



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

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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 \pm SD) exposed to 2 sub-lethal (SL) concentrations, SL1 ($0.36 \mu\text{g l}^{-1}$) and SL2 ($0.18 \mu\text{g l}^{-1}$), 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 ($1.44 \mu\text{g l}^{-1}$) 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

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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-Espéricueta 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 μ m) 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: $28.5 \pm 0.5^\circ\text{C}$, salinity: 30 ± 0.5 g l⁻¹, DO: 6.1 ± 0.4 mg l⁻¹, pH: 8.2 ± 0.2 , NO₂-N: <0.02 mg l⁻¹ and NH₃/NH₄: 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, NO₂-N and NH₃/NH₄ 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, NO₂-N: <0.02 mg l⁻¹ and NH₃/NH₄: 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 (LC₅₀) 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 LC₅₀ 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 LC₅₀ 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 $SGR = [(\ln W_2 - \ln W_1) / (t_2 - t_1)] \times 100$, 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, NO₂-N and NH₃/NH₄ 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 micro-centrifuge 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 10⁶ cells ml⁻¹ were used for the comet assay.

2.7. *In vitro* exposure to hydrogen peroxide

A standard genotoxin, hydrogen peroxide (H₂O₂), 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 H₂O₂ (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 H₂O₂. 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 \pm 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 LC₅₀ values of CPF for juveniles of *Litopenaeus vannamei* were 2.18, 1.98, 1.80 and 1.44 $\mu\text{g l}^{-1}$ at the end of exposure periods of 24, 48, 72 and 96 h, respectively (Table 1). LC₅₀ values decreased with exposure time, and a direct correlation between mortality and toxicant concentration was discernible. The 96 h LC₅₀ concentration of CPF to *L. vannamei* juveniles was determined to be 1.44 $\mu\text{g l}^{-1}$ (95 % confidence limits: upper = 1.47 and lower = 0.86 $\mu\text{g l}^{-1}$; $p < 0.05$). Observed values of LOEC and NOEC were 1.20 and 0.80 $\mu\text{g l}^{-1}$, 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 (LC₅₀) of chlorpyrifos to juveniles of *Litopenaeus vannamei* tested during different exposure periods

Exposure period (h)	LC ₅₀ ($\mu\text{g l}^{-1}$)	95 % confidence limits ($\mu\text{g l}^{-1}$)	
		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 \pm 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 $\mu\text{g l}^{-1}$ (0.18 $\mu\text{g l}^{-1}$)

Parameter	Control	SL1	SL2
Temperature ($^{\circ}\text{C}$)	28.68 \pm 0.30	28.36 \pm 0.37	28.45 \pm 0.38
Dissolved oxygen (mg l ⁻¹)	5.98 \pm 0.20	5.91 \pm 0.22	5.88 \pm 0.15
pH	7.91 \pm 0.20	7.71 \pm 0.10	7.65 \pm 0.12
Salinity (g l ⁻¹)	30 \pm 0.4	30 \pm 0.3	30 \pm 0.3
NO ₂ -N (mg l ⁻¹)	0.02 \pm 0.003	0.02 \pm 0.004	0.02 \pm 0.003

Table 3. Growth (mean \pm 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 \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 (% d ⁻¹)	1.72 \pm 0.32	1.53 \pm 0.40	1.57 \pm 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 H₂O₂ 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: $53.61 \pm 0.71\%$, HL: $49.36 \pm 3.42\%$ and ML: $32.40 \pm 4.97\%$) compared to those exposed to SL2 (GL: $39.25 \pm 3.90\%$, HL: $32.22 \pm 4.21\%$ and ML: $22.66 \pm 2.85\%$) ($p < 0.001$). 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.

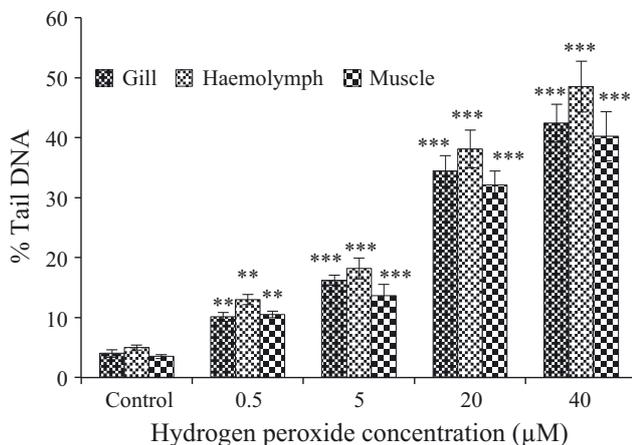


Fig. 1. Percent DNA damage in different cells of *Litopenaeus vannamei* juveniles exposed to different concentrations of hydrogen peroxide. Data are mean \pm SD ($n = 100$ comets). ** $p < 0.01$, *** $p < 0.001$

