

Effect of Insecticide "Chlorpyrifos" on Immune Response of *Oreochromis niloticus*

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Abstract

This study was carried out to determine the level of Chlorpyrifos (CPF), an organophosphorus insecticide, in water of Abbassa and Sahl El-Hussinia fish farms and investigate the sub lethal concentration of this pesticide on immune response of *Oreochromis niloticus* (*O. niloticus*). Water samples were collected from Abbassa and Sahl El-Hussinia fish farms and analyzed using Gas chromatograph (GC) for detection of Chlorpyrifos level. Two hundred and ninety *O. niloticus* with average body weight (35 ± 0.5 g) were collected from Abbassa fish hatchery, Sharkia Governorate, Egypt. One hundred and seventy of these fish were used for the determination of acute toxicity of Chlorpyrifos, while, another 120 fish were used for the determination of the effect of different sub lethal concentrations of Chlorpyrifos (1/8, 1/20 and 1/43 of 96 h LC₅₀) on the immune response of *O. niloticus*. It was found that; the levels of Chlorpyrifos in Abbassa and Sahl El-Hussinia fish farms were 0.008 and 0.0016 mg/L, respectively. The LC₅₀ value of Chlorpyrifos was 0.07mg/L. Sub-lethal concentration of Chlorpyrifos altered the non-specific immunological parameters (namely, total globulin, immunoglobulin M (IgM), lysozyme, nitric oxide, phagocytic activity) and interleukin-1 β (IL-1 β) of *O. niloticus*. It could be concluded that water of Abbassa and Sahl El-Hussinia fish farms have had detectable residue levels of CPF, which was altered the immunological status of *O. niloticus*.

Keywords: Chlorpyrifos, *Oreochromis niloticus*, Residues, Abbassa, Sahl El-Hussinia

Introduction

Aquaculture is considered one of the most important food sources and nutrition for hundred millions of people all over the world. Now, aquaculture provides half of human needs for fish consumption, while, by 2050, fish production is expected to provide nutrition and food security for 9.7 billion of people [1]. Fish culture in Egypt depends mainly on agriculture drainage and wastewater that leads to water pollution by agriculture wastes such as pesticides. The increased amounts of pesticide pollutants are harmful to fish leads to sudden mortalities, while lower levels of pollutants result in accumulation in the aquatic environment leading to other hazards such as immunosuppression, reduced metabolism and damage of the gill tissue [2].

Aquatic pollution is still a problem in many freshwater and marine environments. It had harmful effects for the health of the aquatic organisms especially the fish. The effects of pollutants can be either lethal or sub-

lethal [3]. Pesticides enter water sources through the direct application in aquatic systems for eradication of insects, herbs and mollusks. While, the indirect arrival was by erosion from agricultural lands and agricultural wastewater infiltration and eventually washed into deep-water environments and ecosystem [4].

Chlorpyrifos (CPF) is an organophosphate insecticide that is used widely in most regions. In agriculture, it is used to control insect pest on fruits and grains as a foliar spray or applied directly to soil. The CPF pollute natural water through air drift or surface runoff, leading to accumulation in living organisms in water and mainly in fish [5]. This study aimed to determine the level of CPF in water samples from Abbassa and Sahl El-Hussinia fish farms and to detect the effect of different sub-lethal concentrations of CPF toxicity on general health conditions and the immune response of *Oreochromis niloticus* (*O. niloticus*).

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Material and methods

Water samples

Water samples were collected from Abbassa and Sahl El-Hussinia fish farms for the determination of CPF level using Gas chromatography apparatus (GC) at the Central Agricultural Pesticides Laboratory (CAPL), Dokki, Egypt.

Determination of the LC₅₀

One hundred and seventy fish were used in this experiment; one hundred apparently healthy *Oreochromis niloticus* with an average body weight (35±0.5 g) were obtained from Abbassa fish hatchery, Sharkia, Egypt. Fish were used for the determination of LC₅₀ of 96 h of CPF. The fish were left in fully prepared

glass aquaria (each, 80 x 40 x 30 cm capacity) for 15 days before the beginning of the experiment for acclimation. After this period, the fish were divided into 10 equal groups to determine the zero and hundred percentage of mortalities. All groups of fish were exposed to different concentrations of CPF (Table 1). To determine the actual LC₅₀, another 70 *O. niloticus* were adapted and then they were divided into 7 equal groups. The groups were exposed to various concentrations of CPF (0.02, 0.04, 0.06, 0.08, 0.1, 0.12 and 0.14 mg/L) in groups from 1 to 7, respectively for a period of 96 h. The 96 h LC₅₀ was determined according to Behrens and Karber [6]. The groups were observed at 12 h interval up to 96 h.

