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EVALUATION OF MICRONUCLEI AND HAEMATOLOGICAL PROFILES AS GENOTOXIC ASSAYS IN *CHANNA PUNCTATUS* EXPOSED TO MALATHION

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ABSTRACT
Different genetic biomarkers have been used to evaluate the mutagenic effects of pollutants such as agriculture pesticide and also a great variety of chemicals delivered on the environment by human activities. This way, the aim of present study was to evaluate the effect of agriculture pesticide in *Channa punctatus* through the micronucleus test as cytogenetic parameter and Haematological studies. Live specimen of *Channa punctatus* (n=96) were acclimatised in aquaria before experiments. Fishes were kept separately in 1.0, 2.0 and 4.0 ppm malathion for three days. Control fishes were not subjected to any treatment. Blood collected from caudal vein was smeared on clean slides, fixed in methanol and stained with 2% Giemsa. Mean frequency of micronuclei observed was 0.06±0.007, 0.11±0.009, 0.28±0.014 and 0.42±0.015 in control, 1.0, 2.0, 4.0 ppm malathion respectively. There was decrease in red blood cell (RBC) count (3.24, 2.87 and 1.19, 10^6 mm^{-3}) and haemoglobin content (10.5, 8.63 and 5.63 g/dl) when compared to the control (RBC- 5.36, 10^6 mm^3 and13.6 g/dl). Whereas the number of white blood cells (WBC) increased (4.63, 4.73 and 4.85, mm^3 x 10^3) in malathion treated fishes when compared to the control (4.56 mm^3 x 10^3). There was a progressive increase in the percentage of micronuclei with increases in the intensity of exposure. Study shows that malathion is a clastogenic chemical and these assays can be used as bioassays for monitoring pollution in aquatic medium.

KEYWORDS: Micronucleus test, RBC, WBC, Genotoxicity, Malathion.

INTRODUCTION

The aquatic environment plays a vital role for functioning of ecosystem and is intimately related with human health. The increasing human population and industrial development has worsened the problem of disposal of anthropogenic chemicals and wastes in the aquatic environment. A majority of these contaminants contain potentially genotoxic and carcinogenic substances. These chemicals are responsible for DNA damage in variety of aquatic organisms and fishes causing malignancies, reduced survival of embryos, larvae and adults, eventually affecting the economy of fish production significantly. Genotoxicity not only reduces the 'fitness' (i.e. growth, fertility and fecundity) in wild fish populations, but also pose risk to human health via food chain. Besides, causing mortality, these pollutants can cause genotoxicity in aquatic organisms which can lead to severe consequences in fishes like development of tumors (Folmar *et al.*, 1993). These changes in the genetic material of organisms can be detected by using genotoxicity assay system and Haematological studies. These studies will provide information that would be useful for formulation of strategies and planning regarding conservation of aquatic ecosystems and can be passed on to industries and other agencies for adoption.

Micronucleus test (MNT) is a widely used cytogenetic technique for assessment of chromosomal damage induced by various genotoxicants. Schroder (1966) for the first time studied the formation of micronuclei in mammalian bone marrow cells; subsequently this assay was developed by Schmid (1975) in mammalian systems. The MNi are also known as Howell-jolly bodies in mammals. Like mammalian species, MNT has also been adopted to study genotoxicity in fishes. Fishes provide a suitable model for monitoring aquatic genotoxicity and waste water quality because of their ability to metabolize xenobiotics and accumulate pollutants (Grisolia and Corderio, 2000). Micronuclei in fish could be smaller in size than that suggested by Schmid (1975), because most fish chromosome are much smaller (e.g. 1/10 to 1/30 of the size of principal nucleus). The formation of micronuclei depends on fish species and target tissues and also on environmental conditions (e.g. temperature, season etc) and the kind of pollution involved. The MN assay gives advantages over metaphase analysis of genotoxic response in fish, however, the intra specific factor that may affect the response of MN assays in fishes include age, sex, diet, health, reproductive status and genetic strain.

