Genotoxic Effects of Butataf Herbicide on Nile Tilapia

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ABSTRACT

This study was carried out to assess the capability of butataf herbicide in inducing cytogenetic damage and changes in plasma protein of Nile tilapia, *Oreochromis niloticus*. The 96 hours half lethal concentration (96 hr LC_{50}) of the butataf herbicide (N-Butoxymethyl-2-chloro-2,6-diethylacetanilide) determined for the adults *O. niloticus* was 0.2 ppm. The field concentration of this herbicide was 0.004 ppm which corresponds to 1/50 of the LC_{50}. The present experimental assay was carried out on the 30 day of exposure to the 1/100, 1/50, 1/25 LC_{50} and 1/10 LC_{50} which were used to study the effect of butataf on the rate of cell proliferation as well as the possible chromosomal aberrations of Nile tilapia genome. The results revealed that the butataf herbicide decrease of the rate of cell proliferation (low mitotic index). The rate of cell proliferation reached 1.1% in treated Nile tilapia with the highest dose 0.02 ppm of butataf which compared with the control groups (6.7%). In addition, different types of chromosomal aberrations; i.e., fragments, chromatid breaks. Stickiness, hypo and hyperpolyploidy were observed. The polyacrylamide gel electrophoresis of plasma protein bands revealed that the treatment with butataf herbicide caused striking variation in number, density and mobility of bands as compared to that of the control. The study concluded that butataf may have a genotoxic effect on tilapia fish.

Keywords: genotoxicity, Nile tilapia, butataf herbicide, mitotic index, chromosome aberrations, SDS-PAGE, plasma protein

INTRODUCTION

In order to detect genotoxic activity in an aquatic environment, a cytogenetic investigation on fish was carried out by several authors. Cytogenetic observations were primarily based on chromosomal aberration analysis, although such tests exhibit some disadvantages such as low mitotic activity, the difficulty of finding a sufficient number of metaphases for scoring chromosome aberrations and the limitation of a suitable fish karyotypes (Kligerman, *et al.,* 1975; Hooftman, 1981; Al-Sabti, 1991). The study of toxicity should be more concerned with sublethal effects and the sublethal study has been forced because of need to find the safe concentration of the pollutants (Johnson, 1968). Tooby (1971) recorded many differences in the toxicities of different herbicides even among different forms of the same herbicide. Butataf herbicide is used to eliminate noxious weeds in the rice fields; unsuspected side effects on fish are to be expected (Svobodova *et al.,* 1993). Generally, genotoxic effects of some mutagenic and/or carcinogenic chemicals on fish cells should be done in a dose-response manner. It was proven that there exists a species-response between different species of the same family (Cyprinids) to the same dose of the same chemical (Al-Sabti 1985, 1986). Negative and positive controls must also be carried out to ensure reliable results about the genotoxicity of the chemicals investigated. Since many types of DNA damage caused by mutagens present in water induce alteration in chromosomes, the measurement of chromosomes aberrations offers an acceptable parameter for monitoring mutagenic substances in water. Moreover, chromosomes aberrations selectively count only the primary DNA lesions that are not repaired by the machinery of the cell (Evans 1977& Obe *et al.* 1982). Because of lack of suitable karyotypes in most of fish, testing the genotoxic potential of an agent on fish has to be mainly extrapolated from cytogenetic bioassays which carried out only on

some particular fish species having suitable karyotypes. Therefore, it could be added benefit to find toxicological endpoints other than the conventional ones that could help determine genotoxicity, particularly in those fish that have unsuitable karyotypes for genotoxicity assessment. Meanwhile, evaluation of additional parameters such as a qualitative assay of protein profiles was taken up in two fish models, *O. mossambicus* and *A. testudineus* (Guha and Khuda-Bukhsh, 2002). In this regards, Guha and Khuda-Bukhsh (2003) studied the genotoxic effects in fish *Oreochromis mossambicus* exposed to different doses of β-carotene (0.02, 0.05 and 0.1%,) separately and conjointly with 0.2% ethylmethane sulphonate during five different time periods. The relative efficacy of three doses of 0.02, 0.05 and 0.1%, β-carotene in ameliorating genotoxic effects of 0.2% EMS was also tested after a treatment period of 48hr. The results indicated that ethylmethane sulphonate caused chromosomes aberrations and an apparent alteration of protein synthesis in various tissues. Some of these genotoxic effects of ethylmethane sulphonate appeared to be improved by all three doses of β-carotene, of which the 0.02% dose showed a marginally better efficacy.

