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Lethal and Sub-lethal Interactive Toxicity of Endosulfan and Lindane on Black Tiger Shrimp (*Penaeus monodon* Fabricius)

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ABSTRACT. *Individual and combination effects of endosulfan and lindane on survivability and ecological competence of commercially important shrimp postlarvae of Penaeus monodon Fabricius were studied using spiked-water toxicity tests. Dose-response studies in static, non-renewal tests revealed that NOEC, LOEC and LC_{50} (96 h) of endosulfan for survival effect in spiked-water toxicity tests based on mean concentrations were 0.536, 1.038 and 1.60 µg/l, respectively. The respective statistical endpoints ($p<0.05$) for lindane were 0.617, 1.638 and 3.01 µg/l. The acute mixture effect was additive represented by negative additive index which ranged from -1.04 to 0.25. The whole body homogenate phenoloxidase enzyme activity was significantly ($p<0.05$) affected by endosulfan and lindane compared to respective controls. The effects on enzyme activity were biphasic (up-regulation and down-regulation) for individual pesticides but the mixture effect was monophasic. It was demonstrated that single exposure to endosulfan decreased enzyme activity by 86% while it was 61% for lindane in low-dose phase. Up to 99% increase of enzyme activity was recorded in higher dose range of endosulfan while it was 51% for lindane. The combination of compounds increased the enzyme activity by 61% over the control. The NOEC and LOEC for enzyme depressive effect were 0.274 and 0.536 µg/l for endosulfan and <0.617 and 0.617 µg/l for lindane, respectively. The results demonstrated that mixture effects of endosulfan and lindane on shrimp postlarvae of P. monodon are additive or more than additive thus highlighting the significance of multipollutant effects on survival.*

INTRODUCTION

The widespread environmental contamination of organochlorine pesticides lead to their restrictions on production and use in industrialized countries (UNEP, 2002). The restrictions were imposed in the early 1980s in most of the countries including Sri Lanka. In Sri Lanka, organochlorine pesticides were heavily used in agriculture as well as in public health. Presently, there is a temporal decreasing trend of these pesticides in the environment and biota (Guruge and Tanabe, 2001), except for compounds which have contemporary uses (Monirith *et al*., 2000). Recently, some of the members of organochlorine pesticides such as endosulfan and lindane have received much attention due to the reported environmentally significant contaminant levels in sediment (Leonard *et al*., 2001) and agricultural runoff

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(Wirth *et al*., 2001). Hence, due to continued discharges and leakage of organochlorine pesticides, toxicity assessments with regard to these pollutants and their potential risk to aquatic species are of great importance.

Organochlorine pesticides have been linked to detrimental effects on estuarine biota, ranging from acute exposure leading directly to mortality, to chronic effects. Galloway and Depledge (2001) reviewed the aspects of immunological impairments in invertebrates and showed that some of the complex mixtures such as contaminated sediment, harbor dredging and PCBs have been implicated in causing immunotoxicity in the crustacean *Crangon crangon*. The above review reported recent studies of immunotoxicity on certain crustacean species for several chemical groups including pesticides (eg. chlorpyriphos, atrazine, carbaryl), heavy metals (eg. copper, lead, cadmium), organotins (eg. tributyl-tin, dibutyl-tin) and industrial pollutants (eg. PAHs).

The species dominating the marine shrimp culture in South-East Asia are *Penaeid* shrimps, especially *Penaeus monodon*, commonly known as the black tiger shrimp. The present study was designed focusing on toxicity involving dose-response assessment using *Penaeus monodon* Fabricius as the concerned ecological receptor. The main objective of the study was to investigate potential risk of endosulfan and lindane on shrimp postlarvae with a view to elucidate their significance to aquaculture industry.

