD in *Macrobrachium borellii* showed remarkable changes in enzyme activity (Lavaris et al., 2011). The airborne exposure and salinity stress effect on oxidative stress level studied in two different crab species, *Callinectes danae* and *C. ornatus* (Friere et al., 2011). The effect of river pollutants on metabolic and antioxidant enzyme activity have also reported in prawn *Macrobrachium malcolmsonii* (Manosathyadevan and Selvisabjanayakam, 2013).

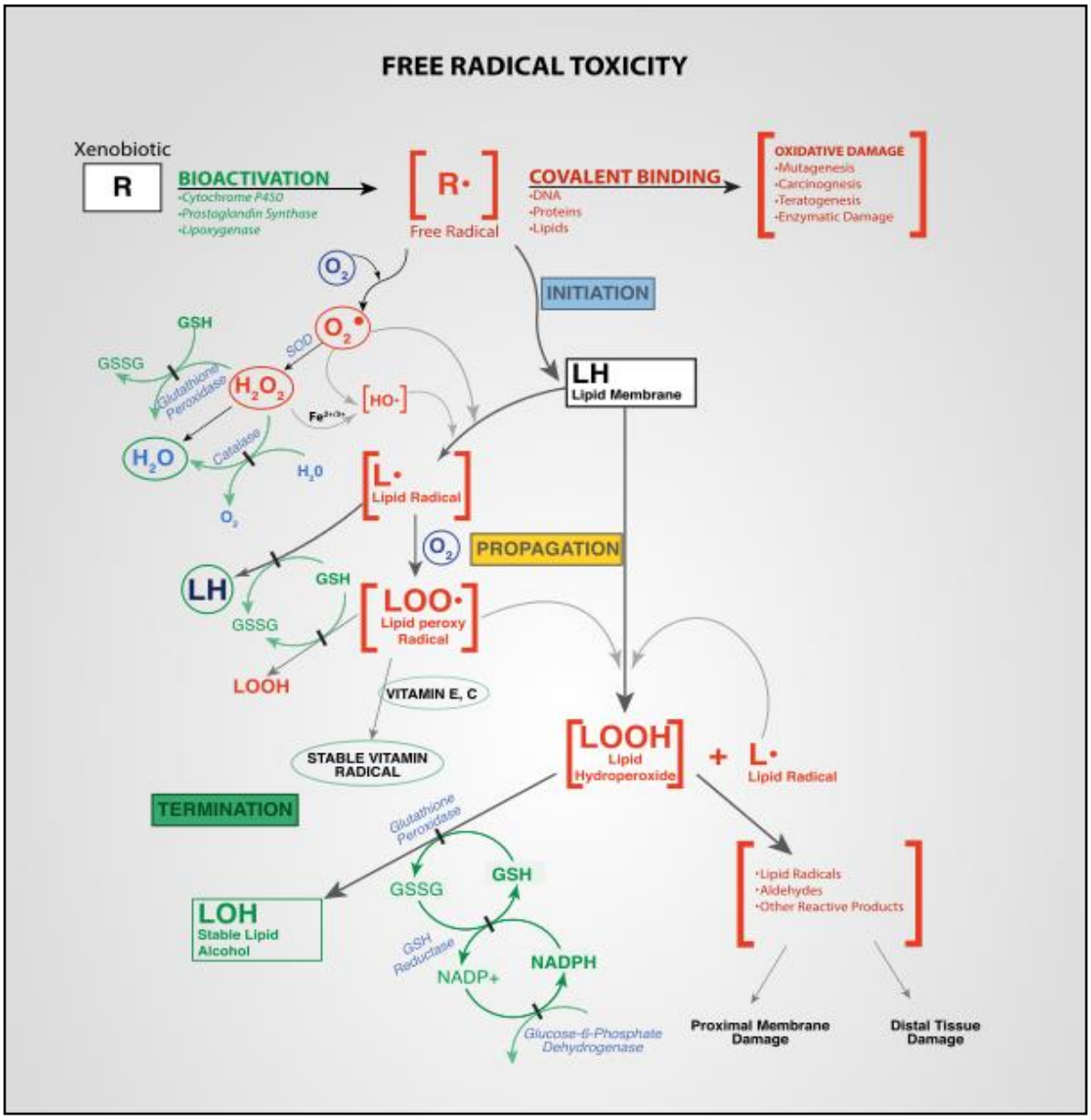


Fig. 1.6 Free radical production inside tissue and subsequent detoxification by cellular enzymes (https://en.wikipedia.org/wiki/Reactive_oxygen_species)

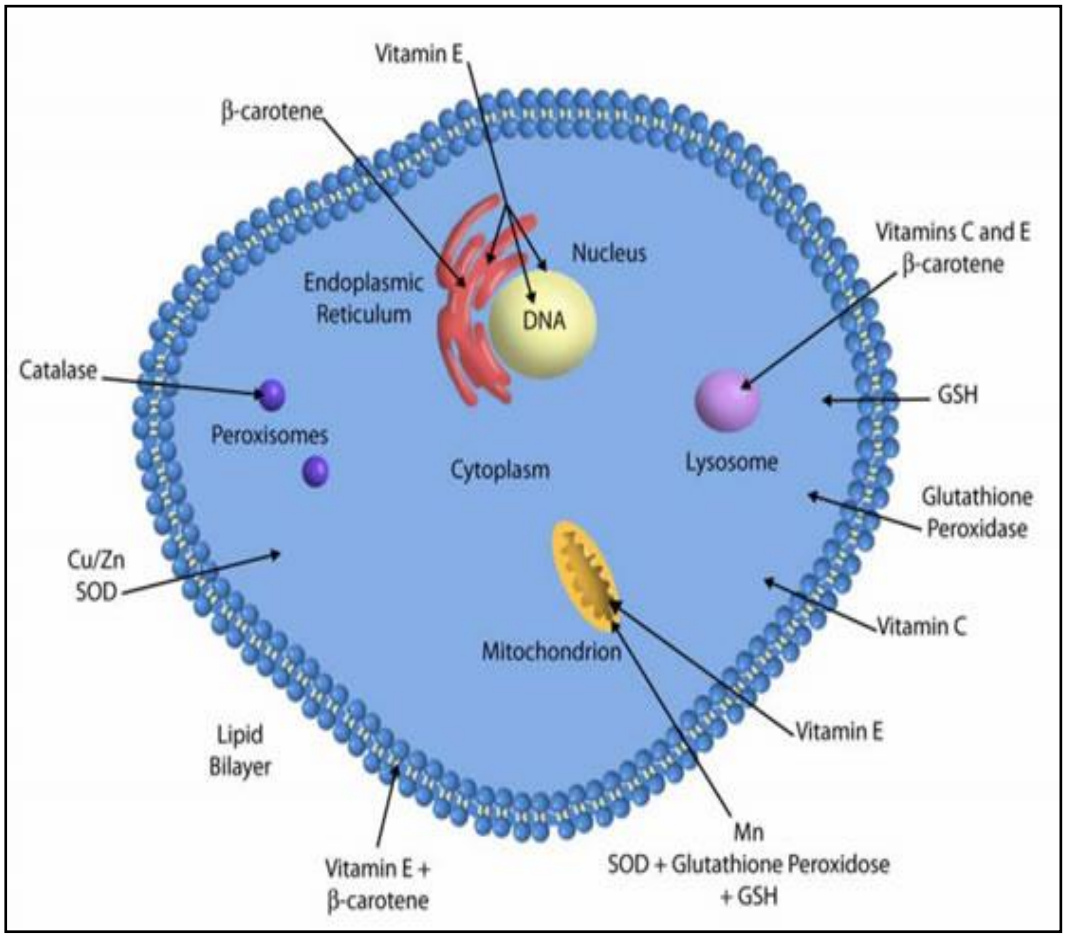


Fig. 1.7 Cellular position of antioxidants and antioxidant enzymes (www.news-medical.net/health/Antioxidant-Enzyme-Systems.aspx)

The effect of pesticides on oxidative stress in terms of evaluation of antioxidant enzyme activity in post larvae and juveniles of *L. vannamei* would help us to understand the antioxidant enzyme activity status against the pesticide exposure. Further, this study will also provide information of the enzyme which acts first after pesticide exposure over a long period of time. As there is a lack of reports on the in vivo effect of pesticide in penaeid shrimps, this study will help us to generate data on the direct impact of pesticide exposure on oxidative stress in one of the commercially important shrimp species *L. vannamei*. Therefore, these studies on the effect of pesticide exposure in two life stages of *L. vannamei* have been presently undertaken

1.8 Gene expression

The gene expression study deals with the collection of information from a gene which is used for the synthesis of functional gene product *viz.* enzymes, proteins. Gene expression analysis was done in almost all forms of life from prokaryotes to eukaryotes i.e viruses, bacteria, fungi, mammals and humans. This process includes transcription, splicing and translation of proteins. During transcription DNA from gene used to produce mRNA copies by using RNA polymerase. Then carried to the ribosome for translation process where actual protein synthesis takes place. These processes are important to controls the structural and functional unit of cells.

In genetics, gene expression always gained the importance to study gene regulation based on environmental changes and also helps in understanding the effect of contaminants to a higher level of the niche (Garcia-Reyero et al., 2008).

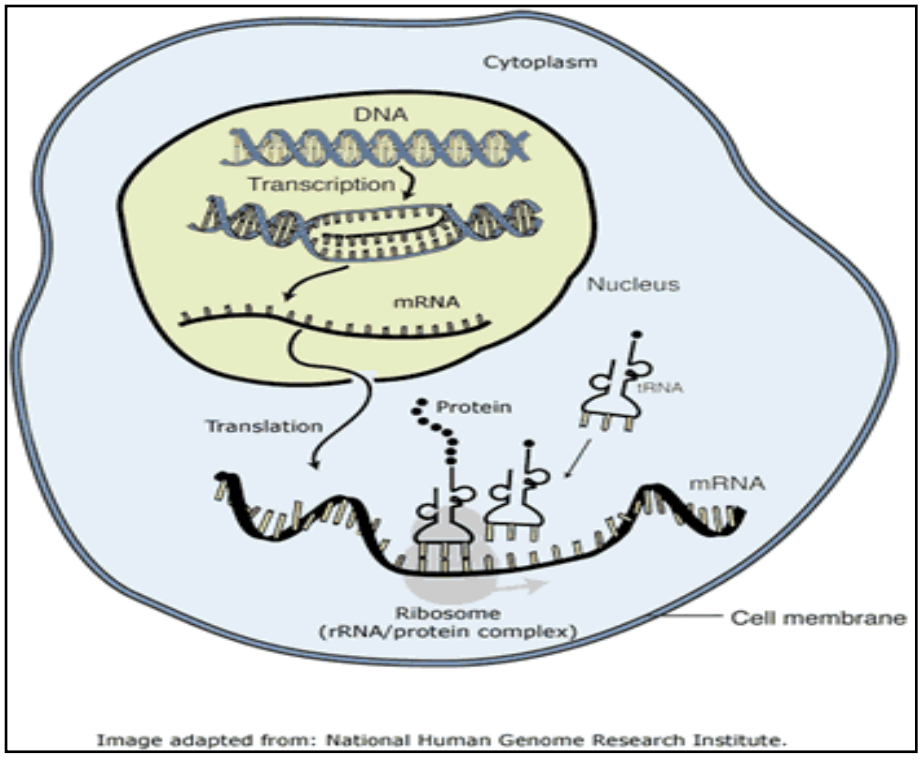


Fig. 1.8 Gene expression and regulation
(www2.le.ac.uk/projects/vgec/highereducation/topics/geneexpression-regulation)

1.8.1 Real Time Polymerase Chain Reaction (RT-PCR)

Study on gene expression will help us to understand the toxic effect of pesticides on organisms at the molecular genetic level. Real Time Polymerase Chain Reaction (RT-PCR) is one of the popular techniques employed for this across the globe. Gene expression studies of Mendoza-Cano and Sanchez-Paz (2013) showed that RT-PCR can be used as an effective molecular technique for the universal detection of the White Spot Syndrome Virus in marine crustaceans in short period of time with high accuracy. Further, studies carried out by Rivera-de et al. (2019) in *L. vannamei* and Jang et al. (2008) in *L. vannamei* and *Fenneropenaeus chinensis e*mploying the RT-PCR technique also showed the efficiency and effectiveness of this molecular technique to study gene expression in targeted organisms. Hence, this technique was selected for the present gene expression study in *L. vannamei*. Due to lack of *in-vivo* study on pesticide effect on gene expression in penaeid shrimp, this study will generate the quality data on gene expression in *L. vannamei* which could be comparable to other penaeid shrimps. Under this objective we have studied the antioxidant enzyme gene expression through RT-PCR which will reveal, these enzyme changes at the genetic level. This study will also co-relate with the oxidative stress level on exposure to pesticide at the gene level.

Objectives of the present study

1. To determine the 96 h LC_{50} values of organophosphate pesticides (Chlorpyrifos and Dimethoate) in *L. vannamei* under laboratory conditions

2. To assess the genotoxic effects of sublethal concentrations of these pesticides in *L. vannamei*

3. To assess the activity level of antioxidant enzymes and quantify the expression of these genes in *L. vannamei* exposed to sublethal concentrations of above pesticides

Chapter 2

Experimental animals (Procurement and maintenance) and Preparation of reagents and stains

2.1 Procurement and maintenance of *Litopenaeus vannamei*

Litopenaeus vannamei Post Larvae (PL) were procured from Skyline Hatcheries, Kumta, Karnataka, India and transported to the Aquaculture laboratory at CSIR-National Institute of Oceanography (NIO), Dona Paula, Goa in polythene bags filled with oxygen. After their arrival at the laboratory, PL were acclimatized by maintaining them at standard laboratory conditions in large Fiberglass Reinforced Plastic (FRP) tanks of 800 L capacity with continuous aeration. Seawater was collected locally, treated by rapid sand filtration, followed by cartridge filtration (20 to 1 µm) and was later on passed through ultraviolet radiation. This UV treated sterile seawater was used for acclimatization as well as for experimental purpose. Adequate aeration being provided using air blowers and optimum water quality parameters [Temperature 28.5 ± 0.5 ºC), salinity (30 ppt), dissolved oxygen (6.1 ±0.4 mg L^{-1}), pH (8.2 ± 0.2), NO_2–N (<0.02 mg L^{-1}) and NH_3/NH_4 (0 mg L^{-1})] were maintained during the acclimation period. Further, during acclimatization period, these PL were fed with *Artemia* nauplii (Plate 2.1 a and b) and PL feed (INVE) (Plate 2.2 a and b) while juveniles (JL) were fed with dry pellet (CP, Aquaculture; proximate composition: 38−40% protein, 5% lipid and 3% fiber) (Plate 2.3 a and b) *ad libitum* twice a day until the commencement of experiment. PL were grown under laboratory conditions until the two desired sizes *viz.* shrimp post larvae (PL14; total length, 12.4 ± 0.5 mm; wet weight, 55 ± 5 mg) (Plate 2.4) and juveniles (JL) (Total length, 52.2 ± 10 mm; wet weight 1.78 ± 1.0 g) (Plate 2.5) were attained and employed for the present study.

Plate 2.1 a: *Artemia* cysts

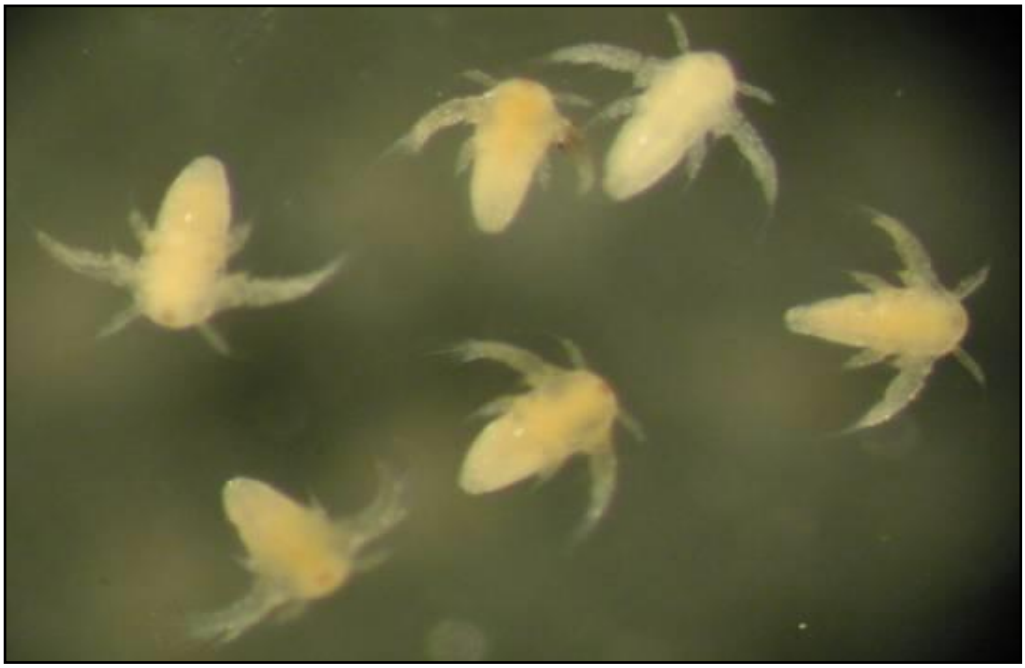


Plate 2.1 b: Newly hatched *Artemia* nauplii

a **b**

Plate 2.2 a, b: Dry feeds used for post larvae

a **b**

Plate 2.3 a, b: Dry feed used for juveniles

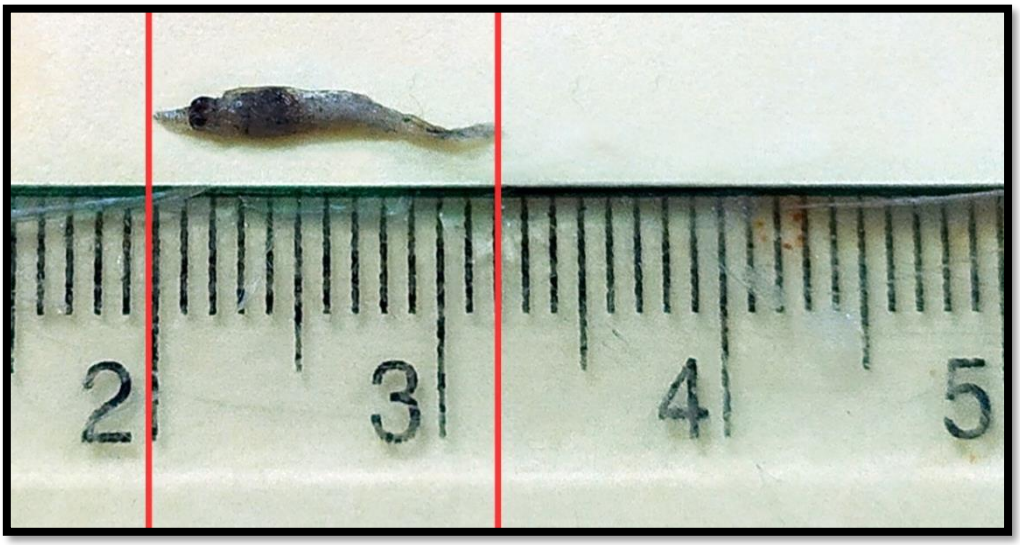


Plate 2.4: Size of post larva (in mm)

Plate 2.5: Size of juvenile (in mm)

2.2 Pesticides

Two commercial-grade organophosphate pesticides with the brand names *viz.* i) PYRIBAN containing 'CHLORPYRIFOS' (20% EC) and ii) TAFGOR containing 'DIMETHOATE' (30% EC) were procured from a local dealer and used for the present study.

Plate 2.6 a: Chlorpyrifos

Plate 2.6 b: Dimethoate

2.3 Preparation of reagents and stains

2.3.1 Preparation of stock solution

Both pesticides *viz.* Chlorpyrifos and Dimethoate were in liquid form. Based on the effective concentration and their solubility in water respective stock solutions were prepared in double distilled water (DDW). Solubility of CPF in water is 2 mg L^{-1} whereas that of DMT is3 mg L^{-1}. According to their solubility in water, 10 ul of respective pesticide dissolved in 1 L DDW to obtain the approximate 2 ppm stock solution.

2.3.1.1 Test solutions

Test solutions of the selected concentrations were prepared by dilution of the respective stock solution by the direct addition of required amount of pesticide stock solution to the test chamber (Tank) containing UV treated sea water. However, Sea water without pesticide were used as controls.

2.3.2 Comet assay:

- **Detergent and acid solution:**

  For washing glassware, a warm soapy solution followed with 4% HCl were used.

- **Phosphate buffered saline (1X, pH 7.4):**

Component	Weight in gram (g)	Molarity (M)
NaCl	8	0.137
KCl	200	0.0027
Na_2HPO_4	1.44	0.01
KH_2PO_4	0.240	0.0018

To 800 ml of double distilled water (DDW) above components were added and pH was adjusted to 7.4. Final volume was made up to 1 L with DDW.

❖ **Lysing solution**

Component	Weight (g)	Molarity (M)
NaCl	73.01	2.5 M
Na_2EDTA	18.7	100 mM
Tris	0.6	10 mM

All the above components were dissolved in 350 ml of Double Distilled Water (DDW). Approximately 4 g of NaOH (pellets) allowed to dissolve in above mixture about 20 min. Adjusted the pH to 10 by HCl and made up the volume to 445 ml with DDW. Rest volume (= 55 ml) adjusted by 10% DMSO and 1% Triton X 100 added freshly according to requirement.

❖ **Unwinding Solution/ Electrophoresis buffer:**

Stock solution

Component	Weight(g)	Molarity (M)
NaOH	200	10 N
EDTA	14.89	200 mM

Dissolved 200 g of NaOH in 500 ml of DDW and 14.89 g of EDTA in 200 ml of DDW separately with pH 10. Store both at room temperature.

Working solution (300 mM NaOH: 1 mM EDTA) for 900 ml:

Stock solution	Volume added
NaOH	27 ml
EDTA	4.5 ml
DMSO	1.8 ml

❖ **Neutralization buffer:**

Component	Weight in gram (g)	Molarity (M)
Tris base	4.845	400 mM

Dissolved Tris base to 90 ml of DD water, adjusted pH to 7.5 and made up to final volume of 100 ml. Store solution at room temperature, but made it chill before use.

❖ **Preparation of Agarose:**

a) 1% Normal Agarose

100 mg of normal agarose was mixed with 9 ml of DDW and boiled until get dissolved. Then added 1 ml of 10X PBS to the above solution and mixed properly. Microwave again and make up the final volume to 10 ml with DD water.

b) 0.5 % Low Melting Point Agarose

50 mg of Low melting point agarose mixed with 9 ml DDW and microwaved to dissolve the agarose. Then added 1 ml of 10X PBS to the solution and mixed properly and heated again. Final volume of 10 ml made up with DDW.

❖ **Saline- Sodium Citrate (2x SSC):**

Component	**Weight (g)**
NaCl	17.5
Sodium citrate	8.8

Added above both to 1000ml of DDW and adjusted the pH to 7.0.

❖ **Trypan Blue (0.4%):**

Dissolved 0.4 g of trypan blue in 100 ml of DDW.

❖ **Ethidium Bromide**

10X stock solution (20 µg ml^{-1}) added 10 mg to 50 ml of DDW and stored at room temperature. 1X working solution- Diluted above in 1:9 ratio with DDW.

2.3.3 Protein estimation:

❖ **Reagent A:** 2% Na_2CO_3 in 0.1 N NaOH

(2 g Na_2CO_3 in 100 ml of 0.1 N NaOH)

❖ **Reagent B:** 0.5% $CuSO_4$ in 1% sodium potassium tartarate

(0.5 g $CuSO_4$ in 50 ml D/W + 1 g sodium potassium tartarateto 50 ml DDW)

❖ **Reagent C:** Mixed both A and B reagent in 50:1 ratio

BSA (Bovin Serum Albumin) standard: 5 mg ml^{-1} (0.05 g of BSA dissolved in 10 ml of DD water)

2.3.4 Antioxidant enzyme activity:

❖ **Stock buffer indicator solution of pH-8.0**

Components	Amount	Total volume
Bromothymol blue	0.025 g	-
NaOH(2N)	0.50 ml	-
Stock buffer	37.5 ml	250 ml

Working buffer indicator solution

Buffer solution	Amount (ml)	Total volume
Stock buffer indicator	119.05	250ml

❖ **Acids and base for adjustment of pH of the buffer**

Components	Mol.Wt. (g)	Normality	Dilution in 100 ml
Hcl (12.4 N)	36.5 (30%)	9.54 N	0.5 ml
NaOH	40	0.05 N	0.2 g

❖ **Preparation of Triton-X-100**

Component	Amount (ml)	Dilution (ml)
Triton-X-100	2.5	100

❖ **Preparation of sucrose solution**

Component	Mol. Wt. (mg)	Amount for 0.25 M in 1 L (g)	Amount for 0.25 M in 250 ml (g)
Sucrose	342.3	85.5	21.375

❖ **Preparation of Phosphate buffer of pH-8**

Components	Mol. Wt. (mg)	Amount for 0.1 M in 1L (g)	Amount for 0.1 M in 250 ml (g)
K_2HPO_4	174.18	17.418	4.35
KH_2PO_4	136.09	13.609	3.40

❖ **Epinephrine (1 mg/ml)**

9 ml of water was taken in a clean test tube. To this 80 µl of 2 N HCl was added and mixed well. Later 10 mg of epinephrine was weighed and immediately added to the above solution. Covered with aluminium foil.

❖ **Carbonate Buffer (0.050 M, pH 10.2)**

0.0390 g of $Na_2EDTA.2H_2O$ and 4.20 g of $NaHCO_3$ added to 800 ml of DDW. Adjusted the pH to 10.2. Transferred the buffer to clean volumetric flask and adjusted the final volume up to 1L.

❖ **Tissue homogenization buffer for catalase and glutathione peroxidase enzyme activity (100 mM, pH 7.4)**

Compounds	**Mol. Wt.**	**Wt. for 100 ml (g)**	**Concentration**
K_2HPO_4	174.18	1.7418	0.1 M
KH_2PO_4	136.09	1.3609	0.1 M
NaCl	58.50	0.5850	0.1 M
EDTA Na_2	372.24	0.0372	1 mM

Working buffer: Phosphate buffer (10 mM, pH 7)

Mixed 10 ml of tissue homogenizing buffer with 90 ml of DDW and adjusted pH to 7

Working buffer (pH 6.5)

Sr. No.	**Compound**	**Mol. Wt.**	**Wt. for 100ml (g)**	**Concentration**
1	K_2HPO_4	174.18	1.742	0.1M
2	EDTA Na_2	372.24	0.0372	1 mM

❖ **0.2M Hydrogen peroxide**

Added 1.13ml from 50% H_2O_2 and final volume adjusted to 100ml with DDW

❖ **Dichromate/acetic acid mixture** (1:3)

Mixed 5% solution of potassium dichromate ($K_2Cr_2O_7$) with glacial acetic acid in 1:3 ratio

❖ **Stock solution of GSH**

Component	Mol. Wt. (g)	Wt. for 1.0 ml (mg)	Concentration	Wt. for 5 ml (30 mM)
Reduced glutathione	307.43	9.223	30mM	46.115mg

❖ **Stock solution of CNDB**

Component	Mol. Wt. (g)	Wt. for 1.0 ml (mg)	Concentration	Wt. for 5 ml (30 mM)
1-chloro-2,4-dinitrobenzene	202.56	6.077	30mM	30.385

Weighed out 6.077 mg of CDNB crystals and added in 1.0ml of 95% Ethanol. Preserved the solution at 4 °C until used further for experiment.

❖ **Sodium phosphate buffer with Ethylene diamine tetra acetic Acid (EDTA), pH 7.0**

Components	Concentration	Volume
Sodium phosphate, monobasic, anhydrous	0.40 mM	100 ml
Ethylenediaminetetraacetic acid (EDTA)	50 mM	

❖ **1 mM sodium azide solution**

Prepared 50 ml in sodium phosphate buffer with EDTA

❖ **B-Nicotinamide adenine dinucleotide phosphate, reduced form**

Used 1.0 mg to prepare the stock solution

❖ **Glutathione reductase enzyme solution**

Used 100 units/ ml to prepare the solution

❖ **200 mM glutathione reduced**

Prepared 5 ml in DDW

❖ **Glutathione peroxidase enzyme solution**

Prepared 1.5-3.0 units/ ml in sodium phosphate buffer

❖ **0.042 % hydrogen peroxide**

Prepared 5 ml in DDW using 30 % H_2O_2

2.3.5 RT-PCR

❖ **DEPC treated water (0.1%)**

To prepare 0.1%DEPC treated water added 1ml of diethyl pyrocarbonate in 1000ml of milliQ water and mixed well. Incubated further at 37^0C for 12 h. Finally autoclaved and cooled at room temperature prior to use.

❖ **75% ethanol**

Added 75 ml of absolute ethanol to 25 ml of autoclaved DDW.

Chapter 3

Acute toxicity (96 h LC_{50}) studies of Chlorpyrifos and Dimethoate in post larvae and juveniles of *L. vannamei*

3.1 Introduction

Toxicity tests are an important component for assessing the effect of single or complex chemical mixture on aquatic ecosystems including the safety of the flora and fauna residing there. In aquatic toxicity tests, groups of selected organisms are exposed to test materials under defined conditions to determine their potential adverse effects. Several standardized toxicity test protocols have been developed for determining the toxicity of chemicals to aquatic species (OECD, 2018). Detailed protocols for toxicity tests with marine and freshwater organisms are available from the United States Environmental Protection Agency (U.S. EPA) and other entities such as the American Society for Testing and Materials (ASTM). These protocols, guide on the application of toxicity tests for assessing toxicity of specific single chemicals, complex effluents and ambient water or sediment samples.

The evaluation of acute lethal toxicity in study animals is a pre-requisite as a prior step to carry out detailed sublethal toxicity studies in the organism. Determination of median lethal concentration (LC_{50}) is one of the parameters most employed to study the acute lethal toxicity of pollutants (Chinedu et al., 2013). The LC_{50} can be interpolated from the curve by drawing a horizontal line from the 50% mortality point on the y-axis to the concentration-response curve and then drawing a line from the point of intersection with the curve to the x-axis whereas the vertical line intersects the x-axis at the LC_{50} value. The LC_{50} under a defined set of environmental conditions can provide useful information to carry out further toxicity studies including sublethal dose effects. From an ecotoxicological point of view, this parameter is often estimated in the more sensitive species and/or stages of an aquatic community and is a potential tool to establish safe concentrations of pollutants in the environment.

Comparison of the toxicity between different taxa confirms the difference in their tolerance capacity based on the hierarchical level, including the reports on the acute toxicity study of Chlorpyrifos (CPF) and Dimethoate (DMT) in toad *Bufo melanostictus* (Jayawardena et al., 2011), brine shrimp *Artemia salina* (Rao et al., 2007), fresh water carp *Cyprinus carpio* (Halappa and David, 2009), Indian minor carp, *Labeo bata* (Samajdar and Mandal, 2015). Some studies reported on acute toxicity effect of CPF and DMT in penaeid shrimp including *P. monodon* and *M. monoceros* (Kumar et al., 2010; Eamkamon et al., 2012; Shoaib and Siddiqui, 2015). In these lines, our present study can add information on baseline data of toxicity of pesticides on an ecologically important species of crustacean *L. vannamei* from penaeid family.

The significant role of water quality parameters influencing the toxicity of different pesticides that ultimately have a harmful effect on food-chain and physiological functions of aquatic fauna has been documented (Anyusheva et al., 2012; Uddin et al., 2016). Climate change has been considered as a global threat and an important challenge for the 21st century (Schiedek et al., 2007). A wide variation in the ambient salinity and temperature in combination with xenobiotic compounds may result in more deleterious effects due to altered chemical fate and transport and changes in physiological response associated with such variations (Schiedek et al., 2007; DeLorenzo et al., 2009). Hence, mostly the standard bioassays alone may not be predictive of the actual pesticide toxicity under variable environmental conditions. Therefore, testing under a wider range of exposure conditions for improving the accuracy of chemical risk assessments becomes imperative. It is well known that a significant deviation in salinity and temperature from optimum ranges may affect osmoregulation and metabolic rates in shrimps. Hence, the effect of salinity and temperature on the quantum of acute toxicity of Chlorpyrifos (CPF) and Dimethoate (DMT) on *L. vannamei* also taken up in this investigation.

3.2 Material and methods

3.2.1 Acute toxicity study (Determination of 96 h LC_{50}) (OECD, 2018)

3.2.1.1 Experimental animals

Shrimp PL (PL14; total length, 12.4 ± 0.5 mm; wet weight, 55 ± 5 mg) and juveniles (JL) (Total length, 52.2 ± 10 mm; wet weight 1.78 ± 1.0 g) were used to study the acute toxicity of DMT and CPF.

3.2.1.2 Range finding tests

Prior to commencement of the definitive tests, separate range finding tests were conducted for PL and JL. The range-finding test is to predict the 96 h LC_{50} values with enough accuracy for the definitive test. It helped in fixing the correct concentrations of pesticides for a definitive test. These concentrations were selected according to the protocol explained in OECD guideline. Initially PL and JL were exposed to both the pesticides varying in the range of (1-100 ppm) concentrations. Based on this initial observations further PL were exposed to various concentrations of CPF (0.1−3 µg L^{-1}) and DMT (10−600 µg L^{-1}), whereas different concentrations of CPF (0.1−5 µg L^{-1}) and DMT (20−1000 µg L^{-1}) were used to expose JL.

3.2.1.3 Definitive tests

Prior to experiments, test chambers were washed with diluted detergent, followed with an acid wash to remove any previous contaminants and then filled with required volumes of above filtered sterile sea water. The water level in test tanks were adjusted to achieve desired loading requirements. Prior to the test, specific volume of stock solutions of pesticides were added to the test chambers and maintained with temperature (28.5 ± 0.5 ºC), salinity (30 ppt), dissolved oxygen (6.1 ±0.4 mg L^{-1}), pH (8.2 ± 0.2). *L. vannamei* of specific sizes were introduced into the test tanks according to the experimental design. Test cambers were observed at regular intervals of once in every 6 h and the dead shrimps were removed to avoid further contamination. Further, the quality of water was also maintained throughout.

3.2.1.4 Validity of test

For the test to be valid the following criteria were fulfilled

- Mortality in control had not exceeded 10% at the end of the test
- Static toxicity procedure was used for the experimental duration of 96 h
- Constant conditions were maintained throughout the test
- Dissolved oxygen was maintained at 60% of air saturation value throughout the test

3.2.1.5 Experimental set up

PL exposed to five different nominal concentrations of CPF (0.4, 0.8, 1.2, 1.6 and 2.0 µg L^{-1}) and DMT (100, 200, 300, 400 and 500 µg L^{-1}) along with separate unexposed controls were used for acute toxicity experiments. The tanks of 2 L capacity were used to maintain stocking density of 10 PL per 1L.

JL exposed to five different nominal concentration of CPF (0.4, 0.8, 1.2, 1.6 and 2.0 µg L^{-1}) and DMT (100, 200, 400, 600 and 800 µg L^{-1}) along with separate unexposed controls were used for acute toxicity experiments. The tanks of 21 L capacity were used to maintain stocking density of 10 JL per 10 L.

Maximum care was taken to maintain the animals with no or minimum disturbance during handling. Specific volume of sea water was measured accurately and required amount of pesticides were added to each test tank to get a specific concentration of pesticide in each tank. Feeding was stopped before24 h of the commencement of acute toxicity test (96 h LC_{50}).

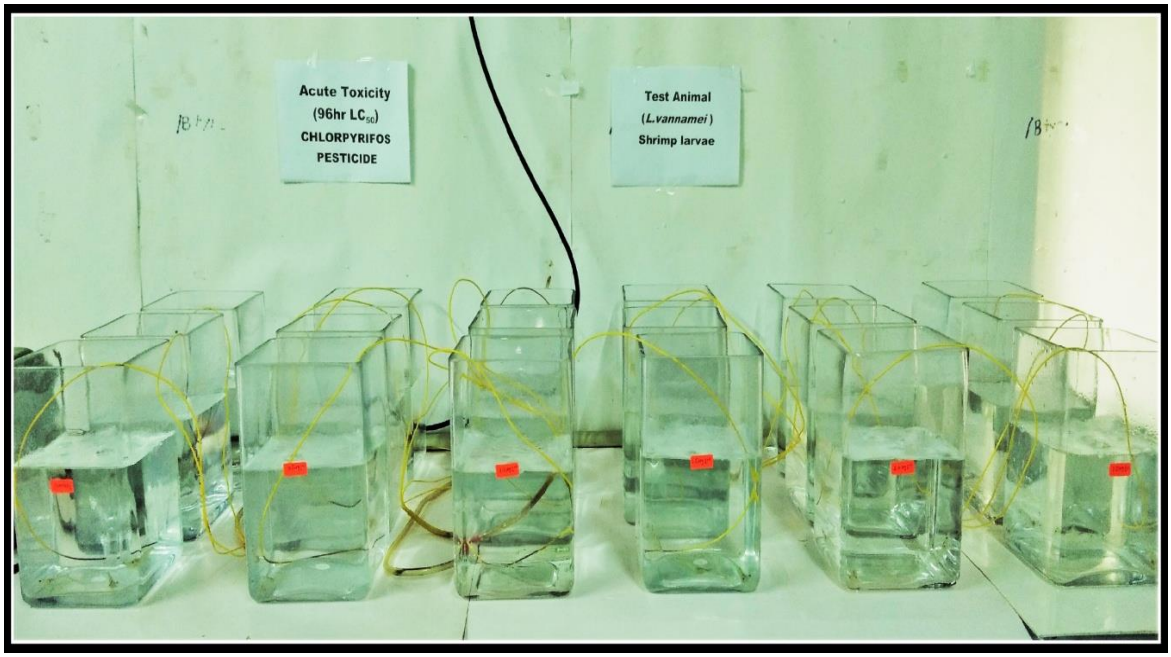


Plate 3.1: Experimental set up for acute toxicity testing of Chlorpyrifos / Dimethoate with post larvae of *L. vannamei*

Plate 3.2 Experimental set up for acute toxicity testing of Chlorpyrifos/ Dimethoate with juveniles of *L. vannamei*

3.2.1.6 Recording of mortality

Mortalities of PL and JL in acute toxicity experiments were recorded at the intervals of 24, 48, 72 and 96 h during the 96 h exposure period. The dead organisms removed immediately on detection to avoid further contamination. The criteria employed for proof of mortality were total lack of body movement, immobility of heart and scaphognathite after repeated prodding with a probe (Lignot et al., 1998).

The cumulative mortalities rates were calculated using the formula defined by Abbott (1925):

$$\% \text{ Corrected} = \left(1 - \frac{\text{n in after treatment}}{\text{n in control}}\right) \times 100$$

n= Number of PL/ JL in tank

The median lethal concentrations (LC_{50}) and their 95% confidence limits for CPF and DMT at different exposure periods (24, 48, 72 and 96 h) were calculated with a computer-based program LDP line software described by Finney (1971). Low observed effective concentration (LOEC) was determined as the lowest concentration that had induced mortality. However, No Observed Effective Concentration (NOEC) was determined as the highest concentration that had not induced mortality. The low observed effective concentration and no observed effective concentration determined based on different concentration of CPF used to expose the PL and JL for different time intervals.

3.2.1.7 Role of salinity and temperature on acute toxicity

Three different salinities *viz.* 15, 25, and 35 ppt and three different temperatures *viz.*15, 25 and 34 ºC were used to study acute toxicity of DMT and CPF. Different salinities were prepared by diluting with fresh water or concentrating natural seawater with sea salt while the temperature remained same as room temperature (28 ± 1 ºC). However, the different temperature experiments were conducted in an air-conditioned room to maintain 15 ºC temperature while the thermostatic aquarium heaters in each aquarium (50 W, temperature range 22−34ºC, Protherm, Italy) were used to maintain 25 and 34 ºC temperature. During different temperature experiments salinity was maintained at 30 ppt. The water quality

parameters such as temperature, DO and pH were recorded daily whereas salinity, NO_2–N, and NH_3/NH_4 were measured prior and at the end of experimental period.

After seven days of acclimatization and rearing at different salinities and temperatures in laboratory, *L. vannamei* PL of PL21 stage (15.2 mm ± 0.3 mm; wet weight, 65 ± 5 mg) and four week old healthy uniform size JL (total length, 53.4 ± 6.4 mm; wet weight, 1.79 ± 0.6 g) without any stress signs or no visual symptoms of diseases were selected.

Prior to experiments, shrimps were not fed for 24 h to avoid faecal contamination. Based on the range finding tests, different concentrations were selected to exposed PL and JL. PL were exposed to five different nominal concentrations of CPF (0.1−1.8 µg L^{-1}) and DMT (50−500 µg l^{-1}). However, JL were exposed to five different nominal concentrations of CPF (0.2−2 µg L^{-1}) and DMT (100−800 µg L^{-1}). Shrimp PL were exposed in 2 L glass jar with 1 L seawater with a stocking density of 10 PL per jar. Whereas JL were exposed in 21 L glass aquaria containing 10 L seawater with a stocking density of 10 JL per aquarium. The experiments were carried out in triplicates along with separate control tanks.

The cumulative mortalities, median lethal concentration, LOEC and NOEC calculated as described above in recording of mortalities (3.2.3).

3.2.1.8 Statistics

Statistical analysis was performed by using computer based GraphPad PRISM 5.0 software (Graph Pad, San Diego, CA, USA). Data obtained from the experiments were expressed as mean ± SD. Data from different treatment groups were assessed by analysis of variance (ANOVA) (Underwood, 1997) with DoE and different concentrations as sources of variation. Variation in water quality parameters from treated and control aquaria were assessed by ANOVA. Appropriate transformations were applied before subjecting the data to statistical analysis. However, data considered statistically significant at $P< 0.05$.

3.3 Results

3.3.1 Range finding tests

Based on the observation of the range finding test, the concentration range of CPF (0.4−2µg L^{-1}) and DMT (100-500 µg L^{-1}) were selected and used to expose PL in definitive tests, whereas

concentration range of CPF (0.4−2 µg L^{-1}) and DMT (100-800 µg L^{-1}) were selected and used to expose JL in definitive tests.

3.3.2 a Acute toxicity of Chlorpyrifos (CPF) to Post larvae (PL)

Cumulative mortalities (%) observed in PL on exposure to different concentrations of CPF are represented in Table 3.1. There were no mortalities observed in control tanks during the 96 h duration of exposure, which indicated that the mortalities recorded in the test solutions were induced by CPF. Moreover, the mortalities observed in treatment tanks increased with the increasing exposure time as well as with the increasing concentrations of CPF (Table 3.1).

The graphical representation of median lethal concentration to PL at different time interval presented in Fig. 3.1. The calculated LC_{50} values of CPF to PL were 2.09, 1.82, 1.31 and 0.88 µg L^{-1} at the end of exposure period 24, 48, 72 and 96 h, respectively and are represented in Table 3.2. It shows the highly toxic nature of CPF as represented of very low value (LC_{50}= 0.88 µg L^{-1}) of median lethal concentration. The data of LC_{50} recorded were subjected to ANOVA to compare values at different time intervals ($P< 0.05$). CPF was found less toxic (LC_{50}= 2.09 µg L^{-1}) at initial exposure time of 24 h compared to the toxicity at 96 h of exposure (LC_{50}=0.88 µg L^{-1}) ($P< 0.05$).

Low Observed Effective Concentration (LOEC) and No Observed Effective Concentration (NOEC) of CPF are represented in Table 3.3. The observed LOEC of CPF to PL was 0.8 µg L^{-1}, whereas the observed NOEC of CPF to PL was 0.4 µg L^{-1}(Table 3.3).

Table 3.1 Cumulative mortalities (%) (Mean ± SD) in post larvae of whiteleg shrimp (*L. vannamei*) exposed to various concentrations of Chlorpyrifos at four time intervals

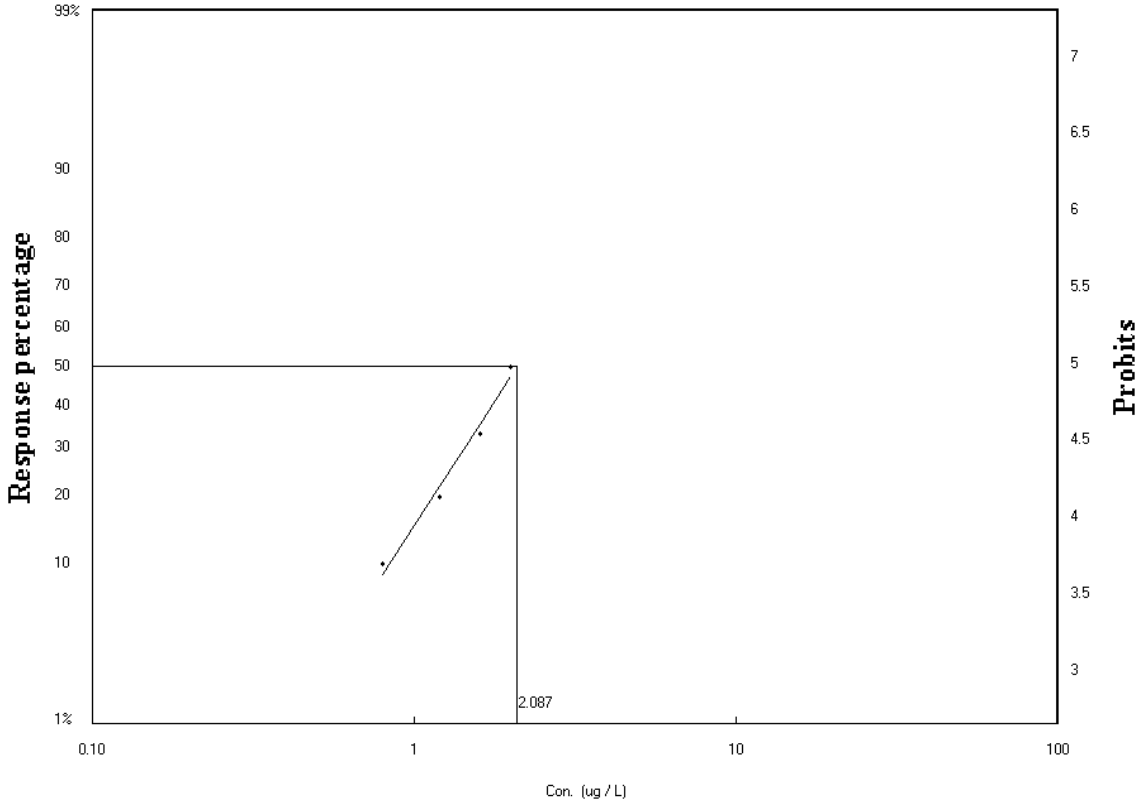
Concentrations (µg L^{-1})	**24 h**	**48 h**	**72 h**	**96 h**
Control	0	0	0	0
0.4	0	0	10 ± 1.1	20 ± 1.11
0.8	10 ± 1.6	20 ± 2.1	30 ± 2.3	40 ± 2.33
1.2	20 ± 2.1	30 ± 2.4	40 ± 3.1	60 ± 4.56
1.6	30 ± 2.8	40 ± 2.5	60 ± 4.23	80 ± 5.31
2.0	50 ± 3.2	60 ± 4.1	70 ± 4.45	90 ± 6.12

Table 3.2 Median lethal concentrations (LC_{50}) of Chlorpyrifos with a 95% confidence interval at the end of four exposure durations (ANOVA, $P< 0.05$, different superscript letters indicate significant difference between each exposure durations)

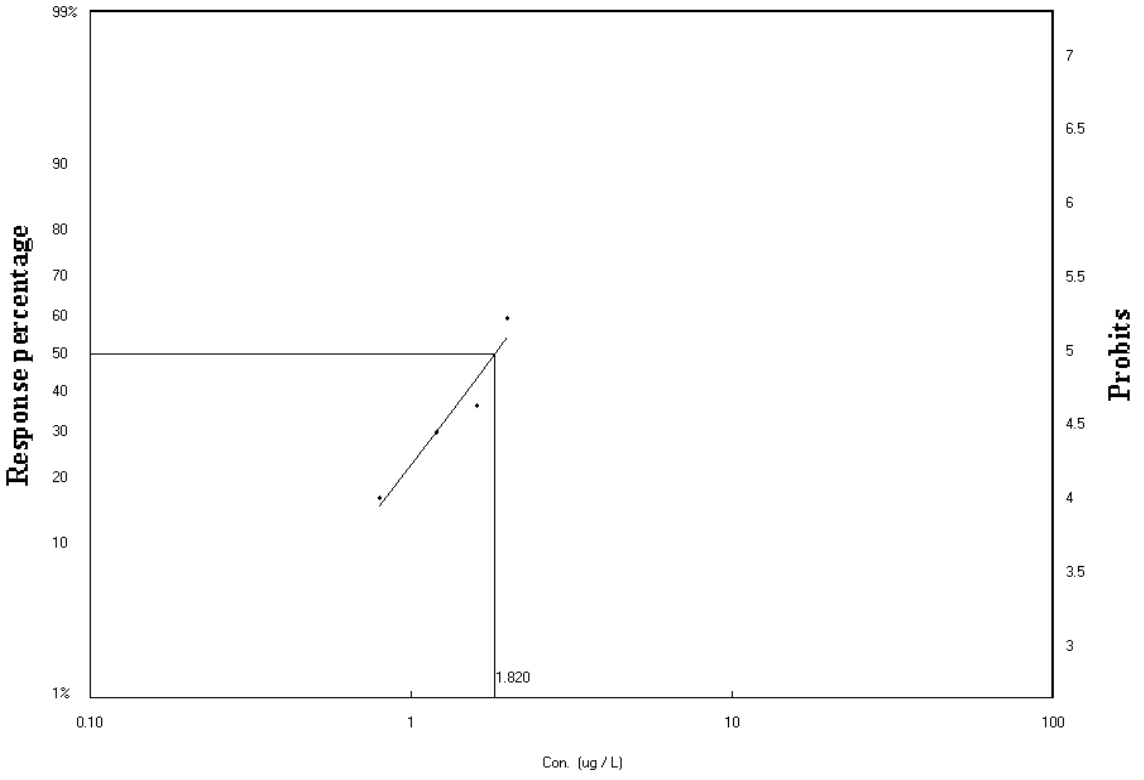
Exposure periods	**LC_{50} (µg L^{-1})**	**Lower limit**	**Upper limit**
24 h	2.09^{a}	1.84	2.56
48 h	1.82^{b}	1.62	2.17
72 h	1.31^{c}	1.19	1.49
96 h	0.88^{c}	0.80	0.97

Table 3.3 Low Observed Effective Concentration (LOEC) and No Observed Effective Concentration (NOEC) values of Chlorpyrifos in *L. vannamei* post larvae

Observed values	**µg L^{-1}**
LOEC	0.8
NOEC	0.4

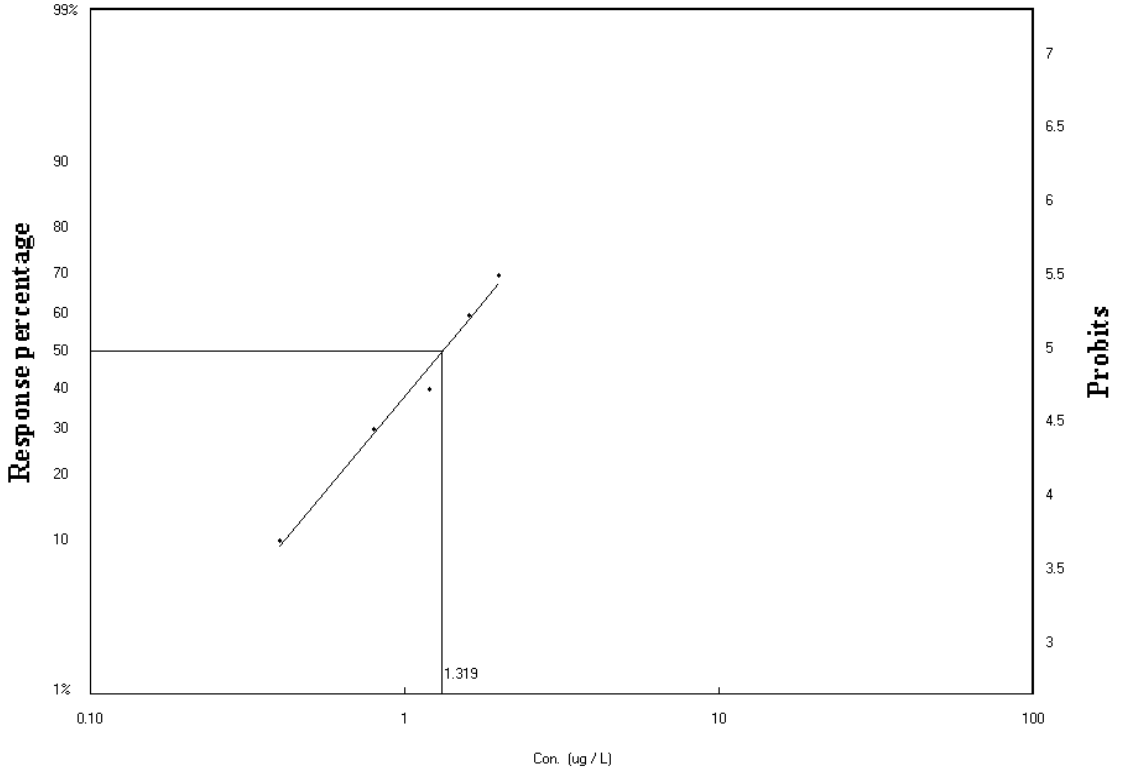


24 h

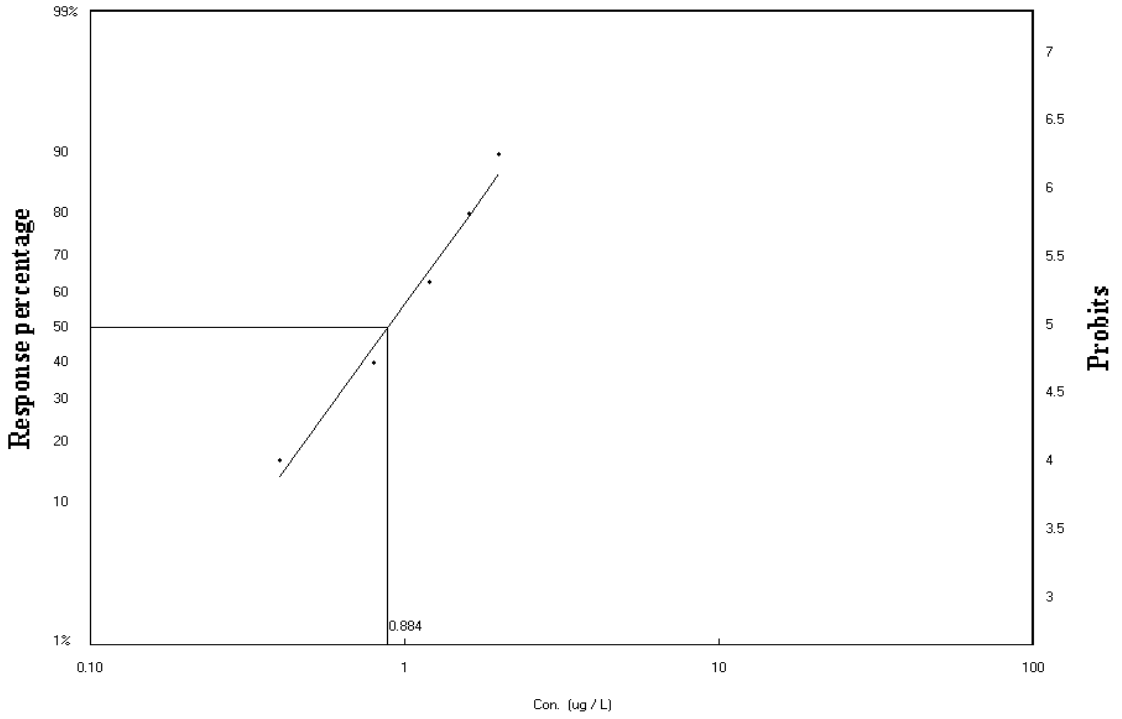


48 h

Fig 3.1 a Graphical representation of the median lethal concentrations (LC_{50}) of Chlorpyrifos to *L. vannamei* post larvae at the end of 24 and 48 h



72 h



96 h

Fig 3.1 b Graphical representation of the median lethal concentrations (LC_{50}) of Chlorpyrifos to *L. vannamei* post larvae at the end of 72 and 96 h

3.3.2 b Acute toxicity of Chlorpyrifos to juveniles (JL)

Cumulative mortalities (%) observed in JL on exposure to different concentrations of CPF are represented in Table 3.4. There were no mortalities observed in control tanks during the 96 h duration of exposure, which indicated that the mortalities recorded in the test solutions were induced by CPF. Moreover, the mortalities observed in treatment tanks increased with the increasing exposure time as well as with increasing concentrations of CPF (Table 3.4).

The graphical representation of median lethal concentration to JL at different time interval is presented in Fig. 3.2. The calculated LC_{50} values of CPF to JL were 2.18, 1.98, 1.80 and 1.44 µg L^{-1} at the end of exposure period 24, 48, 72 and 96 h, respectively and is represented in Table 3.5. It shows the highly toxic nature of CPF as represented by the very low value (LC_{50}= 1.44 µg L^{-1}) of median lethal concentration. The data of LC_{50} recorded were subjected to ANOVA to compare LC_{50}values at different time intervals ($P< 0.05$). CPF was found less toxic at initial exposure time of 24 h (LC_{50}= 2.18 µg L^{-1}) compared to the toxicity at 96 h of exposure (LC_{50}= 1.44 µg L^{-1}) ($P< 0.05$).

Low Observed Effective Concentration (LOEC) and No Observed Effective Concentration (NOEC) of CPF are represented in Table 3.6. The observed LOEC of CPF to JL was 0.8 µg L^{-1}, whereas the observed NOEC of CPF to JL was 0.4 µg L^{-1}(Table 3.6).

