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Manifestation of carbaryl toxicity on soluble protein and histopathology in the hepatopancreas and gills of the prawn, *Macrobrachium malcolmsonii*

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Abstract: *The present study examined alterations in the protein pattern and histopathology of the hepatopancreas and gills of Macrobrachium malcolmsonii following exposure to Sevin®, the commercial grade of carbaryl (1-Naphthylmethyl carbamate) pesticide. Juvenile prawns were exposed to sub-lethal concentrations 5.15 µg l^{-1}, 7.73 µg l^{-1} and 15.47 µg l^{-1} of carbaryl for 21 days. Samples were obtained from the hepatopancreas and gills of prawns. The concentration of buffer soluble protein in tissues of test prawns was significantly ($p<2.5\%$) lower than the control. This suggests that proteins were utilized to generate energy in order to withstand carbaryl induced toxic stress. Polypeptide bands of different molecular mass (150 - 10 kDa) in tissues of test prawns stained less intensely than those in control. This indicates that carbaryl toxicity caused protein degradation in tissues of test prawns. The histology of the hepatopancreas and gills showed few marked pathological changes in prawns exposed to carbaryl. These include rupture of the basal laminae, abnormal infiltration / aggregation of hemocytes in the interstitial sinus of the hepatopancreas and in the hemocoelic space of gill lamellae, fused gill lamellae and necrosis like appearance both in the tubules of the hepatopancreas and gill lamellae. The structural alterations observed in the hepatopancreas and gills of the prawns suggest that carbaryl caused tissue damage in M. malcolmsonii. Protein degradation is a phenomenon in damaging tissues, and thus, under such condition the hepatopancreas and gills of test prawns would not perform their vital functions, which in turn ultimately affect the survival and growth of M. malcolmsonii. The changes noted in protein and histology of test prawns can be taken as biomarkers for monitoring pesticide pollution in aquatic environments. Since this species of prawn is a nutritious delicacy to human being, monitoring aquatic pollution by carbaryl is warranted.*

Key words: *Carbaryl, Prawn, Protein, SDS-PAGE, Histopathology, Hepatopancreas, Gills*
PDF of full length paper is available online

Introduction

Water bodies adjoining the agricultural fields are continuously being contaminated by leaching of pesticides and affects non-target organisms. The carbamate pesticides are used world wide for agricultural pest control operations. Carbaryl (C_{12} H_{11} NO_2) is an ester of N-methyl carbamic acid (N-naphthyl N-methyl carbamate). It is a broad spectrum contact poison and ranks worldwide as one of the most popular insecticides for control of agricultural pests, ectoparasites of human and animals including head lice. Pesticides and heavy metals induced alterations in the biochemistry and physiology of aquatic animals in general (Gupta and Kumar, 2006; Bhide *et al*., 2006; Satyaparameshwar *et al*., 2006; Singh and Singh, 2007; Tilak *et al.,* 2007; Srivastava *et al.,* 2008; Butchiram *et al.,* 2009) including *M. malcolmsonii* (Bhavan and Geraldine, 1997, 2000a,b, 2001, 2002, 2004; Bhavan *et al.,* 2008; Geraldine *et al*., 1999; Kabila *et al*., 2000a,b; Yamuna *et al.,* 2002) has extensively been reported.

