CYTOCHROME P_{450} INDUCTION, HEMATOLOGICAL CHANGES AND MELANOMACROPHAGE ACCUMULATION IN NILE TILAPIA (*Oreochromis niloticus* Linn.) AFTER EXPOSURE TO CHLORPYRIFOS

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ABSTRACT

This study aimed to evaluate some pesticide-induced physiological responses in Nile tilapia (*Oreochromis niloticus* Linn.), which may be developed as biomonitoring indices for organophosphate exposure. Male juvenile fishes were grouped (15 individuals per group) and exposed daily for 20 days to varying concentrations of chlorpyrifos, namely: 0.3 µg/L, 1.0 µg/L, 1.7 µg/L. The different residual doses were administered through daily incorporation of the desired concentrations of the test materials in aquarium water. The control fishes were maintained in the untreated water. The specimens were examined after one, three and ten days following the treatments.

Analysis revealed significant induction in cytochrome P_{450} levels among the treated fishes ($P < 0.05$) even at ten days following the treatment period. The mean corpuscular volume and the mean corpuscular hemoglobin levels also appeared to have been influenced by the treatment. Moreover, a significant increase in heterophils and a decrease in lymphocytes were noted in fishes under 1.6 µg/L treatment level. Histological analysis of the liver tissues showed an apparent proliferation of melanomacrophage centers among fishes exposed to 1.6 µg/L concentration. Induction of cytochrome P_{450} levels denotes detoxifying activity due to the presence of a toxicant, which could cause considerable stress on animals as evidenced by the hematological changes and increase in size of the melanomacrophage centers.

Key words: hematological parameters, cytochrome P_{450} induction, melanomacrophage accumulation, hepatopancreas, *Oreochromis niloticus*, chlorpyrifos,

Abbreviations: OP-organophosphate; NSS-normal saline solution; EDTA-ethylene diaminotetraacetic acid; RBC-red blood cell; WBC-white blood cell; Hgb-hemoglobin; MCV-mean corpuscular volume; MCH-mean corpuscular hemoglobin; MCHC-mean corpuscular hemoglobin concentration; Hgt-hematocrit; MMC-melanomacrophage center;

INTRODUCTION

There are a number of synthetic pesticides that are available for use in residential, industrial, agricultural and aquatic ecosystem protection. One of them is chlorpyrifos, an organophosphate (OP), which exhibits activity against insects and even arthropods found in a wide array of terrestrial and aquatic ecosystems (Racke, 1993).

In the aquatic ecosystem, the amount of chlorpyrifos traced in surface water has been reported to range from non-detectable to aqueous concentrations of 0.04 to 0.134 µg/L (Natale et al. 1988 as cited by Barron and Woodburn, 1995) and 0.2 to 1.6 µg/L (Braun and Frank, 1980 as cited by Barron and Woodburn, 1995). In addition, residual levels of chlorpyrifos in rice paddy ecosystems have been determined by Calumpang et al. (1997) to be at 0.3 to 1.6 µg/L which could still be present in paddy water up to 24 hours after treatment. These studies clearly show that small but measurable quantities of the chemical can be transported from agricultural areas.

Nile tilapia (*Oreochromis niloticus* Linn.) and other fish species are very susceptible to such aquatic chemical pollutants as they try to search for food at the bottom of the rice paddies, ponds and even irrigation canals that serve as their habitats. In fact, they have been considered as good bioindicators for several pollutants such as atrazine (Hussein et al. 1996), oxyflourfen (Hassanein et al. 1999), dieldrin and monocrotophos (Golow and Godzi, 1994; Thangnipon et al. 1995), polychlorinated biphenyls (Ueng et al. 1995), heavy metals such as zinc, cadmium and mercury (Cuvin-Aralar, 1994 as cited by Hassanein et al. 1999) and for studying the effect of various environmental stresses like hypertonic stress (Ayson et al. 1993 as cited by Hassanein et al. 1999) and confinement stress (Auperin et al. 1997 as cited by Hassanein et al. 1999).

Induction of cytochrome P_{450} in fishes has been considered to be a good criterion for monitoring water pollution (Payne et al. 1987). Cytochrome P_{450} is a family of hemoproteins responsible for the metabolism of endogenous materials and a number of foreign chemicals. This enzyme system acts and serves as a route of detoxification of various pollutants and carcinogens. The major interest of monitoring cytochrome P_{450} induction is to detect responses due to OP sublethal exposure at the biochemical level before more serious population effects become apparent. Likewise, hematological parameters can allow a more rapid detection of physiological changes brought about by environmental stressors or pollutants (Torres et al. 1986). Melanomacrophage accumulation is another potential index for monitoring any stressor present in the environment.

