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Changes in the haemolymph of *Fenneropenaeus indicus* exposed to Malathion and endosulfan

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Abstract
Malathion and Endosulfan are two widely used pesticides in agriculture and are persistent in aquatic environment. Aquatic animals inhibiting these environments are continuously exposed to these pesticides. *Fenneropenaeus indicus* is a major shrimp species in coastal regions of Asia and hence it is preferred for aquaculture production. In this study we examined certain changes in the hemolymph of *F. indicus* exposed to sublethal (25 and 50 ng/L) doses of two pesticides, Malathion and Endosulfan for 20 days. Both the pesticides significantly altered the clotting time, total haemocyte count (THC), phenoloxidase (PO), total protein and glucose content of plasma to various levels in the test animals. The changes in all the factors evaluated varied with the exposure time and were also dose-dependent. The clotting time, protein and glucose content of plasma increased during the study period while the phenoloxidase activity and the THC were reduced in the test animals. The study revealed that sublethal levels of Malathion and endosulfan significantly altered the immunological factors thereby reducing the health status of the animals which may result in the invasion of pathogens from the aquatic environment.

Keywords: *Fenneropenaeus indicus,* Malathion, Endosulfan, Phenoloxidase, Haemocyte, Clotting time

1. Introduction
Chemical pesticides are well recognized as an economic approach for controlling pests in agriculture and horticulture [1, 2]. It has been reported that as much as 70% of the chemical formulations used for pest control programmes in agriculture affect non target organisms inhabiting aquatic environment and aquaculture ponds that are fed by the rivers and estuaries [3]. India is the largest manufacturer and consumer of pesticides in south Asia and due to its economic viability, Malathion and endosulfan are commonly used in agriculture system of the country. Malathion (Diethyl 2-[(dimethoxyphosphorothioyl)sulfanyl]butanedioate) is an organophosphate pesticide and is one of the five most commonly used pesticides in India, accounting for 65% of all organophosphate pesticides applied in the field [4]. The environmental concentrations of Malathion in sediment and water of agroecosystem have been reported to vary from 2.62 to 129 µg/kg and from 0.699 to 298 µg/L, respectively [5]. Endosulfan (6, 7, 8, 9, 10, 10-hexachloro-1, 5, 51, 6, 9, 9a - hexahydro - 6, 9 - methano -2, 4, 3-benzodioxathiepin-3-oxide) is an organochlorine insecticide widely used in agriculture to control pests. In India, the agricultural consumption of endosulfan was estimated to be approximately 5,200 metric tons [6] and has been identified as one of the main pesticides found in the waters of major rivers of the country [7].
The concentration and persistence of both Malathion and endosulfan in the aquatic environment exert harmful effect to invertebrates and fishes living in the polluted ecosystem. Malathion is able to alter the immune system in several species of laboratory animals and fishes are well documented [8], but there is a paucity of such research reports in decapod crustaceans. Even though endosulfan increases agricultural crop yields by reducing damages caused by pests, it exert its toxic potential to non target organisms by altering the biological functions [9,10], ultimately affecting the survival of the population. These pesticides may also tend to accumulate in aquatic animals [11] and consumption of contaminated fish can cause severe health problems in human beings [12].
Decapod crustaceans are preferred for aquaculture in coastal areas due to its economic importance. The exposure of pesticides to these animals in their natural habitat and coastal aquaculture systems can cause deleterious effects for their survival. The Indian white shrimp, *Fenneropenaeus indicus* is preferred for aquaculture in traditional brackishwater farms of

several Asian countries. The 96 h LC_{50} level of endosulfan and Malathion for this shrimp is found to be 0.48 and 214 µg L-1, respectively [13]. Although numerous studies have been made concerning the toxic effects of pesticides on *F. indicus* [14, 15], little efforts have been directed at towards their toxic effects at the immune level of the animal. The immune system is central to health and resistance/susceptibility to pathogens in all species. Interestingly, it is also one of the most sensitive and susceptible system to the effect of pesticides. In this study we are reporting the effect of two sublethal levels of endosulfan and Malathion (25 and 50 ng/L) on the immune system of *F. indicus*.

