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Toxicity of phosphamidon on the gills of a freshwater fish, *Anabas testudineus*

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ABSTRACT

An array of wide spectrum chemicals and their combinations are now being used as insecticides, fungicides, herbicides, nematicides, rodenticides and molluscicides. Kuttanad, the rice bowl of Kerala, India (Altitude – sea level; Latitude – 9.9 N and Longitude – 76.2 E) is a region where there is over application of pesticide during the punja cultivation periods. Phosphamidon, Monocrotophos, Henosan and Thymet are the major components of the pesticides being used in Kuttanad. The exposure of fish to pesticides is likely to induce a number of lesions in body organs like gills, liver and kidney. The gills are the most important and sensitive organs of a fish body, which first experience the hazards of, polluted ambient water. As the fish gills are exposed to pesticides, a wide variety of structural changes in fish gills have been reported. They are hypertrophy, hyperplasia, oedematous separation of respiratory epithelium, telengiectasis, stasis, hemorrhage, necrosis, etc. The study shows that the pathological changes are gradually increasing from the lowest concentration to the highest concentration. The present study is aimed at assessing the extent of Phosphamidon induced histological changes in the gills of *Anabas testudineus*, a fresh water food fish widely inhabiting the paddy fields of Kuttanad, Kerala, India, and to arrive at a Maximum Allowable Toxicant Concentration (MATC) and an Application Factor (AF) based on the histopathology. This end point gives an 'early warning' of the damage caused in the fish at the histological level before their mortality. Hence preventive measures can be taken to protect the fishery resources. Based on the index value, the MATC is 3.1623 ppm and the Application Factor (AF)is 0.0804.

Key words*: Pesticide, Histopathology, Gills, MATC, AF.*

Introduction

A wide range of toxicants originating from agricultural and industrial activities have been introduced into the aquatic environment inducing altered fish health and toxicity. Kuttanad, the rice bowl of Kerala, India, is a region where there is over application of pesticide during the punja cultivation period. According to the data compiled by Kuttanad Water Balance Study Project, 485 tonnes of pesticides were applied in Kuttanad on an annual basis of which 370 tonnes were used for the puncha crop alone (KWBSP, 1990). Dimecron, Monocrotophos, Henosan, Thymet, Fernoxan and Nuvacron are the major components of the pesticides being used in Kuttanad. The study of Sruthy *et al.* (2017) found traces of 16 pesticides raising alarm in paddy fields of Kuttanad and which tend to accumulate in organisms and become more concentrated as they pass up the food chain. In such a degraded aquatic environ-

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ment, particularly where pollutants occur at chronic sublethal concentrations, biomarkers are sensitive tools for the early detection of environmental changes (Sulekha and Mercy, 2022; 2021b; 2021a; 2011; 2009; Heier *et al.*, 2009). Biomarkers demonstrate correlations between environmental factors and their outcomes, and they provide insight into both the status of an ecosystem and the status of a given organism (Strzy¿ewska *et al.*, 2015; Sorour and Harbey, 2012; Flores-Lopes and Thomaz, 2011). Fishes and fish gills comprise one of these biomarkers are the most important and sensitive organs of a fish body, which first experience the hazards of, polluted ambient water (Sulekha and Mercy, 2021a; Mitrovic-Tutundzic and Poleksic, 1986; Mallatt, 1985).

Morphological lesions in the gills are easier to detect than functional abnormalities (Velasco-Santamarßa and Cruz-Casallas, 2008), because they may become visible long before behavioural changes in fish can be detected (Heier *et al.*, 2009). Furthermore, the gills serve as early warning signs on the health status of fish. It is believed that the degree of morphological lesions in the gills can delimit the degree of environmental pollution (Flores-Lopes and Thomaz, 2011). Indeed, the concentration of toxic factors in this organ is identical to the level of xenobiotics found in the water that the fish inhabit. In contrast, toxin concentrations in the other organs such as the liver or kidneys depict only the degree of distribution of the toxic compounds (Manera *et al.*, 2016). Therefore the histopathological analysis of gills is a more suitable tool to assess the environmental deterioration. The present laboratory study was conducted for the assessment of pesticide induced pathogenesis in the gills of *Anabas testudineus*, a true denizen of Kuttanad paddy fields, to calculate the sublethal effects of phosphamidon, a widely used pesticide in the paddy fields of Kuttanad, before to their mortality. The maximum allowable toxicant concentration (MATC) and the application factor (AF) are derived from the study for the calculation of safe use of the particular pesticide in the paddy fields. So that we can calculate the safe doze of other similar pesticides, provided their LC50, which can be applied in the field.