This study aimed to establish the major physiological responses in *O. niloticus* due to chlorpyrifos treatments, which may serve as biomonitoring indices for organophosphate exposure.

MATERIALS AND METHODS

Collection, maintenance and treatment of specimens

Sixty male juvenile tilapias (*Oreochromis niloticus* Linn.) obtained from a hatchery in Bay, Laguna were placed in eight aquarium tanks (30 cm x 60 cm x 30 cm) with well-aerated and dechlorinated water. They were acclimatized for two weeks prior to testing and were given fish pellets regularly. Water in the aquaria was changed daily. After the acclimatization period, the individual fish body weight was obtained.

A known brand of insecticide containing 33% chlorpyrifos and 67% aromatic hydrocarbons and other inert ingredients was used. Different treatment doses, with 15 individuals per group, were established as follows: Treatment A (control), Treatment B (0.3 µg/L), Treatment C (1.0 µg/L) and Treatment D (1.6 µg/L). Exposure to the various concentrations was done on a

daily basis for 15 days by incorporating the desired volume of the test material in 25 liters of water inside each tank using a microliter syringe. Water was changed and the tank was thoroughly washed daily.

One, three and ten days after the cessation of treatment, five fish specimens per treatment level for each observation period were sacrificed for analysis. Blood samples from each individual were collected and the liver samples were excised for both enzyme and histological evaluation. Since the weight of some liver samples were found to be inadequate to meet the required volume for microsome sample preparations, liver tissues of fishes sacrificed during the same day were pooled and considered as one treatment group. Microsomal preparation of liver was done disregarding the dose level of treatments.

Hepatic cytochrome P_{450} induction

Liver microsome preparation and cytochrome P_{450} evaluation were done following the procedure described by Omura and Sato (1964 as cited by Elangbam et al. 1989) with some modifications.

Liver samples were put in vials with normal saline solution (NSS) and temporarily stored in crushed ice and later refrigerated at 5°C prior to preparation of microsomal samples. Each sample was transferred into a centrifuge tube with 0.1 M sodium phosphate buffer containing 0.15 M KCl (pH 7.5) in a volume four times the liver weight. Tissue samples were processed in a tissue homogenizer. The homogenates were then centrifuged at 10,000 x g for 25 minutes using a refrigerated centrifuge. The supernatant was recovered and recentrifuged at 11,900 x g for three hours. The microsomal pellets were resuspended in an original weight of 0.073 M potassium phosphate buffer at pH 7.5. All of the above-mentioned steps were carried out at a temperature of 5°C.

The measurement of cytochrome P_{450} levels was done by using a spectrophotometer, which was set to scan from 400-500 nm (1 cm/nm). Carbon monoxide was added for 60 seconds and then the suspension was reduced with sodium dithionite solution. After two minutes, the absorbance spectrum in the range between 400 and 500 nm was measured against a control containing the suspension and buffer without sodium dithionite. The absorption maximum at 450 nm was determined by consideration of the base line in the range between 420 and 500 nm. The enzyme concentration was calculated from the absorbance difference at 450 and 490 nm using the extinction coefficient of 0.91 nmoles/mL.

Hematological tests

Blood was drawn from the heart of the specimens and was put into vacuum blood containers with EDTA.

The red blood cell (RBC) count, hematocrit, white blood cell (WBC) count, and differential WBC count were obtained using the methods of Stoskopf (1993). The cyanomethemoglobin method was employed to get the hemoglobin (Hgb) content. Wintrobe erythrocyte indices such as mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC) and mean corpuscular hemoglobin (MCH) were computed according to Coles (1986).

Histological analysis

The small portion of liver samples were fixed in 10% formalin, dehydrated in a series of ethanol and embedded in paraffin. They were sectioned at 8 μm thickness and stained with hematoxylin and eosin for light microscopy.

Statistical analysis

One-way Analysis of Variance (ANOVA) was done to determine if significant variations occurred in the blood counts and Wintrobe erythrocyte indices among treatment doses. Test of means was performed using LSD (least significant difference),. On the other hand, student's t-test was done on the hepatic cytochrome P450 levels between treated and untreated fishes per observation period.