Table 1: Estimation of zero and hundred % mortalities in Nile tilapia exposed to different levels of Chlorpyrifos (After 96 hours)

Groups (N=10)	Concentration of Chlorpyrifos (mg/L)	Mortality number during 96 hours				Total number	%
		1 st day	2 nd day	3 rd day	4 th day		
1	0	0	0	0	0	0	0
2	0.02	0	0	0	0	0	0
3	0.04	0	1	2	2	5	50
4	0.06	0	1	3	2	6	60
5	0.08	0	2	2	2	6	60
6	0.1	0	3	2	1	6	60
7	0.12	0	2	4	1	7	70
8	0.14	0	3	4	3	10	100
9	0.16	0	5	2	3	10	100
10	0.18	0	6	4	0	10	100

The effect of Chlorpyrifos chronic toxicity on general health condition and immune response of *O. niloticus*

One hundred and twenty apparently healthy fish were obtained from Abbassa fish hatchery, Sharkia Governorate, Egypt, to determine the effect of chronic toxicity of CPF on the immune response of *O. niloticus*. The fish with an average body weight (35±0.05 g) were allocated into four groups; each group had three replicates (10 fish replicate⁻¹). Group 1: control group (not treated with CPF), group 2 were exposed to 1/8 96 h LC₅₀ (0.008 mg/L) of CPF; group 3 were exposed to 1/20 96 h LC₅₀ (0.0035 mg/L) of CPF and group 4 were

exposed to 1/43 96 h LC₅₀ (0.0016 mg/L) of CPF. The water parameters were monitored and were kept within the recommended ranges during the experiment (water temperature=25.5±0.5; pH=7.2±0.5; ammonia=0.02±0.001 mg/L and nitrite=0.017±0.001 mg/L) [7]. Water exchange was performed twice weekly with maintenance of the insecticide level in the water. The fish were fed on basal diet obtained from Fish Research Unit, Faculty of Veterinary Medicine, Zagazig University; at a rate of 3% of body weight, two times daily. Clinical signs and postmortem lesions were recorded during the experimental period (one month).

Innate immune response parameters and serum proteins level

Blood samples (3 samples/group) were obtained by puncturing the caudal blood vessels after 15 and 30 days of the experiment. The blood samples were collected without heparin for serum separation and were centrifuged at 3000 rpm for 15 min then stored at -20°C until analysis. Serum total protein, albumin and globulin levels were assayed [8-10]. Immunoglobulin M (IgM) was determined using ELISA Kit. Lysozyme activity, nitric oxide, phagocytic activity were also determined [11-13].

Expression of IL-1 β by quantitative RT (real time)-PCR.

Total RNA was extracted from the spleen tissue (3 samples/group) of all fish groups after 15 and 30 days of the experiment using easy-REDTM following the manufacturer protocol (iNtRON Biotechnology, South Korea). The complementary DNA was synthesized following the manufacturer's instructions of a Quantitect® Reverse Transcription kit (Qiagen, Germany). Quantitative real-time PCR analysis was performed using SYBR green PCR master mix (StepOnePlus, Applied Biosystem, USA). The target gene chosen was IL-1 β (F: 5'-TGC TGA GCA CAG AAT TCC AG-3'; R: 5'-GCT GTG GAG AAG AAC CAA GC-3'); and EF-1 α as an internal standard (F: 5'-CCT TCA ACG CTC AGG TCA TC-3'; R: 5'-TGT GGG CAG TGT GGC AAT C-3') [14, 15]. The thermal cycling conditions were initial denaturation at 94 °C for 5 min, 40 cycles of amplification (DNA denaturation at 94°C for 15 sec, annealing at 62°C for 30 sec, extension at 72 °C for 30 sec). The final extension at 62°C for 1min. To estimate the variation of gene expression on the different samples, the CT of each sample was compared with that of the control group according to the " $\Delta\Delta Ct$ " method using the following ratio: $(2^{-\Delta\Delta Ct})$. Whereas $\Delta\Delta Ct = \Delta Ct_{reference} - \Delta Ct_{target}$. $\Delta Ct_{target} = Ct_{control} - Ct_{treatment}$ and $\Delta Ct_{reference} = Ct_{control} - Ct_{treatment}$.

