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Original Article

Acute Toxicity of Herbicides on the Survival of Adult Shrimp, *Artemia Franciscana*

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ABSTRACT

Background: Herbicides are widely used in agriculture to kill a large variety of unwanted weeds. However, the application in water ecosystems may pose harmful impacts on the health of aquatic organisms. We studied the acute toxicity of four major types of herbicides on the survival of adult shrimp, *Artemia franciscana* (AF).

Methods: The brine shrimp, AF, was hatched from the commercially encysted dry eggs. The acute toxicity (LC50; 48 hr) of four herbicides, paraquat; 2,4-dichlorophenoxy acetic acid (2,4-DCPA); trifluralin; and glyphosate, was examined by a standard method. We exposed the shrimps to sequentially rising concentrations of each herbicide in triplicate. The mortalities were recorded at 12, 24, 36, and 48 hours after exposure and the LC50 was calculated, using a Probit software.

Results: This study demonstrated that the acute toxicity of these herbicides was significantly different in adult shrimp AF. The lethal concentrations (LC50) of Paraquat, 2,4-DCPA, trifluralin and glyphosate against the shrimp were 2.701, 14.475, 0.446 and 17.431 mg/l, respectively. Trifluralin and paraquat caused the highest lethality at lower LC50 concentration compared to the other two herbicides.

Conclusion: The results demonstrated that increasing herbicides concentration or duration of exposure raised the mortality rate of AF's.

Keyword: Acute Toxicity, Adult Shrimp, Artemiafranciscana, Herbicides.

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INTRODUCION

Pollutants in aquatic ecosystems at concentrations as low as few nanograms can cause damage to living organisms and alter their relationship with the environment. It is important to use sensitive markers to assess the environmental risks associated with pesticide application [1-2].

The brine shrimps, *Artemia franciscana* (AF), are branchiopod crustaceans that inhabit salty water lakes (4–250 g/l) around the world, both inland and on the coast. They are also characterized by such features as temperature tolerance (6-35 °C), short life cycle and high adaptability under adverse environmental conditions [1, 2]. This shrimp has been used in the assessment of the pollution of marine life and saline waters. Various strains and species of AF demonstrate different sensitivity to environmental toxins at different developmental stages [1-2]. The AF's are the main aquatic animals in salty waters and have long been considered as suitable experimental organisms for environmental studies due to the ease of culture, short generation time, and availability of their dormant eggs or cysts for research [3, 4].

In an effort to eradicate weeds, the use of herbicides in modern agriculture has led to serious environmental contaminations, leading to loss or retarded growth and development of many vital aquatic organisms. Herbicides can enter the aquatic ecosystems as a result of terrestrial runoffs, and to a lesser extent, of direct application and aerial spraying [5]. The microbial organisms in freshwaters are directly or indirectly affected by such toxic compounds as glyphosate, paraquat, trifluralin and 2,4-DCPA, the most non-selective herbicides commonly used in agriculture.

Glyphosate is a non-selective herbicide that is generally used in agriculture for the control or destruction of herbaceous plants by altering their protein synthesis [6]. Also, this herbicide is used in fish-ponds, lakes, canals and slow running waters [7]. Due to changes in metabolic, oxidative and hematological factors, glyphosate is able to change the biological stability and cause damage to non-target organisms [6, 7].

Paraquat (1,1-dimethyl-4,4-bipyridininumion) is one of the most common and non-selective herbicides used for eliminating vegetative pests, controlling weeds and aquatic plants, and its presence has been detected in various water sources worldwide [8]. Water contamination with paraquat is harmful to the health of aquatic organisms as it produces superoxide anion that can directly or indirectly cause cell death [8].

2,4-DCPA is an expansive, systemic and phenoxy compound used as the active ingredient of some aquatic herbicides. It is highly water soluble and has low binding affinity to soil, which facilitates its transport to water sources. It is used in ester, acidic or amine salt form universally, but in aqueous environments, it is

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usually found in its free anion form. Degradation of 2,4-DCPA requires microbial presence, light, oxygen and proper pH level. For example, in water, its moieties have a half-life ranging from 15 to 333 days depending on being in an aerobic or anaerobic aquatic system [9].