Table 3.4 Cumulative mortalities (%) (Mean ± SD) in juveniles of whiteleg shrimp (*L. vannamei*) exposed to various concentrations of Chlorpyrifos at four time intervals

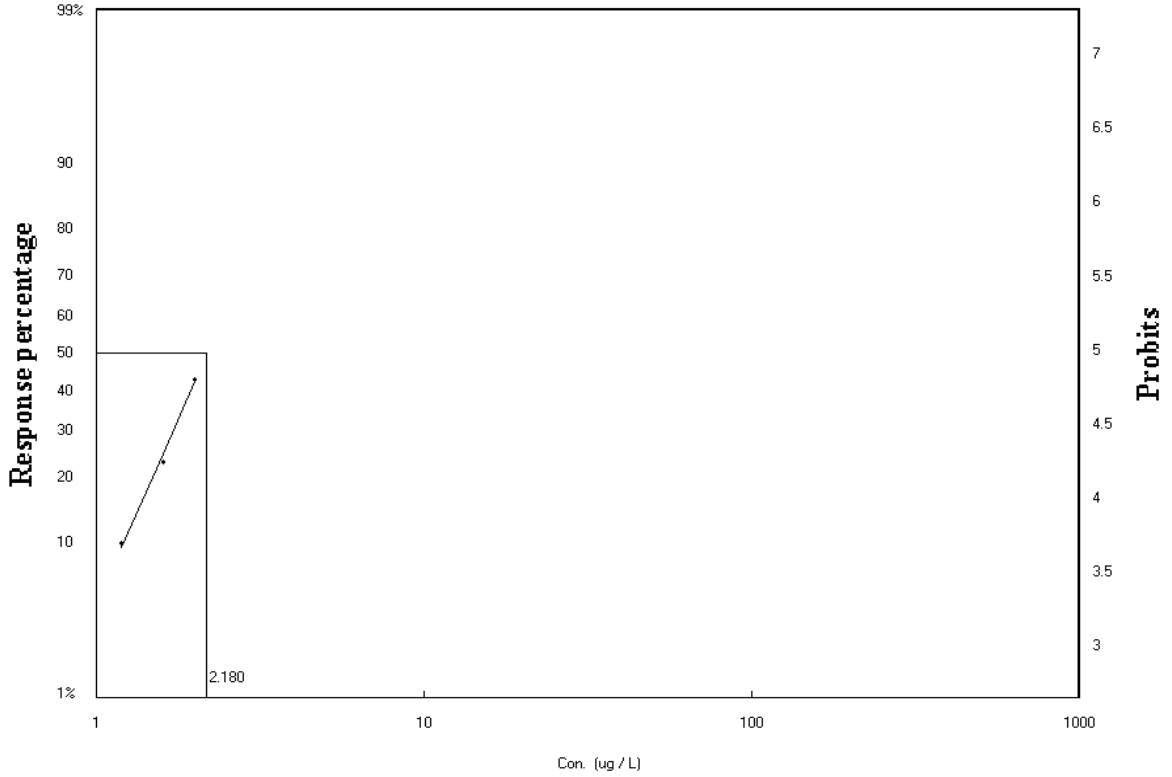
Concentrations (µg L^{-1})	24 h	48 h	72 h	96 h
Control	0	0	0	0
0.4	0		10	10
0.8	0	10 ±0.89	10 ± 0.56	20 ± 1.23
1.2	10 ± 1.05	20 ± 1.43	20 ± 1.23	30 ± 2.10
1.6	20 ± 1.24	40 ± 2.14	50 ± 3.12	60 ± 3.67
2.0	40 ± 2.10	50 ± 3.11	60 ± 3.48	70 ± 4.17

Table 3.5 Median lethal concentrations (LC_{50}) of Chlorpyrifos with a 95% confidence interval at the end of four exposure durations (ANOVA, P< 0.05, different superscript letters indicate significant difference between each exposure durations)

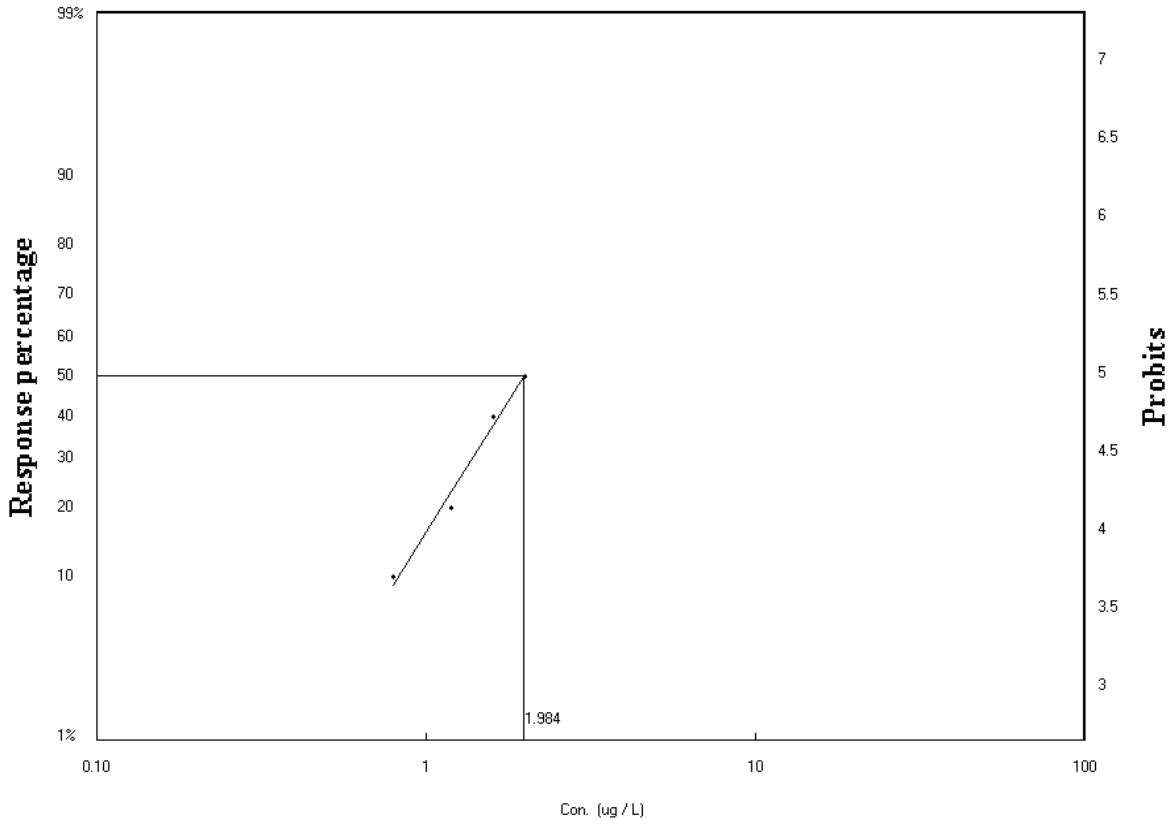
Exposure periods	LC_{50} value	Lower limit	Upper limit
24 h	2.18^{a}	1.97	2.63
48 h	1.98^{a}	1.77	2.36
72 h	1.80^{a}	1.58	2.08
96 h	1.44^{b}	0.84	1.46

Table 3.6 Low Observed Effective Concentration (LOEC) and No Observed Effective Concentration (NOEC) values of Chlorpyrifos in *L. vannamei* juveniles

Observed values	µg L^{-1}
LOEC	0.8
NOEC	0.4

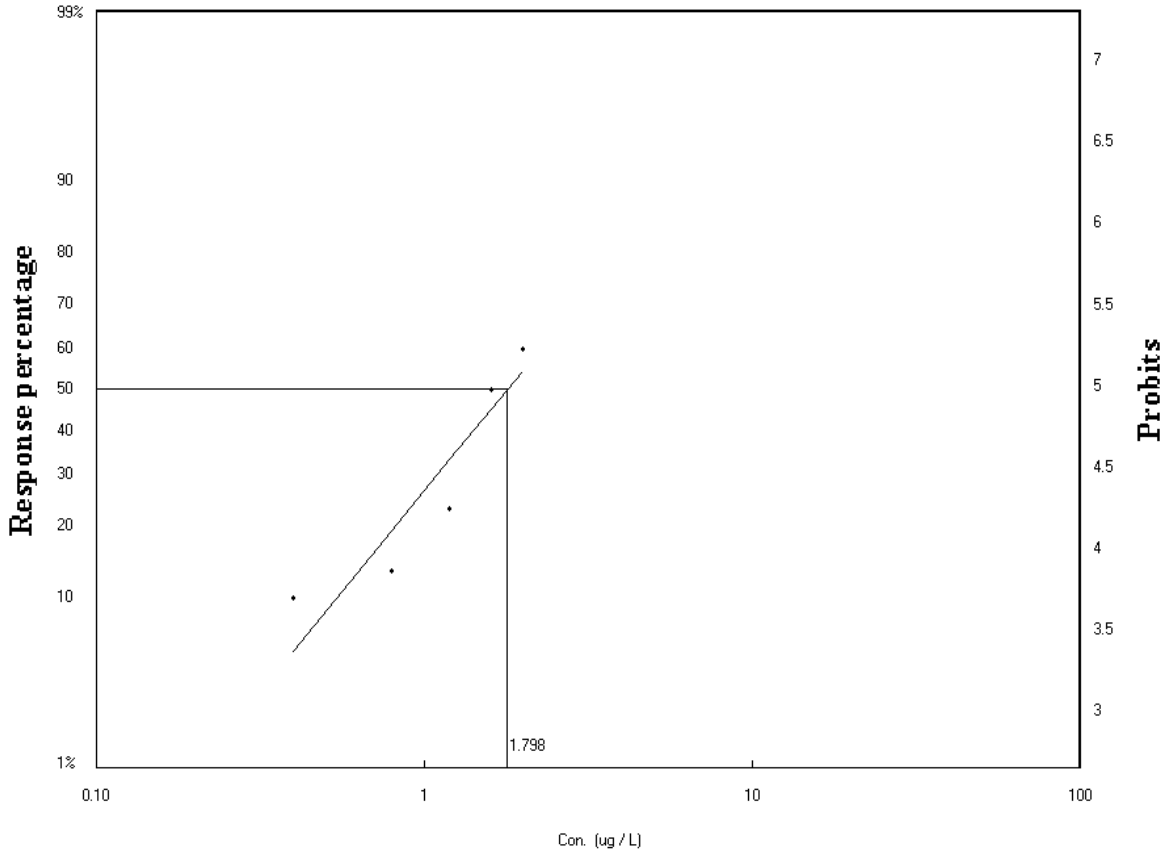


24 h

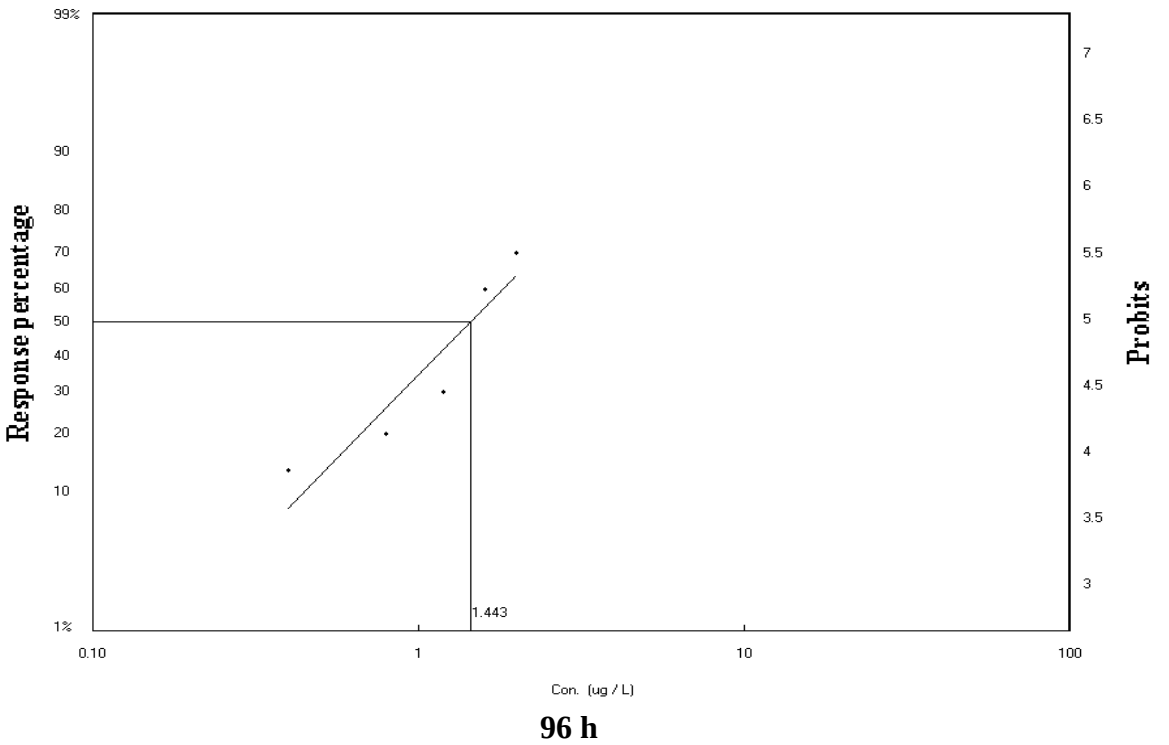


48 h

Fig 3.2 a Graphical representation of the median lethal concentrations (LC_{50}) of Chlorpyrifos to *L. vannamei* juveniles at the end of 24 and 48 h



72 h



96 h

Fig 3.2 b Graphical representation of the median lethal concentrations (LC_{50}) of Chlorpyrifos to *L. vannamei* juveniles at the end of 72 and 96 h

3.3.3 a Acute toxicity of Dimethoate to post larvae

Cumulative mortalities (%) observed in PL on exposure to different concentrations of DMT are represented in Table 3.7. There were no mortalities observed in control tanks during the 96 h duration of exposure, which indicated that the mortalities recorded in the test solutions were induced by DMT. Moreover, the mortalities observed in treatment tanks increased with the increasing exposure time as well as with the increasing concentrations of DMT (Table 3.7).

The graphical representation of median lethal concentration to PL at different time interval is presented in Fig. 3.3. The calculated LC_{50} values of DMT to PL were 480.52, 421.44, 374.79 and 357.84 µg L^{-1} at the end of exposure period 24, 48, 72 and 96 h, respectively and are represented in Table 3.8. The data of LC_{50} recorded were subjected to ANOVA to compare LC_{50} values at different time intervals ($P< 0.05$). DMT was found less toxic at initial exposure time of 24 h (LC_{50}= 480.52 µg L^{-1}) compared to the toxicity at 96 h of exposure (LC_{50}= 357.84 µg L^{-1}) ($P< 0.05$).

Low Observed Effective Concentration (LOEC) and No Observed Effective Concentration (NOEC) of DMT are represented in Table 3.9. The observed LOEC of DMT to PL was 200 µg L^{-1}, whereas the observed NOEC of DMT to PL was 100 µg L^{-1} (Table 3.9).

Table 3.7 Cumulative mortalities (%) (Mean ± SD) in post larvae of whiteleg shrimp (*L. vannamei*) exposed to various concentrations of Dimethoate at four time intervals

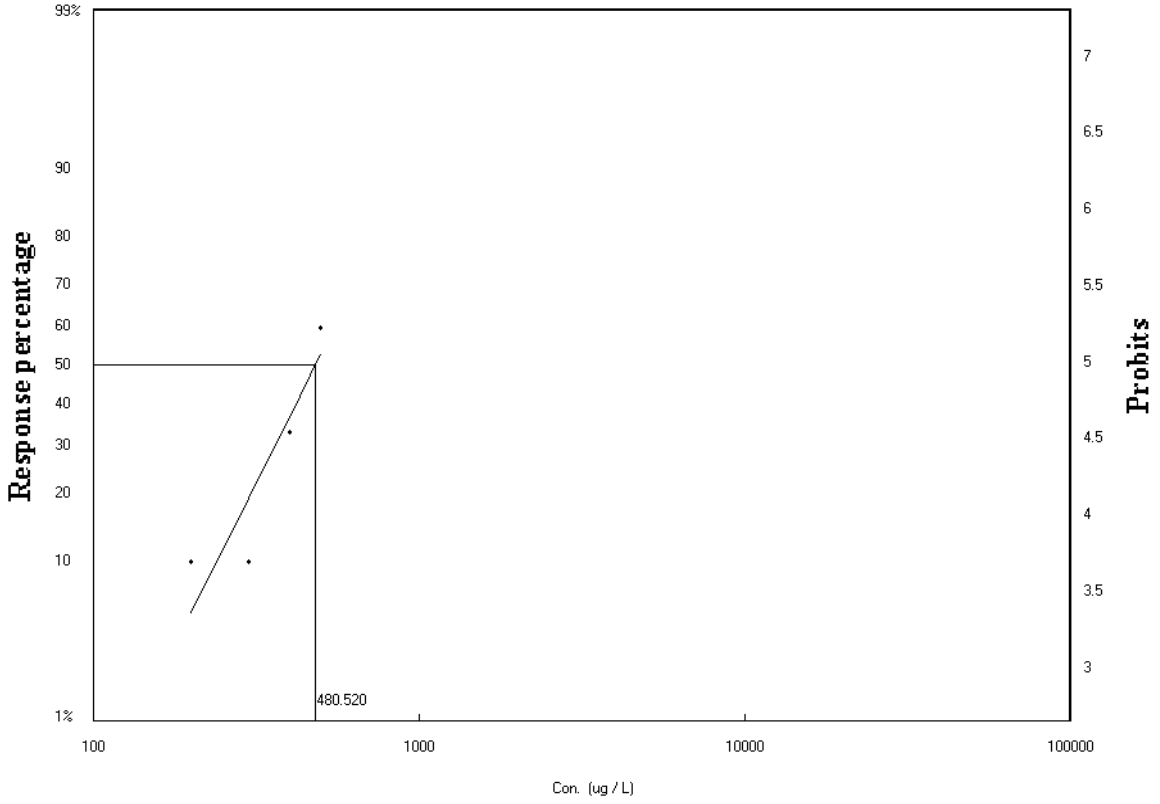
Concentrations (µg L^{-1})	24 h	48 h	72 h	96 h
Control	0	0	0	0
100	0	0	10 ± 0.67	10 ± 0.78
200	10 ± 1.2	10 ± 0.56	20 ± 1.23	20 ± 1.11
300	10 ± 0.97	20 ± 1.13	30 ± 2.15	30 ± 2.14
400	30 ± 2.10	40 ± 2.13	50 ± 3.45	50 ± 3.23
500	60 ± 2.33	70 ± 4.56	80 ± 6.45	80 ± 6.75

Table 3.8 Median lethal concentrations (LC_{50}) of Dimethoate with a 95% confidence interval at the end of four exposure durations (ANOVA, $P< 0.05$, different superscript letters indicate significant difference between each exposure durations)

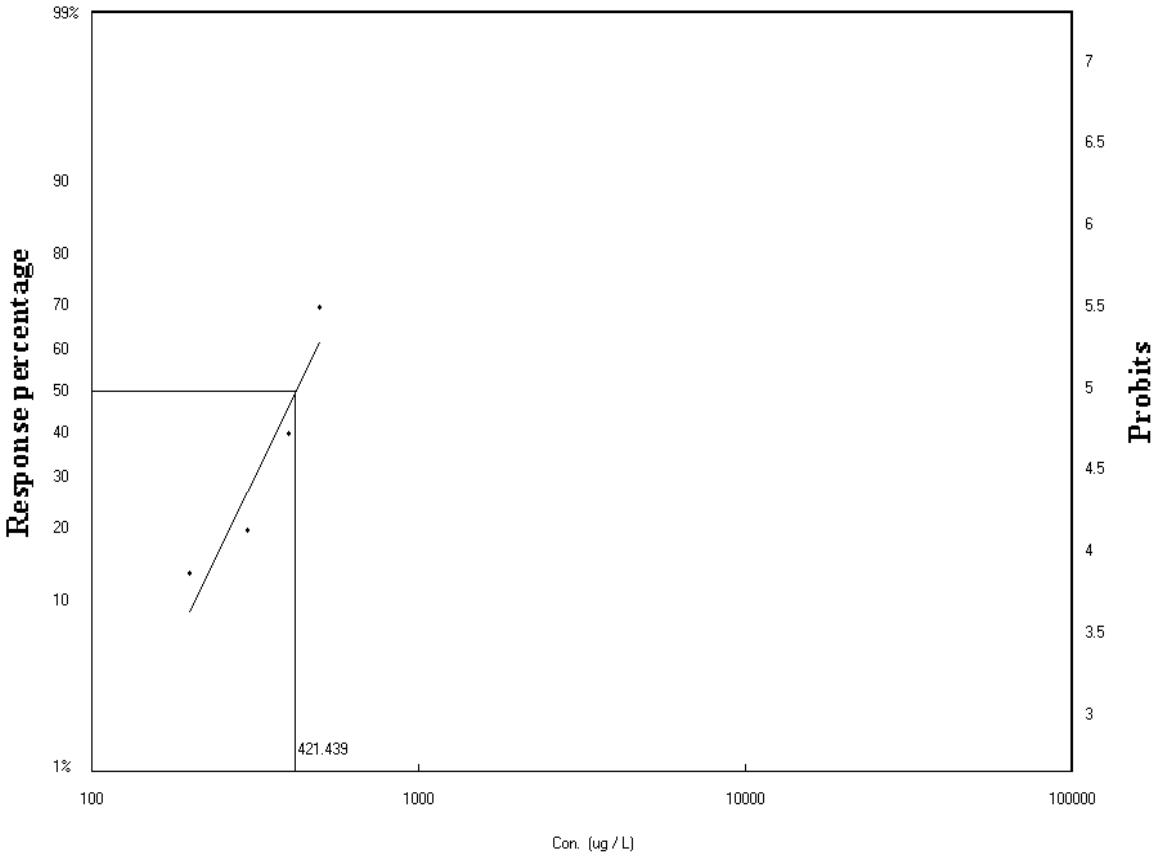
Exposure periods	LC_{50} value	Lower limit	Upper limit
24 h	480.52^{a}	382.11	523.45
48 h	421.44^{b}	384.64	489.21
72 h	374.79^{c}	368.11	452.52
96 h	357.84^{c}	288.21	411.28

Table 3.9 Low Observed Effective Concentration (LOEC) and No Observed Effective Concentration (NOEC) values of Dimethoate in *L. vannamei* post larvae

Observed values	µg L^{-1}
LOEC	200
NOEC	100

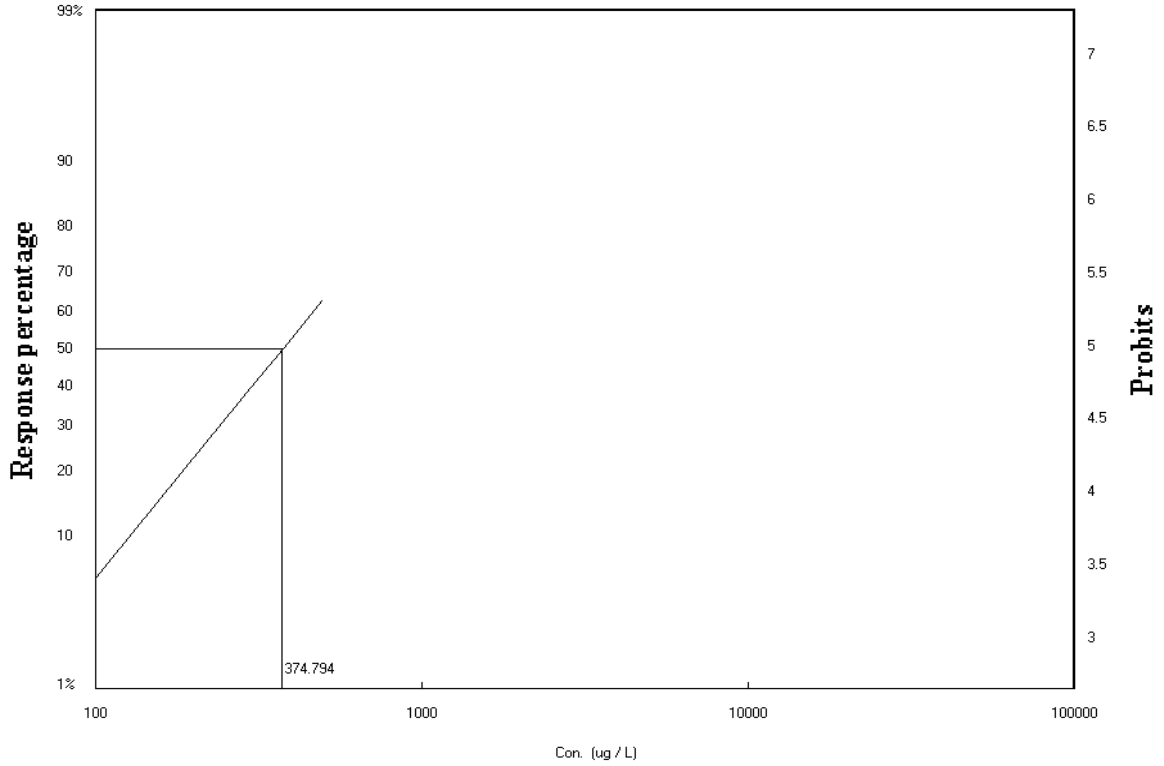


24 h

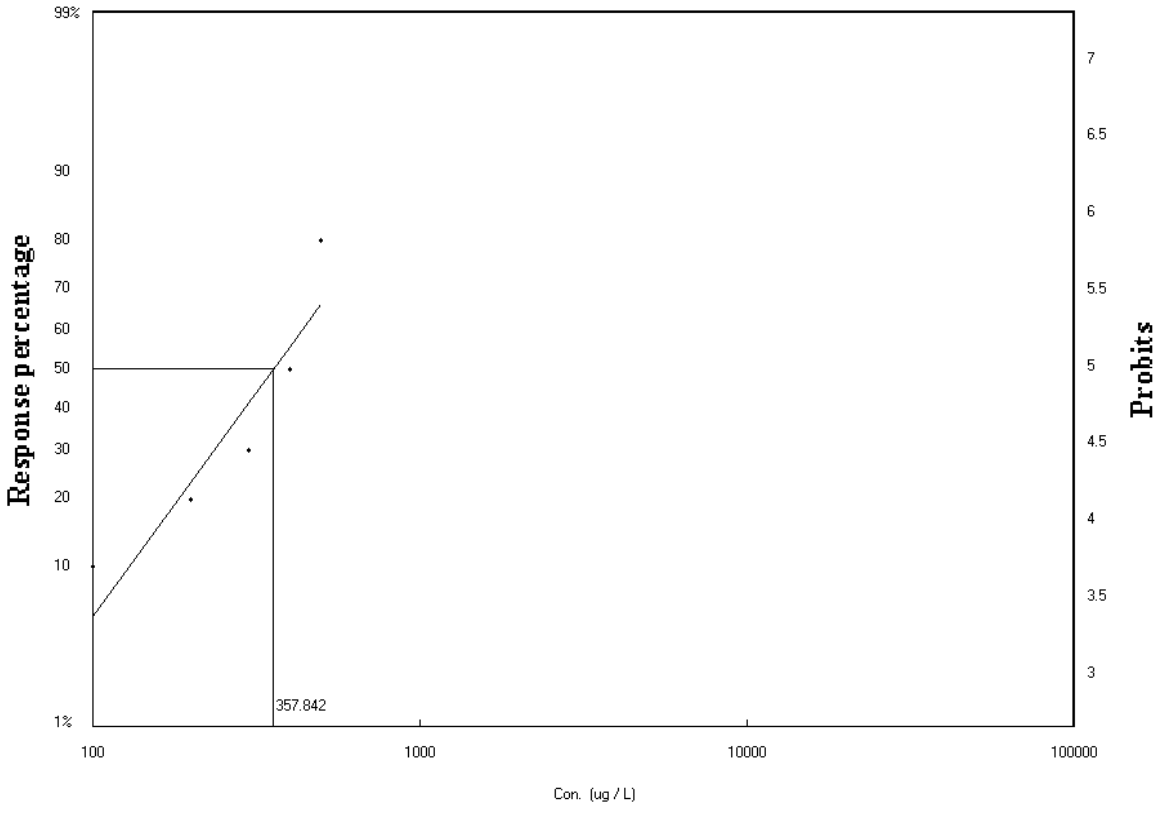


48 h

Fig 3.3 a Graphical representation of the median lethal concentrations (LC_{50}) of Dimethoate to *L. vannamei* post larvae at the end of 24 and 48 h



72 h



96 h

Fig 3.3 b Graphical representation of the median lethal concentrations (LC_{50}) of Dimethoate to *L. vannamei* post larvae at the end of 72 and 96 h

3.3.3 b Acute toxicity of Dimethoate to juveniles

Cumulative mortalities (%) observed in JL on exposure to different concentrations of DMT are represented in Table 3.10. There were no mortalities observed in control tanks during the 96 h duration of exposure, which indicated that the mortalities recorded in the test solutions were induced by DMT. However, the mortalities observed in treatment tanks were increased with the increasing exposure time as well as with the increasing concentrations of DMT (Table 3.10).

The graphical representation of median lethal concentration to JL at different time interval is presented in Fig. 3.4. The calculated LC_{50} values of DMT to JL were 1039.04, 785.37, 733.02 and 563.61 µg L^{-1} at the end of exposure period 24, 48, 72 and 96 h, respectively and are represented in Table 3.11. The data of LC_{50} recorded were subjected to ANOVA to compare LC_{50}values at different time intervals ($P< 0.05$).

Low Observed Effective Concentration (LOEC) and No Observed Effective Concentration (NOEC) of DMT are represented in Table 3.12. The observed LOEC of DMT to JL was 400 µg L^{-1} whereas, the observed NOEC of DMT to JL was 200 µg L^{-1}(Table 3.12)

Table 3.10 Cumulative mortalities (%) (Mean ± SD) in juveniles of whiteleg shrimp (*L. vannamei*) exposed to various concentrations of Dimethoate at time intervals

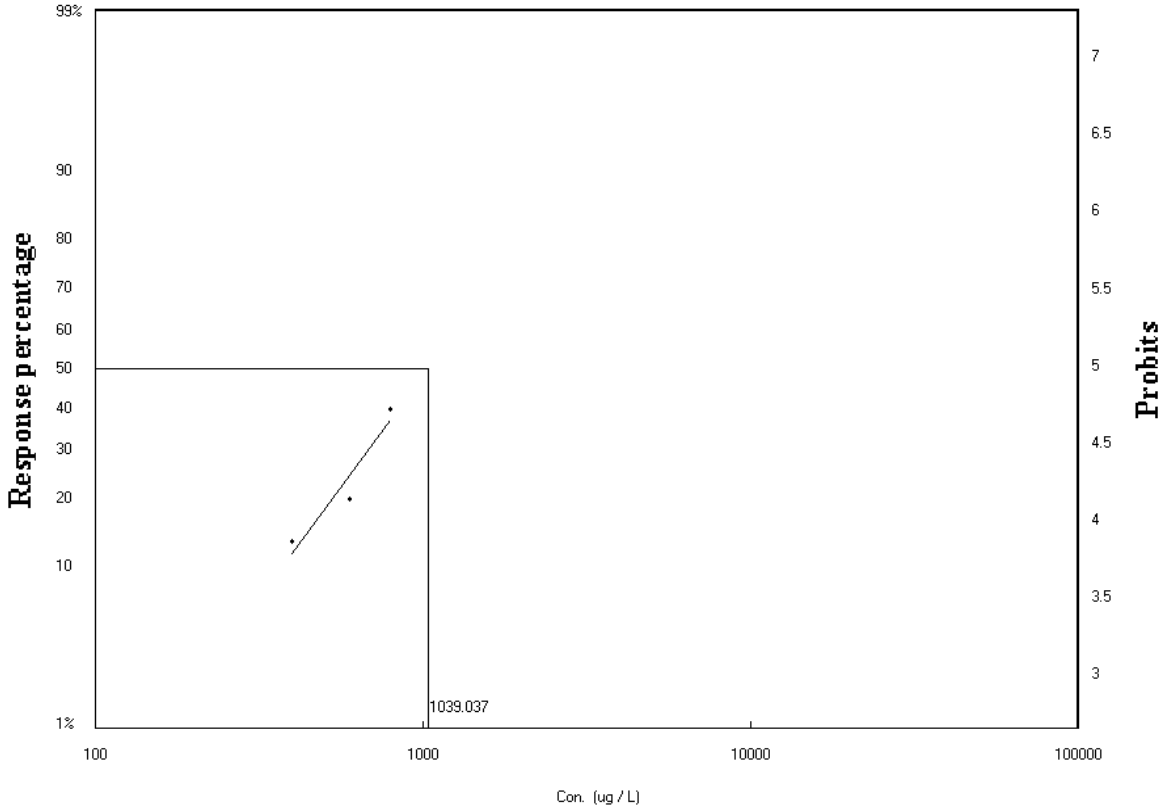
Concentrations (µg L^{-1})	**24 h**	**48 h**	**72 h**	**96 h**
Control	0	0	0	0
100	0	0	0	0
200	0	0	0	10 ± 0.89
400	10 ± 0.92	10 ± 0.45	20 ± 1.34	30 ± 2.11
600	20 ± 2.11	30 ± 2.16	30 ± 2.34	40 ± 2.67
800	40 ± 2.33	50 ± 4.43	60 ± 4.56	80 ±6.45

Table 3.11 Median lethal concentrations (LC_{50}) of Dimethoate with a 95% confidence interval at the end of four exposure durations (ANOVA, P< 0.05, different superscript letters indicate significant difference between each exposure durations)

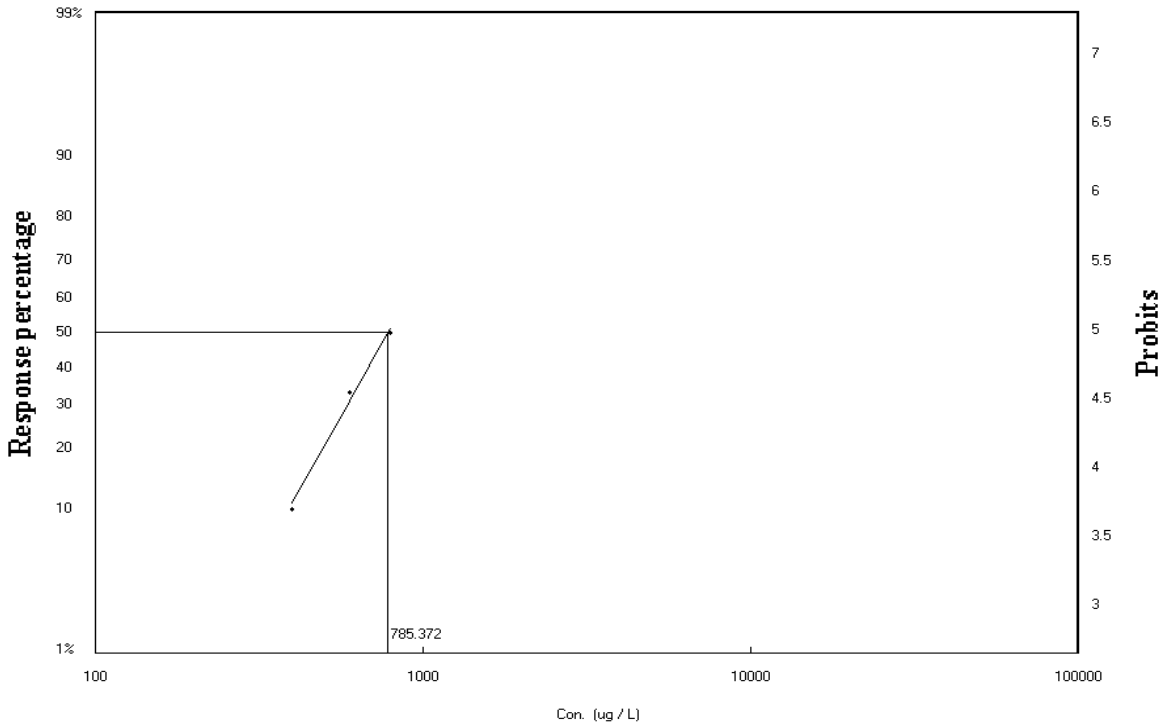
Exposure periods	**LC_{50} value**	**Lower limit**	**Upper limit**
24 h	1039.04^{a}	849.84	1716.81
48 h	785.37^{b}	712.38	916.27
72 h	733.02^{c}	684.14	874.23
96 h	563.61^{d}	456.11	676.45

Table 3.12 Low Observed Effective Concentration **(**LOEC) and No Observed Effective Concentration (NOEC) values of Dimethoate in *L. vannamei* juveniles

Observed values	**µg L^{-1}**
LOEC	400
NOEC	200

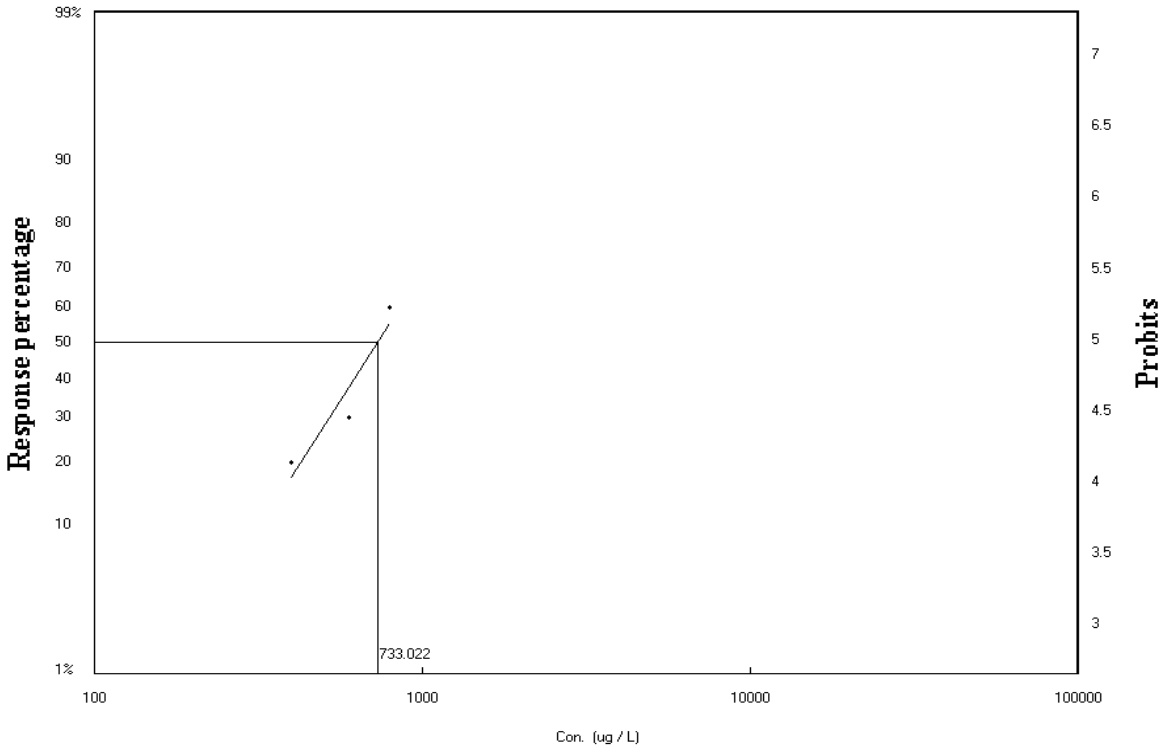


24 h

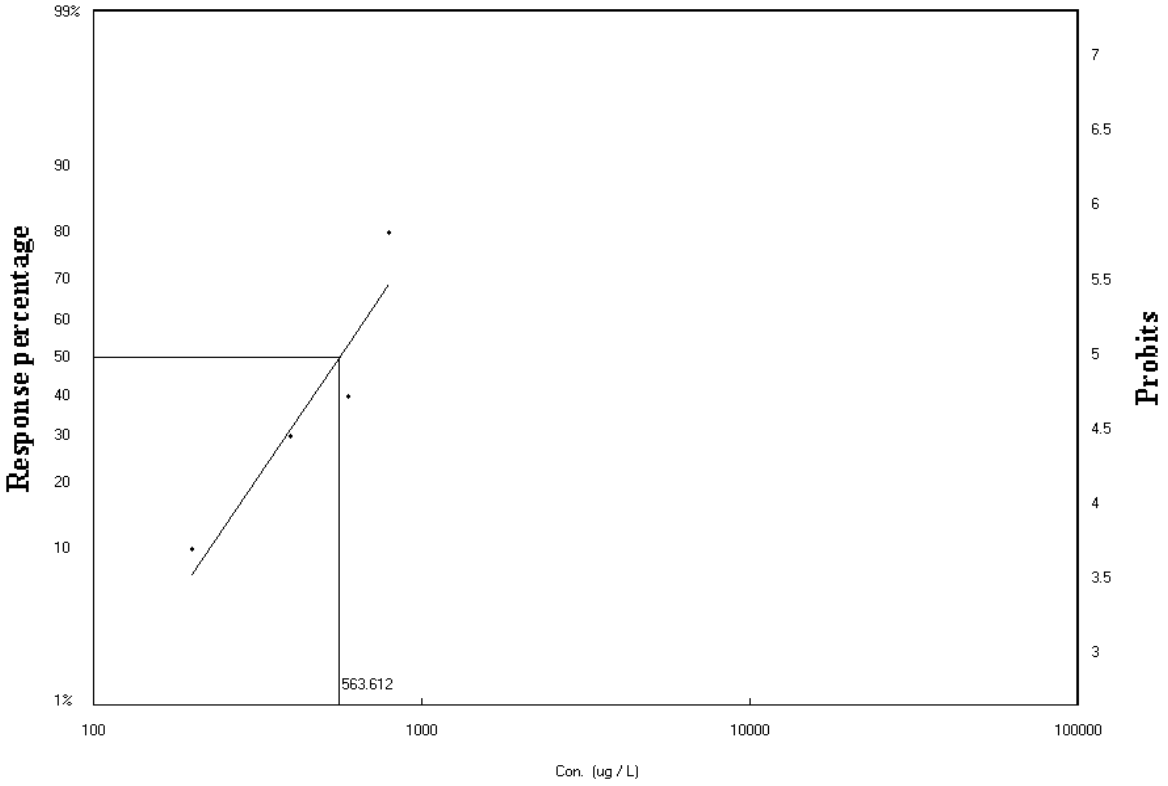


48 h

Fig 3.4 a Graphical representation of the median lethal concentrations (LC_{50}) of Dimethoate to *L. vannamei* juveniles at the end of 24 and 48 h



72 h



96 h

Fig 3.4 b Graphical representation of the median lethal concentrations (LC_{50}) of Dimethoate to *L. vannamei* juveniles at the end of 72 and 96 h

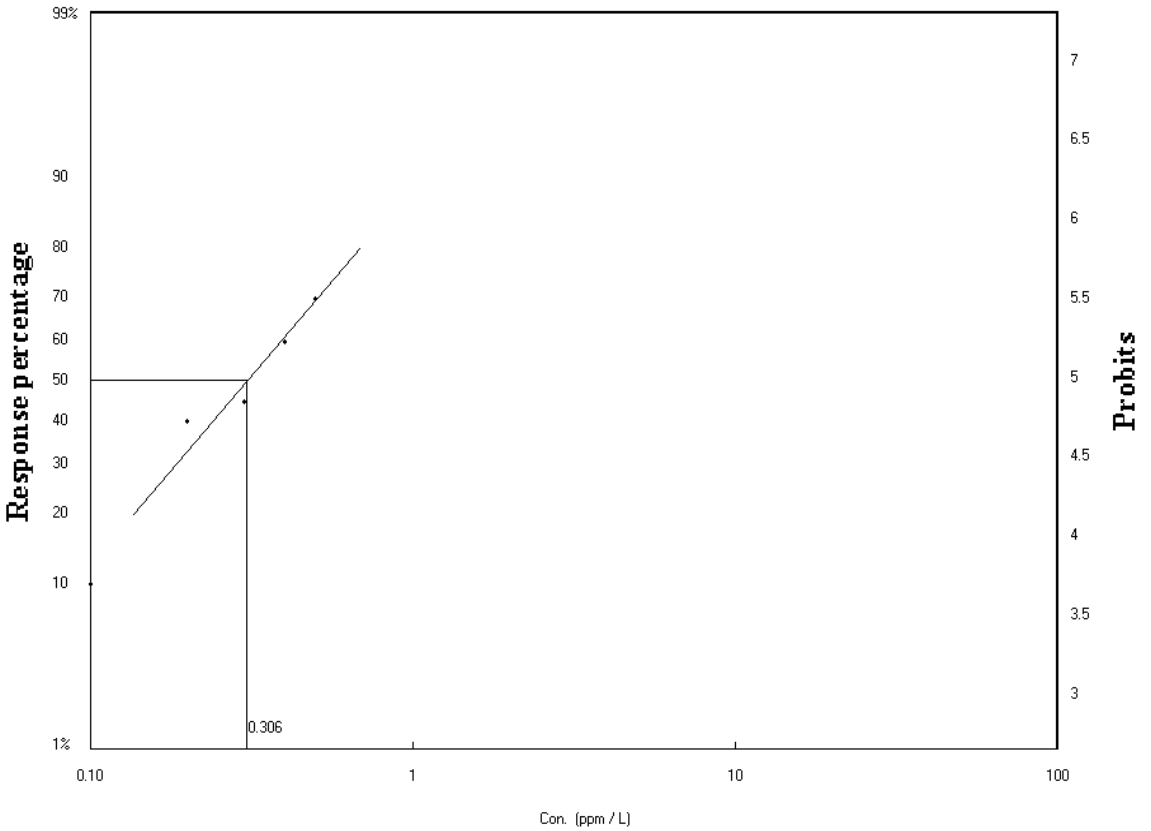
3.3.4 Influence of salinity and temperature on the acute toxicity of Chlorpyrifos and Dimethoate in post larvae and juveniles of *L. vannamei*

3.3.4.1 a Influence of salinity on acute toxicity of Chlorpyrifos in post larvae

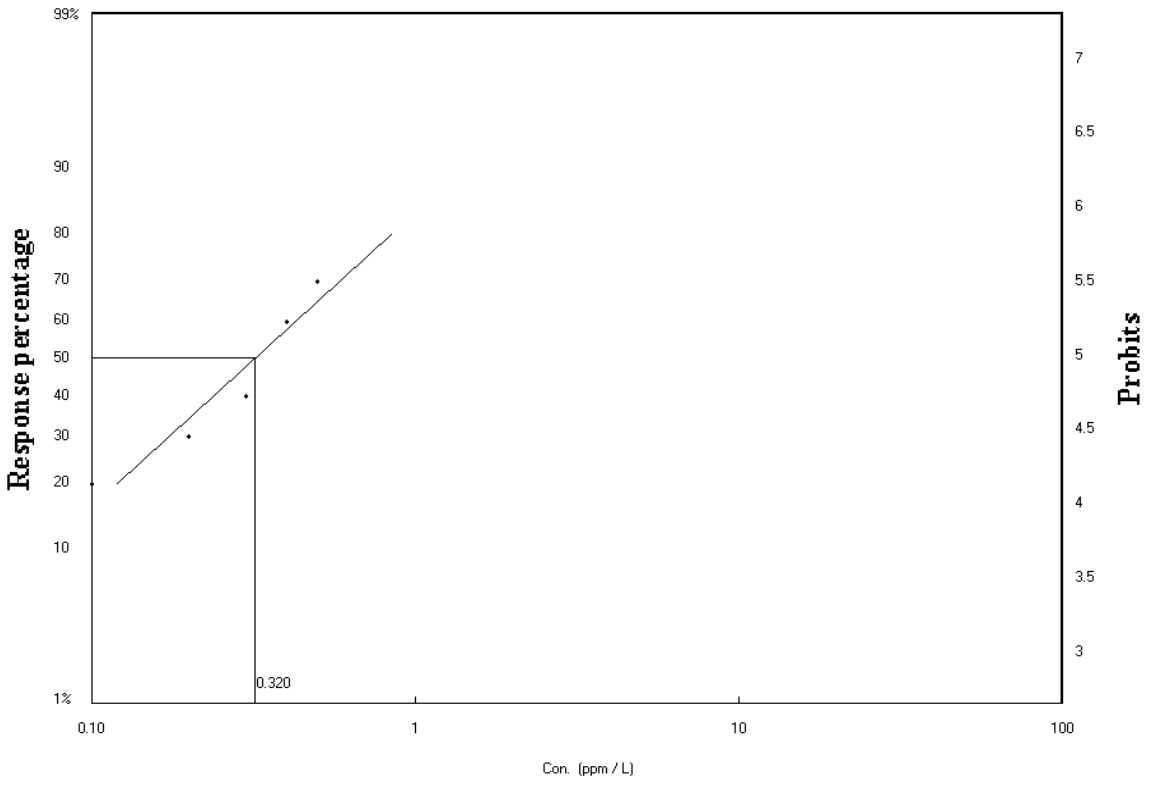
The median lethal concentration (LC_{50}) of CPF to PL at 5, 15 and 25 ppt salinity at the end of 96 h are represented in Table 3.13 and are graphically presented in Fig. 3.5. The 96 h LC_{50} values of the CPF exposed PL found to be 0.31, 0.32 and 0.92 µg L^{-1} at 5, 15 and 25 ppt salinity, respectively. Results of ANOVA followed by Tukey–Kramer multiple comparison tests revealed that the 96 h LC_{50} values of CPF at 25 ppt was found to be significantly lower when compared to 5 and 15 ppt salinity ($P< 0.05$).

Table 3.13 Median lethal concentrations (LC_{50}) of Chlorpyrifos with a 95% confidence interval to post larvae at the salinity of 5, 15 and 25 ppt after 96 h of exposure at 28 ºC temperature (ANOVA, $P< 0.05$, different superscript letters indicate significant difference between each exposure period)

Post larvae	**Chlorpyrifos**	**95% Confidence interval (µg L^{-1})**	
Salinity (ppt)	**96 h LC_{50} (µg L^{-1})**	**Lower limit**	**Upper limit**
5	0.31^{a}	0.16	0.44
15	0.32^{a}	0.24	0.46
25	0.92^{b}	0.75	1.32



5 ppt



15 ppt

Fig 3.5 a Graphical representation of the median lethal concentrations (LC_{50}) of Chlorpyrifos to post larvae after 96 h at salinities of 5 and 15 ppt at 28 ºC temperature

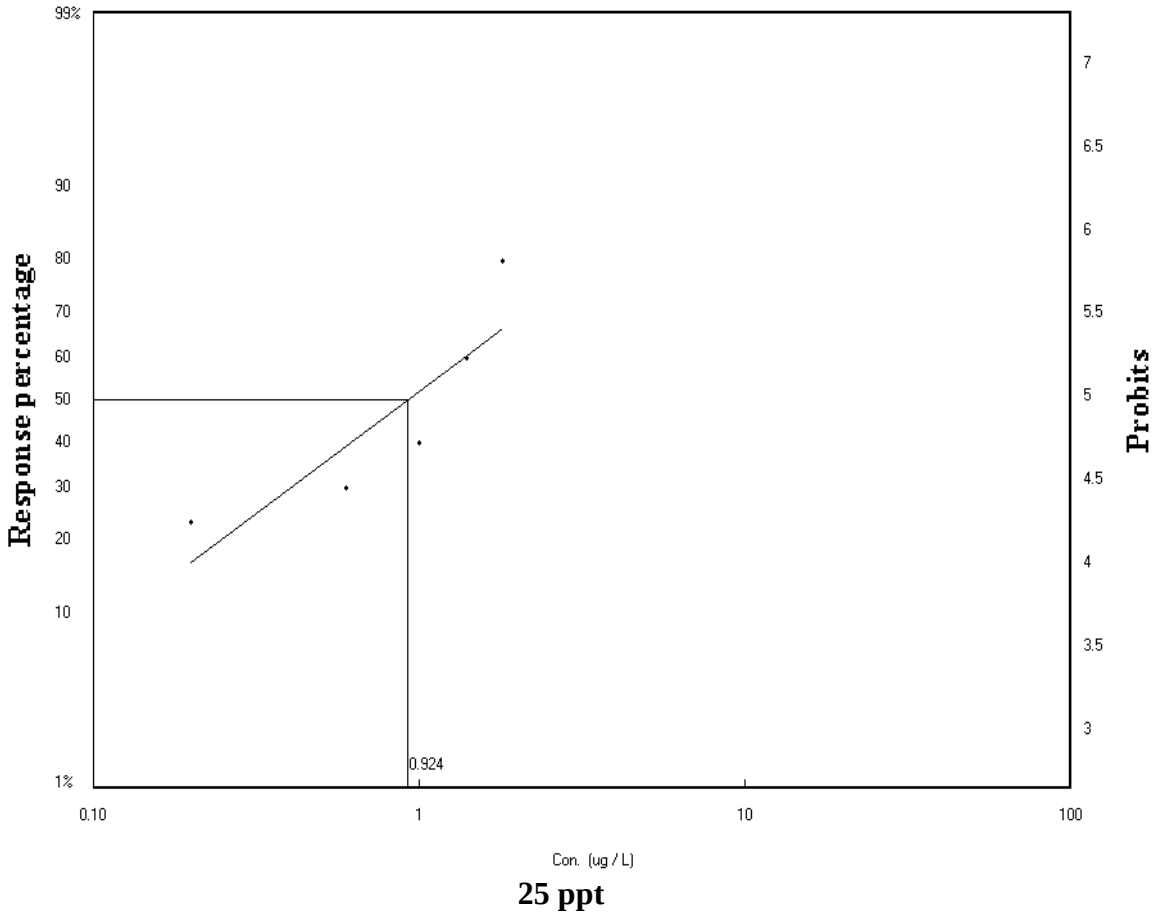


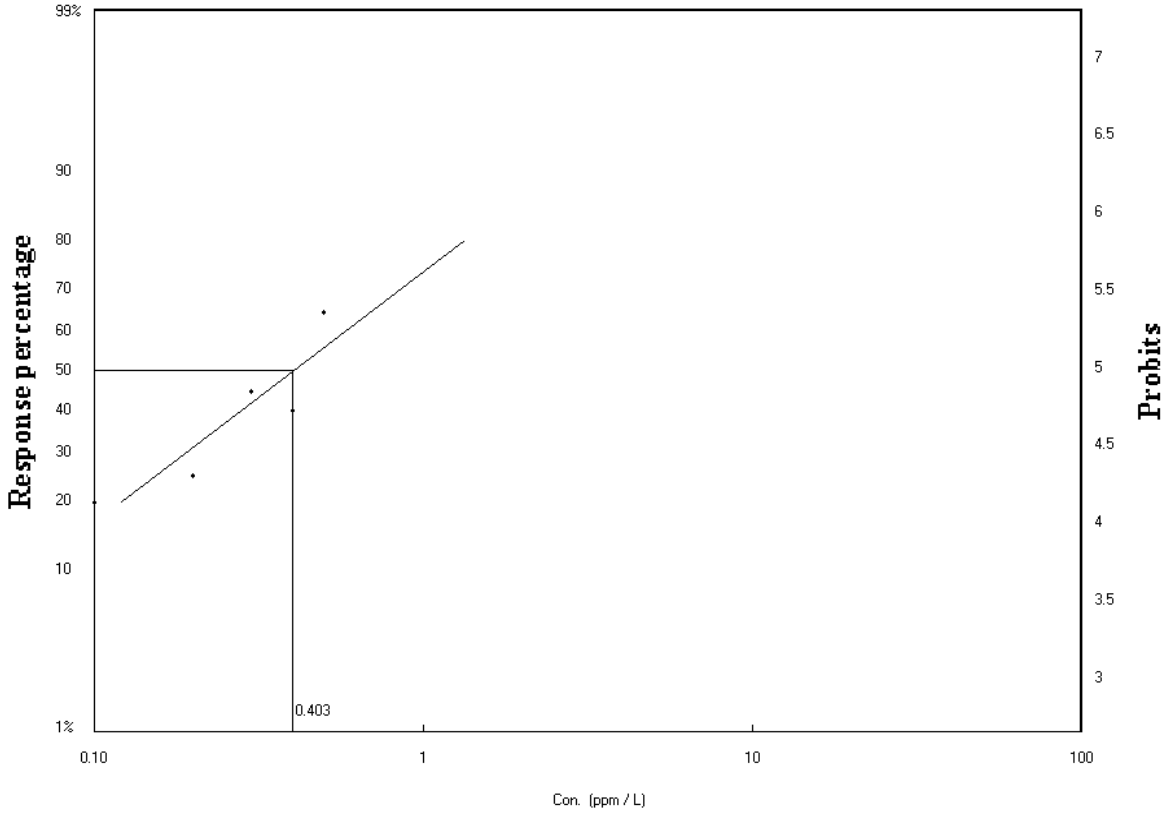
Fig 3.5 b Graphical representation of the median lethal concentrations (LC_{50}) of Chlorpyrifos to post larvae after 96 h at salinity of 25 ppt at 28 ºC temperature

3.3.4.1 b Influence of salinity on acute toxicity of Chlorpyrifos in juveniles

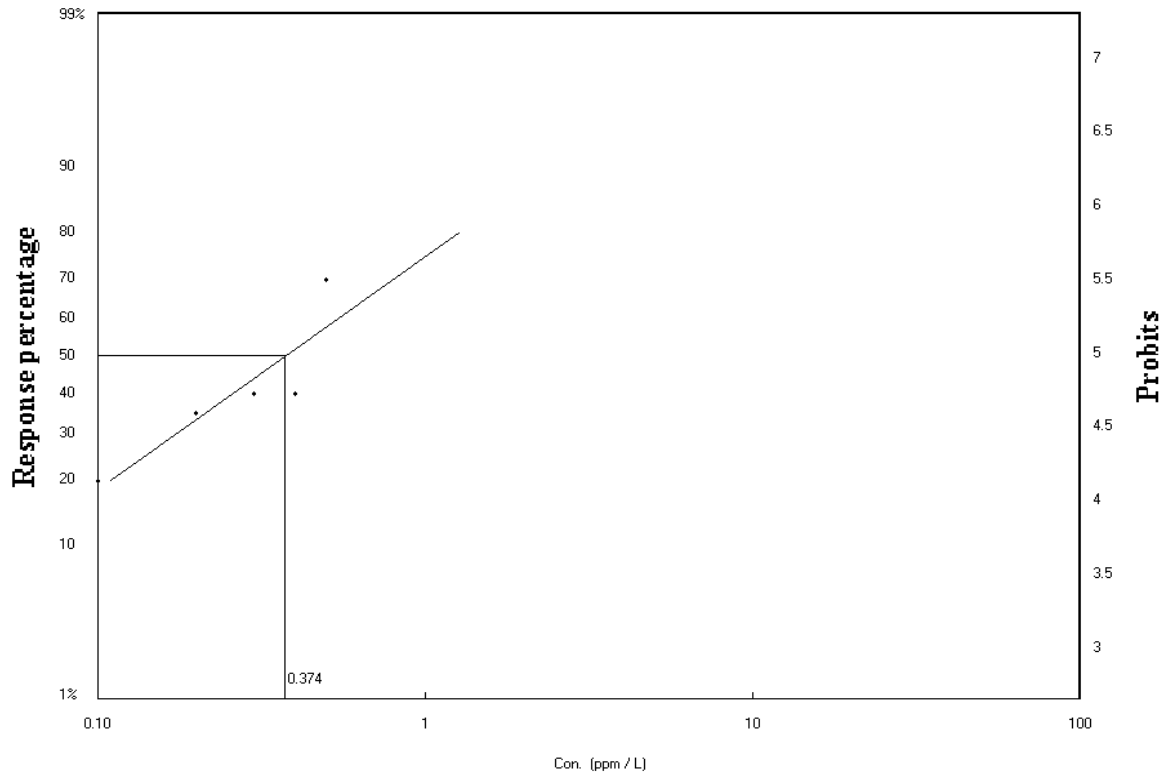
The median lethal concentration (LC_{50}) of CPF to JL at 5, 15 and 25 ppt salinity at the end of 96 h are represented in Table 3.14 and are graphically presented in Fig. 3.6. The 96 h LC_{50} values of the CPF exposed JL found to be 0.40, 0.37 and 1.68 µg L^{-1}at 5, 15 and 25 ppt salinity, respectively. Results of ANOVA followed by Tukey–Kramer multiple comparison tests revealed that the 96 h LC_{50} values of CPF at 25 ppt was found to be significantly lower when compared to 5 and 15 ppt salinity ($P< 0.05$).