In this work two bioassays were employed. They are: chromosomal aberrations (CA) and electrophoretic-plasma protein patterns in Nile tilapia fish which were treated with butataf herbicide.

MATERIALS AND METHODS

Apparently healthy Nile tilapia, *Oreochromis niloticus* specimens with an average body weight of 100 ± 5 g were obtained from Abbassa Fish Farm, Abbassa, Abou-Hammad, Sharkia and they were acclimated in laboratory conditions for two weeks prior to start the experiment. The 96 hour half lethal concentration (96 hr LC_{50}) of the butataf herbicide (N-Butoxymethyl-2-chloro-2,6-diethylacetanilide) was determined according to Behreus and Karbeur (1953) as 0.2 ppm and was applied to

field with a concentration of 0.004 ppm. Five groups of fish were kept for 30 days to evaluate the effect of different sublethal concentrations for butataf herbicide on cytogenetic behavior and plasma protein. Each group consisted of 30 fish was divided into three replicates of ten fish. Then maintained in glass aquaria supplied with de-chlorinated aerated water at a temperature of 26 ±2 ^{o}C, pH 7.2 ± 0.2 and dissolved oxygen 5.5 ± 0.5 mg/l. The first group was kept as control. Other groups of applied fish were exposed to the 1/10, 1/25, 1/50 and 1/100 LC_{50} (0.02, 0.008, 0.004 & 0.002 ppm), respectively. The feeding rate was 3% of the live body weight daily using 30% of protein feed mix.

Cytogenetic analysis

1- Mitotic index was calculated according to Brusick (1980) as in following equation:

Mitotic index = (Number of dividing cells / Number of total examined cells) 100

2- Chromosomal aberrations

Chromosomes preparations were made at the end of the experiment from the gills by modified method of Al-Sabti *et al.* (1983). Fish were injected i.p. (intraperitoneal) with doses of 0.01 ml/g body weight of 0.6% solution of colchicines for 3hr and then gill filament tissues were removed and placed in hypotonic 0.005 N KCl solutions for 20 min. The tissues were snipped and fixed in fresh and cold fixed solution methanol/ glacial acetic acid (3:1) for 40 min. By pastier pipette dropping on a clean and cold (-5 ^{o}C) slide was done. The slides were dried by alcohol lamp and left at room temperature for 3 hr before staining. Slides were stained with 10% Giemsa in phosphate buffer 6.8 pH for 15 min. About 100 well-spread, metaphase figures of control and each of the five treated groups were microscopically examined using oil immersion lens to calculate the number of selected aberrant metaphase and the normal ones.

Electrophoresis analysis

Blood samples were taken from the caudal vein of non anaesthetized fish by sterile syringe with 0.5 ml of the blood

containing EDTA as an anticoagulant. Plasma was obtained by centrifugation at 3000 rpm for 15 min and stored in deep freezer for further electrophoresis analyses. Three samples for each treatment including control were used. These samples were subjected to SDS-PAGE (Sodium dodecyl sulphate-polyacrylamide gel electrophoresis) according to Laemmli (1970) on 9 % separating gel by Tris-glycine running buffer for visualizing different sub-fractions of protein bands.

Statistical analysis

All statistical analyses and genetic dissimilarity were carried out using the Statistical Package for the Social Sciences (SPSS) program for windows (ver. 10.0). Each individual was scored for the presence or absence of band products accordingly; relative molecular weights of each fragment could be measured and scored manually and by **Gel Analyzer Ver. 3, (2007)**.