Statistical analyses

The data were analyzed by comparing the means differences using analysis of variance (One Way ANOVA) using the SPSS 16.0 computer program. A P- value of ≤ 0.05 ($P \leq 0.05$) was considered statistically significant.

Results and Discussion

Chlorpyrifos level in collected water samples

Chlorpyrifos level detected in the water samples collected from Abbassa and Sahl El-Hussinia fish farms were 0.008 and 0.0016 mg/L, respectively. The detectable levels of CPF in the water may be attributed to the escape of this insecticide from agriculture drainage. Nearly similar results were observed by Malhat and Nasr [16] who estimated the residues of organophosphorus pesticides in water samples from different tributaries of the River Nile in Egypt, which are El Menofiya canal water supplies (El Sarsawia, El Bagoria and Bahr Shebin), in addition to El Embaby, El Menofi and Miet Rabiha drainage canals using Gas chromatograph. Only Chlorpyrifos-methyl (41.53 ng l⁻¹) and Prothiphos (30.03 ng l⁻¹) were detected in El-Embaby drain.

Chlorpyrifos 96 h LC₅₀ in Nile tilapia

The results showed that 96 h LC₅₀ of CPF in Nile tilapia was 0.07mg/L (Table, 2). Fish showed abnormal swimming behavior, nervous manifestations, erected pectoral fin (Figure 1A) and erythema on skin with fin rot (Figure 1B) and asphyxia before death. The recorded clinical signs may be attributed to that CPF inhibit acetyl choline esterase by its binding capacity to the enzyme active site, that plays an important role in neurotransmission [5]. This enzyme makes rapid hydrolysis of neurotransmitter acetylcholine to choline and acetate 7. The inhibitory effects lead to nervous manifestations on fish. In another study, it is reported that the 96 h LC₅₀ of CPF in *Tilapia guineensis* was 0.002 mg/L, with increase in operculum beat frequency and tail beat frequencies by increase the exposure time and concentration [17]. In addition, Muttappa *et al.* [18] mentioned that the 96 h LC₅₀ for CPF in *O. mossambicus* was 0.022 ppm. The differences in the value of LC₅₀ may be attributed to species sensitivity, life stage and environmental factors.

Table 2: Actual estimation of 96 h mortalities LC₅₀ in Nile tilapia exposed to different levels of Chlorpyrifos

Groups (N=10)	Concentration (mg/L)	Number of dead fish at 96 hours	a	b	axb	Σaxb
1	0.02	0				
2	0.04	5	0.02	2.5	0.05	
3	0.06	6	0.02	5.5	0.11	
4	0.08	6	0.02	6	0.12	
5	0.1	6	0.02	6	0.12	
6	0.12	7	0.02	6.5	0.13	
7	0.14	10	0.02	8.5	0.17	
						0.7

96 h LC₅₀ =highest dose- Σaxb/n, the formula of calculation was: 96 h LC₅₀=highest dose- Σ axb/n (a=Constant factors between two successive dose, b=the mean of dead fish in each group, c=the number of fish in each group, Σaxb=sum of axb, n=Number of fish in each groups=0.14 0.7/10=0.07mg/L