2. Materials and methods

2.1. Collection and Maintenance of animals

Five hundred live *F. indicus* (total length, 8.52 ± 0.56 cm; weight 6.34 ± 0.15 g) were collected from Muttukadu Creek, Chennai, India. They were acclimatized to the laboratory condition in a cement tank of 5 ton capacity for 2 weeks before initiation of experiments. During the acclimation period the shrimps were fed twice daily with a commercial feed (CP-Aqua, India). The optimum water quality parameters were maintained during the acclimation period (temperature, 25.5 ± 0.5; salinity 20 ± 1.0 ppt; dissolved oxygen 6.0 ± 0.5; pH, 8.0 ± 0.5). Daily water exchange (10%) was carried out to maintain the water quality at optimum level. Only shrimp in the intermoult stage was used for the study and the moult stage was identified by examining the uropod in which partial retraction of the epidermis could be visualized [16]. Shrimps showing no signs of infection or injuries were selected for the experimental purpose.

2.2. Experimental design

Six (200 L capacity) fibreglass tanks, each containing twenty numbers of shrimps form an experimental group. Five such groups, four for the treatment of desired quantity of two pesticides (25 and 50 ng/L) of Malathion and endosulfan and a control group without the toxicant were used for the study. Stock solutions of toxicants, Malathion and endosulfan (Parul chemicals Ltd, Gujarat, India, 50% EC and 35% EC, respectively), were diluted with deionised water to prepare solutions of required concentrations. Water exchange was done daily and replaced with water with the same quantity of pesticides.

2.3. Measurement of immune parameters

2.3.1. Collection of haemolymph

Haemolymph was collected using 2 ml sterile syringe with 26½ gauge needle from the base of pleopod, walking legs and ventral sinus. Haemolymph (1:1 ratio) was collected in anticoagulant (0.114 M trisodium citrate, 0.10 M NaCl, pH 7.00) and transferred to 1.5 ml eppendorf tubes kept on ice. The samples were immediately centrifuged in 700 *g* for 10 m at 4 ºC. The pellets were separated and used for phenoloxidase (PO) assay while the supernatant, plasma, was used for the assay of protein and glucose. To estimate the Total Hemocyte Count (THC), hemolymph was collected in Alsevier solution containing 10% formaldehyde and for determining the clotting time, sample without anticoagulant was collected in 0.5 ml eppendorff tubes kept on ice and immediately used.

2.3.2. Phenoloxidase activity

For PO estimation, the cell pellets were resuspended in sodium cacodylate buffer (10 mM sodium cacodylate, 0.45 M Nacl, 20 mM $CaCl_2$, 30 mM $MgCl_2$, pH 8.0) and homogenized. The resultant solution was centrifuged at 17,400 *g* for 45 min at 4 ºC. The supernatant designated as haemocyte lysate supernatant (HLS) was used as enzyme source, kept at - 20 ºC for further studies. PO activity was measured by recording the formation of dopachrome from L-Dopa after treating with HLS [17]. Briefly, 50 µl of HLS was incubated with same quantity of trypsin (2 mg/ml) in 96 well flat bottom microtitre plate (Tarsons, India) for 20 min at 25 ºC. Then, 100 µl of L-Dopa (3 mg/ml) was added. Absorbance was measured after 5 min at 490 nm in ELISA Reader (Labsystems, USA) for 20 min with 2 min intervals. PO activity is expressed as units; one unit of enzyme was defined as the increase in absorbance of 0.001 min/mg of protein.