RESULTS AND DISCUSSION

After exposure of Nile tilapia; *Oreochromis niloticus* to different concentrations of the butataf herbicide, the 96 hour half lethal concentrations (96 hr LC_{50}) was 0.2 ppm, and field concentration in nature (0.004 ppm) equal to 1/50 of the half lethal concentration are given in Table (1). Consequently, according to Bathe *et al*. (1974) the butataf is considered as highly toxic herbicide to *O. niloticus* fish. During determination of the 96-hr LC_{50}, fish exhibited erratic swimming movements. The skin had a spotted appearance and mucus secretion increased and accumulated on the gills, so the fish exhibited a different respiratory manifestation such as surfaced swimming, opening their mouth with rapid and frequent respiratory movement. The erratic swimming and surfaced fairly frequently movements may be due to hyper-contraction of the muscles which due to cholinesterase inhibition as previously reported by Ferguson (1989)

Table (1): *Different sublethal concentrations in ppm of butataf herbicide for Nile tilapia.*

Concentrations Fish species	96 hr LC_{50} (ppm)	$^{1}/_{100}$ LC_{50} (ppm)	$^{1}/_{50}$ LC_{50} (ppm)	$^{1}/_{25}$ LC_{50} (ppm)	$^{1}/_{10}$ LC_{50} (ppm)
Oreochromis niloticus	0.20	0.002	0.004	0.008	0.02

while Atallah, *et al.* (1997) attributed such changes to the extraordinary need for the oxygen which due to the thick coating of the gills with profuse mucus together with congestion and hyper-plastic epithelium of the secondary lamellae. The mottled appearance of skin and heavy mucus secretion are due to the melanosis (aggregation of melanocytes) happened by the stimulation of α-melanonine stimulating hormone (α-MSH) secretion in fish which was subjected to stressors and irritation of the gills which subjected to pollutants (Satchell, 1984 and Ferguson, 1989).

Cytogenetic analysis

1- Mitotic activity

Cell proliferation in gills of Nile tilapia treated with different concentrations of butataf herbicide as represented by mitotic indices is presented in Table (2). Mitotic indices of the control group showed to be higher than that of treated *O. niloticus.* The results showed a gradual decline in mitotic indices with increasing of the dose of butataf herbicide. This decrease ranged from 3.7 to 1.1 % at all doses of butataf herbicide when compared with that of the control group (6.7%). These results are in agreement with (**Shaban, 1999**) who obtained low mitotic index in both Nile tilapia and common carp after treatment with other toxic compound (Aflatoxin β_1).

2- Chromosome aberrations

As presented in Figure (l a) of this study, metaphase of Nile tilapia, *O. niloticus* consists of 22 pairs with no morphologically distinct sex

chromosomes (Majumdar and McAndrew, 1986).

Table (2): *Mitotic indices of gills cells of Nile tilapia after treatment with butataf herbicide.*

Concentrations	No. of divid. Cells	No. of non-divid. Cells	Total No. of cells	Mitotic indices	Chi square Value
Control	30	416	446	6.7	
$^1/_{100}$ LC_{50}	16	418	434	3.7	4.1*
$^1/_{50}$ LC_{50}	12	430	442	2.7	7.9*
$^1/_{25}$ LC_{50}	6	442	448	1.3	16.8***
$^1/_{10}$ LC_{50}	5	454	459	1.1	19.3***

** Significant at p < 0.05*

****highly significant at p < 0.01*

Tables (2 & 3) and Figures (1 b to d) show the different types of structural and numerical chromosomal aberrations which were obtained after exposure of Nile tilapia to butataf herbicide. The statistical analysis revealed highly significant ($p<0.01$) effect of butataf herbicide in inducing chromosomal aberrations. The total aberrant metaphases in fishes ranged from 18 to 42% after exposure to butataf as compared to the control group (4%). Different types of structural aberrant were represented by chromosomal deletion, chromosomal fragments, chromosomal breaks and chromosomal stickiness, while the numerical aberrations were represented by hyper and hypoploidy. The number of aberrant metaphase was increased with increasing of the dose of butataf herbicide. The most frequent type of aberrations induced at 1/10 dose level was stickiness 15 %. These results nearly are in agreement with (Shaban, 1999) who found that the most frequent type of aberrations increased with increasing the dose of

$AF\beta_1$ in both Nile tilapia and common carp.

