

**In vivo DNA damage in gill, haemolymph and muscle cells of whiteleg shrimp, *Litopenaeus vannamei* (Boone, 1931) on exposure to organophosphorus Pesticide**

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**Running head:** Genotoxic effect of Chlorpyrifos to whiteleg shrimp

**ABSTRACT:**

In response to growing worldwide market demand, intensive shrimp farming, particularly the whiteleg shrimp, *Litopenaeus vannamei* (Boone, 1931) has expanded tremendously. The present study investigated induced DNA damage in gill (GL), haemolymph (HL) and muscle (ML) cells in juveniles of *L. vannamei* (52.2 ± 6.4 mm; 1.78 ± 0.5 g) exposed to two sublethal concentrations, SL1 (0.36 µg l<sup>-1</sup>) and SL2 (0.18 µg l<sup>-1</sup>) of organophosphorus pesticide, chlorpyrifos (CPF) during 21 days of exposure (DoE). The magnitude of DNA damage (% tail DNA) as measured by comet assay at specified intervals (3, 7, 14 and 21 DoE) was found to be tissue-specific, time and dose-dependent (p < 0.05). At the end 21 DoE, % of tail DNA damage (53.61 ± 8.71), (49.36 ± 3.42) and (32.40 ± 4.97) was relatively higher at SL1 compared to SL2 (39.25 ± 3.90), (32.22 ± 4.21) and (22.66 ± 2.85), respectively in GL, HL and ML cells. No significant differences in water quality parameters were found among the treated and control aquaria. The significant reduction in specific growth rates (% growth day<sup>-1</sup>) observed in exposed shrimps indicate that SL concentrations of CPF negatively impacting the growth in *L. vannamei* juveniles. A very low 96 h LC<sub>50</sub> value (1.44 µg l<sup>-1</sup>) indicated sensitivity of *L. vannamei* juveniles to CPF that it could be used as bioindicator species for assessing pesticide pollution. The study results highlight the implications in extending the farming of *L. vannamei* to low-saline inland areas adjacent to traditional agricultural fields.

**KEY WORDS:** Chlorpyrifos; Organophosphorus Pesticide; DNA damage; Comet assay; Whiteleg shrimp; *Litopenaeus vannamei*

## 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. 2018). These products are often used to control pests in the household or in the agricultural fields. As a consequence of non-target toxicity and accumulation in the environment, many hitherto registered pesticide products are either 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 the recent years

Among the major organophosphorus pesticides (OPs), chlorpyrifos (CPF) is one of the most widely used pesticide to control insects in agriculture and 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, however has been its effect on non-target wildlife populations (Frasco et al. 2006, Kumar et al. 2010, Kumar et al. 2017). Such an 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, the world shrimp production, particularly the farming of whiteleg shrimp, *Litopenaeus vannamei* has increased tremendously (Kumar & Engle 2016) and contributed 53% to the total shrimp and prawn production in 2016 (FAO 2018). Owing to 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 saline areas often adjacent to the 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 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 & 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 the minute DNA damage, the single cell gel

electrophoresis or comet assay has become widely used genetic tool (Klobucar 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, Galindo-Reyes et al. 2002, Labrie et al. 2003, Suryavanshi et al. 2009, Mello et al. 2011, Eamkamon et al. 2012, Thi Tu et al. 2012). Unfortunately, studies on long-term *in vivo* assessment of genotoxic effect of OPs in penaeid shrimps, particularly *L. vannamei* are scarce.

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

## **MATERIAL AND METHODS**

### **Experimental shrimps and rearing conditions**

Healthy post larvae (PL14; total length,  $12.4 \pm 0.5$  mm; wet weight,  $55 \pm 5$  mg) of the whiteleg shrimp, *Litopenaeus vannamei* produced from SPF (specific pathogen free) broodstock and negative to WSSV (white spot syndrome virus) as confirmed by PCR (polymerase chain reaction) procured from a commercial shrimp hatchery (Skyline Aqua Hatchery, Kumta, Karnataka) were reared at Aquaculture Laboratory, CSIR-NIO, Goa (India). The seawater used in the rearing of PLs and for pesticide exposure experiments was filtered through three stage filtration system comprising rapid sand filtration, cartridge filtration (20 to 1  $\mu\text{m}$ ) and finally through ultraviolet disinfection. The PLs were reared in 800 l tank for four weeks with a photoperiod of 12 h light: 12 h dark. During rearing period, shrimps were fed *ad libitum* twice a day with commercial shrimp pellet feed (CP-Aquaculture, India; proximate composition, 38 – 40% protein; 5% lipids 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 the help of portable water quality meter (CyberScan Series 600, Eutech Instruments, Singapore). 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 ( $28.5 \pm 0.5^\circ\text{C}$ ), salinity ( $30 \pm 0.5$  g  $\text{l}^{-1}$ ), DO ( $6.1 \pm 0.4$  mg  $\text{l}^{-1}$ ), pH ( $8.2 \pm 0.2$ ),  $\text{NO}_2\text{-N}$  ( $<0.02$  mg  $\text{l}^{-1}$ ) and  $\text{NH}_3/\text{NH}_4$  (0 mg  $\text{l}^{-1}$ ).

## Chemicals

The commercial grade organophosphorus pesticide ('PYRIBAN', AIMCO Pesticides Ltd., Ratnagiri, India) containing chlorpyrifos (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 ( $2 \text{ mg l}^{-1}$ ). All other chemicals used in comet assay were of molecular grade (Sigma-Aldrich, India).

## Acute toxicity experiment

Four-week old laboratory reared active and healthy uniform size juveniles (total length,  $52.2 \pm 6.4$  mm; wet weight,  $1.78 \pm 0.5$  g) without any stress signs (colourless abdomen) and 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  $2 \mu\text{g l}^{-1}$ ) along with a separate control (without pesticide) in triplicate were used for acute toxicity experiment. Shrimp juveniles were exposed in 21 l glass aquaria containing 10 l seawater with a stocking density of 10 juveniles  $\text{aquaria}^{-1}$ . Water quality parameters such as temperature, DO and pH were recorded daily, whereas salinity,  $\text{NO}_2\text{-N}$  and  $\text{NH}_3/\text{NH}_4$  were measured prior and at the end of experimental period; temperature ( $28.4 \pm 0.2^\circ\text{C}$ ), salinity ( $30 \pm 0.5 \text{ g l}^{-1}$ ) DO ( $5.8 \pm 0.6 \text{ mg l}^{-1}$ ), pH ( $8.1 \pm 0.4$ ),  $\text{NO}_2\text{-N}$  ( $< 0.02 \text{ mg l}^{-1}$ ) and  $\text{NH}_3/\text{NH}_4$  ( $0 \text{ mg l}^{-1}$ ). Mortality was recorded during the 96 h exposure period according to the time schedule at 24, 48, 72 and 96 h. 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 of heart and scaphognathite after repeated prodding with a probe.

The cumulative mortality rates were calculated using the formula as defined by Abbott (1925). The median lethal concentrations 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.