2.3.3. Clotting time

The clotting time of haemolymph was determined by the method of Jussila *et al* [18] with slight modifications. Briefly, 50 µl of haemolymph was sucked into a precooled (in ice) plain soda lime glass capillary tube having inner diameter of 1.1-1.2 mm and a length of 7.5 mm. The tube was turned to vertical position with the sample in the upper end. With the influence of gravity, the sample flows down and reaches the other end; the tube was turned 180º to make the position of sample again in the upper end. This was repeated until the flow of haemolymph stops. The time taken by the sample from the initial vertical position to the time when the flow stops was taken as clotting time and is recorded by the timer in NOKIA 2600

2.3.4. Total Haemocyte Count (THC)

A sample of 150 µl of haemolymph was mixed with an Alsiever solution (113 mM glucose, 27.2 mM sodium citrate, 2.8 mM citric acid, 71.9 mM sodium chloride) and 10% formaldehyde (v/v). Haemocyte counting was done with 20 µl of the diluted sample under a phase contrase microscope and a Neubauer hemocytometer.

2.3.5. Protein and glucose estimation

Protein content in plasma and HLS was estimated by Lowry *et al* [19] using Bovine Serum Albumin as standard. The level of glucose in haemolymph was estimated using glucose estimation Kit (Span Diagnostic Pvt. Ltd. Surat, India).

2.4. Statistical analysis

All the data obtained in the present study were analysed statistically by using SPSS software.

3. Results

The various changes in haemolymph parameters of *F. indicus* after exposure to malathion (25 ng/L) is shown in Table 1. The clotting time, plasma glucose and protein component in plasma increased from 10 DoE (Days of Exposure) while PO activity and THC decreased from 10 DoE and 15 DoE, respectively. The clotting time increased from 10, 15 and 20 DoE with a prominent ($p<0.01$) change in mean clotting time (127.67 ± 6.29 s) on 20 DoE. Similarly significant ($p<0.01$) elevation in plasma glucose was recorded from 10 DoE that resulted in an accumulation of 69.15% glucose in plasma of the treated shrimps on 15 DoE than control shrimps (81.35 ± 9.21 mg/100 ml). Meanwhile the PO activity decreased prominently ($p<0.001$), from 10 DoE with the exposure and resulted in a sharp reduction in PO expression of test animals (30.50 ± 2.12 units/min/ mg of protein) than untreated animals (49.14 ± 2.68

units/min/ mg of protein) after 20 days. In contrast, the protein level of plasma varied (p<0.05) from 10 DoE leading to an increase of 21.12% on 20 DoE (113.09 ± 8.00 mg/ml). Exposure to pesticide resulted in a marked (p < 0.01) reduction of THC in shrimp was observed on 15 and 20 DoE.

Table 1: Haematological parameters of healthy and malathion (25 ng/L) exposed *Fenneropenaeus indicus*.

Treatment days	Clotting time (s) (n= 6)	Glucose (mg/100ml; n = 8)	PO activity (units/min/ mg of protein; n = 8)	Protein (mg/ml; n = 8)	THC (X 10^6 cells/ml; n = 6)
Control	96.17 ± 5.72	81.35 ± 9.21	49.01 ± 2.68	93.33 ± 4.15	26.19 ± 2.24
5	94.82 ± 6.75^D	83.68 ± 6.83^D	46.85 ± 4.23^D	98.71 ± 8.39^D	24.47 ± 1.92^D
10	110.33 ± 4.80^A	97.69 ± 6.00^A	36.91 ± 2.42^C	106.03 ± 8.96^B	22.06 ± 3.27^D
15	111.50 ± 6.47^A	132.61 ± 8.55^A	31.11 ± 1.53^C	110.57 ± 7.83^B	19.56 ± 1.77^A
20	127.67 ± 5.95^A	143.27 ± 7.45^A	30.50 ± 2.12^C	113.09 ± 8.00^B	16.20 ± 1.39^A

Each Value is mean ± SD. $^AP < 0.01$; $^BP < 0.05$; $^CP < 0.001$; ^{D} not statistically significant.