M. malcolmsonii is the only commercially important prawn species which inhabits the Cauvery river, an important perennial river of South India. Carbaryl is widely used along the belt of the Cauvery river and also all over India for the control of agricultural crop pests. Therefore, there is possibility for contamination of natural water bodies by this pesticide. Carbaryl is moderately mobile in soils and can be found in the groundwater and surface water due to its widespread use and persistence under acidic conditions (Venkateswarlu *et al.,* 1980). In river water (pH, 7.3-8) carbaryl degrade completely within 2 weeks (Eichelberger and Lichtenberg, 1971). It degrades in distilled water with a half-life of 3.2 hr at pH, 9 and 12.1 days at pH, 7 (Wolfe *et al.,* 1978). It has low solubility in water (Venkateswarlu *et al.,* 1980). The half-life of carbaryl is about 10 days in aqueous suspension at pH 7 (Hassal, 1990). Our previous studies have revealed that carbaryl was toxic to *M. malcolmsonii* and its accumulation in the body resulted in alterations in various biochemical constituents such as total protein, glycogen, free sugar, free amino acid, glutathione S-transferase, acetylcholinesterase, lactate dehyrogenase, and phosphatases (Bhavan *et al*., 1997a,b; Bhavan and Geraldine, 2002). In view of the above, it was quite interesting to study the carbaryl induced changes in buffer soluble protein and histopathology in the hepatopancreas and gills of *M. malcolmsonii* following exposure to sub-lethal concentrations. Moreover, reports pertaining to these aspects are not available in *Macrobrachium* species.

Materials and Methods

Juveniles of *M. malcolmsonii* were collected from the lower anicut (a water regulation canal) of the Cauvery river with the help of State Fisheries Department (Kumbakonam, Thanjavur district, Tamilnadu, India) personnel. Prawns were transported in oxygenated polythene bags and acclimatized to laboratory conditions with groundwater for three weeks in a cement aquarium (capacity: 1000 liter). The physico-chemical characteristics of Cauvery river water and ground water used in the laboratory were estimated by standard

methods (APHA, 2005). The river water had these characteristics: salinity, 1.5%; total hardness, 120.0 mg l^{-1}; pH, 8.3; nitrate, 1.6 mg l^{-1}; chloride, 28.0 mg l^{-1}; ammonia, 0.028 mg l^{-1}; dissolved oxygen, 5.8 mg l^{-1}; BOD, 30.7 mg l^{-1}; COD, 60.7 mg l^{-1} and total solid, 1.8 g l^{-1}. The ground water had these characteristics: salinity, 1.4%; total hardness, 255.0 mg l^{-1}; pH, 8.2; nitrate, 1.6 mg l^{-1}; chloride, 27.0 mg l^{-1}; ammonia, 0.058 mg l^{-1}; dissolved oxygen, 6.7 mg l^{-1}; BOD, 85.0 mg l^{-1}; COD, 147.0 mg l^{-1}; and total solid, 1.7 g l^{-1}).

Sevin® 50% W.P., containing 50% active ingredient of carbaryl (1-Naphthyl methylcarbamate) purchased from a local agrochemical service centre was used. It was directly dissolved in double distilled water to prepare solutions of the required concentration.

96 hr LC_{50} value of carbaryl (Sevin® 50% W.P) to the juveniles of *M. malcolmsonii* as 77.370 µg l^{-1} (Bhavan *et al*., 1997a). Based on this three sub-lethal concentrations (1/15th, 1/10th and 1/5th of the 96 hr LC_{50}) were chosen. Active ingredient of carbaryl present in these three concentrations was computed to be 5.15 µg l^{-1}, 7.73 µg l^{-1} and 15.47 µg l^{-1} respectively.

For the present study the prawns were divided into four groups, each comprising 30 intermoult juveniles (average length: 4.5-5.0 cm and body mass: 1.0-1.25 g). One group served as control; the other groups were exposed to three sub lethal concentrations of carbaryl. Each group comprised of three aquaria (15 liter capacity), with 10 juveniles in each aquarium. The experiment was carried out for 21 days. Water medium was gently siphoned out daily and replaced by medium containing freshly prepared carbaryl solution, with minimal disturbance to the prawns. During the course of the experiment the water medium was not aerated and the animals were fed *ad libitum* with boiled beef liver. Tissues of the prawns such as hepatopancreas and gills were sampled on 21st day of exposure. For buffer soluble protein and SDS-PAGE, 25 prawns in each group were sampled, tissues from 5 prawns were pooled to constitute a single observation, and thus, 5 such observations were made for each group. The remaining 5 prawns were sampled for histopathological observations.