Al Sabti (1986) tested several chemicals (aflatoxin B1, arochlor 1254, benzidene, benzo(a)pyrene, and 20-methylchloanthrene) for their ability to induce micronuclei under laboratory conditions in three cyprinids viz, Common Carp, *Cyprinus carpio*, *tench, Tinca tinca*, and grass carp, *Ctenopharyngodon idella.* Al Sabti and Metacalfe (1995) have reviewed the literature on clastogenic effects of many chemicals and physical agents on fish cells, with emphasis on the induction of the micronuclei in teleost species. Svobodava *et al*. (1997) studied the effect of Malachite green on *Cyprinus carpio* by using micronuclei test. Campana *et al*. (1999) evaluated genotoxicity of the pyrethroid lambda- cyhalothrin using the micronuclei test in erythrocytes of the fish *Cheridon interruptus interruptus.* Sandra *et al*. (1996) used MNT for the *in situ* mutagens in freshwaters in erythrocytes of *Barbus plebejus* from two natural environments.

The count of red blood cells is quite a stable index and the fish body tries to maintain this count within the limits of certain physiological standards using various physiological mechanisms of compensation. Blood cell responses are important indicators of changes in the internal and/or

external environment of animals. In fish, exposure to chemical pollutants can induce either increases or decreases in haematological levels. Their changes depend on fish species, age, the cycle of the sexual maturity of spawners and diseases (Luskova, 1997). Like in warm-blooded animals, changes in the blood parameters of fish, which occur because of injuries of the latter organs or tissues, can be used to determine and confirm the dysfunction or injuries of the latter (organs or tissue). However in the fish, these parameters are more related to the response of the whole organism, i.e. to the effect on fish survival, reproduction and growth. It should be noted that although the mechanisms of fish physiology and biochemical reaction to xenobiotics has not been investigated enough, it is obvious that species differences of these mechanisms exist. Fish live in very intimate contact with their environment, and are therefore very susceptible to physical and chemical changes which may be reflected in their blood components (Wilson and Taylor, 1993). In fish, exposure to chemical pollutants can induce either increases or decreases in haematological levels. Blood tissue truly reflects physical and chemical changes occurring in organism. Therefore, detailed information can be obtained on general metabolism and physiological status of fish in different groups of age and habitat. Early diagnosis is also possible when evaluating haematological data, particularly blood parameters (Folmar, 1993; Luskova, 1997). Furthermore, it should be noted that haematological indices are of different sensitivity to various environmental factors and chemicals (Vosylienë, 1999b). Previous haematological study of nutritional effects, infectious diseases and pollutants brought knowledge that erythrocytes are the major and reliable indicators of various sources of stress (O'neal and Weirich, 2001). This study therefore assessed the micronucleus test and the haematological profile of *Channa punctatus* exposed to malathion.

MATERIALS AND METHODS

Test Chemical

Malathion (EC 50%) manufactured by Insecticides (India) Limited E-443-444, Riico Industrial Area, Chopanki (Bhiwadi) - 301 707, Rajasthan was used during the present course of study. The commercial grade preparation is a yellow coloured liquid containing 50% active ingredients (w/w) and the rest is constituted by inactive ingredients. All procedures were carried out according to the international practices for animal use and care under the control of an institutional ethical committee of the University.