Table 3.14 Median lethal concentrations (LC_{50}) of Chlorpyrifos with a 95% confidence interval to juveniles at the salinity of 5, 15 and 25 ppt after 96 h of exposure at 28 ºC temperature (ANOVA, $P< 0.05$, different superscript letters indicate significant difference between each exposure period)

Juvenile	**Chlorpyrifos**	**95% Confidence interval (µg L^{-1})**	
Salinity (ppt)	**96 h LC_{50} (µg L^{-1})**	**Lower limit**	**Upper limit**
5	0.40^{a}	0.35	0.53
15	0.37^{a}	0.33	0.42
25	1.68^{b}	1.54	2.52

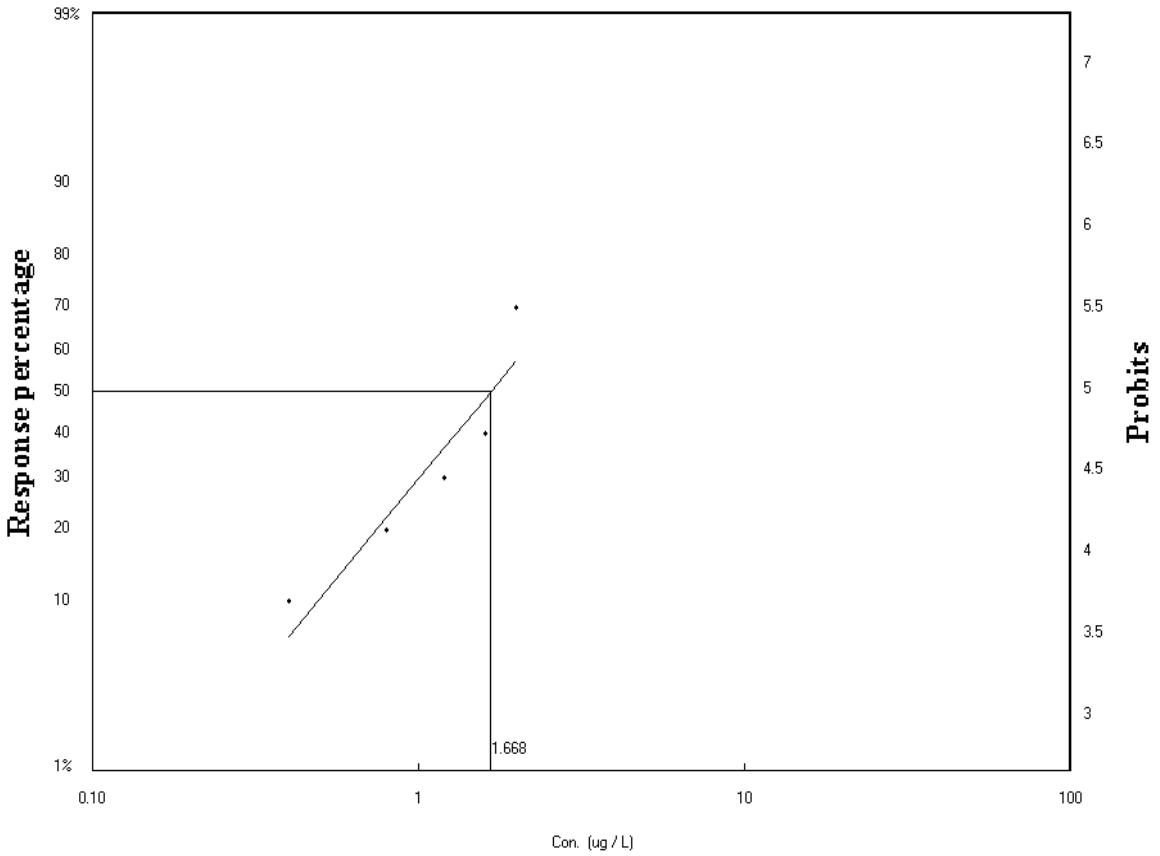


5 ppt



15 ppt

Fig 3.6 a Graphical representation of the median lethal concentrations (LC_{50}) of Chlorpyrifos to juveniles after 96 h at the salinities of 5 and 15 ppt at 28 ºC temperature



25 ppt

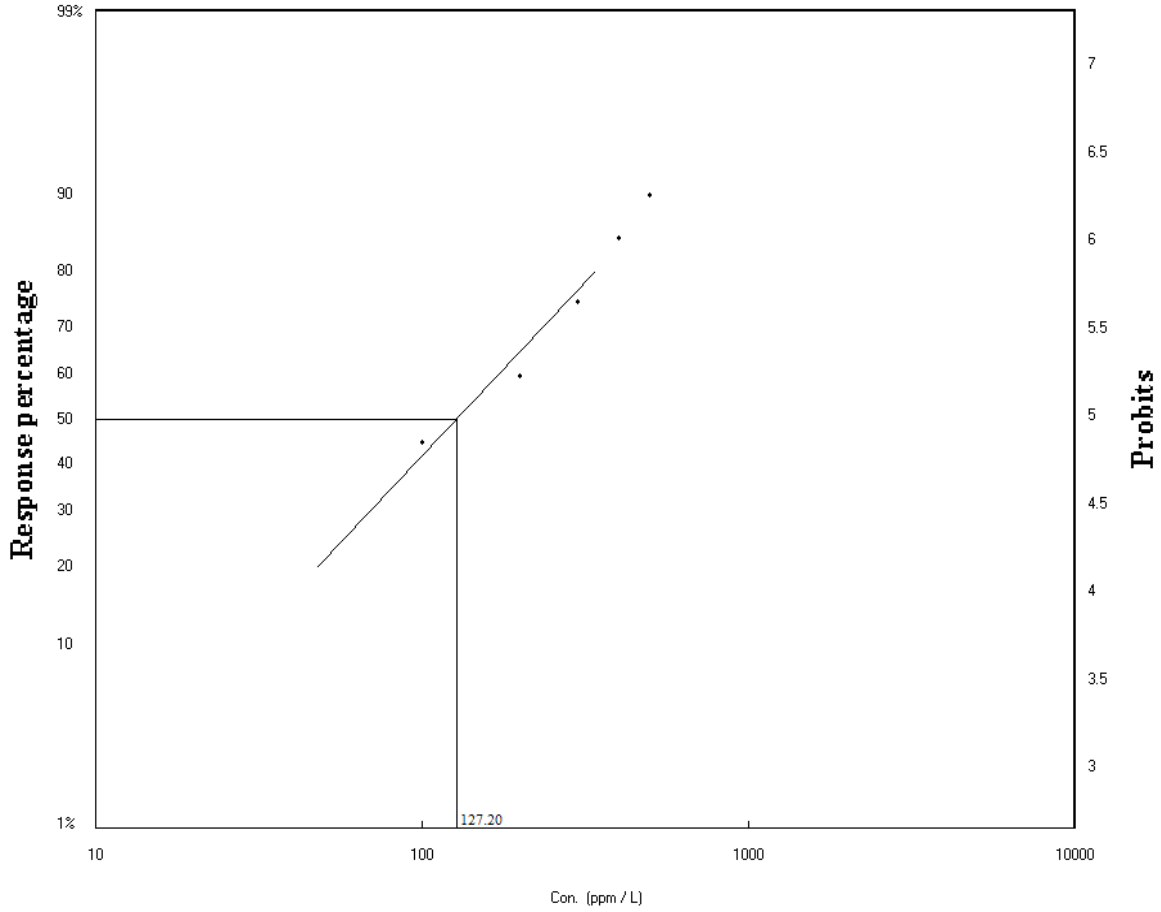
Fig 3.6 b Graphical representation of the median lethal concentrations (LC_{50}) of Chlorpyrifos to juveniles after 96 h at the salinity of 25 ppt at 28 ºC temperature

3.3.4.2 a Influence of salinity on acute toxicity of Dimethoate in post larvae

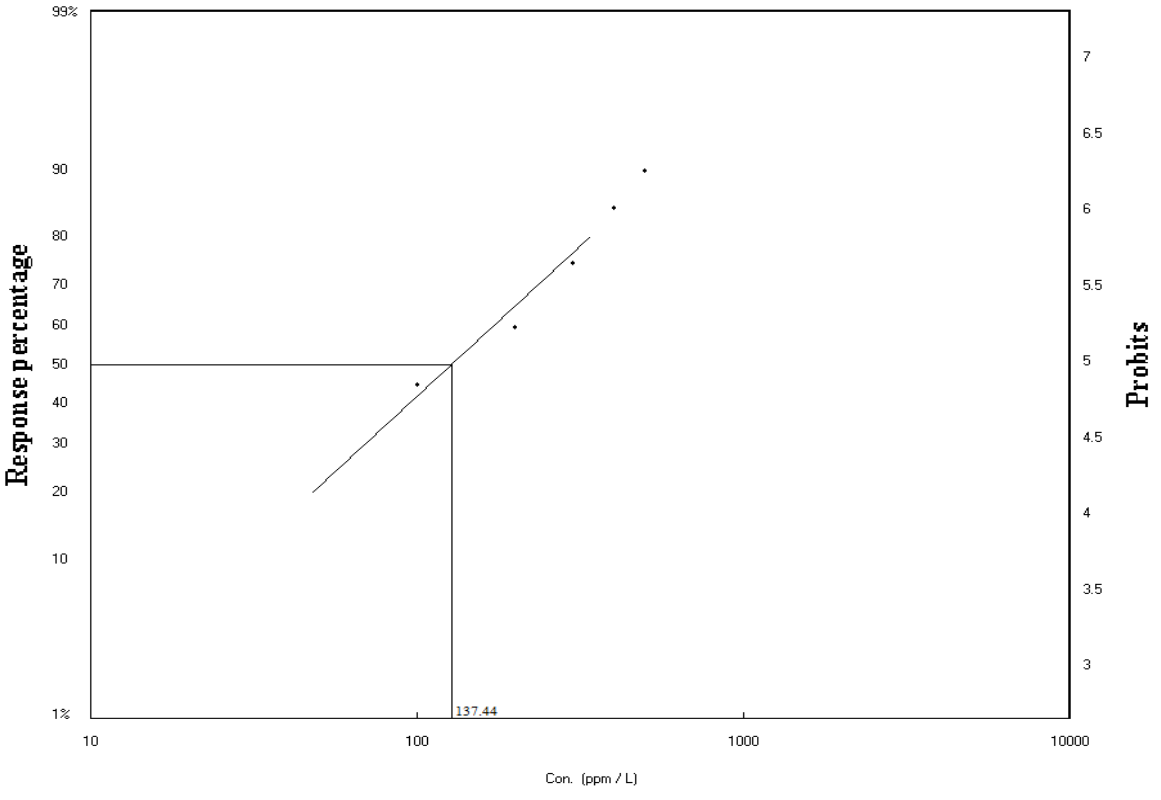
The median lethal concentration (LC_{50}) of DMT to PL at 5, 15 and 25 ppt salinity at the end of 96 h are represented in Table 3.15 and are graphically presented in Fig. 3.7. The 96 h LC_{50} values of the DMT exposed PL found to be 103.11, 321.26 and 182.76 µg L^{-1}at 5, 15 and 25 ppt salinity, respectively. Results of ANOVA followed by Tukey–Kramer multiple comparison tests revealed that the 96 h LC_{50} values of DMT at 25 ppt was found to be significantly lower when compared to 5 and 15 ppt salinity ($P< 0.05$).

Table 3.15 Median lethal concentrations (LC_{50}) of Dimethoate with a 95% confidence interval to post larvae at the salinity of 5, 15 and 25 ppt after 96 h of exposure at 28 ºC temperature (ANOVA, $P< 0.05$, different superscript letters indicate significant difference between each exposure period)

Post larvae	**Dimethoate**	**95% Confidence interval (µg L^{-1})**	
Salinity (ppt)	**96 h LC_{50} (µg L^{-1})**	**Lower limit**	**Upper limit**
5	127.20^{a}	71.22	160.71
15	137.44^{b}	110.13	692.15
25	386.59^{c}	300.53	210.48

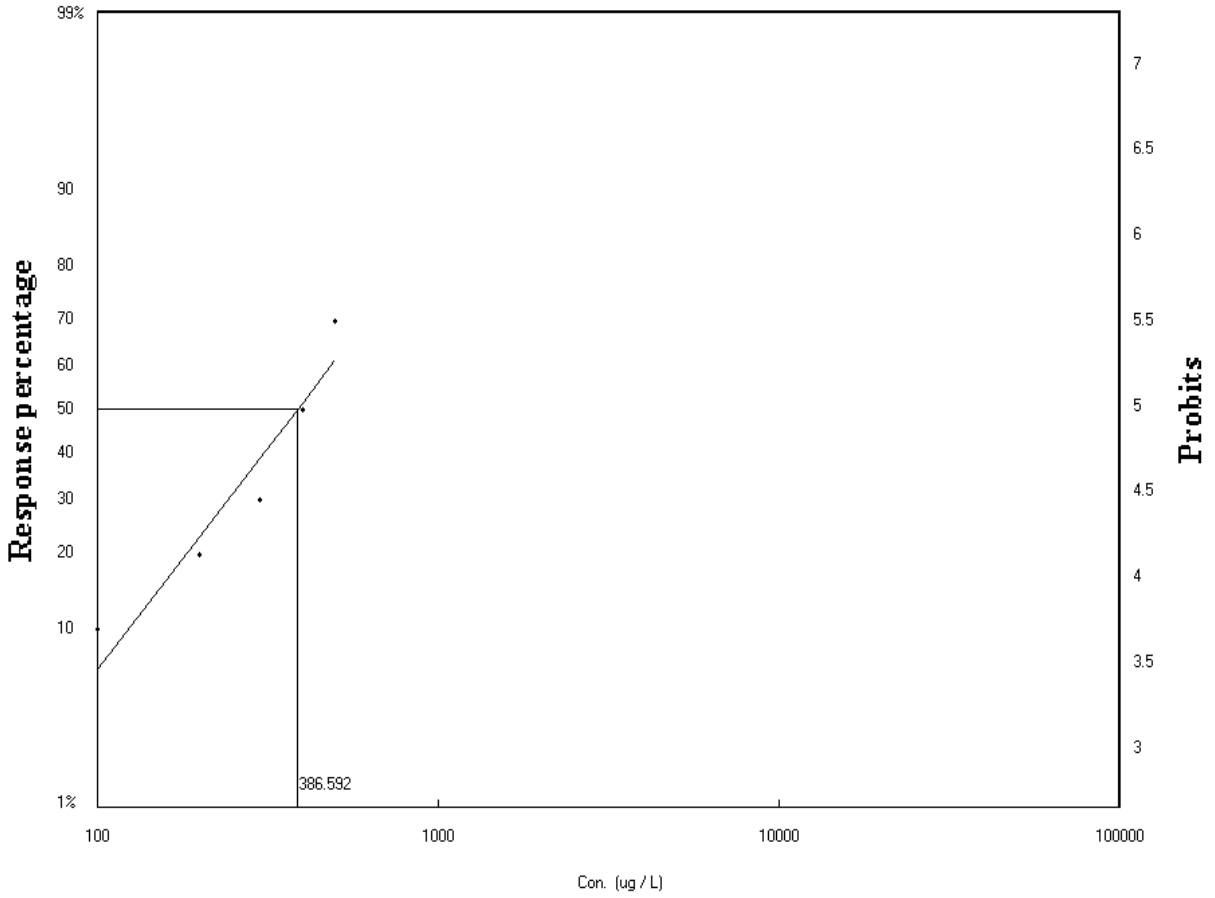


5 ppt



15 ppt

Fig 3.7 a Graphical representation of the median lethal concentrations (LC_{50}) of Dimethoate to post larvae after 96 h at the different salinities of 5 and 15 ppt at 28 ºC temperature



25 ppt

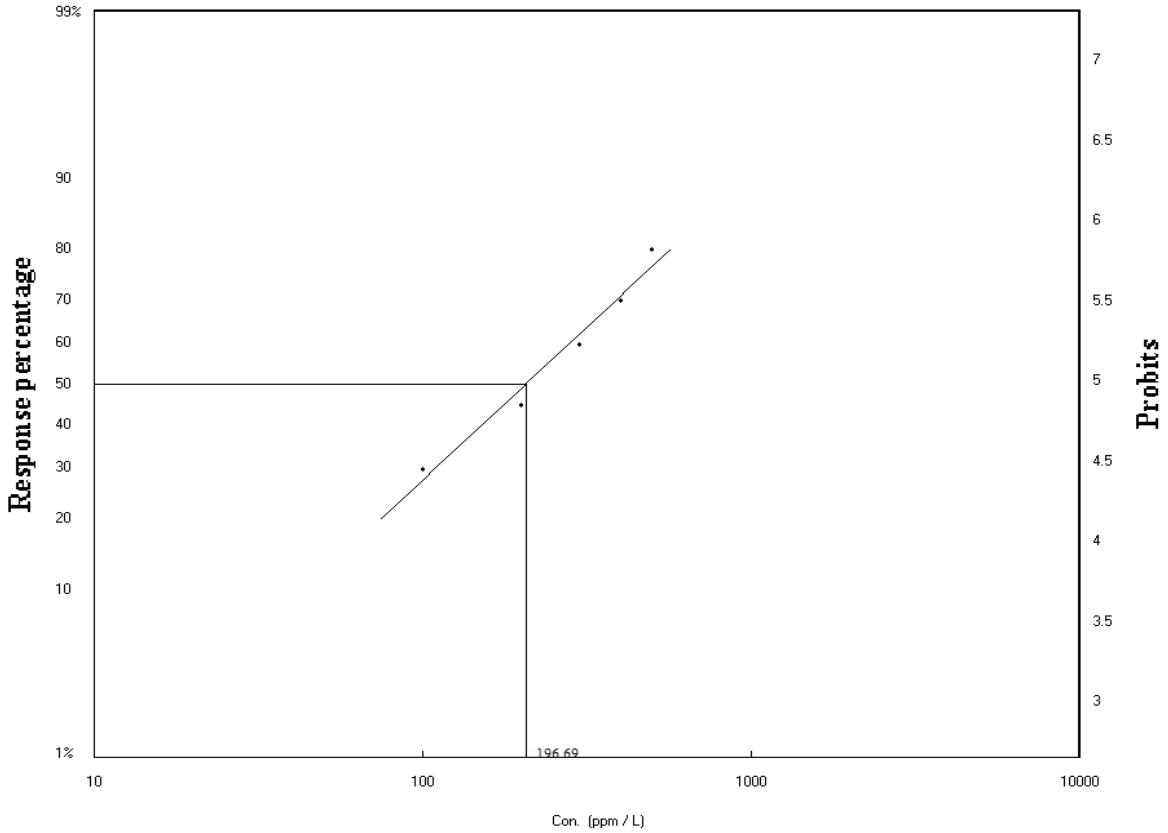
Fig 3.7 b Graphical representation of the median lethal concentrations (LC_{50}) of Dimethoate to post larvae after 96 h at the salinity of 25 ppt at 28 ºC temperature

3.3.4.2 b Influence of salinity on acute toxicity of Dimethoate in juveniles

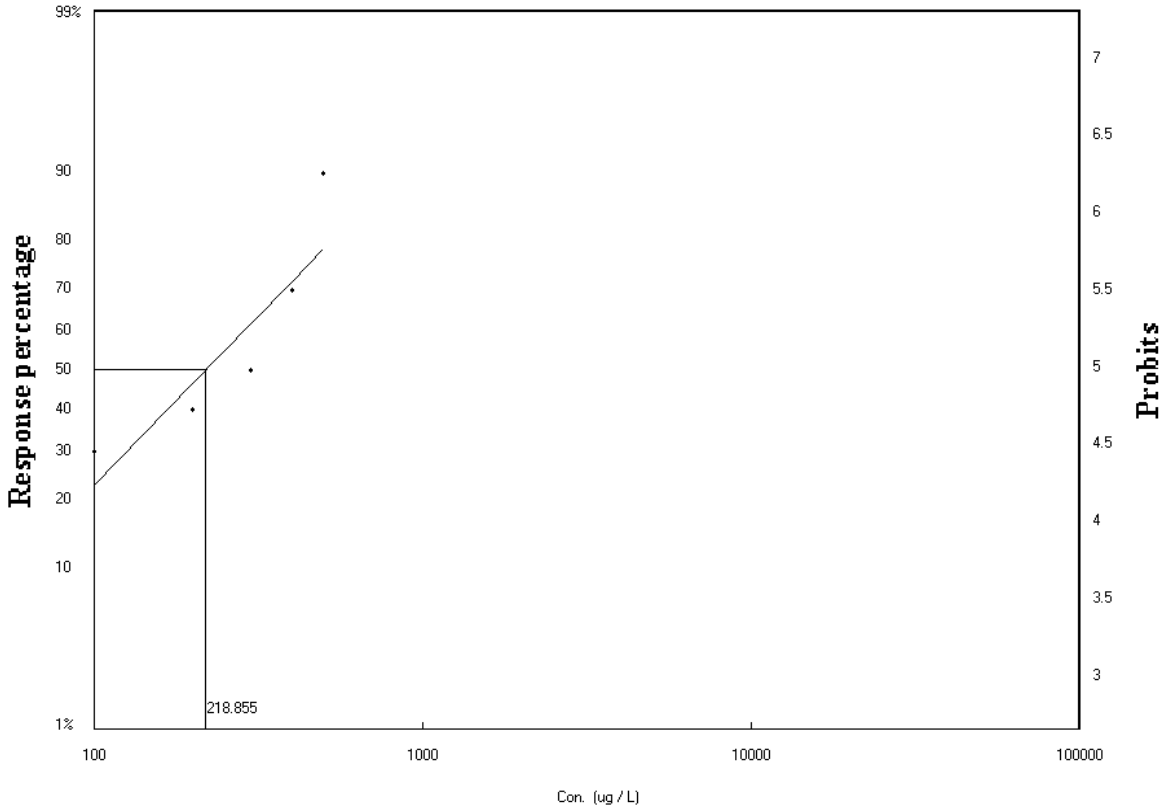
The median lethal concentration (LC_{50}) of DMT to JL at 5, 15 and 25 ppt salinity at the end of 96 h are represented in Table 3.16 and are graphically presented in Fig. 3.8. The 96 h LC_{50} values of the DMT exposed JL found to be 196.69, 218.86 and 691.51 µg L^{-1}at 5, 15 and 25 ppt salinity, respectively. Results of ANOVA followed by Tukey–Kramer multiple comparison tests revealed that the 96 h LC_{50} values of DMT at 25 ppt was found to be significantly higher when compared to 5 and 15 ppt salinity ($P< 0.05$).

Table 3.16 Median lethal concentrations (LC_{50}) of Dimethoate with a 95% confidence interval to juveniles at the salinity of 5, 15 and 25 ppt after 96 h of exposure at 28 ºC temperature (ANOVA, $P< 0.05$, different superscript letters indicate significant difference between each exposure period)

Juvenile	**Dimethoate**	**95% Confidence interval (µg L^{-1})**	
Salinity (ppt)	**96 h LC_{50} (µg L^{-1})**	**Lower limit**	**Upper limit**
5	196.69[a]	161.64	229.51
15	218.86[b]	185.22	298.41
25	691.51[c]	560.44	832.42

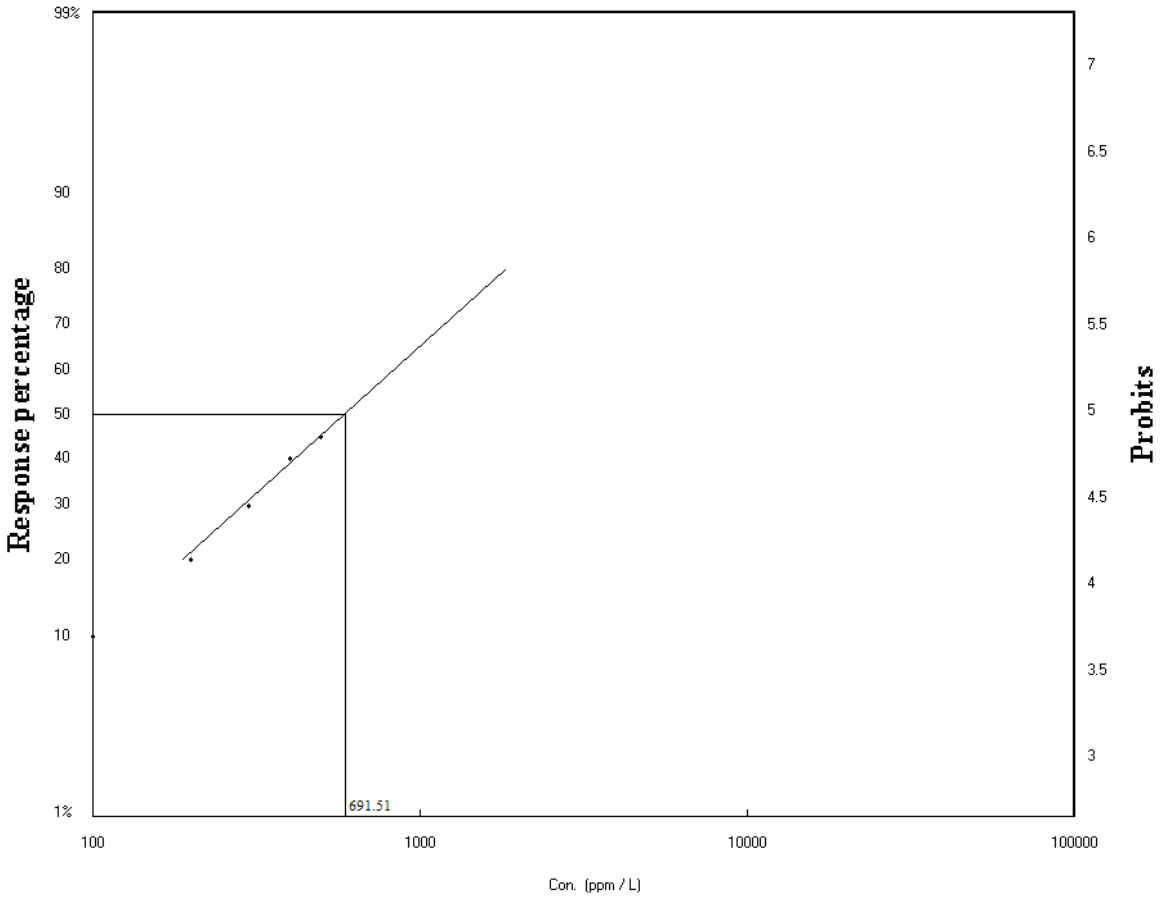


5 ppt



15 ppt

Fig 3.8 a Graphical representation of the median lethal concentrations (LC_{50}) of Dimethoate to juveniles after 96 h at the salinities of 5 and 15 ppt at 28 ºC temperature



25 ppt

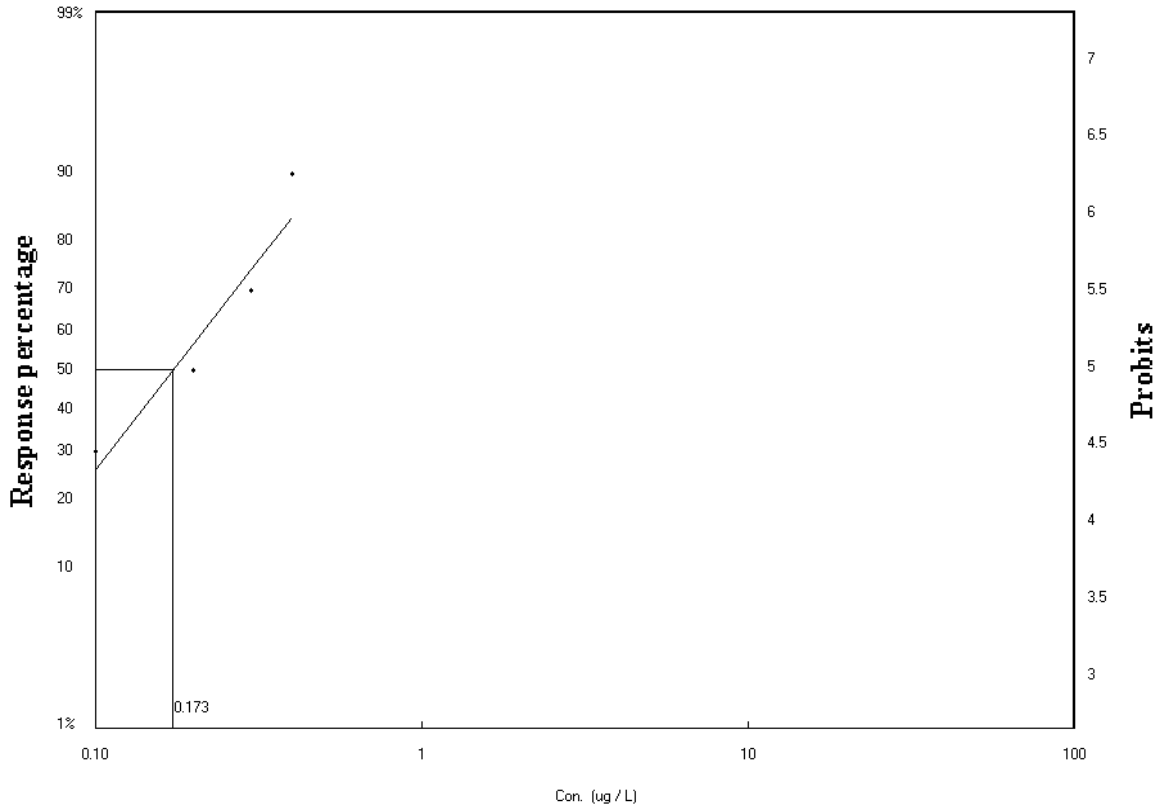
Fig 3.8 b Graphical representation of the median lethal concentrations (LC_{50}) of Dimethoate to juveniles after 96 h at the salinity of 25 ppt at 28 ºC temperature

3.3.4.3 a Influence of temperature on acute toxicity of Chlorpyrifos in post larvae

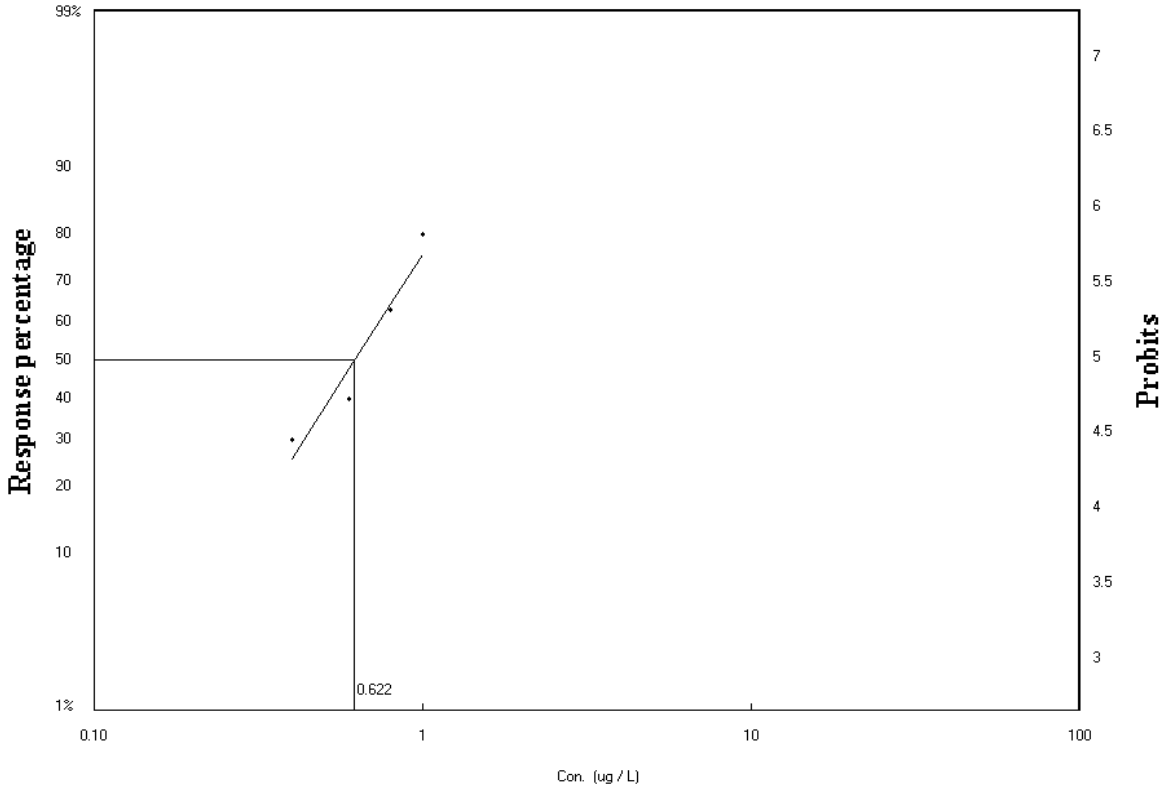
The median lethal concentration (LC_{50}) of CPF to PL at 15, 25 and 34 ºC temperature at the end of 96 h are represented in Table 3.17 and are graphically presented in Fig. 3.9 a and b. The 96 h LC_{50} values of the CPF exposed PL found to be 0.17, 0.62, 0.46 µg L^{-1} at 15, 25 and 34 ºC temperature, respectively. Results of ANOVA followed by Tukey–Kramer multiple comparison tests revealed that the 96 h LC_{50} values of CPF at 25 ºC was found to be significantly higher when compared to 15 and 34 ºC ($P< 0.05$).

Table 3.17 Median lethal concentrations (LC_{50}) of Chlorpyrifos with a 95% confidence interval to post larvae at the temperature of 15, 25 and 34 ºC after 96 h of exposure at 30 ppt salinity (ANOVA, $P< 0.05$, different superscript letters indicate significant difference between each exposure period)

Post larvae	**Chlorpyrifos**	**95% Confidence interval (µg L^{-1})**	
Temperature (ºC)	**96 h LC_{50} (µg L^{-1})**	**Lower limit**	**Upper limit**
15	0.17^{a}	0.12	0.23
25	0.62^{b}	0.56	0.68
34	0.46^{c}	0.40	0.51

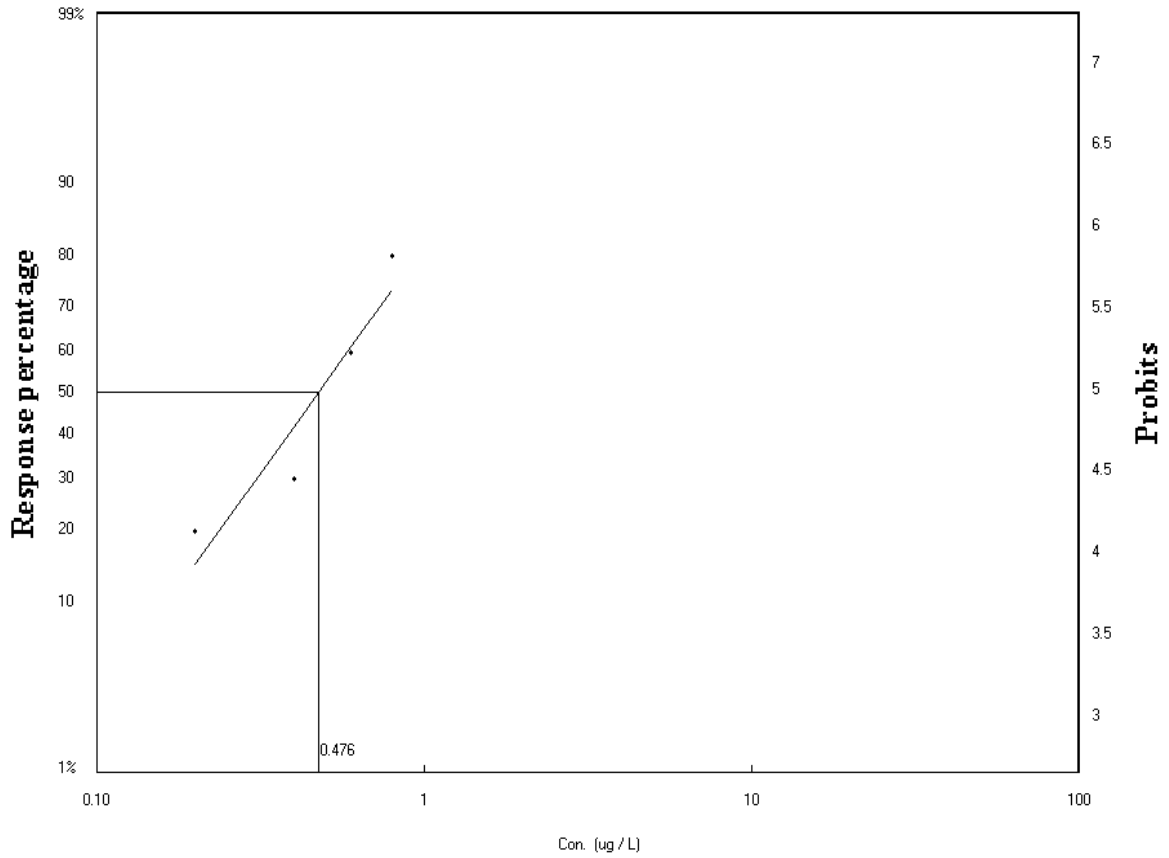


15 °C



25 °C

Fig 3.9 a Graphical representation of the median lethal concentrations (LC_{50}) of Chlorpyrifos to post larvae after 96 h at the temperatures of 15 and 25 °C at 30 ppt salinity



34 °C

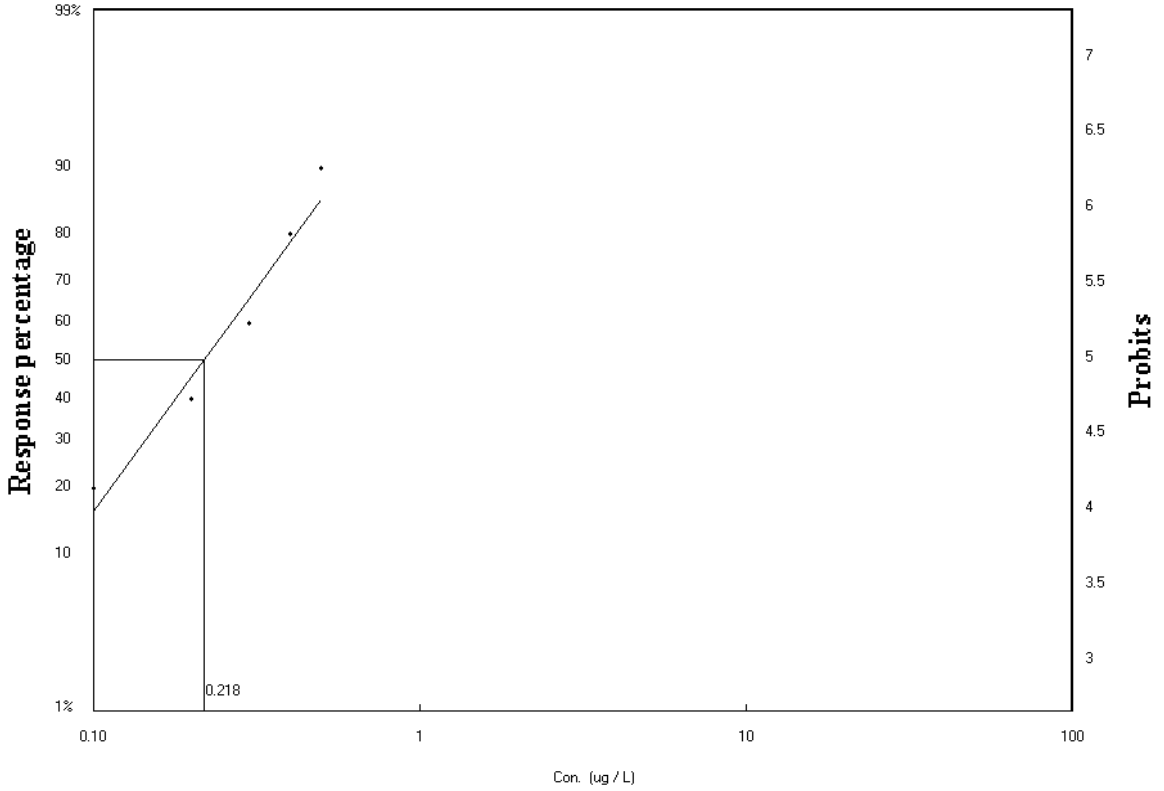
Fig 3.9 b Graphical representation of the median lethal concentrations (LC_{50}) of Chlorpyrifos to post larvae after 96 h at the temperature of 34 °C at 30 ppt salinity

3.3.4.3 b Influence of temperature on acute toxicity of Chlorpyrifos in juveniles

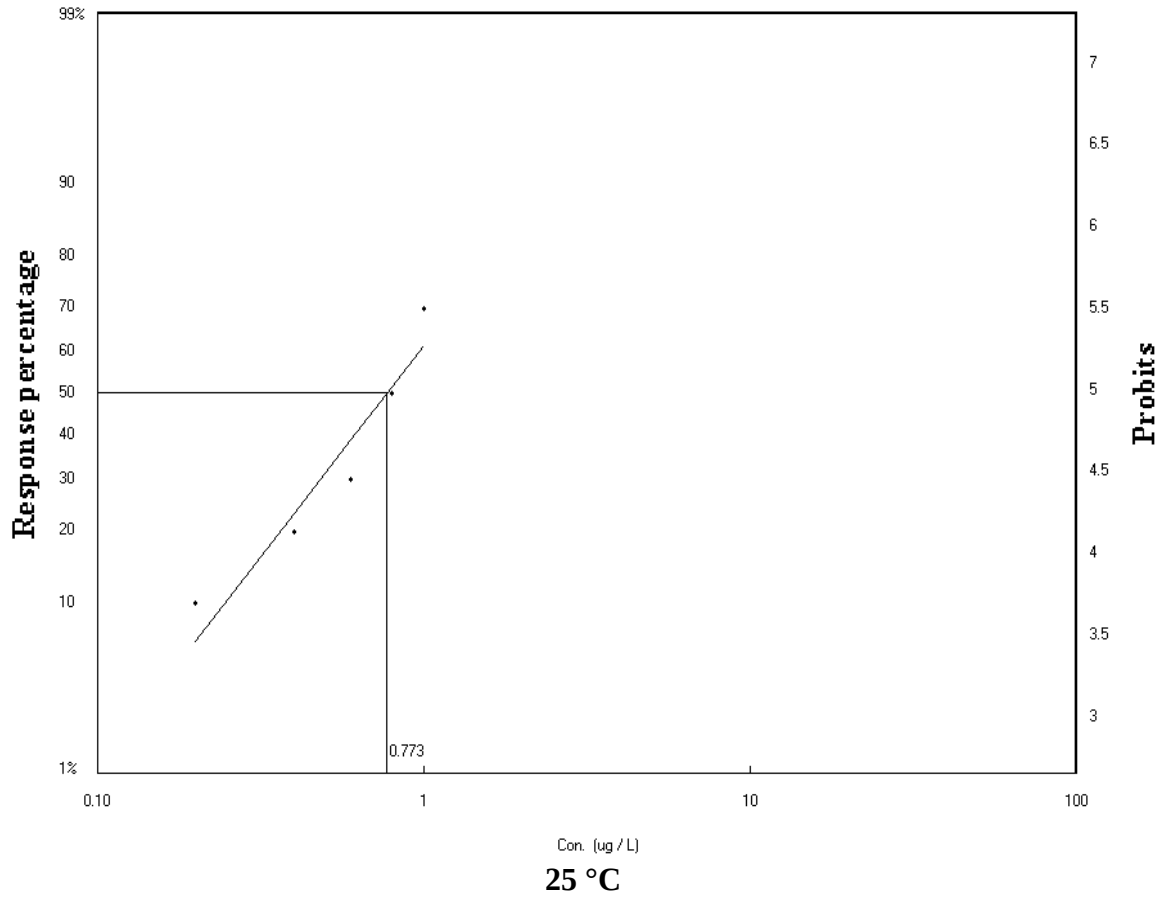
The median lethal concentration (LC_{50}) of CPF to JL at 15, 25 and 34 ºC temperature at the end of 96 h are represented in Table 3.18 and are graphically presented in Fig. 3.10 a and b. The 96 h LC_{50} values of the CPF exposed JL found to be 0.22, 0.77 and 0.65 µg L^{-1} at 15, 25 and 34 ºC temperatures, respectively. Results of ANOVA followed by Tukey–Kramer multiple comparison tests revealed that the 96 h LC_{50} values of CPF at 25 ºC was found to be significantly higher when compared to 15 and 34 ºC ($P< 0.05$).

Table 3.18 Median lethal concentrations (LC_{50}) of Chlorpyrifos with a 95% confidence interval to Juveniles at the temperature of 15, 25 and 34 ºC after 96 h of exposure at 30 ppt salinity (ANOVA, $P< 0.05$, different superscript letters indicate significant difference between each exposure durations)

Juvenile	**Chlorpyrifos**	**95% Confidence interval (µg L^{-1})**	
Temperature (ºC)	**96 h LC_{50} (µg L^{-1})**	**Lower limit**	**Upper limit**
15	0.22^{a}	0.20	0.24
25	0.77^{b}	0.60	1.39
34	0.65^{c}	0.49	0.93

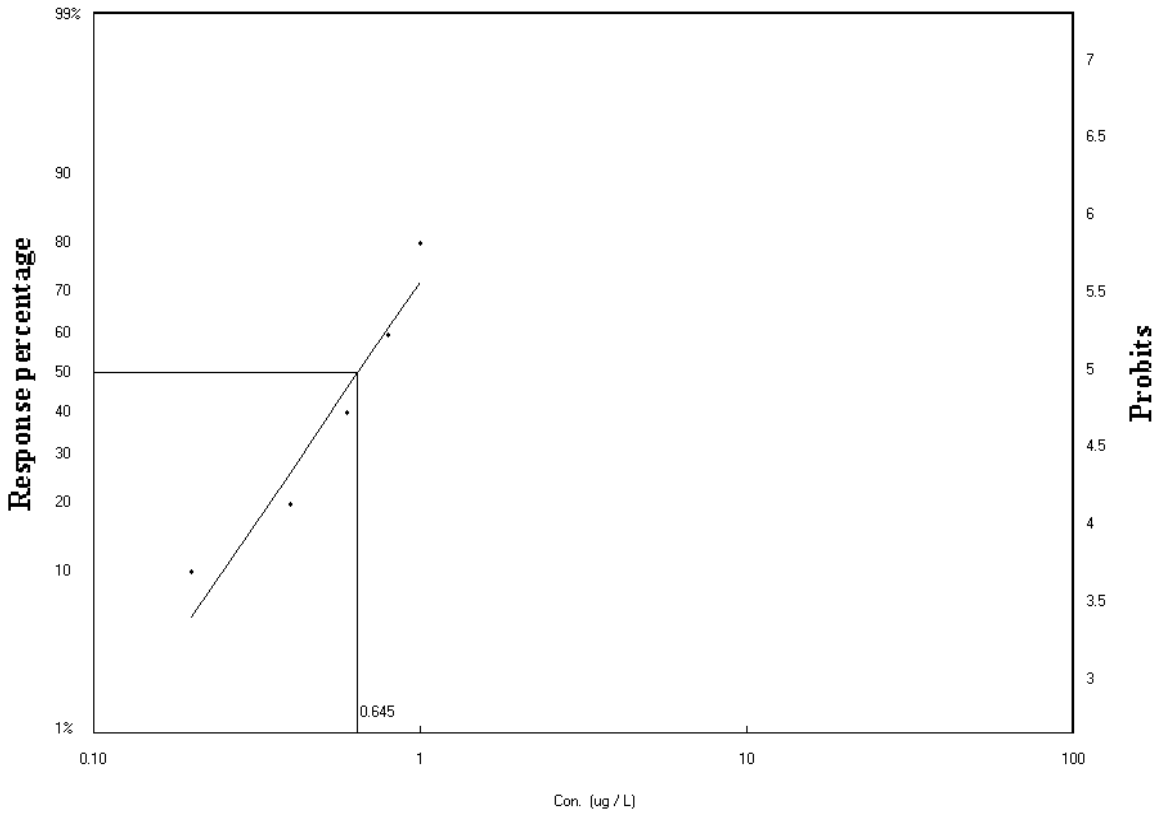


15 °C



25 °C

Fig 3.10 a Graphical representation of the median lethal concentration (LC_{50}) of Chlorpyrifos to juveniles after 96 h at the temperatures of 15 and 25°C at 30 ppt salinity.



34 °C

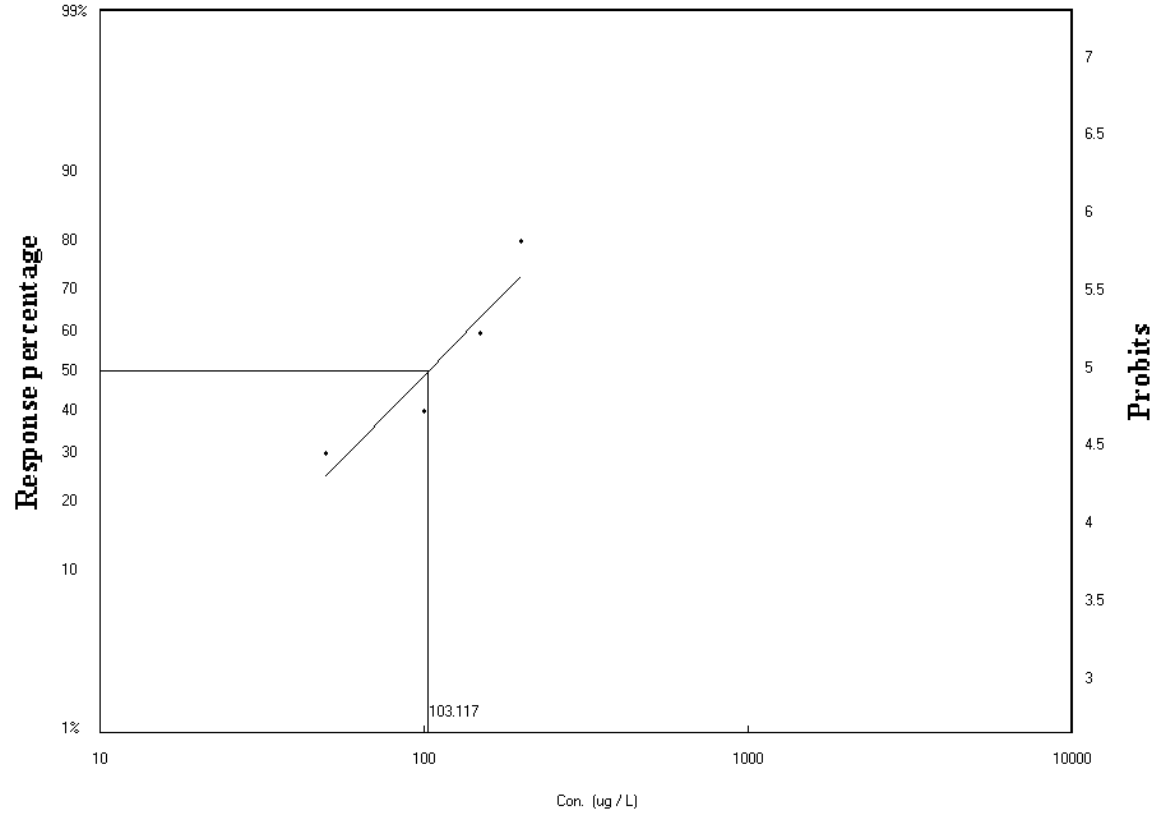
Fig 3.10 b Graphical representation of the median lethal concentration (LC_{50}) of Chlorpyrifos to juveniles after 96 h at the temperature of 34 °C at 30 ppt salinity

3.3.4.4 a Influence of temperature on acute toxicity of Dimethoate in post larvae

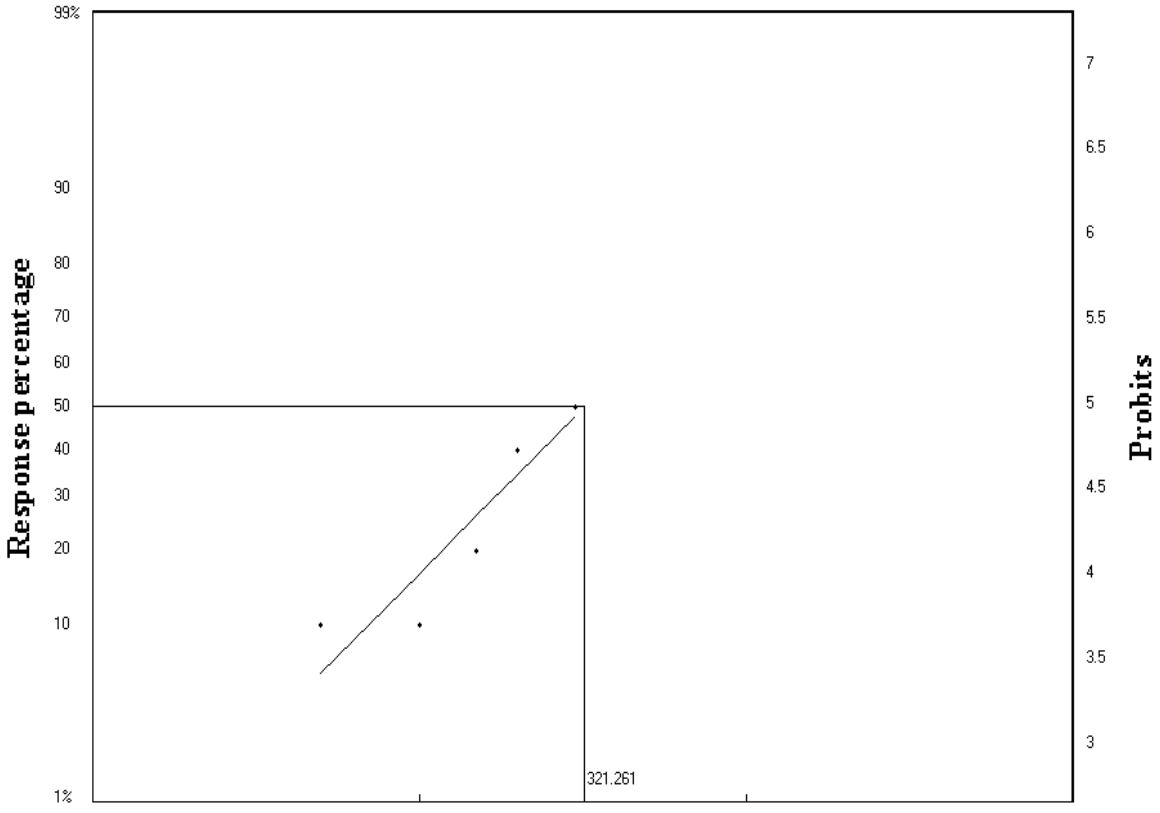
The median lethal concentration (LC_{50}) of DMT to PL at 15, 25 and 34 ºC temperature at the end of 96 h are represented in Table 3.19 and are graphically presented in Fig. 3.11 a and b. The 96 h LC_{50} values of the CPF exposed PL found to be 103.11, 321.26 and 182.76 µg L^{-1} at 15, 25 and 34 ºC temperature, respectively. Results of ANOVA followed by Tukey–Kramer multiple comparison tests revealed that the 96 h LC_{50} values of CPF at 25 ºC was found to be significantly higher when compared to 15 and 34 ºC ($P< 0.05$).

Table 3.19 Median lethal concentrations (LC_{50}) of Dimethoate with a 95% confidence interval to Post larvae at the temperature of 15, 25 and 34 ºC after 96 h of exposure at 30 ppt salinity (ANOVA, $P< 0.05$, different superscript letters indicate significant difference between each exposure durations)

Post larvae	**Dimethoate**	**95% Confidence interval (µg L^{-1})**	
Temperature (ºC)	**96 h LC_{50} (µg L^{-1})**	**Lower limit**	**Upper limit**
15	103.11^{a}	92.11	125.36
25	321.26^{b}	286.45	395.51
34	182.76^{c}	156.46	221.47

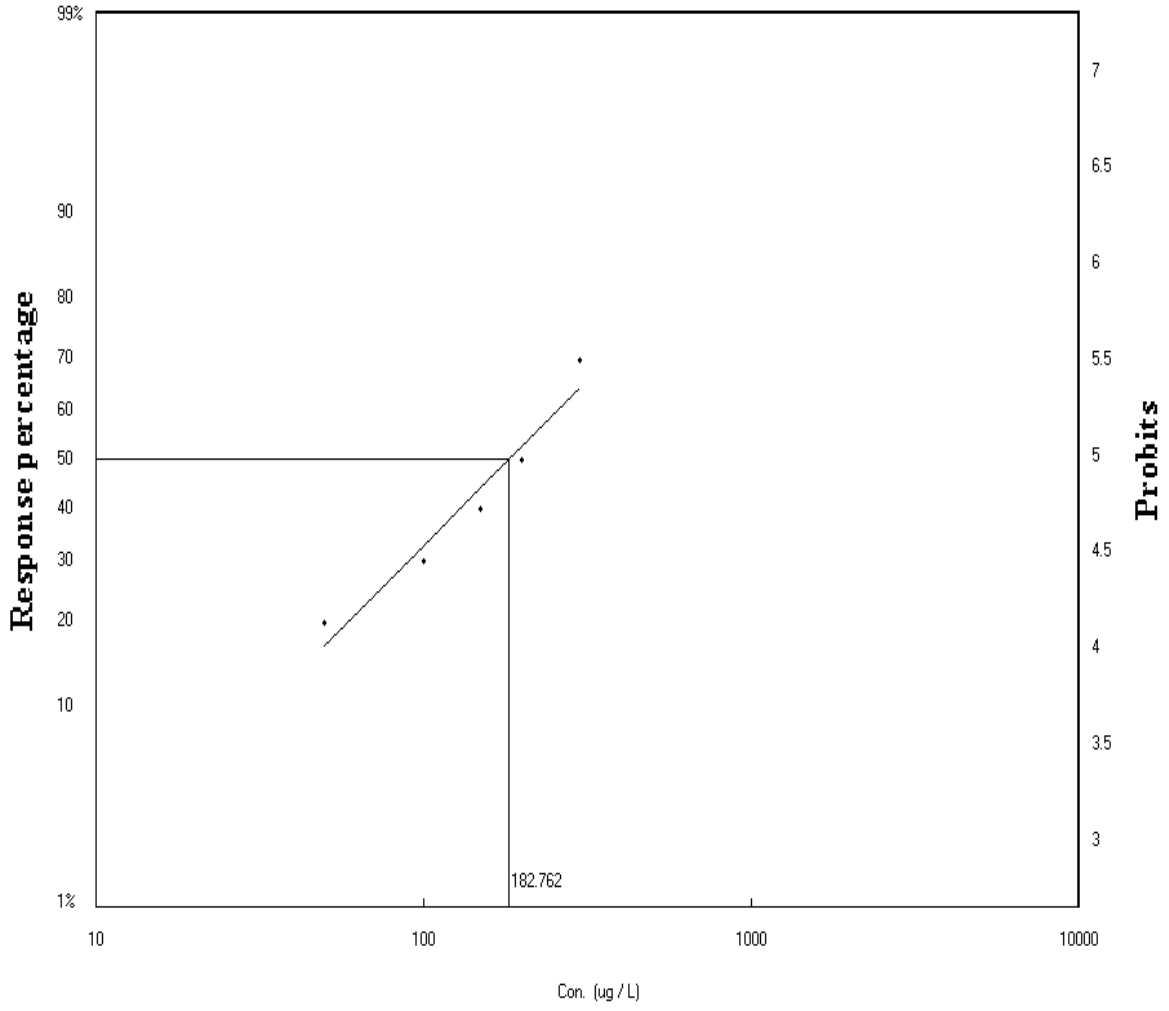


15 °C



25 °C

Fig 3.11 a Graphical representation of the median lethal concentrations (LC_{50}) of Dimethoate to post larvae after 96 h at the temperatures of 15 and 25°C at 30 ppt salinity



34 °C

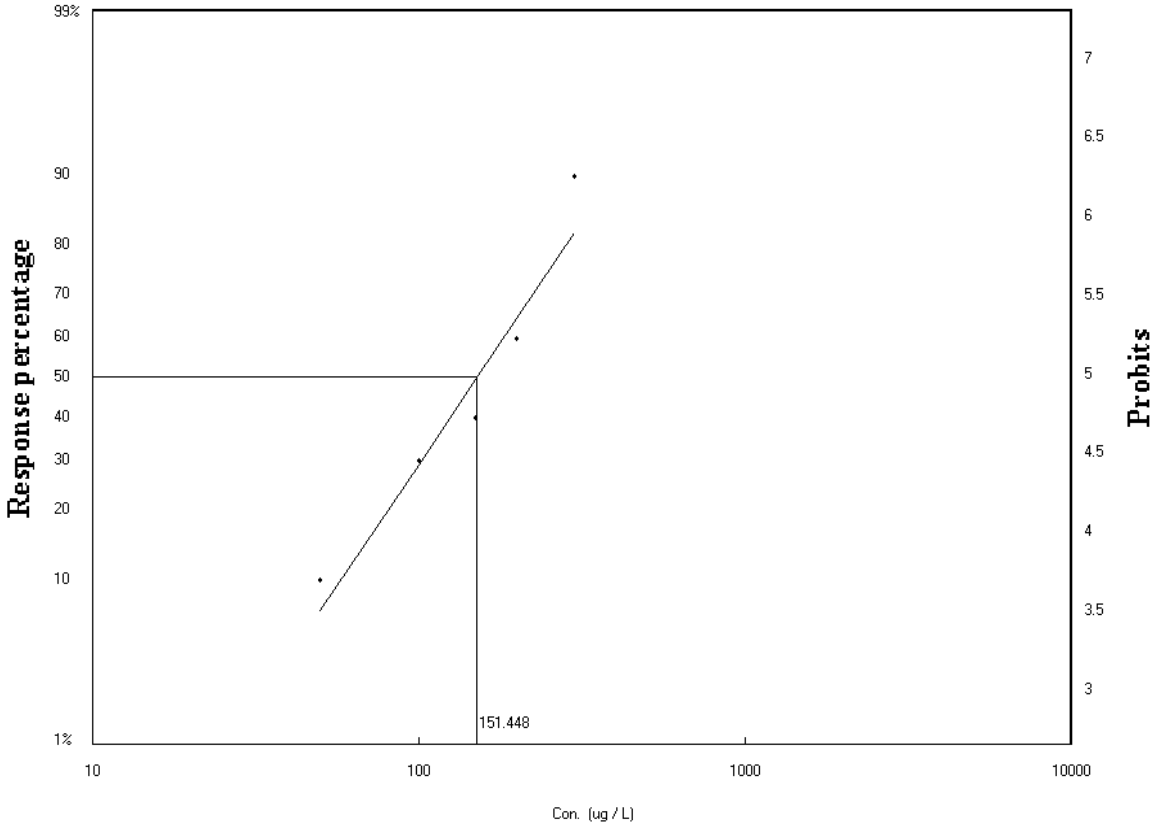
Fig 3.11 b Graphical representation of the median lethal concentrations (LC_{50}) of Dimethoate to post larvae after 96 h at the temperature of 34 °C at 30 ppt salinity

3.3.4.4 b Influence of temperature on acute toxicity of Dimethoate in juveniles

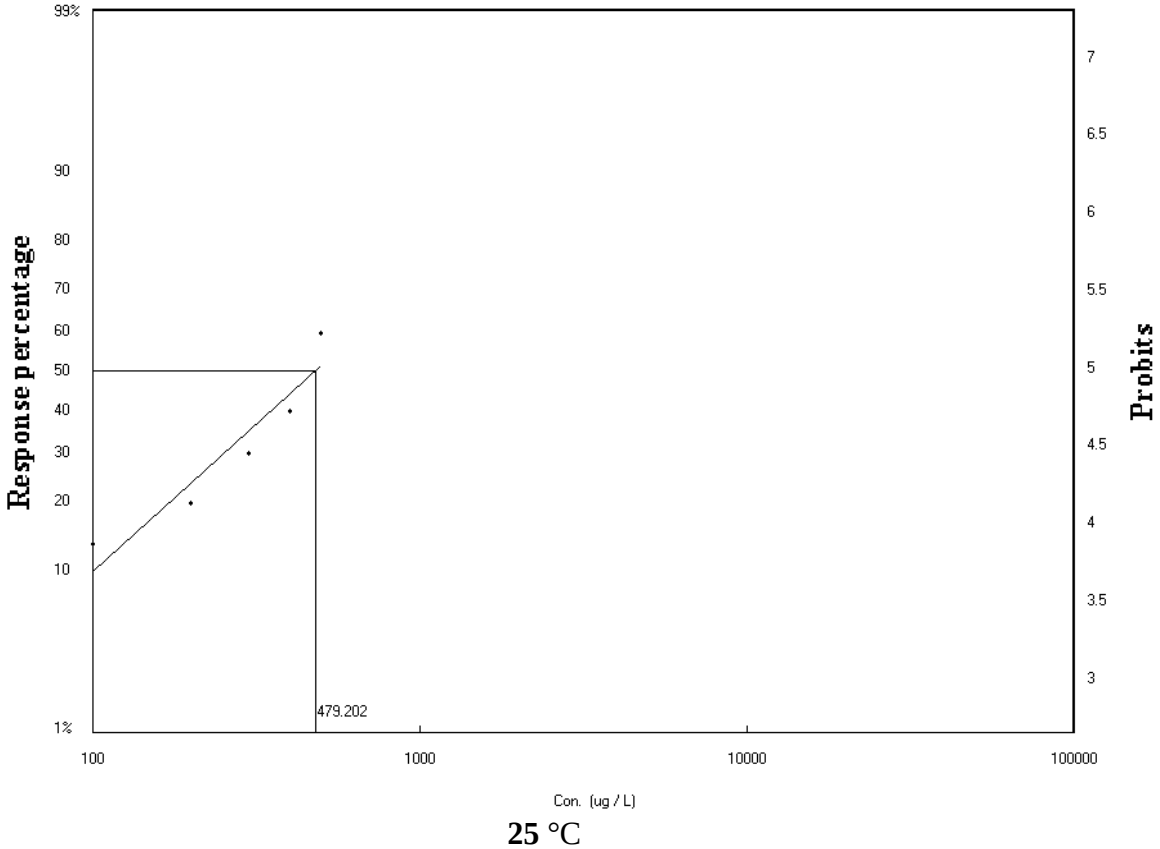
The median lethal concentration (LC_{50}) of DMT to JL at 15, 25 and 34 ºC temperature at the end of 96 h are represented in Table 3.20 and are graphically presented in Fig. 3.12 a and b. The 96 h LC_{50} values of the DMT exposed PL found to be 151.46, 479.20 and 236.74 µg L^{-1} at 15, 25 and 34 ºC temperature, respectively. Results of ANOVA followed by Tukey–Kramer multiple comparison tests revealed that the 96 h LC_{50} values of DMT at 25 ºC was found to be significantly lower when compared to 15 and 34 ºC ($P< 0.05$).