Trifluralin, a dinitroaniline compound, is broadly used as a pre-emergence herbicide that is almost not harmful to mammals but can be extremely toxic to aquatic organisms. The use of trifluralin has been forbidden in the European Union since 2007 (decision 2007/629/EC), because of inadequate research on its aquatic risks, the metabolites toxicity, and the risks to the consumer [10]. However, trifluralin is broadly used in other countries, such as the U.S. and Asia. Indeed, trifluralin was a candidate compound that had been screened for efficacy against pathogenic aquatic fungi and, hence, a possible replacement for malachite green, which is a controversial antimicrobial agent in aquaculture, but a potential carcinogenic compound [10].

Bioassay techniques have been the basis of environmental security and chemicals safety programs [11]. Over the last decade, uses of aquatic bioassays have been extended from water pollution control to the determination of potential toxins to aquatic life. The harmful effect of toxins on aquatic organisms, mostly of freshwater origin, can be tested using bio-indicators and experimental aquatic species, such as AF's [12, 13, 14, 15]. However, limited efforts have been made for using seawater animals as test organisms, and this may well be important for the evaluation of environmental adverse effects on water sources and ponds with high salt contents. Based on our previous observations, we hypothesized that trifluralin and paraquat were more toxic to adult AF's than the other two herbicides. The purpose of this study was to test our hypothesis and to carefully compare the lethal effect of the four generic herbicides: paraquat, 2,4-DCPA, trifluralin and glyphosate on adult shrimp, AF.

MATERIALS AND METHODS

The adult marine AF's (Inve Ltd. Co., Thailand) were hatched from commercially available cysts. They were incubated in artificial seawater at a salt content level of 35 parts per thousand (ppt), 27ºC and pH 8.5 [16]. The first nauplii (crustacean larvae) appeared after 24 hours of incubation at 25ºC under aeration and illumination of around 2000 lux. They were transferred into new seawater tanks and incubated for 24 hr under similar conditions. Then, they were transferred into a multi-well test plates with the respective concentrations of the herbicides diluted in artificial seawater. The toxicity of the herbicides on adult AF's was tested at 12, 24, 36 and 48 hour of exposure at 25ºC. The lethal dose (LC 50) in AF's was determined for each herbicide at selected concentrations and after 12, 24, 36 and 48 hr of exposure [17]. The LC50 was determined by exposing 30 adults AF's per group in triplicates for different herbicides in 5 ml of water for 48 hours. The control group was kept in experimental water samples without herbicides, but with all other conditions kept identical. The adult AF's were then exposed to sequentially rising concentrations (4-6) of each herbicide in triplicates to measure the mortality rates (0-100%). The mortality rate was recorded every 6 hours up to 48 hours. The herbicide concentration to induce 10, 15, 50, 85, 95 or 100 percent mortality was estimated, using Probit software version 1.5 (Environmental Protection Agency, Washington, DC, USA) based on a regression analysis. The LC estimations provided the lower and upper range values at 95% confidence level [18]. The selected herbicide concentrations representing their acute toxicity in AF's are shown in Table 1.

Table 1. Experimental design: Concentration range tested on the adult *A. franciscana.*

Tested herbicides	Concentration range (mg/L)	Number of treatments	Number of replicating	Total exposed Artemia
Paraquat	0.6, 1.25, 2.5, 5, 10, 20, 40	7	3	30
2, 4-DCPA	0.3, 0.6, 2.5, 5, 25, 50, 100, 200	8	3	30
Trifluralin	0.03, 0.125, 0.25, 0.5, 1, 10	6	3	30
Glyphosate	2.5, 5, 10, 25, 50, 100	6	3	30

RESULTS

In this study, 2, 4-DCPA was shown to be the most toxic herbicide toward adults AF's.