Methodology

Live specimens of *Channa punctatus* (n=96) for the present study were collected from local market. After disinfection with a dip of 2% $KMnO_4$ for 15 minutes, four groups of fishes were acclimatized in aquaria for one month before initiation of experiment. Fishes were fed on lab made diet on alternate days during the acclimation period. Toxicity tests were conducted in 10 litre water capacity plastic tanks. A minimum of 8 fish were exposed to each pesticide concentration and each experiment was conducted in triplicate. Water of each tank containing toxicant was changed daily to remove faecal matter and waste metabolite of fish and to maintain the required concentration of pesticide. Appropriate controls were run simultaneously in pesticide - free water. Blood was collected from caudal vein/ heart puncture from fish specimens using heparinised syringe. The fishes were not allowed to feed during the exposure period. Four groups of fishes were kept separately at control, 1.0, 2.0 and 4.0 ppm malathion for 3 days. Blood was collected from heart puncture in a heparinised syringe and thin smear on pre-cleaned slides was made. Slides were fixed by dipping in absolute methanol for 5-10 min then air dried for at least 1 hour and stained in Giemsa stain for 10 min. Slides were air dried overnight and mounted with DPX and observed under microscope using 40/100X objective lenses and the micro nucleated cells were scored. The RBC counts were made by Neubauer Haemocytometer (Shah and Altindag 2004a). Blood was diluted 1:200 with Hayem's solution (Mishra *et al*., 1977). Erythrocytes were counted in the loaded haemocytometer chamber and total numbers were reported as 10^6 mm^{-3} (Wintrobe, 1967). Counting was done in the five smaller squares i.e. in the 1^{st}, 5^{th}, 13^{th}, 21^{st} and 25^{th}. The RBC's on the lower and right sides of a square were added in the total, while those on the upper and left sides were rejected.

WBC counts were made by Neubauer Haemocytometer (Shah and Altindag 2005). Blood was diluted 1:20 with Turk's diluting fluid and placed in haemocytometer. Four large (1 sq mm) corner squares of the haemocytometer were counted under the microscope (Nikon microscope 80i) at 100X. The cells touching the boundary lines were not counted. The total number of WBC was calculated in mm^3 x 10^3 (Wintrobe, 1967).

The principle of Sahli's method is simple: "the haemoglobin contained in a known quantity of blood is converted into acid haematin by means of hydrochloric acid. The colour is then compared with a standard tube containing acid haematin of known strength" (Whitby and Britton, I935). N/10 HCl solution was filled in the graduating tube up to 2 gms mark. The micropipette was filled up by sucking fresh blood upto 20 cmm marks. The blood of micropipette was then added to the N/10 solution in the graduated tube. The acid haematin solution was thoroughly stirred with the help of a glass rod and then allowed to stand for 10 minutes. Afterwards the acid haematin solution was gradually diluted by adding distilled water in a drop wise manner. This was continued till the colour of the acid haematin solution matched with that of the standard sealed tubes. The reading before the colour just fades was taken as the correct and final reading.

Statistical Analysis

Data are expresses as the mean ±S.E. statistical analysis was done using student's t test (2-tailed) with the help of SPSS 18. The level of significance was set at $P < 0.05$.

RESULTS

Micronucleus Test

In *Channa punctatus*, the effect of malathion in increasing concentration at sublethal level (1.0, 2.0, 4 ppm) has been studied. For MNT total 1000 blood cells from control group, 1.0 ppm, 2.0 ppm and 4.0 ppm dose of malathion were screened. The results are presented in (Table-I, Figure-1, 2 and 6). The mean frequency of micronuclei was observed in was 0.06±0.007, 0.11±0.009, 0.28±0.014

and 0.42±0.015 in control, 1.0, 2.0, 4.0 ppm malathion. The result indicated that the percentages of micronuclei increased with increase in concentration of malathion. The values mentioned above showed a significant increase when compared to the control (P<0.05).

Total RBC Count

The erythrocyte count of healthy controls showed a mean value of 5.36 10^6 mm^{-3}. The fishes exposed to sub lethal concentrations of malathion showed mean values of RBC's as 3.24, 2.87 and 1.19, 10^6 mm^{-3} for 1.0, 2.0 and 4.0 ppm treatments respectively. The treatment with malathion was found to inflict a drastic reduction in the total count of RBC's (Table- I, Figure-3). The values mentioned above showed a significant decrease when compared to the control (P<0.05).

