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Comparative toxicities of five herbicides on nauplii of *Artemia franciscana* as an ecotoxicity bioindicator

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Abstract

Artemia is one of the most important test organisms available for ecotoxicity tests. In this study the toxicity of five widely used herbicide formulations including: Paraquat, 2,4-dichlorophenoxy acetic acid (2,4-D), Trifluralin, Glyphosite and - Atrazine were compared using the ecotoxicity bioindicator, *Artemia franciscana.* Acute toxicity (48 h LC50) of five herbicides were determined via OECD standard methods. Artemia were hatched using standard methods and 12 h old nauplii were used for toxicity evaluation of herbicides. Nauplii were exposed to serial concentrations (more than 6 in triplicates) of each herbicid. Mortalities at 12, 24, 36 and 48 hours after exposure were recorded and the LC50 were calculated using the Probit software. The results showed that the mortality rate increased by increasing the exposure time in all herbicide toxicity assays. Besides 48 hours LC50 value of Paraquat, 2,4-D, Trifluralin, Glyphosite and Atarzine were calculated at 15.67, 12.93, 11.87, 164.31 and 61.34 mg L^{-1} in *A. franciscana* respectively. Glyphosate showed significantly less lethality and toxicity while Trifluralin and 2,4-D showed the highest toxicity in *A. franciscana* among the examined herbicides. Then for sustainable agricultural activity, especially in areas that runoff water flows into aquatic ecosystems, it is highly recommended to use environmentally friendly herbicides like –Glyphosate as an alternative to the highly toxic herbicides like Trifluralin, Paraquat and 2,4-D.

Keywords: Toxicity, Herbicide, *Artemia franciscana*, Ecotoxicity bioindicator

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Introduction

Several areas of scientific knowledge, disperse information related to the genus *Artemia* (brine shrimp). An integration of this information is important for toxicologists working with this genus. Artemia is subdivided into six generally recognized bisexual species and a large number of parthenogenetic populations, are characterized by common features such as adaptability to wide ranges of salinity (5-250 g L^{-1}) and temperature (6-35 centigrade), short life cycle, high adaptability to adverse environmental conditions, high fecundity, parthenogenetic reproduction strategy (with nauplii or cysts production), small body size, and adaptability to varied nutrient resources as it is a non-selective filter feeder. In itself, the intrinsic features of this genus turn it into an appropriate organism for use in ecotoxicology, promising reliability, feasibility and cost-effectiveness in research ecotoxicity practices. Artemia is one of the most important test organisms available for ecotoxicity testing and research done so far allows us to state that it is possible to endure several options related to Artemia use in toxicology and ecotoxicology. Its natural tolerance may be seen as an advantage, in comparison to other test organisms, less adapted to a large number of abiotic conditions. Artemia can be subjected to both field and laboratory testing conditions. Moreover tolerance of Artemia specimens makes this genus adaptable to a great variety of testing conditions, in estuarine, marine or hypersaline environments, thus responding to the actual demands of standardized tests for saline ecosystems. Genetic variability among parthenogenetic strains can be transformed into an advantage, through selection of the most appropriate strain, complying with requirements of sensitivity, reproductive features, life history traits, relative abundance and geographical distribution (Nunes *et al.*, 2006).

Khuzestan Province is the most important agricultural center in Iran (Afkhami *et al.*, 2007), because around 30% of Iran's freshwater resources are located in this province.

Toxicity testing of chemicals on bioindicator animals has been used for a long time to detect the potential hazards posed by chemicals to environment and human. Bioassay techniques have been the cornerstone of programs on environmental health and chemical safety (Moraes *et al.*, 2007).

Although herbicides have the potencial to adversely affect the environment, their effects on environment mostly depend on the characteristics of each kind of herbicide. The most popular and widely used herbicide formulations in Iran includes: Paraquat, 2,4-dichlorophenoxy acetic acid (2,4-D), Trifluralin, Glyphosite and Atrazine.