Table 3.20 Median lethal concentrations (LC_{50}) of Dimethoate with a 95% confidence interval to juveniles at the temperature of 15, 25 and 34 ºC after 96 h of exposure at 30 ppt salinity (ANOVA, $P< 0.05$, different superscript letters indicate significant difference between each exposure durations)

Juvenile	**Dimethoate**	**95% Confidence interval (µg L^{-1})**	
Temperature (ºC)	**96 h LC_{50} (µg L^{-1})**	**Lower limit**	**Upper limit**
15	151.45^{a}	110.18	212.00
25	479.20^{b}	404.02	619.27
34	236.74^{c}	209.48	264.86



15 °C



25 °C

Fig 3.12 a Graphical representation of the median lethal concentrations (LC_{50}) of Dimethoate to juveniles after 96 h at the temperature of 15 and 25 °C at 30 ppt salinity

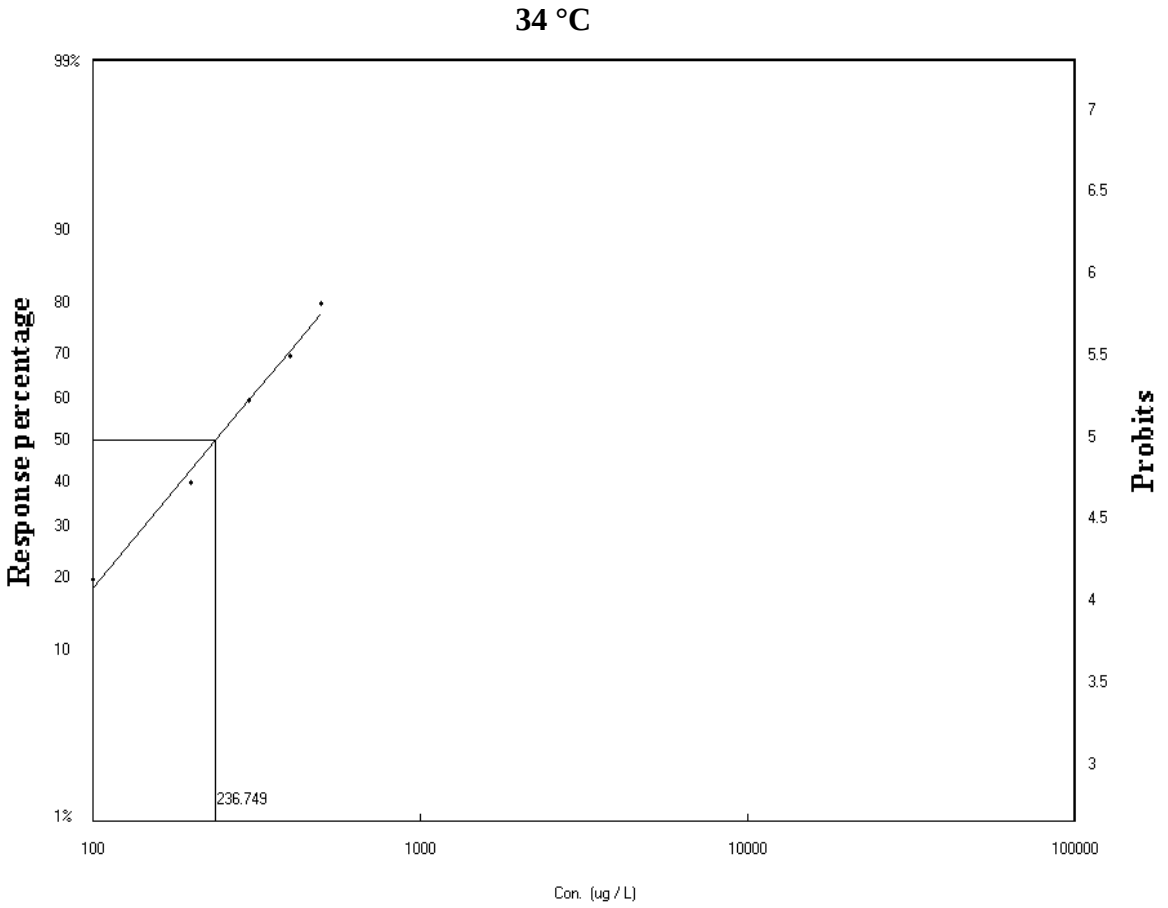


Fig 3.12 b Graphical representation of the median lethal concentrations (LC_{50}) of Dimethoate to juveniles after 96 h at the temperature of 34 °C at 30 ppt salinity

3.4 Discussion

3.4 a Acute toxicity of Chlorpyrifos and Dimethoate

Knowledge on toxicity tolerance of organism to contaminants through acute toxicity studies (96 h LC_{50}) is necessary to understand their response to such contaminants in natural waters. Therefore, the acute bioassay tests (96 h LC_{50}) were carried out to demonstrate the toxicity of CPF and DMT pesticides in *L. vannamei* PL and JL. The present study determined 96 h LC_{50} values of CPF to PL and JL as 0.88 and 1.44 µg L^{-1}, respectively. Whereas the 96 h LC_{50} values of DMT to PL and JL were found to be 357.24 and 563.61 µg L^{-1}, respectively. Earlier studies on the acute toxicity of CPF and DMT in prawns and shrimps showed the toxicity of both pesticides towards the different prawn and shrimp species. Findings of the present investigations agree of these earlier reports on the toxicity of CPF to penaeid group of shrimps, *Metapenaeus monoceros* and *Penaeus monodon.* The 96 h LC_{50} of CPF found to be 1.3 µg L^{-1} and 59.16 nmol L^{-1} in *M. monoceros* and *P. monodon*, respectively (Eamkamon et al., 2012; Shoaib and Siddiqui, 2015). Kumar et al. (2010) reported the 96 h LC_{50} of CPF and DMT in grass shrimp (*Paratya australensis*) as 0.06 µg L^{-1} and 800 µg L^{-1}, respectively. This report is in accordance with present investigation which shows the highly toxic nature of CPF compared to the DMT. The 96 h LC_{50} of CPF and DMT in freshwater prawn (*Macrobrachium rosenbergii*) found to be 0.3 µg L^{-1} for CPF and 102.7 µg L^{-1} for DMT (Satapornvanit et al., 2009) which also proved the highly toxic nature of CPF compared to the DMT. Further, 96 h LC_{50} values of CPF in grass shrimp (*Palaemonetes pugio*) was reported to be 0.44 µg L^{-1} (Key and Fulton, 1993). Furthermore, the acute toxicity of CPF found to be 0.49 µg L^{-1} and 0.004 µg L^{-1} to freshwater prawn (*Palaemonetes argentines*) and *P. australiensis*, respectively (Montagna and Collins, 2007; Kumar et al., 2010). All the above studies reported the toxic nature of CPF and DMT to different prawns and shrimp species and found that CPF is more toxic compared to DMT. Our present observation on the 96 h LC_{50} of CPF and DMT to shrimp (*L. vannamei*) is in par with these reports.

Reports on the toxic effects of various other pesticides on *L. vannamei* provide supporting information on the toxic response of this species. The 48 h LC_{50} values of 19 mg L^{-1} and 1.46 mg L^{-1} have been reported in *L. vannamei* following exposure to two OP pesticides, fenitrothion (Lignot et al., 1998) and methamidophos (Garcia-de La Parra et al., 2006), respectively. Wide variability in the acute toxicity indices in terms of LC_{50} on exposure of *L. vannamei* to four

different organochlorine pesticides (Chlordane, 63µg L^{-1}; DDT, 8.7µg L^{-1}; Lorsban, 4.8 µg L^{-1}; Lindane, 3.9 µg L^{-1}) have been reported by Reyes et al. (1996). A significantly lower acute toxicity index, 9.33 ng L^{-1} has reported in *L. vannamei* on exposure to the permethrin-based insecticide (Mello et al., 2011).

3.4 b Influence of salinity and temperature on acute toxicity of Chlorpyrifos and Dimethoate

In par with the reports on various shrimp species, salinity and temperature are the two very important environmental factors which influence the quality and quantity of the yield of *L. vannamei*. The optimal temperature for the growth of *L. vannamei* has been reported to be size-specific: ~28–30 ºC for PL (Ponce-Palafox et al., 1997), > 30 ºC for small juveniles (5 g) and about 27 ºC for sub-adults (Wyban et al., 1995). Although Roy et al. (2010) and) reported that *L. vannamei* can tolerate a wide salinity range and Boyd, (1989) considered that salinity of 15–25 ppt as ideal for its culture. Considering the large-scale expansion of *L. vannamei* to inland saline areas, the present study assessed the effects of different salinity and temperature regimes on the acute toxicity of two commonly used OPs (CPF and DMT) in two developmental stages (PL and JL) of *L. vannamei*.

Results of the present study indicated that both the CPF and DMT are more toxic at 5 and 15 ppt salinities compared to 25 ppt to PL ($P< 0.05$). Further, in temperature experiments, indicated that CPF and DMT are more toxic at 15 and 34 °C compared to their toxicity at 25 °C to PL ($P< 0.05$). Also, in the case of JL, both CPF and DMT are more toxic at 5 and 15 ppt salinities compared to 25 ppt ($P< 0.05$). Whereas, in temperature variation experiments, CPF and DMT were found to be more toxic at 15 and 34 °C compared to their toxicity at 25 °C to JL ($p < 0.05$). A decrease in the toxicity tolerance to two pesticides (beta-cypermethrin and acephate) in *L. vannamei* at lower salinity levels (5 ppt vs 20 ppt) has also been reported by Wang et al. (2013). Effect of salinity and temperature in influencing toxicity levels on exposure to a fungicide chlorothalonil and the insecticide Scourge® in larval and adult estuarine grass shrimp, *Palaemonetes pugio* also reported by DeLorenzo et al. (2009). During the present study, acute toxicity indices of CPF and DMT in PL and JL at 25 ppt salinity and 25 °C temperature were relatively lower compared to the other tested salinities (5 and 15 ppt) and temperatures (15 and 34 °C). Also, it has been reported that *L. vannamei* reared at low (1 ppt) and high salinity (37 ppt) as well as at low temperature (20 °C), suffered osmotic stress and consumed more oxygen (Bett and Vinatea, 2009). In contrast, shrimps reared at the salinity of 25 ppt and

a temperature of 25–30 °C exhibited stable oxygen consumption (Bett and Vinatea, 2009). Higher temperatures and/ or lower salinities have been reported to cause a significant effect on the oyster species ability to cope with stress induced by toxic substances and the regulation of associated physiological processes involved in the detoxification of hazardous substances (Cherkasov et al., 2006; Lanning et al., 2006). Furthermore, the deleterious effect in shrimps because of thermal and osmotic stress have been reported. The increase in water temperature trigger an outbreak of the *Vibrio* (by weakening the immune response of shrimp) and accompanied reduction in aspartate aminotransferase activity reported by Chien et al. (2003) respectively.

In the present investigation, post-larvae of L. vannamei were found to be more sensitive to both OPs than their juveniles ranging in 1–2 orders magnitude of higher sensitivity. The sensitivity of shrimp larvae to pesticides has been observed to be of 1–3 orders magnitude higher than their adults (Nimmo et al., 1971; Mohammed, 2013; DeLorenzo et al., 2014) and these findings are in agreement with our present findings. Nimmo et al. (1971) studied the distribution of polychlorinated biphenyl Aroclor® 1254 in a penaeid shrimp (the pink shrimp, Penaeus duorarum) and observed higher toxicity in larvae compared to its juveniles. Mohammed et al. (2013) too reported that the early life stages of aquatic organisms are more sensitive than adults (juvenile). DeLorenzo et al. (2014) have reported higher sensitivity of larval stages of two estuarine crustacean species *Americamysis bahia* and *Palaemonetes pugio* towards the insecticide pyrithroids compared to their adults, all the above observations are in par with our findings. Eamkmon et al. (2012) studied the effect of CPF on black tiger shrimp from penaeid group which showed higher sensitivity of juvenile stage towards the pesticide exposure which is in par with our present study on the toxic effect of CPF on juvenile stage of penaeid shrimp L. vannamei. Significantly higher toxicity level has been reported in grass shrimp (*Palaemonetes pugio*) larvae compared to their adults following exposure to CPF (Key and Fulton, 1993). Rao et al. (1985) reported that the higher sensitivity of crustacean larvae to toxicants compared to their adults/ juveniles may be as a consequence of increased moulting frequency during their early life stages. Therefore, higher toxic levels recorded during the present study in post-larvae of L. vannamei might be due to the cumulative stress effect induced by changes in salinity and temperature they are exposed in addition to the toxicity of DMT and CPF.

Present findings suggest that changes in salinity and temperature have altered the toxicity of DMT and CPF in *L. vannamei* and the degree of the toxic effect depended on its life stage

and type of OP. The potential effect of pesticides in farming systems is expected to be much higher than these laboratory-derived results as the presence of sediment enhances the toxicity of pesticides (Holmes et al., 2008). There may be a build-up of OPs in ponds over time and may pose a risk to shrimp aquaculture, due to partitioning of CPF to sediment (Giddings et al., 2014). Owing to their burrowing nature into sediments and detritivore feeding habit, all stages of shrimps are susceptible to sediment-bound contaminants (Hook et al., 2018) and thus pose a greater risk to shrimp culture.

3.5 Conclusion

In conclusion, PL and JL of *L. vannamei* are more sensitive at lower salinity and temperature. These results are of practical significance, as *L. vannamei* is considered to be a candidate species for shrimp farming in low saline inland areas. Relatively higher acute toxicity nature of evaluated pesticides at lower salinities calls for precaution in extending the cultivation of *L. vannamei* in low-salinity areas adjacent to traditional agriculture fields. Changing environment (increase/decrease in salinity and temperature) due to climate change might alter the interactions of xenobiotic compounds, more specifically pesticides resulting in more toxicity to aquatic organisms. The data obtained during the present study could be used as a baseline for monitoring OPs pesticide pollution.

Chapter 4

In vivo DNA damage in *L. vannamei* exposed to sublethal doses of Chlorpyrifos and Dimethoate

4.1 Introduction

New generation pesticides i.e organophosphates (OP) on one hand are being non persistent and degradable in nature benefiting to man by replacing the problematic organochlorine pesticides (OC), but on the other hand are posing differential threats to environment being highly toxic to aquatic organisms and causing long term ecological implications (Hallapa and David, 2009; Kumar et al., 2010; Joseph and Raj, 2011; Tu et al., 2012). Last few decades have witnessed the bad wide effects of OP pesticides on biological and physiological parameters in fresh water and marine aquatic organisms (Rickwood, 2004; Rao et al., 2005; Garcia-de La Parra et al., 2006; Chandrasekara and Pathiratne, 2007; Krishnapriya and Padmaja, 2014). This shows the toxic impact of OPs among a wide range of taxa.

Owing to the growing concern of the harmful effects of genotoxicants and xenobiotic compounds in the aquatic environment, the need for the development of sensitive biomarkers has gained importance (Hayashi et al., 1998, Zeid and Khalil, 2014). Evaluation of DNA damage is one of the rapid and reliable tools for assessing the genotoxic potential of pollutants. Due to its obvious advantages such as rapid detection and high sensitivity in detecting the minute DNA damage, the single cell gel electrophoresis or comet assay has become a widely used genetic tool (Klobucar et al., 2003, Frias-Espericueta et al., 2011). Very few studies have evaluated the toxicity study of different pesticides in penaeid shrimps to date (Reyes et al., 2002; Labrie et al., 2003; Suryavanshi et al., 2009; Mello et al., 2011; Eamkamon et al., 2012; Tu et al., 2012). Unfortunately, very little attention was given to long-term *in vivo* assessment of genotoxic effect of OP in penaeid shrimps.

Therefore, the present study was undertaken to gain a better understanding of the genotoxic potential of two OP (Chlorpyrifos: CPF and Dimethoate: DMT) in penaeid shrimp *L. vannamei* following chronic exposure. It is expected that the outcome of this study will elucidate the sensitivity of *L. vannamei* towards the OP and their potential to afflict the DNA damage thus, making it an excellent bioindicator test species.

4.2 Material and methods

4.2.1 Experimental animals

Shrimp Post Larvae (PL) (PL14; total length, 12.4 ± 0.5 mm; wet weight, 55 ± 5 mg) and Juveniles (JL) (Total length, 52.2 ± 10 mm; wet weight 1.78 ± 1.0 g) were used to study the acute toxicity of DMT and CPF.

4.2.2 Sublethal (SL) dose selection

The sublethal (SL) doses of CPF and DMT were selected based on the 96 h LC_{50} values of respective pesticides to PL and JL. The $1/4^{th}$ and $1/8^{th}$ concentration of the 96 h LC_{50} values were selected as the SL doses for present study. The SL doses used for present study are shown in the following table:

Life stages of *L. vannamei*	**Pesticides**	**SL doses (µg L^{-1})**	
		$1/4^{th}$ of LC_{50}	**$1/8^{th}$ of LC_{50}**
Post larvae	CPF	CPF1= 0.22	CPF2= 0.11
	DMT	DMT1= 89.46	DMT2= 44.73
Juvenile	CPF	CPF1= 0.36	CPF2= 0.18
	DMT	DMT1= 140.9	DMT2= 70.45

4.2.3 Experimental set up

Out of 1200 PL, 600 were used for one pesticide (Chlorpyrifos) and the remaining 600 PL for another pesticide (Dimethoate). These 600 larvae were divided into three groups, each of 200 larvae, one group used as controls and the remaining two groups for two sublethal concentrations of one pesticide. The experiments were conducted in duplicate with 100 larvae in each tank along with control. Randomly selected 600 inter-moult JLs of similar sizes were divided into three groups, each one comprising of 200 JLs. From this, two groups were exposed to two pesticides while one group served as control. Experiments on JLs were also conducted in duplicate with 100 JL per tank. To maintain the constant concentrations of pesticides in test solutions, the entire medium in each aquarium was gently siphoned out daily (10:00 h) and

renewed with the freshly prepared solution of respective SL concentrations. Maximum care was taken to maintain the animals with no or minimum disturbance during handling.

The tests were conducted for 21 days of exposure (DoE), while samples for comet assay, antioxidant enzyme activity and antioxidant enzyme gene expression were collected on 3, 7, 14 and 21 DoE. At each of the time intervals, 10 PL and 10 JL were collected at random for each test. Also, shrimps from the control group were similarly sampled at the same time intervals as the treated ones. During the exposure period, mortality in control tanks did not exceed 5%. To maintain the same stocking density throughout the experiment dead PLs and JLs, if any were replaced with shrimps reared in the same medium separately. During the chronic exposure experiment, PLs were fed at rate of 8–10% of body weight while JLs were fed at a rate of 4–6% of body weight which split across three different feed times (10:00, 15:00 and 20:00 h).

Plate 4.1 Experimental set up for chronic toxicity study with post larvae of *L. vannamei*

Plate 4.2 Experimental set up for chronic toxicity study with juveniles of *L. vannamei*

4.2.4 Exposure duration

PL and JL were exposed for 21 Days of Exposure (DoE) and samples were collected at 3, 7, 14 and 21 DoE from both control and exposed shrimps.

4.2.5 DNA damage study

4.2.5.1 Comet assay (Single cell gel electrophoresis)

DNA damage induced by pesticides was studied employing comet assay or single cell gel electrophoresis.

4.2.5.2 Sample collection

a) **PL:** 5 PL weighing 200 mg were used.

b) **JL:**

Haemolymph (HL): 350 µl of haemolymphs were collected from rostral sinus region of JL using a hypodermic syringe.

Gill (GL): 100 mg of gills were collected from JLs.

Muscles (ML): 200 mg of muscle tissue were collected from JLs.

4.2.5.3 Sample processing

The whole shrimp PL as well as gill and muscle tissues from JL were chopped followed by washing twice with chilled phosphate buffer saline (PBS, Calcium and Magnesium free) and later on transferred to an ice-cold homogenization buffer (1-X Hanks' balanced salt solution, 20 mM EDTA, 10% dimethyl sulphoxide, pH 7.0–7.5). The tissues were homogenized with a Potter-Elvehjem homogenizer to obtain single cell suspension. The obtained homogenates were centrifuged at 3000 rpm at 4 °C for 5 min and pellets were suspended in chilled PBS. On the other hand, HL was collected using heparin as anticoagulant. The extraction of samples were done under dim light and transferred immediately to micro-centrifuge tubes placed on an ice pack to prevent endogenous DNA damage occurring during sample preparation and also to inhibit DNA repair in unfixed samples (Siu et al., 2004).

4.2.5.4 Cell viability test

Prior to the commencement of comet assay, the cell count and cell viability tests were performed using trypan blue dye exclusion method (Strober, 2015) for ensuring enough live cells in cell suspension for carrying out the assay. Those samples exhibiting >90% viability and cell count of a minimum of 10^6 cells ml^{-1} were used for further analysis of comet assay.

4.2.5.5 Validity test

4.2.5.5.1 *In vitro* exposure to hydrogen peroxide

The comet assay was validated by using a standard reference genotoxin, hydrogen peroxide (H_2O_2) as described by Bhagat et al. (2016). Whole shrimp PLs were treated with different concentrations of H_2O_2 (1, 5, 20, 30 and 60 µM) whereas, the organs of juvenile *viz.* GL, ML and HL were incubated with different concentrations of H_2O_2 (0.5, 5, 20 and 40 µM) for 30 min in dark conditions. The different concentrations of H_2O_2 were prepared in PBS at room temperature (28 °C) whereas control samples were incubated without H_2O_2 in PBS. Three replicates were employed for each concentration.

4.2.5.6 Procedure (Singh et al., 1988)

The comet assay was performed as a three-layer procedure (Singh et al., 1988) using one side frosted microscopic slides with slight modification. The unwinding and electrophoresis process were optimized before the actual analysis of shrimp samples. Initially, the slides were cleaned with 100% ethanol and flame dried. Frosted sides of slides were coated with 100 µl of 1% normal melting point agarose (NMPA) and solidified at room temp. About 15-20 µl of cell suspensions were mixed with 80-85 µl of 0.5% low melting point agarose (LMPA) and layered on previously coated with a NMPA (1%). Finally, it was covered with a third layer of 100 µl LMPA. After solidification of gel, the slides were immersed in lysing solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, pH 10 with 10% DMSO and 1% Triton X-100) for 3 h at 4 °C. The slides were then transferred to a horizontal gel electrophoresis unit, immersed in fresh cold alkaline electrophoresis buffer (300 mM NaOH, 1 Mm EDTA, pH > 13) and incubated in the solution for 20-25 min for DNA unwinding. Electrophoresis was carried out for 20 min, using 20 V and 300 mA electric current. The slides were neutralized by immersing in neutralizing buffer (0.4 M Tris-HCl, pH 7.5) for 10 min to remove excess alkali and rinsed with distilled water. Finally, slides were stained with ethidium bromide (15 µg L^{-1}) for 5 min for the visualization of DNA damage. Duplicate slides were prepared per test concentration for different types of cells and respective control groups for all the time intervals.

All the slides were observed under an epifluorescent microscope (Olympus BX51, Japan) under 100X objective. A total of 100 comets were randomly scored per group. The comet images were captured using Image pro AMS 6.0 and analysed by comet assay software project (Casp_1.2.3 beta). The comet parameter *viz*. % tail DNA determined by the software was used for the quantification of DNA damage.

4.2.6 Statistics

Statistical analysis was performed by using computer based GraphPad PRISM 5.0 software (Graph Pad, San Diego, CA, USA). Data obtained from the experiments were expressed as mean ± SD. Data from different treatment groups and different tissues were assessed by analysis of variance (ANOVA) (Underwood, 1997) with DoE and SL concentrations as sources of variation. The Student's *t*-test (Zar, 1996) also carried out to compare the quantum of variation between two groups. Variation in water quality parameters from treated and control aquaria were assessed by ANOVA. Appropriate transformations were applied before subjecting the

data to statistical analysis. If results were found significant, multiple comparisons between different means from control and treated shrimps were then subjected to Tukey–Kramer test high significant differences (HSD) (Zar, 1996). However, four levels of significance were reported *viz.* ns: non significance, $= P< 0.05, #= P< 0.01 and *= P< 0.001.

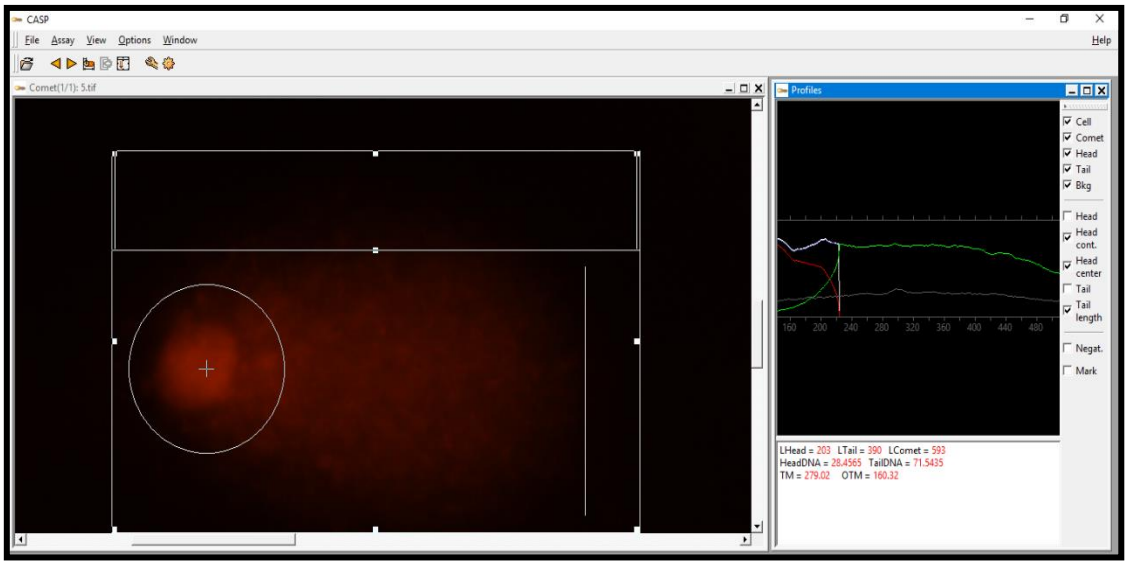
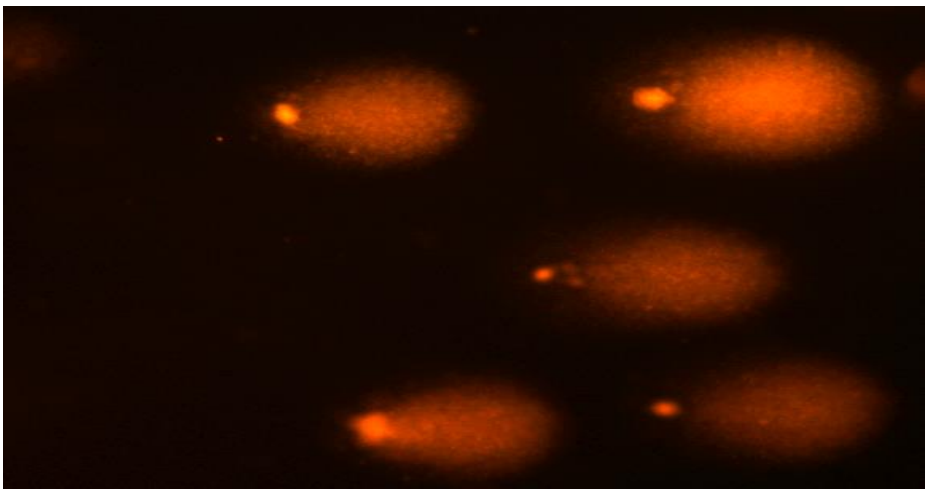
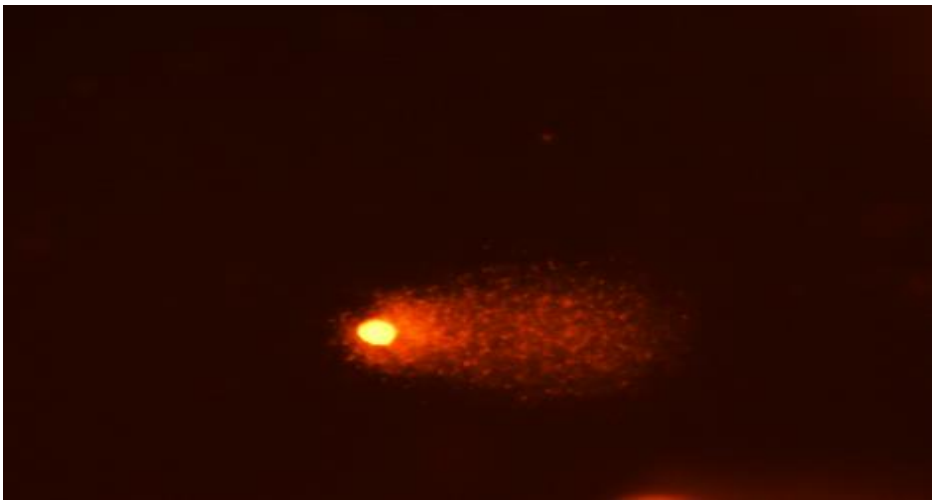


Plate 4.3 Image of nuclear DNA damage as analysed by Casp_1.2.3 beta software

Plate 4.4 Image of nuclear DNA from control specimens of *L. vannamei* (without pesticide exposure). (Scale= 100X magnification)



a



b

Plate 4.5 a, b Images (comets) of nuclear DNA from *L. vannamei* exposed to sub lethal (SL) doses of Chlorpyrifos (CPF)/ Dimethoate (DMT). (Scale= 100X magnification)

4.3 Results

4.3.1 *In vitro* exposure to hydrogen peroxide

The data on the % tail DNA damage in PL and JL (GL, HL and ML cells) on exposure to various concentrations of H_2O_2 are represented in Tables 4.1 and 4.2 respectively and are further shown graphically in Fig. 4.1 and Fig. 4.2, respectively. Results of comet assay showed significant DNA damage in cells of PL as well as GL, HL and ML cells of JL *vis-à-vis* their respective controls. The dose-dependent increase in DNA damage was observed in PL (Table 4.1) and different cell types of JL (Table 4.2). Therefore, the PL and targeted cells of JL showed

a positive response to genotoxin H_2O_2, thereby validating the sensitivity of *L. vannamei* to a genotoxicant. Moreover, the comet assay procedure employed for the DNA damage study also validated and proved to be a sensitive tool.

Table 4.1 Tail DNA (%) in post larvae treated with various concentrations of H_2O_2

Size of animal	Conc. of H_2O_2 (µM)	Tail DNA (%) (Mean ± SD)
Post Larvae	**Control**	3.34 ± 0.08
	1	8.32 ± 1.22
	5	12.21 ± 1.32
	20	33.11 ± 2.23
	30	41.43 ± 3.21
	60	54.13 ± 5.32

Table 4.2 Tail DNA (%) in gill, haemolymph and muscle cells of juveniles incubated with various concentrations of H_2O_2

Size of animal	Conc. of H_2O_2 (µM)	Tail DNA (%) (Mean ± SD)		
Juvenile		**Gill**	**Haemolymph**	**Muscles**
	Control	2.51 ± 0.08	3.26 ± 0.09	1.89 ± 0.01
	0.5	9.87 ± 1.43	13.21 ± 1.11	9.82 ± 1.21
	5	15.43 ± 2.11	18.76 ± 2.12	12.34 ± 1.24
	20	35.12 ± 3.24	38.34 ± 4.12	31.11 ± 3.12
	40	42.54 ± 5.32	48.65 ± 6.11	39.21 ± 3.32

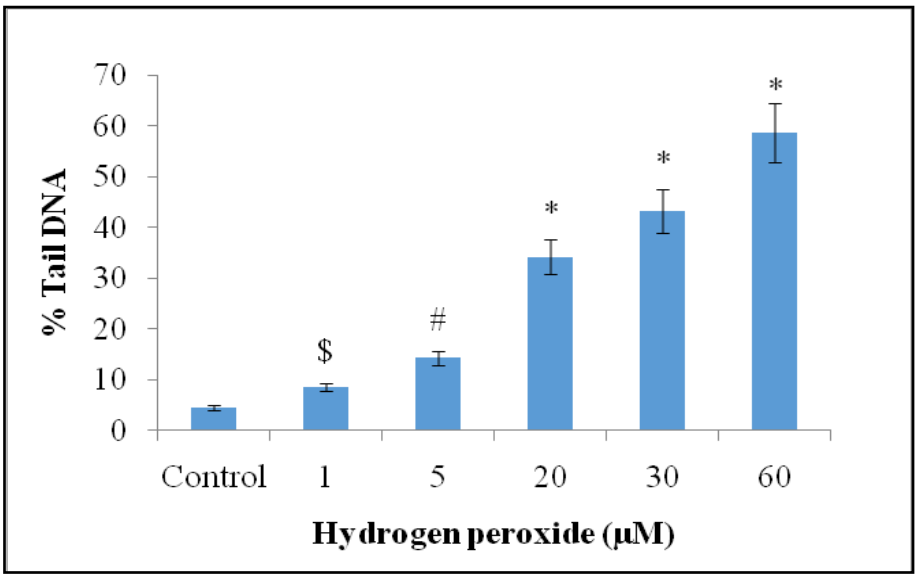


Fig. 4.1 Tail DNA (%) in post larvae incubated with various concentrations of H_2O_2. Values are expressed as Mean ± SD (ANOVA, $ = P< 0.05, # = P< 0.01, *= P< 0.001)

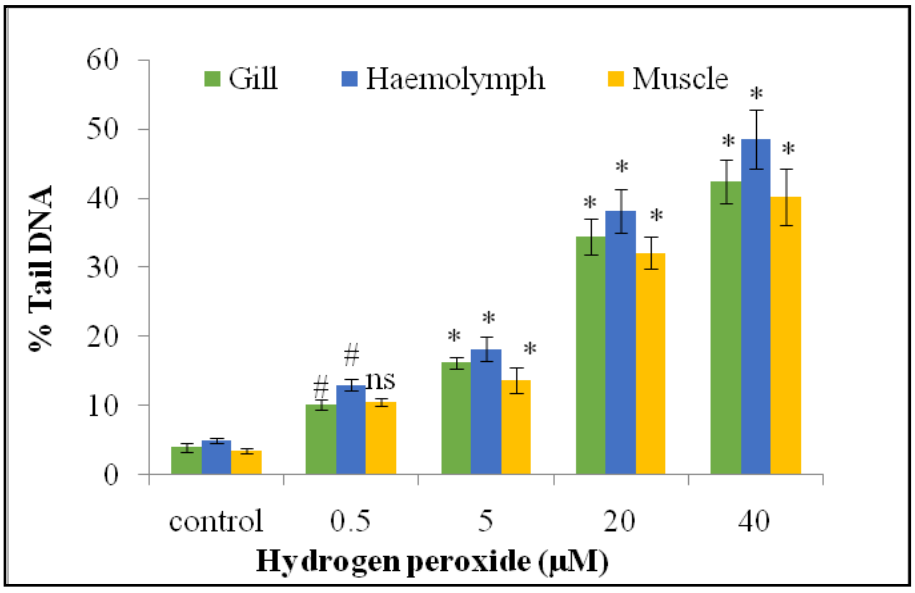


Fig. 4.2 Tail DNA (%) in gill, haemolymph and muscle cells of juveniles exposed to various concentrations of H_2O_2. Values are expressed as Mean ± SD (ANOVA, ns= Non significance, #= P< 0.01, *= P< 0.001)

4.3.2 DNA damage in post larvae (PL) exposed to Chlorpyrifos (CPF) and Dimethoate (DMT)

4.3.2.1 Chlorpyrifos induced DNA damage

CPF induced DNA damage in PL measured at 3, 7, 14 and 21 DoE and comparison between SL doses, CPF1 and CPF2 are represented in Tables 4.3 and 4.4 respectively and are further graphically represented in Fig. 4.3 and Fig.4.4, respectively. Results of ANOVA followed by Tukey-Kramer's multiple comparison tests were carried out to compare DNA damage between treated and control groups at different time intervals. The intensity of DNA damage recorded to be higher in the PL exposed to CPF1 compared to CPF2 at all time intervals (Table 4.3). Higher DNA damage was recorded at 21 DoE for both SL doses (Fig. 4.3). The highest % tail DNA damage recorded exposed to CPF1 was 43.24 ± 6.45 % and CPF2 was 28.23 ± 4.54 % respectively. However, the lowest % tail DNA damage recorded exposed to CPF1 was 15.21 ± 1.56 % and CPF2 was 8.52 ± 1.28 %, respectively. This differentiates the dose-dependent and time-dependent changes in DNA damage on exposure to CPF. Moreover, student-*t* test carried out to compare DNA damage induced by CPF1 and CPF2 for different time intervals ($P< 0.001$) (Fig. 4.4).

Table 4.3 Tail DNA (%) in post larvae exposed to two sublethal doses of Chlorpyrifos (CPF1 = 0.22 µg $L^{-1}$and CPF2 = 0.11 µg L^{-1}) at different time intervals

Size of animal	**Days of exposure (DoE)**	**Tail DNA (%) (Mean ± SD)**		
Post larvae		**Control**	**CPF1= 0.22 µg L^{-1}**	**CPF2= 0.11 µg L^{-1}**
	3	1.22 ± 0.22	15.21 ± 1.56	8.52 ± 1.28
	7	2.08 ± 0.32	30.08 ± 3.48	15.11 ± 2.13
	14	3.09 ± 0.21	35.11 ± 4.89	24.15 ± 3.67
	21	4.22 ± 0.54	43.24 ± 6.45	28.23 ± 4.54

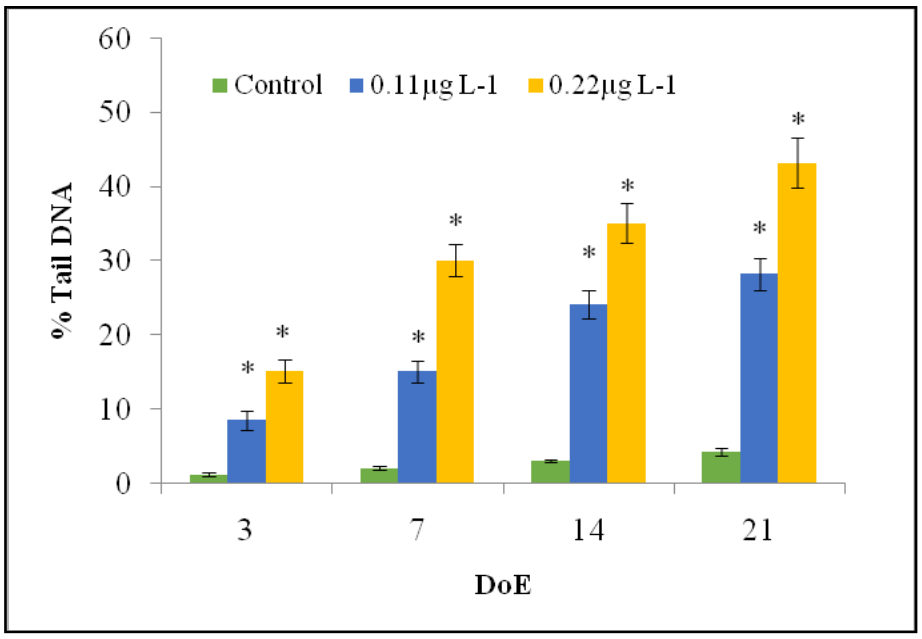


Fig. 4.3 Tail DNA (%) in Post larvae exposed to two sublethal doses of Chlorpyrifos (CPF1= 0.11 µgL^{-1} and CPF2= 0.22 µg L^{-1}). Values are expressed as Mean ± SD (ANOVA, #= P< 0.01, *= P< 0.001)

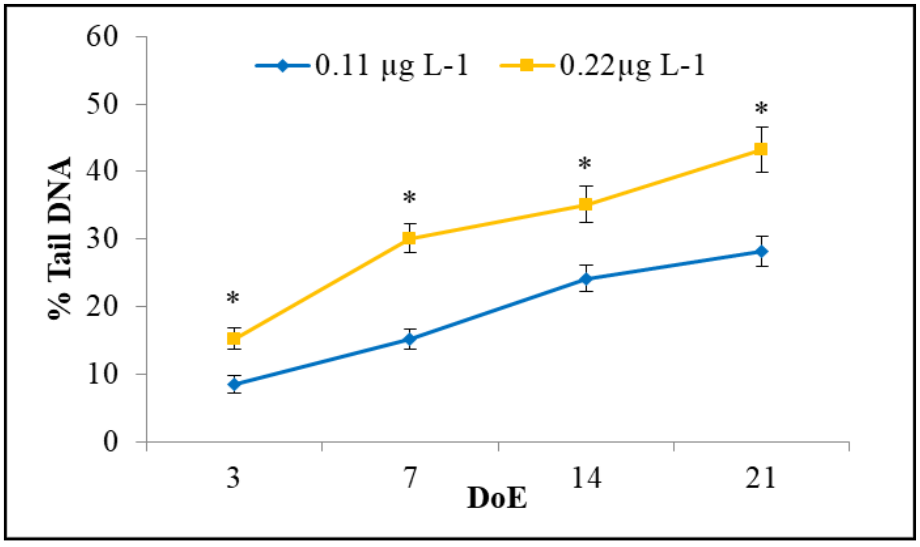


Fig. 4.4 Comparison of the DNA damage induced by CPF1 (0.22 µg L^{-1}) with CPF2 (0.11 µg L^{-1}) in Post larvae on 3, 7, 14 and 21 DoE. Values are expressed as Mean ± SD (Student-*t* test, *= P< 0.001)

4.3.2.2 Dimethoate induced DNA damage

DMT induced DNA damage in PL, measured at 3, 7, 14 and 21 DoE and comparison between SL doses, DMT1 and DMT2 are represented in Tables 4.5 and 4.6 repectively and are further graphically represented in Fig. 4.5 and Fig. 4.6, respectively. Results of ANOVA followed by Tukey-Kramer's multiple comparison tests were carried out to compare DNA damage between treated and control groups at different time intervals. The intensity of DNA damage recorded to be higher in the PL exposed to DMT1 compared to DMT2 at all time intervals (Table 4.4). Higher DNA damage was recorded on 21 DoE for both SL doses. DNA damage compared between the control and exposed shrimps for both SL doses showed a significantly higher DNA damage in exposed shrimp at all time intervals (Fig. 4.5). The highest % tail DNA damage recorded exposed to DMT1 was 38.11 ± 8.32 % and DMT2 was 20.14 ± 5.08 %, respectively. However, the lowest % tail DNA damage recorded exposed to DMT1 was 15.19 ± 1.26 % and DMT2 was 8.28 ± 1.22 %, respectively. This differentiates the dose-and time-dependent changes in DNA damage on exposure to DMT pesticide. Moreover, student-*t* test was carried out to compare DNA damage induced between DMT1 and DMT2 at all time interval ($P< 0.001$) (Fig. 4.6).

Table 4.4 Tail DNA (%) in post larvae exposed to two sublethal doses of Dimethoate (DMT1 = 89.46 µg $L^{-1}$and DMT2 = 44.73 µg L^{-1}) at different time intervals

Size of animal	**Days of exposure (DoE)**	**Tail DNA (%) (Mean ± SD)**		
		Control	**DMT1= 89.46 µg L^{-1}**	**DMT2= 44.73 µg L^{-1}**
Post larvae	3	1.22 ± 0.23	15.19 ± 1.6	8.88 ± 1.56
	7	2.08± 0.37	20.65 ± 3.22	12.12 ± 1.44
	14	3.09± 0.29	27.16 ± 4.13	16.22 ± 3.82
	21	4.22± 0.34	38.11 ± 8.32	20.14 ± 5.08

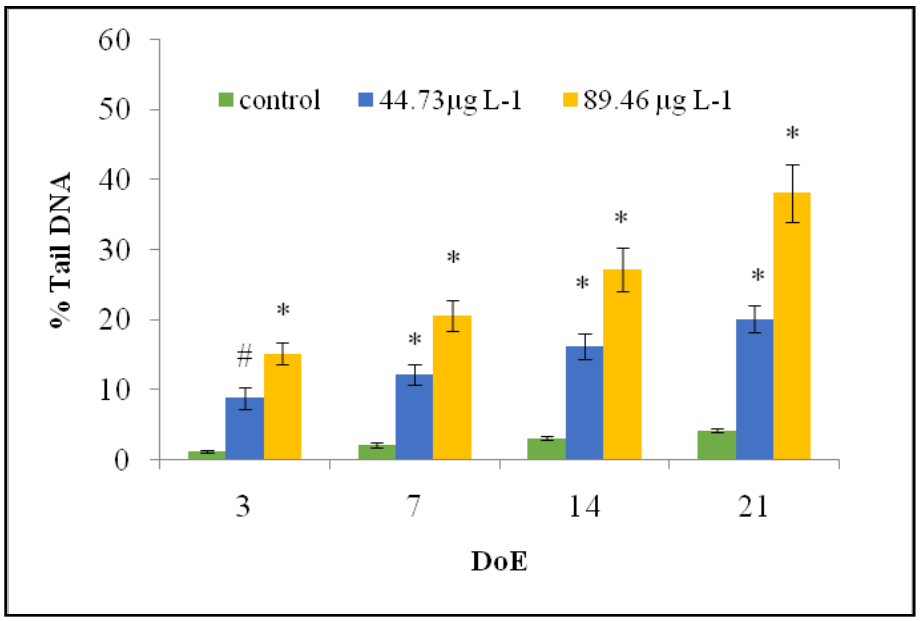


Fig. 4.5 Tail DNA (%) in post larvae exposed to two sublethal doses of Dimethoate (DMT1= 89.46 µg L^{-1} and DMT2= 44.73 µg L^{-1}). Values are expressed as Mean ± SD (ANOVA, #= P< 0.01, *= P< 0.001)

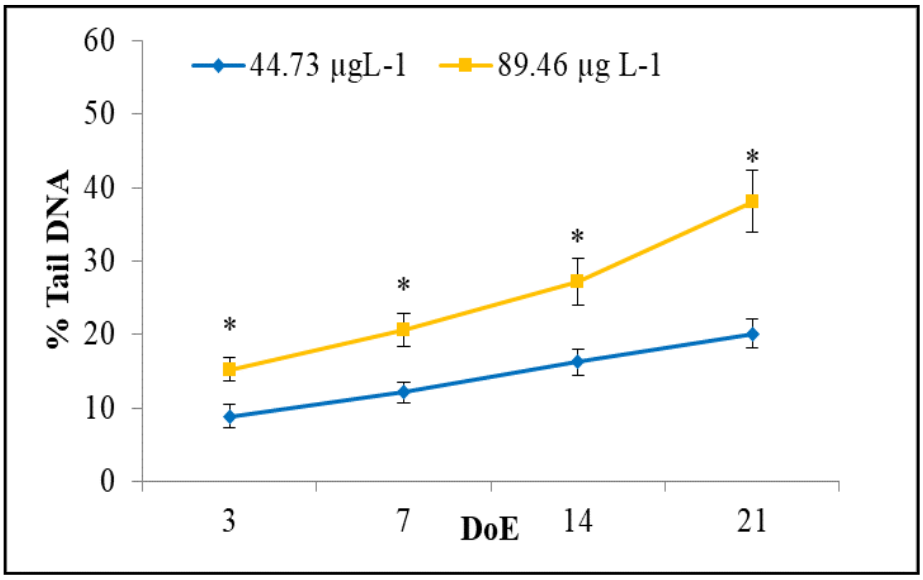


Fig. 4.6 Comparison of the DNA damage induced by DMT1 (89.46 µg L^{-1}) with DMT2 (44.73 µg L^{-1}) in post larvae on 3, 7, 14 and 21Days of exposure (DoE). Values are expressed as Mean ± SD (Student-*t* test, *= P< 0.001)

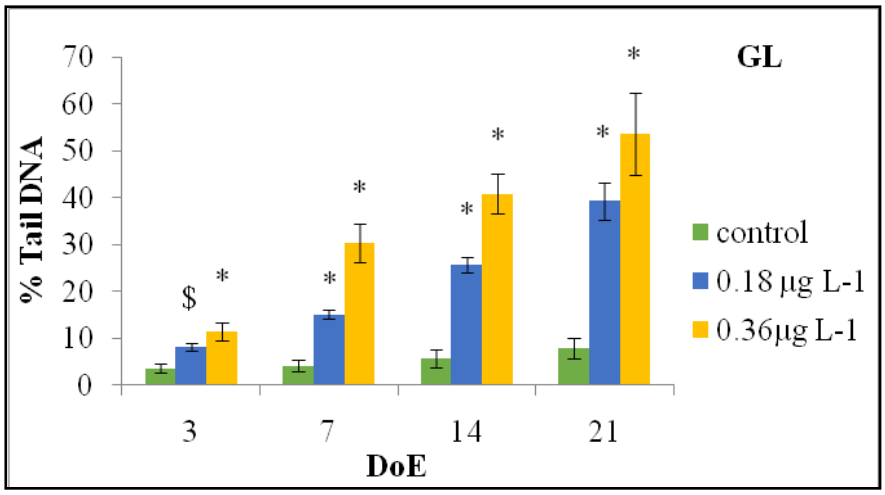
4.3.3 DNA damage in juveniles exposed to Chlorpyrifos and Dimethoate

4.3.3.1 DNA damage in gill (GL), Haemolymph (HL) and muscle (ML) cells of juveniles exposed to Chlorpyrifos (CPF)

The differential response in % tail DNA damage in GL, HL and ML cells exposed to two SL doses of CPF *vs.* control are represented in Tables 4.5 and are further graphically represented in Fig. 4.7 a, b and c, respectively. Results of ANOVA followed by Tukey-Kramer's multiple comparison tests were carried out to compare DNA damage between treated and control groups at different time intervals. The time-dependent and dose-dependent increase in % tail DNA damage was observed in GL, HL and ML cells (Table 4.5). At the end of 21 DoE, JL exposed to CPF1 exhibited significantly higher % tail DNA damage (GL= 53.61 ± 8.71 %, HL= 49.36 ± 3.42 %, ML= 32.40 ± 4.97 %) compared to those exposed to CPF2 (GL= 39.25 ± 3.90 %, HL= 32.22 ± 4.21 % and ML= 22.66 ± 2.85 %). The GL cells of *L. vannamei* exposed to CPF1 and CPF2 doses exhibited a significantly higher % tail DNA damage when compared to cells retrieved from HL and ML (Table 4.5). However, the lower level of % tail DNA damage was recorded in ML cells at CPF1 and CPF2 on comparison with HL and GL cells. Moreover, the levels of DNA damage in different cells followed the order, GL > HL > ML at all time intervals

Table 4.5 Tail DNA (%) in different cells of juveniles of exposed to two sublethal doses of Chlorpyrifos (CPF1= 0.36 µg $L^{-1}$and CPF2= 0.18 µg L^{-1}) at different time intervals

Size of animal	**Days of Exposure (DoE)**	**CPF1= 0.36 µg L^{-1}**			**CPF2= 0.18 µg L^{-1}**		
Juvenile		**Gill**	**Haemolymph**	**Muscles**	**Gill**	**Haemolymph**	**Muscles**
	3	11.54 ± 1.19	8.93 ± 1.25	5.21 ± 1.75	8.12 ± 0.82	7.25 ± 0.77	4.45 ± 0.54
	7	30.31 ± 4.12	18.41 ± 4.6	6.62 ± 1.54	15.14 ± 0.94	10.24 ± 1.83	5.21 ± 0.44
	14	40.83 ± 4.3	29.66 ± 3.87	15.14 ± 3.12	25.66 ± 1.65	22.66 ± 2.85	8.11 ± 1.56
	21	53.61 ± 8.71	49.36 ± 3.42	32.4 ± 4.97	39.25 ± 3.9	32.22 ± 4.21	22.66 ± 2.85



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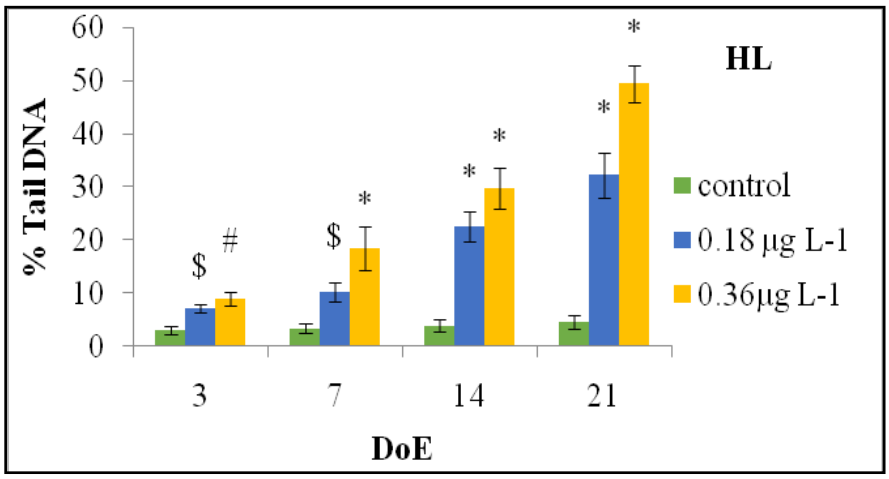

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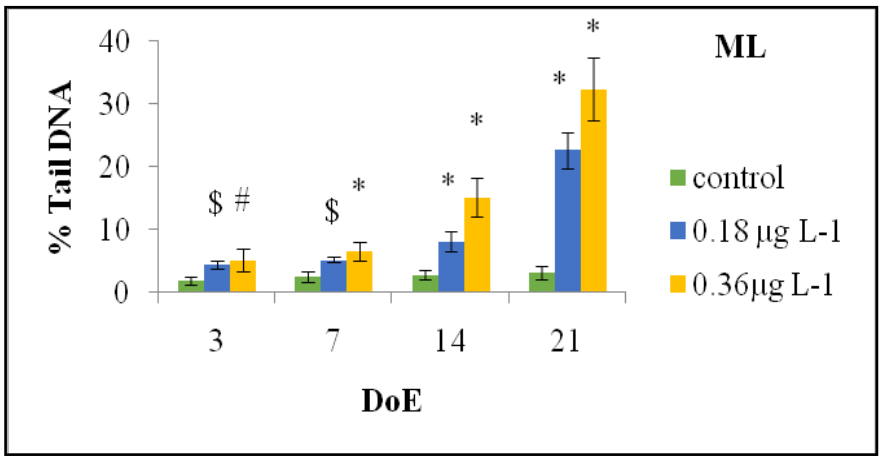
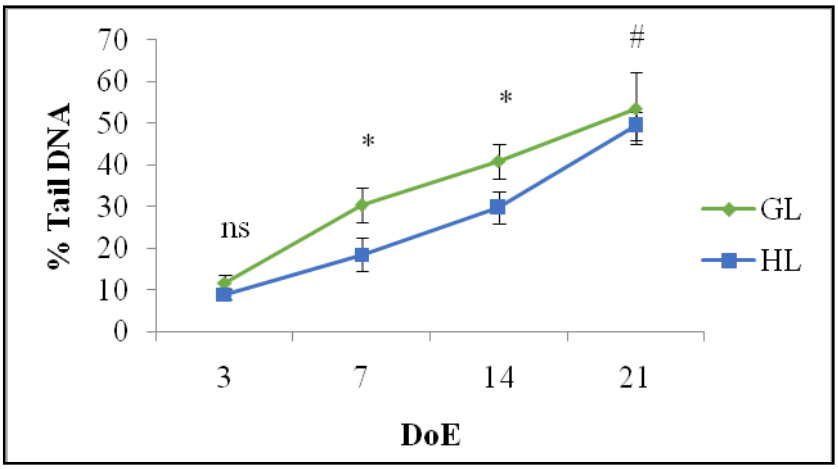


c

Fig. 4.7 a, b, c Tail DNA (%) in different cells of juveniles exposed to two sublethal doses of Chlorpyrifos (CPF1 = 0.36 µg L^{-1} and CPF2 = 0.18 µg L^{-1}) at the end of 3, 7, 14 and 21 DoE. Values are expressed as Mean ± SD (ANOVA, $= P< 0.05, #= P< 0.01, *= P< 0.001)

4.3.3.1.1 Comparative assessment of DNA damage in different cells of juveniles

The multiple comparisons of DNA damage in cells of GL, HL and ML at 3, 7, 14 and 21 Days exposed to CPF1 and CPF2 are represented in Fig. 4.8 a, b, c, Fig. 4.9 a, b, c and Table 4.5 respectively. The DNA damage compared between GL and HL exposed to CPF1 showed higher DNA damage in GL compared to HL at all time intervals except 3 DoE (Fig. 4.8a). On the other hand, DNA damage compared between GL and HL exposed to CPF2 also showed higher DNA damage in GL compared to HL at all time intervals except 3 and 14 DoE (Fig. 4.9a). However, the DNA damage compared between GL and ML exposed to CPF1 and CPF2 showed higher DNA damage in GL compared to ML ($P< 0.001$) at all time intervals (Fig. 4.8b and 4.9b). Moreover, the DNA damage compared between HL and ML at CPF1 (Fig. 4.8c) and CPF2 (Fig. 4.9c) showed higher DNA damage in HL compared to ML at all time intervals except 3 DoE.