Materials and Methods

The experiment on the sublethal toxicity of an organophosphate pesticide, phosphamidon, on the juveniles of *Anabas testudineus* were conducted for 30 days during the period of investigation. The experiment was conducted in the wet lab which has concrete floor with gentle slope, having proper drainage to remove pesticide contaminated water to minimize the hazards. There were provisions for water supply, lighting and adequate ventilation in the shed.

Juveniles of *Anabas testudineus* were collected from pollution free ponds from the natural habitat. The average size of *Anabas testudineus* with7.2 ±0.6cm in total length and 7.50 ± 2.50g in weight were used for phosphamidon exposure. During these periods, they were fed *ad libitum* once a day on fresh clam meat. Phosphamidon is a water soluble organophosphate concentrate containing O-2, Chloro 2 (diethyl carbonyl - 1 - methyl -O vinyl) O, O - dimethyl phosphate as active ingredient in a kilogram of product. This is equivalent to 1000 gm of phosphamidon in a litre of product (w/v). This is also a systemic insecticide - cum - acaricide, effective against sucking, chewing and mining insects on paddy, maize, barly, wheat, cotton, sugar cane, oil seeds, vegetables, fruits, tea and cardamom and against mites on wheat and tea. This is also a product of Hindustan-Ciba - Geigy Limited, Bombay, known under the trade name DIMECRON. This is effective against sucking, chewing and mining insects on paddy, maize, barley, etc.

Based on the LC_{50} value (39.34ppm) obtained (Mercy *et al.*, 2000a), five nominal concentrations of the pesticides were selected for sublethal toxicity studies. Maximum and minimum sublethal concentrations were chosen based on Konar (1969) and Sprague (1973). The concentrations of pesticides used for each sublethal exposure were 0.0 ppm, 0.5 ppm, 1.0 ppm, 2.0 ppm, 5.0 ppm and 10.0 ppm of phosphamidon. Sublethal exposure was done in a static system where water and pesticide medium were renewed every 24 hr to maintain the desired pesticide concentration. A control, free of pesticide, was also maintained in each experiment. All the treatments and the controls were carried out in triplicates. Ten healthy fishes, chosen at random from the acclimated stock and the experiment was done in cement cisterns of 35 litres capacity with the following specifications. Clear filtered fresh water drawn from an open well subjected to a fine filtration using nylon bolting cloth, was used for the experiment and the tanks were filled with 32 litres of water and aerated to saturation prior to use. The

fishes were fed once a day on fresh clam meat *ad libitum*.

Water quality parameters in the experimental tanks were measured by the following methods. Modified standard Wrinkler's method (Strickland and Parsons, 1972) was used for measuring the dissolved oxygen. pH was tested using universal pH indicator solution method. Temperature tested using thermometer with an accuracy of 0.1 °C.

After 30 days of the experiments five specimens from each of the treated as well as the control group were sacrificed and gills, the target organ was dissected out and fixed immediately in Bouin's fluid. This fixed organ was washed, dehydrated, cleared and embedded in paraffin wax. Serial sections of the organ was taken at 3 to 5 µm thickness and stained with Hematoxylin-eosin staining procedures (Stevens, 1982). Detailed histological observations were carried out with the help of a binocular microscope. Based on the histological lesions observed in the gills of these fishes the index values were calculated. The method followed for the analysis is that of Poleksic and Mitrovic-Tutundzic (1994) who has classified the gill lesions based on two criteria

First criterion

The first criterion classified gill lesion, based on the type and location of the damaged gill tissue, into five main groups.

(a) Hypertrophy and hyperplasia of gill epithelia and related changes
(b) Changes in the mucous and/or chloride cells
(c) Gill parasites
(d) Blood vessel changes
(e) Terminal stages

Second criterion

The second criterion - of severity, is based on the scope for repair of the lesions, i.e. the possibility of restoration of normal morphological structure with an improvement in the environmental conditions, or the cessation of pollution.

With regard to this second criterion, gill lesions are classified into three progressive stages:

(a) For the first stage 10^{o}
(b) For the second stage 10^{1}
(c) For the third stage 10^{2}

Of the total of 26 types of gill change, 19 belong to the first stage, 5 to the second and 2 to the third (Poleksic and Mitrovic-Tutundzic, 1994).

The sum of the number of lesion types within each of the three stages multiplied by the stage index as above represents the numerical value of the degree of damage in a single fish gill, i.e.

$$I = \sum_{i=1}^{19} a_i + 10 \sum_{i=1}^{5} b_i + 10^2 \sum_{i=1}^{2} c_i$$

Where I is the degree of changes in a single fish gill

a = first stage alterations
b = second stage alterations
c = third stage alterations

The method of calculating a value of I marks it possible to compare the degree of tissue change in a large number of fish from different situation of pollution, and to correlate the intensity of pollution with the intensity of the changes found.

Application of the mathematical equation to derive categories of gill damage.