LC50 values of paraquat, 2,4-DCPA, trifluralin and glyphosate for AF species are summarized in Table 2. Further, AF's demonstrated a relatively high sensitivity to trifluralin. The AF's mortality rate following exposure to increasing concentrations of the herbicides over 12, 24, 36, and 48 hours, showed that the higher the concentration, the greater the AF mortality rate (Fig. 2). The mortality rate also increased with rising the exposure time. The acute toxicity of paraquat, 2,4-DCPA, trifluralin and glyphosate was determined in AF's after 12, 24, 36 and 48 hours of exposure. The LC50 values at 48 hours were 2.701, 14.475, 0.446 and 17.431 mg/l of the above herbicides, respectively (Table 2). The maximum allowable concentration (MAC) of these herbicides was 0.27, 1.44, 0.044 and 1.74 mg/l, respectively. The maximum and minimum LC50 values were 17.43 mg/l and 0.45 mg/l for glyphosate and trifluralin, respectively. The herbicides toxicity toward AF's in decreasing order were trifluralin > paraquat > 2, 4-DCPA > glyphosate, respectively (Table 3). Trifluralin and paraquat produced the highest lethal effect, i.e., lowest LC50 concentration, in AF's among the herbicides (Figures 1 & 3). The 2, 4-DCPA showed significantly lower lethal effects (highest LC50) than all the other herbicides tested.

Table 2. Specifications of the survey herbicides.

Herbicide	Chemical Name	Supplier	Puriy Rate	Acute Oral LD50 For Rat	Statue
Paraquat	1,1-dimethyl-4,4‘-bipyridinium	Aria Shimi Co, Iran	20%	129-157 MG/KG	Water soluble green liquid
2, 4-DCPA	2-(2,4-dichlorophenoxy)-acetic acid	Shimagro Co, Iran	67.5%	2100 MG/KG	Water-soluble brown liquid
Trifluralin	α-trifluoro-2,6-dinitro-(N,N-dipropyl-p-toluidine),	Aria Shimi Co, Iran	48%	5000 MG/KG	Water soluble orange liquid
Glyphosate	N- phosphonomethyl glycine	Sinochem Co, China	41%	5600 MG/KG	Water-soluble yellow liquid

Table 3. lethal concentrations of herbicides (mg/l) (95% confidence intervals) depending on exposure time for *A. fransiscana*.

Type of Toxicant	**Exposure time (h)**				
	lethal concentration (mg/L)	**12 h**	**24 h**	**36 h**	**48 h**
Paraquat	**LC_{10}**	6.091	2.787	1.225	**0.957**
	LC_{15}	7.675	3.781	1.514	**1.167**
	LC_{50}	20.387	13.731	3.707	**2.701**
	LC_{85}	54.155	49.867	9.076	**6.253**
	LC_{95}	96.098	106.321	15.353	**10.236**
	LC_{100}	182.683	248.263	27.662	**17.776**
2,4-D	**LC_{10}**	42.234	35.699	2.584	**2.544**
	LC_{15}	48.824	40.975	3.661	**3.548**
	LC_{50}	90.142	73.384	15.970	**14.475**
	LC_{85}	166.424	131.428	69.656	**59..053**
	LC_{95}	238.527	185.037	165.373	**134.803**
	LC_{100}	356.971	271.439	435.582	**339.789**
Trifluralin	**LC_{10}**	0.478	0.109	0.090	**0.091**
	LC_{15}	0.530	0.151	0.125	**0.124**
	LC_{50}	0.821	0.604	0.502	**0.446**
	LC_{85}	1.273	2.409	2.020	**1.605**
	LC_{95}	1.646	5.427	4.572	**3.405**
	LC_{100}	2.195	13.475	11.416	**7.907**
Glyphosite	**LC_{10}**	15.031	8.014	6.583	**5.373**
	LC_{15}	18.029	10.359	8.053	**6.729**
	LC_{50}	38.897	30.671	18.881	**17.431**
	LC_{85}	83.918	90.808	44.269	**45.154**
	LC_{95}	131.794	171.731	73.003	**78.950**
	LC_{100}	**218.513**	**350.599**	**127.844**	**147.624**