Buffer soluble protein and SDS-PAGE: Samples were prepared in Tris-HCl buffer – 0.1 M (pH, 8.6). The concentration of buffer soluble protein was estimated by the method of Lowry *et al*. (1951) using spectrophotometer (Systronic-118, Systronics Ltd., Bombay, India). The data were analyzed statistically by adopting student 't' test (Zar, 1984). 12% linear slab gels (15 x 10 cm) were prepared essentially as described by Laemmli (1970). Sample buffer was prepared by the method of Lee and Watson (1995). Each well was uniformly loaded with 200 µg of protein. Electrophoresis was carried out for about 4 hr at 50 V DC for stacking gel and 100 V DC for separating gel in an air conditioned room. After electrophoresis, the gel for the hepatopancreas was stained with silver nitrate (Merril *et al*., 1984). The gel for the gills of the prawns was stained for 24 hr with Coomassie Brilliant Blue R solution and de-stained in methanol (30%), acetic acid (10%) and water (60%). Molecular weight standards (Servo Company, USA) of phosphorylase b (97.4 kDa), bovine serum albumin (67 kDa), ovalbumin (45 kDa) and carbonic anhydrase (29 kDa) were also run parallel in the gel.

Histopathology: The hepatopancreas and gills were dissected out and immediately fixed in Bouin's fixative for 48 hr. Preserved tissues were carefully processed by a routine histological method (Gurr, 1962); dehydrated in alcohol series and embedded in paraffin wax. They were cut into 6 µm thickness by a rotary microtome (Weswox, MT 1090/1090A). Sections were processed and stained by haematoxylin and eosin for observation in the light microscope.

Results and Discussion

Buffer soluble protein and SDS-PAGE: The concentration of buffer soluble protein in the hepatopancreas and gills of test prawns was found to be significantly lower than that of respective controls irrespective of the sub-lethal concentrations of carbaryl (Table 1). The decrease was most pronounced in prawns exposed to 15.47 µg l^{-1} of carbaryl (12.4% in the hepatopancreas and 18.9% in the gills). In prawns exposed to 7.73 µg l^{-1} of carbaryl, the decrease in soluble protein levels were 8.4% in the hepatopancreas and 15.3% in the gills. In the case of 5.15 µg l^{-1} of carbaryl, the decrease was 6.0% in the hepatopancreas and 10.3% in the gills. Among the two tissues tested, the gills exhibited more changes in concentration of buffer soluble protein than hepatopancreas (Table 1). The decline in buffer soluble protein noted in tissues of test prawns was further evident from the quit obvious decline recorded in the staining intensity of various polypeptide bands of molecular mass between 150-10 kDa resolved in the hepatopancreas and gills (Fig. 1,2, Plate I).

The decline in buffer soluble protein indicates the fact that protein was utilized excessively. In our previous studies the concentrations of total carbohydrate and glycogen were found to decreased in tissues of *M. malcolmsonii* exposed to same concentrations (Bhavan and Geraldine, 2002). In the present study as well such decline in total carbohydrate level might have occurred. Thus, physiological compensatory mechanisms were in operation to provide intermediates for deriving energy to cope up with the energy demand induced by stress of carbaryl. Similar reasons have also been suggested in the freshwater field crab, *Barytelphusa guerini* to explain toxicity of methylparathion (Reddy and Rao, 1991) and in *M. malcolmsonii* to explain dichlorvos and endosulfan toxicity (Geraldine *et al*., 1999; Bhavan and Geraldine, 1997, 2001, 2002). Since, the gills are the primary organ for respiration and osmoregulation it is inseparably in contact with the water medium. In our previous study, both accumulation and depuration of carbaryl has been primarily occurred in the gill tissue of *M. malcolmsonii* exposed exactly to similar sub lethal concentrations that were used in the present study (Bhavan *et al*., 1997b). Thus, the gills exhibit more changes in concentration of buffer soluble protein than the hepatopancreas. Hence, the gills can be taken as a readily responding organ to carbaryl toxicity.