## *In vivo* chronic exposure experiment

For understanding the behavioural and physiological changes in animals on exposure to pesticides, sublethal concentrations offer an excellent scope (Edwards 1973). In toxicological studies, chronic tests of shorter duration ( $\sim 21$  days) have been recommended as an alternative to longer chronic tests (Bhavan & Geraldine 2000, Suryavanshi et al. 2009). Based on the result of 96 h  $\text{LC}_{50}$  values ( $1.44 \mu\text{g}$

$\Gamma^{-1}$ ; 95% confidence limits, upper =  $1.46 \mu\text{g l}^{-1}$  and lower =  $0.85 \mu\text{g l}^{-1}$ ;  $p < 0.05$ ), two SL concentrations, SL1 ( $0.36 \mu\text{g l}^{-1}$ ) and SL2 ( $0.18 \mu\text{g l}^{-1}$ ) equivalent to  $1/4^{\text{th}}$  and  $1/8^{\text{th}}$  of 96 h  $\text{LC}_{50}$  value (nominal concentration), respectively computed from a commercial grade CPF were selected for *in vivo* chronic exposure experiment. The selected SL concentrations were lower than LOEC and NOEC values. Static renewal method for toxicity tests with separate control tanks as was followed (Buikema et al. 1982).

For chronic exposure experiment, 300 inter-moult juveniles of uniform size (length,  $52.2 \pm 6.4$  mm; wet weight,  $1.78 \pm 0.5$  g) were divided into three groups, each group comprising of 100 juveniles. Out of three groups, one group formed the control, while two remaining groups were exposed to two SL concentrations of CPF for a period of 21 days of exposure (DoE). In order to maintain the constant concentration of CPF in test solutions, the entire toxic medium in each aquarium tank was gently siphoned out daily (10:00 h) and renewed with 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 preceding day's pesticide remain present in the treatment tanks.

A total of two replicate aquaria (all glass aquaria, 100 l capacity) having 50 l seawater were maintained for each SL concentration and the control group (50 shrimps replicate<sup>-1</sup>). Samples for comet assay were collected on 3, 7, 14 and 21 DoE. On designated sampling occasion, 10 juvenile shrimps from each concentration (five from each replicate tank) were randomly selected and processed for comet assay. Shrimps from the control group were similarly sampled at the same time as treated ones. During exposure period, mortality in control and exposure tanks was minimal (<5%). In order 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 which have been exposed to same DoE. During chronic exposure experiment, the juveniles were fed thrice a day (10:00 h, 15:00 h and 20:00 h) at the rate of 4-6% of body weight. Mean body weight of juvenile *L. vannamei* recorded on every sampling occasion was used to determine specific growth rate, SGR (% day<sup>-1</sup>) over the exposure period of 21 DoE from the formula  $\text{SGR} = [(\ln W_2 - \ln W_1) / (t_2 - t_1)] \times 100$ , where  $W_1$  and  $W_2$  represents 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,  $\text{NO}_2\text{-N}$  and  $\text{NH}_3/\text{NH}_4$  were done weekly following the methods as described previously. Before each usage, probes were thoroughly rinsed with deionised water to remove traces of pesticide adhering to the probes, if any.

## **Sample collection from shrimps**

Tissue samples of gills (GL), haemolymph (HL) and muscles (ML) were pooled from 10 randomly selected shrimps (five from each replicate tank) from treated and control groups for comet assay. Approximately 350 µl of haemolymph was drawn from rostral sinus region of control and CPF exposed shrimps using sterilized hypodermic syringe on 3, 7, 14 and 21 DoE. The GL and ML tissues (~50 mg) were also collected from control and exposed shrimps. The GL and ML tissues were washed twice with chilled phosphate buffer saline (PBS, calcium and magnesium free) and transferred to chilled homogenization buffer (1-X Hanks' Balanced Salt Solution, 20 mM EDTA, 10% dimethyl sulphoxide (DMSO), pH 7.0–7.5). The tissue was homogenized with a Potter-Elvehjem homogenizer to obtain a single cell suspension. The homogenate was centrifuged at 3000 rpm at 4°C for five min and the pellet was suspended in chilled PBS (Dhawan et al. 2008). The extraction of sample was carried out under dim light and transferred immediately to microcentrifuge tube placed on 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).

## **Cell viability assay**

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

## ***In vitro* exposure to hydrogen peroxide**

A standard genotoxin, hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) was used to validate the results obtained by comet assay and response of different cells as described by Bhagat et al. (2016). Freshly detached GL and ML cells and HL from juveniles were treated with different concentrations of H<sub>2</sub>O<sub>2</sub> (0.5, 5, 20 and 40 µM) prepared in PBS for 30 min in dark condition at room temperature (28°C). On the other hand, the control samples were incubated in PBS without H<sub>2</sub>O<sub>2</sub>. Three replicates per concentration were selected for comet assay.

## **Comet assay (single cell gel electrophoresis)**

The comet assay was performed as a three 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 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 first

layer. Finally, it was covered with a third layer of 100  $\mu$ l 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 then 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 neutralized while 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, the slides were stained with ethidium bromide (15  $\mu$ g l<sup>-1</sup>) for 5 min for visualization of DNA damage.

Slides were observed under a fluorescent microscope (Olympus BX51, Japan) at 100X magnification fitted with appropriate filters. Duplicate slides per test concentration per cell type (treated groups) and control group were prepared on each sampled 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 two replicated slides). The comet images were captured using ImagePro AMS 6.0 and analyzed 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.

### **Statistical analysis**

Data obtained from the experiment are expressed as mean  $\pm$  SD. % DNA damage in different treatment groups and tissues were assessed by analysis of variance (ANOVA) (Underwood, 1997) with DoE and SL concentration as sources of variation. Variation in water quality parameters and SGR (% day<sup>-1</sup>) 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 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). Four levels of significance (ns : non significance, \*p < 0.05, \*\*p < 0.01 and \*\*\*p < 0.001) were reported.

## **RESULTS**

### **Acute toxicity study**

There was no mortality observed in 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 pesticide. Mortality in treatment tanks increased with the progress in

exposure time and increase in concentration of CPF. The LC<sub>50</sub> values of CPF for juveniles of *L. vannamei* were 2.18, 1.98, 1.80 and 1.44 µg l<sup>-1</sup> at the end of exposure periods of 24, 48, 72 and 96 h, respectively (Table 1). The LC<sub>50</sub> values decreased with exposure time and a direct correlation between mortality and toxicant concentration was discernible. The 96 h LC<sub>50</sub> concentrations of CPF to *L. vannamei* juveniles was determined to be 1.44 µg l<sup>-1</sup> (95% confidence limits, upper = 1.46 and lower = 0.85 µg l<sup>-1</sup>; p < 0.05). Observed values of LOEC and NOEC were 1.20 µg l<sup>-1</sup> and 0.80 µg l<sup>-1</sup>, respectively.

### **Physico-chemical and growth parameters**

No visible signs of disease nor significant mortality were observed in *Litopenaeus vannamei* juveniles on exposure to two SL concentrations of CPF during 21 DoE. No significant variations in water quality parameters were observed in control and treated tanks (Table 2) during entire experimental routine of 21 DoE (p > 0.05). It was noticeable that juveniles in treated tanks were found to be consuming an adequate amount of ration (visual observation of gut fullness) provided during initial DoE. Beyond 14 DoE, treated shrimps failed to consume a normal amount of food towards the end of experiment while, shrimps in control tanks consumed normal ration of food. Specific growth rates (% day<sup>-1</sup>) 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).

### **In vitro exposure to hydrogen peroxide**

The % tail DNA damage in GL, ML and HL cells on exposure to different concentrations of H<sub>2</sub>O<sub>2</sub> is shown in Fig.1. Results of comet assay showed a significant DNA damage in all studied cells *vis-à-vis* 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.