RESULTS AND DISCUSSION

Hepatic cytochrome P_{450} induction

Analysis of the level of cytochrome P_{450} value (in nmole/mL) of the liver microsomes showed that regardless of the administered doses, significant differences exist between the treated fish and the untreated fish. The t-values obtained were 5.3, 10.0 and 8.2 ($P<0.05$) for the one-day, 3-day and 10-day groups relative to the control. The highest mean value of 0.69±0.036 was obtained from fishes observed one day after cessation of treatment. This was followed by relatively lower mean values of 0.33±0.061 and 0.30±0.04 for fishes analyzed at 3 and 10 days after cessation of treatments, respectively. The mean cytochrome P_{450} value of 0.30 0.07 was observed among individuals under the control group.

The present data suggest that chlorpyrifos, even at very low residual concentrations in water, can increase the metabolic capacities of fish. This is supported by the observed 6-fold increase of enzyme activity relative to the control by those fishes evaluated one day after termination of the 15-day treatments. However, transferring exposed animals into toxicant-free water resulted in a progressive recovery.

Cyctochrome P_{450} in fishes has been documented as criterion for determining water pollution (Payne et al. 1987). The results of the present study clearly demonstrate the inducibility of the enzyme in liver tissues of tilapia by chlorpyrifos. Detection of activity, however, was most pronounced 24 hours after cessation of treatments and progressively was reduced thereafter. There are other reports in the literature showing induction of cytochrome P_{450} in insecticide-treated animals including birds (Miller et al. 1978), rats (Neskovic and Vitrovic, 1977), and different species of fish (Payne et al. 1987). From the present data, one could speculate that tilapia may be relatively sensitive to induction of cytochrome P_{450}. The low concentration levels of the test material had appeared to adequately cause a detectable increase in the level of the liver enzyme. This result suggests that tilapia possesses a sensitive hepatic xenobiotic biotransformation system, which may be taken into account when using the species as an indicator animal in studies of the biological impact of pollutants.

Hematological parameters

Figure 1 shows the results of the evaluation done on the different erythrocyte components among the test fishes. A general increase in RBC counts was apparent, although a much pronounced increase was found among those individuals observed a day after the cessation of treatments. Among these individuals, the RBC counts varied considerably among treatment groups (F-value = 31.48, $P < 0.05$). Treatment D exhibited significantly higher count (2.17×10^6/mL) compared with the control (1.26×10^6/mL). At either 3 days or 10 days after cessation of treatments, however, Treatment B and C individuals already appeared to recover with mean values quite comparable with those of the control. RBC counts of Treatment D remained significantly high even at 3 or 10 days after cessation of treatments. Meanwhile, the mean Hgb values exhibited no significant differences among treatment groups. The same pattern was also observed on hematocrit and on the MCHC values.

The two erythrocyte components, which appeared to have been affected by the treatment, are MCV and MCH. The significant effects, however, were only detectable one day after cessation of the 15-day treatments. During the rest of the observation period, the two components exhibited mean values comparable with those of the control.

The observed increase in RBC count of the treated fishes may be due to the number of circulating immature erythrocytes. Generally, young erythrocytes in fishes are smaller than the mature ones, hence a lower MCV is expected (Buckley, 1976 as cited by Cyriac et al. 1989; and Torres et al. 1986). It is possible that the chlorpyrifos treatment affected the respiration of fishes and had indirectly caused the production and release of young erythrocytes in circulation. This is in response to oxygen demand and carbon dioxide transport. Bradbury et al. (1991) had observed a decrease in arterial oxygen and carbon dioxide in fishes exposed acutely to chlorpyrifos. This phenomenon could be caused by either an increase in metabolic activity or the destruction of the gill membrane leading to faulty gaseous exchange (Davis, 1973 as cited by Cyriac et al. 1989).

The potential influence of chlorpyrifos on erythrocyte metabolism in fish is not yet well established in literature. The present findings, however, suggest that the test chemical exerts some influences on the general RBC metabolism, affecting the fish even after several days of cessation of treatment. This was also apparent with the pattern observed in the hepatic cytochrome P_{450}, where enzyme activity was still traceable even ten day after cessation of treatment, which means that the enzyme system could still be detoxifying or acting upon foreign materials.