MATERIALS AND METHODS

Experimental setup

= forest area
= Urban area
= Grassland area
= Aquaculture area
= Agricultural area
= water body

Toxicity test was conducted in bowl type glass chambers (~2 l) containing 1 l of test tion. Filtered natural seawater at 16 ‰ salinity was used as common diluents of toxicity including the control treatments. Bulk quantities of water sampled into pre-cleaned E bottles were air saturated for more than 3 days prior to use in the experiment. All test nbers were maintained unaerated during the 96 h experimental duration. All treatments replicated three times. The experiments were conducted at 29±2 °C and natural :dark photoperiod of 12:12 h. Static, non-renewal acute toxicity tests were performed in ıbsence of food. Ten-day old shrimp postlarvae were used for toxicity tests according to USEPA (2002). Shrimps were kept fasting during the 24 h acclimation period. Twenty postlarvae of more or less equal length and body fatness were transferred to each testing chamber. Dead animals were removed as soon as they were observed to be dead and/or confirmed by probe test.

Water spike

The concentrations of endosulfan used in the definitive test were selected based on range finding tests conducted taking into account LC_{50} values as reported on other shrimp species (Wirth *et al*., 2001; Key *et al*., 2003). However, a reported LC_{50} value for another *Penaeid* shrimp species (Galindo *et al*., 1996) was used for deciding test concentrations of lindane in the definitive test. The concentrations used in the mixture toxicity tests were based on LC_{50} values of nominal concentrations derived from tests conducted with individual pesticides. Equitoxic concentrations of 50, 25 and 10% less and 100% more tha the LC_{50} values of each pesticide were used. The chemical preparation was done according to Sumith and Parkpian (2008). The solvent effect was ruled out according to Wirth *et al*. (2001).

Test medium parameters

Daily measurements of pH (CONSORT C932 electrochemical analyzer), dissolved oxygen and temperature (METTLER TOLEDO MO128 Dissolved Oxygen Meter equipped with IP67 Probe) were recorded in each test chamber during 9:00 to 11:00 a.m. The actual salinity was measured using a portable refractometer (Model: NOW, Tokyo, Japan). Pre- and post-experimental ammonia and nitrite were determined using standard colorimetric test methods as described by Parsons *et al*. (1984). Unionized ammonia (NH_3-N) was calculated from appropriate temperature, pH and salinity tables (Colt, 2006). Replicated chambers were pooled for pesticide analysis. The extraction of endosulfan and lindane in water samples were conducted according to the procedure of USEPA method 8081B (USEPA, 2000) and analyzed on a gas chromatograph (Hewlett Packard 7673) equipped with an electron capture detector (ECD).

Postlarvae homogenate for phenoloxidase analysis

Whole-body macerated haemolymph samples (frozen at –20 °C) of postlarvae of *P. monodon* were used in the protein and phenoloxidase assay. The surviving postlarvae from replicates were pre-weighed (wet weight) and homogenized in pre-cooled Eppendorf tubes containing 100-400 µl of phosphate buffered saline (PBS) solution and the extract then centrifuged at 6,500 x g for 15 min at 4 °C in a refrigerated centrifuge (Model: K3 System with rotor recognition, CENTURION Scientific Ltd.). The acellular fraction (supernatant) was frozen at –20 °C for further analysis. Detection of PO-activity in acellular fraction sample was carried out by measurement of L-Dopa transformation in dopachrome.

Protein analysis

Bio-Rad Protein Assay Standard II (BSA) was used as the standard. A range of dilutions of standard (30 µl) was prepared (3 replicates) using suitable volumes of buffer solution. Ten micro liters (10 µl) from each dilution and 200 µl of diluted dye reagent (Coomassie® Brilliant Blue G-250) were mixed in 96-well micro plates. Diluted dye reagent was prepared using dye reagent concentrate and distilled water in the ratio of 1:4 and filtered (Whatman #1 filter) to remove particulates. Duplicate measurements were done for sample homogenates for protein analysis. Protein concentration was measured according to the standard work curve.

