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Biochemical Effects of the Herbicide Diclofop-Methyl Bioaccumulation in Freshwater Fish (*Oreochromis niloticus*)

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Abstract: Chronic toxicity and bioacumulation of diclofop-methyl (Iloxan 36% EC) in tilapia fish (*Oreochromis niloticus* was investigated. Fish was exposed to two sub-lethal concentrations 1/10 (0.19 mg L^{-1}, low concentration) and 1/3 (0.63 mg L^{-1}, high concentration) of determined 96 h-LC_{50}, 1.89 mg L^{-1} for 28 days. Results indicated that the bioaccumulation in whole fish was more than muscle. Results of biochemical parameters were indicated that alanine aminotransferases, alkaline phosphatase, acetyl-cholinesterase activities, albumin and glucose levels were increased significantly with both exposed concentrations comparing to control. However, significant increases were shown in aspartate aminotransferases and total protein levels with low concentration. On the other hand, high concentration caused significant increase in urea levels. Meanwhile, creatinine levels were decreased in both exposed concentrations. Noticeable changes in antioxidants biomarkers were recorded, a reduction in SH-protein level and an elevation in lipid peroxidation biomarker were recorded in plasma after treatment with two concentrations.

Key words: Diclofop-methyl, herbicide, bioaccumulation, tilapia fish, control

INTRODUCTION

With the increasing industrialization human being are continuously disturbing the delicate ecological balance in aquatic ecosystems. Pesticides are mainly synthetic organic compounds that are deliberately introduced into the environment to control selected organisms. Despite this benefit, the use of these kind chemicals must be controlled because fractions from these pesticides are released into the environment presenting a potential hazard risk. Herbicides originating from agricultural activity enter the aquatic environment through atmospheric deposition, surface run-off or leaching and frequently accumulate in soft-bottom sediments and aquatic organisms (Miles and Pfeuffer, 1997; Lehotay *et al*., 1998; Kreuger *et al*., 1999). Because of persistence of herbicides in the aquatic environment, some compounds that have long time persistence accumulated in fish, causing toxic effect (Tilak *et al*., 2004). Diclofop-methyl (methyl 2-[4-(2,4-dichlorophenoxy) phenoxy] propanoate) is a selective post-emergence herbicide developed for use in control of wild oats, wild millets and other annual grass weeds in sunflowers, peanuts and dicotyledonous vegetable (Xu *et al*., 2008). Diclofop-methyl has been newly registered in Egypt as herbicide. The available data on behavior of this compound are very few so there are need to study the behavior of this compound in the Egyptian environment. Among the many properties available for describing distributions and environmental behavior of pesticides, the Biological Concentration Factor (BCF) has proven very important as far as the behavior and fate of water-born chemicals in the aquatic environment is concerned (Tao *et al*., 2000). Fish are widely used to evaluate the health of aquatic ecosystems and biochemical changes among fishes serve as biomarkers of environmental pollution (Schlenk and Di-Giulio, 2002). The objective of this study is assessment of 96 h-LC_{50} value, determine the uptake rate, Biocon Centration Factor (BCFs) and to explain the biomarker responses that may

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be caused in tilapia fish exposed to diclofop-methyl with two concentration in aquarium for 28 days under the laboratory conditions.

MATERIALS AND METHODS

Tested herbicide: Diclofop-methyl (Iloxan 36.0% EC) was used in this study. The formulation and active ingredient of diclofop-methyl were obtained from Bayer Ltd.

Tested species: A total of 150 specimens of fish, *Oreochromis niloticus* (90±10 g in weight) were obtained from Al-Abasa fish farm, randomly put in aerated glass aquarium (100 L) for each 10 fish and allowed to acclimatize under laboratory conditions for 14 days. The physico-chemical characters of water were measured according to APHA, were maintained at optimum level such as pH (7.1±0.2 mg L^{-1}), salinity (0), dissolved oxygen (8-8.6 mg L^{-1}), total alkalinity (256±10.8 mg L^{-1}), total hardness as $CaCO_3$ (98.6±2.4 mg L^{-1}), calcium (68±6.5 mg L^{-1}) and magnesium (18±1.2 mg L^{-1}). During the experiment, fish received an adequate human care under constant laboratory conditions (constant and continuous aeration, 12:12 h light/dark cycle, 40-70% humidity at 27±2°C). The fish were fed daily with commercial pellets.