The use of fish chromosomes in genotoxic investigations has been little practiced, in spite of the fact that the fishes make up the largest and most diverse group of vertebrates. Fish have been proven to be important animals in experimental laboratory work not only for cytotoxicological and different genetic studies, but also for biochemical and physiological research. The use of fish cells for the study of genotoxic effects has only recently been explored. Unfortunately, only a small number of fish species are suited for cytogenetic investigations because of their large number of chromosomes and /or their small size; moreover the mitotic index in fish is too low when compared to that in mammals (Al-Sabti 1991).

As fish may act as 'sentinel' organisms for indicating aquatic pollution, several species have been successfully used as test materials for detecting genotoxic activity in the aquatic environment, (Hooftman, 1981; Kligerman, *et al.,* 1975; Manna & Mukherjee, 1989). Analysis of metaphase chromosomes in fish for the occurrence of chromosome aberrations and sister-chromatid exchange (SCE) in order to detect as well as quantify the extent of genotoxicity or point mutation induced by an agent has proven to be useful only in a few fish models (Kligerman, 1982; Manna, 1984). The majority of fish species are not suitable for the chromosomal assay because of their small size and large diploid number of chromosomes without any marked variation in size. Therefore, assay of the peripheral erythrocytes in fish for the occurrence of micronuclei (MN) and different types of nuclear abnormalities has been adopted as a good substitute for the chromosomal assay, and based on information on the frequency of occurrence of micronuclei or nuclei with abnormal shape, a monitoring system for potential genotoxicity of an agent has been proposed (Hooftman & de Raat, 1982; Manna,

et al., 1985; Guha & Khuda-Bukhsh, 2001 and Ramadan, 2005).

Table (3): *Chromosome aberrations in gills cells of Nile tilapia after treatment with butataf herbicide.*

Concentrations	No. of examined metaphase	Total No. of aberration metaphase	Structural chromosomes Aberrations				Numerical chromosomes Aberrations	
			D	F	B	S	Hyperpoly ploidy	Hypopoly Ploidy
Control	100	4	0	1	1	2	0	0
$^1/_{100}$ LC_{50}	100	18	2	4	3	6	2	1
Chi square value		8.1***	1.98	1.75	0.98	1.9	1.98	0.99
$^1/_{50}$ LC_{50}	100	26	3	5	4	9	3	2
Chi square value		14.6***	2.95	2.59	1.75	4.2*	2.95	1.98
$^1/_{25}LC_{50}$	100	35	4	6	5	13	3	4
Chi square value		20.97***	3.9*	3.45*	2.59	7.5**	2.95	3.9*
$^1/_{10}$ LC_{50}	100	42	5	7	6	15	4	5
Chi square value		26.2***	4.88*	4.33*	3.45*	9.2***	3.9*	4.88*