Toxicity of carbaryl in tissues of test prawns might have interfered with the biosynthesis of proteins, which in turn could have resulted in loss of cellular ions and proteins (Eller, 1975; Sola *et al*., 1994). Therefore, breakdown as well as synthesis of proteins may

Table-1: Concentration of buffer soluble protein in tissues of *M. malcolmsonii* exposed to carbaryl (mg g^{-1} wet tissue)

Tissues	Sub lethal concentrations (µg l^{-1})			
	Control	5.15	7.73	15.47
Hepatopancreas	151.430 ± 3.141	142.476 ± 3.223(6.0 %)	138.441 ± 3.166(8.4 %)	132.349 ± 3.081(12.4 %)
Gills	91.452 ± 1.994	82.071 ± 2.462(10.3 %)	77.526 ± 1.620(15.3 %)	74.217 ± 1.670(18.9 %)

Each value is mean ± SD (n=5). All the values are significant at p < 2.5 % level, ('t' test). Values in parentheses are percentage decrease over control

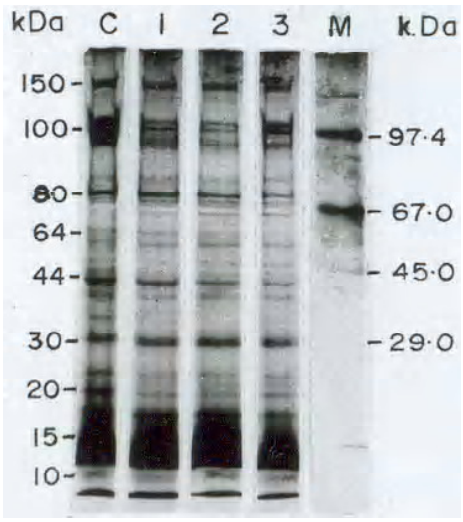

Fig. 1: Electrophoretic profile (12% SDS-PAGE) of proteins from the hepatopancreas of *M. malcolmsonii* exposed to carbaryl. M = Marker protein; C = Control; 1 = 5.15 µg l^{-1} of carbaryl; 2 = 7.73 µg l^{-1} of carbaryl and 3 = 15.47 µg l^{-1} of carbaryl

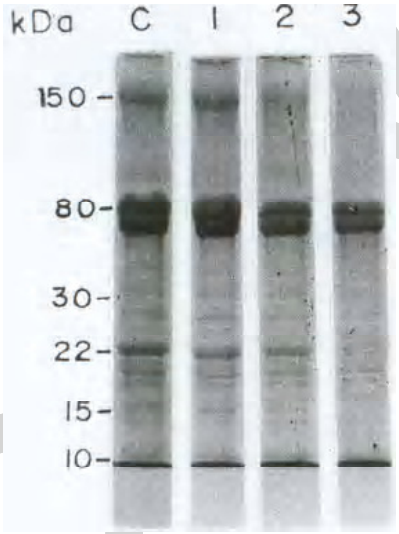

Fig. 2: Electrophoretic profile (12% SDS-PAGE) of proteins from the gills of *M. malcolmsonii* exposed to carbaryl. C = Control; 1 = 5.15 µg l^{-1} of carbaryl; 2 = 7.73 µg l^{-1} of carbaryl and 3 = 15.47 µg l^{-1} of carbaryl

have occurred as a toxicological response in prawns exposed to carbaryl. The breakdown of protein might have dominated over its synthesis. Thus, decline was recorded in concentration of soluble protein and the staining intensity of various polypeptide bands in tissues of *M. malcolmsonii* exposed to carbaryl. The results further suggest protein degradation due to manifestation of carbaryl toxicity. According to Wedler (1987) under stress condition protein denaturation occurs due to the weakening of polar bonds, misfoldings and protein aggregation. Such phenomenon might have been occurred in the present study as well due to toxic stress of carbaryl.