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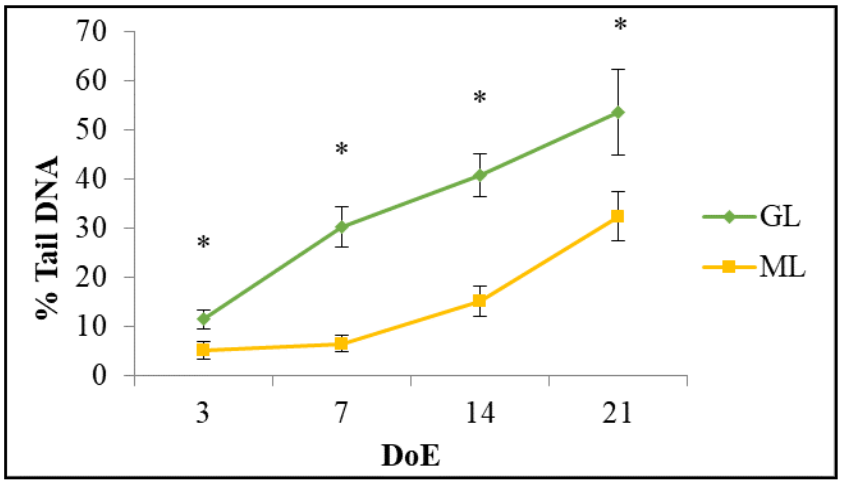

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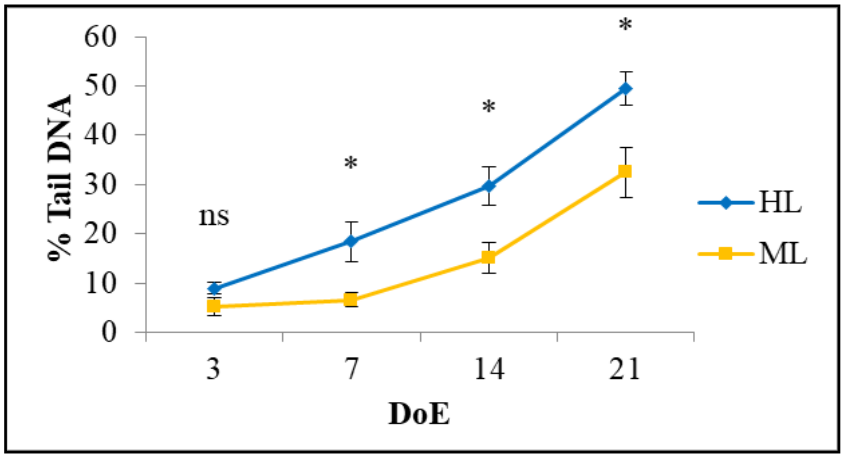
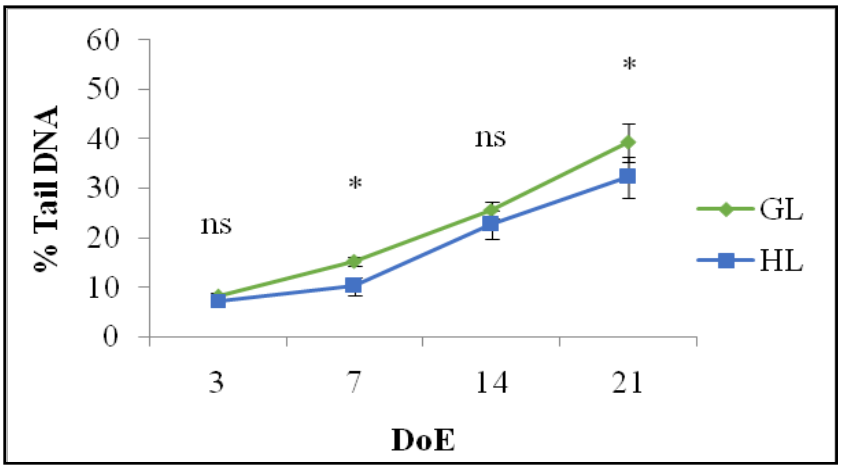


c

Fig. 4.8 a, b, c Comparison of DNA damage induced by CPF1 (0.36 µg L^{-1}) in gill, haemolymph and muscle cells of juveniles at the end of 3, 7, 14 and 21 Days of Exposure (DoE). Values are expressed as Mean ± SD (Student-*t* test, $= P< 0.05, #= P< 0.01, *= P< 0.001)



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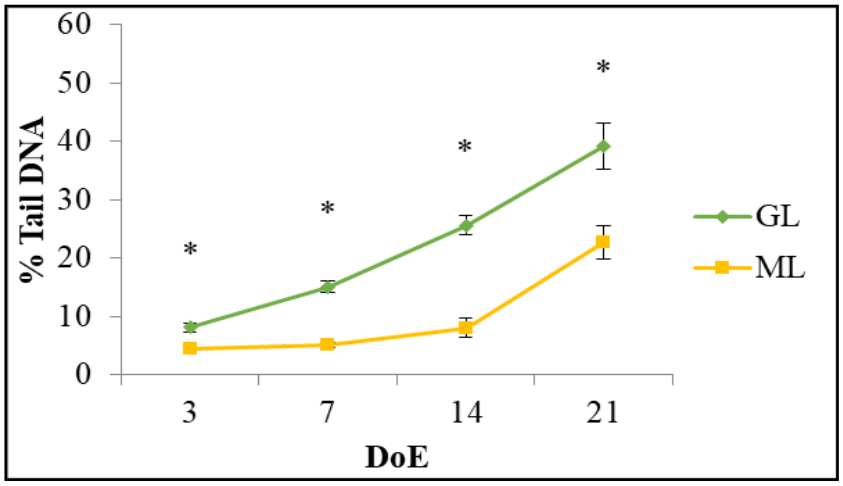

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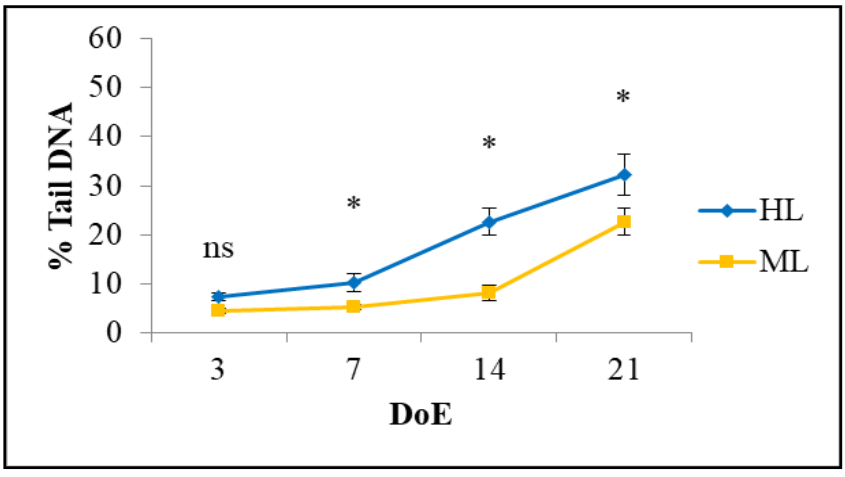


c

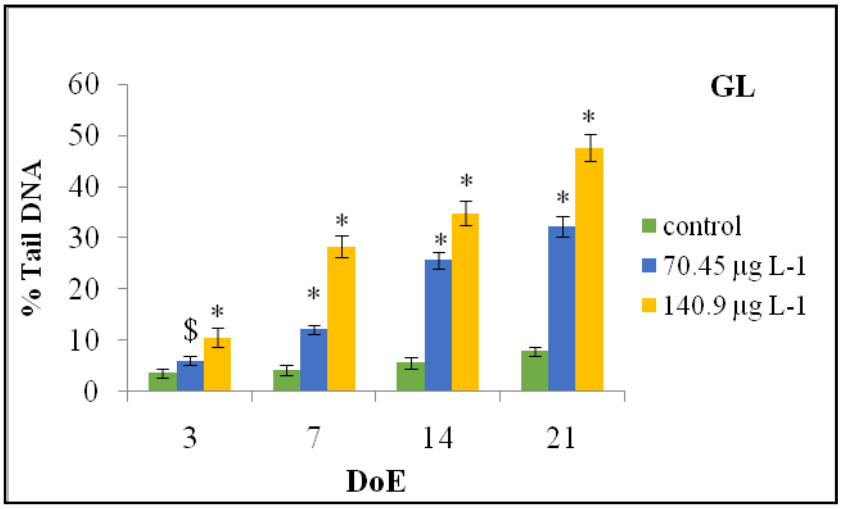
Fig. 4.9 a, b, c Comparison of DNA damage induced by CPF2 (0.18 µg L^{-1}) in gill, haemolymph and muscle cells of juveniles at end of 3, 7, 14 and 21 Days of Exposure (DoE). Values are expressed as Mean ± SD (Student-*t* test, $= P< 0.05, # = P< 0.01, * = P< 0.001, ns= non-significance)

4.3.3.2 DNA damage in gill (GL), haemolymph (HL) and muscle (ML) cells of juveniles exposed to Dimethoate (DMT)

The differential response in % tail DNA damage in GL, HL and ML cells exposed to two SL doses of DMT *vs.* control are represented in Fig. 4.10 a, b, c. Results of ANOVA followed by Tukey–Kramer multiple comparison tests revealed that the % tail DNA damage differed significantly between control and treated groups at all time intervals. The time-dependent and dose-dependent increase in % tail DNA damage was observed in GL, HL and ML cells (Table 4.6). At the end of 21 DoE, JL exposed to DMT1 exhibited significantly higher % tail DNA damage (GL= 47.61 ± 8.71 %, HL= 41.36 ± 3.42 % and ML= 30.40 ± 4.97 %) compared to those exposed to DMT2 (GL= 32.25 ± 3.90 %, HL= 28.22 ± 4.21 % and ML= 20.66 ± 2.85 %). The GL cells of *L. vannamei* exposed to DMT1 and DMT2 doses exhibited significantly higher % tail DNA damage when compared to cells retrieved from HL and ML (Table 4.6). However, the lower level of % tail DNA damage recorded in ML cells at DMT1 and DMT2 on comparison with HL and GL cells (Table 4.6). Moreover, the levels of DNA damage in different cells followed the order, GL > HL > ML at all time intervals.

Table 4.6 Tail DNA (%) in different cells of juveniles exposed to two sublethal doses of Dimethoate (DMT1 = 140.90 µg $L^{-1}$and DMT2 = 70.45 µg L^{-1}) at different time intervals

Size of animal	**Days of Exposure (DoE)**	**DMT1 = 140.90 µg L^{-1}**			**DMT2 = 70.45 µg L^{-1}**		
		Gill	**Haemolymph**	**Muscles**	**Gill**	**Haemolymph**	**Muscles**
Juvenile	3	10.54 ± 1.22	7.93 ± 1.11	4.21 ± 1.22	6.12 ± 1.34	4.25 ± 0.89	3.45 ± 1.01
	7	28.31 ± 3.44	15.41 ± 1.89	5.62 ± 2.13	12.14 ± 1.56	8.24 ± 1.10	4.21 ± 1.02
	14	34.83 ± 4.12	26.66 ± 2.33	12.14 ± 1.43	25.66 ± 2.34	20.66 ± 2.45	6.11 ± 1.23
	21	47.61 ± 8.71	41.36 ± 3.42	30.4 ± 4.97	32.25 ± 3.90	28.22 ± 4.21	20.66 ± 2.85



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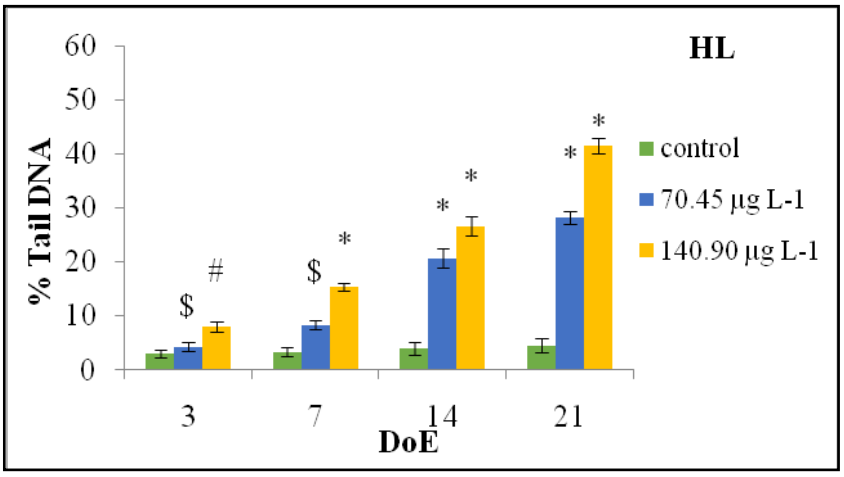

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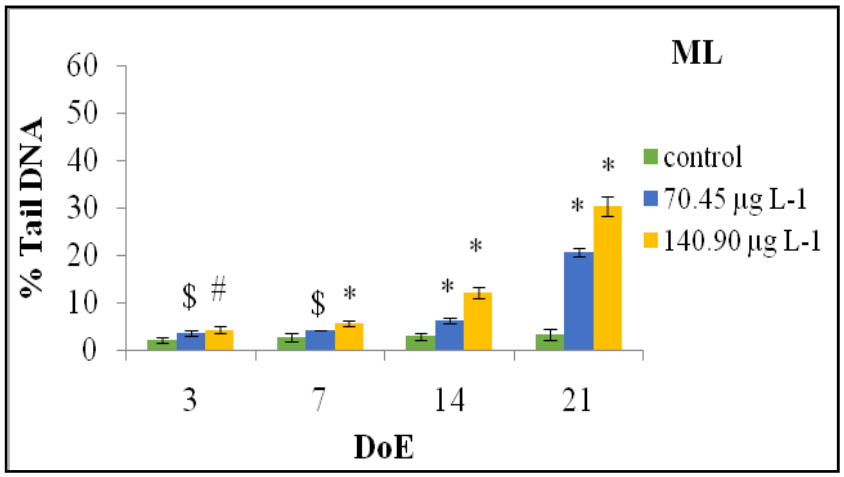
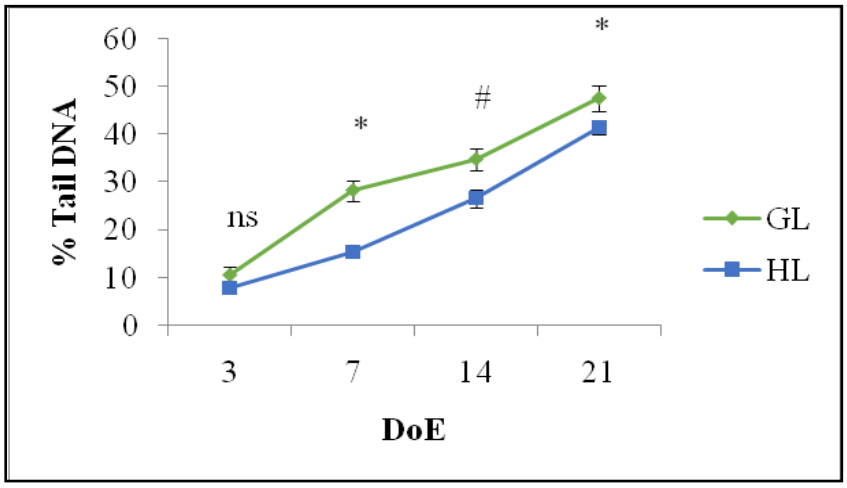


c

Fig. 4.10 a, b, c The % tail DNA in different cells of *L. vannamei* juvenile exposed to two sublethal doses of Dimethoate (DMT1 = 140.90 µg L^{-1} and DMT2 = 70.45 µg L^{-1}) at the end of 3, 7, 14 and 21 Days of exposure (DoE). Values are expressed as Mean ± SD (ANOVA, $ = P< 0.05, # = P< 0.01, *= P< 0.001)

4.3.3.2.1 Comparative assessment of DNA damage in different cells of juveniles

The multiple comparisons of DNA damage in cells of GL, HL and ML at 3, 7, 14 and 21 Days exposed to DMT1 and DMT2 are represented in Fig. 4.11 a, b, c, Fig. 4.12 a, b, c and Table 4.6 respectively. The DNA damage compared between GL and HL exposed to DMT1 and DMT2, showed higher DNA damage in GL compared to HL at all time intervals except 3 DoE (Fig. 4.11 a and Fig. 4.12 a). The DNA damage compared between GL and ML exposed to DMT1 and DMT2, showed higher DNA damage in GL compared to ML at all time intervals (Fig. 4.10 b and 4.12 b). Moreover, the DNA damage compared between HL and ML at DMT1 (Fig. 4.11 c) and DMT2 (Fig. 4.12 c) showed higher DNA damage in HL compared to ML at all time intervals except 3 DoE at DMT2.



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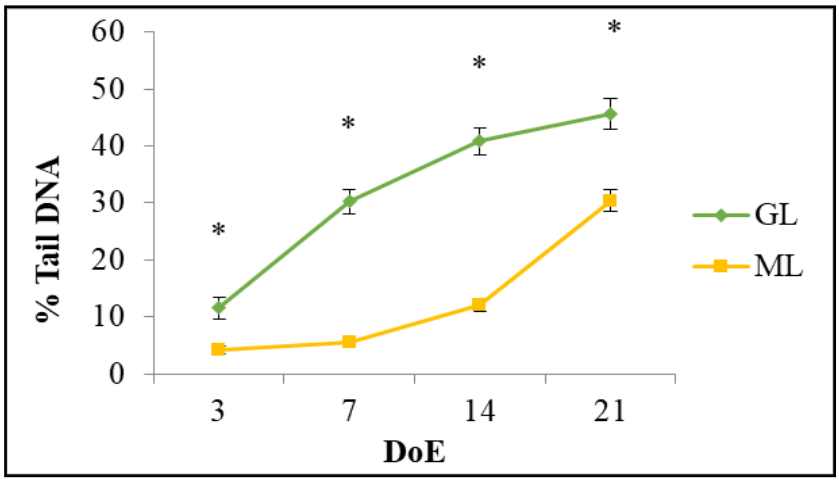

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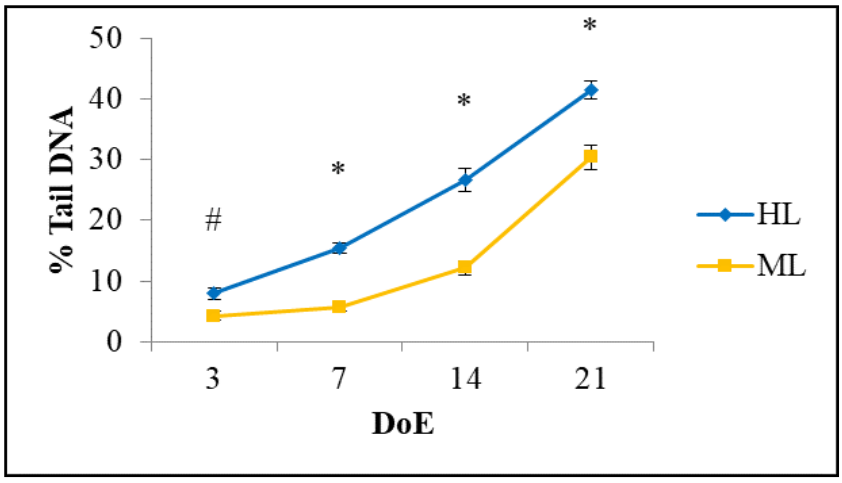
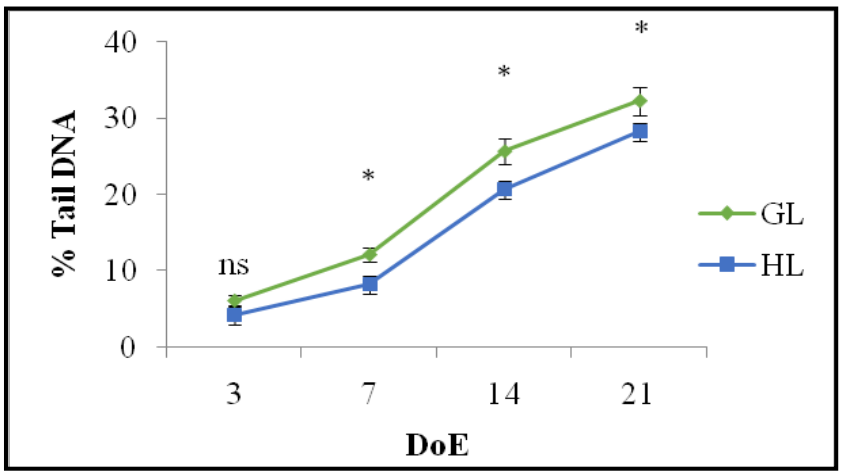


c

Fig 4.11 a, b, c Comparison of DNA damage induced by DMT1 (140.90 µg L^{-1}) in gill, haemolymph, and muscle cells of juveniles at the end of 3, 7, 14 and 21 Days of exposure (DoE). Values are expressed as Mean ± SD (Student-*t* test, $ = P< 0.05, # = P< 0.01, *= P< 0.001



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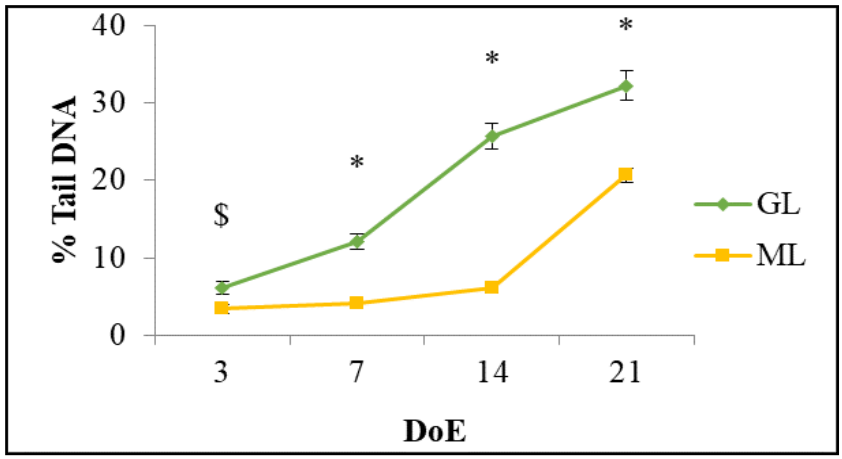

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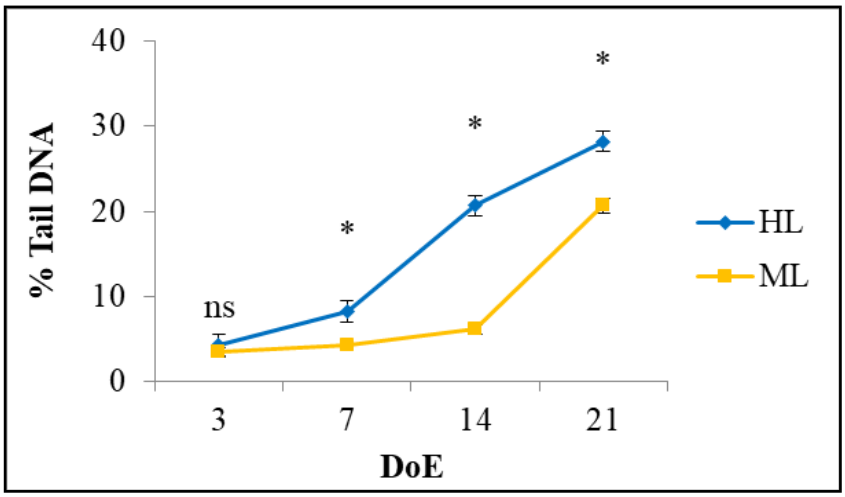

c

Fig. 4.12 a, b, c Comparison of DNA damage induced by DMT2 (70.45 µg L^{-1}) in gill, haemolymph and muscle cells of juveniles at the end of 3, 7, 14 and 21 Days of exposure (DoE). Values are expressed as Mean ± SD (Student-*t* test, $ = P< 0.05, # = P< 0.01, *= P< 0.001, ns= non significance)

4.4 Discussion

In genetic toxicology, comet assay is used as a rapid and strong tool to evaluate the relationship between the DNA damage and genotoxicant exposure. The DNA damage study through comet assay is advantageous as it does not require mitotically active cells (Buschini et al., 2003), which is essential for other cytogenetic methodologies (chromosomal aberration and micronucleus tests). Hence, it has been extensively employed in genotoxicity studies and environmental monitoring programmes (Buschini et al., 2003; Lee and Steinert, 2003; Frias-Espericueta et al., 2011; Dailianis et al., 2014; Ullah et al., 2016; Bhagat et al., 2016). Also, this technique is less expensive and less time consuming over other biomonitoring tools (Zeid and Khalil, 2014). The percentage of DNA in tail region reflects the amount of DNA migrated out of the nucleus and directly related to the quantification of DNA breakage (Singh et al., 1988). The intoxication of CPF and DMT pesticides at chronic level in *L. vannamei* PL and different cells (GL, HL and ML) of JL resulted in a significant increase in % tail DNA throughout the exposure. The time-dependent increase in DNA damage is in accordance with the study carried out in *Cirrhinus mrigala* on exposure to CPF (Sunanda et al., 2016). Moreover, it has been also observed that in dose-dependent study, where exposing animals at ecologically relevant range can be co-related with the survival of organisms (Ullah et al., 2016). Considering for this context, it shows co-relation and clear dose-response effect in increased DNA damage. In comparative aspects between DNA damage induced by CPF and DMT, it showed CPF has a higher potential to induce DNA damage than DMT.

The DNA damage in *L. vannamei* JL was highly tissue-specific, dose-dependent as well as time-dependent. Few previous reports on DNA damage in *L. vannamei* and *L. rohita* are available for comparison (Frias-Espericueta et al., 2011; Mohanty et al., 2011). Both a time-dependent and a dose-dependent increase in DNA damage were observed in haemocytes of *L. vannamei* following exposure to copper has been observed by Frias-Espricueta et al. (2011). On the other hand, a dose-dependent increment in DNA damage in GL cell lines of *P. monodon* exposed to two OP (Malathion and Monotrotophos), have been also reported by Jose et al. (2011). A dose-dependent increment in DNA damage in the blood (30.34 ± 0.76 %), GL (58.17 ± 0.65 %) and liver (47.83 ± 0.30%) tissues of *L. rohita* after 96 h exposure of Phorate has been reported by Mohanty et al. (2011). On the other hand, similar observations of DNA damage concomitant with the increase in dose on exposure to Monocrotophos reported in erythrocytes of fish, *Tilapia mossambicus* (Banu et al., 2001).

In the present study, a dose-dependent increase in % tail DNA damage was observed at both SL doses of CPF and DMT. At the end of 21 DoE, the % tail DNA damage in PL and all three tested cells of JL exposed to CPF1 and DMT1 were significantly ($P< 0.05$) higher compared to those exposed to CPF2 and DMT2. The greater magnitude of variability in DNA damage witnessed in different cells of *L. vannamei* on exposure to two SL doses of CPF could be attributed to the generation of high number of free radicals (Ali et al., 2009). The potential of CPF to induce oxidative stress in organisms and cause over production of Reactive Oxygen Species (ROS) has been documented by Livingstone, (2001). The production of higher amounts of free radicals as a consequence of increased metabolic activities resulting in damage to the protein and nucleic acids on exposure to OP compounds have been reported (Hodgson and Levi, 1996; Mohanty et al., 2011; Bhagat et al., 2016). Therefore, dose-dependant increase in DNA damage observed in *L. vannamei* during the present study might be due to increased production of free radicals at a higher SL dose. However, the lower SL doses also had potential to create the DNA damage in PL and different cells of juveniles, but the higher SL doses induced DNA damage was more noticeable compared to the lower SL doses.

Reyes et al. (2002) studied the diverse toxic effects (including LC_{50}, DNA adducts and breaks and total protein) of various pesticides *viz.* DDT, azinphosmethyl, permethrine, parathion, chlorpyrifos, malathion, endosulfan and carbaryl in the larvae of the shrimp, *Litopenaeus stylirostris.* The results indicated a significant reduction in the DNA and total protein in the larvae exposed to these pesticides. Breaks and adducts were also registered in those larvae exposed to DDT. These indicate that pesticide pollution can induce severe toxic effects which in turn are expected to induce a lower growth rate and other health hazards in shrimp larvae. It is possible that pesticide pollution induced reduction in total protein results in a decrease in larval growth rate and the DNA breaks or adducts have been related to pathologies and carcinogenesis (Reyes et al., 2002). The study carried out by Lavarias et al. (2013) also supports the above findings of toxic effect of OP pesticide. Exposure of freshwater prawn *Macrobrachium borellii* to OP pesticide Fenitrothion induced significant changes in their various biochemical parameters. The pesticide induced oxidative stress in the prawns altered their biochemical composition and consequently induced DNA damage in them.

Out of the three cell types used to assess the potential DNA damage, the cells from GL showed a higher degree of DNA damage than those retrieved from HL and ML irrespective of SL doses. Further, an increment in DNA damage in GL of *L. vannamei* JL with the DoE was also observed. Such tissue-specific DNA damage observed in the present study is in

congruence with the previous study by Ali et al. (2009), who reported that CPF inflicts more damage in GL cells than lymphocyte cells of freshwater fish, *C. punctatus*. In crustaceans, GL are important organs of respiration as well as of osmoregulation (Pequeux, 1995). Significant damage to osmoregulatory effectors, particularly gills, by OP contamination has been demonstrated by histopathological studies (Pawar and Katdare, 1984; Baticados and Tendencia, 1991). Higher DNA damage observed in GL cells might be due to respiration activity of juvenile shrimps which allows the constant flow of pesticide water over gill surface (Dzwonkowska and Hubner, 1986; Ali et al., 2009), whereas haemolymph receives contaminant when chemicals enter into the circulatory system (Ali et al., 2009). Increased rate of respiration in JL of *L. vannamei* following SL exposure of four different organochlorine pesticides has been documented by Reyes et al. (1996). In comparison with other cells, the GLs are relatively more susceptible and sensitive to the injury caused by xenobiotic compounds due to inefficient detoxification system in shrimps and prawns compared to fishes (Hayashi et al., 1998; Cavas and Ergene-Gozukara, 2003, 2005; Mohanty et al., 2011). Furthermore, an impairment of the GL structure and ultra-structure in JL of *Penaeus monodon* after 96 h exposure to lethal concentrations to OP pesticide, gusathion A has been documented by Baticados and Tendencia (1991). It has been hypothesized that the cytochrome P450-1A (a protein family involved in the biotransformation of organic chemicals) present in fishes and crustaceans plays an important role in hydrocarbon metabolism and has greater significance in detoxification and protection against toxic injury caused by xenobiotic compounds (Haaschet al., 1989; Goksoyret al., 1991; Reyes et al., 2002; Koenig et al., 2012). In contrast, the same protein present in molluscs and crustaceans has relatively a lower detoxification capacity than fishes (Livingstone 1989, 1992; Koenig et al., 2012). Furthermore, it must be emphasized that physicochemical interactions between various types of organisms and rearing medium may result in a variable response in different cells on exposure to xenobiotic compounds (Ali et al., 2009). High variability in genotoxic response observed in different cells during the present study might be due to their differential ability in responding to CPF exposure.

In aquatic environments, the effects on structural and functional units caused by SL concentrations of xenobiotics are higher than inducing the mortality (Sancho et al., 2003). A study conducted by the US Environmental Protection Agency laboratory (EPA, 1981) highlighted that the penaeid shrimps are more sensitive to pesticides compared to fishes or molluscs. Therefore, it appears that economically important non-target organisms, particularly shrimps are relatively more sensitive to several xenobiotic compounds in their ecosystem (Krieger, 2001). Furthermore, the presence of pesticide in rearing water weakens the shrimp

immune system and cause an out break of infectious diseases at lower concentrations (Roque et al., 2005; Mello et al., 2011). A 27-53% shell softening in *P. monodon* JL during 96 h exposure to OP pesticide, Gusathion A has been reported by Baticados and Tendencia (1991). Moreover, significantly higher mortality in *L. vannamei* JL after combined exposure to the OP insecticide, methyl parathion and the bacterium *Vibrio parahaemolyticus* in comparison to each stressor individually has been experimentally documented by Labrie et al. (2003).

Considering the sensitivity of shrimps to pesticides (due to their phylogenetic similarities with insects, particularly the nervous and muscular systems), the chronic exposure of pesticides to the biological units (DNA damage) could result in the production of poor nutritional and commercial quality product (Mello et al., 2011). This is corroborated by the considerable DNA damage recorded during the present study, specifically in ML cells, the main edible body part of shrimp. A significantly marked decrement in protein content (28–42%) in JL of *L. vannamei* on exposure to four organochlorine pesticides has been reported by Reyes et al. (1996). A progressive depletion in total protein content in GL, ML, hepatopancreas and brain on the exposure (28 DoE) to SL doses of CPF has been documented by Narra et al. (2013). Furthermore, significant alterations in major biochemical constituents in penaeid shrimp (*Metapenaeus monoceros*) on exposure to SL doses of endosulfan pesticide has also been reported (Suryavanshi et al., 2009). Moreover, the metabolic alteration and reduction in protein content have a direct impact on the growth rate of larval development that has been observed by Reyes et al. (2002). Therefore, it appears that the SL doses of CPF and DMT are capable of inflicting DNA damage, growth of shrimps and lowering the nutritional quality of commercially important shrimp species.

4.5 Conclusion

Based on the degree of DNA damage recorded in PL and different cells (GL, ML and HL) of JL it implies that *L. vannamei* are highly sensitive in detecting the genotoxic effects induced by OP. We can also conclude that the DNA damage in the present study may be associated with oxidative stress on pesticide exposure leading to relatively higher DNA damage compared to the control. The comet assay proved to be a simple, reliable and inexpensive bio-marker tool to evaluate DNA damage at cell level. The present work contributes towards acknowledgment on potential pesticide effects on penaeid shrimp. Therefore, to monitor the presence of environmental or occupational contaminants, this study opens new prospective to use *L. vannamei* as a suitable bio-indicator organism for pesticide toxicity study.

The results also point to the potential risks involved in the extension of *L. vannamei* farming to inland areas, where pesticides are routinely used. Standard toxicity bioassays may not be predictive of actual pesticide toxicity under variable environmental conditions. Hence, testing under a wider range of exposure conditions could improve the accuracy of chemical risk assessments. Further studies assessing the immune responses in different shrimp life stages and varying culture conditions on exposure to OP would advance our current understanding of shrimp nutritional pathology. Such information would facilitate the development of diagnostic tools for better decision making in aquaculture management.

Chapter 5

Antioxidant enzyme activity and their gene expression in *L. vannamei* exposed to Chlorpyrifos and Dimethoate

5.1 Introduction

Although the pesticides are often intentional releases includes their surface runoff and leaching into the environment through various processes which contaminate the environment mainly the aquatic habitat (Farombi et al., 2008; Tripathi and Shasmal, 2011; Ramesh et al., 2018). Such release of pesticides in the aquatic environment creates the oxidative stress in aquatic organisms through the generation of reactive oxygen species ROS (Livingstone, 2001; Modesto and Martinaze, 2010; Dogan et al., 2011).

Oxidative stress arises in the body when there is an imbalance between the production of ROS and the antioxidant defence system. Therefore, when the production of ROS exceeds the rate of their removal and causes the peroxidation of lipid biomolecules within cells (Hernandez et al., 2013; Weindiger and Kozlov, 2015). ROS, the free radicals are atom or molecule containing one or more unpaired electrons in their outer shell. The free radicals and ROS are generated from normal metabolism or metabolism of other xenobiotic chemicals including pesticides and airborne pollutants including industrial chemicals (Farombi, 2008; Blanco-Ayala et al., 2014). The free radical's generation results into oxidation of the proteins, DNA and steroid components, enzyme inactivation as well as peroxidation of lipids in cell membranes which on further decomposition threaten the cell integrity (Borg and Schaich, 1984; Tuzmen et al., 2008; Barski et al., 2011).

When a toxic chemical enters an organism, it activates several biochemical and physiological mechanisms in that organism to rectify the toxic stress caused by that particular chemical compound (Raja and Puvaneswari, 2017). The biological mechanism of activation mainly includes activity of the antioxidant enzyme system involved in the oxidation of various substances which play an important role as a catalyst during the process. Thus, the interference of the enzyme activities may lead to harmful results. Hence, the quantum of enzyme activity in aquatic animals may serve as early indicators of toxicity of pesticides, heavy metals and other pollutants (Kabila, 1996; Yamuna, 1997; Richterova et al., 2015). The antioxidant enzymes prevent the production of ROS eventually closing of lipid peroxidation cycle (Lushchak, 2011). Further, the antioxidant defence system includes the pool of free radical, Superoxide, hydrogen peroxide and hydroxyl radicals scavenging enzymes *viz*. superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx). The CAT enzyme catalyses hydrogen peroxide into water and oxygen molecules whereas SOD and GPx catalyse the dismutation of superoxide to hydrogen peroxide and oxygen molecules (Storey, 1996; Kryuko et al., 2003). An increase and

decrease in the activity of these enzymes play a crucial role to maintain cell homeostasis and ultimately the health of organisms (Ojha et al., 2011; Poljsak et al., 2013). Antioxidant enzymes activity level can be used as bioindicators of pesticide pollution. Therefore, in the present investigation of antioxidant activities of enzyme *viz*. CAT, SOD and GPx were studied in post larvae (PLs) and juveniles Gill (GL), haemolymph (HL) and muscles (ML) of *L. vannamei* on exposure to sublethal (SL) dose of two OPs pesticides *viz.* Chlorpyrifos (CPF) and Dimethoate (DMT).

Organisms exhibit a characteristic response to a stressor that may be measured through a variety of parameters which include the metabolic components in blood, liver and muscle tissues, DNA damage, enzyme activity and gene expression. The gene expression study deals with the collection of information from a gene which is used for the synthesis of a functional gene product, protein. Gene expression study is involved in almost all forms of life from prokaryotes to eukaryotes i.e., viruses, bacteria, fungi, mammals and humans. This process includes transcription, splicing and translation of protein. These processes are important to control the structural and functional unit in cells. The gene regulation serves the major importance and contributed towards the function of a gene in the cell and ultimately in organisms.

In order to prevent the introduction or spread of diseases in crustaceans, a rigorous testing is required prior to their shipment from the countries where pathogens were found previously. Further, importing crustaceans may also wish to perform additional testing prior to accepting shipments (Walker and Winton, 2010). As such, assays for the detection of crustacean pathogens are an important part of any aquatic diagnostic laboratory portfolio. Sensitive molecular assays are often considered to be the best methods for the surveillance of samples destined for import or export (Sri Widada et al., 2004; Hernandez-Herrera et al., 2007; Poulos et al., 2008). More specifically, quantitative PCR (qPCR) is often the preferred molecular assay, as the technique is very sensitive and the results can usually be obtained within a few hours to a few days from the reception of the samples (Andrade et al., 2007; Mendoza-Cano and Sanchez-Paz, 2013). Studies on the effect of *Vibrio harvae* on the expression SOD gene in *L. vannamei* employing the technique of RT-PCR showed the upregulation of the gene which indicate the activation of gene on exposure to environmental contamination by a pathogen (Rivera-de et al., 2019). Jang et al. (2008) effectively used the real-time PCR method to quantify the load of white spot syndrome virus (WSSV) and hepatopancreatic parvovirus (HPV) in the shrimp and pond water in which fleshy shrimp, *Fenneropenaeus chinensis*, and Pacific white

shrimp, *Litopenaeus vannamei*, were reared. These reports and studies show the sensitivity and accuracy of Real Time PCR technique for the gene expression studies in *L. vannamei* for the present investigation. Owing to the great concern towards the gene regulation, our present study also focuses on the expression of antioxidant enzyme genes through Real-Time PCR in *L. vannamei* post larvae and juveniles. This chapter deals with the investigations on the antioxidant enzyme activity and their gene expression in *L. vannamei* exposed to two OP pesticides, Chlorpyrifos and Dimethoate.

5.2 Material and methods

5.2.1 Experimental animals

Shrimp PL (PL14; total length, 12.4 ± 0.5 mm; wet weight, 55 ± 5 mg) and juveniles (JLs) (Total length, 52.2 ± 10 mm; wet weight 1.78 ± 1.0 g) were used to study the acute toxicity of DMT and CPF.

5.2.2 Sublethal (SL) dose selection

The sublethal (SL) dose of CPF and DMT was selected based on the 96 h LC_{50} values of respective pesticides to PLs and JLs. The $1/4^{th}$ concentration of the 96 h LC_{50} value was selected as the SL dose for present study. The SL dose used for present study is mentioned in the following table:

Life stages of *L. vannamei*	**Pesticides**	**LC_{50} Value ($\mu g\ L^{-1}$)**	**SL dose ($\mu g\ L^{-1}$)**
			$1/4^{th}$ of LC_{50}
PL	CPF	0.88	CPF1= 0.22
	DMT	357.84	DMT1= 89.46
JL	CPF	1.44	CPF1= 0.36
	DMT	563.61	DMT1= 140.9

5.2.3 Experimental set up

Out of 1200 PL, 600 were used for one pesticide (Chlorpyrifos) and the remaining 600 PL for another pesticide (Dimethoate). These 600 larvae were divided into three groups, each of 200 larvae, one group used as controls and the remaining two groups for two sublethal concentrations of one pesticide. The experiments were conducted in duplicate with 100 larvae in each tank along with control. Randomly selected 600 inter-moult JLs of similar sizes were divided into three groups, each one comprising of 200 JLs. From this, two groups were exposed to two pesticides while one group served as control. Experiments on JLs were also conducted in duplicate with 100 JL per tank. To maintain the constant concentrations of pesticides in test solutions, the entire medium in each aquarium was gently siphoned out daily (10:00 h) and renewed with the freshly prepared solution of respective SL concentrations. Maximum care was taken to maintain the animals with no or minimum disturbance during handling.

The tests were conducted for 21 days of exposure (DoE), while samples for comet assay, antioxidant enzyme activity and antioxidant enzyme gene expression were collected on 3, 7, 14 and 21 DoE. At each of the time intervals, 10 PL and 10 JL were collected at random for each test. Also, shrimps from the control group were similarly sampled at the same time intervals as the treated ones. During the exposure period, mortality in control tanks did not exceed 5%. To maintain the same stocking density throughout the experiment dead PLs and JLs, if any were replaced with shrimps reared in the same medium separately. During the chronic exposure experiment, PLs were fed at rate of 8–10% of body weight while JLs were fed at a rate of 4–6% of body weight which split across three different feed times (10:00, 15:00 and 20:00 h).

Sample collection

a) **PL:** PL weighing 200-500 mg were used.
b) **JL:**

Haemolymph (HL): 350 µl of haemolymphs were collected from rostral sinus region of JL using a hypodermic syringe.

Gills (GL): 100 mg of gills were collected from JLs.

Muscles (ML): 200 mg of muscle tissue were collected from JLs.

These samples were divided into two parts and the first part is used for antioxidant enzyme activity and the other part for gene expression studies

Plate 5.1: Experimental set up for chronic toxicity study with post larvae of *L. vannamei*

Plate 5.2 Experimental set up for chronic toxicity study with juveniles of *L. vannamei*

5.2.4 Antioxidant enzyme activity:

Antioxidant enzyme activity of catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx) were assessed by following standard protocols.

5.2.4.1 Protein estimation (Lowry et al., 1951)

5.2.4.1.1 Principle

The principle behind the Lowry method is to determine protein concentrations in the reactivity of the peptide nitrogen[s] with the copper [II] ions under alkaline conditions and the subsequent reduction of the Folin-Ciocalteau phosphomolybdic phosphotungstic acid to heteropolymolybdenum blue by the copper-catalysed oxidation of aromatic acids.

5.2.4.1.2 Procedure

0.25 g of PL, GL and ML tissues from JLs were homogenized in PBS while 250 µl of HL was diluted with PBS. The obtained homogenates were transferred into the centrifuge tube into which 2 ml of 1N NaOH was added. All the tubes were incubated for 10 min in an 80-90 ºC water bath. Then centrifugation was carried out at 5000 rpm for 5 min. From each tube 0.5 ml sample was pipetted out and equal amount of distilled water (D/W) was added.

BSA standards were prepared with increasing concentrations according to the following table along with blank.

BSA standards (mg/ml)	H_2O added (µl)	Total volume (µl)	Final concentration (µg/ml)
0	250	250	0
20	230	250	100
40	210	250	200
80	170	250	400
120	130	250	600
160	90	250	800
200	50	250	1000

5 ml of reagent C was added to the above tubes of standards and tissue samples and mixed well by vortex and tubes were incubated in dark for 10 min. 0.5 ml of Folin Ciocalteu (FC) (Diluted FC reagent 1:1 ratio with D/W) was added to tubes. Further tubes were incubated in dark for 20 min. Finally, absorbance was recorded at 750 nm. Standard graph of concentration *vs* absorbance was plotted. The amount of protein present in the given sample was estimated from the standard graph.

5.2.4.2 Antioxidant enzyme assays

5.2.4.2.1 Catalase enzyme activity (CAT) (Sinha, 1972)

Principle: CAT activity was measured spectrophotometrically at 583 nm using 0.06 M hydrogen peroxide (0.01M, pH-7 phosphate buffer) as a substrate. Samples were incubated with H_2O_2 (substrate) for different periods. The principle is based on the reduction of dichromate into acetic acid to chromic acetate (green) when heated in the presence of H_2O_2. Further, the remaining H_2O_2 is determined by adding mixed reagent having Cr_2O_7/H+ followed by heating and measuring the resulting green colored chromic acetate spectrophotometrically at 583nm.

Sample processing: Tissues were homogenized with 4 ml of phosphate buffer (0.1M, pH- 7.4) using high-speed Ultra Turrax homogenizer for 1 min while the HL was mixed with PBS. The homogenates were then transferred into the pre-cooled centrifuge tubes, followed with centrifugation at 10,000 rpm for 1 h at 4 ºC. The resulting supernatant was pipetted out carefully which was used further for analysis.

Controls and treated samples were prepared as 0.1 ml of enzyme followed by 4.5 ml H_2O_2 with incubation for 1 min. The reaction was stopped by adding 2 ml of Cr_2O_7/H+ mixture. This reaction mixture was incubated in a water bath for the development of green coloured complex at 85 ºC for 10min. The final volume to 7ml was adjusted by adding required quantity of triple distilled water (0.5 ml for control and 0.4 ml for treated samples). Finally, absorbance was recorded at 583nm.

Calculations:

CAT Activity = $\frac{[(OD_{control} - OD_{sample})/9.2059]}{\text{Incubation time (min)* volume of enzyme added * Protein conc.}}$

$= \frac{[(OD_{control} - OD_{sample})/9.2059]}{1*0.1*\text{Protein conc.}(mgml^{-1})}$

Catalase (CAT) activity was expressed in terms of µmoles of H_2O_2 consumed$min^{-1}mg^{-1}$ of protein.

5.2.4.2.2 Superoxide dismutase (SOD) (Misra and Fridovich, 1972)

All tissue samples were weighed and homogenized in 1 ml of phosphate buffer (0.1M, pH 7.4) while the HL was mixed with the PBS. Obtained homogenates were transferred into test tubes. To each test tube, 0.2 ml of ice-cold chloroform and 0.15 ml of ice-cold ethanol were added, followed by addition of 1 ml distilled water. Then, the whole solution was mixed thoroughly. The mixture was centrifuged at 3000 rpm for 10 minutes and the supernatant was used for enzyme activity.

3.5 ml of carbonate buffer (pH 10.2) was pipetted out into the different test tubes. To these 5, 10, 15, 20, 30, 40, 50, 60, 70 µl aliquots of above supernatant was added and mixed well. To the above mixture, 200 µl of epinephrine was added and the total volume was made up to 4 ml. Finally, the change in their optical density was measured at 480 nm at 0, 5 and 10 minutes.

U/ml of enzyme = $1000\ \mu l^{-1}$ of enzyme causing 50% inhibition

U/mg of protein = (1000 µl of enzyme causing 50% inhibition) $protein^{-1}$ concentration

5.2.4.2.3 Glutathione peroxidase (GPx) (Paglia and Valentine, 1967)

Tissues were homogenized in 0.1 M Phosphate buffer (pH 7.4) in 1:4 ratio while the HL was mixed with PBS in the same ratio and transferred into test tubes. After homogenization, the tubes were centrifuged at 18,000 rpm for 30 min at 4 ºC temperature. Supernatants were removed and stored in cold condition for further assay.

The cocktail was prepared by adding 9.20 ml + 0.10 ml + 0.05 ml of above supernatant in β-NADPH and mixed by inversion and adjusted to pH 7.0 at 25 ºC with 1 M HCl / 1 M NaOH.

The test solution was prepared by adding reaction cocktail of 3 ml + 0.05 ml glutathione peroxidase enzyme solution whereas the blank was prepared by using sodium phosphate buffer 0.05 ml + 3 ml reaction cocktail, followed mixing by inversion and equilibrated temperature to 25 ºC. Absorbance was monitored at 340 nm until it reaches a constant level using a thermostatic spectrophotometer. Finally, 0.05 ml of H_2O_2 was added to test and blank tubes immediately after mixing and recorded the decrease in absorbance at 340 nm.

Calculations:

$$\text{Units*/ml enzyme} = \frac{[\Delta A_{340nM}\text{min}^{-1}\ \text{Test} - \Delta A_{340nM}\text{min}^{-1}\ \text{Blank}]\ (2)\ (3.1)\ (df)}{(6.22)\ (0.05)}$$

2 = 2 µmoles of GSH produced per µmole of β-NADPH oxidized

3.1 = Total volume (in ml) of assay

df = Dilution factor

6.22 = Millimolar extinction coefficient of β-NADPH at 340 nm

0.05 =Volume (in ml) of enzyme used

$$\text{Units/mg protein} = \frac{\text{Units/ml enzyme}}{\text{mg protein/ ml enzyme}}$$

(One unit equals to the oxidation of 1.0 µmole of reduced glutathione to oxidized glutathione by H_2O_2 per minute at pH 7.0 at 25 °C.)

5.2.5 Antioxidant enzyme gene expression

Expression levels of the genes of antioxidant enzymes catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx) were assessed by quantitative Real Time PCR (qRT-PCR).

5.2.5.1 Total RNA extraction

5.2.5.2.1 Homogenization:

Whole PLs as well as the GL and ML tissues of JLs were homogenized with liquid nitrogen in mortar and pestle to obtain powder form. Then, 1 ml of TRIZOL reagent was added per 100 mg of tissues/ 100 µl of HL sample and incubated at room temperature for 10 min.

5.2.5.2.2 Phase separation

To carry out phase separation 0.2 ml of 1-bromo 3-choloropropane (BCP) was added per 1 ml of TRIZOL reagent used. The samples were mixed for 15 seconds and incubated further at room temperature for 5 min. Samples were centrifuged at 12,000 rpm for 15 minutes at 4 ºC. After centrifugation, the mixture was separated into three layers i.e. lowermost red phenol-chloroform phase, white film interphase containing DNA and protein and a colourless upper aqueous phase containing total RNA. The upper aqueous phase was carefully transferred without disturbing the interphase into a fresh tube.

5.2.5.2.3 RNA precipitation

To the above tubes, isopropanol was added to precipitate the total RNA using 0.5 ml of isopropanol per 1 ml of TRIZOL reagent used. Total RNA forms gel-like pellet on the side/bottom of the tube. Tubes were incubated at room temperature for 10 min and centrifuged again at 10,000 rpm for 10 min at 4 ºC.

5.2.5.2.4 RNA wash

The supernatant was removed completely through repeated pipetting without disturbing pellet. The RNA pellet was washed with 75% ethanol by adding 1 ml of 75% ethanol per 1 ml of TRIZOL reagent used for the initial homogenization. The samples were mixed and centrifuged at 7500 rpm for 5 min at 4 ºC. After centrifugation, supernatant ethanol was removed followed by air dry of RNA pellet for 3-5 min. Maximum care was taken not to dry RNA pellet completely due to its further effect on solubility.

5.2.5.2.5 Redissolving RNA

The RNA pellet was dissolved into DEPC treated water by passing samples a few times through pipette tip.

5.2.5.2.6 NanoDrop analysis

The total RNA concentration was quantified by using NanoDrop™ 2000/c (Thermo Fisher Scientific).

For quantification and purity of total RNA sample of 2 µl used for the analysis. A260/ A280 ratio of ~2.1 considered pure RNA samples and ratio of <1.8 indicates potential DNA or protein contamination. The A260/ A230 ratio should also be above 2.0. A low ratio indicates contamination with the wash solutions.

5.2.5.2.7 cDNA synthesis

cDNA synthesis from total RNA was carried out by using PrimeScript™ 1st Strand cDNA Synthesis kit by TAKARA.

In 10 µl of reaction mixture contains Oligo dT Primer+ dNTP Mixture+ Template RNA. The reaction mixture incubated for 5 min at 65 ^{0}C. Template RNA Primer Mixture 10 µl added 5X PrimeScript Buffer+ 4 RNase Inhibitor + PrimeScriptRTaseto prepare 20µl final reaction mixture. Incubated the reaction mixture for 60 min at 42 ^{0}C. Inactivated the enzyme by incubating at 95℃ for 5 min then cooled on ice.

5.2.5.2.8 Gene amplification

Once the cDNA synthesis was completed, the gene amplification of targeted genes *viz.* SOD, CAT and GPx were carried out with the help of SYBR Premix EX Taq™ II (TliRNae\seH Plus) kit, TAKARA by using specific primers while β-actin used as a house keeping gene.

The relative fold of induction was calculated using comparative CT method for relative quantification. The amplifications were carried out in a 96 well plate in a total volume of 25µl using 4 µl of cDNA, 200nM of each gene-specific primers pair with SYBR Premix EX Taq™ II in an ABI 7500 Real-Time PCR system. Cycling parameters were 95 ºC for 15 min, followed by 40 cycles of 95 ºC for 15s and 60 °C for 1 min. The specificity of 5 PCR products was confirmed by dissociation curve analysis performed at the end of qPCR by continuously heating from 55 ºC to 95 ºC with an increment of 0.1 ºC. Data were analysed using the ABI 7500

software version 2.0.1. The relative fold of induction was determined by ΔΔ CT method. The fold change was calculated using the formula 2- ΔΔ CT.

Primers:

SOD (Zhou et al., 2010): F-CGTAGAGGGTATTGTCGT
R-TTGAAATCATACTTGAGGG

CAT (Guo et al., 2013): F-TCAGCGTTTGGTGGAGAA
R-GCCTGGCTCATCTTTATC

GPx (Zhou et al., 2010): F-AGGGACTTCCACCAGATG
R-CAACAACTCCCCTTCGGTA

β-actin (Guo et al., 2013): F-GCCCATCTACGAGGGATA
R-GGTGGTCGTGAAGGTGTAG

5.2.6 Statistics

Statistical analysis was performed by using computer based GraphPad PRISM 5.0 software (Graph Pad, San Diego, CA, USA). Data obtained from the experiments were expressed as mean ± SD. Data from different treatment groups and different tissues were assessed by analysis of variance (ANOVA) (Underwood, 1997) with DoE and SL concentrations as sources of variation. Variation in water quality parameters from treated and control aquaria were assessed by ANOVA. Appropriate transformations were applied before subjecting the data to statistical analysis. A p-value less than 0.05 (typically ≤ 0.05) is considered statistically significant.

5.3 Results

5.3.1 Studies on Post larvae (PL)

5.3.1.1 Antioxidant enzyme activity and their gene expression in post larvae exposed to Chlorpyrifos (CPF)

The antioxidant activity of the enzymes SOD, CAT and GPx on 3, 7, 14 and 21 DoE were recorded in PL on exposure to SL dose of CPF1 are represented in Fig. 5.1, 5.2 and 5.3, respectively. Data obtained were subjected to ANOVA to compare treated and control groups at different time intervals. In CPF1 exposed PL, the highest activity of SOD measured was 0.15 U mg^{-1} protein and the lowest was 0.12 U mg^{-1} protein (Fig. 5.1). The highest level of CAT enzyme activity recorded in CPF1 exposed PL was 76.30 µmole H_2O_2 min^{-1} mg^{-1} protein and the lowest was 73.33 µmole H_2O_2 min^{-1} mg^{-1} protein (Fig. 5.2). Highest GPx enzyme activity measured was 128.91 U mg^{-1} protein and lowest was 74.32 U mg^{-1} protein in CPF1 exposed PLs (Fig. 5.3). The profile of enzyme activity in control and treated groups at different time intervals has provided in table 5.1.

The SOD activity was highest on 3 DoE which then decreased on 7 and 14 DoE and later on again increased on 21 DoE. The CAT activity was lower on 3 DoE which gradually increased till 21 DoE. However, higher GPx activity in CPF1 exposed PL observed on 3 DoE which later on showed decrease in activity till 21 DoE. The different level of activity observed in SOD, CAT and GPx in chronic exposure shows their alternate roles of scavenging activity to minimize the level of ROS production on exposure to the pesticides. Also, decrease in activity observed in all the three enzymes could be due to the suppression of that particular enzyme for specific duration due to continuous exposure of pesticides whereas increase in activity of enzymes observed which shows the activation of enzymes on exposure to pesticides.

Table 5.1 Antioxidant activity of Superoxide dismutase (SOD), Catalase (CAT) and Glutathione peroxidase (GPx) in post larvae exposed to Chlorpyrifos (CPF1= 0.22 µg L^{-1})

Life stage of *L. vannamei*	**DoE**	**Enzyme activity**					
		SOD (U mg^{-1} protein)		**CAT (µmole H_2O_2 min^{-1} mg^{-1} protein)**		**GPx (U mg^{-1} protein)**	
		Control	**CPF (0.22 µg L^{-1})**	**Control**	**CPF (0.22 µg L^{-1})**	**Control**	**CPF (0.22 µg L^{-1})**
Post Larvae	3	0.081 ± 0.003	0.15 ± 0.01	71.32 ± 2.34	72.33 ± 2.45	110.9 ± 2.56	128.91± 4.56
	7	0.083 ± 0.005	0.13 ± 0.02	72.29 ± 2.43	74.49 ± 3.56	111.1 ± 2.78	112.4± 5.36
	14	0.084 ± 0.004	0.11 ± 0.01	70.54 ± 3.24	75.7 ± 5.43	112.5 ± 3.45	89.45± 5.67
	21	0.081 ± 0.003	0.14 ±0.01	72.33 ± 3.45	76.33 ± 4.23	112.67 ± 2.89	74.32± 5.87

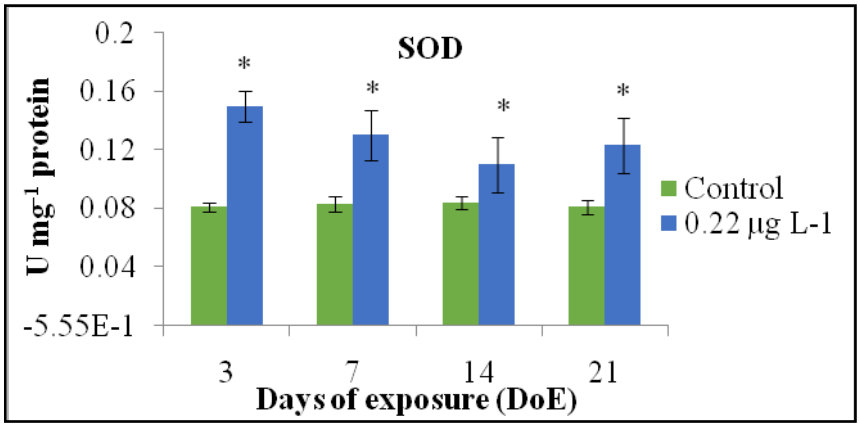

Fig. 5.1 Superoxide dismutase (SOD) activity in post larvae exposed to sublethal dose of CPF1 = 0.22 µg L^{-1}. Values are expressed as Mean± SD (ANOVA, *= P< 0.05)

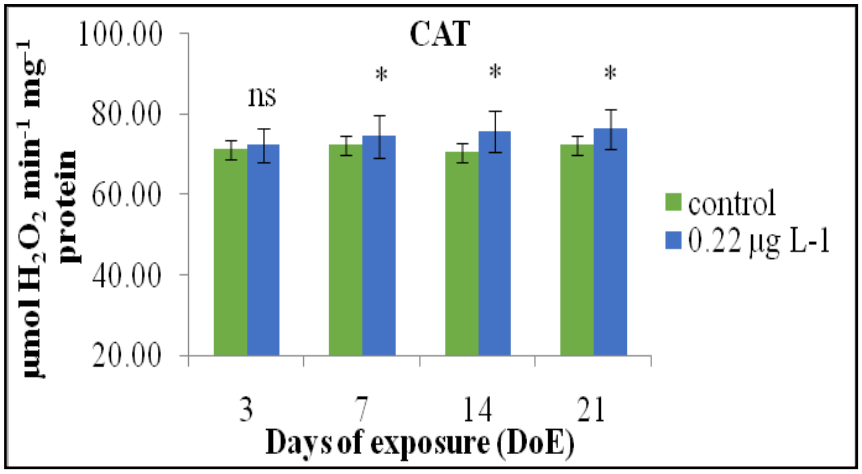

Fig. 5.2 Catalase (CAT) activity in post larvae exposed to sublethal dose of CPF1 = 0.22 µg L^{-1}. Values are expressed as Mean± SD (ANOVA, ns = non significance, *= P< 0.05)

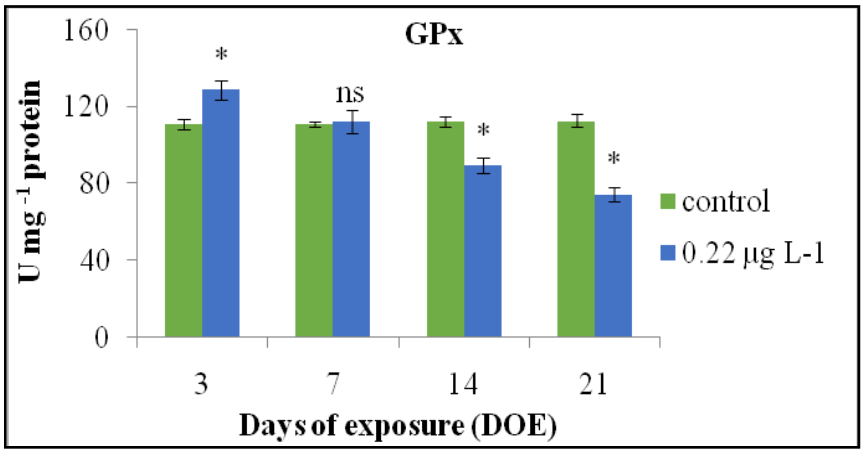

Fig. 5.3 Glutathione peroxidase (GPx) activity in post larvae exposed to sublethal dose of CPF1 = 0.22 µg L^{-1}. Values are expressed as Mean± SD (ANOVA, ns= non significance, *= P< 0.05)

Antioxidant enzyme genes *viz.* SOD, CAT and GPx expressed in control and CPF exposed PL is represented in Fig. 5.4. The measured gene expression was calculated as fold changes over control. Data were subjected to one-way ANOVA to compare levels of expression between control and treated PL at 3, 7 14 and 21 DoE. The differential response of SOD, CAT and GPx genes were recorded on exposure to SL dose of CPF. The SOD gene was down-regulated on 3 (0.54 ± 0.12) and 7 (0.72 ± 0.14) DoE whereas up-regulated on 14 (1.67 ± 0.11) and 21 (1.75 ± 0.13) DoE against control at CPF1 ($P< 0.05$). In exposed PL, the CAT gene expression was down-regulated on 3 DoE (0.73 ± 0.22) whereas up-regulated on 7 (1.48 ± 0.21), 14 (1.82 ± 0.22) and 21(4.17 ± 0.24) DoE against control (1 ± 0.08). Further, the GPx enzyme gene was up-regulated at all time intervals *viz.*3 (6.14 ± 0.38), 7 (8.58 ± 0.59), 14 (6.11 ± 0.12) and 21 (1.13 ± 0.11) in CPF1 exposed PL compared against the control (1 ± 0.08).