The changes in haemolymph parameters of *F. indicus* after treatment with malathion (50 ng/L) is shown in Table 2. The clotting time of haemolymph increased significantly (p<0.01) from 10 DoE and resulted in the elevation of clotting time by 26.2, 41.86 and 48.71% on 10, 15 and 20 DoE, respectively. Alternatively, the treatment resulted in prominent (p<0.01) accumulation of plasma glucose on all sampling days. Approximately, 50% increase in glucose content of plasma was observed on 15 DoE (62.94%) and 20 DoE (70.04%) in the test shrimps than that of the control animals (84.30 ± 4.39 mg/100ml). In contrast, PO activity, declined on all the four sampling days. The PO expression was reduced by 18.19, 34.36, 46.03 and 52.05% on 5, 10, 15 and 20 DOE, respectively, while, the protein content of plasma elevated prominently (p<0.05) from 10 DoE which resulted in an accumulation of protein in plasma (17.53%) after 20 DoE. The exposure to malathion also resulted in significant (p<0.01) variation in THC that reduced by 18.24, 31.23 and 47.18% on 10, 15 and 20 DoE, respectively compared to untreated animals (25.93 ± 2.47 X10^6 cells ml-1).

Table 2: Haematological parameters of healthy and malathion (50 ng/L) exposed *Fenneropenaeus indicus*.

Treatment days	Clotting time (s) (n= 6)	Glucose (mg/100ml; n = 8)	PO activity (units/min/ mg of protein; n = 8)	Protein (mg/ml; n = 8)	THC (X 10^6 cells/ml; n = 6)
Control	94.16 ± 4.90	84.30 ± 4.39	45.96 ± 2.59	96.52 ± 5.32	25.93 ± 2.47
5	107.16 ± 5.77^D	108.92 ± 6.11^A	37.54 ± 2.30^B	104.73 ± 6.38^D	24.38 ± 2.82^D
10	118.83 ± 6.36^A	125.30 ± 7.11^A	29.56 ± 2.44^C	110.63 ± 6.60^B	21.20 ± 3.57^D
15	133.50 ± 7.94^A	137.36 ± 8.07^A	23.80 ± 2.80^C	112.14 ± 7.54^B	17.83 ± 2.72^D
20	140.00 ± 5.25^A	143.35 ± 7.04^A	21.72 ± 1.14^C	113.44 ± 7.21^B	15.25 ± 2.67^A

Each Value is mean ± SD. $^AP < 0.01$; $^BP < 0.05$; $^CP < 0.001$; ^{D} not statistically significant.

The observed changes in haemolymph components of *F. indicus* exposed to endosulfan (25 ng/L) are shown in Table 3. There was no change in clotting time of test animals upto 10 DoE, later increased significantly (p<0.01) on 15 DoE (34.40%) and 20 DoE (41.66%). Similarly, the glucose level in plasma of experimental shrimps increased prominently (p<0.05) from 10 DoE resulting in the accumulation of this component by 48.03 and 68.43% than the control shrimps (74.22 ± 8.09 mg/ml) on 15 DOE and 20 DOE, respectively. The phenoloxidase activity of *F. indicus* decreased on all sampling days, significantly (p<0.001) on 15 DoE (20.2%) and 20 DoE (37.3%). Meanwhile, the protein content of the plasma showed no variation till 15 DoE but express marked (p < 0.05) increase on 20 DoE.

Table 3: Haematological parameters of healthy and endosulfan (25 ng/L) exposed *Fenneropenaeus indicus*.

Treatment days	Clotting time (s) (n= 6)	Glucose (mg/100ml; n = 8)	PO activity (units/min/ mg of protein; n = 8)	Protein (mg/ml; n = 8)	THC (X 10^6 cells/ml; n = 6)
Control	98.33 ± 4.20	74.22 ± 4.09	46.70 ± 2.04	93.62 ± 9.91	25.76 ± 2.54
5	108.01 ± 7.56^D	79.39 ± 6.73^D	42.33 ± 2.48^B	100.25 ± 6.75^D	24.04 ± 3.44^D
10	111.33 ± 7.39^D	83.25 ± 7.37^B	41.01 ± 1.92^A	102.81 ± 6.29^D	24.91 ± 2.41^D
15	132.16 ± 5.37^A	109.87 ± 7.56^A	36.60 ± 2.70^A	102.17 ± 8.58^D	22.84 ± 2.67^B
20	139.33 ± 6.08^A	125.05 ± 8.90^A	29.46 ± 2.36^C	114.08 ± 8.33^B	21.71 ± 2.24^B

Each Value is mean ± SD. $^AP < 0.01$; $^BP < 0.05$; $^CP < 0.001$; ^{D} not statistically significant.