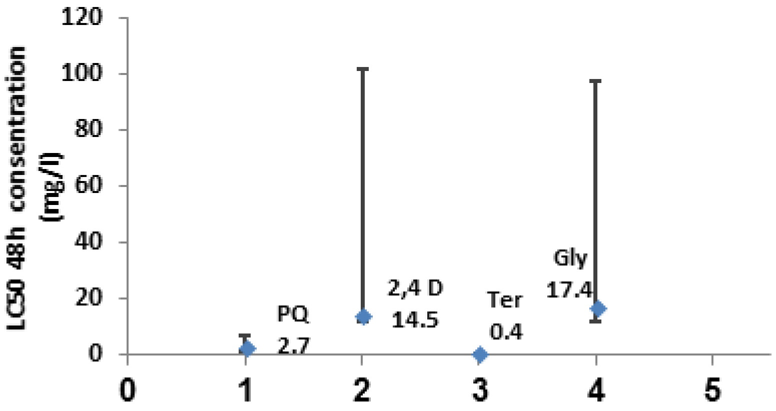

Figure 1. Comparison of LC50 48h concentrations of selected herbicides in *A. franciscana.*

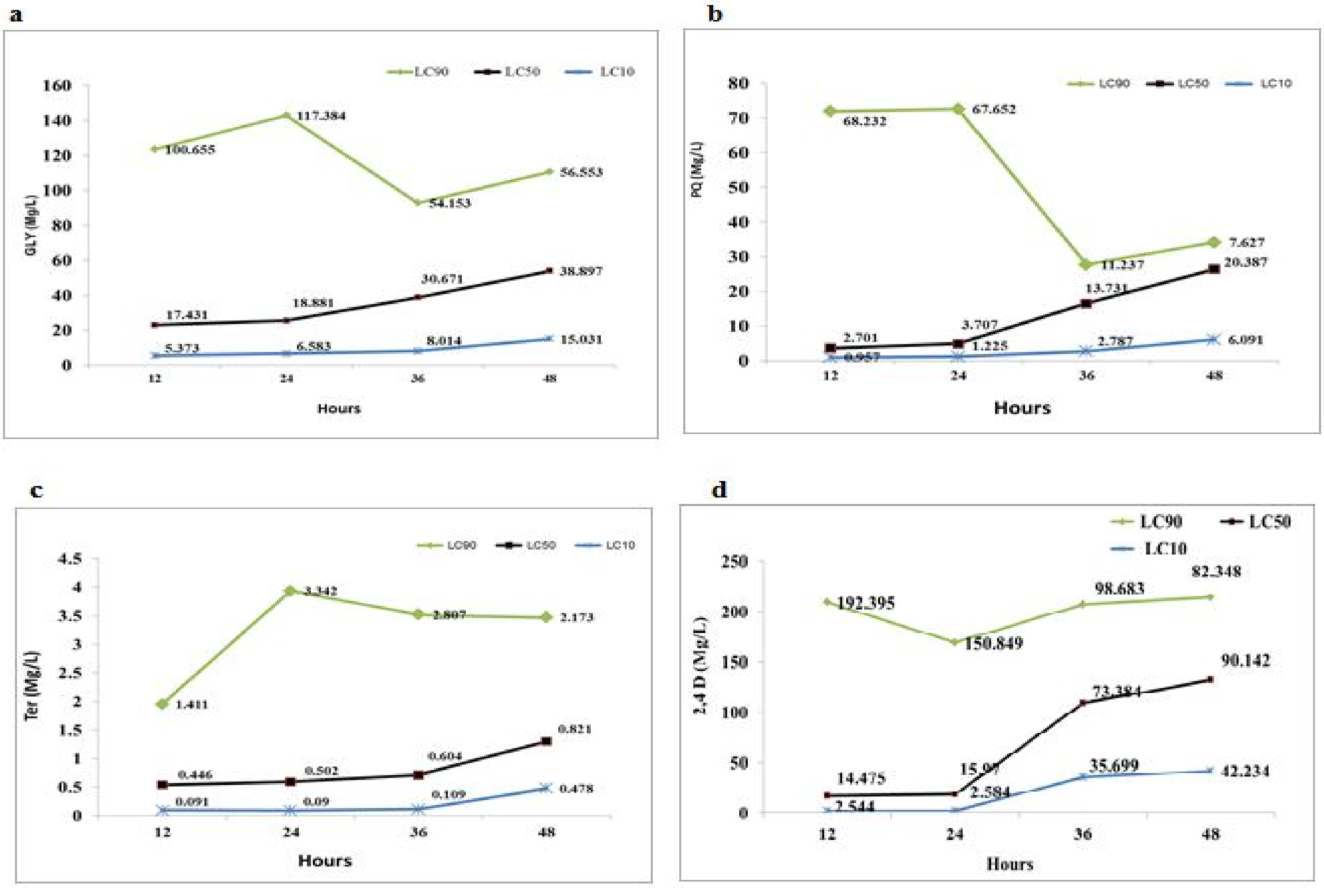

Figure 2. Mortality rate (LC 10, 50, 90 during 48 hours) of *A. fransiscana* in herbicide treatments.

Figure 3. View of adult male and female A. Fransiscana (with egg bags). 48 hours after exposure to paraquat (1) and trifluoralin (2). The accumulations of herbicides are indicated with black arrows.

DISCUSSION

The contamination of waters by herbicides is a potential threat to the productivity and mortality of fish. They can affect environmental quality of life and impair the essential ecosystem by reducing the aquatic species diversity, modifying food chains and nutrient cycling, and changing the stability and resilience of the ecosystems [18]. These natural features are the important reasons for making herbicides harmful to aquatic species and human environment [17].

Various species of AF's are invaluable test organisms for eco-toxicity research and enable us to design viable experimental methods to study eco-toxicology [1]. Some researchers [19, 20] have considered AF's as a potential bioaccumulation vector of environmental contaminants, due to their role in food chains. These studies have indicated that bioaccumulation of chlorpyrifos (an agricultural pesticide) was likely to occur under laboratory conditions, thus, could be considered a potential risk against aquatic animals [1].

The toxicity of various echotoxins such as herbicides and their detrimental consequences on aquatic species was mostly assayed by 48 hr LC50 in bio-indicators and test organisms. We studied the 48-hr LC50 of several commonly used agricultural herbicides and our results showed that all tested herbicides were toxic to AF's. The results indicated that although toxicity of the herbicides toward AF's was different, there was a positive correlation not only with the herbicide concentration but also with the exposure time. This result was in agreement with the observations of Alishahi *et al.