Glyphosate is one of the most globally recognized pesticides used around the world (commercial names: Roundup, Rodeo). Glyphosate is a broadspectrum herbicide that inhibits the synthesis of essential amino acids. It is widely used to control undesirable weeds in

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agriculture (e.g., Roundup-Ready corn ,soybeans and cane farms), forestry, aquatic habitats, and residences. Roundup is the most prevalently used formulation, containing both the active ingredient (Glyphosate) as well as a surfactant (e.g., POEA; polyethoxylated tallowamine) that allows penetration of plant cuticles (Relyea, 2005).

Paraquat (PQ) is a nonselective and nonsystemic herbicide that is frequently used world wide (Salazar-Lugo *et al*., 2011). PQ has been proved to be highly toxic to humans and animals and many cases of acute PQ poisoning or deaths have been reported over the past few decades. Superoxide- and singlet oxygen-catalyzed lipid peroxidation is the possible biochemical mechanism of PQ toxicity. Due to its high water solubility and extensive use, PQ may enter surface water as a result of spray drift, leaching from soil and water, or running off from agriculture (Junguo *et al.,* 2014).

Trifuralin is a dinitroaniline compound used as a selective preemergence herbicide. Trifuralin can get into aquatic ecosystems in runoff and by underground waters, similar to other herbicides used near the water. Its effects on biotic components of a freshwater ecosystem (phyto-, zooplankton, and bottom fauna) in concentrations which could be reached in aquatic environment have been demonstrated (Poleksic and Karan, 1999).

The herbicide 2,4 Dichllorophenoxyacetic acid (2,4-D) has been used extensively in modern agriculture and, despite its short half-life in soil or aquatic environments, toxicological studies suggest a great potential for inducing undesirable effects affecting non-targeted organisms (Chinalia *et al.,* 2007). It is known, that 2,4-D causes changes in the animal nervous system through complex formation with acetylcholine and inhibition of AChE (Benli *et al.,* 2007).

Atrazine is one of the most consumed and major herbicides in the world in the past four decades. In Iran, Atrazine has been used as a herbicide on cotton, sugar cane, corn, etc. farms in Golestan and the southern provinces especially Khuzestan. These are major fish farming regions in the country. Atrazine is also absorbed through leaves but has a 0% transference rate. Atrazine has the trade name of Gesaprim in Iran distributed in the market with weight purity of 80% formulation. In pure water, the half-life of Atrazine is considered to be three days, whereas in seawater, this period is 30 days (Abdali *et al.,* 2011).

The application of various herbicides and chemical fertilizers in Khuzestan Province is much higher than international standards. Therefore, it can be claimed that the province's surface water and groundwater can probably be contaminated with different pesticides (Banaee *et al*., 2011). Although there is little information about the toxicity of herbicides in surface water in Iran, toxicity evaluation of herbicides in surface waters using bioindicators is a serious nessesity to residents' health and aquatic ecosystems. The aim of the

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present study was the comparison of toxicity of five widely used herbicide formulations using ecotoxicity bioindicator, *Artemia franciscana* to find the most environmentally friendly herbicide for sustainable agricultural activities. To the best of our knowledge, this work is the first study comparing the toxicity of highly used herbicides using ecotoxicity bioindicator, *A. franciscana*.

Materials and methods

The nauplii of the marine copepod *A. fransiscana* (company of LTD INVE (Thailand)) were hatched from commercially available cysts. The cysts were incubated in artificial seawater (Instant Ocean) at a salinity of 35 ppt, -27 ºC and - pH 8.5. The first nauplii usually appeared after 24 h of incubation at -25 ºC under aeration and illumination of around 2000 Lux light. They were transferred into fresh seawater and incubated for 24 h under similar conditions. Then, they were transferred into a multi-well test plates with the respective concentrations of the tested formulations, prepared in artificial seawater. The toxicity of the formulations on *A. fransiscana* nauplii was tested at 12, 24, 36 and 48 h of exposure at - 25 ºC. The rate of toxicity, based on LC_{50}, was determined as the concentration in which the tested formulation caused 50% mortality of Artemia nauplii after 12, 24, 36 and 48 h of exposure. For all selected herbicides, range-finding tests were performed for the determination of the appropriate concentrations, used in the definitive tests.