### **Comparative evaluation of genotoxicity**

Results of ANOVA followed by Tukey–Kramer test multiple comparison test revealed that the % 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 two SL concentrations of CPF *vs.* control was discernible (Fig. 2a, b and c). A time and dose-dependent increment in % tail DNA damage was observed in GL, HL and ML cells (Fig. 2a, b and c). 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 control group at all time intervals ( $p < 0.001$ ). The % tail DNA damage in HL (Fig. 2b) and ML cells (Fig. 2c) also followed a similar trend.

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

## DISCUSSION

### Acute Toxicity

Although due to their relative non-persistence, OPs have replaced organochlorine compound pesticides in agricultural activities, their residues, however have been detected in a variety of medium such as water, marine organisms, in plant and plant products, fish tissues, sediments, in prawn and prawn pond water (Barcelo et al. 1990, Varo et al. 1998, Amaraneni & Pillai 2001, Kumar et al. 2004, Amaraneni 2006, Rao et al. 2007, Halappa & David 2009; Burgos-Hernández et al. 2006, 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, Roque et al. 2005, Rao et al. 2005, Rao 2006, Aniladevi Kunjamma et al. 2008, Halappa & David 2009, Samajdar & Mandal 2015). Such a 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 the 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 the xenobiotic compound.  $LC_{50}$  values of CPF to *Litopenaeus vannamei* juveniles observed in this study (2.18, 1.98, 1.80 and 1.44  $\mu\text{g l}^{-1}$  at 24 h, 48 h, 72 h and 96 h, respectively) are

much lower than those previously reported laboratory derived acute toxicity indices of pesticides containing CPF for other fish and crustacean species. For instance, the freshwater fish *Channa punctatus*, 811.98  $\mu\text{g l}^{-1}$  (Ali et al. 2009); *Labeo bata*, 106.94  $\mu\text{g l}^{-1}$  (Samajdar & Mandal 2015); freshwater shrimp, *Paratya australiensis*, 0.004  $\mu\text{g l}^{-1}$  (Kumar et al. 2010), penaeid shrimp, *Penaeus monodon*, 59.16  $\text{nmol l}^{-1}$  (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 indicated that CPF is highly toxic to juveniles of *L. vannamei* as reflected in the very low 96 h  $\text{LC}_{50}$  value of 1.44  $\mu\text{g l}^{-1}$ . A wide variability in the acute toxicity indices on exposure of *L. vannamei* juveniles to four different organochlorine pesticides (Chlordane, 63; DDT, 8.7; Lorsban, 4.8; Lindane, 3.9  $\mu\text{g l}^{-1}$ ) has been reported by Galindo-Reyes et al (1996). A significantly lower acute toxicity index (9.33  $\text{ng l}^{-1}$ ) has also been reported in *L. vannamei* juveniles on exposure to the permethrin-based insecticide (Mello et al. 2011). Furthermore, the 48 h  $\text{LC}_{50}$  value of 19  $\text{mg l}^{-1}$  and 96 h  $\text{LC}_{50}$  value of 1.46  $\text{mg l}^{-1}$  have been reported with juveniles of *L. vannamei* following exposure to two OP pesticides, fenitrothion (Lignot et al. 1998) and methamidophos (Garcia-de la Parra et al. 2006), respectively. On the basis of the observation made in the present study (96 h  $\text{LC}_{50}$ , 1.44  $\mu\text{g l}^{-1}$ ) 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 the CPF pesticide toxicity.

### **Genotoxicity evaluation in gill, muscle and haemolymph cells**

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 & Steinert, 2003, Frías-Espericueta et al. 2011, Dailianis et al. 2014, Bhagat et al. 2016, Ullah et al. 2015). 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 comet assay (Lee & Steinert 2003).

The % tail DNA damage in *L. vannamei* juveniles as assessed through comet assay was highly tissue-specific and dose and time-dependent (Fig 2a, b, c). 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 ( $30.34 \pm 0.76\%$ ), gill ( $58.17 \pm 0.65\%$ ) and liver ( $47.83 \pm 0.30\%$ ) tissues of *L. rohita* after 96 h exposure of phorate has been reported by Mohanty et al. (2011). On the other hand, both time-dependent and dose-dependent increase in DNA damage in haemocytes of *L. vannamei* ( $40.08 \pm 5.64\%$ ) following exposure to copper has been

observed by Frias Espricueta et al (2011). A dose-dependent increment in DNA damage in gill cell lines of *P. monodon* exposed two OPs (malathion,  $40.81 \pm 9.10\%$  and monocrotophos,  $38.85 \pm 13.68\%$ ) 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 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 three tested tissue cells of *L. vannamei* exposed to SL1 concentration (GL,  $53.61 \pm 8.71$ ), (HL,  $49.36 \pm 3.42$ ) and (ML,  $32.40 \pm 4.97$ ) was significantly higher (Fig. 2a, b, c;  $p < 0.05$ ) compared to those exposed to SL2 concentration (GL,  $39.25 \pm 3.90$ ), (HL,  $32.22 \pm 4.21$ ) and (ML,  $22.66 \pm 2.85$ ) (Fig. 2a, b, c). The greater magnitude of variability in DNA damage witnessed in different cells of *L. vannamei* on exposure to two SL concentrations of CPF could be attributed to the generation of high amount of free radicals. The potential of CPF to induce oxidative stress in organisms and cause over production of ROS (reactive oxygen species) has documented by Livingstone (2001). The production of higher amounts of free radicals is a consequence of increased metabolic activities resulting in damage to the protein and nucleic acids on exposure to OP compounds have been reported (Hodgson & Levi 1996, Mohanty et al. 2011, Bhagat et al. 2016). Therefore, a dose-dependant 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 higher SL concentration.

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 concentrations (Fig 3a, c and Fig. 4a, c). An increment in DNA damage in GL of *L. vannamei* juveniles with the DoE was also observed. Such a 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 freshwater fish, *C. punctatus*. In crustaceans, gills 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 & 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 juveniles of *L. vannamei* following sublethal exposure of four 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 inefficient detoxification system in shrimps and prawns compared to fishes (Hayashi et al. 1998, Cavas & Ergene-Gozukara 2003, 2005, Mohanty et al. 2011). Furthermore, an impairment of the gill structure and ultrastructure in juveniles of *Penaeus monodon* after 96 h exposure to lethal concentrations to OP pesticide, gusathion A has been documented by Baticados & Tendencia (1991). It has been hypothesized 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, Goksoyr et al. 1991, Galindo-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, 1991, James et al. 1998, Koenig et al. 2012). Furthermore, it must be emphasised that physico-chemical interactions between various types of organism and rearing medium may result in variable response in different cells on exposure to xenobiotic compounds (Ali et al. 2009). A high variability in genotoxic response observed in different cells during 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 a number of xenobiotic compounds in their ecosystem (Krieger 2001). Furthermore, the presence of pesticide in rearing water weakens shrimp immune system and cause an outbreak of infectious diseases at lower concentrations (Roque et al. 2005, Mello et al. 2011). A 27-53% shell softening in *P. monodon* juveniles during 96 h exposure to OP pesticide, gusathion A has been reported by Baticados & Tendencia (1991). Furthermore, a 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 present study, specifically in ML cells – main edible body part of shrimp. Significant difference ( $p < 0.05$ ) in SGR (% day<sup>-1</sup>) recorded between control and treated groups (SL1 and SL2) of *L. vannamei*

juveniles during 21 days of exposure supports the contention. A significantly marked decrement in protein content (28–42%) in juveniles of *L. vannamei* on exposure to four 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 the exposure (28 DoE) to sublethal concentrations 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). Therefore, it appears that the SL concentrations of CPF are capable of inflicting DNA damage, 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 the laboratory derived results due to water-sediment interactions as Holmes et al. (2008).reported that the presence of sediment enhances the toxicity of pesticides