Acute toxicity study: The 96 h-LC_{50} of diclofop-methyl were determined according to Organization for Economic Cooperation and Development (OECD) guidelines (Weil, 1952; OCED, 1992) using sixty tilapia fish *Oreochromis niloticus*.

Bioaccumulation study: Bioaccumulation of diclofop-methyl in fish was monitored during the experimental period 28 days under laboratory conditions. After acclimatization freshly collected fish were divided into three groups of 30 fish in each group. Group 1 and 2 fish were exposed to diclofop-methyl with concentrations 1/10 and 1/3 96 h-LC_{50} as 0.19 and 0.63 mg L^{-1}, respectively and unexposed group as control (OECD, 1996).

Residual analysis

Sample preparation: Water samples (500 mL) were collected from low and high concentrations at 1, 7, 14, 28 days during the exposure period. Water samples were extracted according the method described by (Madsen *et al*., 2003). Fish samples were collected during the experimental period, 10 g of homogenate samples extracted with 20 mL 1% acidic acetonitrile in 50 mL polypropylene centrifuge next centrifugation at rpm 4000/10 min. The 5 mL of supernatant were transformed to clean 15 mL polypropylene centrifuge tube then add 1 g $MgSO_4$ anhydrous, 1 g sodium acetate dihydrate and 1 g NaCl and then centrifugation at 5000 rpm/5 min. The 3 mL of supernatant were concentrated to 1 mL for analysis.

Chromatographic analysis: Samples were analysed by Agilent 7890 (USA) Gas Chromatography Coupled with Electron Capture Detector (GC-ECD) equipped with capillary column HP-5 (30 m×0.25 mm×0.25 μm) and nitrogen gas was mobile phase at flow rate 2 mL min^{-1} with temperature program started at 180°C hold on 1 min and increasing to 220 in rate 25°C min^{-1} hold on 2 min and increasing to reach 245°C in rate 3°C min^{-1}. The mean recovery values from spiked samples with diclofop-methyl standard were ranged from (90-93) and (85-91) for water and fish, respectively.

Biochemical analysis: Blood samples were drawn from caudal vein with heparinized needles and syringes at the end of the experimental period 28 days. The blood samples were centrifuged at 5000 rpm for 10 min at 4°C. Plasma was collected and frozen at -40°C for assay of biomarker responses and protein electrophoresis. Biochemical markers were measured in plasma samples; Aspartate aminotransferases (AST) and Alanine aminotransferases (ALT) activities by Reitman and Frankel (1957), Alkaline Phosphatase activity (ALP) (Belfield and Goldberg, 1971), albumin content (Doumas *et al*., 1971), plasma glucose concentration (Barham and Trinder, 1972), urea level (Fawcett and Scott, 1960), creatinine (Schirmeister, 1964), Acetylcholinesterase (AChE) (Ellman *et al*., 1961) and Malondaldehyde (MDA) (Ohkawa *et al*., 1979).

Total plasma SH-proteins were evaluated spectrophotometrically at 412 nm using DTNB as a reagent. SDS-PAGE electrophoresis of plasma and muscle proteins was carried out according to the method of (Laemmli, 1970).

RESULTS AND DISCUSSION

Bioaccumulation in fish is influenced by factors specific to the chemical component, the environmental conditions, the exposure route and the species. Models must capture the combined effects of these factors in bioaccumulation through a set of parameters.

The results are showed the accumulation of diclofop-methyl residues in two fish groups exposed to 0.19 and 0.63 mg L^{-1} as one tenth and one third of 96 h-LC_{50}, respectively. Results clearly showed that the bioaccumulation of diclofop-methyl was in order whole