Phenoloxidase analysis

A range of dilutions of acellular fraction samples (30 µl) was prepared using suitable volumes of buffer solution. Ten micro liters (10 µl) from each dilution and 200 µl of diluted dye reagent were mixed in 96 well micro plates. After 10 min of reaction period, the reactant absorbance values (OD_{ABS}) were measured by spectrophotometry at 595 nm using VERSAmax tunable microplate reader supported by Softmax Pro computer software program. Twenty microliters (20 µl) of undiluted haemolymph samples were distributed in a 96-well micro plate and successively added 30 µl of cocodylate buffer (CAC), 50 µl of PO activity modulator (1% trypsine) and incubated for 10 min at room temperature. Then, 130 µl of CAC buffer and 50 µL of 5 mM L-Dopa were and absorbance was measured by spectrophotometry at 490 nm. Triplicate measurements were done for enzyme analysis. The maximum rate of enzyme activity during 30 min reaction period at 28.2±0.2 °C measured at

3 min intervals was taken as the basic data input for further calculations. Enzyme activity was expressed as units, where one unit represents the change in absorbance per min and normalized to the protein (mg) content (Hernández-López *et al*., 1996). The mean of 3 absorbance values of phenoloxidase activity for every 3 min interval during 30 min were recorded for calculations. Change in phenoloxidase activity of shrimp postlarvae after different chemical treatments were calculated as the change (activation and/or suppression) in percentage of phenoloxidase activity compared to respective controls, which were set at 100%.

Data processing and statistical analysis

Animal survivability was counted at 24, 48, 72 and 96 hours of exposure for LC_{50}, LOEC and NOEC calculations. The Lowest Observed Effect Concentration (LOEC) was the lowest concentration that had statistically significant mortality or adverse effect. The No Observed Effect Concentration (NOEC) was the highest concentration that had no statistically significant mortality or adverse effect (Key *et al*., 2003). The toxic unit concept deduced by Marking (1977) for determining the additive index was used for the assessment of binary mixture effect. Trimmed Spearman-Karber method (Hamilton *et al*., 1977) was used for LC_{50} and their respective 95% confidence limit calculations using a software program (Version 5.1). Automatic trimmed Spearman-Karber estimates were conducted and where possible ten percent (10%) trimmed estimates were considered. Animals lost due to escape from the test solution were excluded from calculating LC_{50} values (Buikema *et al*., 1980). The data for survivability and postlarval phenoloxidase activity were analyzed using SPSS (Version 11.5) statistical software program. Means were tested for homogeneity of variances before the test. Comparisons of homogeneous subsets were conducted with one-way analysis of variance (ANOVA) supported by Duncan's multiple range tests in Post Hoc. Mean differences were considered significant at $p<0.05$.

RESULTS AND DISCUSSION

Acute toxicity

The daily water quality parameters fluctuated within a range of 25.0-28.0 °C, 6.66-7.77 pH and 5.59-3.33 mg/l Dissolved Oxygen during the entire 96 h test period. The acute 96 h LC_{50} and their 95% confidence limits of endosulfan and lindane on shrimp postlarvae exposed to individual chemicals in water are shown in Table 1. The LC_{50} diminished with time (= increasing toxicity) and the difference between 24 h LC_{50} and 96 h LC_{50} values of all tests were significantly different ($p<0.05$). Figure 1 shows the diminishing pattern of LC_{50} with best fit power correlation ($R^2>0.9$) with the exposure period. The difference between the minimum and maximum values in the endosulfan in spiked-water treatment was narrower (19.7%) than the other treatment; *viz*., 44.1% in the lindane spiked-water test. Dose-response assessment revealed that NOEC and LOEC of endosulfan for survival effect in spiked-water toxicity tests based on mean concentrations were 0.536 and 1.038 µg/l respectively. The respective statistical endpoints ($p<0.05$) for lindane were 0.617 and 1.638 µg/l. Acute toxic effects of endosulfan and lindane are not surprising, as being neurotoxic organochlorine pesticides, they induce hyperexcitability progressing towards the production of convulsions and death (Vale *et al*., 2003). Control survival in all spike tests was ≥90% allowing for the validity of the test (USACE, 1998).