## CONCLUSION

The results of acute toxicity test (96 h LC<sub>50</sub>) showed high toxic potential of CPF to juveniles of *Litopenaeus vannamei* and could serve as baseline data for entry and high risk of such pesticides into aquacultural system. Based on the degree of DNA damage recorded in different cells (GL, ML and HL) implies that juveniles of *L. vannamei* are highly sensitive in detecting genotoxic effects induced by OPs, particularly CPF. The results also point to the potential risks involved in extension of farming *L. vannamei* 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 the immune responses in different shrimp life stages and varying culture conditions on exposure to OPs would advance our current understanding in shrimp nutritional pathology. Such an information would facilitate the development of diagnostic tools for better decision making in aquaculture management.

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## LITERAURE CITED

- Abbott WS (1925) A method of computing the effectiveness of an insecticide. *J Econ Entomol* 18: 265–267
- APHA (1992) Standard methods for the examination of water and wastewater. American Public Health Association, Washington, D.C. 5220 D, p.5–9.
- Ali D, Nagpure NS, Kumar S, Kumar R, Kushwaha B, Lakra WS (2009) Assessment of genotoxic and mutagenic effects of chlorpyrifos in freshwater fish *Channa punctatus* (Bloch) using micronucleus assay and alkaline single cell gel electrophoresis. *Food Chem Toxicol* 47: 650–656
- Amaraneni SR, Pillai RR (2001) Concentrations of pesticide residues in tissues of fish from Kolleru Lake in India. *Environ Toxicol* 16: 550–556
- Amaraneni SR (2006) Distribution of pesticides, PAHs and heavy metals in prawn ponds near Kolleru Lake wetland, India. *Environ Int* 32: 294–302
- Aniladevi Kunjamma KP, Philip B, Bhanu SV, Jose J (2008) Histopathological effects on *Oreochromis mossambicus* (Tilapia) exposed to chlorpyrifos. *J Environ Res Dev* 2:553–559
- Araldi RP, de Melo TC, Mendes TB, de Sá Júnior PL, Nozima BH, Ito ET, and others (2015) Using the comet and micronucleus assays for genotoxicity studies: A review. *Biomed Pharmacother* 72:74–82
- Banu BS, Danadevi K, Rahman MF, Ahuja YR, Kaiser J (2001) Genotoxic effect of monocrotophos to sentinel species using comet assay. *Food Chem Toxicol* 39: 361–366
- Barcelo D, Porte C, Cid J, Albaigés J (1990) Determination of organophosphorus compounds in Mediterranean coastal waters and biota samples using gas chromatography with nitrogen-phosphorus and chemical ionization mass spectrometric detection. *Int J Environ Anal Chem* 38:199–209

- Baticados MaCL, Tendencia EA (1991) Effects of Gusathion A on the survival and shell quality of juvenile *Penaeus monodon*. *Aquaculture* 93:9–19
- Bhagat J, Sarkar A, Ingole BS (2016) DNA damage and oxidative stress in marine gastropod, *Morula granulata* exposed to phenanthrene. *Water Air Soil Pollut* 227: 114 [https://doi: 10.1007/s11270-016-2815-1](https://doi.org/10.1007/s11270-016-2815-1)
- Bhardwaj T, Sharma JP (2013) Impact of pesticides application in agriculture industry: An Indian scenario. *Int J Agr Food Sci Technol* 4:817–822
- Bhavan PS, Geraldine P (2000) Histopathology of the hepatopancreas and gills of the prawn *Macrobrachium malcolmsonii* exposed to endosulfan. *Aquat Toxicol* 50:331–339
- Buikema AL, Niederlehner BR, Cairns JJr (1982) Biological monitoring. Part IV. Toxicity testing. *Water Res* 16:239–262
- Burgos-Hernández A, Zapién MGL, Madrid MLA, Sifuentes COG, Gil CIM, Burgos ECR, Olivas RR (2006) Presence of insecticides in shrimp farms adjacent to the Sea of Cortes: detection, quantification, and toxicity testing. *Eur Food Res Technol* 222: 380–384
- Buschini A, Carboni P, Martino A, Poli P, Rossi C (2003) Effects of temperature on baseline and genotoxicant induced DNA damage in haemocytes of *Dreissena polymorpha*. *Mutat Res* 537: 81–92
- Butcherine P, Benkendorff K, Kelaher B, Barkla BJ (2018) The risk of neonicotinoid exposure to shrimp aquaculture, *Chemosphere* (2018), doi: <https://doi.org/10.1016/j.chemosphere.2018.10.197>.
- Cavas T, Ergene-Gozukara S (2003) Evaluation of the genotoxic potential of lambda cyhalothrin using nuclear and nucleolar biomarkers on fish cells. *Mutat Res* 534: 93–99
- Cavas T, Ergene-Gozukara S (2005) Induction of micronuclei and nuclear abnormalities in *Oreochromis niloticus* following exposure to petroleum refinery and chromium processing plant effluents. *Aquat Toxicol* 74:264–271

- Chandrasekara LWHU, Pathiratne A (2007) Body size related differences in the inhibition of brain acetylcholinesterase activity in juvenile Nile tilapia (*Oreochromis niloticus*) by chlorpyrifos and carbosulfan. *Ecotox Environ Safe* 67:109–119
- Das S, Adhya TK (2015) Degradation of chlorpyrifos in tropical rice soils. *J Environ Manag* 152:36–42
- Dailianis S, Tsarpali V, Melas K, Karapanagioti HK, Manariotis ID (2014) Aqueous phenanthrene toxicity after high frequency ultrasound degradation. *Aquat Toxicol* 147:32–40
- Dhawan A, Bajpayee M, Patel S, Pandey AK, Shukla RK, Parmar D (2008) *In vivo* and *in vitro* alkaline single cell gel electrophoresis/comet assay. In: Dhawan A, Shanker R (eds) Protocol manual: Alexander Hollaender workshop on genetic toxicology, Indian institute of toxicology research, p 12–23
- Dzwonkowska A, Hubner H (1986) Induction of chromosomal aberrations in the Syrian hamster by insecticides tested *in vivo*. *Arch Toxicol* 58:152–156
- Eamkamon T, Klinbunga S, Thirakhupt K, Menasveta P, Puanglarp N (2012) Acute toxicity and neurotoxicity of chlorpyrifos in black tiger shrimp, *Penaeus monodon*. *Environ Asia* 5:26–31
- Edwards CA (1973) *Environmental Pollution by Pesticides*. Plenum Press, New York.
- Environmental Protection Agency (1981) Acephate, aldicarb, carbophenothion, DEF, EPN, ethoprop, methyl parathion, and phorate: Their acute and chronic toxicity, bioconcentration potential, and persistence as related to marine environments. EPA 600/4-81-023. Gulf Breeze, FL.
- FAO (2018) *The State of World Fisheries and Aquaculture– Meeting the sustainable development goals*. Rome. Licence: CC BY-NC-SA 3.0 IGO, pp. 227
- Finney DJ (1971) *Probit Analysis*. University Press, Cambridge, p 333.
- Frasco MF, Fournier D, Carvalho FL, Guilhermino (2006) Cholinesterase from the common prawn (*Palaemon serratus*) eyes: catalytic properties and sensitivity to organophosphate and carbamate compounds. *Aquat Toxicol* 77: 412–421