Effects of I values are denoted as follows

***I* values**	**Effects**
0-10	Functionally normal gills
11-20	Slightly to moderately damaged gills
21-50	Moderately to heavily damaged gills
>100	Irreparably damaged gills.

Based on these I values, obtained for the gills,No Observable Effect Concentration (NOEC - it is the concentration at which there is no significant change in the histology from that of the control) and Least Observable Effect Concentration (LOEC - in which there are significant changes in the histological structure from that of the control) was determined. Concentrations which showed I values less than 10 (functionally normal) were taken as NOEC and concentrations that gave I values between 10 and 20 were taken as LOEC.

Based on these NOEC and LOEC values Maximum Allowable Toxicant Concentration (MATC- the concentration in which the animal can tolerate for survival and reproduction without much effect on its metabolic activities) was calculated using the formula

$$MATC = (NOEC \times LOEC)^{1/2}$$

From this MATC value Application Factor (AF) was calculated using the formula(Mount and Stephan, 1967)

$$AF = \frac{MATC}{48hr\ LC_{50}}$$

The AF, which is worked out for one species can be used for other related species also, provided their

48hr LC_{50} is known. From this the MATC for latter species can be conveniently found out. This will be an important tool for the farmers in deciding the concentrations of pesticides before its application into the paddy fields.

An evaluation of histopathological changes in the vital organs of fish exposed to different concentrations of pesticides would help in assessing the magnitude of pesticide induced pathogenesis in fish.

Results

Physico-chemical parameters

Weekly mean temperature, pH and DO values ranged from 27.0 to 28.71°C, 7.3 to 7.6 and 6.1 to 7.1 $mg.l^{-1}$ respectively.

Structure of the gill of Anabas testudineus

A. testudineus is characterized by small number of short gill filaments. The gill filaments are lined externally with a thick stratified epithelium referred to as the primary epithelium or the gill filament epithelium. *A.testudineus* is a fresh water species, so the chloride cells, the characteristic of marine water species is few in number or absent. The secondary lamellae that are the actual sites of gaseous exchange are arranged on the two sides of a filament. This lamella is lined by a double-layered squamous cells epithelium. Internal to the epithelium is the lamellar blood sinuses, lined and spanned by pillar cells of contractile function. A marginal blood channel, lined by endothelium, occurs within the apex of the lamellae. The gills are characterised by equally spaced secondary lamellae, intact cellular layers and no sign of fusion between neighbouring lamellae.

The details of the number of fishes examined under the phosphamidon exposure and the pattern of damages noticed in each fish are summarised in the Table 1.

Histopathological observations

Control: Minor change could be observed was hypertrophy in few lamellae (Fig. 1A). The index value calculated is 0.6 (Table 2).

0.5 ppm: Histology of the gills of fishes exposed to this concentration exhibited hypertrophy and thinning of secondary lamellae (Fig. 1-B), hyperplasia approximately half the length of secondary lamellae and oedematous separation of respiratory epithelium (Fig 1-C). Distortion of secondary lamellae (Fig. 1-C) and shortening of secondary lamellae (Fig. 1-D) were also exhibited by a number of lamellae. The index value calculated is 3.6 (Table 2).

1.0 ppm: Certain lamellae exhibited fusion of the tips of the secondary lamellae (Fig. 1-E). Shortening of secondary lamellae and oedematous separation of respiratory epithelia from both sides of the secondary lamellae in certain regions (Fig.1-F) and thinning of secondary lamellae (Fig.1-G) were also observed in this exposure. Sometimes the secondary lamellae were fused approximately half of its length due to hyperplasia (Fig. 1-G). Hypertrophy was rarely noted in a few fishes (Fig. 1-G). Hyperplasia and oedematous separation of respiratory epithelium were the common and conspicuous changes in the lamellae of gills exposed to this concentration. The index value calculated is 5.0 (Table 2).

2.0 ppm: The gill lamellae of fishes in this sublethal concentration frequently depicted hyperplasia, oedematous separation of respiratory epithelium and shortening of secondary lamellae. Hyperplasia was exhibited in different ranges. Sometimes it extended approximately upto half the length of the secondary lamellae and in certain other cases several secondary lamellae were completely fused together. Severe oedema separated the respiratory epithelia from both the sides of the secondary lamellae and at certain regions it led to the fusion of the tips of secondary lamellae. Thinning and shortening of secondary lamellae and filamental blood vessel enlargement were rarely noted. Slight distortion of secondary lamellae was also observed in certain filaments (Fig. 1-H). The index value calculated is 6.4 (Table 2).