*, [17] who reported the highest toxicity of herbicides toward *L. esocinus* belonged to trifluralin, and the mortality rate of exposed fish to herbicides enhanced either by increasing herbicides concentration or the duration of exposure.

Trifluralin and paraquat were more toxic than the others since AF's demonstrated a relatively high sensitivity to trifluralin, indicating the higher impact of these herbicides on AF's environment which can be transferred to the primary consumers, i.e., humans. Trifluralin has been screened for efficacy against pathogenic and aquatic fungi and may be a possible replacement for malachite green, a controversial antimicrobial agent used in aquaculture [21]. Trifluralin is virtually nontoxic to mammals but can be highly toxic to aquatic organisms [22]. In this study, the environmental factors, herbicides concentration and AF's species were similar to those reported in previous studies. Also, the differences we found in herbicides' toxicity toward AF's were in agreement with those reported in the literature for other crustaceans [21, 22] and pointed out the resistance of the AF species against acute exposure to the organophosphate herbicides.

Our 48 hr. LC50 values were supported by findings from other researchers [23,24,25] who reported that AF's were the least sensitive organisms tested. Also, Varo´ *et al.* [22] has reported that the three strains of AF's and five species of *A. parthenogenetica* did not show a LC50 below the maximum concentration tested (18 mg/L). Similar responses have been shown in the larvae of AF's exposed to some antibiotics [26].