Total WBC Count

The results of the total count of white blood cells revealed that the blood of the control fish showed a mean value of 4.56 mm^3 x 10^3. The fishes exposed to sub lethal concentrations of malathion showed mean values of WBC as 4.63, 4.73 and 4.85, mm^3 x 10^3 for 1.0, 2.0 and 4.0 ppm treatments respectively (Table I, Figure-4). The values of WBC count increased with increase in the concentration of malathion. The values mentioned above showed a significant increase when compared to the control (P<0.05).

Estimation of Haemoglobin

The control fishes showed mean value of 13.6 g/dl for haemoglobin. The fishes exposed to sub lethal concentrations of malathion showed mean values of haemoglobin as 10.5, 8.63 and 5.63 g/dl at 1.0, 2.0 and 4.0 ppm treatments respectively **(**Table-I, Figure-5). The values for treatments showed a significant decrease when compared to the control at (P<0.05).

TABLE I -Total Count of RBC's, WBC's, Haemoglobin and Micronuclei in the Control and Malathion Treated *Channa punctatus*

Variable	Control (Mean±SE)	1ppm (Mean±SE)	2ppm (Mean±SE)	4ppm (Mean±SE)
RBC (10^6/mm^3)	5.36*± 0.09	3.24 ± 0.07	2.87* ± 0.05	1.19* ± 0.01
WBC (10^3/mm^3)	4.56* ± 0.01	4.63 ± 0.01	4.73* ± 0.01	4.85* ± 0.02
Haemoglobin (g/dL)	13.06 ± 0.25	10.5 ± 0.23	8.63* ± 0.19	5.63* ± 0.15
Micronuclei	0.06 ± 0.007	0.11* ± 0.009	0.28* ± 0.014	0.42* ± 0.015