5.0 ppm: The secondary lamellae of most of the fishes in this concentration exhibited oedematous separation of respiratory epithelia, hyperplasia and telangiectasis.The oedematous separation of respiratory epithelia from both the sides of the secondary lamellae and shortening of secondary lamellae (Fig. 1-I) and thinning of secondary lamellae (FIg.2-K) were frequently observed. All the lamellae of some fishes exhibited hypertrophy (Fig.2-J). In most of the fishes, the secondary lamellar hyperplasia fused the secondary lamellae approximately half the length of it due to hyperplasia (Fig.2-K). Slight stasis could rarely be noted in a few lamellae of all the fishes (Fig.2-K). The adjacent telangiectasis fused the tips of the secondary lamellae and distortion of secondary lamellae was also noticed in certain regions (Fig.2-L). The index value calculated is 17.4 (Table

Table 1. The intensity of alterations in each *A. testudineus* treated with phosphamidon

Concentrations (ppm)	C					0.5					1.0					2.0					5.0					10.0				
No. of fishes	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
GILL ALTERATIONS																														
FIRST STAGE																														
1. Hypertrophy of respiratory epithelium	+	+	+	-	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	+	+	-	-	-	-
2. Irregular hyperplasia of epithelial cells	+	+	+	-	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-	+	+	-	-	—	-
3. Lifting of respiratory epithelium	-	-	-	-	-	+	+	—	-	-	+	+	+	-	-	+++	+++	+	+	-	+++	+++	++	+	+	+++	+++	+++	+++	++
4. Thinning of respiratory epithelium	-	-	-	-	-	++	+	+	-	-	++	+	+	-	-	++	++	+	-	-	++	++	+	+	-	+++	+++	++	+	++
5. Hyperplasia from the base to approximately half the length of the secondary lamellae	-	-	-	-	-	+	+	-	-	-	+	+	+	+	-	++	+	+	-	-	+++	+++	++	+	+	+++	+++	+++	++	++
6. Fusion of tips of secondary lamellae	-	-	-	-	-	-	-	-	-	-	++	++	+	-	-	++	+	+	+	+	++	++	+	+	+	-	-	-	-	-
7. Fusion of several secondary lamellae	-	-	-	-	-	+	+	+	-	-	+	+	+	-	-	++	++	+	+	-	+++	+++	+++	+	+	+++	+++	+++	++	+
8. Shortening of secondary lamellae	-	-	-	-	-	-	-	-	-	-	+	+	+	-	-	++	+	+	+	-										
9. Lamellar teleangiectasis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	++	+	+	-	-	++	++	+	+	+
10. Filamental blood vessel enlargement	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	++	+	+	-	-	-	-	-	-	-	+++	++	+	+	+
SECOND STAGE																														
1. Complete fusion of all the secondary lamellae	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	++	++	+	+	+
2. Stasis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	++	++	+	+	-	++	++	+	+	+
THIRD STAGE																														
1. Necrosis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+++	+++	+++	+++	++

2).

10.0 ppm: Most of the fishes of this concentration exhibited necrosis, a severe irreparable branchial alteration. All the fishes in this exposure showed the oedematous separation of respiratory epithelium, stasis and necrosis (Fig. 2-M). The darkly stained pyknotic nuclei were observed in most of the lamellae of few fishes (Fig. 2-M). The complete fusion of several secondary lamellae and fusion of approximately half the length of secondary lamellae due to hyperplasia of primary lamellar cells could be observed in a few fishes (Fig. 2-N). Filamental blood vessel enlargement and infiltration of blood cells into the capillaries of secondary lamellae were also depicted (Fig. 2-N). Shortening of secondary lamellae (Fig. 2-N) and in some cases the complete

