Research Article

Bioaccumulation of different concentrations of Butachlor in the Zebrafish (*Danio rerio*)

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Abstract

The herbicides used in agriculture threaten aquatic ecosystems and biodiversity on a global scale. There are several reports on the residues of currently used herbicides in the tissues of aquatic organisms. This study aimed to determine the effect of concentration on the accumulation of Butachlor in liver tissue in zebrafish fish exposed to sub-lethal concentrations of the toxin. In this study, we investigated the losses and residual toxins in the water and the liver of the zebrafish exposed to sub-lethal concentrations of Butachlor herbicide. This study was performed on 680 zebrafish. Initially, to determine the LC_{50}-96 h value, a preliminary pilot study was performed, according to which, the main experiment was then conducted considering four treatments each with three replicates for 30 days. The experimental groups included T1 (exposure to 40% of the LC_{50}-96 h concentration of Butachlor), T2 (exposure to 60% of LC_{50}-96 h), T3 (exposure to 80% of LC_{50}-96 h), and control (C). Mortality was recorded daily. Sampling was conducted from water and liver tissues on the first day and the days 15 and 30 to determine Butachlor residues using gas chromatography (GC). The results showed that Butachlor residue in water was associated with the concentration of the toxin and the exposure time, (T3>T2>T1 groups; $p < 0.05$). According to the results, the highest mortality and residual Butachlor in the liver tissue were related to the T2>T3>T1> C groups, respectively, on days 15 and 30 after exposure ($p < 0.05$). According to the results of this study, Butachlor herbicide can accumulate in liver tissues of zebrafish even when it is used in low concentrations. Also the behavioral and clinical features following Butachlor use included restlessness, rapid respiration, air swallowing at the surface of the water, loss of balance, and disoriented swimming was observed. Regarding the importance of fish as the protein source in humans' food, this phenomenon can be a potential threat to human health. Therefore, it is necessary to reduce the application of this toxin and replace it with alternative compounds.

Keywords: Butachlor, Herbicide, Zebrafish, Bioaccumulation

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Introduction

Agricultural toxins like pesticides are known as one of the main water pollutants around the world (Naveed *et al*., 2011) The use of chemicals such as herbicides, pesticides, and insecticides in modern agricultureis inevitable for pest control, high yield, and public health management, especially in developing countries (Bhaskara Tataji and Vijaya Kumar, 2016). This has led to a remarkable surge in the use of synthetic chemicals to eliminate weeds and pests for the last two decades. As a consequence, these compounds accumulate in agricultural products, running and groundwater resources, and soil (Ghaffar *et al.*, 2015).

Butachlor is among the first herbicides introduced to be utilized in rice fields. It has the chemical formula of 2-Chloro-2',6'-diethyl-N-(butoxymethyl) acetanilide (Tilak *et al.*, 2007). This herbicide is used in rice fields to control perennial and some broadleaf weeds (Ateeq *et al.*, 2002), and according to available reports, the annual use of this toxin in Asia has been estimated about 4.5×10^7 kg (Abigail *et al.*, 2015). In this regard, several reports are indicating the presence of residual Butachlor in soil, groundwater, and surface water at concentrations ranging from 0.1 to 1.4 µg/L) (Mamun *et al.*, 2009; Shi *et al.*, 2011; Van Toan *et al.*, 2013).

This is important considering that even at sublethal concentrations, Butachlor can cause disorders such as respiratory distress and tissue poisoning and accumulation, affecting cellular biochemical pathways and ultimately inhibiting the function or reducing the level of Acetylcholinesterase (Tilak *et al.*, 2007). Studies have been conducted on Butachlor poisoning in fish and other aquatic organisms, including the studies of (Geng *et al.*, 2005) and Geng *et al.* (2010) on *Rana zhenhaiensis*, Ateeq *et al.* (2006) on *Clarias batrachus*, Peebua *et al.* (2007) and Nwani *et al.* (2013) on *Oreochromis niloticus* and *Tilapia zillii*, and finally Chang *et al.* (2013) on zebrafish (*Danio rerio*).