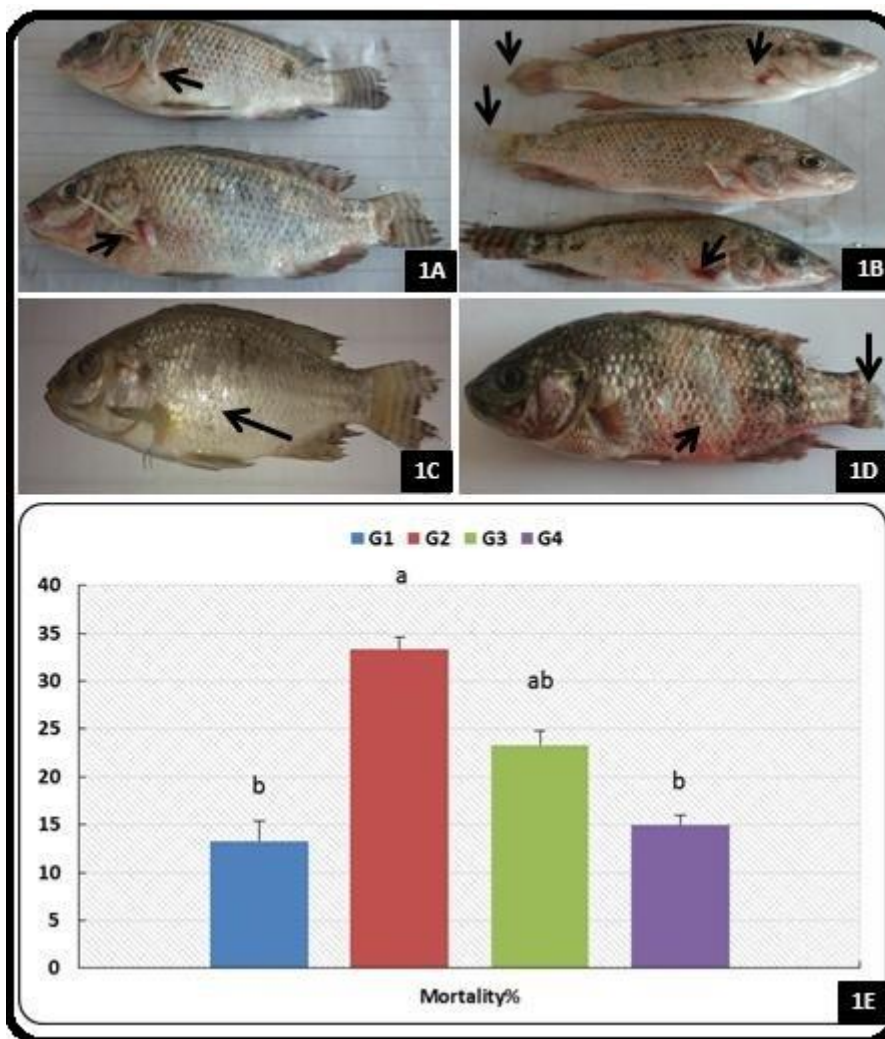


Figure 1: Clinical signs of *O. niloticus* exposed to A). Acute toxicity of Chlorpyrifos that showing pectoral fin erection and B). Fin rot and erythema. C). *O. niloticus* that exposed to 1/20 96 h LC₅₀ of Chlorpyrifos suffered from fin rot and increase of mucus secretions D). *O. niloticus* that exposed to 1/8 96 h LC₅₀ (0.008 mg/L) of Chlorpyrifos suffered from erythema on skin and fin rot. E). Mortality rate at the end of experimental period (30 days). The bars with different superscripts (a, b, and c) are significantly different.

The chronic toxicity effect of Chlorpyrifos on general health condition and non-specific immune parameters of *O. niloticus*

Fish exposed to 1/8 96 h LC₅₀ (0.008 mg/L) of CPF (group 2) showed significant high mortality rate with signs of erythema on skin and fin rot followed by group 3 (fish exposed to 1/20 96 h LC₅₀, 0.0035 mg/L of CPF) with fin rot and increased mucus secretions compared to control group (Figure

1C, 1D and 1E). Increased mortalities with increased concentrations of CPF was observed. Chindah *et al.* [17] who found that *Tilapia guineensis* exposed to sub lethal concentrations of CPF (0.0125 mg/L; 0.025 mg/L; 0.05 mg/L and 0.1 mg/L) showed increased mortality rates with elevated concentration level and exposure time obtained similar results.