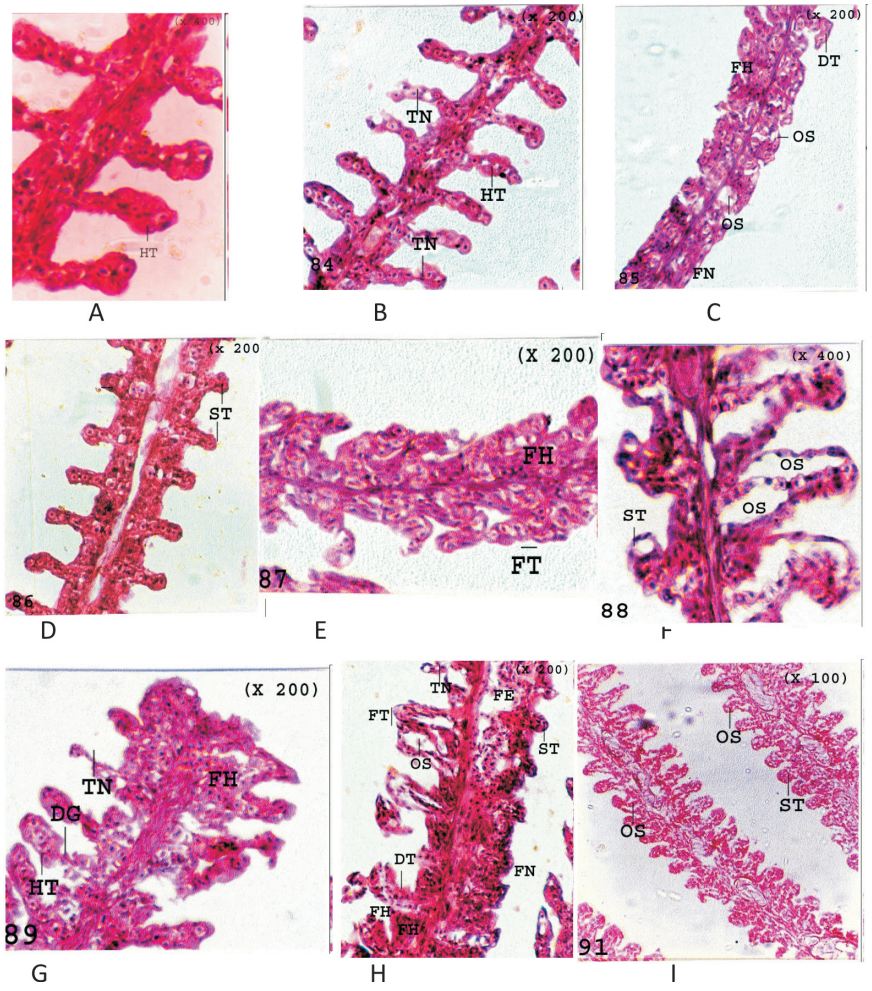

Fig. 1. Gills of *A. testudineus* treated with phosphamidon: [A]control - hypertrophy (HT). H + E x 400. **[B]**0.5 ppm - hypertrophy (HT) and thinning of secondary lamellae (TN). H + E x 200. **[C]**0.5 ppm - distortion of secondary lamellae (DT), hyperplasia approximately half the length of secondary lamellae (FH), oedematous separation of respiratory pithelium (OS) and fusion of several secondary lamellae (FN). H + E x 200. **[D]** 0.5 ppm - shortening of secondary lamellae (ST). H + E x 200. **[E]**1.0 ppm - hyperplasia approximately half the length of secondary lamellae (FH) and fusion of tip of secondary lamellae (FT). H + E x 200.**[F]** 1.0 ppm - oedematous separation of respiratory epithelium (OS) and shortening secondary lamellae (ST). H + E x 400. **[G]**1.0 ppm hypertrophy (HT), hyperplasiaapproximately half the length of secondary lamellae (FH), thinning of secondary lamellae (TN) and degeneration of epithelial cells (DC). H + E x 200. **[H]** 2.0 ppm - thinning of secondary lamellae (TN), filamental blood vessel enlargement (FE), fusion of tips of secondary lamellae (FT), shortening of secondary lamellae (ST), odematous separation of respiratory epithelium (OS), distortion of secondary lamellae (DT), fusion of several secondary lamellae (FN) and hyperplasia approximately half the length of secondary lamellae (FH). H + E x 200.**[I]** 5.0 ppm - oedematous separation of respiratory epithelium (OS) and shortening of secondary lamellae (ST). H + E x 100.

loss of secondary lamellae from one side (Fig. 2-O, P)of the filament was also frequently observed. Thinning of secondary lamellae (Fig. 2-P), stasis (Fig. 2-O) and Telangiectasis (Fig.2-Q)could also be noticed.Almost all the lamellae of few fishes exhibited necrosis Telangiectasis (Fig. 2-O,Q). Due to exfoliation, the secondary lamellae resembled a slender stick like structure (Fig. 2-P). The index value calculated is 116.8 (Table 2).

The histopathological studies made on the gills of *A. testudineus* exposed to different sublethal concentrations showed a dose-related degeneration and necrosis. The NOEC is considered as 2.0 ppm and the LOEC is considered as 5.0 ppm (Table 3). Based on the index values, the calculated MATC was 3.1623 ppm. From this value the AF calculated as:

AF = MATC / 48 h LC_{50} = 3.1623 / 39.34 = 0.0804 (Table 3)

Table 2. The index values of the gill alterations (following Poleksic and Mitrovic-Tutundizic, 1994)

A. testudineus treated with phosphamidon	
Treatment (ppm)	Index value
0.0	0.6
0.5	3.6
1.0	5.0
2.0	6.4
5.0	17.4
10.0	116.8