One of the animal models used in studies on environmental pollutants is zebrafish (*Danio rerio*), which is used as a vertebrate model in studying the toxicity of various chemical compounds, including herbicides (Chang *et al.*, 2013; Hill *et al.*, 2005; Huang *et al.*, 2020; Sipes *et al.*, 2011). Zebrafish have several characteristics, including small size, extra uterine embryo development, short reproductive cycle, and transparent embryos, delivering it a valuable laboratory model in this field (Feitsma and Cuppen, 2008).

Regarding the harmful effects of herbicides on growth and physiological changes in aquatic animals (Hedayati and Gerami, 2014; Vajargah and Hedayati, 2017) and the availability of simple and accurate bioassay methods for evaluating acute and chronic poisoning, we aimed to assess the effects of sub lethal concentrations of Butachlor on the survival of zebrafish and measure residuals of the toxin in the water and tissues of the fish.

Materials and methods

Experimental animal and culture condition

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In this study, 680 adult zebrafish with the mean weight of 0.25±0.05 g and the mean length of 2.5±0.05 cm were prepared from the Razef Ornamental Fish Breeding Center (Tehran-Iran), affiliated with the Faculty of Veterinary Medicine, Tehran University of Science and Research, Tehran, Iran. For adaptation, the newly purchased fish were kept under laboratory conditions in 120 L aquariums at the ambient temperature of 25±1°C and the light cycle of 12-hour light/12-hour dark for two weeks before the onset of the experiment. During the adaptation, the fish were fed twice daily based on 2% of the body weight (BW) by commercial feed (Crude protein 28%, crude fat 3%, crude fiber 4%, and moisture 10%). Thirty percent of water exchange was applied daily.

Determining Butachlor lethal concentration

Butachlor, under the brand name of Macheti (60% purity), was purchased from Ariya Shimi Chemicals and Agricultural Materials Co. (Tehran, Iran). Acute toxicity assay to determine the LC_{50}96 h values of Butachlor was conducted with a definitive test in a semi-static system in the laboratory as per the standard methods (Federation and Association, 2005). To determine the LC50 of this herbicide the total count of 80 adult zebrafish was randomly divided into four treatments (three experimental and control) each with two replicates (10 fish per group) and hosted in glass tanks containing 67 liters of chlorine-free and aerated tap water. These groups were then exposed to different concentrations of Butachlor (0, 0.05, 0.5, 0.1, and 0.10 mL) for 96 hours. Losses were recorded daily and were reported at the end of 96 hours. The LC50 of Butachlor was determined following the probit analysis method described by (Finney, 1971). During the experiment, 70% of the water was replaced daily, and the desired Butachlor concentration was adjusted each time. During this study, the behavioral changes of the fish were screened; dead fish were quickly removed from the tank, and the number of losses was recorded daily.

Experimental design

At this step, 600 zebrafish were randomly allocated to four groups (i.e., T1, T2, T3, and control (C) each with three replicates) in twelve tanks (Contains 120 liters of water), 50 fish per tank. In this regard, T1, T2, and T3 experimental groups were exposed to 40%, 60%, and 80% of the LC_{50}-96 h concentration of Butachlor herbicide, for 30 days respectively. The control (C) was not exposed to Butachlor. During the experiment, 70% of the water of each tank was changed daily, and the desired concentration of Butachlor was readjusted. During the study, the behavioral changes compared with the control group (including changes in activity, swimming behavior, food intake and opercular movement) of the fish were assessed; dead fish were removed immediately, and recorded daily. To determine the residual of Butachlor in the water and fish tissues, sampling was performed on days 0, 15, and 30 after exposure to the toxin.