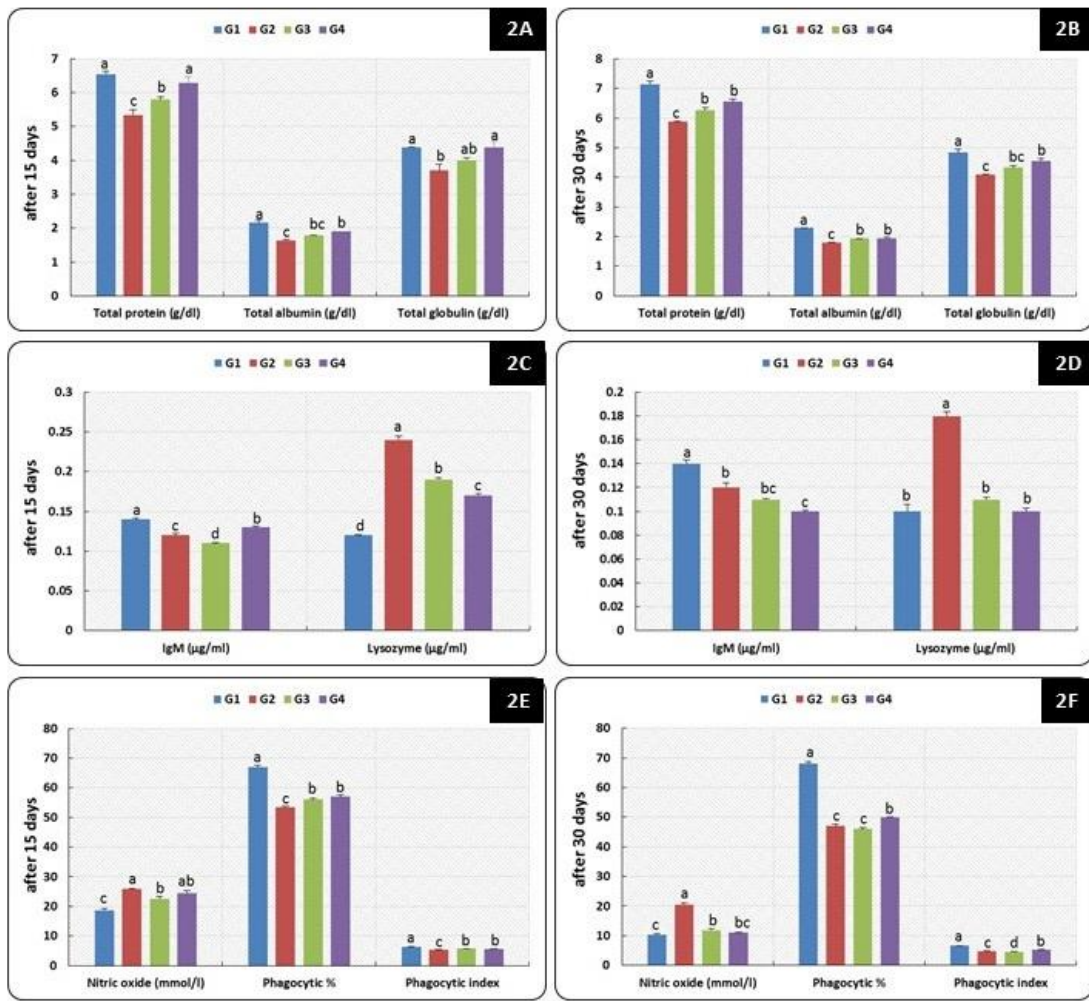


Figure 2: Effect of the different concentrations of Chlorpyrifos on A and B). Total serum protein (g/dl), albumin (g/dl) and globulin (g/dl) of *O. niloticus* after 15 and 30 days, respectively. C and D). IgM (µg/mL) and lysozyme activity (µg/ml) of *O. niloticus* after 15 and 30 days, respectively. E and F). Nitric oxide (mmol/L), phagocytic % and phagocytic index of *O. niloticus* after 15 and 30 days, respectively. The bars with different superscripts (a, b, c and d) are significantly different (P < 0.05).