Values Represents Mean ±SEM of Micronuclei Significant at *P < 0.001

FIGURE 1. Figure showing Micronuclei exposed to malathion

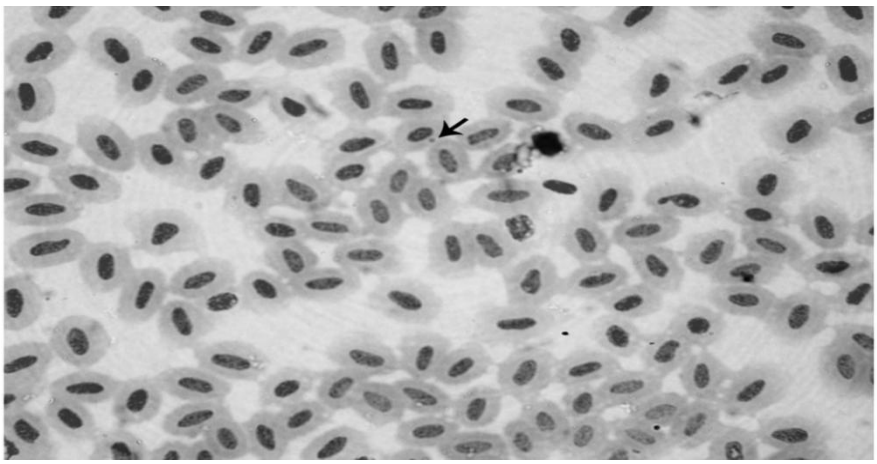


FIGURE 2. Figure showing Micronuclei in budding cell exposed to malathion

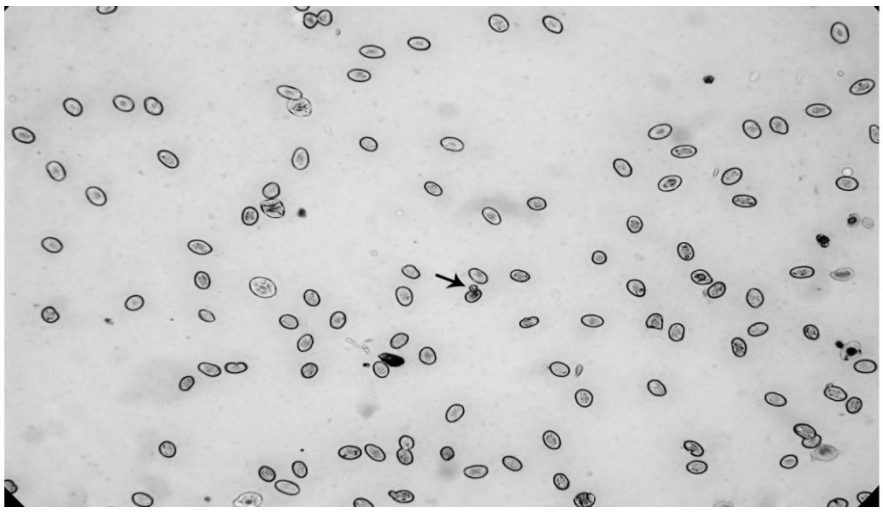


FIGURE 3. Graphical representation of total Count of RBCs in the Control and Malathion Treated *Channa punctatus*

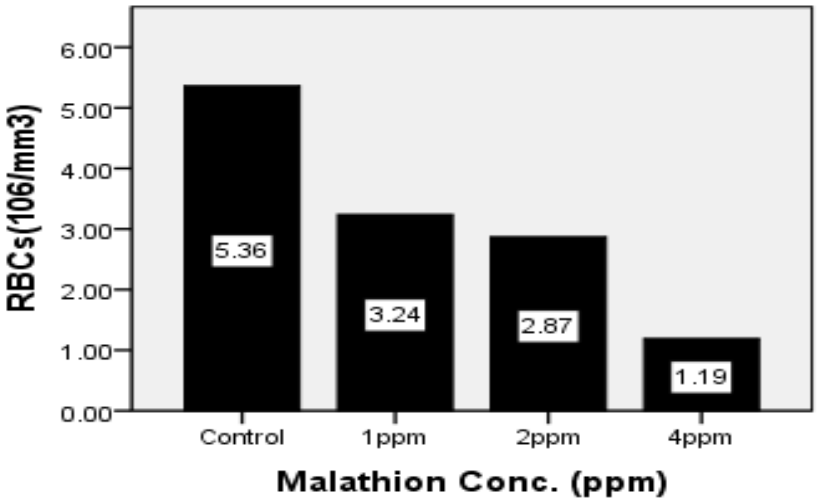


FIGURE 4. Graphical representation of total Count of WBCs in the Control and Malathion Treated *Channa punctatus*

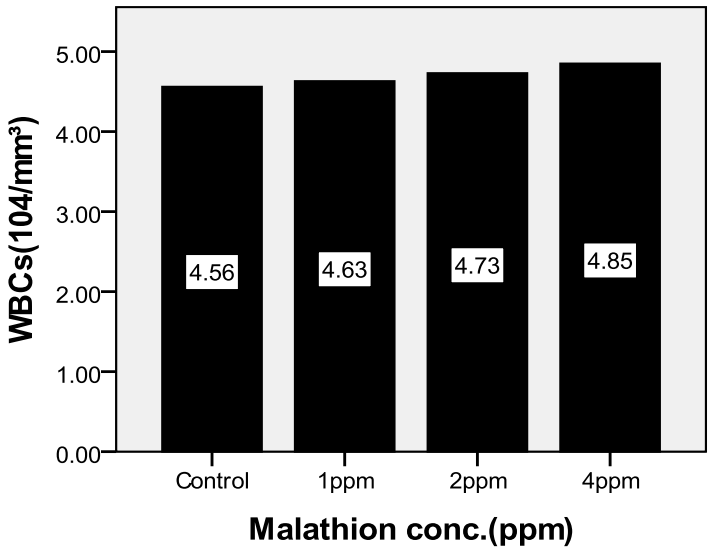


FIGURE 5. Graphical representation of total Count of Haemoglobin in the Control and Malathion Treated *Channa punctatus*