- Frías-Espericueta MG, Aguilar Juárez M, Voltolina Lobina DF, Paniagua Chávez CG (2011) Effect of Cu on haemocytic DNA of the white shrimp, *Litopenaeus vannamei*, assessed by the comet assay. *J World Aquacult Soc* 42: 586–590
- Galindo-Reyes JG, Jasso AM, Lizarraga CV (1996) Toxic effect of organochlorine pesticides on *Penaeus vannamei* shrimps in Sinaloa, Mexico. *Chemosphere* 33:567–575
- Galindo-Reyes JG, Leyva NR, Millan OA, Lazcano GA (2002) Effects of pesticides on DNA and protein of shrimp larvae *Litopenaeus stylirostris* of the California Gulf. *Ecotox Environ Safe* 53:191–195
- García-de la Parra LM, Bautista-Covarrubiasa JC, Rivera-de la Rosaa N, Betancourt-Lozanoa M, Guilhermino, N (2006) Effects of methamidophos on acetylcholinesterase activity, behavior and feeding rate of the white shrimp (*Litopenaeus vannamei*). *Ecotox Environ Safe* 65:373–380
- Girón-Pérez MI, Santerre A, Gonzalez-Jaime F, Casas-Solis J, Hernández-Coronado M., Peregrina-Sandoval J, Takemura A, Zaitseva G (2007) Immunotoxicity and hepatic function evaluation in Nile tilapia (*Oreochromis niloticus*) exposed to diazinon. *Fish Shellfish Immunol* 23: 760–769
- Goksoyr A, Andersson T, Buhler DR, Stegeman JJ, Williams DE, Forlin L (1991) Immunochemical cross reactivity of  $\beta$ -naphtho-avone-inducible cytochrome P450 (P450 1A) in liver microsomes from different fish species and rat. *Fish Physiol Biochem* 9: 1–13
- Haasch ML, Wejksnora PJ, Stegeman JJ, Lech JJ (1989) Cloned rainbow trout liver P450 complementary DNA as a potential environmental monitor. *Toxicol Appl Pharm* 98:362–368
- Halappa R, David M (2009) Behavioural responses of the freshwater fish, *Cyprinus carpio* (Linnaeus) following sublethal exposure to chlorpyrifos. *Turk J Fish Aquat Sci* 9:233–238
- Hayashi M, Ueda T, Uyeno K, Wada K, Kinai N, Saotome K et al. (1998) Development of genotoxicity assay systems that use aquatic organisms. *Mutat Res* 399:125–133
- Hodgson E, Levi PE (1996) Pesticides: an important but underused model for the environmental health sciences. *Environ Health Persp* 104:97–106

- Holmes RW, Anderson BS, Phillips BM, Hunt JW, Crane DB, Mekebre A, Connor V (2008) State-wide investigation of the role of pyrethroid pesticides in sediment toxicity in California's urban waterways. *Environ Sci Technol* 42: 7003–7009
- Hook SE, Doan H, Gonzago D, Musson D, Du J, Kookana R, Sellars MJ, Kumar A (2018) The impacts of modern-use pesticides on shrimp aquaculture: An assessment for north eastern Australia. *Ecotoxicol Environ Safe* 148: 770–780
- James MO, Boyle SM (1998) Cytochromes P450 in crustacea. *Comp Biochem Phys C* 121:157–172
- Jose S, Jayesh P, Mohandas A, Philip R, Singh ISB (2011) Application of primary haemocyte culture of *Penaeus monodon* in the assessment of cytotoxicity and genotoxicity of heavy metals and pesticides. *Mar Environ Res* 71:169–177
- Klobucar GIV, Pavlica M, Erben R, Papes D (2003) Application of the micronucleus and comet assay to mussel *Dreissena polymorpha* haemocytes for genotoxicity monitoring of freshwater environments. *Aquat Toxicol* 64:15–23
- Krieger R (2001) *Handbook of Pesticide Toxicology agents*. San Diego: Academic Press.
- Koenig S, Fernandez P, Sole M (2012) Differences in cytochrome P450 enzyme activities between fish and crustacea: Relationship with the bioaccumulation patterns of polychlorobiphenyls (PCBs). *Aquat Toxicol* 108:11–17
- Kumar A, Nadda G, Shanker A (2004) Determination of chlorpyrifos 20% EC (Dursban 20 EC) in scented rose and its products. *J Chromatogr A* 1050:193–199
- Kumar A, Correll R, Grocke S, Bajet C (2010) Toxicity of selected pesticides to freshwater shrimp, *Paratya australiensis* (Decapoda: Atyidae): use of time series acute toxicity data to predict chronic lethality. *Ecotox Environ Safe* 73: 360–369
- Kumar G, Engle CR (2016) Technological advances that led to growth of shrimp, salmon and tilapia farming. *Rev Fish Sci Aquac* 24:136–152

- Kumar U, Berliner J, Adak T, Rath PC, Dey A, Pokhare SS et al. (2017) Non-target effect of continuous application of chlorpyrifos on soil microbes, nematodes and its persistence under sub-humid tropical rice-rice cropping system. *Ecotox Environ Safe* 135:225–235
- Labrie L, Roque A, Gomez-Gil B, Turnbull JF (2003) Effect of methyl parathion on the susceptibility of shrimp *Litopenaeus vannamei* to experimental vibriosis. *Dis Aquat Organ* 57:265–270
- Lee RF, Steinert S (2003) Use of the single cell gel electrophoresis/ Comet Assay for detecting DNA damage in aquatic (marine and freshwater) animals. *Mut Res* 544:43–64.
- Lignot JH, Charmantier G, Cochard JC (1998) Effect of an organophosphorus insecticide, fenitrothion, on survival, osmoregulation, and acetylcholinesterase activity in different life stages of two penaeid shrimps: *Penaeus stylirostris* and *Penaeus vannamei* (crustacean, decapoda). *J. Shellfish Res* 17: 1251–1258
- Livingstone DR (1989) Cytochrome P450 and oxidative metabolism in molluscs. *Xenobiotica* 19:1041–1062
- Livingstone DR (1991) Organic xenobiotic metabolism in marine invertebrates. In: Gilles R (ed) *Advances in Comparative and Environmental Physiology*, Springer-Verlag, Berlin, pp. 45–185
- Livingstone DR (2001) Contaminant stimulated reactive oxygen species production and oxidative damage in aquatic organisms. *Mar Pollut Bull* 42:656–666
- Matsumoto ST, Mantovani MS, Malagutti MII, Dias AL, Fonsca IC, Martin-Morales MA (2006) Genotoxicity and mutagenicity of water contaminated with tannery effluents as evaluated by the micronucleus test and comet assay using the fish, *Oreochromis niloticus* and chromosome aberrations in onion root-tips. *Genet Mol Biol* 29:148–158
- Mello GLD, Junior HA, Garcia S, Vinatea L (2011) Acute toxicity of pyrazosulfuron-ethyl and permethrin to juvenile *Litopenaeus vannamei*. *Acta Sci Biol Sci* 33:1–6