The fish groups exposed to 1/8 and 1/20 LC₅₀ 96 h of CPF (groups 2 and 3) showed decreased levels of serum total proteins, albumin and globulin after 15 and 30 days of the experiment. While, group 4 (fish exposed to 1/43 96 h LC₅₀, 0.0016 mg/L of CPF) showed no significant differences in the levels of serum total proteins, globulin and decrease level of albumin compared with control after 15 days of the experiment. The same group showed diminished levels of total proteins, albumin and globulin after 30 days of the experiment (Figure 2A and 2B). Low values of total proteins may be attributed to inhibition of RNA synthesis by CPF leading to disturbing the protein metabolism and inhibition of hepatic metabolizing enzymes. Stress conditions due to pesticide pollution resulted in catabolism of the proteins to provide energy to withstand these stress conditions with degradation of proteins into free amino acids for different metabolic activities [19, 20]. These results are in agreement with those obtained by Ramesh and Sarvanan [21] in *C. carpio* exposed to CPF and results obtained by Bhanu, and Deepak [22] who reported a decrease in the serum protein in *C. carpio* exposed to sub lethal concentration of Cypermethrin for 28 days. The decreased serum albumin and globulin levels due to CPF exposure may be due to liver damage caused by this pesticide or to the decrease in protein synthesis in the liver [22].

The IgM level decreased after 15 and 30 days of the experiment in groups 2, 3 and 4 compared with the control group (Figure 2C and 2D). The IgM is the most important immunoglobulin in fish, which is altered by pesticide pollution; lowered levels of IgM may be attributed to the effect of CPF as a stress factor on fish leading to diminished levels of IgM. These results coincide with that recorded in plasma IgM level in *O. niloticus* and *C. carpio*, which were exposed to 0.051 mg/mL

and 75 µg/L of CPF, respectively [23, 24]. Concerning the level of lysozyme and nitric oxide, increased lysozyme level in groups 2, 3 and 4 after 15 days of the experiment compared with control group was observed (Figures 2C, 2D, 2E and 2F). After 30 days, the lysozyme level was significantly increased in groups 2 and 3, while there were no significant differences in group 4 compared to the control group. The lysozyme is one of the humoral innate immune factors; which is considered as a natural antibiotic of fish [25]. *O. niloticus* exposed to CPF at a rate of 0.102 and 0.255 mg/L revealed an increase in the activity of lysozyme level; but the lower concentration (0.051 mg/mL) of such pesticide did not produce any change on the lysozyme activity [26]. However, Wang *et al.* [27] reported that exposure of *C. carpio* to CPF (75 µg/L) decreased the levels of lysozyme in plasma and spleen.

Increased level of nitric oxide under the influence of CPF may be attributed to that; fish body produces protective mechanism against the stress of pesticide. This result is in agreement with that obtained by Díaz-Resendiz *et al.* [25] who reported increased production of nitric oxide significantly after exposure to pesticide. In addition, Xu *et al.* [26] reported that CPF (1.16, 11.6 and 116 µg/L) induce an increase in the level of nitric oxide in *C. carpio*. The phagocytic % and phagocytic index were significantly decreased in all experimental groups compared with control after 15 and 30 days of the experiment (Figure 2E and 2F). This may be attributed to that CPF suppress the action on fish phagocytic cells as the pollution by pesticide is considered to be abiotic stress factor. This result is nearly similar with that obtained by Girón-Pérez *et al.* [28] who reported that exposure of Nile tilapia to Diazinon decreased the phagocytic index of mononuclear cells.