Index values represents average of 5 fishes

Discussion

Anabas testudineus exposed to sublethal concentrations of phosphamidon obviously exhibited dose–dependent pathological lesions and the maximum severity was noticed in the highest sublethal concentrations. The overall changes observed due to the exposure to different concentrations of phosphamidon are hypertrophy, hyperplasia, fusion of secondary lamellae, oedematous separation of respiratory epithelium, lamellar telangiectasis, stasis, infiltration of blood cells into the secondary lamellae, filamental blood vessel enlargement, complete loss of second-

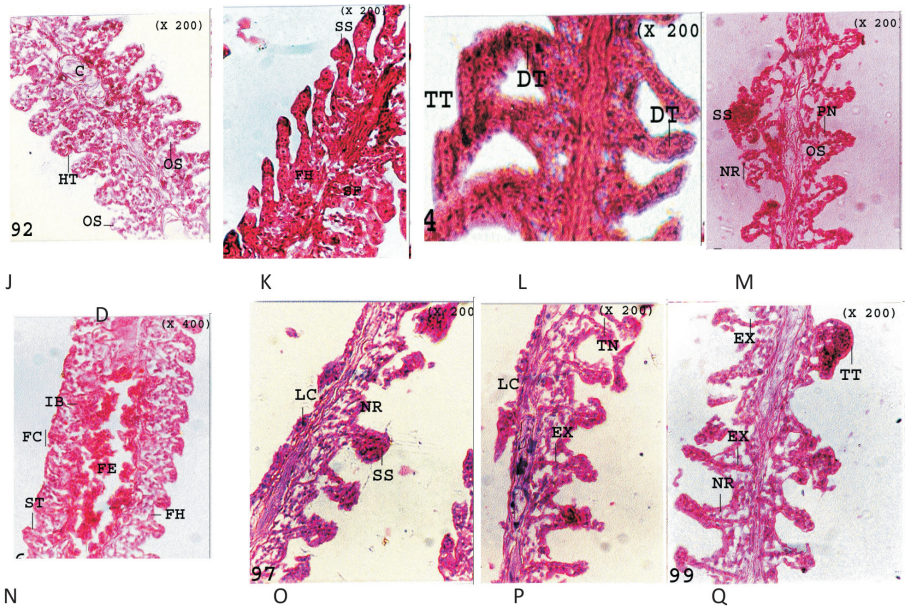

Fig. 2. Gills of *A. testudineus* treated with phosphamidon:[J] 5.0 ppm - hypertrophy (HT), oedematous separation of respiratory epithelium (OS), and cartilage (C). H + E x 200. **[K]** 5.0 ppm - stasis (SS), hyperplasia approximately half the length of secondary lamellae (FH) and thinning of secondary lamellae (TN). H + E x 200.**[L]** 5.0 ppm - distortion of secondary lamellae (DT) and telangiectasis (TT). H + E x 200. **[M]** 10.0 ppm - oedematous separation of respiratory epithelium (OS), stasis (SS), pyknotic nuclei (PN) and necrosis (NR). H + E x 200. **[N]**10.0 ppm complete fusion of secondary lamellae (FC), infiltration of blood cells into the secondary lamellae (IB), filamental blood vessel enlargement (FE), shortening of secondary lamellae (ST) and hyperplasia approximately half the length of secondary lamellae (FH). H + E x 200.**[O]** 10.0 ppm - complete fusion of secondary lamellae (LC), stasis (SS) and necrosis (NR). H + E x 200.**[P]** 10.0 ppm - complete loss of secondary lamellae (LC), thinning of secondary lamellae (TN) and exfoliation (EX). H + E x 200. **[Q]** 10.0 ppm - telangiectasis (TT), exfoliation (EX) and necrosis (NR). H + E x 200.

ary lamellae and necrosis.

Some authors have divided morphological changes into two types: lesions that result from direct damage by irritating factors and lesions that result from fish defence mechanisms (Baskar, 2014; Poleksic and Mitrovic-Tutundzic, 1994). Based on findings from Velasco-Santamarßa and Cruz-Casallas (2008), it can be assumed that epithelial lifting, epithelial cell hyperplasia and hypertrophy, and partial adhesions of the gill lamellae are examples of fish defence mechanisms. These lesions result in an increased distance between the environment and the blood, and thus, they create an additional barrier for environmental factors (Baskar, 2014; Poleksic and Mitrovic-Tutundzic, 1994). Consequently, the epithelial surface available for diffusion is decreased, and contaminant absorption into the blood is reduced. This defensive mechanism is triggered in fish in response to toxins, irritants and factors that induce environmental stress (Baskar, 2014).

Hypertrophy of the respiratory epithelium was observed in the gills of some of the fishes in the control group and 0.5, 1.0 and 5.0 ppm of phosphamidon treated *A. testudineus*. This increases the distance across which water born irritants must diffuse to reach the blood stream (Mallatt, 1985). Since this lesion was found in the lower sublethal concentrations, it is apparent that rather than reflecting direct toxicant action, this alteration can be interpreted as defense response of the fish to the irritants as evidenced by Sulekha and Mercy (2022); Baskar (2014); Velasco-Santamarßa and Cruz-Casallas (2008); Poleksic and Mitrovic-Tutundzic (1994).