The lethal concentration (LC50) was tested by exposing 30 nauplii per group (in triplicates). The control group was kept in experimental water without herbicides with all of the other conditions kept constant.

Then the nauplii were exposed to 4-6 sequential rising concentrations of each herbicide (in triplicates) in a way that zero and 100% mortality was recorded after 48 hours in selected concentrations. The mortality rate was recorded every 6 hours and until 48 hours. The concentration of herbicides to induce 10, 15, 50, 85, 95 and 100 percent mortality (LC10, LC15, LC50, LC85, LC95, LC100) was estimated after 12, 24, 36, and 48 hours using probit software version 1.5 designed by U.S. EPA. This software estimates LC concentration based on regression between mortality rate and log of toxin concentration. LC estimation is presented with lower and upper range with 95% confidence level (Aydin and Kuprucu, 2005). The selected herbicide concentrations for estimating their acute toxicity in *A. fransiscana* are -shown in Table 2.

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Table 1: Specifications of the test herbicides

Herbicide	Chemical Name	Supplier	Purity Rate	Acute Oral LD50 For Rat	Statue
Paraquat	1,1-dimethyl-4,4'-bipyridinium	Aria Shimi Co, Iran	20%	129-157 mg kg^{-1}	Water soluble green liquid.
2, 4 - D	2-(2,4-dichlorophenoxy)-acetic acid	Shimagro Co, Iran	67.5%	2100 mg kg^{-1}	Water soluble brown liquid
Trifluralin	α-trifluoro-2,6-dinitro-N,N-dipropyl-p-toluidine),	Aria Shimi Co, Iran	48%	5000 mg kg^{-1}	Water soluble orange liquid
Glyphosate or Roundup	N- phosphonomethyl glycine	Sinochem Co, China	41%	5600 mg kg^{-1}	Water soluble yellow liquid
Atrazine	Chloro-4-ethylamino-6-isopropylamino-1-3-5-triazinre	Moshkfam Co, Iran	80%	1350 mg kg^{-1}	Water soluble white powder

Table 2: Experimental design table: concentration ranges tested on the Nauplii

Tested herbicides	Concentration range (mg L^{-1})	Number of treatments	Number of replicating	Total exposed Nauplii
Paraquat	10,20,40,80,160,200	6	3	90
2, 4 - D	40,80,200,400	4	3	90
Trifluralin	10,20,40,80,200	5	3	90
Glyphosate	40,80,200,400,800	5	3	90
Atrazine	40,80,200,400	4	3	90

Result

The mortality rate increased along with increasing the exposure time in all herbicide toxicity assays. Besides the rate of mortality has a positive correlation with the concentration of all herbicides. Results showed that the 48 hours LC50 values (Median lethal concentration) of Paraquat, 2,4-D, Trifluralin, Glyphosite and Atarzine were calculated at 15.67, 12.93, 11.87, 164.31 and 61.34 mg L^{-1} in *A. franciscana*, (Table 3) and MAC of these herbicides were 1.56, 1.3, 1.2, 16.43 and 6.13 mg L^{-1}, respectively. The greatest LC50 was 164.31 mg L^{-1} for Glayphosate and the least was 11.87 mg L^{-1} for Trifluralin. Therefore, the toxicity rate of these herbicides in *A. franciscana* were higher in Trifluralin, 2,4-D, and Paraquat, respectively.

The -Glyphosate showed significantly less lethality (higher LC50 concentration) than other tested herbicides. Trifluralin and 2,4-D showed the highest lethality (lower LC50 concentration) in *A. franciscana* among the examined herbicides (Fig. 1).