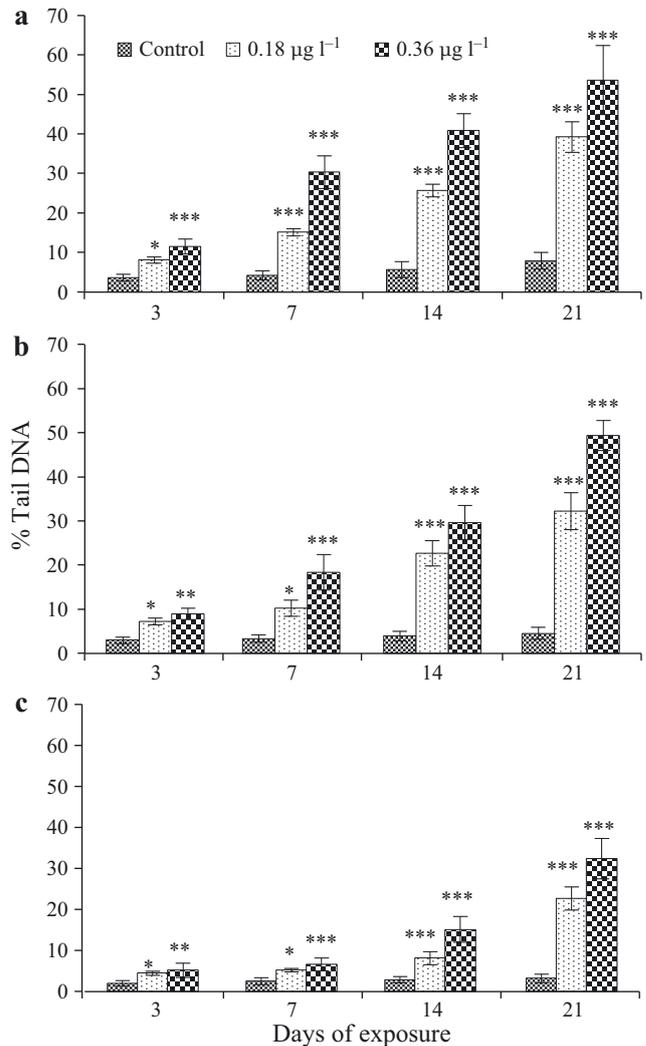


Fig. 2. Percent tail DNA in different cells of *Litopenaeus vannamei* juveniles exposed to 2 sublethal concentrations (SL1 = $0.36 \mu\text{g l}^{-1}$, SL2 = $0.18 \mu\text{g l}^{-1}$) of chlorpyrifos at the end of 3, 7, 14 and 21 d of exposure in (a) gill cells, (b) haemolymph and (c) muscle cells. Data are mean \pm 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