- Mohanty G, Mohanty J, Nayak AK, Mohanty S, Dutta SK (2011). Application of comet assay in the study of DNA damage and recovery in rohu (*Labeo rohita*) fingerlings after an exposure to phorate, an organophosphate pesticide. *Ecotoxicol* 20:283–292
- Narra MR, Kodimiyala R, Rao JV, Begum G (2013) Evaluation of the biochemical stress response to chlorpyrifos in tissue of the edible crab *Barytelphusa guerini*: withdrawal of exposure improves the nutritional value. *Z Naturforsch C* 68: 318–326
- Pavlaki MD, Araujo MJ, Cardoso DN, Silva AR, Cruz A, Mendo S et al. (2016) Ecotoxicity and genotoxicity of cadmium in different marine trophic levels. *Environ Pollut* 215:203–12
- Pawar KR, Katdare M (1984) Effects of sub-lethal and lethal concentrations of fenitrothion, BHC, and carbofuran on behaviour and oxygen consumption of the FW prawn *Macrobrachium kistnensis* (Tiwari). *Arch Hydrobiol* 99: 398–403
- Persoone G, Vanhaecke P, Gobbers E (1985) Evaluation of the impact of parathion, methyl parathion, fenitrothion, and fenthion on the aquatic environment. Report of the ECC XI/785/83, 579 p.
- Pequeux A (1995) Osmotic regulation in crustaceans. *J Crustacean Biol* 15: 1–60
- Rao JV, Rani CHS, Kavitha P, Rao RN, Madhavendra SS (2003) Toxicity of chlorpyrifos to the fish *Oreochromis mossambicus*. *B Environ Contam Tox* 70:985–992
- Rao JV, Ghousia B, Pallela R, Usman PK, Nageswara Rao R (2005) Changes in behavior and brain acetylcholinesterase activity in mosquito fish, *Gambusia affinis* in response to the sublethal exposure to chlorpyrifos. *Int J Environ Res Pub Health* 2:478–483
- Rao JV (2006) Toxic effects of novel organophosphorus insecticide (RPR-V) on certain biochemical parameters of euryhaline fish, *Oreochromis mossambicus*. *Pestic Biochem Phys* 86:78–84
- Rao JV, Kavitha P, Jakka NM, Sridhar V, Usman PK (2007) Toxicity of organophosphates on morphology and locomotor behavior in brine shrimp, *Artemia salina*. *Arch Environ Contam Toxicol* 53:227–232

- Roque A, Abad S, Betancourt-Lozano M, Parra LMG, Baird D, Guerra-Flores AL, Gomez-Gil B (2005) Evaluation of the susceptibility of the cultured shrimp *Litopenaeus vannamei* to vibriosis when orally exposed to the insecticide methyl parathion. *Chemosphere* 60:126–134
- Roy LA, Davis DA, Saoud IP, Boyd CA, Pine HJ., Boyd CE (2010) Shrimp culture in inland low salinity waters. *Rev Aquacult* 2:191–208
- Samajdar I, Mandal DK (2015) Acute toxicity and impact of an organophosphate pesticide, chlorpyrifos on some haematological parameters of an Indian minor carp, *Labeo bata* (Hamilton 1822). *Int J Environ Sci* 6:106–113
- Sancho E, Fernandez-Vega C, Ferrando MD, Andreu-Moliner E (2003) Eel ATPase activity as biomarker of thiobencarb exposure. *Ecotox Environ Safe* 56:434–441
- Serrano R, Hernandez F, Pena JB, V Dosda Canales J (1995) Toxicity and bioconcentration of selected organophosphorus pesticides in *Mytilus galloprovincialis* and *Venus gallina*. *Arch Environ Contam Toxicol* 29:284–290
- Singh NP, McCoy MT, Tice RR, Schneider EL (1988) A simple technique for quantification of low-levels of DNA damage in individual cells. *Exp Cell Res* 175:184–191
- Siu WHL, Cao J, Jack RW, Wu RSS, Richardson BJ, Xu L, Lam PKS(2004) Application of the comet and micronucleus assays to the detection of genotoxicity in haemocytes of the green-lipped mussel (*Perna viridis*). *Aquat Toxicol* 66: 381–392
- Sultana R, Ali W, Ameer F, Munshi AB, Nasir M (2012) Accumulation of pesticide residues by shrimp, fish and brine shrimp during pond culture at Ghorabari (District Thatta). *J Chem Soc Pakistan* 34: 541–549
- Suryavanshi U, Sreepada RA, Ansari ZA, Nigam S, Badesab S (2009) A study on biochemical changes in the penaeid shrimp, *Metapenaeus monoceros* (Fabricius) following exposure to sublethal doses of organochlorine pesticide (endosulfan). *Chemosphere* 77:1540–1550

- Thi Tu H, Silvestre F, Meulder BD, Thome JP, Phuong NT, Kestemont P (2012) Combined effects of deltamethrin, temperature and salinity on oxidative stress biomarkers and acetylcholinesterase activity in the black tiger shrimp (*Penaeus monodon*). *Chemosphere* 86:83–91
- Ullah S, Begum M, Dhama K, Ahmad S, Hassan S, Alam I (2016) Malathion induced DNA damage in freshwater fish, *Labeo rohita* (Hamilton, 1822) using alkaline single cell gel electrophoresis. *Asian J Anim Vet Adv* 11: 98–105
- Underwood AJ (1997) *Experiments in Ecology: their logical and interpretation using Analysis of Variance*. Cambridge University Press. Cambridge, UK p. 504
- Varo I, Serrano R, Navarro JC, Lopez FJ, Amat F (1998) Acute lethal toxicity of the organophosphorus pesticide chlorpyrifos to different species and strains of *Artemia*. *B Environ Contam Toxicol* 61:778–785
- Zar JH (1996) *Biostatistical Analysis*. Prentice Hall, Upper Saddle River, New Jersey, USA. p. 481
- Zeid EHA, Khalil ALSA (2014) Effects of acute fenitrothion insecticide exposure on DNA damage and oxidative stress biomarkers and health of Nile Tilapia fingerlings, *Oreochromis niloticus* L. *World J Fish Mar Sci* 6:361–370

**Table 1:** Median lethal concentrations (LC<sub>50</sub>) of chlorpyrifos (CPF) to juveniles of *Litopenaeus vannamei* tested for different periods of exposure.