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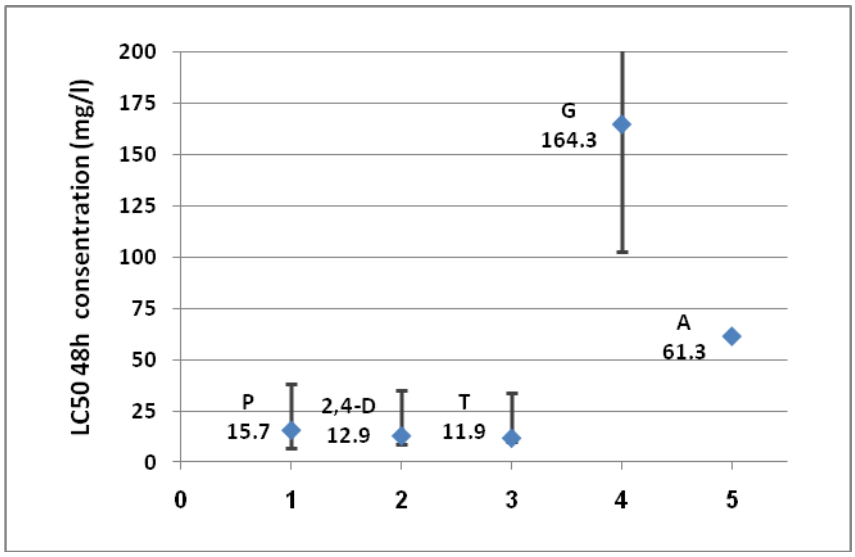

Figure 1: Comparison of LC50 96 h concentrations of selected herbicides in *Artemia franciscana.*

Table 3: lethal concentrations of herbicides (mg L^{-1}) (95% confidence intervals) depending on exposure time for *Artemia fransiscana*.

Type of Toxicant	lethal concentration (mg L^{-1})	Exposure time (h) 12 h	24 h	36 h	48 h
Paraquat	LC_{10}	22.19	11	6.3	4.53
	LC_{15}	28.05	14.57	8.14	5.74
	LC_{50}	75.66	47.73	24.12	15.67
	LC_{85}	204.04	156.31	71.42	42.74
	LC_{95}	365.28	313.65	135.10	77.03
	LC_{100}	540.26	684.27	275.86	149.01
2,4-D	LC_{10}	39	17.86	4.83	2.14
	LC_{15}	58.17	24.97	7.12	3.02
	LC_{50}	315.8	102.81	36.7	12.93
	LC_{85}	1714.129	423.29	188.84	55.36
	LC_{95}	4627.12	971.49	494.08	129.98
	LC_{100}	14072.66	2463.58	1451.02	180.97
Trifluralin	LC_{10}	15.63	9.09	2.57	1.7
	LC_{15}	20.50	12.14	3.71	2.47
	LC_{50}	64.5	41.26	17.54	11.87
	LC_{85}	202.91	140.18	82.86	57.06
	LC_{95}	397.63	287.40	206.15	143.42
	LC_{100}	844.80	642.27	572.17	402.61
Glyphosite	LC_{10}	103.4	83.4	54.8	49.04
	LC_{15}	133.33	105.87	69.49	61.80
	LC_{50}	390.62	290.33	189.74	164.31
	LC_{85}	1144.34	796.15	518.01	436.85
	LC_{95}	2150.72	1439.38	934.11	775.59
	LC_{100}	4360.36	2794.10	1808.03	1475.27
Atrazine	LC_{10}	88.83	13.03	2.91	5.38
	LC_{15}	166.35	27.78	5.74	8.57
	LC_{50}	2360.61	683.31	101.22	61.34
	LC_{85}	33496.57	16801.94	1781.86	438.87
	LC_{95}	158945.37	110095.12	9595.91	1393.10
	LC_{100}	909317.85	904130.29	63258.64	5080.11