The observed changes in haemolymph components of *F. indicus* exposed to endosulfan (50 ng/L) are shown in Table 4. The clotting time was increased prominently (p<0.01) by 25, 45 and 57% on 10 DoE, 15 DoE and 20 DoE as compared with the control animals (99.34 ± 5.35 s). The glucose content of plasma also increased significantly (p<0.001) from 10 DOE that resulted in an accumulation of 94.6% glucose in the haemolymph (144 ± 8.29 mg/100ml) on 20 DoE than the control shrimps (74.84 ± 4.21 mg/100ml). At the same time, the PO activity showed a marked (p<0.001) reduction from 10 DoE (37.93 ± 1.58 units) to 20 DoE (25.31 ± 4.35 units) resulting in a lowering of 45.8% activity after 20 DoE than untreated shrimps (46.76 ± 2.97units). The protein content increased significantly (p<0.05) on the 10 DoE and reached a maximum accumulation after 20 DoE compared with that of the control animals (95.19 ± 3.81 mg/ml). The THC reduced prominently (p < 0.05) from DoE showing a decline in the number from 25.76 ± 2.54 X 10^6 (control) to 19.71 ± 2.24 X 10^6 cells/ml (test shrimps) after 20 DOE with the exposure of endosulfan (50 ng/L).

Table 4: Haematological parameters of healthy and endosulfan (50 ng/L) exposed *Fenneropenaeus indicus*.

Treatment days	Clotting time (s) (n= 6)	Glucose (mg/100ml; n = 8)	PO activity (units/min/ mg of protein; n = 8)	Protein (mg/ml; n = 8)	THC (X 10^6 cells/ml; n = 6)
Control	99.34 ± 5.35	74.84 ± 4.21	46.76 ± 2.97	95.19 ± 3.81	24.65 ± 3.45
5	102.50 ± 6.04^D	81.88 ± 6.05^D	41.23 ± 2.45^A	101.27 ± 3.69^D	23.04 ± 2.16^D
10	124.66 ± 8.65^A	101.71 ± 6.55^C	37.93 ± 1.58^C	106.40 ± 3.72^B	22.15 ± 2.48^D
15	144.16 ± 8.01^A	133.32 ± 7.25^C	31.21 ± 2.97^C	111.33 ± 4.23^A	18.53 ± 2.20^A
20	156.17 ± 7.87^A	144.39 ± 8.29^C	25.31 ± 2.35^C	116.58 ± 4.62^A	16.80 ± 2.04^A

Each Value is mean ± SD. $^A P < 0.01$; $^B P < 0.05$; $^C P < 0.001$; ^{D} not statistically significant.

4. Discussion

Crustaceans, like other invertebrates mainly rely on the innate immune defence mechanism, mediated by the circulating haemocytes [1]. Impairment of immune system can lead to pathogenic invasion and hence the depletion of the stock in the environment and collapse of the food chain. Disease resistance is a successful determinant of species integrity and hence, whole ecosystem stability. In the present study, *F. indicus* under the influence of stress from both levels of endosulfan and Malathion took more time for the haemolymph to clot. The haemolymph clotting time has been observed to change in the presence of bacteria or environmental pollutants [18, 20, 21]. Crustaceans have an open circulatory system in which the haemolymph carries out several physiological functions. One of these functions is the transport of molecules such as the respiratory protein haemocyanin followed by the clotting protein and other humeral components. In the presence of pollutants this property could be altered that result in the elevation of clotting time [18].