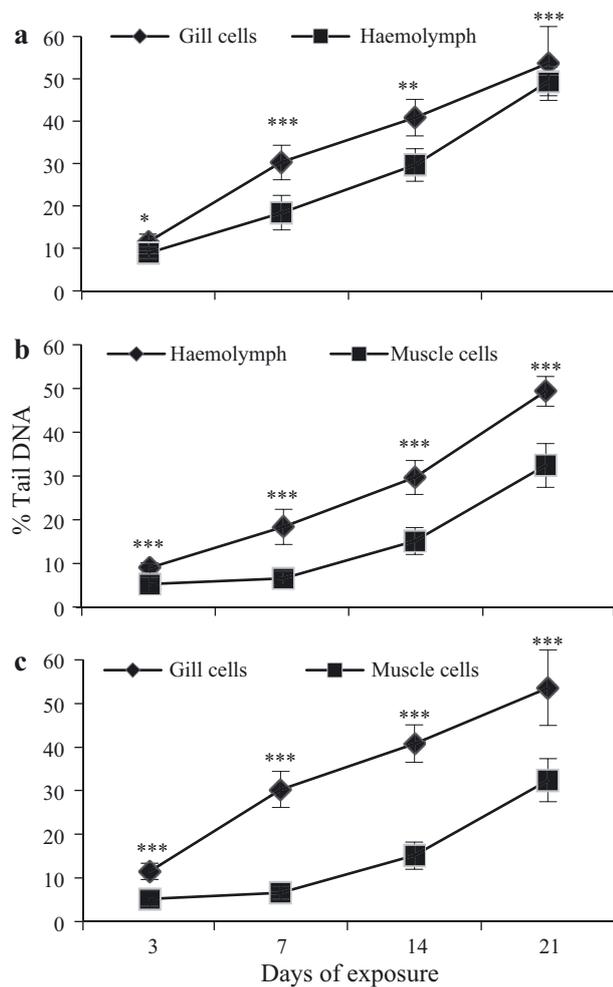


Fig. 3. Comparison of % DNA damage induced by sublethal concentration SL1 ($0.36 \mu\text{g l}^{-1}$) of chlorpyrifos in gill, haemolymph, and muscle cells of *Litopenaeus vannamei* juveniles at the end of 3, 7, 14 and 21 days of exposure. (a) Gill vs. haemolymph, (b) haemolymph vs. muscle, (c) gill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

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,

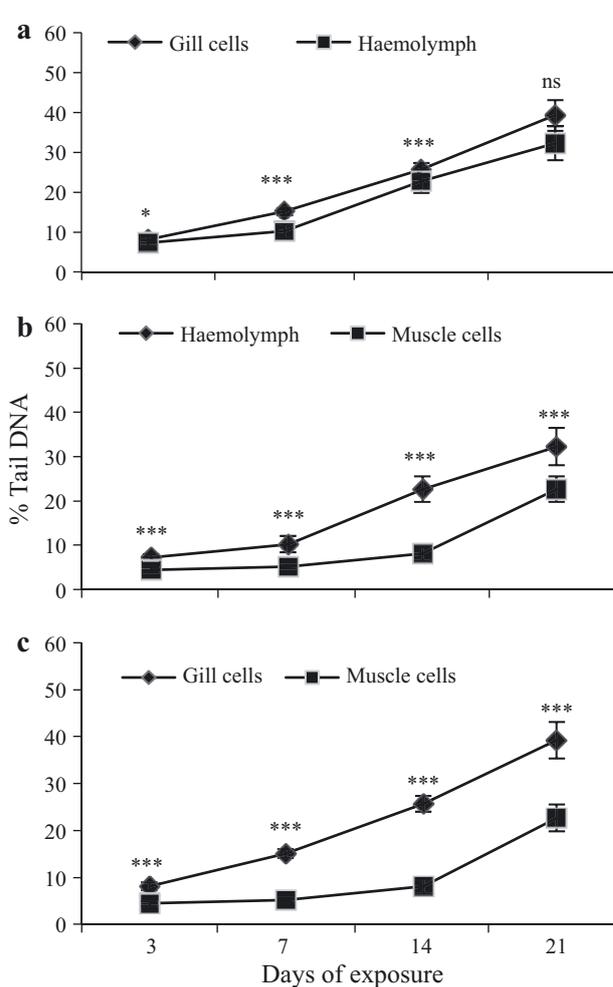


Fig. 4. Comparison of % DNA damage induced by sublethal concentration SL2 ($0.18 \mu\text{g l}^{-1}$) of chlorpyrifos in gill, haemolymph, and muscle cells of *Litopenaeus vannamei* juveniles at the end of 3, 7, 14 and 21 days of exposure. (a) Gill vs. haemolymph, (b) haemolymph vs. muscle, (c) gill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

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 LC₅₀) is an important tool to assess the toxic tolerance range of particular species to xenobiotic compounds. LC₅₀ 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 LC₅₀ 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 LC₅₀ value of 19 mg l⁻¹ and 96 h LC₅₀ 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 LC₅₀, 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 mosambicus* (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 LC₅₀) 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.

Acknowledgements. The authors are grateful to the director of the CSIR-National Institute of Oceanography, Goa, India, and the head of the Zoology Department, Goa University, Goa, for encouragement and use of facilities. The authors also thank Dr. Aswani Volety, University of North Carolina, Wilmington, NC, USA, and 3 anonymous reviewers for their valuable suggestions in improving the manuscript. This represents contribution No. 6334 of the CSIR-National Institute of Oceanography, Goa, India.

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