Measuring Butachlor residues in water

The measurement of residuals of Butachlor in water was performed according to the instructions provided by the Environment

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Protection Organization of Iran for measuring chlorine toxins in the water. To extract the organic phase, 1 liter of the sample water was initially added to 80mL of normal hexane and 1mL of a solution containing PCB-29, PCB 198, ε-HCH, and endosulfan Id4 (with the concentration of 20 ng/mL), as the internal standard, in the decanter. The mixture was shaken on three occasions (one minute each time) until the formation of two-phase. The lower phase was transferred to another decanter and again shaken after being mixed with 80mL of normal hexane to form a new biphasic (organic and aqueous) solution. After calculating the efficiency of the extraction process, in order to concentrate the samples, their volume was reduced to 10-15 mL by being placed on a rotary evaporator at a speed of 90 r/min and below 30°C temperature. This volume was further reduced to 1 mL using nitrogen gas. A fluorouracil column was used to separate the F1, F2, and F3 fractions. Finally, to re-concentrate the samples, their volume was initially reached 10-15 mL on a rotary evaporator and then 1 mL using nitrogen gas. Finally, 1 µL of the concentrated fractions of F1, F2, and F3 was injected into the Gas Chromatography–Electron Capture Detector (GC-ECD) device for residual Butachlor analysis (Barnhoorn and van Dyk, 2020).

Measuring bioaccumulation of Butachlor in tissues

Butachlor residues in liver tissues were measured according to the instructions provided by the Environment Protection Organization of Iran for measuring chlorine toxins in Biota samples. First, liver tissue samples were dried in a freeze-drier, and the frozen samples were homogenized in an electric mixer for two minutes. Then the samples were weighed and poured into a thimble, and then 1mL of a solution containing polychlorinated biphenyls-29 (PCB29), polychlorinated biphenyls-198 (PCB198), Hexachlorocyclohexane (ε-HCH), and endosulfan Id4 (with the concentration of 20 ng/mL), as the internal standard, was added. The extraction was performed utilizing 250 mL of the hexane solvent and a number of boiling stones for eight hours. Then the extraction output was calculated, and the lipid content of the samples was determined in a10-µL volumeapplying the hot-plate method. Then the sample was poured into a 500 mL decanter funnel, and 40-50 mL hexane was added to dilute it. Next, the hexane phase was separated, and to prevent fat contamination of the hexane phase, it was mixed with sodium sulfate. To concentrate the samples, the volume of the resulting organic phase was initially reached 10-15 mL on a rotary evaporator and then 1mLusing nitrogen gas. The fractions of F1, F2, and F3 were separated using a fluorouracil column. Finally, the samples were re-concentrated by reducing their volume to 10-15 mLon a rotary evaporator and then to 1 mL with nitrogen gas. In the end, 1 µL of the concentrated fractions of F1, F2, and F3 was injected into the GC/ECD device for residual Butachlor analysis (Barnhoorn and van Dyk, 2020).

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Statistical analysis

The LC50 of Butachlor was determined following the probit analysis method. Data analysis was conducted in SPSS software version 18 using the one-way analysis of variance (ANOVA) and the LSD test to determine any significant difference in the means of quantitative variables between the study groups. The results were expressed as mean ±standard deviation, and a P-value of <0.05 was considered as an indicator of a statistically significant observation.

Results

Butachlor lethal concentration and behavioral responses

According to the results of daily losses of the fish, the LC_{50}-96h value of Butachlor was calculated as 0.05 mL in 67 L of water, as determined after being exposed to the different concentrations of the toxin (i.e., 40, 60, and 80%) (Table 1). The behavioral and clinical features following Butachlor treatment included restlessness, rapid respiration, air swallowing at the surface of the water, loss of balance, and disoriented swimming.

Table 1. The survival of the zebrafish exposed to different concentrations of Butachlor

Exposed concentration (mL)	Number of fish	Number of fish alive at different time intervals (hours)				Mortality (%)
		24	48	72	96	
0.05	20	18±1.41a[b]	20±2.82[a]	18±1.41[a]	8±141[a]	50
0. 1	20	20±1.41[a]	13±0.00[b]	11±1.41[b]	8±0.00[a]	75
0.15	20	17±0.00b[c]	13±141[b]	8±0.00[c]	6±0.00[a]	70
0.02	20	15±0.00[c]	8±0.00[c]	7±0.00[c]	1±0.00[b]	90

Behavioral responses and losses after exposure to sub-lethal concentrations of Butachlor

The death toll of the fish exposed to Butachlor toxin on days 15th and 30th is presented in Figure 1. According to the results, the mortality rate was directly related to the exposure time, but not to the concentration of the toxin. The highest mortality rate was observed in the T2 group ($p<0.05$).