The glucose content in the hemolymph was increased prominently at elevated levels (50 ng/L) of pesticides and also proportional to the time of exposure of the test animals. Similar observations were reported in other crustaceans as a result of toxicity due to heavy metals, environmental changes and pesticides [22, 23]. During stress, shrimps use carbohydrate as a source of energy [24] and this reflects in elevation of glucose level in the haemolymph as a method to combat the stress exerted by the pesticides. A possible reason for this phenomenon could be due to the transport of glucose component from hepatopancreas and muscle towards hemolymph, resulting in the reduction of glycogen reserves in these organs. Breakdown of glycogen as a result of glycogenolysis for energy production through glycolytic pathway to meet high energy demands due to pesticide and heavy metal toxicity was reported in invertebrates. Elevated levels of blood glucose and decreased glycogen content in hepatopancreas and muscle was reported in *Macrobrachium malcolmsonii* [25] *Scylla serrata* [26], *Oziotelphusa senex senex* [27], *Uca marionis* [28] and in *Daphnia magna* [29] exposed to heavy metals and pesticides, indicated that shrimps could detect hypoxia by moving glucose before aerobic pathways are used and this response could be a strategy to prepare for anoxia [30].

The phenoloxidase activity was reduced in all the test animals after exposure to both levels of endosulfan and Malathion. The observation in *F. indicus* was found to be similar to the PO activity of *Mytilus edulis* [31] and *Crassostrea gigas* [32] exposed to copper and mercury. In another study, exposure to harbour dredged spoils results in reduced PO activity and THC in *Crangon crangon* [33]. Therefore, the results of the present study can be assumed under the response of reduced THC. Plasma proteins play a vital role in the immunity of crustaceans. It not only correlate with the infection of pathogen [34] but also with environmental stress [25, 35]. Several immune molecules have been purified from crustaceans such as β glucan binding proteins, lipopolysaccharide binding proteins and clotting protein [36]. In the present study, significant increase in plasma proteins was observed in *F. indicus* when external medium contain elevated levels of endosulfan and Malathion. In decapods, elevation of plasma proteins was reported in *Barytelphusa guerini* [37] and *Macrobrachium malcolmsonii* exposed to endosulfan [25, 35]. The observed increase in haemolymph plasma protein of *F. indicus* could be realized as a method to maintain the homeostasis of the animal due to the loss of certain enzymes in tissues which have precise physiological functions in the normal animal [25, 35].

The circulating haemocytes are associated with cellular defence and stress conditions generally result in impaired immune capability of the host [21]. The THC is one of the most widely used parameter for the assessment of crustacean health status, despite large individual variability in cell numbers. It is well known that life cycle, food intake, disease outbreak, pollutants and environmental stress affect the circulating haemocyte count of crustaceans, both in quantity and quality [38]. In the present experiment, Malathion and endosulfan exposed *F. indicus* have less number of haemocytes than the control shrimps. Changes in environmental factors like hypoxia [39], temperature [40] and presence of Polychlorinated Biphenyls [33] also results in a reduced hemocyte number. A possible reason for the reduction in the number of hemocytes in *F. indicus* could be associated with the displacement of hemocytes from haemolymph to organs affected by pesticides.

5. Conclusion

In conclusion, the study revealed that the sublethal (25 and 50 ng/L) levels of Malathion and endosulfan in the ambient water significantly altered the immune parameters of the *F. indicus* leading to drastic changes in the metabolic pathway in haemolymph. The total protein, glucose content of the plasma, and cloting time increased significantly while the total haemocyte count (THC) and phenoloxidase (PO) activity decreased drastically at both the level of pesticides within 20 DoE. These changes in the immune system of *F. indicus* can provide favorable condition for pathogens present in the aquatic environment to invade the animals and can cause mass mortality. To undertake health monitoring on shrimp farms, and other aquatic environment it is necessary to monitor the relationships between environmental conditions and normal or abnormal values of immune responses of shrimp. This will help to develop strategies to avoid diseases outbreak of in aquatic environment.

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