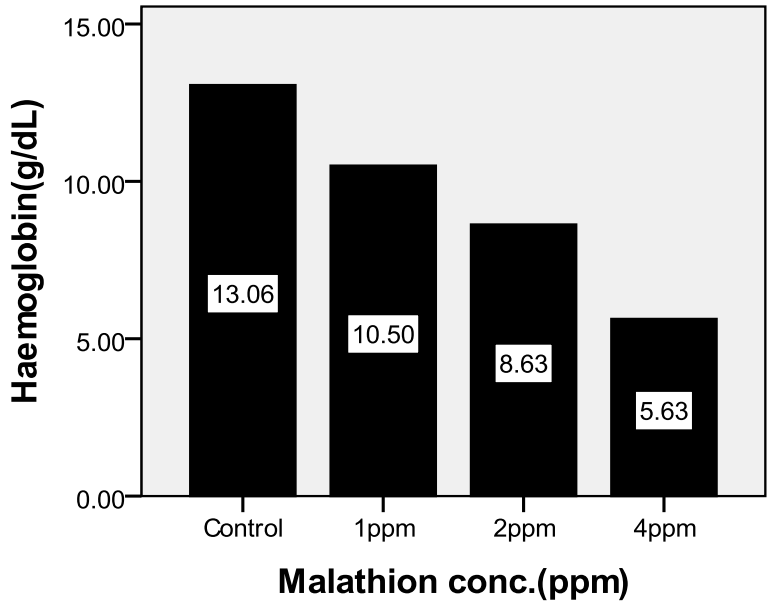
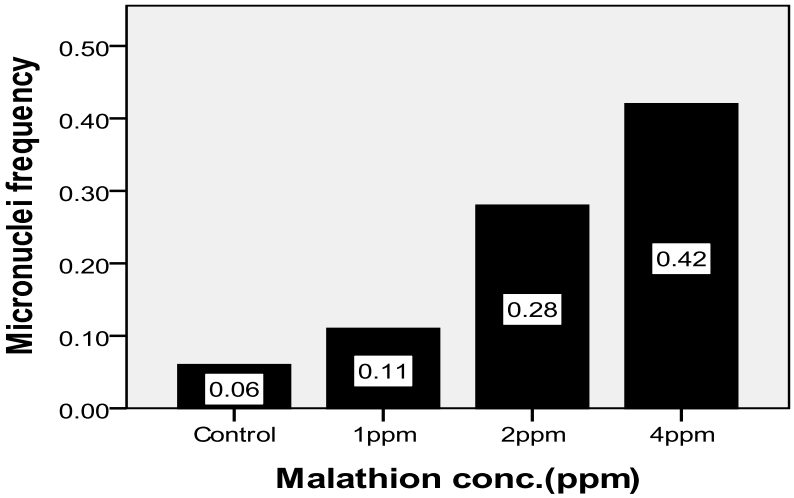


FIGURE 6. Graphical representation of Percentage of Micronuclei at different Concentration of Malathion in blood cells of *Channa punctatus*