Exposure period	Median lethal concentrations (LC <sub>50</sub> values)		
	µg l <sup>-1</sup>	Lower limit	Upper limit
24 h	2.18	1.98	2.63
48 h	1.98	1.78	2.36
72 h	1.80	1.40	1.93
96 h	1.44	0.86	1.47

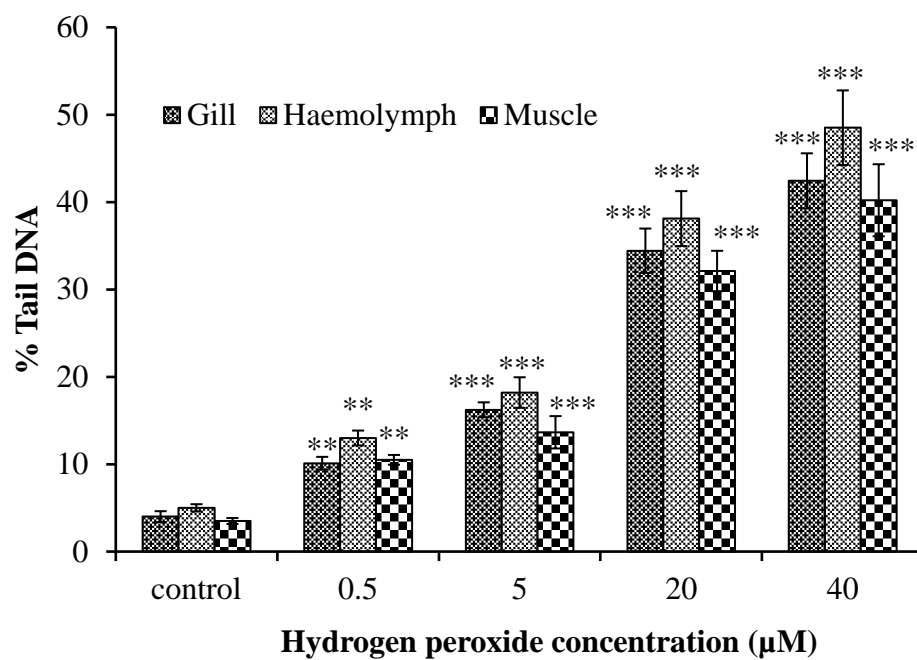
**Table 2.** Data on water quality parameters recorded (mean ± SD) in treatment and control aquaria with juveniles of *L. vannamei* during chronic exposure experiment (21 DoE) of chlorpyrifos (CPF)

Parameter	Control	SL1	SL2
Temperature (°C)	28.68 ± 0.30	28.36 ± 0.37	28.45 ± 0.38
Dissolved Oxygen (mg l <sup>-1</sup> )	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 <sup>-1</sup> )	30 ± 0.4	30 ± 0.3	30 ± 0.3
NO <sub>2</sub> -N (mg l <sup>-1</sup> )	0.02 ± 0.003	0.02 ± 0.004	0.02 ± 0.003

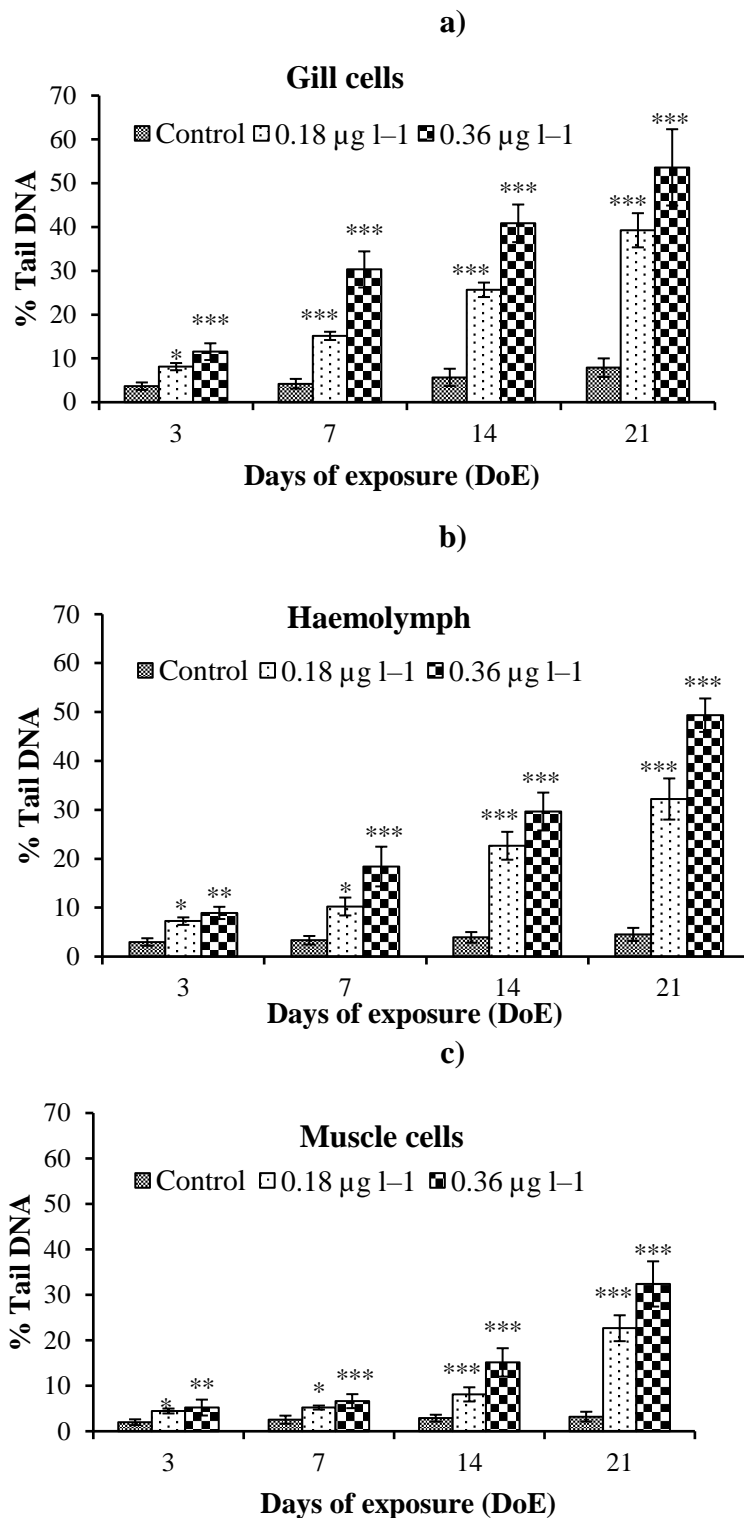
**Table 3.** Data on growth of juveniles of *Litopenaeus vannamei* recorded in treatment and control aquaria (mean  $\pm$  SD) during chronic exposure experiment with chlorpyrifos (CPF).

<b>Growth parameters</b>	<b>Control</b>	<b>SL1</b>	<b>SL2</b>
Initial weight 0 DoE (g)	1.79 $\pm$ 0.04	1.79 $\pm$ 0.056	1.79 $\pm$ 0.06
Final weight 21 DoE (g)	4.10 $\pm$ 0.05	3.75 $\pm$ 0.045	3.82 $\pm$ 0.05
Specific Growth rate (% day <sup>-1</sup> )	1.72 $\pm$ 0.32	1.53 $\pm$ 0.40	1.57 $\pm$ 0.38