It was also observed that the expression of SOD gene was constitutive in nature and the levels kept on increasing from initial days of the exposure until the end. Similarly, expression of CAT gene continued to increase with time. Moreover, the GPx gene had maximum level of expression on 7 DoE and then declined towards the end of exposure period.

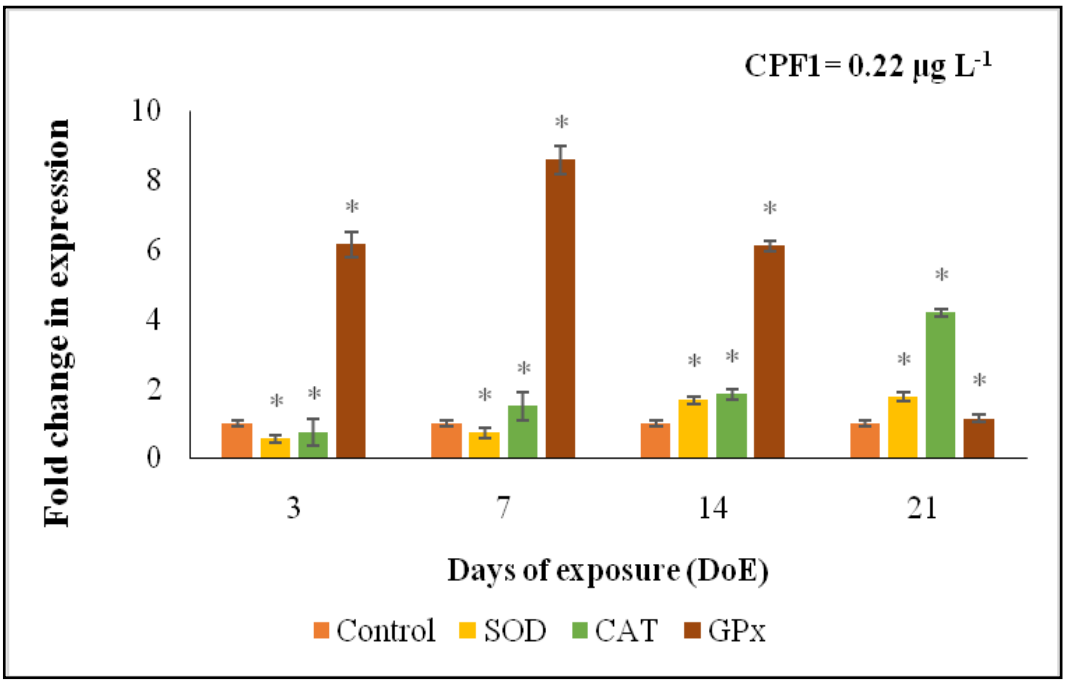

Fig. 5.4 Level of expression of Superoxide dismutase (SOD), Catalase (CAT) and Glutathione peroxidase (GPx) genes in post larvae exposed to sublethal dose of CPF1= 0.22 µg L^{-1}. Values are expressed as Mean ± SD (ANOVA, *= P< 0.05)

5.3.1.2 Antioxidant enzyme activity and their gene expression in post larvae exposed to Dimethoate (DMT)

The antioxidant enzyme activity of SOD, CAT and GPx on 3, 7, 14 and 21 DoE were recorded in PL on exposure to SL dose of DMT1 are represented in Fig. 5.5, 5.6 and 5.7, respectively. Data were subjected to ANOVA to compare treated and control groups at different time intervals. In DMT1 exposed PL, the highest activity of SOD measured was 0.12 U mg^{-1} protein and the lowest was 0.09 U mg^{-1} protein (Fig. 5.5). The activity was observed to be higher on 3 DoE and then till 21 DoE (Table 5.2). Moreover, the activity levels of SOD enzyme in DMT1 exposed PL compared against control showed a higher activity at all the time intervals studied.

The highest level of CAT enzyme activity measured was 74.01 µmole H_2O_2 min^{-1} mg^{-1} protein and the lowest was 70.50 µmole H_2O_2 min^{-1} mg^{-1} protein in DMT1 exposed PLs (Fig. 5.6). The CAT activity was measured lower on 3 DoE and increased till 21 DoE (Table 5.2).

Moreover, the CAT activity in control and treated groups compared it showed higher activity in treated once against control on 14 and 21 DoE whereas, equivalent on 3 and 7 DoE.

In the case of DMT1 exposed PL, GPx enzyme activity was observed to be highest was 126.14 U mg^{-1} protein and lowest was 72.31 U mg^{-1} protein. Further, the GPx activity was higher on 3 DoE and decreased till 21 DoE (Table 5.2). On the other hand, GPx activity in DMT1 exposed PL compared against control, showed higher activity on 3 DoE, equivalent on 7 DoE and lower activity on 14 and 21 DoE (Fig. 5.7).

Table 5.2 Antioxidant activity of superoxide dismutase (SOD), Catalase (CAT) and glutathione peroxidase (GPx) in post larvae exposed to Dimethoate (DMT1= 89.46 µg L^{-1})

Size of animal	**DoE**	**Enzyme activity**					
Post Larvae		**SOD (U mg^{-1} protein)**		**CAT (µmole H_2O_2 min^{-1} mg^{-1} protein)**		**GPx (U mg^{-1} protein)**	
		Control	**DMT (89.46 µg L^{-1})**	**Control**	**DMT (89.46 µg L^{-1})**	**Control**	**DMT (89.46 µg L^{-1})**
	3	0.081 ± 0.004	0.12 ± 0.005	71.32 ± 3.45	70.51 ± 2.56	110.9 ± 4.78	126.14 ± 3.89
	7	0.083 ± 0.003	0.108 ± 0.004	72.29 ± 4.56	72.07 ± 3.56	111.1 ± 3.56	111.76 ± 2.78
	14	0.084 ± 0.002	0.103 ± 0.006	70.54 ± 3.56	73.08 ± 4.53	112.5 ± 4.24	86.56 ± 1.89
	21	0.081 ± 0.001	0.09 ± 0.008	72.33 ± 2.45	74.01 ± 2.67	112.67 ± 2.34	72.32 ± 2.34

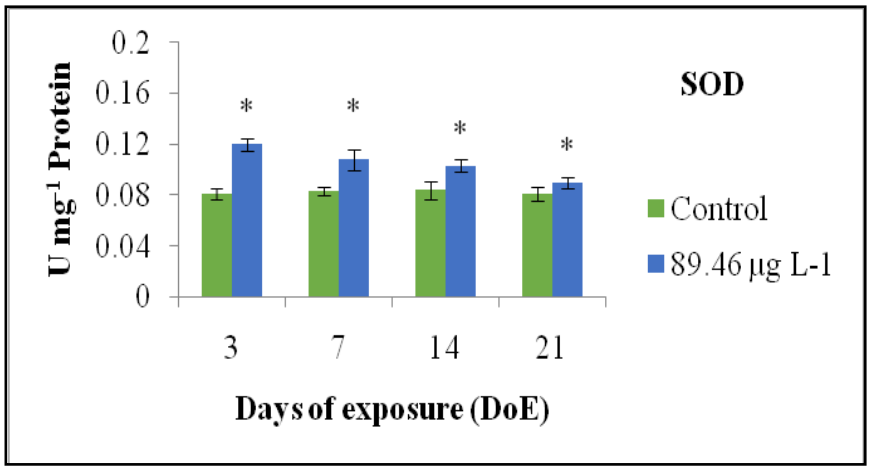

Fig. 5.5 Superoxide dismutase (SOD) activity in post larvae exposed to sublethal dose, DMT1= 89.46 µg L^{-1}. Values are expressed as Mean ± SD (ANOVA, *= P< 0.05)

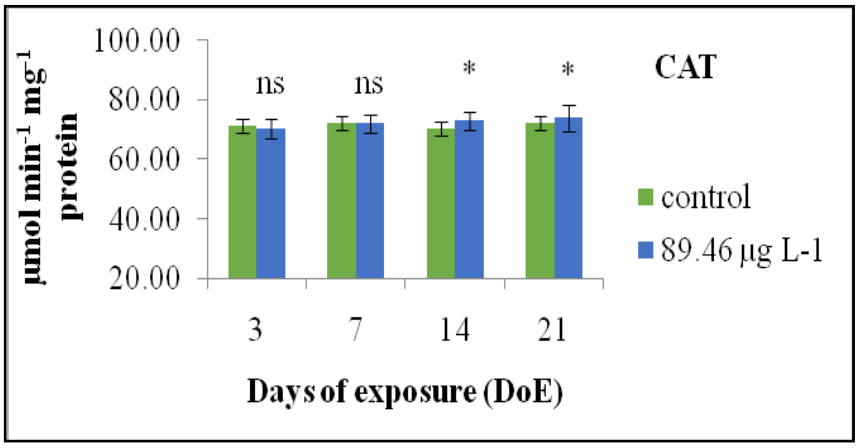

Fig. 5.6 Catalase (CAT) activity in post larvae exposed to sublethal dose, DMT1= 89.46 µg L^{-1}. Values are expressed as Mean ± SD (ANOVA, ns= non significance, *= P< 0.05)

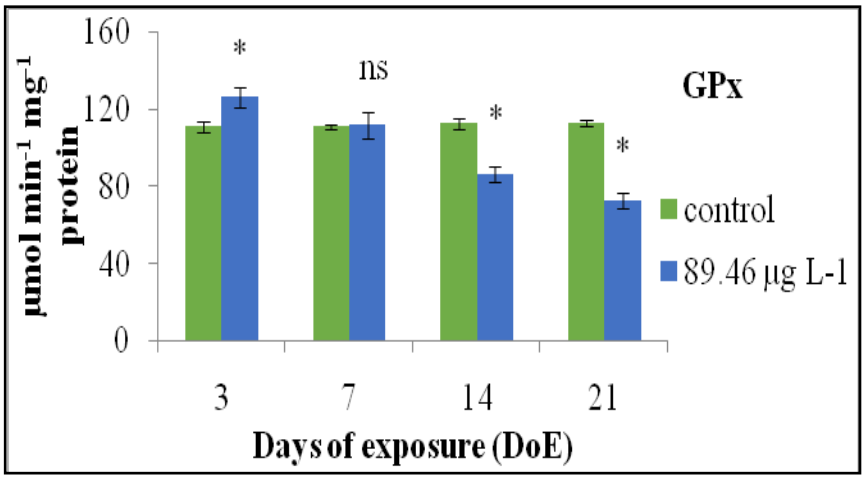

Fig. 5.7 Glutathione peroxidase **(**GPx) activity in post larvae exposed to sublethal dose, DMT1= 89.46 µg L^{-1}. Values are expressed as Mean ± SD (ANOVA, ns= non significance, *= P< 0.05)

Antioxidant enzyme genes *viz.* SOD, CAT and GPx expressed in control and DMT exposed PL is presented in Fig. 5.8. The expressions of different genes are shown as fold changes versus control. Data were subjected to one-way ANOVA to compare levels of expression between control and treated PL at 3, 7 14 and 21 DoE. The differential response of SOD, CAT and GPx genes were recorded on exposure to SL dose of DMT. At DMT1, expression of SOD gene was down-regulated on 3 (0.32 ± 0.14) and 7 (0.58 ± 0.11) DoE followed by up-regulation on the 14 (1.52 ± 0.13) and 21 (1.62 ± 0.13) DoE as compared to control (1± 0.08). In DMT1 exposed PL, expression of CAT gene was down-regulated on 3 (0.52 ± 0.13) DoE while up-regulated on 7 (1.53 ± 0.15), 14 (1.4 ± 0.11) and 21 (2.31 ± 0.18) DoE compared to control (1 ± 0.06). However, expression of GPx in DMT1 exposed PL, was up-regulated on 3 (4.69 ± 0.21), 7 (5.23 ± 0.28) and 14 (2.66 ± 0.16) DoE and down-regulated on 21 (0.68 ± 0.17) DoE.

It was also observed that the SOD gene expression gradually increased from initial days of the exposure until the end. Similar trends of increase in expression with time were also observed for CAT gene. Moreover, upregulation of GPx was measured on 7 DoE which was followed by declining levels of expression towards the end days of exposure.

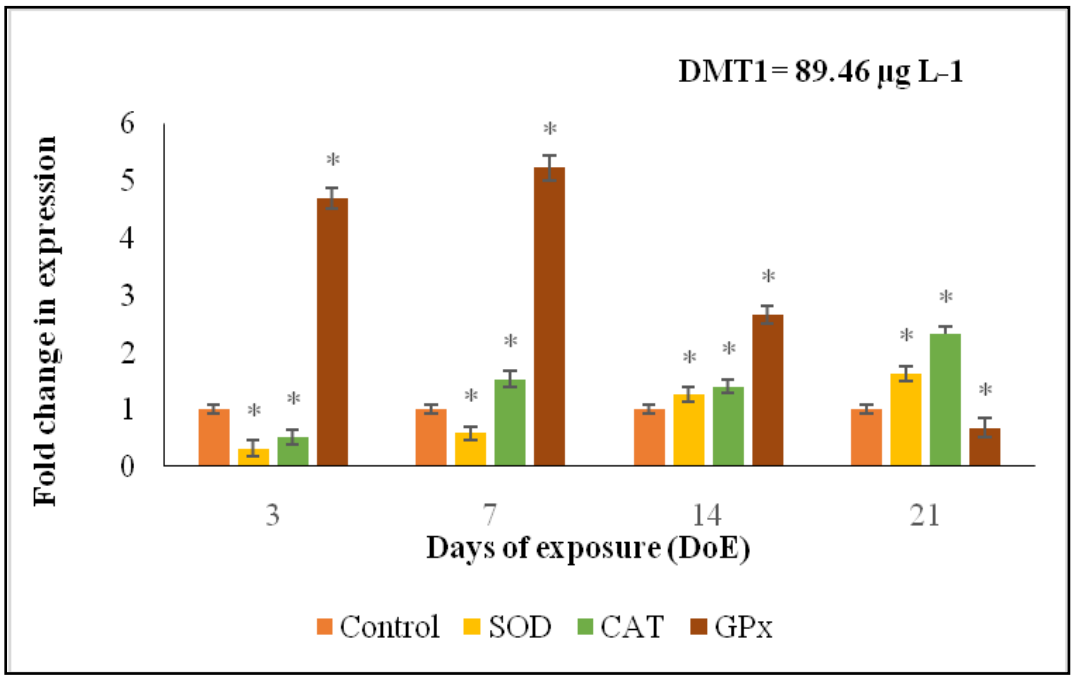

Fig. 5.8 Level of expression of Superoxide dismutase (SOD), Catalase (CAT) and Glutathione peroxidase (GPx) genes in post larvae exposed to sublethal dose of DMT1 = 89.46 µg L^{-1}. Values are expressed as Mean ± SD (ANOVA, *= P< 0.05)

5.3.2 Studies on juveniles (JL)

5.3.2.1 Antioxidant enzyme activity and their gene expression in gill (GL) exposed to Chlorpyrifos (CPF)

The antioxidant enzyme activity of SOD, CAT and GPx on 3, 7, 14 and 21 DoE were recorded in GL exposed to SL dose CPF1 are represented in Fig. 5.9, 5.10 and 5.11, respectively. Data were subjected to ANOVA, to compare treated and control groups at different time intervals. The highest SOD activity measured exposed to CPF1 was 0.25 U mg^{-1} protein whereas, the lowest was 0.15 U mg^{-1} protein. In CPF1 exposed GL, the SOD activity was higher on 3 DoE which decreased till 21 DoE (Table 5.3). Moreover, the activity level of SOD enzyme in CPF1 exposed GL showed higher activity against control at all time intervals (Fig. 5.9).

The highest activity of CAT observed exposed to CPF1 was 85.3 µmole H_2O_2 min^{-1} mg^{-1} protein whereas, the lowest was 63.7 µmole H_2O_2 min^{-1} mg^{-1} protein. In CPF1 exposed GL, the CAT activity was higher on 3 DoE whereas, lower on 7, 14 and 21 DoE compared to control (Fig. 5.10 and Table 5.3).

The highest GPx enzyme activity recorded exposed to CPF1 was 140.91 136.14U mg^{-1} protein whereas, the lowest was 106.32 U mg^{-1} protein. The GPx enzyme activity was found higher on 3, 7 and14 DoE whereas, lower on 21 DoE in CPF1 exposed GL compared against control (Fig. 5.11 and Table 5.3).

Table 5.3 Antioxidant activity of superoxide dismutase (SOD), Catalase (CAT) and glutathione peroxidase (GPx) in gill of juveniles exposed to Chlorpyrifos (CPF1= 0.36 µg L^{-1})

Size of animal	**DoE**	**Enzyme activity**					
Juvenile (Gill)		**SOD (U mg^{-1} protein)**		**CAT (µmole H_2O_2 min^{-1} mg^{-1} protein)**		**GPx (U mg^{-1} protein)**	
		Control	**CPF (0.36 µg L^{-1})**	**Control**	**CPF (0.36 µg L^{-1})**	**Control**	**CPF (0.36 µg L^{-1})**
	3	0.13 ± 0.008	0.25 ± 0.02	81.32 ± 3.45	85.3 ± 4.23	123.9 ± 2.56	140.91 ± 4.11
	7	0.14 ± 0.007	0.20 ± 0.04	82.29 ± 2.98	70.49 ± 2.45	121.1 ± 3.11	136.4 ± 4.23
	14	0.14 ± 0.005	0.18 ± 0.03	80.54 ± 3.24	63.7 ± 1.45	122.5 ± 3.45	129.45 ± 3.89
	21	0.12 ± 0.009	0.15 ± 0.04	82.33 ± 4.21	75.33 ± 2.45	122.67 ± 3.13	106.32 ± 2.98

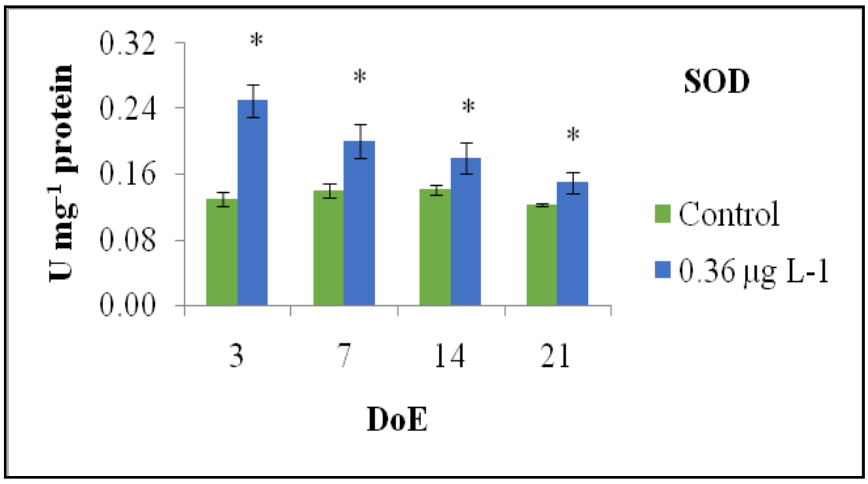

Fig 5.9 Superoxide dismutase (SOD) activity in gill of juveniles exposed to sublethal dose of CPF1 = 0.36 µg L^{-1}.Values are expressed as Mean ± SD (ANOVA, *= P< 0.05)

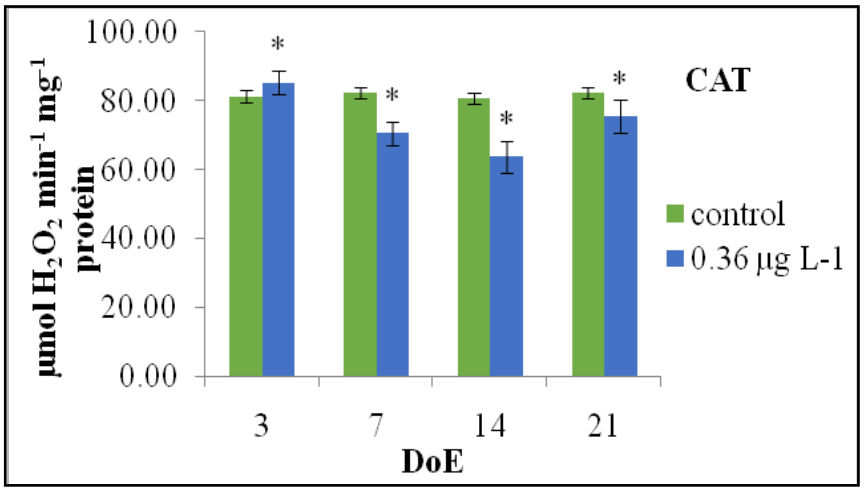

Fig. 5.10 Catalase (CAT) activity in gill of juveniles exposed to sublethal dose of CPF1= 0.36 µg L^{-1}.Values are expressed as Mean ± SD (ANOVA, *= P< 0.05)

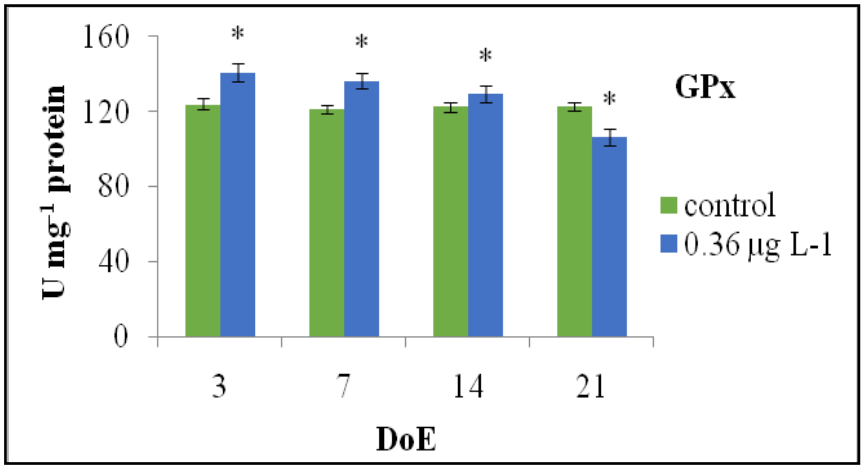

Fig. 5.11 Glutathione peroxidase (GPx) activity in gill of juveniles exposed to sublethal dose of CPF1= 0.36 µg L^{-1}.Values are expressed as Mean ± SD (ANOVA, *= P< 0.05)

Antioxidant enzyme genes *viz.* SOD, CAT and GPx expressed in control and CPF exposed GL are represented in Fig. 5.12. Data was subjected to one-way ANOVA to compare between control and treatments at 3, 7, 14 and 21 DoE. The differential response of SOD, CAT and GPx genes were recorded on exposure to SL dose of CPF and discussed the same. In CPF1 exposed GL, expression of SOD gene was up-regulated at all time intervals *viz.* 3 (1.85 ± 0.15), 7 (4.64 ± 0.34), 14 (2.32 ± 0.19) and 21 DoE (1.24 ± 0.17) compared to control (1 ± 0.14). Expression of CAT gene in CPF1 exposed GL was also up-regulated at all time interval studied *viz.* 3 (1.45 ± 0.16), 7 (1.58 ± 0.14), 14 (1.42 ± 0.15) and 21 DoE (3.08 ± 0.27) compared to control (1 ± 0.09) ($P<0.05$). Levels of GPx gene in CPF1 exposed GLs, was up-regulated on 3 (4.03 ± 0.56), 7 (5.45 ± 1.22) and 14 (2.14 ± 0.22) DoE whereas, down-regulated on 21 (0.92 ± 0.09) DoE compared to control (1 ± 0.11).

It was also observed that SOD gene had the maximum expression on 7 DoE and minimum on 3, 14 and 21 DoE in CPF1 exposed GL. In case of CAT gene, constitutively maximum levels of expression were recorded until the end. Moreover, the expression of GPx gene on exposure to CPF1 observed that initially it expressed at minimum level then increased on 7 DoE and later on decreased till the end.

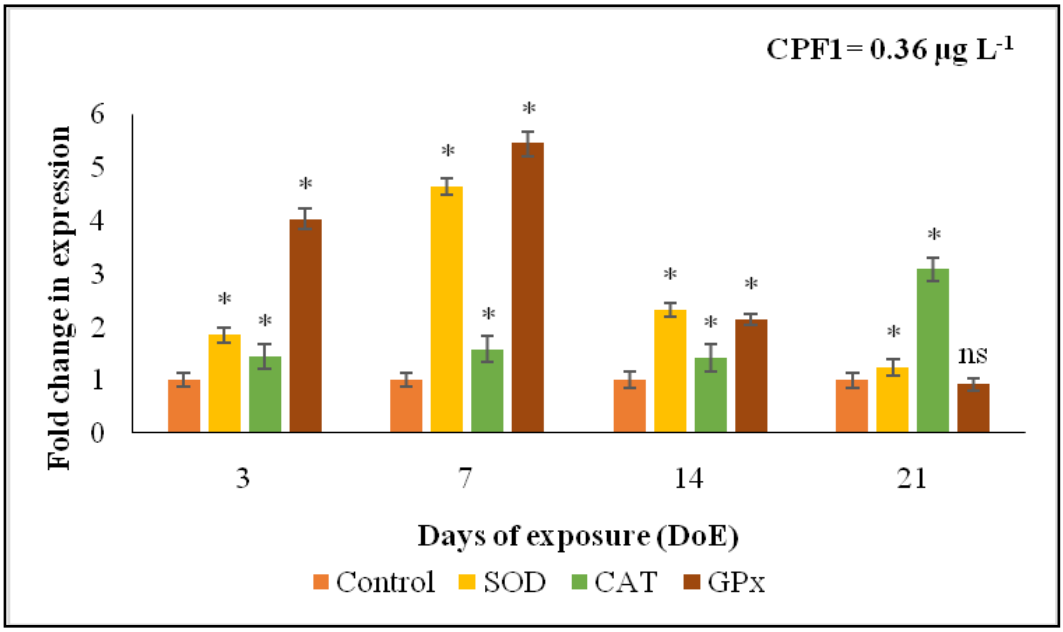

Fig. 5.12. Level of expression of Superoxide dismutase (SOD), Catalase (CAT) and Glutathione peroxidase (GPx) genes in gill of juvenile exposed to sublethal dose, CPF1= 0.36 µg L^{-1}. Values are expressed as Mean ± SD (ANOVA, *= $P< 0.05$)

5.3.2.2 Antioxidant enzyme activity and their gene expression in haemolymph (HL) exposed to Chlorpyrifos (CPF)

The antioxidant enzyme activity of SOD, CAT and GPx on 3, 7, 14 and 21 DoE were measured in HL exposed to SL dose of CPF1 are represented in Fig. 5.13, 5.14 and 5.15, respectively. Data were subjected to ANOVA to compare treated and control groups at different time intervals. The SOD activity recorded highest activity exposed to CPF1 was 0.18 U mg^{-1} protein whereas, the lowest was 0.12 U mg^{-1} protein. The SOD activity was higher on 3 DoE while gradually decreased till 21 DoE in the CPF1 exposed HL (Table 5.13). Moreover, the activity level of SOD enzyme in CPF1 exposed HL compared against control, it showed higher activity in treated HL at all time intervals (Fig. 5.10).

The highest activity of CAT was recorded exposed to CPF1 was 68.3 µmole H_2O_2 min^{-1} mg^{-1} protein on 3 DoE whereas, the lowest was 52.7 µmole H_2O_2 min^{-1} mg^{-1} protein in CPF1 treated HL on 21 DoE. In CPF1 exposed HL, the CAT activity was higher on 3 DoE and then decreased on 7 and 14 DoE and later on increased again on 21 DoE (Table 5.14). Moreover, the activity level of CAT enzyme in CPF1 compared against control higher activity on 3 and 7 DoE, lower activity on 14 DoE and equivalent on 21 DoE (Fig. 5.14).

The highest GPx enzyme activity exposed to CPF1 measured was 128.91 U mg^{-1} protein whereas, the lowest was 98.9 U mg^{-1} protein. In CPF1exposed HL, the GPx enzyme activity measured higher on 3 DoE which gradually decreased till 21 DoE (Table 5.15). Moreover, the activity level of GPx enzyme in CPF1 exposed HL showed higher activity on 3 DoE, lower on 14 and 21 DoE whereas, equivalent on 7 DoE compared to control (Fig. 5.15).

Table 5.4 Antioxidant activity of superoxide dismutase (SOD), Catalase (CAT) and glutathione peroxidase (GPx) in haemolymph of juveniles exposed to Chlorpyrifos (CPF1= 0.36 µg L^{-1})

Size of animal	**DoE**	**Enzyme activity**					
Juvenile (Haemolymph)		**SOD (U mg^{-1} protein)**		**CAT (µmole H_2O_2 min^{-1} mg^{-1} protein)**		**GPx (U mg^{-1} protein)**	
		Control	**CPF (0.36 µg L^{-1})**	**Control**	**CPF (0.36 µg L^{-1})**	**Control**	**CPF (0.36 µg L^{-1})**
	3	0.11 ± 0.008	0.18 ± 0.007	60.32 ± 2.34	68.3 ± 2.45	110.9 ± 3.56	128.91 ± 4.12
	7	0.10 ± 0.007	0.17 ± 0.008	60.29 ± 2.30	65.49 ± 2.46	111.1 ± 3.45	112.4 ± 4.23
	14	0.09 ± 0.006	0.13 ± 0.009	60.54 ± 2.34	52.7 ± 1.89	112.5 ± 3.78	98.9 ± 3.33
	21	0.11 ± 0.005	0.12 ± 0.006	61.33 ± 2.23	60.33 ± 2.33	112.67 ± 3.11	78.82 ± 3.67

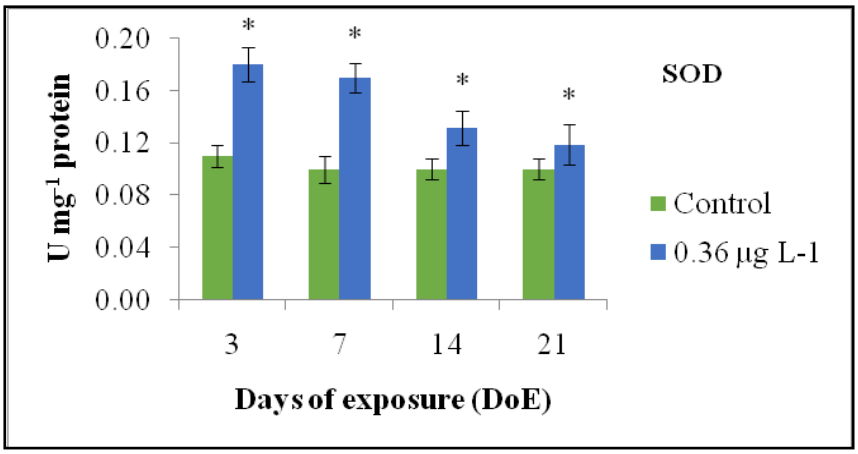

Fig 5.13 Superoxide dismutase (SOD) activity in haemolymph of juveniles on exposure to sublethal dose of CPF1= 0.36 µg L^{-1}. Values are expressed as Mean ± SD (ANOVA, *= P< 0.05)

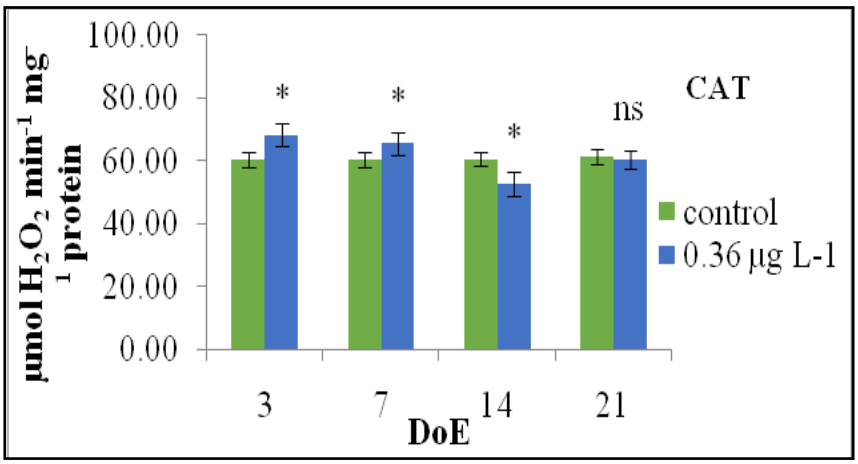

Fig. 5.14 Catalase (CAT) enzyme activity in haemolymph of juveniles exposed to sublethal dose, CPF1= 0.36 µg L^{-1}. Values are expressed as Mean ± SD (ANOVA, ns= non significance, *= P< 0.05)

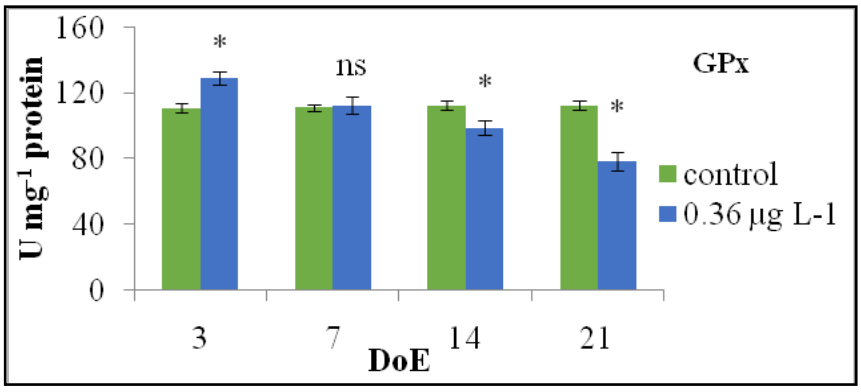

Fig. 5.15 Glutathione peroxidase **(**GPx) enzyme activity in haemolymph of juveniles exposed to sublethal dose, CPF1= 0.36 µg L^{-1}. Values are expressed as Mean ± SD (ANOVA, ns= non significance, *= P< 0.05)

Antioxidant enzyme genes *viz.* SOD, CAT and GPx expressed in control and CPF exposed HL is in Fig. 5.16. Data were subjected to one-way ANOVA to compare between controls and treated at 3, 7, 14 and 21 DoE. The differential response of SOD, CAT and GPx genes were recorded on exposure to SL dose of CPF and discussed the same. In CPF1 exposed HL, the SOD gene was down-regulated at 3 (0.87 ± 0.01) and 21 (0.64 ± 0.11) DoE whereas up-regulated on 7 DoE (1.24 ± 0.14) and equivalent on 14 (0.99 ± 0.26) DoE with respect to control (1 ± 0.15). Expression of CAT gene in CPF1 exposed HL was down-regulated on 3 (0.75 ± 0.09) DoE followed by up-regulation on 7 (1.4 ± 0.25) and 14 (1.12 ± 0.18) DoE and equivalent on 21 (0.98 ± 0.13) DoE with respect to control (1 ± 0.15). The GPx enzyme gene expression in CPF1 exposed HL, was up-regulated on 3 (1.83 ± 0.21) and 7 (1.2 ± 0.14) DoE whereas down-regulated on 14 (0.84 ± 0.13) and 21 (0.54± 0.10) DoE with respect to control (1 ± 0.13).

It was also observed that expression of SOD gene was minimum on 3 DoE and reached maximum level on 7 DoE followed by a decline till the end. Apparently, CAT and SOD genes demonstrated similar trends in expression. However, expression of GPx gene was maximum on initial days of exposure later on decreased till the end of exposure.

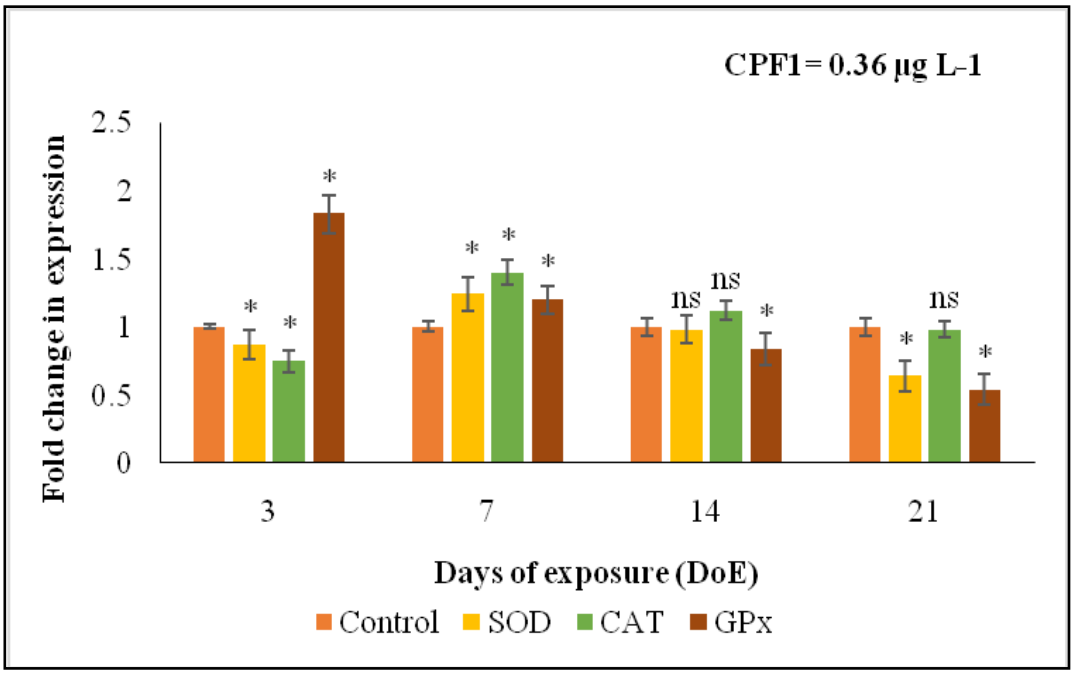

Fig. 5.16 Level of expression of Superoxide dismutase (SOD), Catalase (CAT) and Glutathione peroxidase (GPx) genes in gill of juvenile exposed to sublethal dose of CPF1= 0.36 µg L^{-1}. Values are expressed as Mean ± SD (ANOVA, *= P< 0.05)

5.3.2.3 Antioxidant enzyme activity and their gene expression in muscles (ML) exposed to Chlorpyrifos (CPF)

The antioxidant enzyme activity of SOD, CAT and GPx on 3, 7, 14 and 21 DoE were recorded in ML exposed to SL dose of CPF1 are represented in Fig. 5.17, 5.18 and 5.19, respectively. Data obtained were subjected to ANOVA to compare treated and control groups at different time intervals. The SOD activity recorded highest on exposure to CPF1 was 0.09 mg^{-1} protein on 3 DoE whereas, the lowest was 0.07 U mg^{-1} protein on 21 DoE. The SOD activity was higher on 3 DoE which gradually decreased till 21 DoE in the treated ML (Table 5.5). Moreover, the activity level of SOD enzyme in CPF1 exposed ML when compared against control it showed higher activity in treated once compared to the control at all time intervals (Fig. 5.17).

The highest activity of CAT measured on exposure to CPF1 was 58.3 µmole H_2O_2 min^{-1} mg^{-1} protein whereas, the lowest was 39.33 µmole H_2O_2 min^{-1} mg^{-1} protein. The CAT activity was higher on 3 DoE while gradually decreased till 21 DoE in CPF1 exposed ML (Table 5.5). Moreover, the activity level of CAT enzyme in CPF1 exposed ML when compared against control it showed higher activity on 3 DoE and lower on 7, 14 and 21 DoE against the control (Fig. 5.18).

The highest GPx enzyme activity on exposure to CPF1 measured was 110.1 U mg^{-1} protein whereas, the minimum was 62.18 11 U mg^{-1} protein. The GPx enzyme activity measured maximum on 3 DoE while gradually decreased till 21 DoE in the treated ML (Table 5.5). Moreover, the activity level of GPx enzyme in CPF1 exposed ML compared against control it showed higher activity on 3 DoE, fell to normal level on 7 DoE and minimum on 14 and 21 DoE (Fig. 5.19).

Table 5.5 Antioxidant enzyme activity of superoxide dismutase (SOD), Catalase (CAT) and glutathione peroxidase (GPx) in muscles of juveniles exposed to Chlorpyrifos (CPF1= 0.36 µg L^{-1})

Size of animal	**DoE**	**Enzyme activity**					
Juvenile (Muscles)		**SOD (U mg^{-1} protein)**		**CAT (µmole H_2O_2 min^{-1} mg^{-1} protein)**		**GPx (U mg^{-1} protein)**	
		Control	**CPF (0.36 µg L^{-1})**	**Control**	**CPF (0.36 µg L^{-1})**	**Control**	**CPF (0.36 µg L^{-1})**
	3	0.07 ± 0.003	0.09 ± 0.004	51.32 ± 2.24	58.3 ± 2.33	89.9 ± 3.45	110.1 ± 3.16
	7	0.07 ± 0.003	0.08 ± 0.003	50.29 ± 2.45	47.49 ± 1.78	84 ± 3.23	84.56 ± 4.23
	14	0.06 ± 0.003	0.08 ± 0.004	49.54 ± 1.11	42.7 ± 1.65	84.12 ± 3.45	72.12 ± 2.78
	21	0.06 ± 0.003	0.07 ± 0.004	50.33 ± 1.56	39.33 ± 1.34	83.88 ± 4.11	62.18 ± 1.91

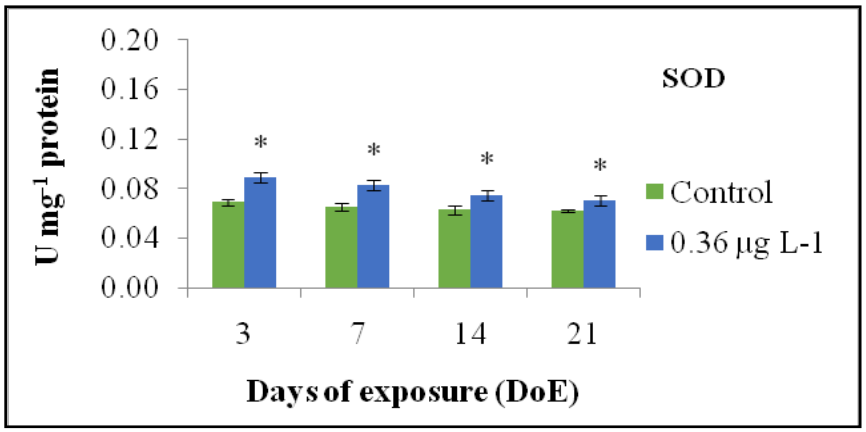

Fig. 5.17 Superoxide dismutase (SOD) enzyme activity in muscles of juveniles exposed to sublethal dose, CPF1= 0.36 µg L^{-1}. Values are expressed as Mean ± SD (ANOVA, *= P< 0.05)

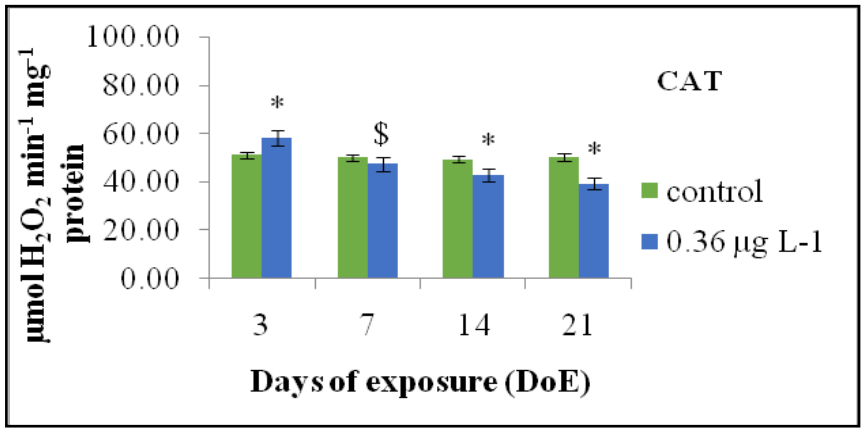

Fig. 5.18 Catalase (CAT) enzyme activity in muscles of juveniles exposed to sublethal dose, CPF1= 0.36 µg L^{-1}. Values are expressed as Mean ± SD (ANOVA, *= P< 0.05)

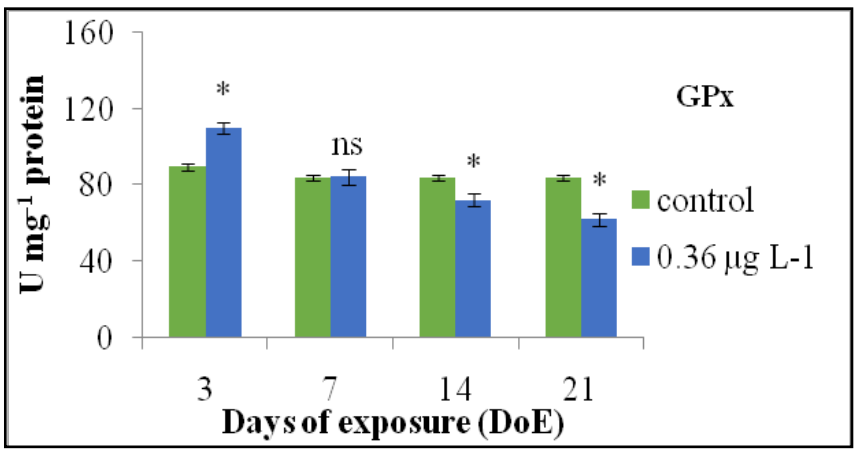

Fig. 5.19 Glutathione peroxidase (GPx) enzyme activity in muscles of juveniles exposed to sublethal dose, CPF1= 0.36 µg L^{-1}. Values are expressed as Mean ± SD (ANOVA, ns= non significance, *= P< 0.05)

Antioxidant enzyme genes *viz.* SOD, CAT and GPx expressed in control and CPF exposed ML is represented in Fig. 5.20. Data subjected to one-way ANOVA to compare between control and treated ML at 3, 7 14 and 21 DoE. The differential response of SOD, CAT and GPx genes were recorded on exposure to SL dose of CPF and discussed the same. In CPF1 exposed ML, the SOD gene was down-regulated at all time intervals *viz*. 3 (0.28 ± 0.08), 7 (0.85 ± 0.09), 14 (0.62 ± 0.06) and 21 (0.38 ± 0.01) compared to control (1 ± 0.11). Expression of CAT gene in CPF1 exposed ML was down-regulated on 3 DoE (0.24 ± 0.02) expression on 7 DoE (1.08 ± 0.14) equivalent to control and again down-regulates on 14 (0.84 ± 0.09) and 21 (0.54 ± 0.01) DoE as compared to control (1± 0.11). Moreover, the GPx gene in CPF1 exposed ML was down-regulated at all time intervals *viz*. 3 (0.45 ± 0.09), 7 (0.75 ± 0.06), 14 (0.23 ± 0.02) and 21 (0.12 ± 0.01) compared to control ($P< 0.05$).

It was also observed that the CPF induced SOD, CAT and GPx gene expression were minimum in comparison to control at all time intervals.

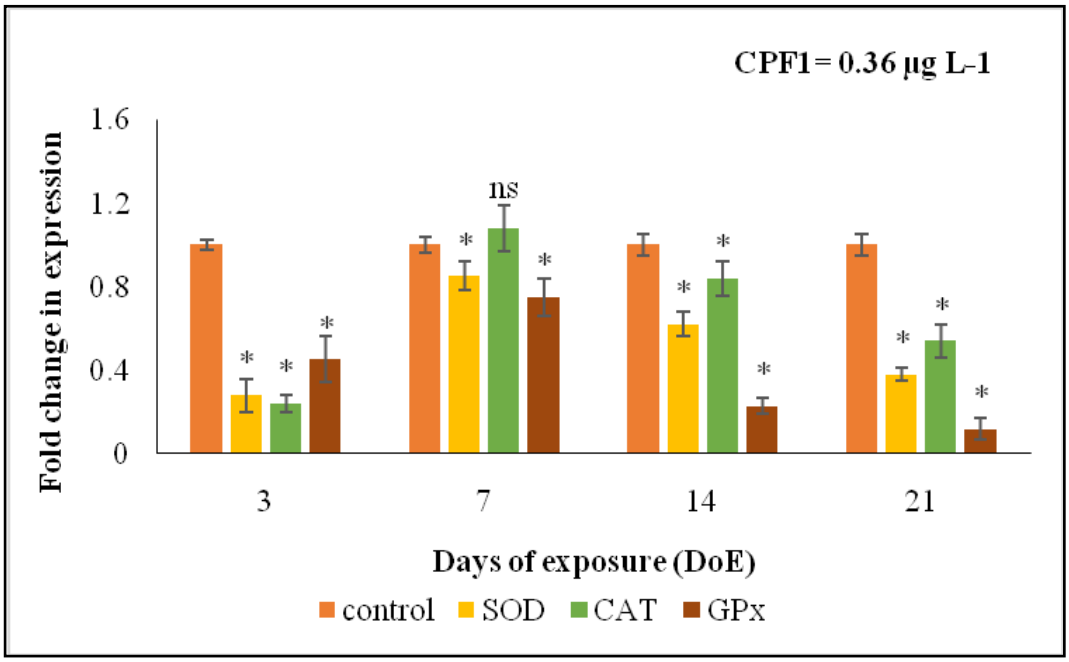

Fig. 5.20 Level of expression of Superoxide dismutase (SOD), Catalase (CAT) and Glutathione peroxidase (GPx) genes in muscles of juvenile exposed to sublethal dose, CPF1= 0.36 µg L^{-1}. Values are expressed as Mean ± SD (ANOVA, *= $P< 0.05$)

5.3.2.4 Antioxidant enzyme activity and their gene expression in gill (GL) exposed to Dimethoate (DMT)

The antioxidant enzyme activity of SOD, CAT and GPx on 3, 7, 14 and 21 DoE were recorded in GL on exposed to SL dose of DMT1 are represented in Fig. 5.21, 5.22 and 5.23, respectively. Data were subjected to ANOVA to compare treated and control groups at different time intervals. The SOD activity measured highest exposed to DMT1 was 0.24 U mg^{-1} protein on 3 DoE whereas, the lowest was 0.13 U mg^{-1} protein on 21 DoE (Fig. 5.21). In DMT1 exposed GLs, the SOD activity was higher on 3 DoE which gradually decreased till 21 DoE (Table 5.6). Moreover, the activity level of SOD enzyme in DMT1 exposed GL compared against control showed higher activity at all time intervals studied (Fig. 5.21).

In DMT1 exposed GLs, the activity of CAT was recorded highest on exposure to DMT1 was .51 µmoleH_2O_2 min^{-1} mg^{-1}protein whereas, the lowest was 59.08 µmole H_2O_2 min^{-1} mg^{-1} protein. The CAT activity was higher on 3 DoE whereas lower on 7, 14 and 21 DoE compared to control in DMT1 exposed GL (Table 5.6). Moreover, the activity level of CAT enzyme in CPF1 exposed GL compared against control it showed lower activity on 7, 14 and 21 DoE whereas, equivalent on 3 DoE (Fig. 5.22).

The GPx enzyme activity recorded highest exposed to DMT1 was 136.14U mg^{-1} protein whereas, the lowest was 94.32 U mg^{-1} protein. In DMT1 exposed GLs, the GPx enzyme activity measured higher on 3 DoE which decreased till 21 DoE (Table 5.6). Moreover, the activity level of GPx enzyme in CPF1 exposed GL compared against control it showed maximum activity on 3 DoE, equivalent on 7 DoE and minimum activity on 14 and 21 DoE (Fig. 5.23).

Table 5.6 Antioxidant enzyme activity of superoxide dismutase (SOD), Catalase (CAT) and glutathione peroxidase (GPx) in gill of juveniles exposed to Dimethoate (DMT1= 140.9 µg L^{-1})

Size of animal	**DoE**	**Enzyme activity**					
Juvenile (Gill)		**SOD (U mg^{-1} protein)**		**CAT (µmole H_2O_2 min^{-1} mg^{-1} protein)**		**GPx (U mg^{-1} protein)**	
		Control	**DMT (140.9 µg L^{-1})**	**Control**	**DMT (140.9 µg L^{-1})**	**Control**	**DMT (140.9 µg L^{-1})**
	3	0.13 ± 0.008	0.24 ± 0.01	81.32 ± 1.64	79.51 ± 3.44	123.9 ± 3.86	136.14 ± 4.11
	7	0.14 ± 0.008	0.19 ± 0.01	82.29 ± 1.62	63.07 ± 3.26	121.1 ± 3.56	124.99 ± 3.98
	14	0.14 ± 0.006	0.16 ± 0.01	80.54 ± 1.62	59.08 ± 2.89	122.5 ± 2.89	118.56 ± 3.67
	21	0.12 ± 0.007	0.13 ± 0.01	82.33 ± 1.59	64.01 ± 3.45	122.67 ± 2.67	94.32 ± 2.33

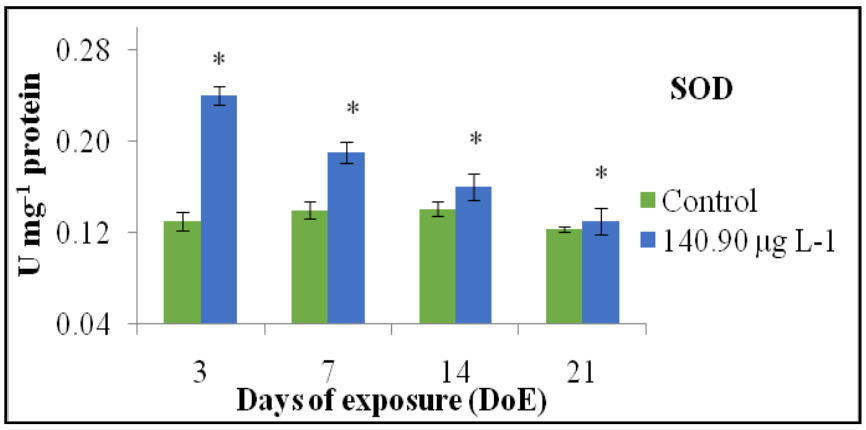

Fig. 5.21 Superoxide dismutase **(**SOD) enzyme activity in gill of juveniles exposed to sublethal dose, DMT1= 140.9 µg L^{-1}. Values are expressed as Mean ± SD (ANOVA, *= P< 0.05)

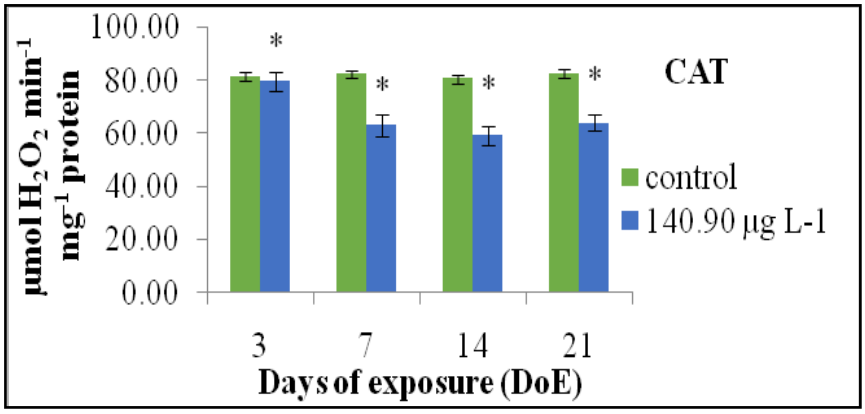

Fig. 5.22 Catalase (CAT) enzyme activity in gill of juveniles exposed to sublethal dose, DMT1= 140.9 µg L^{-1}. Values are shown as mean ± SD (ns= non significance, *= P< 0.05)

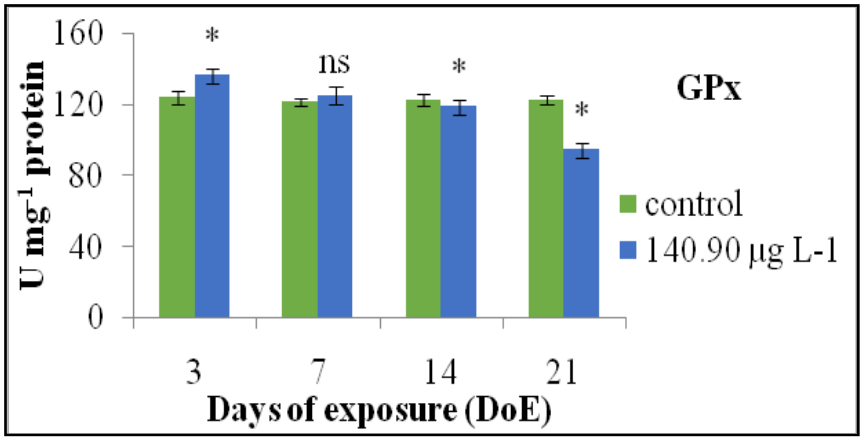

Fig. 5.23 Glutathione peroxidase (GPx) enzyme activity in gill of juveniles exposed to sublethal dose, DMT1= 140.9 µg L^{-1}. Values are expressed as Mean ± SD (ANOVA, ns= non significance, *= P< 0.05)

Antioxidant enzyme genes *viz.* SOD, CAT and GPx expressed in control and DMT exposed GL is represented in Fig. 5.24. Data were subjected to one-way ANOVA in order to compare levels of expression between controls and treated at 3, 7, 14 and 21 DoE. The differential response of SOD, CAT and GPx genes were recorded exposed SL dose of CPF and discussed the same. In DMT1 exposed GL, the SOD gene was up-regulated at all time intervals *viz.* 3 (1.75 ± 0.18), 7 (1.45 ± 0.14), 14 (1.3 ± 0.11) and 21 (1.14 ± 0.13) compared to control (1 ± 0.14). Further, expression of CAT gene in DMT1 exposed GL, was up-regulated at 21 (1.58 ± 0.12) whereas down-regulated on 14 (0.68 ± 0.07) whereas expression was equivalent with control (1 ± 0.14) on 3 (0.98 ± 0.09) 7 (1.11 ± 0.09) The GPx enzyme gene expression, was up-regulated on 3 (1.95 ± 0.21), 7 (2.64 ± 0.25) and DoE, down-regulated on 21 (0.84 ± 0.11) DoE whereas expression was equivalent with control (1 ± 0.14) on 14 (1.11 ± 0.12).