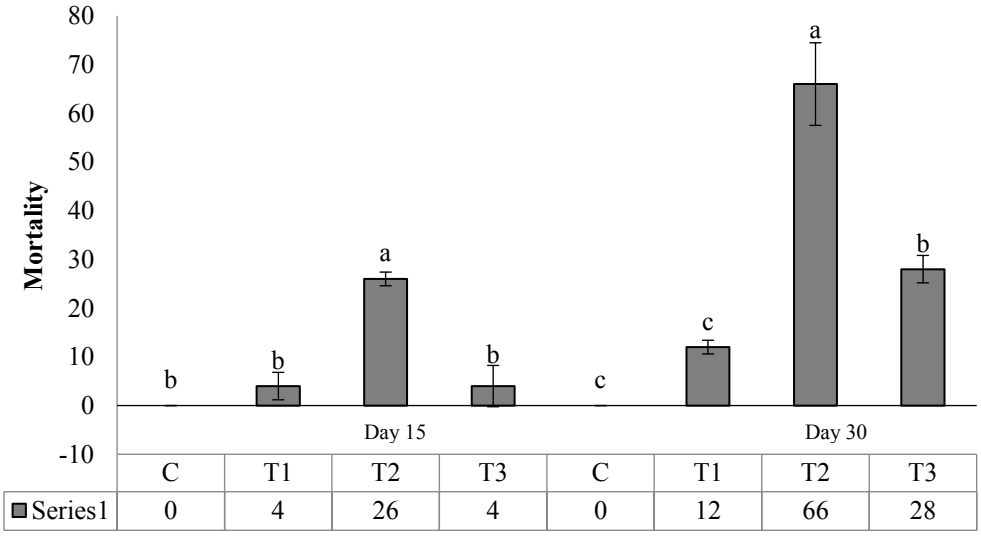

Figure 1. Zebrafish mortality on different days after exposure to Butachlor.

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Butachlor residue in water

As shown in Figure 2, Butachlor was not detected in the tanks' water on day 0, but its concentration gradually increased on days 15 and 30. Based on these results, on day 15, Butachlor remnants increased in water in a dose-dependent manner so that the highest increase was seen in the T3 group ($p < 0.05$). On day 30, Butachlor level in water increased dose-dependently in all groups, with the highest increase being related to the T3 group ($p < 0.05$).

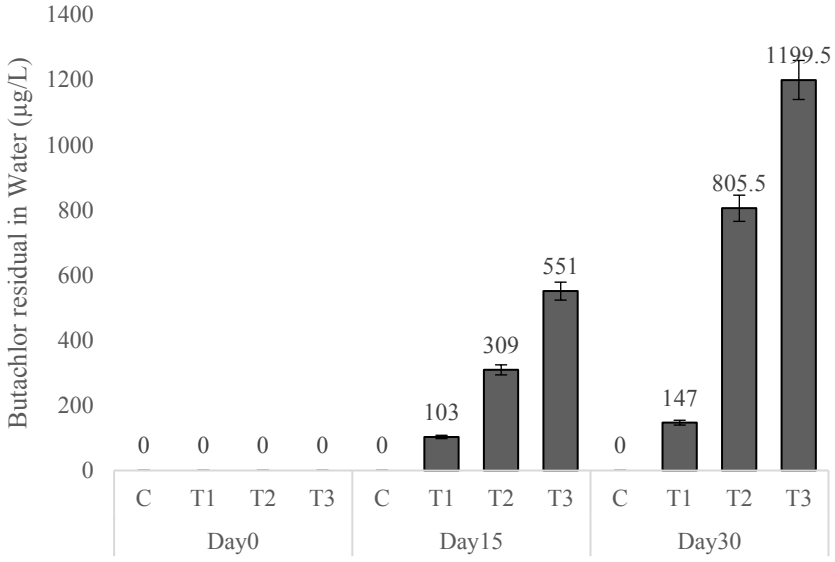

Figure 2. Butachlor residual in water (µg/L) on different days of exposure in different groups.