** Significant at $p < 0.05$*

****highly significant at $p < 0.01$*

D = Chromosome deletion

F = Chromosome fragments

B = Chromosome breaks

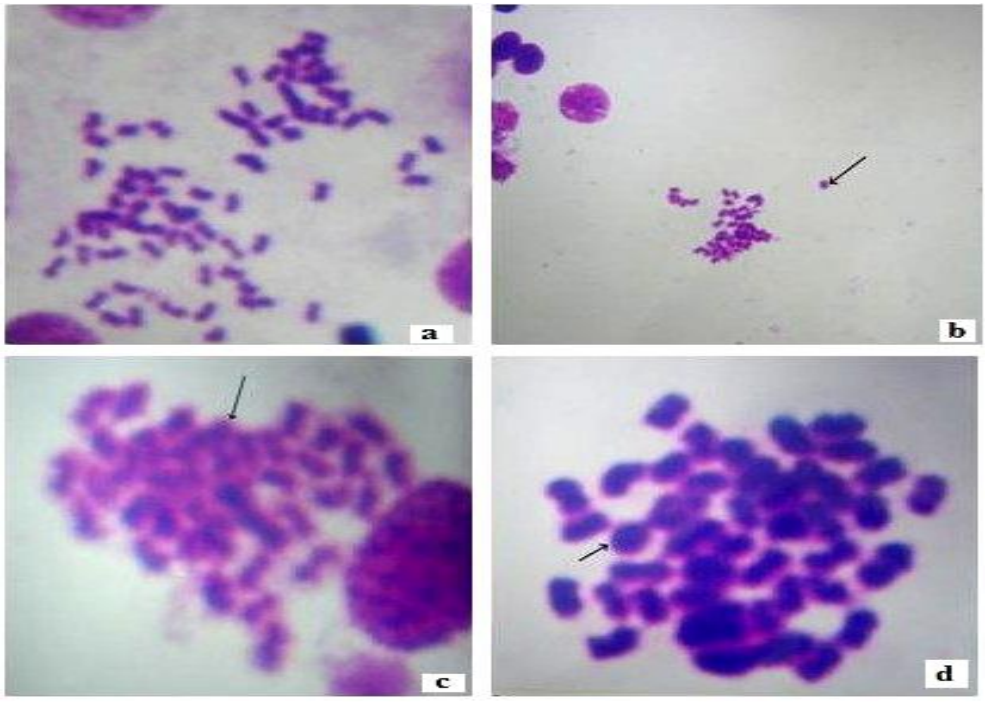
S = Chromosome stickiness

Electrophoresis analysis

The genotoxic effect was also tested using the polyacrylamide gel electrophoresis of plasma protein bands in Nile tilapia after exposure to sublethal concentrations of butataf herbicide (1/100, 1/50, 1/25 & 1/10 LC_{50}). As shown in Figure (2) the number of bands of plasma protein decreased significantly in fish exposed to butataf herbicide. This trend was increased with increasing of toxicity butataf herbicide (12-9 bands), when compared to the control group (13 bands). Therefore some bands disappeared after exposed the fish to chronic toxicity of herbicides. These results are in agreement with

Sharf-Eldeen and Abdel-Hamid (2002) mentioned that expose the fish

Figure (1): ***Photomicrograph slides of gills cells showing normal metaphase (a); chromosome fragments (b); chromosome stickiness (c) and ring chromosome (d) of Nile tilapia.***



to copper metal induced a disappearance of some protein fractions and changed the relative electrophoresis mobilities that indicated genetic mutation. Figure (2) shows the most changes were observed in the range from 66 to 14 kDa. Disappeared bands from treatments were noticed in the range of 66 to 45 kDa while appearance bands in were observed in the treatments in the range of between 45 to 14 kDa. Four of mono-morphic bands in all samples at molecular weights of 203.42, 109.7, 76.04 and 53.94 kDa were detected. While two unique bands in 1/25 LC_{50} and 1/10 LC_{50} concentrations at molecular weight 197.1 and 38.45 kDa were observed. On the other hand, the polymorphic bands were 9,8,7,6 and

5, respectively in control and treatments. Table (4) represents the dissimilarity matrix there was positive correlation for the interaction

Table (4): *Dissimilarity matrix among fish groups treated with butataf herbicide*

Case	control	1/100 LC_{50}	1/50 LC_{50}	1/25 LC_{50}	1/10 LC_{50}
Control	0.11				
1/100 LC_{50}	0.46	0.21			
1/50 LC_{50}	0.54	0.35	0.16		
1/25 LC_{50}	0.56	0.46	0.34	0.14	
1/10 LC_{50}	0.59	0.55	0.39	0.34	0.19

between increases of doses and treatments. The highest value of dissimilarity was that obtained in case of control and 1/10 LC_{50} (0.59).

In this regard, Rizkalla *et al.* (2006) studied the effect of copper, cadmium, zinc and their combinations in water under controlled laboratory conditions on carp fish. The differences in protein banding pattern by electrophoretic mobility indicating differences in molecular mass, charge, and/or shape. It is well established that the protein synthesis is undergone to genetic control and the impairment of DNA by the genotoxic agent would affect gene expression and protein synthesis. Although, strictly speaking, the appearance or disappearance of protein bands may not always be related to cytogenetical changes in general, these changes may indeed reflect to a certain extent, or may have some correlations with the 'stress-proteins' or 'heat-shock proteins' that unfailingly appear in response to chemical or physical insult Manna and Mukherjee (1986). It showed also that time of exposure and concentration of compound enhanced the protein content. According to (Guha and Khuda-Bukhsh, 2003), a critical analysis of gel electrophoretic band profiles of total protein was perhaps not considered by earlier workers to be of practical value in genotoxicity testing, as they either preferred only suitable fish models for their studies, or else depended solely on induction of

micronuclei as valid indicators of genotoxicity where chromosomal studies proved to be difficult. It is in the latter cases that the protein pattern could be of some additional benefit.