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Discussion

The toxicity of herbicides was a real threat for human health. Toxicity testing of herbicides on bioindicator animals such as Artemia has been used to detect the potential hazards posed by chemicals to environment and human (Moraes *et al*., 2007). In this study all tested herbicides were toxic for *A. franciscana* such that median lethal concentration after 48 h exposure (48 h LC50) of five herbicides: Paraquat, 2,4-D, Trifluralin, Glyphosite and Atrazine in *A. franciscana* were 15.67, 12.93, 11.87, 164.31 and 61.34 mg L^{-1} respectively. Artemia can be subjected to both field and laboratory testing conditions. Tolerance of Artemia specimens makes this genus adaptable to a variety of important testing conditions, in estuarine, marine or hypersaline environments, thereby, responding to the real demands of standardized tests for saline ecosystems (Nunes *et al.,* 2006).

The results of this study showed that although the toxicity of the five tested herbicides were different for *A. franciscana*, their toxicity showed positive correlation not only to herbicide concentration, but also to exposure duration. The most toxic tested herbicide was Trifluralin, which is used widely in agriculture particularly in cane farms for controlling the growth of unwanted plants. Trifluralin and 2,4-D were more toxic than the other evaluated herbicides. Recently 2,4-D has been accepted as an efficient and affordable herbicides in large scale agriculture farms particularly cane, corn and rice farms in Iran (Banaee *et al*., 2011).

Regarding the similar efficient concentration of all tested herbicides, the difference between the toxicity of herbicides can be based on their different compounds. Based on probably similar mechanism of herbicides toxicity in Artemia and vertebrate animals it can be concluded that Glyphosate is ten fold lesser toxic compared with PQ, 2-4-D and Trifluralin for aquatic invertebrates and vertebrates. These results can be generated even in terrestrial animals. Because Cox (1998) observed that fish and aquatic invertebrates were more sensitive to Glyphosate toxicity than terrestrial organisms. In a similar work, Falis *et al*. (2014) found that 500 mg L^{-1} of Glyphosate induced no lethality in brine shrimp during the 96h exposure. All environmental parameters, herbicide concentration and examined Artemia source were quite similar in this study based on standard methods, so the differences in herbicides toxicity can just be referred to as the toxicity mechanism of each herbicide in Artemia. The results of this work, like other toxicity assessment studies, showed somehow different results compared with similar reports, but it is worth considering that, toxicity of chemicals to aquatic organisms has been shown to be affected by different factors including: Artemia species, water biochemical parameters like salinity, temperature, pH, light intensity and naplii intensity.

Artemia naplii has been used as a salt water pollutant biomarker in many

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works. Varo *et al*. (2002) investigated the acute toxicity of the organophosphorous pesticides dichlorvos and chlorpyrifos in two different species of Artemia (*A. salina and A. parthenogenetica*). In addition, the in an *in vivo* study the effect of these two pesticides on cholinesterase (ChE) activity of both *A. salina* and *A. parthenogenetica* was also determined. They concluded that these organophosphorous peticides cause 95% reduction in ChE activity of *A. parthenogenetica* nauplii compared with the control group and this reduction was significantly higher in *A. parthenogenetica* than in *A. salina*. They also found that these organophosphorous pesticides have higher toxicity (lower median lethal concentrations, 48h LC_{50}) in *A. parthenogenetica* nauplii compared to *A. salina* (Varo *et al.,* 2002).

In agreement with a later report Guzzella *et al*. (1997) compared the response of freshwater organisms (*Daphnia magna* and *D. pulex, Gammarus pulex*, *G. lacustris*, *G. fasciatus*, *Simocephalus* sp., *Asellus aquaticus*.) and marine organisms (*Brachionus plicatilis* and Artemia sp.) to organophosphorous pesticides (OPs). Their results showed that aquatic organisms living in higher salinity were less sensitive to these pesticides than freshwater aquatic species. Although the chelation of effective components of pesticides occurs in high salinity, the euryhaline character of marine species can help them have a greater osmoregulation capacity which contributes to a greater resistance to toxic effects of Ops (Guzzella *et al.,* 1997).