It was also observed that the SOD gene was expressed at maximum levels in DMT1 exposed GL on initial days of exposure and continued to decrease till the end. However, expression of CAT gene increased from initial days of the exposure until the end. Further, the expression of GPx was minimum on initial days, increased on 7 DoE and later decreased till the end.

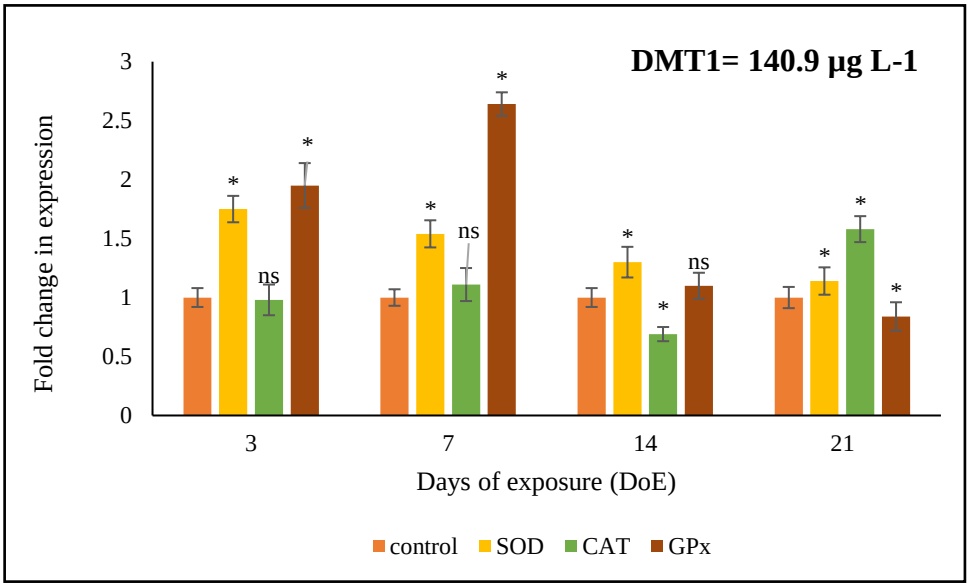

Fig. 5.24 Level of expression of Superoxide dismutase (SOD), Catalase (CAT) and Glutathione peroxidase (GPx) genes in gill of juveniles exposed to sublethal dose, DMT1= 140.9 µg L^{-1}. Values are expressed as Mean ± SD (ANOVA, *= P< 0.05)

5.3.2.5 Antioxidant enzyme activity and their gene expression in haemolymph (HL) exposed to Dimethoate (DMT)

The antioxidant enzyme activity of SOD, CAT and GPx on 3, 7, 14 and 21 DoE were measured in HL exposed to SL dose of DMT1 are represented in Fig. 5.25, 5.26 and 5.27, respectively. Data obtained were subjected to ANOVA to compare treated and control group at different time intervals. In DMT1 exposed HL, the SOD activity recorded highest on exposed to DMT1 was 0.17 U mg^{-1} protein whereas, the lowest was 0.12 U mg^{-1} protein. In DMT1 exposed HL, the SOD activity was higher on 3 DoE which decreased till 21 DoE (Table 5.7). Moreover, the activity level of SOD enzyme in DMT1 exposed HL compared against control showed higher activity at all time intervals (Fig. 5.25).

In DMT1 exposed HL, the highest activity of CAT was recorded exposed to DMT1 was 65.51 µmole H_2O_2 min^{-1} mg^{-1} protein whereas, the lowest was 41.08 µmole H_2O_2 min^{-1} mg^{-1} protein. In DMT1 exposed HL, the CAT activity was higher on 3 DoE then decreased on 7 and 14 DoE which later on increased again on 21 DoE (Table 5.7). Moreover, the activity level of CAT enzyme in CPF1 exposed HL showed a higher activity and lower on 7, 14 and 21 DoE compared against the control (Fig. 5.26).

In DMT1 exposed HL, the highest GPx enzyme activity exposed to DMT1 was 126.14 U mg^{-1} protein whereas, the lowest was 73.32 U mg^{-1} protein on 21 DoE. The GPx enzyme activity was higher on 3 DoE which decreased till 21 DoE in DMT1 exposed HL (Table 5.7). Moreover, the activity level of GPx enzyme in DMT1 exposed HL compared against control it showed higher activity on 3 DoE, equivalent on 7 DoE and lower on 14 and 21 DoE (Fig. 5.27).

Table 5.7 Antioxidant enzyme activity of superoxide dismutase (SOD), Catalase (CAT) and glutathione peroxidase (GPx) in haemolymph of juveniles exposed to Dimethoate (DMT1= 140.9 µg L^{-1})

Size of animal	**DoE**	**Enzyme activity**					
Juvenile (Haemolymph)		**SOD (U mg^{-1} protein)**		**CAT (µmole H_2O_2 min^{-1} mg^{-1} protein)**		**GPx (U mg^{-1} protein)**	
		Control	**DMT (140.9 µg L^{-1})**	**Control**	**DMT (140.9 µg L^{-1})**	**Control**	**DMT (140.9 µg L^{-1})**
	3	0.11 ± 0.007	0.17 ± 0.009	60.32 ± 2.34	65.51 ± 2.89	110.9 ± 2.86	126.14 ± 4.32
	7	0.10 ± 0.008	0.16 ± 0.008	60.29 ± 2.35	52.07 ± 2.11	111.1 ± 3.22	109.99 ± 4.22
	14	0.09 ± 0.01	0.12 ± 0.007	60.54 ± 2.30	41.08 ± 1.89	112.5 ± 2.90	92.11 ± 4.56
	21	0.11 ± 0.01	0.12 ± 0.06	60.33 ± 2.28	50.01 ± 2.34	112.67 ± 3.45	73.32 ± 2.56

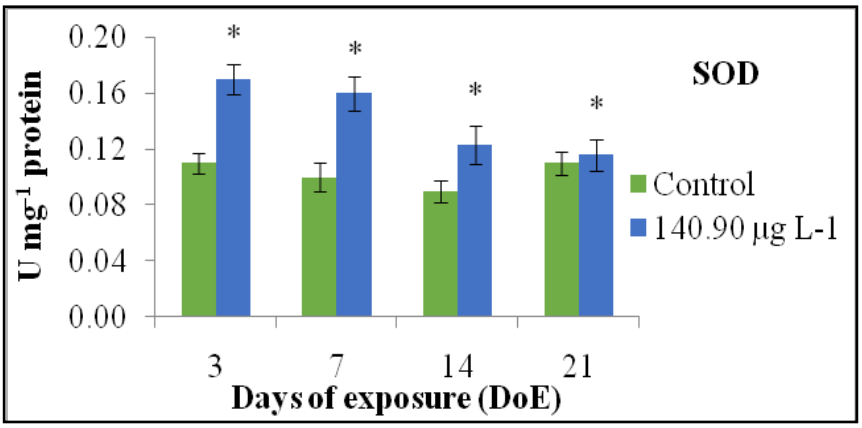

Fig. 5.25 Superoxide dismutase (SOD) enzyme activity in haemolymph of juveniles exposed to sublethal dose, DMT1= 140.9 µg L^{-1}. Values are expressed as Mean ± SD (ANOVA, *= P< 0.05)

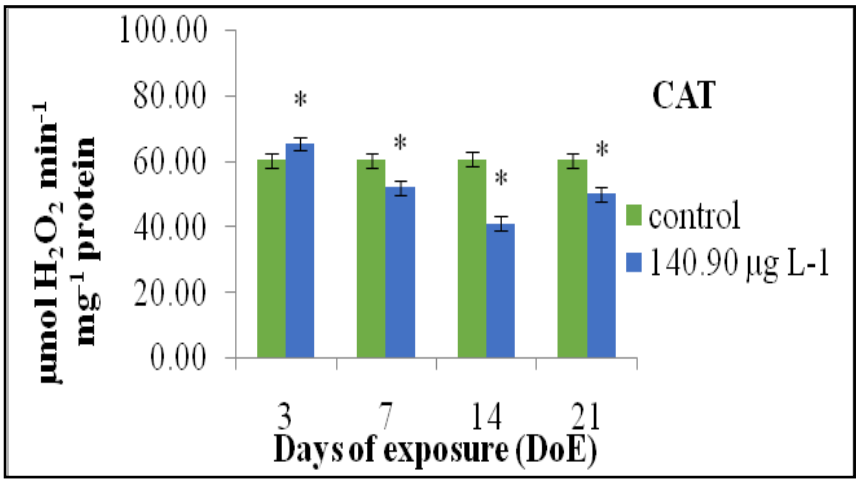

Fig. 5.26 Catalase (CAT) enzyme activity in haemolymph of juveniles exposed to sublethal dose, DMT1= 140.9 µg L^{-1}. Values are expressed as Mean ± SD (ANOVA, *= P< 0.05)

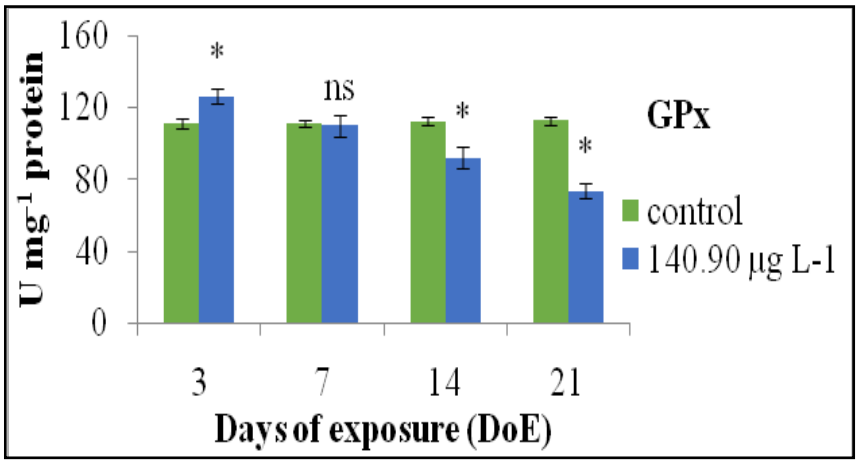

Fig. 5.27 Glutathione peroxidase (GPx) enzyme activity in haemolymph of juveniles exposed to sublethal dose, DMT1= 140.9 µg L^{-1}. Values are expressed as Mean ± SD (ANOVA, ns= non significance, *= P< 0.05)

Antioxidant enzyme genes *viz.* SOD, CAT and GPx expressed in control and DMT exposed HL is represented in Fig. 5.28. Data were subjected to one-way ANOVA to compare between controls and treated at 3, 7, 14 and 21 DoE. The differential response of SOD, CAT and GPx genes were recorded on exposure to SL dose of DMT and discussed the same. In DMT1 exposed HL, SOD gene was down-regulated at 3 (0.75 ± 0.11), 14 (0.45 ± 0.09) and 21 (0.51 ± 0.04) whereas expression was equivalent with control on 7 (0.98 ± 0.10). Further, CAT gene expression in exposed HL was down-regulated on 3 (0.42 ± 0.06) and 21(0.8 ± 0.04) DoE whereas up-regulated on 7 (1.34 ± 0.17) and equivalent with control on 14 (1.09 ± 0.11) DoE. Moreover, the GPx enzyme gene expression in DMT1 exposed HL was up-regulated on 3 (1.75 ± 0.21) and 7 (1.18 ± 0.14) DoE whereas down-regulated on 14 (0.74 ± 0.18) and 21 (0.5 ± 0.09) DoE with respect to control.

It was also observed that SOD was expressed at minimum level on initial days of exposure and reached maximum level on 7 DoE and later decreased until the end. CAT gene was also expressed at lower level on 3 DoE while reached maximum level on 7 DoE and later on again decreased till the end. However, GPx gene was expressed maximum on 3 DoE and kept on decreasing until the end.

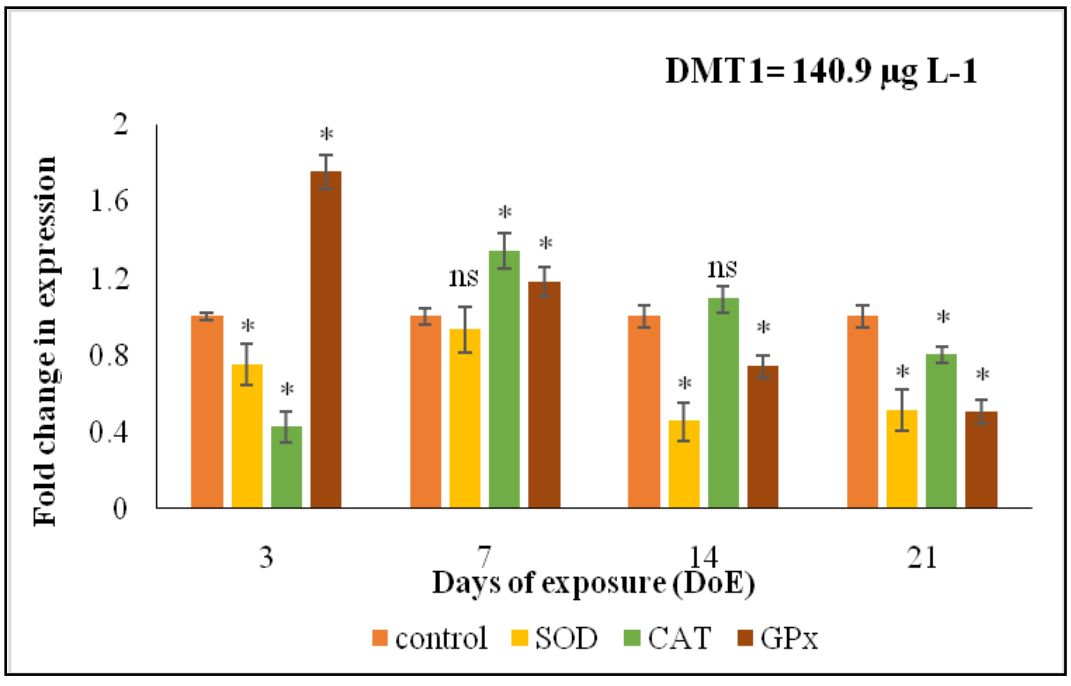

Fig. 5.28 Level of expression of Superoxide dismutase (SOD), Catalase (CAT) and Glutathione peroxidase (GPx) genes in haemolymph of juveniles exposed to sublethal dose, DMT1= 140.9 µg L^{-1}. Values are expressed as Mean ± SD (ANOVA, *= P< 0.05)

5.3.2.6 Antioxidant enzyme activity and their gene expression in muscles (ML) exposed to Dimethoate (DMT)

The antioxidant enzyme activity of SOD, CAT and GPx on 3, 7, 14 and 21 DoE recorded in ML exposed to SL dose of DMT1 are represented in Fig. 5.29, 5.30 and 5.31, respectively. Data obtained were subjected to ANOVA to compare treated and control groups at different time intervals. In DMT1 exposed ML, the SOD activity recorded highest activity exposed to DMT1 was 0.08 U mg^{-1} protein whereas, the lowest was 0.06 U mg^{-1} protein. In DMT1 exposed ML, the SOD activity was higher on 3 DoE which decreased till 21 DoE (Table 5.8). Moreover, the activity level of SOD enzyme in DMT1 exposed ML compared against control it showed higher activity at all time intervals except 21 DoE (Fig. 5.29).

In DMT1 exposed ML, the highest activity of CAT 56.75 µmole H_2O_2 min^{-1} mg^{-1} protein whereas, the activity lowest was 41.45 µmole H_2O_2 min^{-1} mg^{-1} protein. In DMT1 exposed ML, the CAT activity was higher on 3 DoE then decreased on 7 and 14 DoE which later again increased on 21 DoE (Table 5.8). Moreover, the activity level of CAT enzyme in DMT1 exposed ML compared against control showed higher activity on 3 and lower on 7, 14 and 21 DoE (Fig. 5.30).

In DMT1 exposed ML, the highest GPx enzyme activity exposed to DMT1 was 109.34 U mg^{-1} protein whereas, the lowest was 58.11 U mg^{-1} protein. In DMT1 exposed ML, the GPx enzyme activity measured higher on 3 DoE which decreased till 21 DoE (Table 5.8). Moreover, the activity level of GPx enzyme in DMT1 exposed HL compared against control showed higher activity on 3 DoE and lower for remaining time intervals (Fig. 5.31).

Table 5.8 Antioxidant enzyme activity of superoxide dismutase (SOD), Catalase (CAT) and glutathione peroxidase (GPx) in muscles of juveniles exposed to Dimethoate (DMT1= 140.9 µg L^{-1})

Size of animal	**DoE**	**Enzyme activity**					
Juvenile (Muscles)		**SOD (U mg^{-1} protein)**		**CAT (µmole H_2O_2 min^{-1} mg^{-1} protein)**		**GPx (U mg^{-1} protein)**	
		Control	**DMT (140.9 µg L^{-1})**	**Control**	**DMT (140.9 µg L^{-1})**	**Control**	**DMT (140.9 µg L^{-1})**
	3	0.07 ± 0.003	0.08 ± 0.002	51.32 ± 1.24	56.75 ± 1.80	89.9 ± 2.86	109.34 ± 3.22
	7	0.07 ± 0.002	0.08 ± 0.003	50.29 ± 1.45	43.24 ± 1.67	84 ± 2.76	78.94 ± 3.11
	14	0.06 ± 0.003	0.07 ± 0.003	49.54 ± 1.34	41.45 ± 1.65	84.12 ± 2.80	68.22 ± 2.89
	21	0.06 ± 0.003	0.06 ± 0.004	50.33 ± 1.50	45.13 ± 1.45	83.88 ± 2.78	58.11 ± 3.08

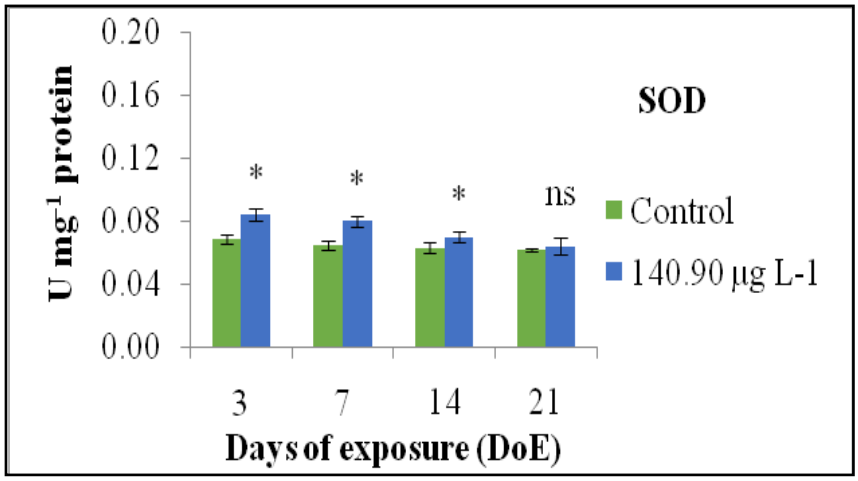

Fig. 5.29 Superoxide dismutase (SOD) enzyme activity in muscles of juveniles exposed to sublethal dose, DMT1= 140.9 µg L^{-1}. Values are expressed as Mean ± SD (ANOVA, ns= non significance, *= P< 0.05)

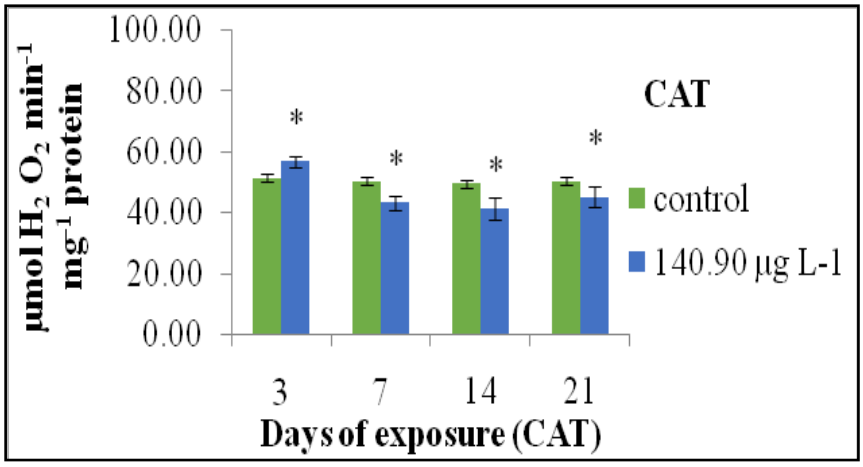

Fig. 5.30 Catalase (CAT) enzyme activity in muscles of juveniles exposed to sublethal dose, DMT1= 140.9 µg L^{-1}. Values are expressed as Mean ± SD (ANOVA, *= P< 0.05)

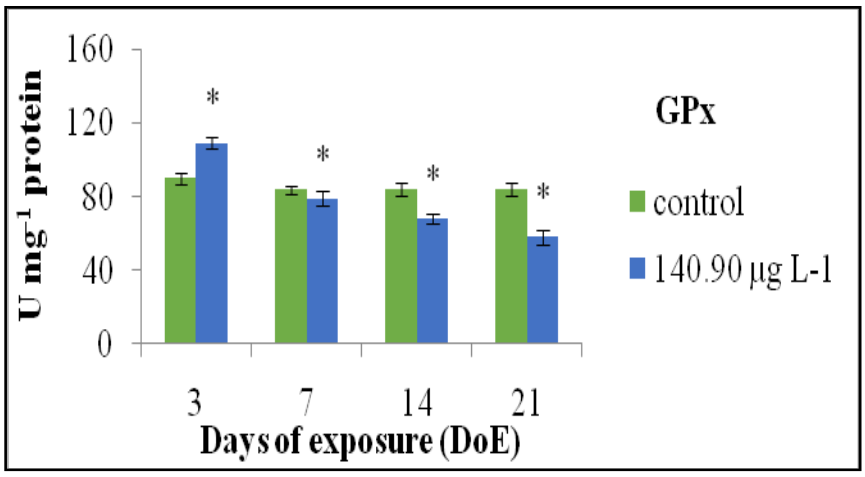

Fig. 5.31 Glutathione peroxidase **(**GPx) enzyme activity in muscles of juveniles exposed to sublethal dose, DMT1= 140.9 µg L^{-1}. Values are expressed as Mean ± SD (ANOVA, *= P< 0.05)

Antioxidant enzyme genes *viz.* SOD, CAT and GPx expressed in control and DMT exposed ML is represented in Fig. 5.32. Data were subjected to one-way ANOVA to compare between controls and treated ML at 3, 7 14 and 21 DoE. The differential response of SOD, CAT and GPx genes were recorded on exposure to SL dose of DMT and discussed the same. In DMT1 exposed ML, the SOD gene was down-regulated in at all time intervals *viz.* 3 (0.098 ± 0.005), 7 (0.51 ± 0.09), 14 (0.23 ± 0.12) and 21 (0.23 ± 0.11) with respect to the control (1 ± 0.11). In DMT1 exposed ML, the CAT gene expression was down-regulated at 3 (0.079 ± 0.008), 14 (0.75 ± 0.12) and 21 (0.53 ± 0.11) DoE whereas, equivalent to control on 7 (1.02 ± 0.13) DoE. Moreover, the GPx gene in DMT1 exposed ML was also down-regulated at all time intervals *viz.* 3 (0.21 ± 0.08), 7 (0.7 ± 0.08), 14 (0.11 ± 0.06) and 21 (0.045 ± 0.007) compared to control (1 ± 0.11).

It was also observed that the DMT pesticides induced SOD, CAT and GPx gene expression were almost inhibited at all time intervals. Further, all the genes were expressed at minimum level in ML compared to the GL and HL.

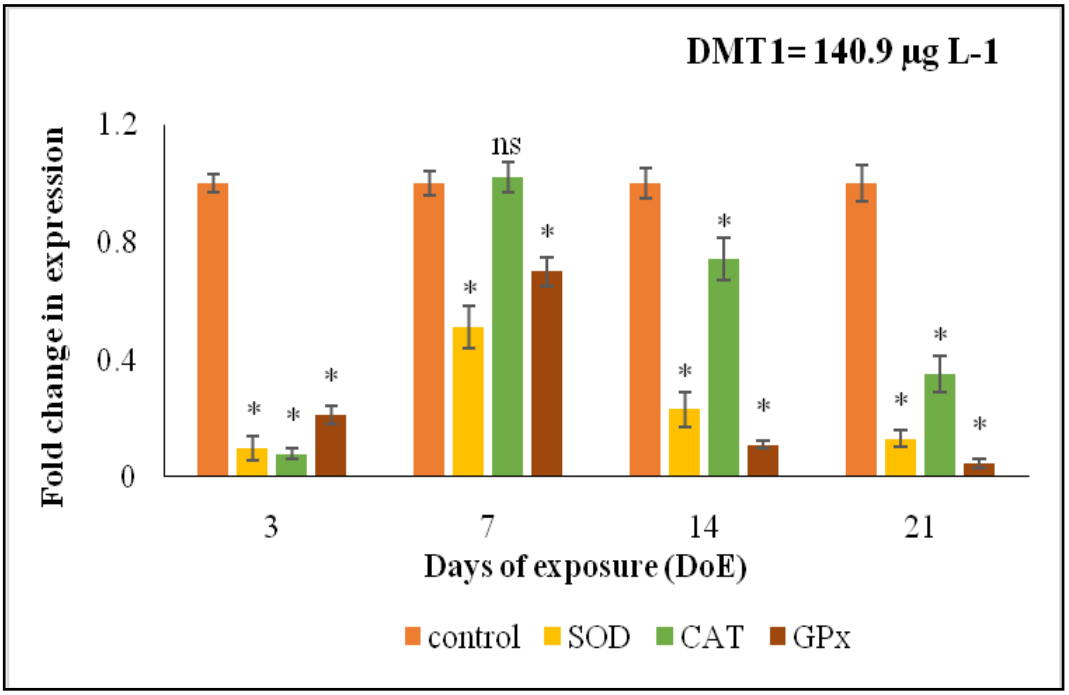

Fig. 5.32 Level of expression of Superoxide dismutase (SOD), Catalase (CAT) and Glutathione peroxidase (GPx) genes in muscles of juvenile exposed to sublethal dose of DMT1= 140.9 µg L^{-1}. Values are expressed as Mean ± SD (ANOVA, *= P< 0.05)

5.4 Discussion

Whenever an organism is exposed to an environmental contaminant, it induces oxidative stress in that organism which in turn generates the Reactive oxygen species (ROS) such as superoxide anion ($O_2 \cdot -$), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH). In the case of invertebrates, they have an adaptive immune system which is regulated by highly specific antigen-antibody receptors and rely on the efficient working of innate immune defence to protect from foreign invaders and generated ROS inside the body (Shoaib and Siddiqui, 2015). Most cells have acquired protective mechanisms to maintain the lowest level of ROS inside the cells. It mainly includes the non-enzymatic (glutathione, Ascorbic acid, vitamin E and C, beta carotene and tocopherol) and enzymatic system *viz.* superoxide dismutase (SOD), catalase (CAT), glutathione peroxidise (GPx), etc. (Kalender et al., 2010). From that SOD scavenges the superoxide anions and converts them into hydrogen peroxide and oxygen whereas, CAT and GPx convert further hydrogen peroxide into water and oxygen (Wang et al., 2009).

The antioxidant system of organisms counteracts the effects of ROS and plays a crucial role in protecting cells from oxidative stress (Parrilla-Taylor and Zenteno-Savín, 2011). The antioxidant enzymes are the first line defence against ROS. The present study was carried out to analyse the activity of the antioxidant enzymes *viz.* SOD, CAT and GPx in PL and JL stages of *L. vannamei* exposed to SL concentration of CPF and DMT pesticides. The present study contributes to the elucidation of the mechanisms that allow them to counteract ROS production and to prevent oxidative damage.

The pesticides are known to induce the oxidative stress through the generation of reactive oxygen species (Vuillaume et al., 1987; Rai and Sharma, 2007; Mehta et. al., 2009; Heikal et al., 2011). Many of these are hydrophobic interactions which bind more likely to the lipid layer of cells and cause the lipid peroxidation which leads to cell damages (Kalender et al., 2010; Heikal et al., 2011). With respect to lipid peroxidation, ROS is also associated with the damage of protein and DNA in cells (Anusuya and Hemalatha, 2014). Hence, ROS affecting the DNA in cells eventually affects the enzyme activity and expression of genes. Therefore, the increased and decreased level of antioxidant enzyme activity in *L. vannamei* observed after exposure of pesticides.

During the present study, activity level of SOD enzyme was high at initial days of exposure and decreased as exposure period exceeded. The possible reason of increase in activity of SOD

enzyme could be due to the engagement of SOD enzyme to dismutase the superoxide anion into hydrogen peroxide. On the other hand, CAT enzyme activity was also high at initial days of exposure then decreased and later again increased. The increase in activity of CAT enzyme in initial days could be due to the engagement of CAT enzyme to convert hydrogen peroxide into water molecules whereas decrease in activity detected could be due to the saturation of hydrogen peroxide leading the deactivation or suppression of CAT enzymes for a duration of time. Later, again increase in activity of CAT enzyme observed this shows the increase in CAT enzyme activity by organism to detoxify the hydrogen peroxide production. Further, the GPx enzyme activity was also high at initial exposure then gradually kept on decreasing until the last day of exposure in shrimp PL and GL, HL and ML of the JL. The GPx activity pattern shows the activation of the enzymes to detoxify the hydrogen peroxide and decrease in activity indicates the suppression in activity due to over production of ROS in body due to pesticide exposure.

The reaction mechanism of SOD, CAT and GPx enzymes on the exposure to pesticides differ according to the composition of pesticides, type of an organism and the target part of organism (Oruc and Usta, 2007). A tissue specific response of SOD, CAT and GPx activities from MLs and hepatopancreas of *L. vannamei* under hypoxic condition have been reported by Parrilla-Taylor and Zenteno-Savin (2011). In the absence of literature about the effect of pesticides on enzyme activities in *L. vannamei* and other crustacean species, available reports on fish species have been discussed here. Farombi et al. (2008) reported that SOD activity levels in African catfish, *Clarias gariepinus* GL observed were decreased on exposure to herbicide, Butachlor after 24 h of exposure and activity decreased by 34% compared to the other studied organs *viz.* liver, kidney and heart. The study carried out by Barski et al. (2011) on the toxicity of CPF and enrofloxacin on rat blood for four weeks revealed a decrease in SOD activity levels and at the same time authors reported an increase in CAT and GPx activity. Furthermore, a study carried out by Helen et al. (2018) on the effect of sublethal doses of dichlorvos on enzyme activity of juvenile catfish, *Clarias gariepinus* observed that CAT activity was decreased at day 1^{st} and 5^{th}, but increased on day 10^{th} and 15^{th} at both sub lethal concentrations. Das et al. (2019) has observed a decrease in the activity levels of SOD, CAT and GPx enzymes of mud shrimp, *Austinogebia edulis* when exposed to heavy metal cadmium. These observations are in par with observations made during the present study.

Many studies have reported the oxidative stress produced by pesticides and their effect on antioxidant enzyme activity in different organisms. SOD is the first enzyme to be indulged to

rectify the pollutant produced ROS in organisms (Winston and Di Guilio, 1991). The SOD enzyme has been recognized as to convert superoxide anion into oxygen and hydrogen peroxide and plays a major role in the immunity system to keep the organism healthy (Wang and Chen, 2006; Mohankumar and Ramsamy, 2006; Cheng et al., 2008). Reduction in the SOD activity in GLs of Nile Tilapia, *Oreochromis niloticus* exposed to SL dose of DMT has been previously reported (Ajitha and Jayprakash, 2016). Furthermore, Bagnyukova et al. (2006) observed the transient reduction in SOD after 6 h of exposure of glyphosate-based herbicide to the goldfish, *Prochilodus lineatus*. This could be explained by the generation of intracellular space by ROS (Sharma et al., 2012) and initiation of an active role of SOD enzyme to which it swiftly terminates the harmful radicals as a part of the immune system of the body. Furthermore, the constant level of increase in the ROS generation and at the same time inadequate supply of SOD to detoxify these ROS, results into the decrease level of SOD enzymes in organisms.

During the present study, the CAT enzyme activity observed to be increased at the end of exposure period of 21 DoE of Chlorpyrifos and Dimethoate pesticides. A similar observation was reported by Ajitha and Jayaprakash (2016) on the exposure of methyl parathion in different tissues of freshwater fish, *Catla catla.* The GPx activity was observed to be higher compared to the SOD and CAT in PL and JL stages of *L. vannamei* in the initial exposure and subsequent reduction in the later part of exposure. Furthermore, GPx activity has been used as important indices for determining the oxidative stress (Blokhnia et al., 2003; David et al., 2018). The decrease in GPx activity has been observed in the fish, *Cyprinus carpio* on exposure to simazine (Stara et al., 2012). Similarly, the decrease in GPx activity on exposure to organophosphorus pesticide, DMT on Nile tilapia has been reported Ajitha and Jayaprakash (2016). The CAT and GPx enzymes are involved in the transformation of hydrogen peroxide (H_2O_2) to water (H_2O) (Baud et al., 2004). The H_2O_2 produced from SOD activity was also converted into water by CAT and GPx enzymes. The increase in CAT and GPx activities in both PL and JL stages of *L. vannamei* during the present study might be related to the detoxification of produced H_2O_2 from oxidative stress caused by CPF and DMT. It has been also reported that SOD-CAT system represents the first-line defence against the oxidative stress caused due to environmental pollutants (Pandey et al., 2003). Also, SOD-CAT enzymes are more sensitive and respond quickly to protect animals from oxidative stress damage (Rao, 2006). The results observed in the present study correlate with the other study carried out on different organisms.

In the present study, it has been observed that CPF is not only being highly toxic but also produces higher oxidative stress than DMT pesticide in PL and JL of *L. vannamei*. The noticeable changes observed during the present study in SOD, CAT and GPx activity shows that it might be related to the defence action carried out against ROS production due to the entry of pesticides inside the body of *L. vannamei*. This explains the organophosphorus pesticides produce the potential oxidative stress in penaeid shrimp, *L. vannamei*.

Any physical or chemical stress can cooperatively activate or induce expression of the antioxidant enzymes. The acid and alkaline pH stress have induced the gene expression of cMnSOD, CAT, GPx and TRx in the hepatopancreas of *L. vannamei* has been reported (Wang et al., 2009). The oxygen stress triggered the expression of cMnSOD and CAT in *L. vannamei* and found that the hypoxic conditions down regulated the genes compared to the normoxic conditions (Zhang et al., 2013). Moreover, the study carried out by Moser et al. (2012) also found the decrease in gene expression of SOD and CAT on exposure to white spot syndrome virus (WSSV) in *L. vannamei*. Results from these earlier studies corroborate with our findings of down regulation of Mn SOD, CAT on initial days of exposure to CPF and DMT in PL and JLs (GL, HL and ML). SL dose of CPF and DMT also caused the down regulation of GPx gene as exposure period prolonged in PL and JL (GL, HL and ML) i.e., either on 14 and 21 DoE or both. Furthermore, the GL were affected more as compared to the HL and ML in our present study, plausibly this could be since GL are in direct and continuous exposure to the pesticide. Thus, pesticides induced the higher levels of gene expression in GLs compared to the HL and ML. Earlier study carried out by Jing et al. (2017) had showed that the gene expression in GL was induced early as compared to liver in the mudskipper (*Boleopthalmus pectinirostris*) when exposed to the different level of lead concentrations. This observation agrees with our results of a higher level of antioxidant enzyme gene expression in GL compared to HL and ML cells.

In our previous chapters we have studied effect of SL dose of both pesticides on DNA damage. When we compared between the DNA damage, enzyme activity and gene expression of SOD, CAT and GPx, it was observed that as the DNA damages increased the enzyme activity and gene expression of SOD and GPx was down-regulated. Hence, we can infer the positive co-relation of DNA damage and antioxidant enzyme activity and their gene expression. Similar observation was also reported by Wang et al. (2009), who explained the DNA damage induced the active expression of CAT and GPx in *L. vannamei* on exposure to acute pH. It has been observed that penaeid prawns are generally more sensitive than fish and molluscs to most pesticides. They have been proposed as indicators of estuarine health due to their worldwide

distribution (García-de la Parra et al., 2006). Earlier studies have also shown that pesticide exposures are responsible for the oxidative stress in crustaceans (Pandey et al., 2001; Machado and Fanta 2003; Bianchini and Monserrat, 2007; Li et al., 2007; Simsek-Koprucu et al., 2008). Antioxidant enzyme systems have been investigated in aquatic organisms to find biochemical biomarkers that could be used in environmental monitoring systems (Farombi et al., 2008; Peric et al., 2017). Alterations found in the activity of antioxidant enzymes of *L. vannamei* (CAT, GPx, and GST) in this study after exposure to two sublethal concentrations of chlorpyrifos, suggest that these changes could be their adaptive responses to ROS.

Our results also suggest that the expression of these genes can serve as the biomarker for stress especially pesticide pollution. Among these genes, GPx displayed a high sensitivity than SOD and CAT in PL and JL on exposure to CPF and DMT. From our study, GPx seems one of the antioxidant enzymes involved in pesticide stress response, as it was noticeable expressed high in PLs and GL, HL and ML of JL. The glutathione s-transferase has been suggested to use a biomarker gene in *L. vannamei* on thermal and acidic pH stress (Zhou et al., 2010). Previously it was reported that MnSOD gene is low abundant in whiteleg shrimp but gets expressed significantly on pH and thermal stress (Wang et al., 2009; Zhou et al., 2010). Our study also showed the expression of the SOD gene on exposure to SL dose of CPF and DMT. The expression of these genes indicates that pesticide exposure has the potential to induce considerable oxidative stress in *L. vannamei*.

5.5 Conclusion

In the present investigation, noticeable changes were observed in various antioxidant enzyme activity in both the PL and JL stages of *L. vannamei* on exposure to SL concentrations of the pesticides, CPF and DMT. The activity of antioxidant enzymes were in considerably higher levels in shrimps exposed to SL dose of CPF compared to those exposed to DMT. Thus, being highly toxic, CPF has higher potential than DMT to induce enzyme activity in PL and JL. Further, both the pesticides, *viz.* CPF and DMT induced alterations in level of the expression of the antioxidant enzyme genes in both the PL and JL stages of *L. vannamei*. Amongst the two pesticides studied, *viz.* CPF and DMT, CPF has induced a higher level of gene expression in PLs and GL, HL and ML of JL. High oxidative stress noted in the PL could affect negatively the process of their development which in turn will further decrease their growth rate and drastically increase the time taken for reaching adult stage the growth development of *L. vannamei* life cycle.

Oxidative stress as revealed by changes in antioxidant enzymes activities and their gene expression in PL of *L. vannamei* were prominent in pesticide exposed shrimps and may cause damage which may lead to further interference in their development stages. In the case of *L. vannamei* JL, where GL, ML tissue and HL were studied, it has been observed that GL tissues showed remarkable changes in the SOD, CAT and GPx activity and related gene expression in comparison to the HL and ML tissue. The GL showed higher oxidative stress in *L. vannamei*, which may be due to the fact that the GL are continuously getting exposed to rearing medium and assist in respiration. The second higher activities were observed in the HL as it receives toxicant through the circulatory system. ML tissue is an important edible part of shrimp and it has been observed that pesticide induced oxidative stress in shrimps leads to cell death and eventually results in shrimps of poor nutritional quality. Therefore, this study enlightens the oxidative stress caused by exposure of SL concentrations of two organophosphorus pesticides which can be used for monitoring pesticide pollution in the farming systems particularly for penaeid shrimps. Due to lack of enough detoxification system in shrimps the pesticide exposed shrimps exhibited reduced enzyme activity and also suppressed expression of related genes at a certain critical period of pesticide exposure.

Summary

The present study focused on understanding of the toxic side effects of two Organophosphorus (OP) pesticide on two life stages of *L. vannamei viz.*, post larvae (PL) and Juveniles (JL)in terms of acute toxicity, genotoxicity and oxidative stress indices. This study revealed the highly toxic potential of these two commonly used OP pesticides *viz.* Chlorpyrifos (CPF) and Dimethoate (DMT). Chronic exposure to these pesticides created oxidative stress which was assessed and quantified by antioxidant enzyme activity of CAT, SOD and GPx in post larvae and different cells of juveniles (Gills (GL), haemolymph (HL) and muscles (ML)). The oxidative stress created due to pesticide can lead to DNA damage this can be related to the higher level of DNA damage observed in study animal. It also indicates the lack of efficient defence system in shrimp on exposure to pesticide as no DNA repair occurred during exposure period. This study recommends carrying out biomonitoring practices at regular basis at shrimp culture ponds to assess and quantify the status of pollution of ponds by pesticides.

Acute toxicity studies:

In acute toxicity studies, it has been observed that OP found highly toxic nature as reflected in low dose of median lethal concentration (LC_{50}) of both the studied pesticides. In comparison of both the pesticides the CPF has proved to be more toxic than DMT to *L. vannamei*. Further, it has also seen that larval stage is highly sensitive in comparison to juvenile stage to both the pesticides.

Considering the importance of salinity and temperature regime during the culture period of shrimps, the effect of salinity and temperature on the acute toxicity of CPF and DMT were studied. Among the three salinities *viz.* 5, 15 and 25 ppt studied in this investigation it was observed that the CPF and DMT showed highly toxic nature at 5 and 15 ppt compared to the 25 ppt. Further, in temperature based acute toxicity study it was observed that CPF and DMT were more toxic at 15 and 34 ºC compared to 25 ºC. These observations are alike in nature in both the PL and JL. This study also suggests that salinity 25 ppt and temperature 25 ºC could be used as ideal condition for shrimp culture in general and specifically to *L. vannamei*.

Genotoxicity:

Evaluation of DNA damage has become one of the rapid and reliable tools for assessing the genotoxic potential of pollutants. In the present study, the genotoxic potential of CPF and DMT were evaluated by employing comet assay. Chronic exposure of sublethal doses of CPF

and DMT pesticides resulted in a significant increase in DNA damage in *L. vannamei* PL and different cells of JLs during 21 days of exposure period. DNA damage observed to be increased against control at 3, 7, 14 and 21 DoE in pesticide exposed PLs and different cells of JL. Time-dependent as well as dose-dependent increment in DNA damage observed. Higher DNA damage in PL at a chronic level predicts further risk during development. Therefore, the present study can be employed to infer the possible consequences of pesticide exposure in early developmental stage.

The DNA damage was observed to be highly tissue specific. Further, a dose-dependent as well as time-dependent increase in DNA damage observed in JL. The GLs were more affected than HL and ML cells. Thus, resulted in higher DNA damage in GL cells compared to HL and ML cells at all time intervals. The higher DNA damage in GL cells could be related to the continuous exposure of gills to the pesticide. The results showed that the sublethal doses of CPF and DMT have great potential to cause DNA damage in both life stages. It was also observed that sublethal doses of CPF has higher potential to cause DNA damage than sublethal doses of DMT. Further, comet assay found to be a very rapid and sensitive tool to identify the DNA damage.

Antioxidant enzyme activity and their gene expression:

Both the pesticides induced considerable changes in the activity of all the antioxidant enzymes studied *viz.* SOD, CAT and GPx. Higher changes in enzyme activities changes were noticed on exposure to CPF compared to the DMT in PL and JL. During the present study, it was observed that SOD activity increased initially which then decreased and later again increased again by 21 DoE. CAT activity gradually increased throughout the exposure. However, the GPx activity was observed to be decreased over the period of exposure. Thus, a differential response of these enzymes was observed on exposure to both pesticides in PL and JL. In JL, GL were more affected than their HL and ML tissues. This could be due to direct exposure of gills to the pesticides. The induction of DNA damage and the reduction of enzyme activity can be correlated with the generation of reactive oxygen species, which may lead to the higher cellular damages and low antioxidant enzyme activity.

Significant fold changes in the expression of SOD, CAT and GPx genes were observed in PL and GL, HL and ML of JL on exposure to CPF and DMT pesticides. The highest level of gene expression was recorded in GL compared to HL and ML tissue on exposure to both the

pesticides. Further, CPF induced higher level of expression of antioxidant enzyme genes compared to those of DMT. The GL were highly affected in JL compared to their HL and ML. However, all genes were expressed at lower level in ML compared to GL and HL. Hence, the GL could be used as target organ to study the effect of aquatic pollution. Further, *L. vannamei* species can be used as a potential test animal among the diverse members of penaeid shrimp family for toxicity study and biomonitoring of aquatic pollution.

List of Abbreviations

***L. vannamei*:** *Litopenaeus vannamei*

PL: Post larvae

JL: Juvenile

FRP: Fibreglass Reinforced Plastic

DoE: Days of Exposure

L: Litre

OP: Organophosphorus pesticide

OC: Organochlorine

CPF: Chlorpyrifos

DMT: Dimethoate

SL: Sublethal

SOD: Superoxide dismutase

CAT: Catalase

GPx: Glutathione peroxidise

GL: Gill

HL: Haemolymph

ML: Muscle

LOEC: Low observed effective concentration

NOEC: No observed effective concentration

ppt: Parts per thousand

ppm: Parts per million

DDW: Double distilled water

h: Hour

g: Gram

M: Molarity

mM: Millimolar

BSA: Bovin Serum Album

N: Normality

ml: Millilitre

Mol. Wt.: Molecular weight

Conc.: Concentration

EDTA: Ethylenediaminetetraacetic acid

DEPC: Diethyl pyrocarobonate

LMPA: Low melting point agarose

NMPA: Normal melting point agarose

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In vivo DNA damage in gill, haemolymph and muscle cells of whiteleg shrimp *Litopenaeus vannamei* on exposure to organophosphorus pesticide

Ashwini P. Pawar[1,2], Sushant V. Sanaye[1], Soorambail Shyama[2], Rayadurga A. Sreepada[1,*], Jacky Bhagat[2], Praveen Kumar[2], Rakhee D. Sinai Khandeparker[1]

[1]Aquaculture Laboratory, Biological Oceanography Division, CSIR-National Institute of Oceanography, Goa 403 004, India
[2]Department of Zoology, Goa University, Taleigao Plateau, Goa 403 206, India

ABSTRACT: In response to growing worldwide market demand, intensive shrimp farming, particularly of whiteleg shrimp *Litopenaeus vannamei*, has expanded tremendously. The present study investigated induced DNA damage in gill (GL), haemolymph (HL) and muscle (ML) cells in juveniles of *L. vannamei* (length: 52.2 ± 6.4 mm; weight: 1.78 ± 0.5 g; mean ± SD) exposed to 2 sublethal (SL) concentrations, SL1 (0.36 µg l^{-1}) and SL2 (0.18 µg l^{-1}), of the organophosphorus pesticide chlorpyrifos (CPF) during 21 d of exposure (DoE). The magnitude of DNA damage (% tail DNA) as measured by the comet assay at specified intervals (3, 7, 14 and 21 DoE) was found to be tissue specific and time and dose dependent ($p < 0.05$). At the end of the experiment, at 21 DoE, % tail DNA damage was relatively higher at SL1 (53.61 ± 8.71, 49.36 ± 3.42 and 32.40 ± 4.97%) compared to SL2 (39.25 ± 3.90, 32.22 ± 4.21 and 22.66 ± 2.85%) in GL, HL and ML cells, respectively. No significant differences in water quality parameters were found among treated and control aquaria. The significant reduction in specific growth rates (% growth d^{-1}) observed in exposed shrimps indicated that SL concentrations of CPF negatively impacted growth in *L. vannamei* juveniles. A very low 96 h median lethal concentration (1.44 µg l^{-1}) indicated sensitivity of *L. vannamei* juveniles to CPF, suggesting that the species could be used as a bioindicator for assessing pesticide pollution. The study results highlight the implications of extending the farming of *L. vannamei* to low-salinity inland areas adjacent to traditional agricultural fields.

KEY WORDS: Chlorpyrifos · Organophosphorus pesticide · DNA damage · Comet assay · Whiteleg shrimp · *Litopenaeus vannamei*

1. INTRODUCTION

Recently, increased attention has been focused on studying the adverse effects of toxic commercial products on target and non-target food organisms (Matsumoto et al. 2006, Halappa & David 2009, Pavlaki et al. 2016, Butcherine et al. 2019). These products are often used to control pests in the household or in agricultural fields. As a consequence of non-target toxicity and accumulation in the environment, many previously registered pesticide products either are banned or their use has been restricted (Ullah et al. 2016). Hence, the production and usage of permitted second-line pesticides have increased abruptly in recent years.

Among the major organophosphorus pesticides (OPs), chlorpyrifos (CPF) is one of the most widely used pesticides to control insects in agriculture and

*Corresponding author: sreepada@nio.org

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horticulture crops such as cotton, rice, pasture and vegetables in India (Rao et al. 2003, Bhardwaj & Sharma 2013, Das & Adhya 2015). CPF is a broad-spectrum chlorinated OP that affects the nervous system of an organism. In spite of obvious advantages such as low persistence and rapid biodegradation in the aquatic environment, one major concern has been its effect on non-target wildlife populations (Frasco et al. 2006, Kumar et al. 2010, 2017). The entry of CPF into natural water bodies has been observed to cause deleterious effects including DNA damage in economically important non-target organisms (Chandrasekara & Pathiratne 2007).

The penaeid shrimps are economically and nutritionally important shrimp species. In recent years, world shrimp production, particularly the farming of whiteleg shrimp *Litopenaeus vannamei* (Boone, 1931), has increased tremendously (Kumar & Engle 2016) and contributed 53% to total shrimp and prawn production in 2016 (FAO 2018). Its remarkable ability to grow and survive in a wide range of salinities (1 to 50 psu) has made *L. vannamei* a choice shrimp species for aquaculture (Roy et al. 2010). The extension of farming marine shrimps to inland low-salinity areas often adjacent to agriculture fields increases the potential risk of pesticide toxicity to the farmed *L. vannamei* due to runoff and/or spray drift (Roque et al. 2005).

Owing to growing concern about the harmful effects of genotoxicants and xenobiotic compounds in the aquatic environment, the development of sensitive biomarkers has gained importance (Hayashi et al. 1998, Zeid & Khalil 2014). Evaluation of DNA damage has become one of the rapid and reliable tools for assessing the genotoxic potential of pollutants. Due to obvious advantages such as rapid detection and sensitivity in detecting minute DNA damage, the single cell gel electrophoresis or comet assay has become a widely used genetic tool (Klobučar et al. 2003, Frías-Espericueta et al. 2011, Araldi et al. 2015). Very few studies have examined the acute toxicity of different pesticides in penaeid shrimps to date (Galindo-Reyes et al. 1996, 2002, Labrie et al. 2003, Suryavanshi et al. 2009, Mello et al. 2011, Eamkamon et al. 2012, Thi Tu et al. 2012). Unfortunately, long-term *in vivo* studies assessing of the genotoxic effect of OPs in penaeid shrimps, particularly *L. vannamei*, are scarce.

The present study was undertaken to gain a better understanding of genotoxic effects following chronic exposure (21 d) to a commercial-grade OP (containing CPF) in juveniles of *L. vannamei*. The outcome of this study is expected to elucidate the cell-specific sensitivity of *L. vannamei* towards the OP and its potential to inflict DNA damage, thus making *L. vannamei* an excellent bioindicator test species. Considering the very limited information on the *in vivo* chronic effects of pesticide exposure in cultured shrimp species, the results of the present study are expected to provide an overview of the long-term effects of SL concentrations of CPF, which could aid decision making for improving farm productivity, sustainability and profitability.

2. MATERIALS AND METHODS

2.1. Experimental shrimps and rearing conditions

Healthy post-larvae (PL14) (n = 3000; total length: 12.4 ± 0.5 mm, wet weight: 55 ± 5 mg) of the whiteleg shrimp *Litopenaeus vannamei*, produced from specific pathogen-free broodstock and negative to white spot syndrome virus as confirmed by PCR, were procured from a commercial shrimp hatchery (Skyline Aqua Hatchery, Kumta, Karnataka) and reared at the Aquaculture Laboratory, CSIR-National Institute of Oceanography, Goa, India. The seawater used in the rearing of PL and for pesticide exposure experiments was filtered through a 3-stage filtration system comprising rapid sand filtration, cartridge filtration (20 to 1 µm) and, finally, ultraviolet disinfection. The PL were reared in 800 l tanks for 4 wk under a photoperiod of 12 h light:12 h dark. During the rearing period, shrimps were fed ad libitum twice a day with commercial shrimp pellet feed (CP Aquaculture; proximate composition: 38–40% protein, 5% lipid and 3% fibre). Excreta, uneaten feed and sloughed exoskeletons were removed by siphoning every day. Water quality measurements (temperature, dissolved oxygen [DO], salinity and pH) were taken daily with a portable water quality meter (CyberScan Series 600, Eutech Instruments). All other water quality parameters were analysed following methods as described in APHA (1992). The measured water quality parameters were within the recommended optimum range for rearing of *L. vannamei* (temperature: 28.5 ± 0.5°C, salinity: 30 ± 0.5 g l^{-1}, DO: 6.1 ± 0.4 mg l^{-1}, pH: 8.2 ± 0.2, NO_2-N: < 0.02 mg l^{-1} and NH_3/NH_4: 0 mg l^{-1}).

2.2. Chemicals

The commercial-grade OP Pyriban (AIMCO Pesticides), containing CPF (effective concentration: 20% w/w) as an active ingredient, was used in this study.

The pesticide, which is in liquid form, was diluted with deionised water to prepare a stock solution of active CPF (2 mg l^{-1}). All other chemicals used in the comet assay were of molecular grade (Sigma-Aldrich).

2.3. Acute toxicity experiment

Four-week-old laboratory-reared active and healthy juveniles of uniform size (total length: 52.2 ± 6.4 mm, wet weight: 1.78 ± 0.5 g) without any stress signs (colourless abdomen) or visual symptoms of disease were selected for acute toxicity experiments. At the start of the experiment, the shrimp were fasted for 24 h. Five different nominal exposure concentrations of CPF (0.4, 0.8, 1.2, 1.6 and 2 µg l^{-1}) along with a separate control (without pesticide) in triplicate were used for the acute toxicity experiment. Shrimp juveniles were exposed in 21 l glass aquaria containing 10 l seawater with a stocking density of 10 juveniles per aquarium. Water quality parameters such as temperature, DO and pH were recorded daily, whereas salinity, NO_2-N and NH_3/NH_4 were measured prior to and at the end of the experimental period (temperature: 28.4 ± 0.2°C, salinity: 30 ± 0.5 g l^{-1}, DO: 5.8 ± 0.6 mg l^{-1}, pH: 8.1 ± 0.4, NO_2-N: <0.02 mg l^{-1} and NH_3/NH_4: 0 mg l^{-1}). Mortality was recorded during the 96 h exposure period according to the time schedule at 24, 48, 72 and 96 h. Dead organisms, if any, were removed immediately upon detection to avoid any type of bacterial contamination. The criteria for proof of mortality were total lack of body movement and immobility of heart and scaphognathite after repeated prodding with a probe (Lignot et al. 1998).

Cumulative mortality rates were calculated using the formula as defined by Abbott (1925). Median lethal concentrations (LC_{50}) and their 95% confidence limits for CPF for different exposure periods (24, 48, 72 and 96 h) were calculated with a computer-based program described by Finney (1971). A lowest observed effect concentration (LOEC) was determined as the lowest concentration that had statistically significant mortality. A no observable effect concentration (NOEC) was determined as the highest concentration that had no statistically significant mortality.

2.4. *In vivo* chronic exposure experiment

For understanding the behavioural and physiological changes in animals on exposure to pesticides, sublethal (SL) concentrations offer an excellent scope (Edwards 1973). In toxicological studies, chronic tests of shorter duration (~21 d) have been recommended as an alternative to longer chronic tests (Bhavan & Geraldine 2000, Suryavanshi et al. 2009). Based on 96 h LC_{50} values (1.44 µg l^{-1}; 95% confidence limits: upper = 1.47 µg l^{-1} and lower = 0.86 µg l^{-1}; $p < 0.05$), 2 SL concentrations, SL1 (0.36 µg l^{-1}) and SL2 (0.18 µg l^{-1}), equivalent to one-quarter and one-eighth of the 96 h LC_{50} value (nominal concentration), respectively, computed from a commercial-grade CPF, were selected for the *in vivo* chronic exposure experiment. The selected SL concentrations were lower than LOEC and NOEC values. The static renewal method for toxicity tests with separate control tanks was followed (Buikema et al. 1982).

For the chronic exposure experiment, 300 intermoult juveniles of uniform size (length: 52.2 ± 6.4 mm, wet weight: 1.78 ± 0.5 g) were divided into 3 groups, each comprising of 100 juveniles. Of the 3 groups, 1 group formed the control, while the 2 remaining groups were exposed to 2 SL concentrations of CPF for a period of 21 d of exposure (DoE). To maintain a constant concentration of CPF in the test solutions, the entire toxic medium in each aquarium tank was gently siphoned out daily (10:00 h) and renewed with a freshly prepared solution of respective SL concentrations of CPF. Care was taken that the disturbance caused to the shrimps was minimal. Before each renewal, tanks were thoroughly washed to ensure that no traces of the preceding day's pesticide remained present in the treatment tanks.

Two replicate aquaria (all-glass aquaria, 100 l capacity) containing 50 l seawater were maintained for each SL concentration and the control group (50 shrimps $replicate^{-1}$). Samples for the comet assay were collected after 3, 7, 14 and 21 DoE. On the designated sampling occasion, 10 juvenile shrimps from each concentration (5 from each replicate tank) were randomly selected and processed for the comet assay. Shrimps from the control group were similarly sampled at the same time as treated ones. During the exposure period, mortality in control and exposure tanks was minimal (<5%). To maintain the same stocking density throughout the experiment, dead juveniles, if any, were replaced with similar-sized shrimps reared separately in the same medium and which had been exposed to the same DoE. During the chronic exposure experiment, juveniles were fed at a rate of 4–6% of body weight split across three different feed times (10:00, 15:00 and 20:00 h). Mean body weight of juvenile *L. vannamei* recorded on every sampling occasion was used to determine the

specific growth rate, SGR (% d^{-1}), over the exposure period of 21 DoE from the formula SGR = [(ln W_2 − ln W_1) (t_2 − t_1)] × 100, where W_1 and W_2 represent the initial and final body weight of *L. vannamei* juveniles at time points t_1 (start of experiment) and t_2 (time of sampling), respectively. During the chronic exposure experiment, water quality parameters, temperature, DO and pH were measured daily, whereas measurements of salinity, NO_2-N and NH_3/NH_4 were done weekly following the methods as described in the previous subsection. Before each usage, probes were thoroughly rinsed with deionised water to remove traces of pesticide adhering to the probes, if any.

2.5. Sample collection from shrimps

Samples of gill (GL), muscle (ML) and haemolymph (HL) cells were pooled from 10 randomly selected shrimps (5 from each replicate tank) from treated and control groups for the comet assay. Approximately 350 µl of haemolymph was drawn from the rostral sinus region of control and CPF-exposed shrimps using a sterilised hypodermic syringe after 3, 7, 14 and 21 DoE. GL and ML tissues (~50 mg) were also collected from control and exposed shrimps. GL and ML tissues were washed twice with chilled phosphate-buffered saline (PBS, calcium and magnesium free) and transferred to chilled homogenisation buffer (1X Hanks' Balanced Salt Solution, 20 mM EDTA, 10% DMSO, pH 7.0–7.5). The tissue was homogenised with a Potter-Elvehjem homogeniser to obtain a single cell suspension. The homogenate was centrifuged at 3000 rpm at 4°C for 5 min, and the pellet was suspended in chilled PBS (Dhawan et al. 2008). Sample extraction was carried out under dim light, and samples were transferred immediately to a microcentrifuge tube placed on an ice pack to prevent endogenous DNA damage occurring during sample preparation and also to inhibit DNA repair in the unfixed cells (Siu et al. 2004).

2.6. Cell viability assay

Prior to commencement of the comet assay, cell count and cell viability checks were performed using a trypan blue dye exclusion test for ensuring adequate living cells in the cell suspension. Cell suspensions exhibiting >90% viability and a cell count of a minimum of 10^6 cells ml^{-1} were used for the comet assay.

2.7. *In vitro* exposure to hydrogen peroxide

A standard genotoxin, hydrogen peroxide (H_2O_2), was used to validate the results obtained by the comet assay and the response of different cells as described by Bhagat et al. (2016). Freshly detached GL, ML and HL cells from juveniles were treated with different concentrations of H_2O_2 (0.5, 5, 20 and 40 µM) prepared in PBS for 30 min in dark conditions at room temperature (28°C). Control samples were incubated in PBS without H_2O_2. Three replicates per concentration were selected for the comet assay.