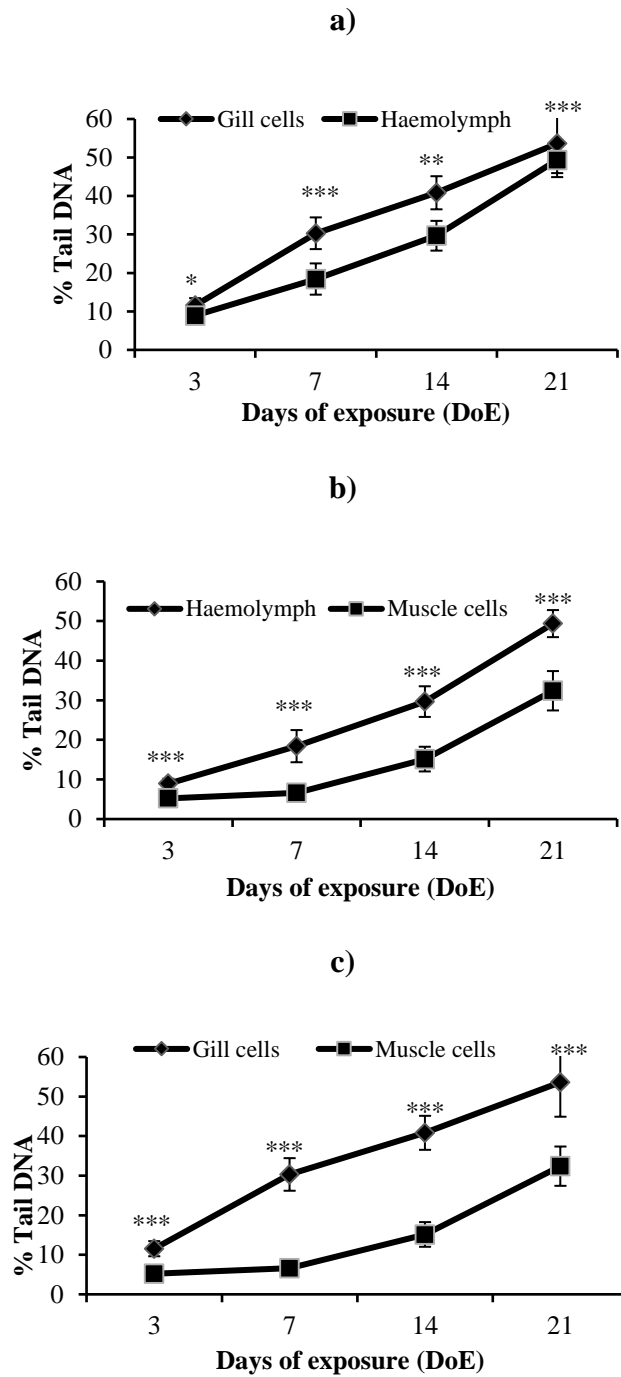




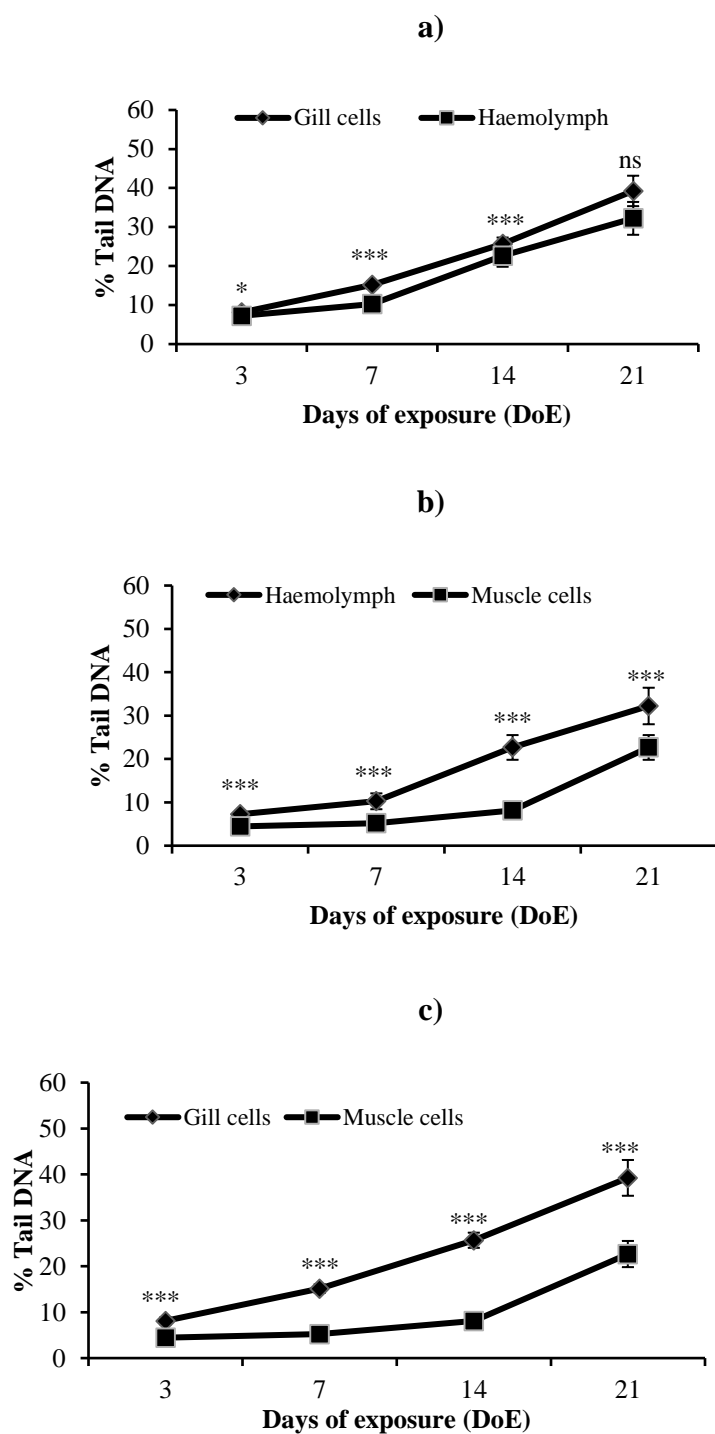
**Fig. 1.** The % DNA damage in different cells of *L. vannamei* juveniles exposed to different concentrations of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>). Each point denotes mean and vertical bars represent standard deviation (*n* = 100 comets). \* = *p* < 0.05, \*\* = *p* < 0.01 and \*\*\* = *p* < 0.001



**Fig. 2** a, b & c. % tail DNA in different cells of *L. vannamei* juveniles exposed to sublethal (SL) concentrations. (SL1 = 0.36 µg l<sup>-1</sup> and SL2 = 0.18 µg l<sup>-1</sup>) of chlorpyrifos (CPF) at the end of 3, 7, 14 and 21 days of exposure (DoE). Data shown is mean and vertical bars represent standard deviation ( $n = 100$  comets). \* =  $p < 0.05$ , \*\* =  $p < 0.01$  and \*\*\* =  $p < 0.001$ .



**Fig. 3** a, b & c. Comparison of % DNA damage induced by SL1 ( $0.36 \mu\text{g l}^{-1}$ ) of chlorpyrifos (CPF) in GL, HL, and ML cells of *L. vannamei* juveniles at the end of 3, 7, 14, and 21 DoE. Each point denotes mean and vertical bars represent standard deviation \* =  $p < 0.05$ , \*\* =  $p < 0.01$  and \*\*\* =  $p < 0.001$ .



**Fig. 4** a, b & c. Comparison of % DNA damage induced by SL2 ( $0.18 \mu\text{g l}^{-1}$ ) of chlorpyrifos in GL, HL, and ML cells of *L. vannamei* juveniles at end of 3, 7, 14, and 21 DoE. Each point denotes mean and vertical bars represent standard deviation. ns : non significant, \* =  $p < 0.05$ , \*\* =  $p < 0.01$ , \*\*\* =  $p < 0.001$ .