In the present study, mild to acute hyperplasia could be observed in accordance with the increasing concentration of pesticideswhich resulted in the interlamellar to interfilamentar fusion. This reaction may increase the epithelial thickness so as to retard or prevent the entry of toxic ions into the blood stream as reported by Sulekha and Mercy (2021a). It is a natural protective response but it diminishes the amount of vulnerable gill surface area, which apparently disrupted respiratory function culminating in hypoxemia (Velasco-Santamarßa and Cruz-Casallas, 2008; Mallat, 1985).

Oedematous separation or lifting of respiratory epithelium from the basement membrane could be observed in *A. testudineus* exposed to all the concentrations of phosphamidon. The separation of respiratory epithelium due to the lifting was very mild in the lower concentrations but the intensity and frequency increased towards the exposure to higher concentrations. The lifting of epithelium could serve as a defense function as these alterations increased the distance across which waterborne irritants must diffuse to reach the blood stream (Sulekha and Mercy, 2021a; Velasco-Santamarßa and Cruz-Casallas, 2008; Mallatt, 1985).

The current study agreed with the observation of Sulekha and Mercy (2021a) and Poleksic and Mitrovic-Tutundzic (1994) the lamellar telangiectasis and stasis are the blood vessel changes. These vascular damages, however, usually found only in animals that were near death. The current study agreed with Mallatt (1985), that the cells comprising branchial blood vessel were relatively resistant to irritant substances and this results vascular damage and it usually found only in animals were near death or exposed to very high doses of pollutants. Thus, the lamellar telangiectasis and stasis were observed in higher concentrations (5.0 and 10.0 ppm) of phosphamidon and it can be considered as the branchial defense response to pesticides as an inflammatory reaction (Sulekha and Mercy, 2021a and Mallatt, 1985).

Histopathological changes in the gills of fishes exposed to higher concentrations were predominated by an extensive degeneration and necrosis of the branchial epithelium resulting in gradual degradation and sloughing of this layer (Paperna and Van As, 1983). In the present study, 10.0 ppm phosphamidon treated *A. testudineus* showed necrosis. According to Temmink *et al.* (1983), necrosis and rupture of the branchial epithelium are believed to reflect "the direct deleterious effects of irritants." Mallatt (1985) reported that epithelial necrosis and rupture are, the most dose-dependent of branchial lesion types, as they are clearly more often reported

Table 3. Maximum allowable toxicant concentration and its corresponding application factor calculated based on the histopathological analysis of *A. testudineus* treated with phosphamidon

Fish species	Pesticides	NOEC (ppm)	LOEC (ppm)	MATC (ppm)	48 hrLC50 (ppm)	AF
A.testudineus	Phosphamidon	2.0	5.0	3.1623	39.34	0.0804

under lethal than under sublethal conditions as in the present study.

The damaged respiratory surface by the destruction of epithelium together with the epithelial lifting increased the diffusion distance between water and blood. This ultimately resulted in inadequate gas exchange. It is presumed that the excessive accumulation of haemocytes in the lamellae has probably impaired their function by altering the blood flow pattern. This in turn resulted in the failure of lamellar circulation and ultimately respiratory collapse. This finally resulted in the death of fishes (Chandy and Kolwalkar, 1984). Infiltration of blood cells in to the central core of primary lamellae and into the respirtatory lamellae were observed in the gills of *A. testudineus* exposed to 10.0 ppm phosphamidon. Ramamurthy *et al.* (1987) also could find such disintegration of blood cells in the blood vessel that passed through the main gill arch. These can be considered as distinct degenerative changes as they were mainly found in the gills exposed to higher concentrations of pesticides.

Distortion of secondary lamellae was one of the frequently observed alterations in the current study. This alteration could be observed in 0.5, 2.0, and 5.0 ppm treated *A. testudineus,* which may be due the weakening of pillar cells. Several scientists have been reported the distortion or curling of secondary lamellae (Sulekha and Mercy, 2021a; Mallatt 1985; Kumaraguru *et al.,* 1982; Kumar and Pant, 1981).

Shortening of secondary lamellae was a characteristic change in all the present treatments. According to Randi *et al.* (1996), the shortening of secondary lamellae could be induced merely by starvation. In fact, cellular involutive processes were common in the starved fish during the long-term assays. Wood and Yasutake (1957) found histopathological changes such as shortening and hypertrophy of secondary lamellae leading to inter-lamellar obliteration in the gills of fish undergoing food deficiency. Many scientists reported the shortening of secondary lamellae due to various reasons (Sulekha and Mercy, 2021a; Ramani, 2001; Wood and Yasutake, 1957).