Figure 2 summarizes the pattern observed on the different leukocyte components. Analysis of WBC count showed no significant difference among the treatment groups during the 3 periods of observation. This finding corroborates with those of Riva and Flos (1993) who indicated that certain individual stressors exert no significant influence on the total leukocyte count of rainbow trout. Though, a constant WBC count was obtained, WBC differential ratios were observed to be significantly affected.

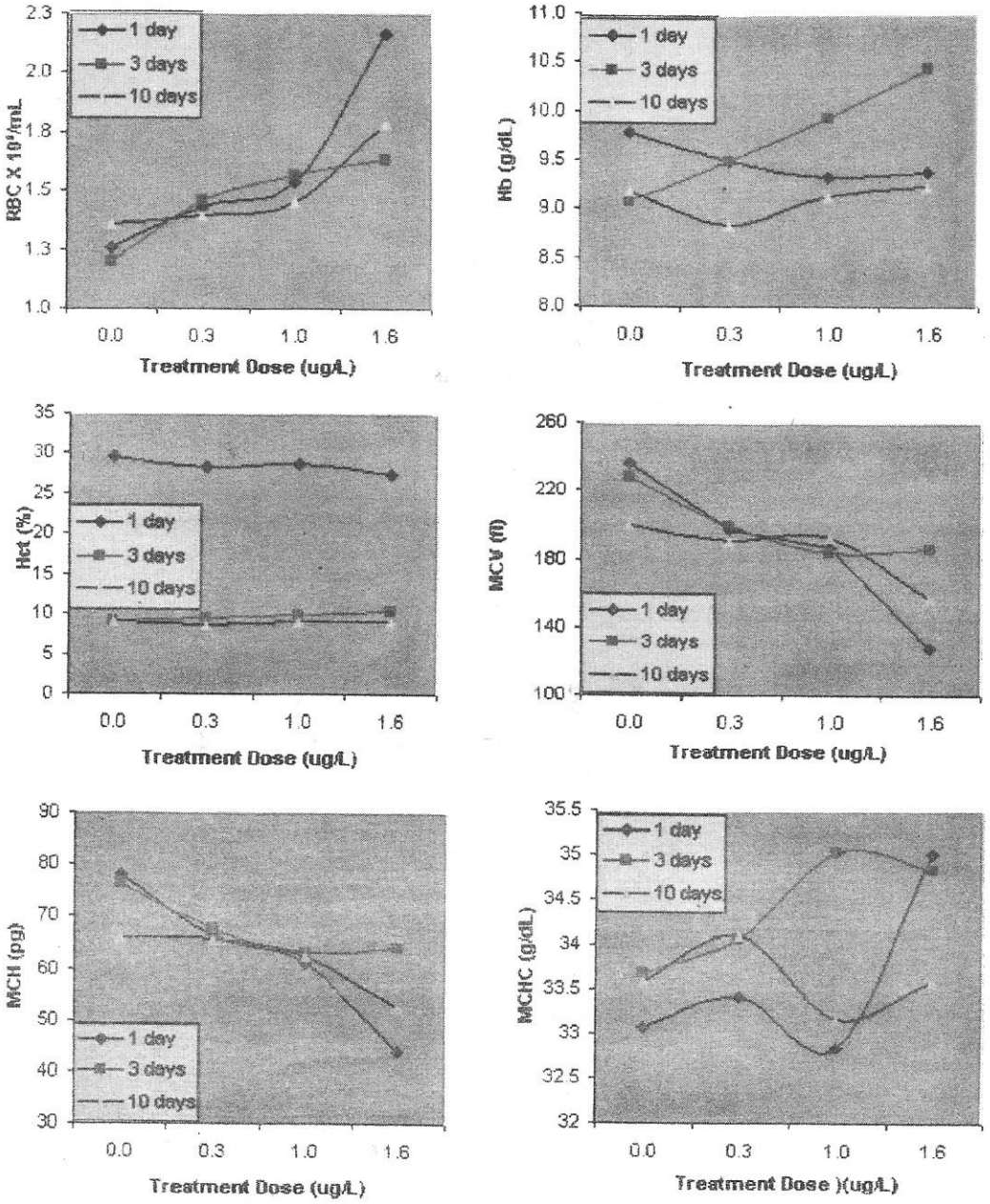

Figure 1. Mean values of the different erythrocyte components (RBC count, Hct, MCH, Hgb, MCV and MCHC) of fishes observed 1, 3 and 10 days after cessation of residual chlorpyrifos treatments.