These results essentially substantiate other acute toxicity experiments using juvenile stages of shrimp species. Lombardi *et al*. (2001) demonstrated that acute toxicity of endosulfan to freshwater prawn *Macrobrachium rosenbergii* De Man was higher in renewal tests than static tests. Low toxicity of 96 h LC_{50} at 0.93 (0.69-1.21) µg/l in static to 0.20 (0.04-0.38) µg/l in renewal tests suggests endosulfan transformation into less toxic hydrolytic products (Cotham and Bidleman, 1989). Key *et al*. (2003) reported that grass shrimp postlarvae (*Palaemonetes pugio*) showed mortality endpoints of LC_{50}, LOEC and NOEC for endosulfan in static renewal tests as 2.56 (1.82-3.59), 1.25 and 0.63 µg/l, respectively. Since these estimations have been done based on nominal concentrations, these results are comparable to those of similar context. If the results are brought in perspective, for comparison purposes, black tiger shrimp postlarvae (*P. monodon*) may be considered equal or more sensitive than grass shrimp postlarvae (*P. pugio*).

Table 1. Acute toxicity of endosulfan and lindane on shrimp postlarvae *P. monodon* in static non-renewal tests during 96 h.

Chemical (µg/l)	LC_{50} (based on nominal concentration)[1]	LC_{50} (based on mean concentration)[1]	LC_{50} (based on final concentration)[1]
Endosulfan	3.01 (2.35-3.86)	1.60 (1.23-2.08)	0.16 (0.12-0.21)
Lindane	5.23 (4.23-6.47)	3.01 (2.51-3.60)	0.81 (0.61-1.09)

[1]LC_{50} values expressed as µg/l and data within brackets are their respective 95% lower and upper confidence limits.

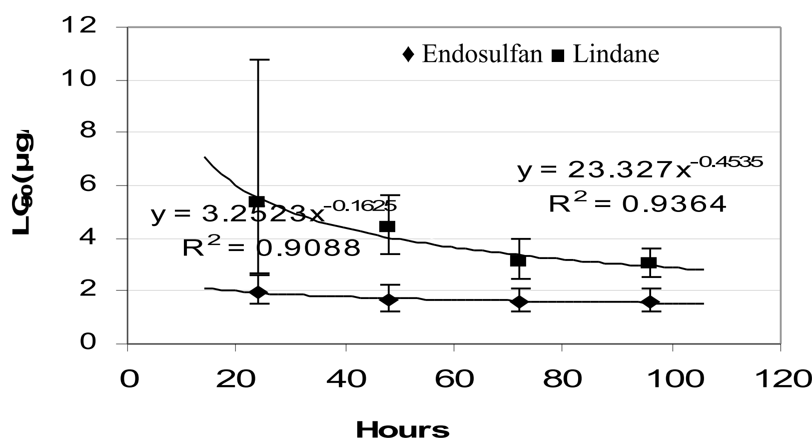

Figure 1. Relationship of LC_{50} with the exposure period for shrimp postlarvae *P. monodon* during the 96 h spiked-water toxicity tests. Error bars represent 95% lower and upper confidence intervals.

Acute mixture effect

From the individual and binary mixture LC_{50} values of endosulfan and lindane, negative value for additive index (S) was obtained (Table 2). Since the value overlaps zero, which is the hypothetical value corresponding to the additive effect, the overall effect of endosulfan and lindane in a mixture represented "additive". According to Brown (1988) any chemical mixture that simply exerts its action without apparent interaction at all can be termed as "additive" which described the situation when the toxicity actions are independent of either component.

Sub-lethal toxicity on phenoloxidase

To assess the immunocompetence, measurement of haemolymph phenoloxidase activity has been suggested by Hernández-López *et al*. (1996) and Gagnaire *et al*. (2004). In order to assess the immunocompetence against endosulfan and lindane, measurements of whole-body homogenate phenoloxidase activity was used (Córdoba-Aguilar *et al*., 2006). Endosulfan and lindane produced a biphasic effect on phenoloxidase enzyme activity depending on the concentration in spiked-water tests (Figure 2).

Table 2. Acute toxicity and additive indices for individual and binary mixtures of endosulfan and lindane on black tiger shrimp postlarvae in spiked-water test matrices.