Histopathology: The hepatopancreas of control prawns showed the well-organized glandular tubular structure. A single layer of epithelial cells was found lining the tubules. The cells showed normal differentiation into E-cells at the narrow distal end of the tubule, R-cells and F cells a short distance away from the distal region and B-cells in the middle and proximal regions of the tubules (Plate I-1). Some of the E-cells exhibited mitotic stages as they proliferate and give rise to the other types of cells. The B-cells exhibited large apical secretary granules. The R-calls structurally resemble typical digestive, absorptive and storage cells. The F-cells were found to be non-vacuolated and deeply stained. The interstitial sinuses (IS) between tubules and basal laminae (BL) were normal (Plate I-1).

The crustacean hepatopancreas is a sensitive organ and liable to injury by pesticides and other water borne pollutants (Baticados *et al*., 1987; Vogt, 1987; Jirvanichpaisal and Miyasaki, 1994; Bhavan and Geraldine, 2000a). In the present study, carbaryl was found to alter the normal microstructure of the hepatopancreas with the following specific pathological changes in *M. malcolmsonii* (Plate I-2,4). Rupture of the basal laminae (RBL) was observed in test prawns exposed to 5.15 µg l^{-1} carbaryl (Plate I-2). Interstitial sinus with abnormal infiltration / aggregation of hemocytes (AIH) was seen in test prawns exposed to 7.73 µg l^{-1} of carbaryl (Plate I-3). Necrotic hepatopancreatic tubules (NHT) filled with tissue debris (TD) was the most note-worthy changes recorded in the hepatopancreas of test prawns exposed to 15.47 µg l^{-1} of carbaryl (Plate I-4). Similar histopathological observations have been reported in the marine prawn *Penaeus vannamei* exposed to benlate (methyl 1-(butylcarbamoyl)-2-benzimidiasole carbamate), a commercial product of benomyl (Lightner *et al*., 1996), in *Penaeus stylirostris, P. vannamei* and *Penaeus monodon* due to aflatoxicosis (Bautista *et al*., 1994) and also in *M. malcolmsonii* exposed to endosulfan (Bhavan and Geraldine, 2000a).

The noted histopathological changes in the hepatopancreas may be due to accumulation of carbaryl (Bhavan *et al.,* 1997b) since this organ is the centre of storage, metabolism and detoxification. The rupture of basal laminae (RBL) observed in the hepatopancreatic tubules (Plate I-2) suggest that tissue integrity was affected in prawns due to exposure to carbaryl. Abnormal infiltration of hemocytes