fish>muscle. Further, the accumulation in group 2 which exposed to one third of LC_{50} was higher than group 1 which exposed to one tenth of LC_{50} indicating that the positive relation between pesticides concentration and BCF in fish. The highest level of accumulation for diclofop-methyl (BCF = 7.05 and 9.21) was found in the whole fish for group 1 and 2, respectively. However, the muscle recorded the low values (BCF = 1.61 and 2.15) for group 1 and 2, respectively. These results are in line with those of Pratap and Singh (2008) whose monitor the total Hexachlorocyclohexane (HCH) and total Dichlorodiphenyltrichloroethane (DDT), aldrin, endosulfan and chlorpyrifos in liver and brain of fishes and they found that the tissue bioaccumulation of these pesticides were higher. Recently more attention has been given to tissue-specific contaminant distribution (Monosson *et al*., 2003; Sapozhnikova *et al*., 2005). The accumulation of diclofop-methyl in fish muscle was in low concentration might be due to the low amount of lipids in fish muscle (Ferrando *et al*., 1991; Larsson *et al*., 1991). As shown in Table 1 significant increase of AST at low concentration ($p \leq 0.001$), ALT and alkaline phosphatase activates in both experimental groups ($p \leq 0.001$) were found compared to the controls. These elevations of liver enzymes in the plasma may be due to the particularly damage liver tissue or alterations in the permeability of cell membrane and increased synthesis or decreased catabolism of aminotransferases. These results are in agreement with Begum and Vijayaraghavan (1996) who reported that AST and ALT were increase in fish, *Clarias batrachus* which exposed to dimethoate. These elevation of liver enzymes in the plasma may be due to the particularly damage liver tissue or alterations in the permeability of cell membrane and increased synthesis or decreased catabolism of aminotransferases. Since, the interaction between contaminants and biomolecules is the first step in the generation of toxic effects, understanding biochemical alterations induced by the exposure to pollutants may contribute to the prediction of toxic effects that may occur later at higher levels of biological organization. Significant increase of T. protein in fish group exposed to low concentration ($p \leq 0.05$) and there was an increase in glucose contents with tested concentrations of diclofop was observed compared to the control. Furthermore, a compensatory production of enzymes lost as a result of tissue necrosis or to meet increased demand to detoxify the pollutants might necessitate enhanced synthesis of enzyme proteins (Gill *et al*., 1990). One of the characteristics of pesticides is stimulate the sympathetic nervous system during stress leads to enhanced release of catecholamine, glucagon and growth hormone which result in promotion of gluconeogenesis, glycogenolysis, insulin resistance and constitution of hyperglycemia (Mechanick, 2006), these facts can explain significant elevation in plasma glucose as a result of herbicide intoxication which recorded in this study.

Also higher albumin level was observed in plasma of both exposed groups. While, urea level in plasma was higher in fish treated with high concentration only compared to control ($p \leq 0.001$), suggesting an impairment of kidney functions.

These effects could also be attributed to the changes in the threshold of tubular re-absorption, renal blood flow and glomerular filtration rate (Zurovsky and Haber, 1995). Meanwhile, significant decrease in creatinine levels comparing to control group with high concentration more than low concentration as shown in Table 1. The reduction in creatinine levels could be attributed to the significant loss of muscle mass in treated fish (Lees and Grean, 1994).

Data in Table 2, reported that the stimulation of AchE activity in exposed fish groups compared with control (137% at low and 134% at high concentration ($p \leq 0.001$) was recorded this may be explained by the coupling between diclofop-methyl and AChE, active substrate (Acetylcholine) or with its receptor. On the other hand significantly decrease in total SH-Proteins levels of both exposed groups (low concentration more than high concentration $p \leq 0.001$) this induction was negatively correlated with the increase of Malondaldehyde content MDA level as the end product of lipid peroxidation.

Table 1: Lever and kidney function biomarkers of fish exposed to diclofop-methyl for 28 days

Parameters	Control	Group 1 (0.19 mg L^{-1})	Group 2 (0.63 mg L^{-1})
Liver function			
AST (u L^{-1})	35.13±2.37	76.17±6.38**	46.11±3.96
ALT (U L^{-1})	24.81±1.56	33.35±2.34*	36.26±2.42**
ALP (U L^{-1})	10.52±1.03	17.27±1.39**	14.72±1.01*
T. protein (g dL^{-1})	3.33±0.14	3.91±0.08*	3.69±0.17
Albumin (g dL^{-1})	0.5±0.04	2.32±0.21**	1.18±0.12**
Glucose (mg dL^{-1})	133.91±11.32	189.64±16.57*	220.8 ±15.40**
Kidney function			
Urea (g dL^{-1})	0.25±0.019	0.25±0.02	0.46±0.01**
Creatinine (mg dL^{-1})	0.93±0.03	0.67±0.01**	0.77±0.07*