Another study [27] examined the exposure of three different life stages of *A. salina* to several phenolic compounds and concluded that the sensitivity to the chemicals increased as the test organism grew in age. These findings show that aquatic animals of defined ages must be used for toxicity assays, since the expression is directly related to the development age of the organisms [27].

In this study, AF's were kept under constant experimental water conditions because physiological parameters, such as quality, temperature, pH, dissolved oxygen in water and the turbidity, the concentration and types of herbicides and the exposure time greatly influence the study results [14,17]. Besides, the toxicity of a micropollutant depends on the medium composition. The salt concentration, hardness and pH of the medium greatly influence the experimental results. Generally, the herbicides tend to be less effective in media with high salt content [22, 28].

In the present study, trifluralin was more toxic toward adult AF's than other herbicides. The LC50 at 48hr of trifluralin was reported 19 µg/L in bluegill fish (*Lepomis macrochirus*),19 µg/L in *Mola mola*, 1 mg/l in *Cyprinus carpio* and 0.56 mg/l in *Daphnia magma*, respectively [17]. The toxicity mechanisms of trifluralin is related to its effects on genes, which cause DNA breaks, loss of genetic material, immunotoxicity and mutations leading to cell death. Besides, trifluralin causes hemoglobin oxidation, red blood cell destruction, and damage to the kidney and liver.

Our results indicated that paraquat was the second toxic herbicides toward AF's and our findings are consistent with those reported by previous studies [8, 29, 30]. Paraquat residues in surface water may be absorbed through the gills, skin and digestive system of fish [8]. Absorbed paraquat is distributed through the blood to all organs and tissues of fish. Due to the lipophilic property of this herbicide, it accumulates mainly in fatty tissues [29, 30]. Although paraquat toxicity toward fish is low, exposure to high doses or prolonged contact with low concentrations of paraquat may also cause severe toxicity to vital organs, primarily the liver, gills and kidney [8].

The reported toxic concentrations of glyphosate ranged from 5 to 415 mg/L in the cladoceran *Daphnia magna* [31]. At 6.5 mg/L *D. magna* can be affected after exposure to commercial formula Faena® and 140 mg/L with glyphosate, while the freshwater rotifer *Lecane quadridentata* can be affected with 13 mg/L of Faena® and 140 mg/L of glyphosate.

Glyphosate is the active ingredient in a range of common broad-spectrum systemic herbicide formulations. Glyphosate-based pesticides are currently among the most widely used chemicals in agriculture but these herbicides have also been approved for domestic weed control [33]. Glyphosate affects target plants by inhibiting the enzyme, synthase of the Shikimate pathway (EPSP). This enzyme is not present in animals, and the environmental toxicity to non-target organisms has been considered low [33]. However, adverse effects have been reported in a range of non-target organisms including bacteria, algae, crustaceans, amphibians, and fish [33, 34]. Glyphosate may inhibit specific enzymes in invertebrates such as phosphoenolpyruvate and alkaline phosphatase but may also alter expression of stress-response genes [35]. Glyphosate is a chelating agent and before its herbicidal properties were discovered, it was patented as a metal chelator. In the environment, glyphosate may act as a ligand and form complexes with divalent metal ions including zinc, iron, manganese, and copper. It has been suggested that glyphosate-metal complexes will have decreased environmental mobility due to its precipitation [35].

CONCLUSION

In support of our study's hypothesis, we produced experimental evidence that trifluralin and paraquat were indeed more toxic to adult AF's than the other two herbicides used in this study. Respective industries should be encouraged to examine the possibility of reducing the application of these herbicides because of their potency to harm non-target aquatic organisms. Also, for unwanted weeds, especially in areas that fish ponds and agricultural farms use the same water sources, it is highly recommended to use glyphosate as an alternative to trifluralin and paraquat. To the best of our knowledge, this study is the first one to compare the toxicity of the four commonly used herbicides toward adult AF's.

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CONFLICT OF INTEREST

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article

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