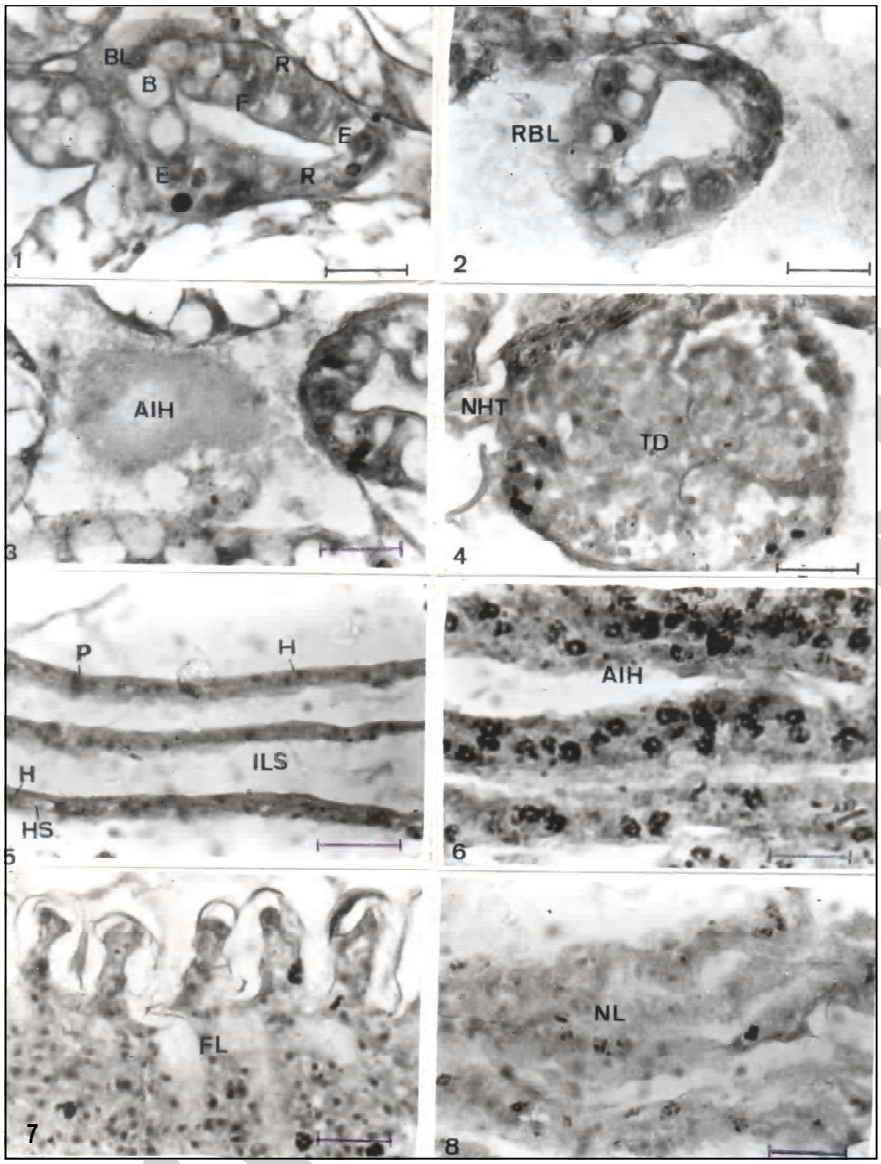

Plate - I

1: Microphotograph of the hepatopancreas of control prawn showing normal hepatopancreatic tubules with embryonic cells (E-cells), absorptive cells (R-cells), secretory cells (B-cells) and fibrillar cells (F-cells). The interstitial sinuses (IS) and basal laminae (BL) are appeared normal, x 400 (bar = 10x). 2-4: Microphotographs of the hepatopancreas of test prawns exposed to carbaryl, 2- The tubule epithelium showing ruptured basal laminae (RBL), x 400 (bar = 10x). 3- The interstitial sinus is filled with abnormal infiltration of hemocytes (AIH), x 400 (bar = 10x). 4- A necrotic hepatopancreatic tubule (NHT) filled with tissue debries (TD), x 400 (bar = 10x). 5- Microphotograph of the gills of control prawn showing arrangement of gill lamellae (L) with uniform inter-lamellar space (ILS), optimum number of hemocytes (H) in the hemocoelic space (HS) and pillar cells (P), x 400 (bar = 10x). 6-8: Microphotographs of the gills of test prawns exposed to carbaryl, 6- showing abnormal infiltration of hemocytic (AIH) in the hemocoelic space, x 400 (bar = 10x), 7- fused gill lamellae (FL), x 400 (bar = 10x), 8- necrotic gill lamellae (NL), x 400 (bar = 10x)

in the interstitial sinuses (AIH) noted in the hepatopancreas of test prawns (Plate I-3) suggest that the mechanism of cellular/ host defence was in operation to neutralize the tissue damage caused by carbaryl and since hemocytes are the most important form of cellular defence in crustaceans (Bodhipaksha and Weeks-Perkins, 1994). The formation of necrotic hepatopancreatic tubules (NHT) recorded in test prawns (Plate I-4) indicates the fact that the distortion, disintegration and death of cells occurred in the hepatopancreas of *M. malcolmsonii* exposed to the highest sub-lethal concentration of carbaryl. Therefore, carbaryl toxicity affects the normal integrity and caused tissue damage in the hepatopancreas of *M. malcolmsonii*.