The present study showed that the use of protein profiles as an additional genotoxic bioassay may be helpful in genotoxicity studies along with the commonly tested parameters and particularly in fishes with unsuitable karyotypes. Finally, the apparent antagonistic action of butataf herbicide is significant, because if it can antagonize more dangerous mutagenic agents, it may have limited use as an antimutagenic agent, particularly in heavily polluted, confined water, where fishes are already exposed to various other mutagens. Effects of a mutagen on protein structure/synthesis can also give important keys, which may have direct or indirect implications for their health and yield in response to different agents.

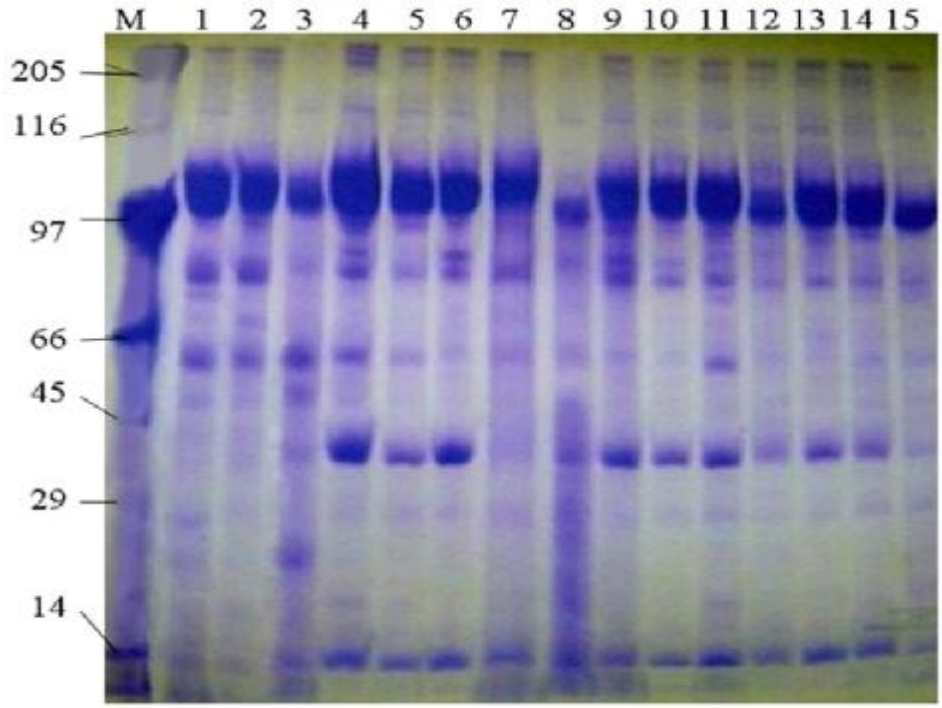


Figure (2): *Polyacrylamide gel electrophoresis profile of plasma protein bands, 1-3 (Control) 4 - 6 (1/100 LC_{50}) 7-9 (1/50 LC_{50}) 10-12 (1/25 LC_{50}) 13-15 (1/10 LC_{50})*