Values represent means±SEM (n = 5); *Significant differences versus control at p = 0.05; **Significant differences versus control at $p \leq 0.01$

Table 2: Influence of plasma acetylcholinestrase and antioxidant (T. SH-protein and MDA) of fish exposed to diclofop-methyl for 28 days

Parameters	Control	Group 1 (0.19 mg L^{-1})	Group 2 (0.63 mg L^{-1})
AchE activity (µmol/min/mL)	115.70±5.05	158.8±1.42**	155.28±5.24**
Activity (%)	-	137	134
T. protein (g dL^{-1})	444.71±38.68	193.6±9.33**	160.88±14.66**
Creatinine (mg dL^{-1})	8.62±0.09	13.47±0.35**	23.57±0.37**

Values represent means±SEM (n = 5); *Significant differences versus control at $p \leq 0.05$; **Significant differences versus control at $p \leq 0.01$

Table 3: Influence of plasma acetylcholinestrase and antioxidant (T. SH-protein and MDA) of fish exposed to diclofop-methyl for 28 days

	Plasma				Muscle		
M.W (kD)	Control	Low	High	M.W (kD)	Control	Low	High
94**	6.97	-	-	43**	0.08	-	-
88	-	10.30	-	39**	0.24	-	-
86**	5.07	-	-	16	-	0.57	-
84	-	-	11.40	14	-	0.24	-
70	7.12	-	22.20	13	-	0.38	-
56	3.46	13.20	-	12	-	0.51	-
43	9.87	2.04	-	11*	-	1.25	0.49
30*	-	4.88	11.40	10##	0.50	1.69	1.41
24	-	4.25	-	9##	0.44	2.72	2.57
18**	4.43	-	-	8	-	1.48	22.50
16*	-	0.26	2.06	7##	2.69	8.29	5.53
15##	1.47	2.03	1.96	6##	2.50	7.20	3.30
14#	4.16	0.79	1.03	5##	24.80	31.80	39.70
13	4.16	2.99	-	4##	11.70	33.30	31.70
11**	0.74	-	-	-	-	-	-
10**	0.33	-	-	-	-	-	-
9	0.31	0.06	-	-	-	-	-
8	7.35	5.21	-	-	-	-	-
7	10.60	6.18	-	-	-	-	-
6	2.95	4.15	-	-	-	-	-
5##	17.40	19.00	20.50	-	-	-	-
Total bands	16.00	14.00	7.00	Total bands	8.00	12.00	8.00

*Appear; #Amount decreased; **Disappear; ##Amount increased

Glutathione depletion is considered a biomarker of environmental stress as observed in fish stressed by environmental pollutants.

Malondaldehyde content (MDA) is a majoroxidation product of peroxidized polyunsaturated fatty acids and increased MDA content is an important indicator of lipid peroxidation. The results also showed the resultant induction of antioxidant could not quench the generated ROS induced by the toxicant (Saez and Bannister, 1990). The electrophoretic separation of proteins that presented in Table 3 observed alterations in plasma proteins. There are five bands disappear (band 94, 86, 18, 11 and 10 kD) while two bands (band 30 and 16 kD) are new in both treated groups compared with control. On the other hand, total number of protein muscles in group exposed to low concentration was more than high concentration and control groups, four consequence bands (band 16, 14, 13 and 12 kD) were appear with low concentration only. While there are two bands appear (band 11 and 8 kD) or disappear (43 and 39 kD) in both treated groups. This pattern of proteins was obtained from electrophoresis could help to understood the physiological and genetic effects and the action of chemicals such as pesticide (Rizkala *et al*., 1997).

CONCLUSION

The results clearly indicated that the effect of low concentration on AST, ALP and AChE activities and level of albumin and total protein were more than the effect of high concentration. In contract, influence of ALT activity and glucose, urea and MDA contents were highly with high concentration compared with low concentration. Additionally, influence of muscle protein pattern was hard with low concentration. These results were explained by Palaniappan and Karthikeyan who recommended that one of the most important properties of a toxic pollutant is its ability to accumulate in the tissues of organisms. Over a long period, the pollutants present in the environment at very low levels may accumulate within the body of aquatic species by various mechanisms to the extent that they exert. Researchers suggest that further studies are needed to study the environmental fate of this new herbicide diclofop-methyl.

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