The gills of control prawns showed uniform arrangement of lamellae (L) with uniform inter-lamellar space (ILS) and normal

hemocoelic space (HS) with hemocytes (H) in the hemolymph circulation (Plate I-5). Pillar cells (P), which are specialized epithelial cells, were found to extend into the lamellar sinus at intervals and to abut similar cells extending from the opposite surface (Plate I-5).

Toxic substances damage the gill tissues first, thereby reducing the oxygen consumption and disrupting the osmoregulatory function in crustaceans (Ghate and Mulherkar, 1979). Exposure of *M. malcolmsonii* to carbaryl resulted in following notable structural alterations in the gill lamellae (Plate I-6 to 8). Abnormal infiltration of hemocytes (AIH) in the hemocoelic space was observed in test prawns exposed to 5.15 µg l^{-1} of carbaryl (Plate I-6). Fused gill lamellae (FL) was seen in test prawns exposed to 7.73 µg l^{-1} of carbaryl (Plate I-7). In test prawns exposed to15.47 µg l^{-1} carbaryl, the gill lamellae were obliterated by the proliferated and infiltrated cells, causing distension and gross enlargement (Plate I-8), and such gill lamellae appeared to be necrotic (NL). Similar lesions have also been reported for *Macrobrachium kistensis* exposed to copper sulphate (Ghate and Mulherkar, 1979), in *Macrobrachium idae* exposed to mercury (Victor *et al*., 1990), in *Macrobrachium lamarrei* exposed to hospital wastes (Kaliamurthy *et al*., 1994) and in *M. malcolmsonii* exposed to endosulfan (Bhavan and Geraldine, 2000a).

The observed abnormal infiltration of hemocytes (AIH) in the hemocoelic space of gill lamellae (Plate I-6) indicates recruitment of hemocytes in host defence reaction to destroy carbaryl (Bodhipaksha and Weeks-Perkins, 1994). The tissue inflammation caused during host defense reaction led to fusion of gill lamellae (FL), which in turn led to hyperplasia and clubbing of entire gills in *M. malcolmsonii* exposed to carbaryl (Plate I-7). The inflammatory changes reduce the vulnerable surface area of the gills in order to maintain its basic functions (Mallatt, 1985). However, such conditions, while helping to slow down toxicant uptake, it could result in severe hamper in its vital functions, which in turn could led to dysfunctional or even nonfunctional gills, and eventually result in asphyxia (Mitchell *et al*. 1978; Tamse *et al*., 1995). The lesions noted in the gills of test prawns led to formation of necrotic like gill lamellae (NL) as a result of severe toxic effect of carbaryl (Plate I-8), which indicates the fact that the normal tissue integrity in the gills of *M. malcolmsonii* was distorted.

In the present study, the observed decrease in both buffer soluble protein as well as the staining intensity of several polypeptide bands, the rupture of basal laminae (RBL), abnormal infiltration of hemocytes in the interstitial sinuses (AIH) of the hepatopancreas and in the hemocoelic space of the gill lamellae and formation of fused gill lamellae (FL) were all represented the constitution of primary physiological compensatory (adaptive) mechanisms in *M. malcolmsonii* to withstand the effects of carbaryl up to the lowest and intermediate sub-lethal levels. However, the progressive decline in buffer soluble protein and the staining intensity of various polypeptide bands, the necrosis in the hepatopancreas (NHT) and gills (NL) of *M. malcolmsonii* exposed to the highest sub-lethal concentration of carbaryl (15.47 µg l^{-1}) represent severe toxicological symptoms due to non-compensatory (non-adaptive) state. Therefore, it is suggested that such changes would lead to a progressive loss of basic biological functions of the hepatopancreas and gills in *M. malcolmsonii* exposed to carbaryl. These biochemical and histopathological changes can definitely be taken as biomarkers for indication of pesticide pollution in natural aquatic environments. Since *M. malcolmsonii* is recognized as one of the potential sources of protein and nutritious delicacy to mankind, it is imperative that it should be prevented from deterioration by possible carbaryl pollution. Hence, monitoring of the indiscriminate applications of carbaryl is warranted.

Acknowledgments

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