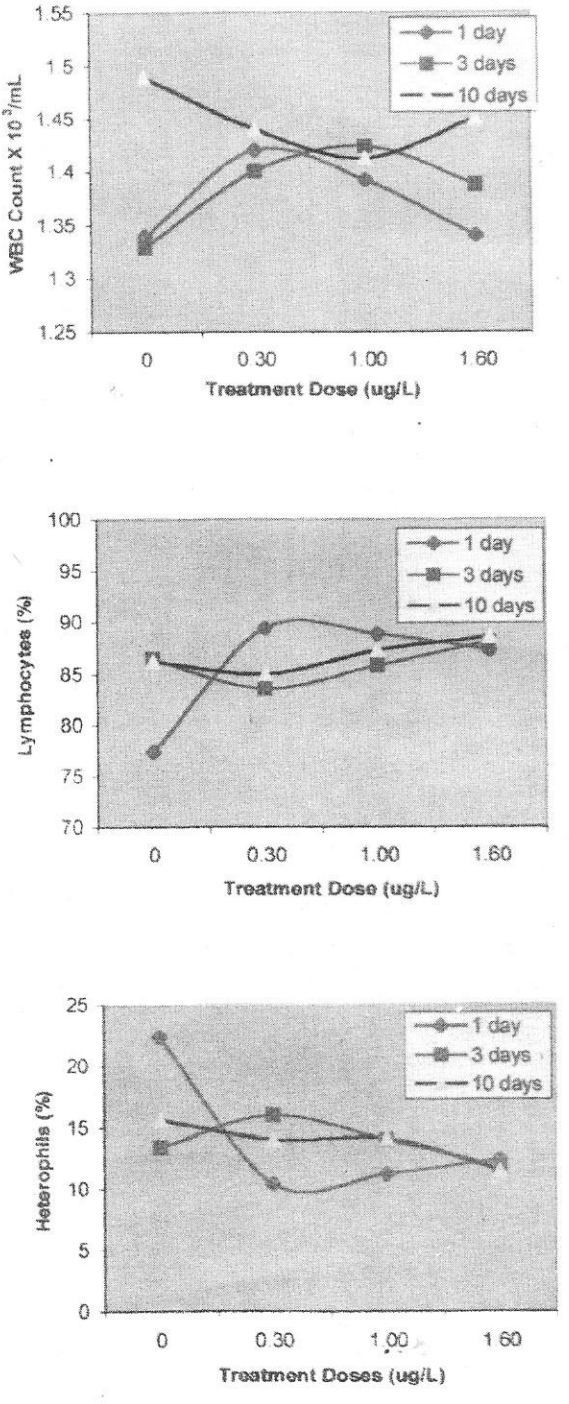

Figure 2. Mean percent values observed among the three leukocyte components (WBC count, lymphocytes and heterophils) of chlorpyrifos-treated fishes at 1, 3 and 10 days after cessation of treatments.

Results also showed that lymphocytes had the highest percentage among the different types of leukocytes. However, a significant decrease in lymphocytes was observed in fish under Treatment D (77.4%) as compared with the control (89.4%), Treatment B (88.8%), and Treatment C (87.2%) with F values = 5.40, $P<0.05$. This pattern was only observed a day after cessation of treatments, Meanwhile, an increase of 22.80% in heterophils was also noted in Treatment D one day after cessation of treatments. Three days and ten days after the cessation of treatments, values for the different WBCs of the treated group were comparable with that of the control.

Histological Analysis of the Liver

Figure 3 illustrates liver tissue samples of the test fishes. Intact liver cells or hepatocytes, which are polygonal with fount nuclei, are evident among individuals under the control group. Sinusoids are found situated between plates of hepatic cells. On the other hand, darkly staining pancreatic tissues can be found embedded along the portal vein in the liver. Thus, hepatopancreas is present in this species of tilapia. Another difference from vertebrate livers is the fact that no Kupffer cells exist in the fish liver (Stoskopf, 1993).