Butachlor bioaccumulation in liver tissue

Butachlor bioaccumulation in zebrafish liver is presented in Figure 3. Accordingly, Butachlor was not detected in the liver on day 0. However, in the experimental groups exposed to Butachlor, the highest and lowest levels of the toxin were observed in the T2 and T1 groups, respectively ($p < 0.05$).

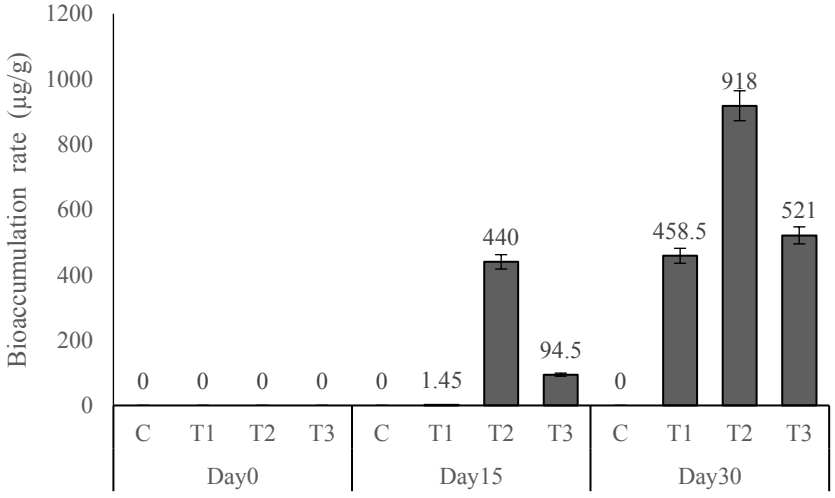

Figure 3. Bioaccumulation of Butachlor in zebrafish liver tissue (µg/g of tissue weight) on different days.

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Discussion

Population growth and increasing demand for food have led to the expansion of agriculture and subsequently a rise in the use of pesticides, leading to the contamination of water with these pollutants. The acute and chronic toxicities of the pesticides used in agriculture are widely investigated in experiments on non-target organisms (Santos *et al.*, 2010), and fish are among the most important non-target organisms to assess contamination with these toxins, including environmental pollution with herbicides (Reindl *et al.*, 2015). Fish are exposed to these toxins through gills, skin and by feeding on contaminated sources (Clasen *et al.*, 2018).

In the present study, no clinical symptoms were observed in the fish exposed to sub-lethal concentrations of Butachlor; however, in the zebrafish acutely exposed to Butachlor (during the experiment for determining the LC_{50}-96 h value), symptoms such as restlessness, rapid respiration, air swallowing at the surface of the water, loss of balance, and disoriented swimming were observed, which are similar to the symptoms reported by Ghaffar *et al.* (2015) in the *Labeo rohita* fish exposed to Butachlor. Respiratory symptoms in the fish exposed to Butachlor have also been reported by other researchers, such as Guo *et al.* (2010) in *Pleuronectiformes* and Nwani *et al.* (2010) in *Tilapia zillii*. Moreover, irregular swimming patterns after Butachlor poisoning were described in rainbow trout in a study by Altinok *et al.* (2012).

According to our results, exposure to lethal (the preliminary pilot experiment) and sub-lethal doses of Butachlor herbicide increased the mortality rate in fish, which was zero in the control group. This was attributable to the accumulation of Butachlor in the fish bodies, resulting in biochemical and pathological impairments and ultimately death, even at sub-lethal concentrations (Tilak *et al.*, 2007).