Test compound	LC_{50} (µg/l) [1]	Confidence limits 95%	Additive Index and Range[2]
Endosulfan	1.60	1.23-2.08	-0.27
Lindane	3.01	2.51-3.60	(-1.04 to 0.25)
Endosulfan +	1.06†	0.54-2.08	
Lindane	1.84††	0.90-3.74	

[1] LC_{50} based on the mean concentration of test compounds at the end of the experiment after 96 h.
[2] Calculations based on Marking (1977).
† LC_{50} of endosulfan in the presence of lindane.
†† LC_{50} of lindane in the presence of endosulfan.

Enzyme depression

There was no significant difference in phenoloxidase activity of the shrimp postlarvae exposed to the lowest spiked concentration of endosulfan (E1/2) and the untreated control after 96 h. Obviously, endosulfan concentrations at E1 and E2 caused a significant ($p<0.05$) depression of phenoloxidase activity in the shrimp postlarvae compared to the control. The magnitudes of depression of phenoloxidase activity of shrimp postlarvae were 72 and 86% on exposure to E1 and E2 of endosulfan relative to control after 96 h, respectively (Figure 2a).

In the lindane experiment, treatment effects on phenoloxidase activity in shrimp postlarvae were significantly different ($p<0.05$) and biphasic effect was also observed as that of endosulfan. While low concentrations (L1-L5) were depressive for enzyme activity by 38-61%, higher concentrations increased enzyme activity in comparison to the control. The highest depression of phenoloxidase activity (61%) was observed at L3 of lindane over the 96 h period and the enzyme depressive activity was observed over lindane concentrations from L1 through L5 (Figure 2b). For enzyme depressive activity, LOECs were determined to be 0.536 µg/l of endosulfan ($p<0.05$) and 0.617 µg/l of lindane ($p<0.05$) compared to

respective controls. Accordingly, NOEC established for endosulfan was 0.274 µg/l while a concentration <0.617 µg/l was estimated for lindane. Surprisingly, NOECs for survival effect were similar to LOECs for depression effect on phenoloxidase activity by endosulfan and lindane. It implies that the effect on enzyme activity was more sensitive than on survival.

Enzyme stimulation

It is also apparent in both experiments that the relative phenoloxidase activity tends to increase significantly at spike concentration of E5 of endosulfan (=5 µg/l of nominal concentration) ($p<0.05$) while for lindane it was at L10 (10 µg/l) ($p<0.05$). Even though there is a trend of enzyme activation on concentration-dependent manner over lindane concentrations from L5 through L10, a significant increase ($p<0.05$) of enzyme activation (51%) was recorded only at L10, over the respective control during the 96 h period. Similarly, the highest enzyme activation of 99% over the control was recorded at E5 where nominal endosulfan concentration was 5 µg/l. The highest exposure concentration of endosulfan (10 µg/l) caused complete mortality of shrimp postlarvae at the end of 96 h period and therefore enzyme activity was measured only for 4 concentrations.

Sub-lethal mixture effect

The data (Figure 2c) indicates that phenoloxidase enzyme activity increased (53-61%) in 3 out of 5 mix concentrations tested. Significant difference ($p<0.05$) of the increase of phenoloxidase activity of mix concentrations was observed from EL3 to EL5 when compared with the control.

A general pattern of enzyme depression by endosulfan and lindane was observed when they were individually applied at low doses but when they were in mixture the effect was reversed. The individual "pure" chemical effect and the mixture effect were in favor of enhancing the enzyme activity at higher doses. By considering the mean water concentrations during 96 h and the enzyme triggering levels for shrimp postlarvae, a significant enzyme up-regulation was observed over endosulfan concentration of 2.680 µg/l and 6.019 µg/l of lindane when they act individually. When they were in combination, a significant up-regulation occurred at mixed concentration of endosulfan at 0.814 µg/l and lindane at 1.416 µg/l. Apart from that, apparent enzyme activation occurred at higher concentrations, which are physiologically intolerant for shrimp survival (*i.e.* >1.7 times of LC_{50}) and therefore, there is a low practical relevance in this observation. However, for up-regulating the enzyme activity, the difference between the effect concentrations were narrowed by 69 and 78% for endosulfan and lindane, respectively when they were in combination, and therefore possible positive or negative implications on shrimps exposed to combination of chemicals cannot be ignored.