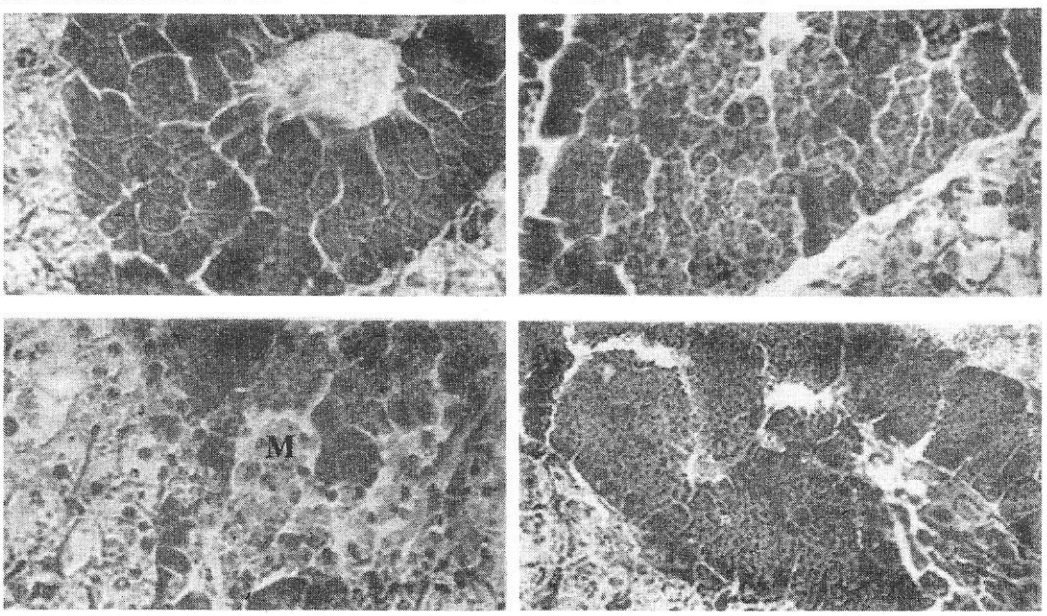

Figure 3. Cross sections of liver of Nile tilapia (*Oreochromis niloticus*) from the control (top left) and Treatment D (1.6 ug/L of chlorpyrifos) groups observed 1 day (top, right), 3 days (bottom, left), and 10 days (bottom, right) after cessation of chlorpyrifos treatments with varying degrees of melanomacrophage center formation (M), 400x.

Liver sections from fish exposed to all the treatment doses show no severe damages and are quite comparable with that of the control. However, brown structures abound near the pancreatic tissues. Histological evaluation clearly showed that residual concentrations of chlorpyrifos induce melanomacrophage center (MMC) formation in the liver of tilapia. MMCs are quite active in the accumulation of phagocytized foreign materials and debris from cells that are to be destroyed, detoxified or reused (Blazer and Dethloff, 2000). A number of studies have reported that MMCs may vary in number and size depending on the level and type of environmental stressors, such as contaminants and disease (Storskopf, 1993). They have observed these centers in kidneys, spleen and liver of some teleost fish species that are exposed to polluted or contaminated waters. It is not surprising that MMCs were observed most in the hepatopancreas of fish exposed to the highest residual chlorpyrifos concentration of 1.6 ug/L. These centers were also present in the other two treatment doses, but only a few brown structures (i.e., smaller MMCs) could be seen.

CONCLUSION

The present study demonstrates that the residual concentrations of chlorpyrifos (i.e. the residue levels detectable in rice paddy water) have the ability to cause a marked induction of cytochrome P_{450} in the liver of tilapia.

Low concentration levels of chlorpyrifos also influence some hematological parameters of tilapia. Fish exposed to different levels of treatments exhibited an increased RBC count, which may be detectable even ten days after cessation of treatments. Wintrobe erythrocytes like MCV and MCH were found to be significantly decreased. On the other hand, hematocrit, hemoglobin content and MCHC were found not to be significantly affected. Analysis also shows that the chemical had no effect on the total WBC count, although a significant decrease in lymphocytes (lymphopenia) and an increase in heterophils (heterophilia) occurred in some treated fishes a day after cessation of treatment.

Histological examinations revealed no apparent abnormalities on the liver structure. The residual doses of chlorpyrifos, however, induced the formation of MMCs in the hepatopancreas of exposed fish. No MMC was seen in the control.

The results of the study suggest that the residue level of 1.6 ug/L chlorpyrifos could cause considerable stress on the exposed fish. Prolonged stress can be detrimental to natural populations of tilapia living in rice paddies. If this level is traced in rice paddies, it can be speculated, that the survival of fish can still be endangered if chlorpyrifos residues remain in paddy waters.

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