DISCUSSION

Malathion has been reported to be more toxic to insects and fish than to mammals due to lack of hydrolytic enzymes in the former (Krueger *et al.*, 1960). The hydrolysis of the oxygen analogue of this pesticide (malaoxon) proceeds very slowly in fish (Areechon & Plumb, 1990). Malathion, inhibits the hydrolysis of acetyl-choline and also/or deposits as such or as its oxygen analogue (malaoxon). This complicates the cytoplasmic nature of these cells which leads to vacuolizations and may further aggravate the toxic effect leading to death of the cells. At present, more than 1000 chemicals have been classified as pesticides and studies using different models have indicated that some of them have genotoxic properties (Zeljezic and Garaj-Vrhovac 2002). Fish and aquatic invertebrates have been considered to be efficient and cost effective model systems for studying the toxic, mutagenic, and carcinogenic potential of pollutants (Braunbeck *et al.* 2005) due to their ability to metabolize, concentrate, and store water-borne pollutants (Osman *et al.* 2007). As such, little information regarding the genotoxic effects of malathion is available for fish. *In vivo* and *in vitro* studies on other subjects have indicated that malathion has the potential to produce genotoxic effects in mammalian systems (Flessel 1993) although the reports are conflicting (Blasiak *et al.* 1999). All test concentrations of malathion used in the present study induced a significantly higher number of MNi compared to the control. Further, the MN induction increased significantly with the advancement of the concentrations. A concentration dependent increase and time-dependent decrease in MN induction due to chlorpyrifos exposure has been reported earlier in the same species (Ali *et al.* 2008). However, in some studies, both concentration- and time-dependent increases in MN induction have also been reported due to chemical exposure in fish (Bahari *et al.* 1994). Although, the MN test has been found to be a sensitive assay to evaluate genotoxic compounds in fish under controlled conditions as an index of cumulative exposure (Bolognesi *et al.* 2006), it might suffer variations according to clastogen, test organism, and the life cycle of the cells (Grisolia and Cordeiro 2000). Further, as the pre-existing mature (and nondividing) erythrocytes would predominate in the blood, the detection of induced MN in mature blood cells will be at a low frequency in the lower concentration. Studies on MN induction in humans and animals indicated malathion to be genotoxic. The technical-grade malathion has the potential to produce chromosomal changes, including chromosome aberrations and MN induction in test animals (Hoda and Sinha 1991). A significant difference in frequency and distribution of MN was observed between the malathion exposed workers and control workers (Garaj-Vrhovac and Zeljezic 2002).

Present investigation on the genotoxic potential of malathion suggested a serious concern about its potential danger to aquatic organisms, especially to fish, and indirectly to human beings.

In recent years haematological variables have been used more to determine the sublethal concentrations of pollutants (Wedemeyer and Yasutake, 1977). Gill *et al* (1991) on the other hand suggested that the fish experience respiratory difficulty when they confront toxic environment and try to compensate for the reduced oxygen uptake at the gill surface by increasing the level of blood constituents concerned with oxygen uptake and delivery. However, a prolonged exposure exhausts the haematopoietic potential revealed by lowered RBC count and haemoglobin. An anaemic condition is generally indicated by the tendency of lower RBC count and haemoglobin content as seen in fishes exposed to environmental pollution (Ramesh 2001). In the fish *Channa punctatus* exposed to malathion haemoglobin percentage decreased significantly. This indicates that malathion caused anaemia. This may be due to a decreased rate of production of red blood cells or an increased loss of these cells. The results of the present investigation show that malathion treatment inflicted a drastic reduction in the total count of RBC's. The reduction was dosage dependent. However, the number of WBCs was deviating significantly from normal values. The significant decrease in the WBC count may be due to a generalised stress response (Ruparelia et al. 1990). White blood cells play a

major role in the defence mechanism of the fish and consist of granulocytes, monocytes, lymphocytes and thrombocytes. Leucocyte count showed greater and quite different pattern change due to malathion exposure when compared with the erythrocyte levels of the control group. Blood of all experimental groups contained higher concentrations of leucocytes than those of controls. An increase in lymphocyte number may be the compensatory response of lymphoid tissues to the destruction of circulating lymphocytes (Shah and Altindag, 2005). Gill and Pant, (1985) have reported that the stimulation of the immune system causes an increase in lymphocytes due to injury or tissue damage.

It has been observed that, there was a progressive increase in the number of micronuclei with increases in the intensity of exposure to malathion, whereas the number of RBCs and haemoglobin percentage decreased significantly from normal values. However, the number of WBCs was deviating significantly from normal values. Present investigation shows that malathion caused immunological impairments in *Channa punctatus*, which suggests that malathion may weaken the immune system and may result in severe physiological problems, ultimately leading to death of the fishes.

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