Phenoloxidase activity in arthropods including crustaceans has been described (Sritunyalucksana and Söderhäll, 2000). Xenobiotics such as benzalkonium chloride (Cheng *et al*., 2003) and copper sulfate (Cheng and Wang, 2001) have been shown to be modulatory on phenoloxidase activity in shrimp species. One of the possible mechanisms includes enzyme elicitor function of endosulfan and lindane at sub-lethal concentrations with obvious

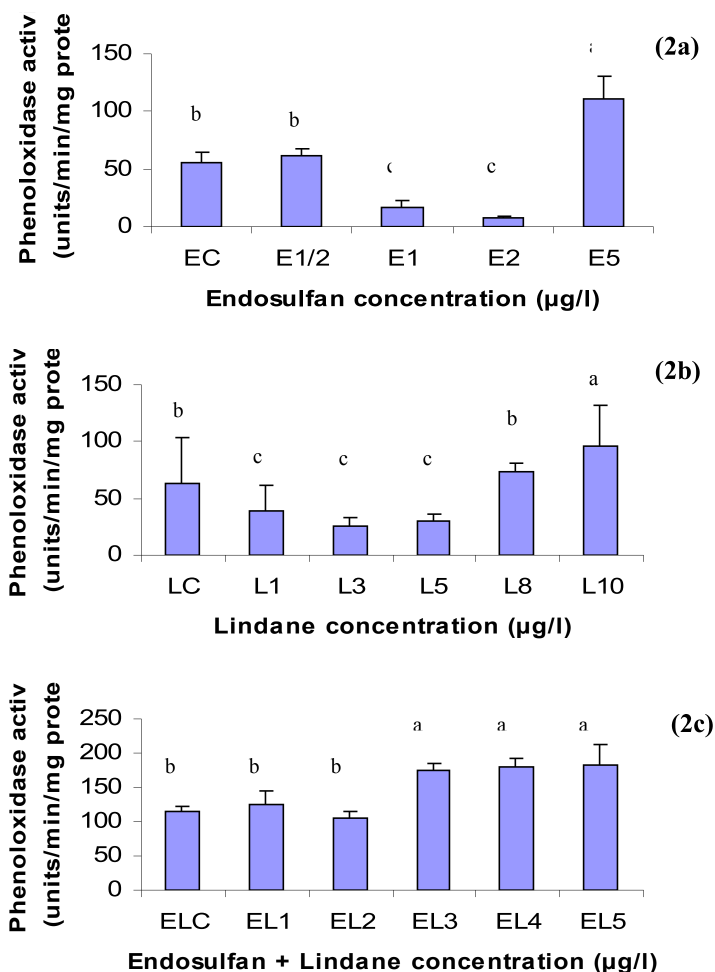

Figure 2. Effect of endosulfan (a), lindane (b) and binary mixture of endosulfan and lindane (c) on phenoloxidase activity on shrimp postlarvae (*P. monodon*) during 96 h spiked-water toxicity test.

Notes: "E" denotes endosulfan spike in water and therefore, E1/2 = 0.5 µg/l spike E1 = 1.0 µg/l spike E2 = 2.0 µg/l spike E5 = 5.0 µg/l spike.). "L" denotes lindane spike in water and therefore, L1 = 1.0 µg/l spike L3 = 3.0 µg/l spike L5 = 5.0 µg/l spike L8 = 8.0 µg/l spike L10 = 10.0 µg/l spike. "EL" denotes water spike with endosulfan+lindane (respectively) and therefore, EL-1 = 0.3+0.5 µg/l spike EL-2 = 0.75+1.25 µg/l spike EL-3 = 1.5+2.5 µg/l spike EL-4 = 3.0+5.0 µg/l spike EL-5 = 6.0+10.0 µg/l spike. Bars are means+SE. Bars with different letters are significantly different ($p<0.05$).