In the present study, the addition of even small amounts (Concentration Please) of Butachlor led to a significant increase in the accumulation of the toxin in the water. In this regard, several studies have been dedicated to detect Butachlor in water (Mamun *et al.*, 2009; Shi *et al.*, 2011; Van Toan *et al.*, 2013). In fact, the ability of pesticides to accumulate and persist in water resources has led them to be regarded as the main water pollutants worldwide (Naveed *et al.*, 2011).

Regarding Butachlor residue in liver tissue, the results of the present study showed that prolonged exposure to the lethal concentrations of Butachlor increased its accumulation in the liver tissue of the fish. Consistently, pesticides residues have been detected in the fish body, those bred in the vicinity of rice fields (Clasen *et al.*, 2018; Teng *et al.*, 2013; Zhang *et al.*, 2016). In line with our results, Rossi *et al.* (2020) reported that the accumulation of herbicides in fish tissues was directly associated with the duration of exposure.

The results showed that Butachlor bioaccumulation in liver tissue is not

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dependent on applied concentration. In other words, an increase in the concentration of the toxin did not lead to a risein toxin concentration in the liver tissue. The highest amount of Butachlor bioaccumulation in the liver tissue was observed in its middle concentration (i.e., the T2 group). This finding was consistent with the results of Palaniappan and Karthikeyan (2009), showing higher bioaccumulation of chromium in the kidneys tissue of *Cirrhinus mrigala* fish when exposed to the lower concentration of toxin. In this regard, the higher bioaccumulation of Butachlor in the fish of the T2 group is accompanied by a higher mortality rate in this group (compared to the T3 group) due to greater tissue damages. Consistent with our findings, Jin *et al.* (2010) in their study observed dose-independent tissue damage in the zebrafish exposed to atrazine, suggesting that a higher concentration of the toxin is not necessarily associated with greater tissue damage.

In general, the reason why Butachlor is accumulated in fish, making this organism a powerful indicator of environmental pollution, is the lack of the carboxylesterase enzyme, reducing the catabolism and elimination of pesticides in fish. Therefore, toxins remain in fish and are detectable for a longer time (Rossi *et al.*, 2020). However, according to existingevidence, the toxicity of chemicals to aquatic organisms is influenced by temperature, pH, dissolved oxygen content, fish size, age, and species, water quality, and the concentration and formulation of chemicals (Gupta *et al.*, 1981; Nwani *et al.*, 2010). Further, factors such as different absorption rates, tissue fat content, and toxins' chemical structure, solubility, and metabolic patternsaffect the amount of their residues in the body (Tilak *et al.*, 2007). Considering that in our study all the physical and chemical factors of water and the concentration and formulation of the toxin were the same in the experimental groups, the higher bioaccumulation of Butachlor in the T2 group compared to the T3 group could not be related to the above-mentioned parameters. Nevertheless, factors such as age, sex, and size of the fish could have had impacts on residual Butachlor to some extent; any great impact by these factors can also be ruled out due to the considerable and statistically significant differences observed on different days between the studied groups.

In conclusion in our study it is observed that despite the fact of lower concentration of Butachlor in T2 group, higher bioaccumulation of toxin in the fishes was reported. Similar results have been reported for different pollutants (Graney Jr *et al.*, 1984; Kazempoor *et al.*, 2021; Pérez-Parada *et al.*, 2018). However, the authors recommend conducting further studies to scrutinize the role of immune responses in the accumulation of Butachlor in the fish exposed to the sub-lethal concentrations of this toxin.

The results of our study indicated that the Butachlor herbicide could cause behavioral change, bioaccumulation in liver tissue and cause mortality, even when used in low concentrations. As a result, it disrupts the physiological and biochemical functions of fish. Based on the results of this study, it is

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highly advisable to limit the use of Butachlor in the environment, especially near water resources, to avoid its serious adverse effects on our health.

Conflict of interest

Authors have no conflict of interest on this work.

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