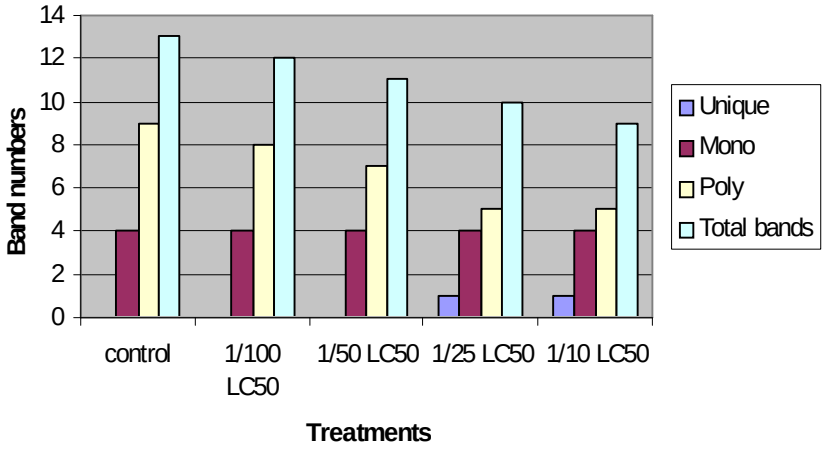


Figure (3): *Polymorphism diagram of plasma protein bands by polyacrylamide gel electrophoresis*

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تأثير السمية الوراثية لمبيد الحشائش البيوتاتاف على البلطي النيلى

أشرف عبد الرحمن رمضان
المعمل المركزي لبحوث الثروة السمكية- بالعباسة- مركز البحوث الزراعية

نظراً لاستخدام البيوتاتاف كمبيد عشبي لمقاومة الحشائش في حقول الأرز كان لابد من دراسة خطورته على الأسماك التي تعيش في مياهها. ودفعنا ذلك لدراسة مدى خطورة هذا المبيد على أسماك البلطي النيلى و التي تعد واحدة من أهم الأسماك النيلية في مصر. و قد استهدف البحث دراسة التغيرات السيتولوجيه و طرز التفريد الكهربي الناجمة من استخدام هذا المبيد على أسماك البلطي النيلى لمدة ٣٠ يوم. حيث تم تعيين الجرعة النصف مميتة خلال ٩٦ ساعة فوجدت أنها تساوي ٠.٢ جزء في المليون. وعرضت مجموعات من الأسماك محل الدر اسه إلى ١٠٠/١ و ٥٠/١ و ٢٥/١ و ١٠/١ جزء في المليون من الجر عه النصف مميتة وتمت المقارنة مع المجموعة الضابطة. و قد أظهرت نتائج البحث انخفاض معدل الانقسام الميتوزي بزيادة التركيز من ٦.٧ إلى ١.١ ٪. وارتفاع معدل التشوهات الكروموسوميه و إن بدت أعلاها في صورة فجوات و كسور ونقص والتصاقات.

وفى هذه الدراسة تم فصل بروتينات بلازما الدم بطريقة التفريد الكهربي و لوحظ نقصا في عدد الحزم بزيادة التركيز من ١٣ حزمه في المجموعة الضابطة إلى ١٢ و ١١ و ١٠ و ٩ حزمه على التوالي. كما لوحظ ٤ حزم مشتركه عامة توجد في جميع العينات عند وزن جزيئي ٢٠٣.٤٢ و ١٠٩.٧ و ٧٦.٠٤ و ٥٣.٩٤ , و حزمتين منفردتين توجد في التركيز الثالث و الرابع عند وزن جزيئي ١٩٧.١ و٣٨.٤٥ , كما توجد حزم غير مشتركه (متباينة) عبر كل

العينات بأعداد ٩ و ٨ و ٧ و ٦ و ٥ للخمس مجموعات. و كانت أعلى درجة تباعد (اختلاف) بين المجموعة الضابطة و تركيز ١٠/١ الجر عه النصف مميتة وبلغت ٠.٥٩ .

وبهذا فان النتائج تشير إلى أن مبيد الحشائش " البيوتاتاف" يمثل خطرا كبيرا في إحداث تغيرات وراثية (تشوهات كروموسوميه – تأثير على التعبير الجيني الخاص بالبروتينات قيد الاختبار) مما أدى إلي تغير طرز التفريد الكهربي للبروتينات في الأسماك مشيراً إلى خطورته على الجهاز الو راثي للأسماك التي تعيش في المياه الملوثه بهذا المبيد. لذلك يجب البحث عن بدائل أخرى أكثر أمانا وسلامة منه للحفاظ على الثروة القومية و الصحة العامة للمواطنين.