decrease of phenoloxidase activity in a manner as described by Sritunyalucksana and Söderhäll, 2000. In this context, effect of enzyme elicitors and subsequent enzyme depression has been further demonstrated by Zhang *et al.* (2005). Decreased phenoloxidase activity in endosulfan exposure can be associated with direct and indirect reaction with the enzyme itself. It has been shown that sulphur-containing compounds such as thio-urea and

sodium sulfite can inhibit phenoloxidase enzyme activity in calm *Ruditapes philippinarum* which has resemblance to crustacean phenoloxidase (Cong *et al*., 2005). The possible release of sulfite moiety from endosulfan (Sutherland *et al*., 2000) and the action of bisulfite inhibition of phenoloxidase (Ferrer *et al*., 1989) are documented. The presence of sulfite functional group in endosulfan which made it distinctive from the rest of the organochlorine pesticides (Hoechst, 1993) may be an excellent example to demonstrate structure-activity relationship. Conversely, there is a possibility that haematocyte destruction could lead to exert signaling for excessive phenoloxidase secretion and consequent elevated enzyme activity. There are number of reports showing impaired haematological functions and damage induced by endosulfan and lindane (George and Ambrose, 2004; Siang *et al*., 2007). The activation of molecular oxygen and subsequent toxicity induced by quinone related compounds (Rabideau, 2001) further support the above facts since quinone is an intermediate in the phenoloxidase activation in the course of synthesis of melanin (Söderhäll and Cerenius, 1998). In the above connection, the present study confirms that mixture of endosulfan and lindane has oxidative responses greater than those of individual chemicals. A relationship between immunosuppression and disease has been established in a number of studies including freshwater prawn (*Macrobrachium rosenbergii*), kuruma shrimp (*Marsupenaeus japonicus*) and black tiger shrimp (*P. monodon*) (Cheng *et al*., 2003; Pholdaeng *et al*., 2005; Cheng *et al*., 2007). In the ecological perspective, chemical stress factors that affect immunological competence are particularly important because they can increase host's susceptibility to infectious diseases (Galloway and Depledge, 2001). Pholdaeng *et al*. (2005) demonstrated beneficial effect of improving immune responses and increase disease resistance in juvenile black tiger shrimp (*P. monodon*) when the phenoloxidase activity is increased by 48% than that of control. Alternatively, concerns have also been expressed that high levels of active phenoloxidases can cause deleterious effects on cellular components by producing toxic intermediates (Söderhäll and Cerenius, 1998). However, there were no definite physiological activity levels of phenoloxidase found in the literature to demonstrate the difference between beneficial and detrimental effects of phenoloxidase in shrimp and surprisingly for any arthropod.

CONCLUSIONS

For investigating potential toxicity of pesticides on aquatic organisms, toxicity testing approaches based on pure chemicals and in combination are employed. This research is the first to derive evidence of acute toxicity data in terms of LC_{50} for endosulfan and lindane on shrimp postlarvae *P. monodon* Fabricius. The results in terms of acute toxicity data for *P. monodon* cannot be compared with the published data for other shrimp species for comparative sensitivity due to differential testing approaches used by different researchers. However, if the results were considered in perspective, black tiger shrimp postlarvae (*P. monodon*) may be considered equal or more sensitive than grass shrimp postlarvae (*Palaemonetes pugio*) which has been the most commonly studied species. Also, this study is the first to report that endosulfan and lindane are involved in shrimp *P. monodon* immunomodulatory function. The innate immunity parameter, phenoloxidase enzyme activity, was influenced by endosulfan and lindane in different ways. Essentially, endosulfan and lindane were additively involved in causing lethal toxicity on shrimp postlarvae which can be explained by their modes of actions. However, a range of concentrations of combined chemical effects on phenoloxidase activity was shown to be in opposite reaction to their individual chemical effects. While individual effects engaged in up-regulation and down-

regulation of the enzyme activity, the mixture effects were always up-regulating. The present study confirms that mixture of endosulfan and lindane has oxidative responses greater than those of individual chemicals. This implied that irrespective of the most pronounced mode of action of a chemical, there can be other functional effects and multiple interactions with biophysiological functions which may obscure the true effect of a chemical. Immunocomptence parameters were often concomitantly expressed with other physiological functions and the present study further warrants a range of assays to measure the true impact of immunocompetence and their relationship with the observed up-regulation and down-regulation of phenoloxidase activity.

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