Thinning of secondary lamellae appeared in A. testudineus treated with almost all the concentrations of phosphamidon. Thinning of secondary lamellae was reported previously by Sulekha and Mercy (2021a) and Poleksic and Mitrovic-Tutundzic (1994). In the present study, this thinning may be caused by the degeneration of secondary lamellar epithelial cells as suggested by Sulekha and Mercy (2021a).

Maximum allowable toxicant concentration (MATC)

Eventhough the pesticides are directly applied against the pests in agricultural fields, it adversely affects the non-target organisms like fishes inhahiting in the paddy fields. In the present study, NOEC, LOEC and MATC values of phosphamidon of *A. testudineus* - a native fish of Kuttanad was calculated based on the histopathological studies on the gills. The intensity of pathology is quantified using the index values suggested by Poleksic and Mitrovic-Tutundzic (1994). According to them, gills having index value less than 10 are functionally normal and those between 10 - 20 are slightly to moderately damaged gills. Hence the concentration which showed index value less than 10 are taken as NOEC and between 10-20 are taken as LOEC. In the present study, the NOEC and LOEC so obtained are given in table 3 and the MATC is thus calculated. It seems that these MATC values are very sensitive when compared to the MATC values of the same pesticides on other fishes such as rohu using other parameters (Ramani, 2001).

These NOEC, LOEC and MATC values so obtained for the species of fishes can also be used to assess tentative water quality status for the pollutants in the natural environment, as the fishes chosen for the study are the most representative test animals depicting the field situation of Kuttanad water bodies. Due to their non migratory nature they are prone to all sorts of pollutants added to the aquatic environment of Kuttanad.

The application factor derived from the laboratory studies can be directly applied to the field situations, as they are the representative species of Kuttanad. Their usefulness lies predominantly in the insight, which they provide on the toxic action of the contaminant, which can be helpful in establishing the relevance of the effect for fitness and survival. This knowledge of fundamental toxicological and pathological processes is not only important for the regulators of chemicals that are potentially aquatic pollutants, but also for researchers involved in field studies.

Histopathology as a tool for sublethal toxicity studies

In ecotoxicology, the effect of toxic compounds on

the fate and dynamics of populations and biomass is studied. This is a difficult and complicated process, and therefore, parameters used for measurement are qualitative and relatively basic, such as survival and mortality. With acute toxicity tests these parameters are quite appropriate; but for long-term effects, caused by sub lethal concentrations, these parameters are difficult to ascertain.

The primary objective of environmental hazard analysis and risk assessment is to arrive at a concentration level, which can protect environmental life even after long-term exposures. In almost all the previous works in toxicity studies, the end points used are mortality, survival and growth. In contrast, the present study, the histopathological parameters are evaluated to obtain the NOEC, LOEC and the MATC of the toxicants used.

Attempts to arrive at NOEC and LOEC values using histopathological parameters are very rare. Wester and Canton (1987) Wester *et al.* (1988) and Wester and Roghair, (1994) have carried out histopathological studies on *Poecilia reticulata* and *Oryzias latipes* exposed to different toxicants. Wester and Roghair (1994) have evaluated utility of this histopathological approach in aquatic toxicology studies, with special emphasis on sublethal effects occurring after chronic exposure. They have reported that histopathology can reveal specific structural toxic effects in a variety of organs of fish. They also reported that this is a useful tool in the risk assessment of toxic chemicals that may be discharged to the aquatic environment. They have compared the no-observable effect concentration (NOEC) for general toxicological parameters and histopathology in *P. reticulata* and *O. latipes* and found that for some compounds significant differences exist in the comparative sensitivity of routine aquatic toxicology parameters and histopathology. The NOEC obtained in histopathological studies are more sensitive than the NOEC obtained in other general studies (Wester and Roghair, 1994).

Hence it is suggested that histopathological studies on the gills of fishes could be used as a relatively sensitive method for estimating the harmful effects of different concentration of a specific pollutant and the long term damage to fish exposed to different levels for a limited period as reported by Poeksic and Mitrovic-Tutundzic (1986). It also helps in the water quality monitoring and provides an 'early warning' of the conditions of fishes.

Conclusion

1. The maximum allowable toxicant concentration (MATC) arrived at in the laboratory studies, based on the histopathology of gills, for the two pesticides are very sensitive values.
2. The application factors derived from this sublethal study can be directly applied to the field situations.
3. Histopathology can be used as a tool in assessing the intensity of pesticides even at the sublethal levels.
4. This will be an important tool for the farmers in deciding the concentrations of pesticides before its application into the paddy fields.

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