Besides Guzzella *et al*. (1997) demonstrated that the *Artemia* sp. and *B. plicatilis* 24h EC50 values for OPs were different from about 1 mg L^{-1} to more than 300 mg L^{-1}. They classified toxicological effects of the OPs in *Artemia* sp. in the following order: chlorpyrifos>azinphos-methyl> fonofos >diazinon>parathion-methyl> azinphos-methyl > omethoate.

In the present study the 48 h LC50 of Paraquat was estimated to be 15.67 ppm in naplii of *A. franciscana*. To the best of our knowledge it is the first report of toxicity evaluation via the artemia biomarker. Yeo (1967) reported that Paraquat at 1.0 and 3.0 ppm was toxic to small mouth bass (*Micropterus dolomieui*) and to mosquito fish (*Gambusia affinis*) in plastic pools. In another study Babatunde and Oladimeji (2014) reported similar results for Paraquat toxicity rates in *Oreochromis niloticus*. Acute toxicity (96 h LC50) of Paraquat was 12.25 mg L^{-1}.

Although reports on toxicity evaluation of herbicides in marine pollution biomarkers are scarce, various studies focused on toxicity of herbicides on different fish species. Farah *et al*. (2004) defined the LC50 values of 2,4-D as 81 ppm for Asian catfish (*Heteropneustes fossilis*) and 122 ppm for walking catfish (*Clarias batrachus)*, 107 ppm for snakehead (*Channa punctatus*) and 302 ppm for *Culex pipiens fatigans*. In a similar study, Sarikaya *et al*. (2002) found LC50 value of 2,4-D in tench (*Tinea tinea*) as 48 mg L^{-1} Sarikaya and

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Yilmaz (2003) reported 96 h LC50 value for *Cyprinus carpio* as 63.24 mg L^{-1}.

In the current research the 48-h LC50 for the Glyphosate in *A. franciscana* was estimated as 164.31 mg L^{-1}. As mentioned toxicity of herbicides were higher in freshwater organisms than in marine aquatic species, as 17.8 mg L^{-1} in *Gambusia yucatana* (Rendón *et al*., 2005) and 120 mg L^{-1} in *Oncorhynchus mykiss* (Giesy *et al.,* 2000).

Our results showed moderate toxicity of Atrazine in artemia nauplii (48 h LC_{50}=61.34 mg L^{-1}). There are no reports on toxicity of Atrazine in artemia biomarker, but Bathe *et al*. (1973), Neskovic *et al*. (1993) and Hussein *et al*. (1996) reported LC50 of 16.0, 18.8 and 9.37 mg L^{-1} for *Lepomis macrochirus* (Blue gill sunfish), *C. carpio* and *O. niloticus,* respectively exposed to Atrazine.

In our work 48h LC50 of Trifluralin in artemia was estimated as 11.87 mg L^{-1} while in a similar study on some fish species different LC50 values were reported including: 19 µg L^{-1} in bluegill fish (*Lepomis macrochirus)*, 19 µg L^{-1} in *Mola mola*, 1 mg L^{-1} in *C. carpio*. Gangolli (1999) evaluated Trifluralin toxicity using fresh water pollutant biomarker, *Daphnia magma*, and found 48 hours LD50 of Trifluralin as 0.56 mg L^{-1}. The lower LD50 of Trifluralin in Daphnia compared with Artemia can be regarded as the negative correlation of pesticide toxicity and water salinity. The higher ion concentrations in salt water can chelate some active matherials in pesticides. Then lower toxicity of herbicids in salt water causes more tolerance in salt water bioindicators like artemia to their toxicity.

The application of various herbicides and chemical fertilizers in Iran specially in Khuzestan Province is much higher than international standards. Although herbicidic capacity of evaluated herbicides were similar, their toxicity rates to salt water ecotoxicity bioindicator, *A. franciscana*, were significantly different. Therefore, it is highly recommended to use environmentally friendly or green herbicides like Glyphosate as an alternative to highly toxic herbicides like Trifluralin, Paraquat and 2,4-D in agricultural activity.

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