2.8. Comet assay (single cell gel electrophoresis)

The comet assay was performed as a 3-layer procedure (Singh et al. 1988) using conventional microscopic slides with slight modification. The unwinding and electrophoresis process was optimised before actual analysis of exposed shrimp samples. Slides for analysis were first cleaned with 100% ethanol, flame dried and then coated with a first layer of 200 µl normal agarose (1%). Simultaneously, 15 µl of cell suspension was mixed with 85 µl of 0.5% low melting point agarose and coated on the first layer. Finally, the slides were covered with a third layer of 100 µl low melting point agarose. After solidification of the gel, the slides were immersed in lysing solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris pH 10 with 10% DMSO and 1% Triton X-100 added fresh) for 3 h at 4°C. Subsequently, the slides were placed in a horizontal gel electrophoresis unit immersed in fresh cold alkaline electrophoresis buffer (300 mM NaOH, 1 mM EDTA, pH >13) for DNA unwinding for 25 min. Electrophoresis was carried out for 20 min, using 20 V and 300 mA electric current. The slides were neutralised while immersing in neutralising buffer (0.4 M Tris-HCl, pH 7.5) for 10 min to remove excess alkali and rinsed with distilled water. Finally, the slides were stained with ethidium bromide (15 µg l^{-1}) for 5 min for visualisation of DNA damage.

Slides were observed under a fluorescence microscope (Olympus BX51) at 100× magnification fitted with appropriate filters. Duplicate slides per test concentration per cell type (treated groups) and control group were prepared on each sampling occasion. A total of 100 randomly selected cells observing several fields of the slide were scored for each cell type (50 cells from each of the 2 replicated slides). The comet images were captured using ImagePro AMS 6.0 and analysed by comet assay scoring software (Casp_1.2.3 beta). The comet parameter (viz. % tail

DNA determined by the software) was used for the quantification of DNA damage.

2.9. Statistical analysis

Data obtained from the experiment are expressed as mean ± SD. The % DNA damage in different treatment groups and tissues was assessed by ANOVA (Underwood 1997) with DoE and SL concentration as sources of variation. Variation in water quality parameters and SGR (% d^{-1}) between treated and control aquaria was assessed by ANOVA. Appropriate transformations were applied before subjecting the data to ANOVA. If ANOVA results were found significant, multiple comparisons between different means of % DNA damage in control and treated shrimps were then made by Tukey-Kramer's test of highly significant differences (Zar 1996). Statistical analysis was performed by using computer-based GraphPad Prism 5.0 software (GraphPad Software). Four levels of significance (ns: not significant, $p < 0.05$, $p < 0.01$ and $p < 0.001$) were reported.

3. RESULTS

3.1. Acute toxicity study

No mortality was observed in the control tanks during the 96 h duration of the test, which indicates that the test conditions were appropriate and, thus, mortality recorded in the test solutions could have been induced by the pesticide. Mortality in the treatment tanks increased with the progress in exposure time and increase in concentration of CPF. The LC_{50} values of CPF for juveniles of *Litopenaeus vannamei* were 2.18, 1.98, 1.80 and 1.44 µg l^{-1} at the end of exposure periods of 24, 48, 72 and 96 h, respectively (Table 1). LC_{50} values decreased with exposure time, and a direct correlation between mortality and toxicant concentration was discernible. The 96 h LC_{50} concentration of CPF to *L. vannamei* juveniles was determined to be 1.44 µg l^{-1} (95% confidence limits: upper = 1.47 and lower = 0.86 µg l^{-1}; $p < 0.05$). Observed values of LOEC and NOEC were 1.20 and 0.80 µg l^{-1}, respectively.

3.2. Physico-chemical and growth parameters

No visible signs of disease or significant mortality were observed in *L. vannamei* juveniles on exposure to 2 SL concentrations of CPF during 21 DoE. No significant variations in water quality parameters were observed in control and treated tanks (Table 2) during the entire experimental routine of 21 DoE ($p > 0.05$). It was noticeable that juveniles in treated tanks were found to consume an adequate amount of ration (visual observation of gut fullness) provided during the initial DoE. Beyond 14 DoE, treated shrimps failed to consume a normal amount of food towards the end of experiment, whereas shrimps in control tanks consumed a normal ration of food. Specific growth rates (% d^{-1}) in *L. vannamei* juveniles at the end of 21 DoE in control and treated groups (SL1 and SL2) differed significantly (Table 3; $p < 0.05$).

Table 1. Median lethal concentrations (LC_{50}) of chlorpyrifos to juveniles of *Litopenaeus vannamei* tested during different exposure periods

Exposure period (h)	LC_{50} (µg l^{-1})	95% confidence limits (µg l^{-1})	
		Lower limit	Upper limit
24	2.18	1.98	2.63
48	1.98	1.78	2.36
72	1.80	1.40	1.93
96	1.44	0.86	1.47

Table 2. Water quality parameters (mean ± SD) recorded in treatment and control aquaria with juveniles of *Litopenaeus vannamei* during chronic exposure experiment (21 d of exposure) with chlorpyrifos. SL1 (2): sublethal concentration 0.36 µg l^{-1} (0.18 µg l^{-1})

Parameter	Control	SL1	SL2
Temperature (°C)	28.68 ± 0.30	28.36 ± 0.37	28.45 ± 0.38
Dissolved oxygen (mg l^{-1})	5.98 ± 0.20	5.91 ± 0.22	5.88 ± 0.15
pH	7.91 ± 0.20	7.71 ± 0.10	7.65 ± 0.12
Salinity (g l^{-1})	30 ± 0.4	30 ± 0.3	30 ± 0.3
NO_2-N (mg l^{-1})	0.02 ± 0.003	0.02 ± 0.004	0.02 ± 0.003

Table 3. Growth (mean ± SD) of juveniles of *Litopenaeus vannamei* recorded in treatment and control aquaria during chronic exposure experiment with chlorpyrifos. DoE: days of exposure. SL1 (2) as in Table 2

Growth parameter	Control	SL1	SL2
Initial weight: 0 DoE (g)	1.79 ± 0.04	1.79 ± 0.056	1.79 ± 0.06
Final weight: 21 DoE (g)	4.10 ± 0.05	3.75 ± 0.045	3.82 ± 0.05
Specific growth rate (% d^{-1})	1.72 ± 0.32	1.53 ± 0.40	1.57 ± 0.38

3.3. *In vitro* exposure to hydrogen peroxide

The % tail DNA damage in GL, ML and HL cells on exposure to different concentrations of H_2O_2 is shown in Fig. 1. Results of the comet assay showed significant DNA damage in all studied cells vis-a-vis the control ($p < 0.05$), thereby validating the response of different cells of *L. vannamei* to the genotoxin and comet assay procedure employed during the present study.

3.4. Comparative evaluation of genotoxicity

Results of ANOVA followed by Tukey-Kramer's multiple comparison test revealed that % tail DNA damage differed significantly between control and treated groups at all time intervals ($p < 0.001$). The differential response in % tail DNA damage in GL, HL and ML cells following exposure to 2 SL concentrations of CPF vs. control was discernible (Fig. 2). A time- and dose-dependent increment in % tail DNA damage was observed in GL, HL and ML cells (Fig. 2). At the end of 21 DoE, *L. vannamei* juveniles exposed to SL1 exhibited significantly higher % tail DNA damage (GL: 53.61 ± 0.71%, HL: 49.36 ± 3.42% and ML: 32.40 ± 4.97%) compared to those exposed to SL2 (GL: 39.25 ± 3.90%, HL: 32.22 ± 4.21% and ML: 22.66 ± 2.85%) ($p < 0.001$). The levels of DNA damage in different cells measured at all time intervals followed the order GL > HL > ML. GL cells of *L. vannamei* exposed to SL1 and SL2 doses exhibited a significantly higher level of % tail DNA damage (Fig. 2a) when compared to the control group at all time intervals ($p < 0.001$). The % tail DNA damage in HL (Fig. 2b) and ML (Fig. 2c) cells also followed a similar trend.

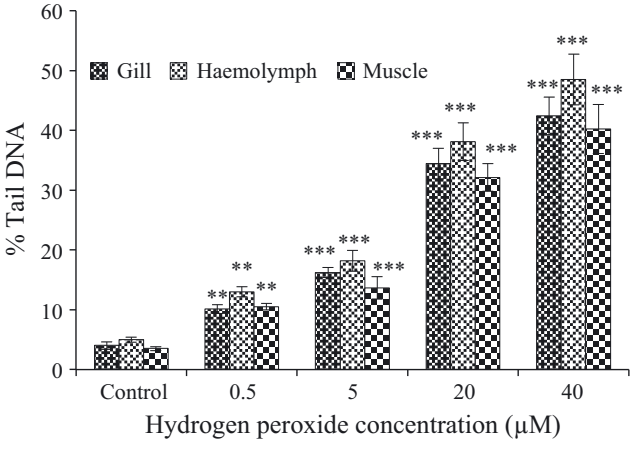

Fig. 1. Percent DNA damage in different cells of *Litopenaeus vannamei* juveniles exposed to different concentrations of hydrogen peroxide. Data are mean ± SD (n = 100 comets). $^{**}p < 0.01$, $^{***}p < 0.001$

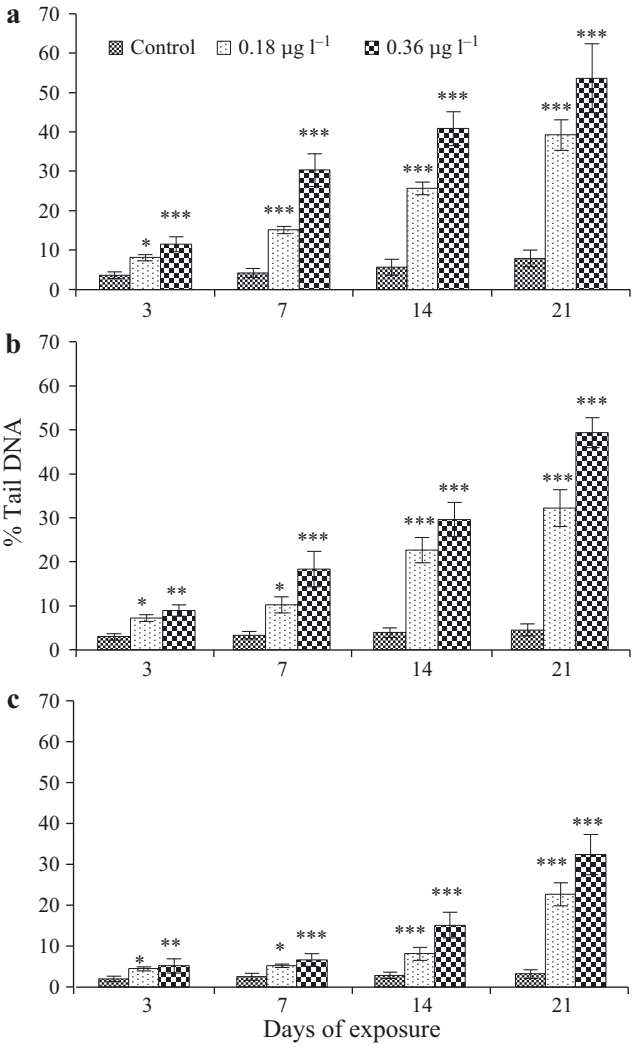

Fig. 2. Percent tail DNA in different cells of *Litopenaeus vannamei* juveniles exposed to 2 sublethal concentrations (SL1 = 0.36 μg l^{-1}, SL2 = 0.18 μg l^{-1}) of chlorpyrifos at the end of 3, 7, 14 and 21 d of exposure in (a) gill cells, (b) haemolymph and (c) muscle cells. Data are mean ± SD (n = 100 comets). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$

A comparative evaluation of DNA damage in GL, HL and ML cells exposed to 2 SL concentrations revealed striking differences. A highly significant difference in measured levels of % tail DNA damage in GL, HL and ML cells (Fig. 3) of juveniles exposed to the SL1 concentration on all DoE ($p < 0.001$) was noticeable. On the other hand, % tail DNA damage in GL and HL cells (Fig. 4a) of juveniles exposed to the SL2 concentration also showed a significant difference ($p < 0.01$) until 14 DoE. The difference in the levels of DNA damage beyond 14 DoE, however, was insignificant ($p > 0.01$). Comparative assessment of the levels of % tail DNA damage in GL, ML and HL cells (Fig. 4) of juveniles exposed to the SL2 concentration

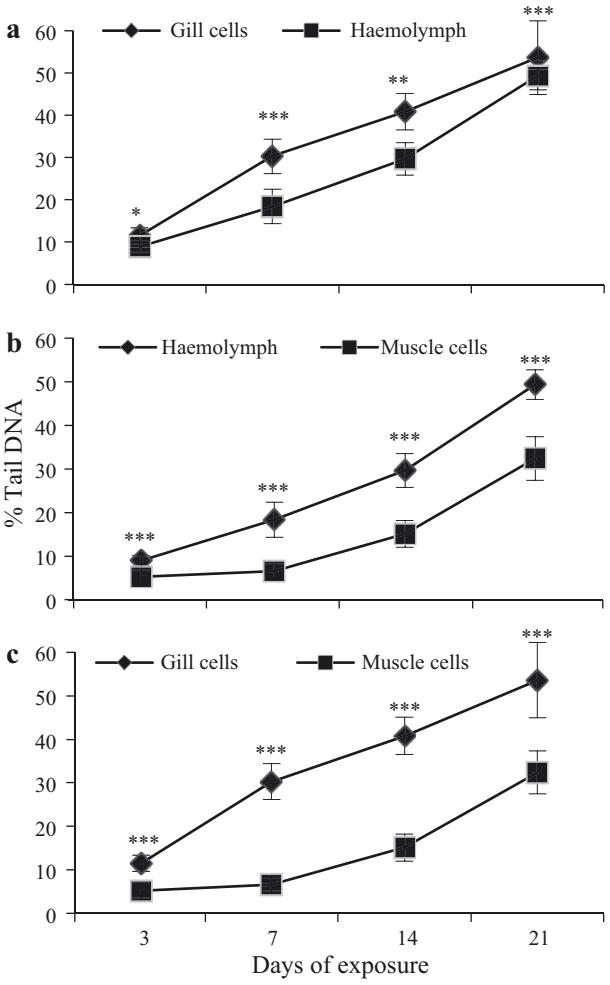

Fig. 3. Comparison of % DNA damage induced by sublethal concentration SL1 (0.36 µg l^{-1}) of chlorpyrifos in gill, haemolymph, and muscle cells of *Litopenaeus vannamei* juveniles at the end of 3, 7, 14 and 21 days of exposure. (a) Gill vs. haemolymph, (b) haemolymph vs. muscle, (c) gill vs. muscle. Data are mean ± SD. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$

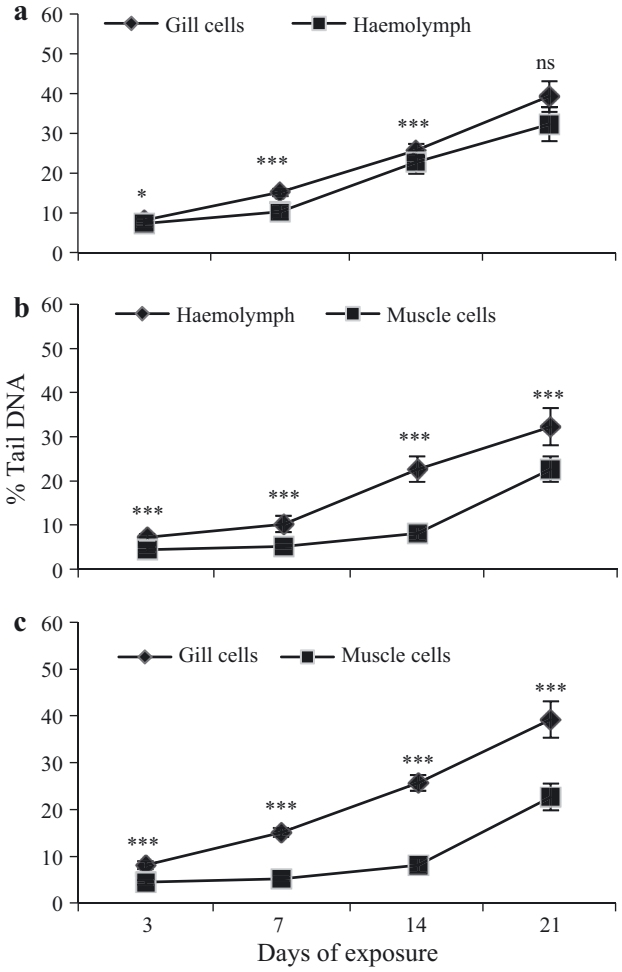

Fig. 4. Comparison of % DNA damage induced by sublethal concentration SL2 (0.18 µg l^{-1}) of chlorpyrifos in gill, haemolymph, and muscle cells of *Litopenaeus vannamei* juveniles at the end of 3, 7, 14 and 21 days of exposure. (a) Gill vs. haemolymph, (b) haemolymph vs. muscle, (c) gill vs. muscle. Data are mean ± SD. ns: not significant, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$

showed a significant difference on all DoE ($p < 0.001$). Overall, of the 3 cell types evaluated in the present study, the cells from GL showed a significantly higher degree of DNA damage than those retrieved from HL and ML at both tested SL concentrations ($p < 0.001$).

4. DISCUSSION

4.1. Acute toxicity

Although OPs have replaced organochlorine compound pesticides in agricultural activities because of their relative non-persistence, their residues have been detected in a variety of media such as water, marine organisms, plants and plant products, fish tissues, sediments and prawns and prawn pond water (Barceló et al. 1990, Varó et al. 1998, Amaraneni & Pillai 2001, Kumar et al. 2004, Amaraneni 2006, Burgos-Hernández et al. 2006, Rao et al. 2007, Halappa & David 2009, Sultana et al. 2012, Hook et al. 2018). High variability in the laboratory-derived acute toxicity indices of different OPs in a number of aquatic organisms (molluscs, crustaceans and fishes) is discernible (Persoone et al. 1985, Baticados & Tendencia 1991, Serrano et al. 1995, Lignot et al. 1998, Rao et al. 2005, Roque et al. 2005, Rao 2006, Kunjamma et al. 2008, Halappa & David 2009, Samajdar & Mandal 2015). Such wide variability in the acute ecotoxicity indices for various non-target aquatic

organisms could be attributable to numerous factors, such as the formulation of the OPs, species, stage of development, environmental conditions and exposure period (Girón-Pérez et al. 2007).

The acute toxicity study (96 h LC_{50}) is an important tool to assess the toxic tolerance range of particular species to xenobiotic compounds. LC_{50} values of CPF to *Litopenaeus vannamei* juveniles observed in this study (2.18, 1.98, 1.80 and 1.44 µg l^{-1} at 24, 48, 72 and 96 h, respectively) are much lower than previously reported laboratory-derived acute toxicity indices of pesticides containing CPF for other fish and crustacean species, such as the freshwater fish *Channa punctatus* (811.98 µg l^{-1}) (Ali et al. 2009) and *Labeo bata* (106.94 µg l^{-1}) (Samajdar & Mandal 2015), freshwater shrimp *Paratya australiensis* (0.004 µg l^{-1}) (Kumar et al. 2010), penaeid shrimp *Penaeus monodon* (59.16 nmol l^{-1}) (Eamkamon et al. 2012) and freshwater crab *Barytelphusa guerini* (0.21 ppm) (Narra et al. 2013). During the present study, the results of acute toxicity testing indicated that CPF is highly toxic to juveniles of *L. vannamei*, as reflected in the very low 96 h LC_{50} value of 1.44 µg l^{-1}. A wide variability in acute toxicity indices on exposure of *L. vannamei* juveniles to 4 different organochlorine pesticides (chlordane: 63 µg l^{-1}, DDT: 8.7 µg l^{-1}, Lorsban: 4.8 µg l^{-1}, lindane: 3.9 µg l^{-1}) has been reported by Galindo-Reyes et al. (1996). A significantly lower acute toxicity index (9.33 ng l^{-1}) has also been reported in *L. vannamei* juveniles on exposure to a permethrin-based insecticide (Mello et al. 2011). Furthermore, the 48 h LC_{50} value of 19 mg l^{-1} and 96 h LC_{50} value of 1.46 mg l^{-1} have been reported with juveniles of *L. vannamei* following exposure to 2 OP pesticides, fenitrothion (Lignot et al. 1998) and methamidophos (García-de la Parra et al. 2006), respectively. On the basis of the observation made in the present study (96 h LC_{50}, 1.44 µg l^{-1}) and previously reported results (Galindo-Reyes et al. 1996, Ali et al. 2009, Kumar et al. 2010, Narra et al. 2013, Samajdar & Mandal 2015), it may be concluded that *L. vannamei* juveniles are highly sensitive to CPF pesticide toxicity.

4.2. Genotoxicity evaluation in gill, muscle and haemolymph cells

DNA damage study through the comet assay is advantageous as it does not require mitotically active cells (Buschini et al. 2003), which is essential for other cytogenetic methodologies (chromosomal aberration and micronucleus tests). Hence, it has been extensively employed in genotoxicity studies and environmental monitoring programs (Buschini et al. 2003, Lee & Steinert 2003, Frías-Espericueta et al. 2011, Dailianis et al. 2014, Bhagat et al. 2016, Ullah et al. 2016). Cells from haemolymph, embryo, gills and digestive glands from aquatic invertebrates such as oysters, mussels, clams and shrimps have been assessed for *in vivo* studies by employing the comet assay (Lee & Steinert 2003).

The % tail DNA damage in *L. vannamei* juveniles as assessed through the comet assay was highly tissue specific and dose and time dependent (Fig. 2). Few previous reports on DNA damage in *L. vannamei* and *L. rohita* are available for comparison (Frías-Espericueta et al. 2011, Mohanty et al. 2011). A dose-dependent increment in DNA damage in blood (30.34 ± 0.76%), gill (58.17 ± 0.65%) and liver (47.83 ± 0.30%) tissues of *L. rohita* after 96 h exposure of phorate has been reported by Mohanty et al. (2011). On the other hand, both a time- and dose-dependent increase in DNA damage in haemocytes of *L. vannamei* (40.08 ± 5.64%) following exposure to copper has been observed by Frías-Espericueta et al. (2011). A dose-dependent increment in DNA damage in gill cell lines of *P. monodon* exposed to 2 OPs (malathion: 40.81 ± 9.10% and monocrotophos: 38.85 ± 13.68%) has been reported by Jose et al. (2011). Similar observations of DNA damage concomitant with the increase in dose on exposure to monocrotophos has been reported in erythrocytes of the fish *Tilapia mossambicus* (Banu et al. 2001).

In the present study, a dose-dependent increment in % tail DNA damage was observed at both tested SL concentrations. At the end of 21 DoE, the recorded % tail DNA damage in all 3 tested tissue cells of *L. vannamei* exposed to the SL1 concentration (GL: 53.61 ± 8.71%, HL: 49.36 ± 3.42% and ML: 32.40 ± 4.97%) was significantly higher (Fig. 2; $p < 0.05$) compared to those exposed to the SL2 concentration (GL: 39.25 ± 3.90%, HL: 32.22 ± 4.21% and ML: 22.66 ± 2.85%) (Fig. 2). The greater magnitude of variability in DNA damage witnessed in different cells of *L. vannamei* on exposure to 2 SL concentrations of CPF could be attributed to the generation of a high amount of free radicals. The potential of CPF to induce oxidative stress in organisms and cause overproduction of reactive oxygen species has been documented by Livingstone (2001). The production of higher amounts of free radicals as a consequence of increased metabolic activities resulting in damage to protein and nucleic acids on exposure to OP compounds has been reported (Hodgson & Levi 1996, Mohanty et al. 2011, Bhagat et al. 2016). Therefore, a

dose-dependent increase in DNA damage observed in different cells of *L. vannamei* juveniles during the present study might be due to increased production of free radicals at the higher SL concentration.

Of the 3 cell types used to assess potential DNA damage, the cells from GL showed a higher degree of DNA damage than those retrieved from HL and ML irrespective of SL concentration (Figs. 3a,c & 4a,c). An increment in DNA damage in GL of *L. vannamei* juveniles with DoE was also observed. Such tissue-specific DNA damage observed in the present study is in congruence with the previous study by Ali et al. (2009), who reported that CPF inflicts more damage in gill cells than lymphocyte cells of the freshwater fish *C. punctatus*. In crustaceans, gills are important organs of respiration as well as osmoregulation (Péqueux 1995). Significant damage to osmoregulatory effectors, particularly gills, by OP contamination has been demonstrated by histopathological studies (Pawar & Katdare 1984, Baticados & Tendencia 1991). Higher DNA damage observed in GL cells might be due to the respiration activity of juvenile shrimps which allows the constant flow of pesticide water over gill surface (Dzwonkowska & Hübner 1986, Ali et al. 2009), whereas haemolymph receives the contaminant when chemicals enter into the circulatory system (Ali et al. 2009). Increased rate of respiration in juveniles of *L. vannamei* following SL exposure of 4 different organochlorine pesticides has been documented by Galindo-Reyes et al. (1996). In comparison with other cells, the gills are relatively more susceptible and sensitive to the injury caused by xenobiotic compounds due to the inefficient detoxification system in shrimps and prawns compared to fishes (Hayashi et al. 1998, Çava & Ergene-Gözükara 2003, 2005, Mohanty et al. 2011). Furthermore, an impairment of the gill structure and ultrastructure in juveniles of *P. monodon* after 96 h exposure to lethal concentrations of the OP pesticide Gusathion A has been documented by Baticados & Tendencia (1991). It has been hypothesised that the cytochrome P450-1A (a protein family involved in the biotransformation of organic chemicals) present in fish plays an important role in hydrocarbon metabolism and has greater significance in detoxification and protection against toxic injury caused by xenobiotic compounds (Haasch et al. 1989, Goksøyr et al. 1991, Galindo-Reyes et al. 2002, Koenig et al. 2012). In contrast, the same protein present in molluscs and crustaceans has a relatively lower detoxification capacity than it has in fishes (Livingstone et al. 1989, Livingstone 1991, James & Boyle 1998, Koenig et al. 2012). Furthermore, it must be emphasised that physico-chemical interactions between various types of organisms and rearing media may result in variable responses in different cells on exposure to xenobiotic compounds (Ali et al. 2009). A high variability in genotoxic response observed in different cells during the present study might be due to their differential ability in responding to CPF exposure.

In aquatic environments, the effects on structural and functional units caused by SL concentrations of xenobiotics are higher than those inducing mortality (Sancho et al. 2003). A study conducted by the US Environmental Protection Agency laboratory (EPA 1981) highlighted that the penaeid shrimps are more sensitive to pesticides compared to fishes or molluscs. Therefore, it appears that economically important non-target organisms, particularly shrimps, are relatively more sensitive to a number of xenobiotic compounds in their ecosystem (Krieger 2001). Furthermore, the presence of pesticides in rearing water weakens the shrimp immune system and causes an outbreak of infectious diseases at lower concentrations (Roque et al. 2005, Mello et al. 2011). A 27 to 53% shell softening in *P. monodon* juveniles during 96 h exposure to the OP pesticide Gusathion A has been reported by Baticados & Tendencia (1991). Furthermore, significantly higher mortality in *L. vannamei* juveniles after combined exposure to the OP insecticide methyparathion and to the bacterium *Vibrio parahaemolyticus* than to each stressor individually has been experimentally documented by Labrie et al. (2003).

Considering the sensitivity of shrimps to pesticides (due to their phylogenetic similarities with insects, particularly the nervous and muscular systems), the chronic exposure of pesticides to the biological units (DNA damage) could result in the production of poor nutritional and commercial quality product (Mello et al. 2011). This is corroborated by considerable DNA damage recorded during the present study, specifically in ML cells—the main edible body part of shrimps. A significant difference ($p < 0.05$) in SGR (% d^{-1}) recorded between control and treated groups (SL1 and SL2) of *L .vannamei* juveniles during 21 DoE supports the contention. A significantly marked decrement in protein content (28–42%) in juveniles of *L. vannamei* on exposure to 4 organochlorine pesticides has been reported by Galindo-Reyes et al. (1996). A progressive depletion in total protein content in gills, muscles, hepatopancreas and brain on exposure (28 DoE) to SL concentrations of CPF has been documented by Narra et al. (2013). Furthermore, significant alterations in major biochemical constituents in the penaeid shrimp *Metapenaeus*

monoceros on exposure to SL doses of endosulfan pesticide has also been reported (Suryavanshi et al. 2009). Therefore, it appears that the SL concentrations of CPF are capable of inflicting DNA damage, suppressing growth of shrimps and lowering the nutritional quality of commercially important shrimp species. The potential effect of pesticides in farming systems is expected to be much higher than laboratory-derived results due to water–sediment interactions, as Holmes et al. (2008) reported that the presence of sediment enhances the toxicity of pesticides

5. CONCLUSIONS

Results of the acute toxicity test (96 h LC_{50}) showed high toxic potential of CPF to juveniles of *Litopenaeus vannamei* and could serve as baseline data for the entry and high risk of such pesticides into aquaculture systems. The degree of DNA damage recorded in different cells (GL, ML and HL) implies that juveniles of *L. vannamei* are highly sensitive to genotoxic effects induced by OPs, particularly CPF. The results also point to the potential risks involved in extending *L. vannamei* farming to inland areas, where pesticides are routinely used. Standard toxicity bioassays may not be predictive of actual pesticide toxicity under variable environmental conditions, and hence testing under a wider range of exposure conditions could improve the accuracy of chemical risk assessments. Further studies assessing immune responses in different shrimp life stages and varying culture conditions on exposure to OPs would advance our current understanding of shrimp nutritional pathology. Such information would facilitate the development of diagnostic tools for better decision making in aquaculture management.

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Effects of salinity and temperature on the acute toxicity of the pesticides, dimethoate and chlorpyrifos in post-larvae and juveniles of the whiteleg shrimp

Ashwini Pandurang Pawar[a,b], Sushant Vilas Sanaye[a], Soorambail Shyama[b], Rayadurga Anantha Sreepada[a,*], Ajit Sharadrao Dake[a]

[a] Aquaculture Laboratory, Biological Oceanography Division, CSIR-National Institute of Oceanography, Goa, 403004, India
[b] Department of Zoology, Goa University, Taleigao Plateau, Goa, 403206, India

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ABSTRACT

In this article, the acute toxicity of the organophosphorus pesticides (OPs), dimethoate (DMT) and chlorpyrifos (CPF) in post-larvae, and juveniles of *Litopenaeus vannamei* at different ambient salinity (5, 15 and 25) and temperature (15, 25 and 34 °C) regimes were compared with standard exposure conditions (temperature, 27 °C and salinity, 20). Chlorpyrifos was significantly more toxic to both life stages ($P < 0.05$). Post-larvae were more sensitive than juveniles to both OPs in all altered salinities and temperatures ($P < 0.05$). Acute toxicity indices (96 h LC_{50} values) showed that lower salinities (5 and 15) and temperature (15 °C) induced significantly higher pesticide toxicity ($P < 0.05$) in both life stages. Very low acute toxicity indices of DMT and CPF at varying levels of salinity and temperature highlight the importance of monitoring OPs pollution in low-saline shrimp aquaculture ponds. Results of the present study provide baseline information for monitoring pesticide pollution in low-saline shrimp aquaculture ponds which are influenced by changes in climatic conditions.

1. Introduction

Organophosphorus pesticides (OPs) have largely replaced organochlorine compounds (OCs) for controlling insects in the intensive agriculture and horticulture because of their rapid degradability (Roque et al., 2005; Halappa and David, 2009). However, the effect of these rapidly degrading OPs on non-target organisms comparable OCs has been a major concern (Kumar et al., 2010; Joseph and Raj, 2011; Tu et al., 2012; Kumar et al., 2017). The primary mode of action of OPs is to inhibit the acetylcholinesterase enzyme which regulates the functioning of the nervous system (Díaz-Resendiz et al., 2015) leading to the hyperactivity and paralysis in the organisms, ultimately culminating into their death (Key and Fulton, 1993; Garcıa-de la Parra et al., 2006). Many reports describing the detrimental effects of OPs on biological and physiological parameters in a wide range of economically important non-target freshwater and marine organisms, and abundance of benthic invertebrates in shrimp ponds is slowly beginning to emerge (Rao et al., 2005; Garcıa-de la Parra et al., 2006; Eamkamon et al., 2012; Krishnapriya and Padmaja, 2014; Uddin et al., 2016; Hook et al., 2018; Pawar et al., 2019).

Recently, shrimp aquaculture has emerged as a fastest-growing food sector and has become an activity of economic importance in many countries of the world. Due to high demand in foreign markets, production of farm-raised shrimp, especially the whiteleg shrimp, *Litopenaeus vannamei*. has expanded rapidly worldwide. *L. vannamei* was the highest harvested crustaceans in the world with a total production of 4.16 million tonnes, contributing to 53% of the total crustacean production (~7.86 million tonnes) in 2016 (FAO, 2018). Owing to its remarkable ability to grow and survive in varying salinity and temperature conditions and relatively faster growth during shorter culture period (Ponce-Palafox et al., 1997; Roy et al., 2010), the farming of *L. vannamei* has expanded to inland low-saline areas in many Asian countries such as China, Thailand, Vietnam, Bangladesh and Indonesia including India. Such an extension in the farming of *L. vannamei* to inland low-saline regions often adjacent to the agriculture fields enhances the potential risk of pesticide toxicity to the farmed stock due to runoff and/or spray drift (Roque et al., 2005; Pawar et al., 2019).

Salinity and temperature are two most prominent abiotic water quality parameters that affect the toxicity of xenobiotics to aquatic organisms (Brecken-Folse et al., 1994; DeLorenzo et al., 2009) because they regulate the bioavailability of a contaminant in the water. Changes occurring in the global climate particularly, variability in water

* Corresponding author at: Aquaculture Laboratory, Biological Oceanography Division, CSIR-National Institute of Oceanography, Dona Paula, Goa, 403004, India.
E-mail address: sreepada@nio.org (R.A. Sreepada).

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temperature and salinity could exacerbate detrimental effects of pollutants on aquatic organisms and as a consequence of altered chemical fate and transport, and changes in physiological response may enhance their toxicity (Lin and Chen, 2003; DeLorenzo et al., 2009; Tu et al., 2012; DeLorenzo et al., 2013; Mehta, 2017). The fluctuations in water temperature in natural water bodies and farming systems depend on air temperature, water depth, pond design, evaporation rate and water management. On the other hand, salinity varies in accordance with seasons, increases during the summer and decreases after rainfall (Tu et al., 2012). Temperature exerts serious physiological effects on living systems and directly influences the tolerance limits of aquatic biota to toxic substances (Mehta, 2017). Salinity is another critical factor that influences the distribution and maintenance of life in aquatic ecosystems (Wang et al., 2013). Therefore, the physiological effects induced by fluctuations in temperature and salinity may potentially altering ecotoxicity of pollutants (DeLorenzo et al., 2013; Velasco et al., 2019).

Sub-optimal temperature or unsuitable salinity may interact in an antagonistic, additive or synergistic manner with toxicants thereby significantly altering the tolerance of aquatic animals (DeLorenzo et al., 2009; Tu et al., 2012; Santos et al., 2014; Patra et al., 2015; Velasco et al., 2019). Influence of salinity and temperature on the tolerance to toxicity of pesticides in a variety of crustaceans species including shrimps has been reported (Staton et al., 2002; DeLorenzo et al., 2009; Tu et al., 2012; Wang et al., 2013). Due to their phylogenetic similarities with insects, crustaceans in general and the penaeid shrimps, in particular, have been proved to be more sensitive to ecotoxicological effects of pesticides (Nimmo et al., 1971; Couch, 1978; Cripe, 1994; Galindo-Reyes et al., 1996; Roque et al., 2005; Mello et al., 2011; Eamkamon et al., 2012; Pawar et al., 2019). Furthermore, higher sensitiveness of early life stages to pesticides than adults has also been widely documented (Juarez and Sanchez, 1989; Hutchington et al., 1998; Pawar et al., 2019).

The ecotoxicity data for most commonly used pesticides conducted under standard test conditions is widely available. However, data derived from standard bioassays is often not comparable because of differences in test design, lack of information on the fluctuating environmental conditions, life stages of commercially important species. Furthermore, physico-chemical factors (salinity and temperature) may alter the bioavailability of chemicals and therefore affect the toxicity (Brecken-Folse et al., 1994). With this background, the present study examined the effects of varying salinity and temperature regimes on the acute toxicity of two commonly used OPs (dimethoate and chlorpyrifos) in post-larval (PL) and juvenile (JL) stages of *L. vannamei*. The outcome of the study has practical implications because of *L. vannamei* is a promising shrimp species for farming in low saline inland areas often influenced by diffuse source run-off from agriculture.

2. Materials and methods

2.1. Experimental shrimps and rearing conditions

Approximately 6000 healthy SPF (specific pathogen-free), 12 days old post-larvae (PL_{12}) stage) of the whiteleg shrimp, *Litopenaeus vannamei* (total length, 11.4 ± 0.2 mm; wet weight, 52 ± 0.5 mg) were procured from a commercial shrimp hatchery (Skyline Aqua Hatchery, Kumta, Karnataka). Post-larvae (PL_{12}) transported in oxygen-filled bags containing seawater (salinity, 20) to the Aquaculture Laboratory, CSIR-NIO, Goa (India). Upon arrival, shrimps were reared and acclimated under laboratory conditions in circular fibre reinforced plastic (FRP) tank (capacity, 2000 L) until they reached the PL_{20} stage. The seawater used for the rearing of post-larvae and later for acute toxicity experiments was treated by rapid sand filtration, cartridge filtration (20 to 1 μm) and passed through ultraviolet radiation. During rearing of post-larvae, EPAC XL feed (INVE Aquaculture Inc., USA) was used.

2.2. Shrimps acclimation

Shrimps (PL_{12}) reared at salinity (20 ± 0.5) and temperature (27 ± 1 °C) were divided randomly into two batches. One batch of shrimps was further divided and adjusted gradually (stepwise decrease/increase in salinity of 2 per day) to three different target salinity regimes (5, 15 and 25) in three separate 800 L FRP tanks for each salinity group. Target salinities were obtained by a mixture of dechlorinated tap water with natural seawater. The selected salinity values fell within the range in which *L. vannamei* is cultured. Salinity was checked at regular intervals by hand-held refractometer (Atago, Japan). Another batch of acclimated shrimps was further divided randomly into three groups at the same temperature and then gradually adjusted to target temperatures (15, 25 and 34 °C). Temperature experiments were conducted in an air-conditioned room (air temperature, 16 °C). Target temperatures were achieved by stepwise decrease/increase in water temperature with the help of aquarium heaters (100 W, temperature range 22 − 34 °C, Protherm, Italy). The temperature of rearing water tanks was also monitored regularly with the help of calibrated thermometer.

For conducting acute toxicity experiment with juveniles of *L. vannamei*, acclimated post-larvae at three target salinities and temperature were further grown for four weeks with a photoperiod of 12 h light: 12 h dark. During this rearing period, shrimps were fed with commercial shrimp pellet feed (CP-Aquaculture, India; proximate composition, 38–40% protein; 5% lipids and 3% fibre) at the rate of 6% of total biomass split into three rations a day. Excreta, uneaten feed and sloughed exoskeletons were removed by siphoning every day. Temperature, dissolved oxygen (DO), salinity and pH were measured daily with a portable water quality meter (CyberScan Series 600, Eutech Instruments, Singapore). All other water quality parameters (NO_3–N, NO_2–N and NH_3/NH_4) were analysed following methods described in APHA (2005).

2.3. Chemicals

The technical-grade of OPs, dimethoate (O, O-dimethyl S-methyl carbamoyl methyl phos-phorodithioate; DMT) and chlorpyrifos (O, O-diethyl O-3, 5, 6-trichloro-2-pyridyl phospho-rothionate; CPF), respectively marketed under the brand names, 'TAFGOR' (Rallis India Ltd., Akola, India) and 'PYRIBAN' (AIMCO Pesticides Ltd., Ratnagiri, India) were used for the study. The technical-grade of DMT and CPF having effective concentrations of 30% and 20%, respectively were diluted with deionised water to prepare stock solutions of active DMT (2 mg L^{-1}) and CPF (2 mg L^{-1}).

2.4. Experimental design

For each pesticide, the tests were conducted under standard exposure conditions (temperature, 27 °C and salinity, 20) per life stage in triplicate For altered exposure conditions, eighteen experimental tanks were obtained by combining three salinities (5, 15 and 25) and temperatures (15, 25 and 34 °C) with three triplications per pesticide per life stage resulting in total of 72 experimental units. The parameters measured were the mortalities in post-larvae and juveniles of *L. vannamei* recorded at the end of 24, 48, 72 and 96 h of exposure.

2.5. Acute toxicity experiments

To determine acute toxicicity indices (96 h LC_{50} values) for DMT and CPF in PL and JL stages of *L. vannamei*, aqueous semi-static renewal tests by following the standard procedures of APHA (2005).. For determining the acute toxicity indices (96 h LC_{50}) in post-larvae (PL_{20}) of *L. vannamei*, acclimated and adjusted shrimps (PL_{20}, 15.2 mm ± 0.3 mm; wet weight, 65 ± 5 mg) to three salinities (5, 15 and 25) and temperatures (15, 25 and 34 °C) regimes were randomly selected. The average size of *L. vannamei* juveniles used for the experiment was of

Table 1
The recorded mortality (%) in post-larvae and juveniles of *Litopenaeus vannamei* for each tested concentration (μg L^{-1}) of dimethoate (DMT) and chlorpyrifos (CPF) at three different salinities and temperatures after 96 h of exposure. Mortality data of three replicates (mean ± SD).

Post-larvae of *Litopenaeus vannamei*

Treatment	DMT		CPF		Treatment	DMT		CPF	
Salinity	Conc. (μg L^{-1})	Mortality (%)	Conc. (μg L^{-1})	Mortality (%)	Temp (ºC)	Conc. (μg L^{-1})	Mortality (%)	Conc. (μg L^{-1})	Mortality (%)
5	100	43.3 ± 3.1	0.1	13.3 ± 1.1	15	50	30.0 ± 1.9	0.1	30.0 ± 2.1
	200	60 ± 4.2	0.2	20.0 ± 1.5		100	40.0 ± 2.5	0.2	50.0 ± 3.1
	300	83.3 ± 6.1	0.3	50.0 ± 2.4		150	60.0 ± 4.1	0.3	70.0 ± 4.2
	400	90.0 ± 7.1	0.4	60.0 ± 3.1		200	80.0 ± 5.9	0.4	90.0 ± 5.1
	500	100.0 ± 8.1	0.5	83.3 ± 5.9		300	100.0 ± 8.1	0.5	100.0 ± 6.7
15	100	40 ± 2.3	0.1	10.0 ± 1.0	25	50	10.0 ± 1.1	0.2	10.0 ± 1.00
	200	63.3 ± 3.5	0.2	20.0 ± 1.1		100	10.0 ± 1.1	0.4	30.0 ± 1.14
	300	70.0 ± 2.9	0.3	40.0 ± 1.7		150	20.0 ± 1.5	0.6	40.0 ± 2.87
	400	90.0 ± 5.5	0.4	60.0 ± 2.8		200	40.0 ± 3.1	0.8	63.3 ± 4.1
	500	100.0 ± 6.5	0.5	80.0 ± 4.8		300	50.0 ± 2.7	1.0	80.0 ± 6.1
25	100	10.0 ± 1.1	0.2	23.3 ± 1.2	34	50	20.0 ± 1.6	0.2	20.0 ± 1.4
	200	20.0 ± 2.3	0.6	30.0 ± 2.1		100	30.0 ± 2.7	0.4	30.0 ± 2.1
	300	30.0 ± 2.5	1.0	40.0 ± 2.9		150	40.0 ± 3.5	0.6	63.3 ± 6.0
	400	50.0 ± 2.6	1.4	60.0 ± 3.8		200	50.0 ± 4.2	0.8	80.0 ± 6.6
	500	70.0 ± 3.1	1.8	80.0 ± 4.7		300	70.0 ± 5.3	1.0	100.0 ± 8.0

Juveniles of *Litopenaeus vannamei*

Treatment	DMT		CPF		Treatment	DMT		CPF	
Salinity	Conc. (μg L^{-1})	Mortality (%)	Conc. (μg L^{-1})	Mortality (%)	Temp (ºC)	Conc. (μg L^{-1})	Mortality (%)	Conc. (μg L^{-1})	Mortality (%)
5	100	30.0 ± 1.9	0.1	10 ± 0.59	15	50	10.0 ± 1.1	0.1	30.0 ± 2.1
	200	43.3 ± 3.2	0.2	13.3 ± 1.1		100	30.0 ± 2.1	0.2	40.0 ± 2.5
	300	60.0 ± 4.7	0.3	40 ± 2.9		150	40.0 ± 3.1	0.3	53.3 ± 3.5
	400	80.0 ± 6.1	0.4	53.3 ± 3.7		200	60.0 ± 4.2	0.4	70.0 ± 4.5
	500	90.0 ± 7.1	0.5	60.0 ± 4.9		300	90.0 ± 6.2	0.5	90.0 ± 6.5
15	100	30.0 ± 2.1	0.1	10.0 ± 1.0	25	100	13.3 ± 1.2	0.2	10.0 ± 1.0
	200	40.0 ± 2.9	0.2	20.0 ± 1.3		200	20.0 ± 1.1	0.4	20.0 ± 1.1
	300	50.0 ± 3.7	0.3	40.0 ± 3.1		300	30.0 ± 1.9	0.6	30.0 ± 2.1
	400	70.0 ± 4.7	0.4	50.0 ± 4.7		400	40.0 ± 2.5	0.8	50.0 ± 2.97
	500	90.0 ± 6.1	0.5	70.0 ± 5.1		500	60.0 ± 3.6	1.0	70.0 ± 4.8
25	100	0 ± 0.00	0.2	10.0 ± 1.0	34	100	20.0 ± 1.3	0.2	0 ± 0.00
	200	10.0 ± 0.9	0.6	20.0 ± 1.7		200	40.0 ± 2.2	0.4	23.3 ± 2.1
	400	20.0 ± 1.2	1.0	30.0 ± 2.7		300	60.0 ± 5.5	0.6	40.0 ± 3.5
	600	30.0 ± 2.1	1.4	40.0 ± 3.3		400	70.0 ± 6.2	0.8	60.0 ± 4.6
	800	70.0 ± 4.2	1.8	70.0 ± 5.1		500	80.0 ± 6.5	1.0	83.3 ± 7.1

53.4 ± 6.4 mm in total length and 1.79 ± 0.6 g in wet weight. Before being subjected to acute toxicity experiments, shrimps fasted for 24 h.

The results of the range-finding experiments for standard exposure conditions and altered exposure conditions, were used as criteria for the definitive tests to select five nominal exposure concentrations of DMT and CPF. The exposure concentrations (μg L^{-1}) of each OPs used for assessing acute toxicity in the PL stage of *L. vannamei* at different salinity and temperature regimes are presented in Table 1. Each treatment had separate controls (without pesticides) in triplicate. Post-larvae (PL_{20}) were exposed in 3 L glass jar with 2 L medium with 20 PL jar^{-1}, whereas juveniles (JL) were exposed in 21 L glass aquaria containing 10 L medium with 10 JL $aquaria^{-1}$. Each jar was covered with a glass disc and aerated via 2 mm glass tubing. During the exposure period of 96 h, each test medium was renewed every 24 h under the same conditions (Buikema et al., 1982). Shrimps were not fed during the exposure period and experiments were conducted in a natural light cycle (12 h light: 12 h dark).

Water quality parameters such as temperature, salinity, DO and pH were recorded daily, before each media change, whereas NO_3–N, NO_2–N and NH_3/NH_4 were measured at the beginning and the end of the experimental period. Probes of portable water quality meter were thoroughly rinsed with double distilled water before every use to remove traces of pesticide adhering to the probes. According to the schedule at the end of 24, 48, 72 and 96 h of exposure, mortalities in post-larvae and juveniles were recorded during the 96 h exposure period (Table 1). The dead organisms, if any were removed immediately on detection, in order to avoid any type of bacterial contamination. The criteria for proof of mortality were total lack of body movement, immobility and showed no response when touched with a glass rod (Lin and Chen, 2003). The cumulative mortality rates at the end of exposure periods of 24, 48, 72 and 96 h were calculated using the formula as defined by Abbott (1925). The concentration response of test organisms was determined for LC_{50} value (median lethal concentration) for each treatment and their 95% confidence limits for an exposure period of 96 h for each treatment were calculated with a computer-based programme described by Finney (1971).

2.6. Data presentation and analysis

All the experiments were performed in three replicates per treatment. Mortality data obtained from the experiments are presented as mean ± SD (Table 1). Measured variables were tested to normality and homogeneity by Cochran's test (Winer et al., 1991). Prior to statistical analysis. acute toxicity data were $\log_{10}(x + 1)$ transformed. One-way analysis of variance (ANOVA, Underwood, 1997) was used to assess the differences in acute toxicity indices between standard exposure and altered exposure conditions and within different treatments. If ANOVA found significant differences, multiple comparisons between different

Table 2
Acute toxicity values with 95% confidence limits (in parentheses) for post-larvae and juveniles of *Litopenaeus vannamei* to dimethoate (DMT) and chlorpyrifos (CPF) at different salinities and temperatures. 96 h LC_{50} values for each test condition is based on percent control mortality. Different superscript letters in the same column significantly differ ($P < 0.05$) from standard conditions (temperature, 27 °C and salinity, 20) and between tested salinities and temperatures.

Post larvae of *Litopenaeus vannamei*					
Salinity	DMT (μg L^{-1})	CPF (μg L^{-1})	Temperature (°C)	DMT (μg L^{-1})	CPF (μg L^{-1})
5	127.2 (71.2–150.0)[a]	0.31 (0.16–0.44)[a]	15	103.11 (92.11–125.36) [a]	0.17 (0.12–0.23) [a]
15	137.44 (110.13–160.71)[b]	0.32 (0.24–0.46)[a]	25	321.26 (286.45–395.51) [b]	0.62 (0.56–0.68) [b]
25	386.59 (300.53– 692.15)[c]	0.92 (0.75–1.32)[b]	34	182.76 (156.46–221.47)[c]	0.46 (0.32–0.60) [c]
Standard	257.84 (278.21–311.20)[d]	0.68 (0.50–0.87)[c]	Standard	257.84 (278.21–311.20)[d]	0.68 (0.50–0.87)[d]
Juveniles of *Litopenaeus vannamei*					
Salinity	DMT	CPF	Temperature (°C)	DMT	CPF
5	196.69 (161.64–229.51) [a]	0.40 (0.35–0.53) [a]	15	151.45 (110.18–212.00) [a]	0.22 (0.12–0.31) [a]
15	218.86 (185.22–298.41)[b]	0.37 (0.33–0.42)[a]	25	479.20 (404.02–619.27) [b]	0.77 (0.60–1.39) [b]
25	691.51 (560.44–832.42)[c]	1.68 (1.54–2.52)[b]	34	236.74 (209.48–264.86) [c]	0.65 (0.49–0.93) [c]
Standard	463.61 (356.11–576.35)[d]	0.73 (0.69–1.06)[c]	Standard	463.61 (356.11–576.35) [d]	0.73 (0.69–1.06) [d]

means amongst treatments were then made by Tukey–Kramer test high significant differences (HSD) (Zar, 1996). Statistical analysis was performed by using computer-based GraphPad PRISM 5.0 software (Graph Pad, San Diego, CA, USA). Significance in all statistical tests was judged at a '*P*' = 0.05 level.

3. Results

No significant variations in water quality parameters were observed in control and treated tanks during the entire experimental routine ($P > 0.05$). The results showed that the significant water quality parameters during the experiment fell within the optimal limits for *L. vannamei*. No mortality observed in the control group either in salinity or temperature based acute toxicity experiments ensured that the observed effects were due to the action of OPs. The 96 h LC_{50} values at standard test conditions (temperature, 27 °C and salinity, 20) for DMT and CPF to post-larvae were 257.84 and 0.68 μg L^{-1}, respectively. Relatively higher 96 h LC_{50} values of 463.61 and 0.73 μg L^{-1}, respectively for DMT and CPF were obtained for juveniles of *L. vannamei* (Table 2). The 96 h LC_{50} values of DMT and CPF to PL stages were 127.2, 137.44 and 386.59 μg L^{-1} and 0.31, 0.32 and 0.92 μg L^{-1} at 5, 15 and 25 salinities, respectively (Table 2). The 96 h LC_{50} values at different temperatures (15, 25 and 34 °C), respectively were 103.11, 321.26 and 182.76 μg L^{-1} for DMT and 0.17, 0.62 and 0.46 μg L^{-1} for CPF (Table 2).

The 96 h LC_{50} values of DMT and CPF obtained for altered salinity and temperature regimes for two life stages of *L. vannamei* differed significantly ($P < 0.05$) from standard exposure conditions (Table 2). A comparison of 96 h LC_{50} values for post-larvae of *L. vannamei* between adjacent salinities showed the statistical difference ($P < 0.05$) between all tested salinities in case of DMT (Table 2). However, differences in 96 h LC_{50} values were significant ($P < 0.05$) between salinities 5 and 25 but not between salinities 5 and 15 ($P < 0.05$) for CPF. On the other hand, a comparison of 96 h LC_{50} values obtained for both DMT and CPF was significant ($P < 0.05$) in all tested temperature regimes (Table 2).

Acute toxicity experiment conducted with juveniles of *L. vannamei* at three different salinities (5, 15 and 25) and temperatures (15, 25 and 34 °C) resulted in relatively higher 96 h LC_{50} values for DMT compared to CPF (Table 2). Juveniles of *L. vannamei* were found to be less sensitive to both OPs than post-larvae of *L. vannamei* in all tested salinity and temperature regimes. The 96 h LC_{50} values of DMT and CPF pesticides at 25 salinity and 25 °C temperature differed significantly ($P < 0.05$) when compared to salinities of 5 and 15 and temperatures of 15 and 34 °C. Relatively, CPF was found to be more toxic compared to DMT to both life stages (PL and JL) of *L. vannamei* in all tested salinity and temperature regimes (Table 2).

4. Discussion

The organophosphate pesticides, DMT and CPF are widely used to restrict insect pests in a range of commercial crops. As these products are extensively used in the market, many toxicological effects on economically important non-target freshwater, marine organisms and abundance of benthic invertebrates in shrimp have been reported (Rao et al., 2005; Garcıa-de la Parra et al., 2006; Eamkamon et al., 2012; Krishnapriya and Padmaja, 2014; Uddin et al., 2016; Hook et al., 2018; Pawar et al., 2019). However, their direct or indirect effects on commercially important shrimp species, particularly under variable salinity and temperature was first addressed in the present study. The 96 h LC_{50} values of DMT and CPF in PL and JL stages of *L. vannamei* recorded during present study are roughly comparable with those previously laboratory-derived for other crustacean species (Roast et al., 1999; Montagna and Collins, 2007; Kumar et al., 2010; Satapornvanit et al., 2009; Narra et al., 2013; Pawar et al., 2019) because of differences in size and life stage, type of rearing water (freshwater or seawater/brackishwater), duration of exposure and interaction of pesticides with biotic/abiotic factors (DeLeronzo et al., 2014; DeLeronzo, 2015).

The two tested OPs significantly differed in their toxicity to both early life stages (PL and SL) of *L. vannamei* in all salinity and temperature regimes. CPF was a highly toxic pesticide indicated by the low acute toxicity indices. CPF was found to be an approximately 400 500 fold and an approximately 400–600 fold more toxic to PL and JL stages of *L. vannamei* compared to DMT in altered tested salinity and temperature conditions. Such a vast intra-species variation for the OPs tested may be due to the lipophilic nature of CPF (Vlahovic et al., 2017). As a lipophilic molecule, CPF's permeation into the lipid-rich tissues of aquatic animal is relatively higher. This lipophilic nature of CPF results in elevated levels of bioaccumulation and causes severe damage or death of the organism (Essumang et al., 2009; Banaee, 2012).

Post-larvae of *L. vannamei* were more sensitive to both OPs than juveniles. Post-larvae showed 1–2 orders magnitude higher sensitive than juveniles to both OPs. The sensitivity of shrimp larvae to pesticides has been observed to be 1–3 orders magnitude higher than adults (Nimmo et al., 1971; Mohammed, 2013; DeLorenzo et al., 2014). Our results are in agreement with previous studies. Significantly higher toxicity levels in grass shrimp (*Palaemonetes pugio*) larvae compared to adults following exposure to CPF has been reported (Key and Fulton, 1993). The sensitivity of crustacean larvae is relatively more prone to toxicants than adults as a consequence of increased moulting frequency during their early life stages (Rao et al., 1985). Therefore, higher toxic

levels recorded during the present study in post-larvae of *L. vannamei* might be due to cumulative stress effect induced by changing salinity and temperature in addition to the toxicity of DMT and CPF.

The two tested OPs was significantly more toxic to both PL and JL stages of *L. vannamei* at 5 and 15 salinities and 15 °C and 34 °C temperatures than at 25 salinity and 25 °C temperature ($P < 0.05$), respectively. A decrease in the toxicity tolerance on exposure to two pesticides (beta-cypermethrin and acephate) in *L. vannamei* at lower salinity levels (5 vs. 20) have been reported by Wang et al. (2013). Similarly, increase in herbicide, atrazine toxicity with decreasing salinity in copepod nauplii (*Eurytemora affinis*) has been reported by Hall et al. (1994). Induction of osmotic stress and consumption of higher oxygen in *L. vannamei* reared at lower salinity (1) and higher salinity (37) as well as lower temperature (20 °C) has been reported (Bett and Vinatea, 2009). In contrast, increase in toxicity in larval and adult estuarine grass shrimp, *P. pugio* on exposure to fungicide chlorothalonil and the insecticide Scourge® concomitant with the increase in salinity and temperature has been reported by DeLorenzo et al. (2009). Furthermore, an increase in salinity resulted in enhancement of toxicity of pesticides and insecticides such as DMT, aldicarb and tebufenozide in brine shrimp (*Artemia* sp) (Song and Brown, 1998). These results suggest that the interactive effects between salinity and pesticides, particularly in crustaceans, are far from clear. Therefore, it appears that the changes in salinity altering the toxicity of pesticides would depend on exposure factors such as duration, acclimation, species, life stage and the specific characteristic of the individual pesticide (DeLorenzo, 2015).

Being one of the regulatory processes of physiological activities, the temperature affects the intensity of biochemical reaction, molecular mobility and the membrane exchange processes (Ozoh, 1992). A comparison of 96 h LC_{50} values for both and JL stages of *L. vannamei* between adjacent temperatures revealed that an increase in temperature from 15 to 25 °C decreased the acute toxicity of both DMT and CPF. However, further increase in rearing temperature from 25 to 34 °C resulted in enhanced toxicity of DMT and CPF to PL and JL stages of *L. vannamei*. Increasing temperature resulting in enhanced toxicity on grass shrimp (*Palaemonetes* spp.) on exposure to OPs has been reported (Breken-Folse et al., 1994). An increase in metabolic rate at higher temperatures results in increased water movement across the gills and increased pesticide uptake (DeLorenzo, 2009). Higher temperatures have been reported to significantly affect the species' ability to cope with stress induced by toxic substances (Cherkasov et al., 2006; Lanning et al., 2006) and the regulation of associated physiological processes involved in the detoxification of hazardous substances. Furthermore, the deleterious effect in shrimps as a consequence of thermal and osmotic stress has been reported (Chien et al., 2003; Moser et al., 2012). The increase in water temperature triggering an outbreak of the *Vibrio* has been reported. This may be due to weakening the immune response of shrimp, and an accompanied reduction in aspartate aminotransferase and alanine aminotransferase activities (Chien et al., 2003; Moser et al., 2012).

The findings of the study suggest that changes in salinity and temperature altered the toxicity of DMT and CPF in *L. vannamei* and the degree of the effect depended on its life stage and type of OP. The potential effect of pesticides in farming systems is expected to be much higher than these laboratory-derived results as the presence of sediment enhances the toxicity of pesticides (Holmes et al., 2008). There may be a build-up of OPs in ponds over time and may pose a risk to shrimp aquaculture, due to partitioning of CPF to sediment (Giddings et al., 2014). Owing to their burrowing nature into sediments and detritivore feeding habit, all stages of shrimp are susceptible to sediment-bound contaminants (Hook et al., 2018), thus posing a greater risk to shrimp culture. In conclusion, acute toxicity to PL and JL stages occurred at very low concentrations of OPs, especially at lower salinities calls for precaution in extending the cultivation of *L. vannamei* in low-salinity areas adjacent to traditional agriculture fields.

Declatation of Competing Interest

The authors have declared that no competing interests exist.

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Data availability statement

All relevant data are within the manuscript.

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