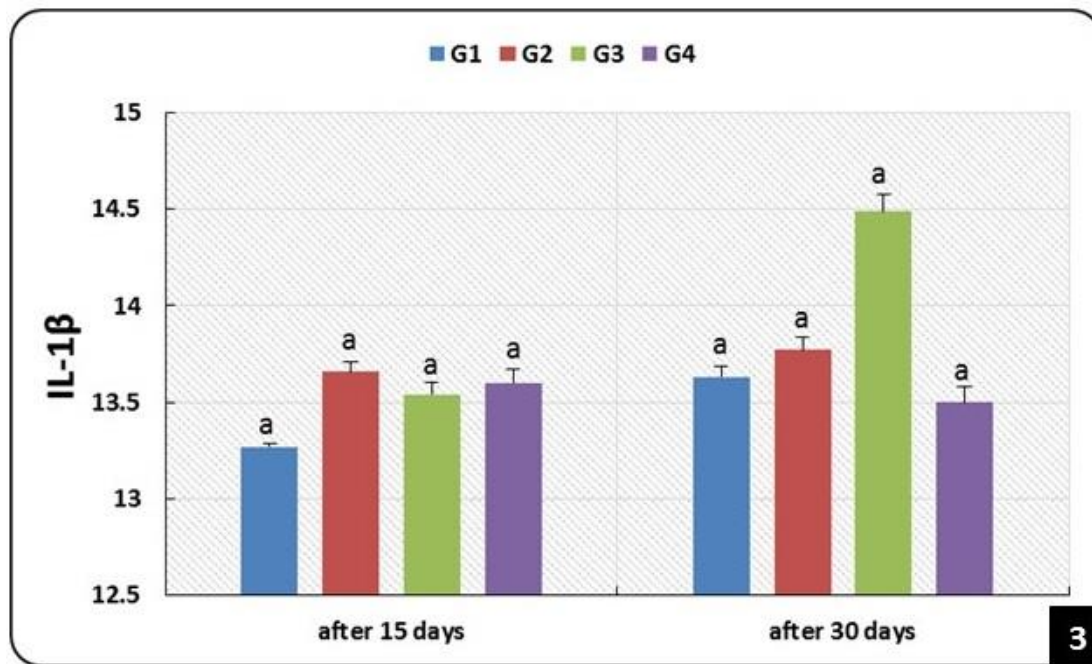


Figure 3: Effect of the different concentrations of Chlorpyrifos on IL-1 β gene expression of *O. niloticus* after 15 and 30 days (G: Group).

The mRNA expression level of the immune genes IL-1 β is shown in Figure 3, which indicated insignificant difference in the expression of IL-1 β between the experimental groups but with slight increase in G2 and G3 after 15 and 30 days of the experiment, respectively. The IL-1 β is one of the cytokines in fish that respond to water pollution by pesticide. Similar results were obtained by Wang *et al.* [29] who reported that exposure of *C. carpio* to CPF (1.16, 11.6 and 116 $\mu\text{g/L}$) for 24 h evoked increased expression of mRNA of IL-1 β .

Conclusion

It could be concluded that water of Abbassa and Sahl El-Hussinia fish farms have had detectable residue levels of CPF. This insecticide has a toxic effect and altered the immunological parameters of *O. niloticus*.

Conflict of interest

The authors declare no conflict of interest.

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الملخص العربي

تأثير مبيد "الكلوربيريفوس" على الاستجابة المناعية في اسماك البلطي النيلي.

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تهدف هذه الدراسة الي تقدير مستوي المبيد الفسفوري الكلوربيريفوس في مياه مزارع العباسة وسهل الحسنية المستخدمة في الاستزراع السمكي كذلك معرفة تأثير التركيز تحت المميت لهذا المبيد الحشري على الاستجابة المناعية لأسماك البلطي النيلي. تم تجميع عينات مياه من مزارع العباسة وسهل الحسنية وتحليلها بواسطة جهاز الجاز كروماتوجراف. تم استخدام مائتين وتسعون سمكة بلطي نيلي بمتوسط وزن ٣٥ جرام من مفرخ العباسة بمحافظة الشرفية، مصر. تم استخدام مائه وسبعون سمكة في تحديد نصف الجرعه المميتة لمبيد الكلوربيريفوس ومائه وعشرون سمكة في معرفة تأثير الجرعات تحت المميتة لهذا المبيد (٨/١، ٢٠/١ و ٤٣/١ من الجرعة نصف المميتة لمدة ٩٦ ساعة) على الاستجابة المناعية لأسماك البلطي النيلي. وجد ان مستوي مبيد الكلوربيريفوس في مياه العباسة وسهل الحسنية هي ٠.٠٠٠٨ و ٠.٠٠١٦ مللي جرام/لتر على التوالي، وان الجرعة النصف مميتة لمبيد الكلوربيريفوس لمدة ٩٦ ساعة كانت ٠.٠٧ مللي جرام/لتر. جرعات الكلوربيريفوس تحت المميتة ادت الي تغيرات معنويه في المعايير المناعية غير المتخصصة لأسماك البلطي النيلي (نسبة الجلوبيولين، الاميونوجلوبيولين م، الليزوزيم، اكسيد النيترات، القدرة على الالتهام) والتعبير الجيني لحجين الانترليوكين ١ بيتا. وفي النهاية يتضح ان مياه المزارع السمكية بكل من العباسة وسهل الحسنية تحتوي على متبقيات من مبيد الكلوربيريفوس والذي له أثر في تغيير الحالة المناعية لأسماك البلطي النيلي.