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Toxicity and Bioconcentration of Chlorpyrifos in Aquatic Organisms: *Artemia parthenogenetica* (Crustacea), *Gambusia affinis*, and *Aphanius iberus* (Pisces)

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Presently, organophosphorus pesticides (OPs), carbamates, pyrethroids and triazines have largely replaced the organochlorine compounds in agricultural practices. They have the advantage of being more biodegradable and having less persistence in the enviroment. Chlorpyrifos [O,O-diethyl-O-(3,5,6-trichloro-2-pyridil) phosphorothioate, CAS RN 2921-88-2, Dursban, Lorsban, Spannit] is a widely used organophosphorus insecticide in the countries of the European Union (>50000 Kg per year) (UNEP 1991).

With regard to the impact of the organophosphorus pesticide chlorpyrifos, this compound has been studied by several authors demonstrating its high toxicity and bioconcentration ability in different groups of aquatic organisms (Serrano et al. 1997; Varó et al. 1998). As a step towards a broader study on bioaccumulation of chlorpyrifos in trophic webs, we report herein the toxicity and bioconcentration of chlorpyrifos in two species of fish (*Gambusia affinis* and *Aphanius iberus*) that are inhabiting surface coastal waters vulnerable to pesticide pollution, and in a crustacean (*Artemia parthenogenetica*) commonly used as prey organism in aquaculture.

MATERIALS AND METHODS

The culture procedures used for crustaceans and fish in this study were as follows. *A. parthenogenetica* [diploid (PD), La Mata, Alicante] cysts were hatched in seawater (38 g/L) at 28 °C under continuous illumination and aeration. One group of newly hatched nauplii was acclimated to 20°C for 24h in an incubator under conditions of continuous illumination and aeration before the acute toxicity test was performed. A second group was reared to adult in 2 L glass flasks at 20 ± 0.5 °C. The animals were fed *ad libitum* with the alga *Tetraselmis suecica* and the medium was changed every 2-3 days until the different developmental stages were reached. *G. affinis* (mosquitofish) adults were collected from a marsh (Castellón, Spain) and *A. iberus* (fartet) adults were supplied by Valencian Institution. The animals were acclimated in 100 L aquaria filled with filtered brackish water (salinity : 4 g / L) for 21 days at natural temperature (21°C) and photoperiod (October, 0°, 40°N). The mortality of the stock animals was less than 1%.

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Acute toxicity tests with *Artemia* were performed using Corning multiwell plates. Given the low solubility of chlorpyrifos (93-99% purity, Dr. Ehrenstorfer Reference Materials, Germany) in sea water, several stock solutions in acetone were made. Test solutions were prepared in volumetric flasks using filtered seawater (0.2 µm filters) and an appropriate quantity of each stock solution. Each experiment was carried out with six different concentrations of the pesticide plus two controls (clean seawater and seawater with acetone). After previous tests to determine the toxicity of chlorpyrifos, the nominal concentrations ranged from 1 to 100 µg/L and 0.1 to 18 mg/L. Actual concentrations in water were checked for one replicate (Table 1).

Thirty nauplii, 10 juveniles or 4 adults of *Artemia* per well (at least 3 plates for each group) were exposed to 1, 4, and 10 mL respectively of the appropriate concentration of the pesticide. The plates were covered with parafilm and left in an incubator at 20 ± 0.5°C for 24 h, in darkness. The animals were not fed during toxicant exposure. The test end point was death, established by the total lack of movements during 10 seconds of observation under a dissection microscope.

The test solutions used for the fish were prepared in brackish water. They were obtained by pipeting the appropriate quantity of each stock solutions per liter of brackish water. Triplicate groups of 5 fish were kept in glass flasks with 800 mL of filtered brackish water (0.2 µm filters) containing the desired pesticide concentrations for both toxicity and bioconcentration tests. The daily renewal method was used for both toxicity and bioconcentration tests.

Dead specimens were removed from the aquaria twice daily. Animals were not fed during tests and were maintained at natural photoperiod for November (0°, 40°N), at 23 ± 0.5 °C of temperature. Nominal concentrations ranged from 1 to 3200 µg/L. The toxicity experiments were carried out maintaining fish for 72 or 96 h at different concentrations of chlorpyrifos and the lack of movement was the criteria for animal death determination. Percentages of mortality at 24, 48, 72 or 96 h were converted to probits. The LC50 values were calculated by means of the regression probit module of SPSS Systems (SPSS Inc, 1989-1992) at the 95 % confidence.

Bioconcentration tests were carried out maintaining the animals without food during different times (up to 48 h for *Artemia* juveniles and up to 72 or 96 h for *A. iberus* and *G. affinis*, respectively) on pesticide concentrations which did not produce mortality during acute toxicity tests: 0.5, 1, 10 and 100 µg/L for *Artemia* juveniles, 100 µg/L for *G. affinis* and 3.2 µg/L for *A. iberus*. Only *Artemia* juveniles were used for bioconcentration tests because they are less sensitive to fast conditions, and they present a good sized-prey for fish. As a routine, the actual concentration of the pesticide in water was determined at the start and at the end of each test and showed a reasonable agreement with the nominal concentrations. A liquid-liquid extraction method with dichloromethane was used to extract chlorpyrifos from samples of water. The chlorpyrifos was

analysed by GC (NPD) (Serrano et al. 1997). The recoveries from spiked water at 1 mg/L level were 90±6%. Limit of detection was 0.1 µg/L.

After collection, organisms were frozen and the whole body of *Artemia* and fish was analysed following the procedure outlined in Hernández et al. (1998). Samples were thawed at room temperature, triturated, and mixed with anhydrous sodium sulfate. The extraction was carried out with acetonitrile:acetone (10:1, v/v) (pesticide residue analysis quality, Scharlau, Barcelona, Spain) by means of a blender (Ultraturrax). Automated cleanup of the extracts was carried out by normal phase-HPLC. Fat-free sample extracts were analyzed by GC. Recoveries of the procedure at 200 µg/L were 101±8 % and 92±6 % for *Artemia* juvenils and *A. iberus*, respectively. Detection limit of the procedure was found to be 0.3 ng/g. GC analysis was performed on a Hewlett Packard 5890 series II (Avondale, USA) with nitrogen-phosphorus detector, equipped with an HP 7673 autosampler. Splitless injections of 2 µL were performed on a fused silica HP Ultra 2 capillary column coated with cross linked 5 % phenyl methyl-silicone with a length of 25 m x 0.20 mm ID and a film thickness of 0.33 mm. Helium was used as carrier gas at a flow of 0.5 mL/min and as make up gas at a flow of 30 mL/min. The oven temperature was programmed as follows: 90°C during 1 min, 30°C/min to 180°C, and 4°C/min to 270°C with a final hold for 20 min. Quantitation was carried out using an external standard method.

RESULTS AND DISCUSSION

The actual chlorpyrifos concentrations in the seawater and brackish water in the experimental aquaria showed a reasonable agreement with the nominal concentrations, suggesting a constant chlorpyrifos concentration in water (Table 1).

The 24h-LC50 mean values obtained for nauplii, juveniles and adults of *Artemia* and the 72 and/or 96h-LC50 value calculated for *A. iberus* and *G. affinis* are shown in Table 2. *Artemia* nauplii did not attain 50 % mortality at the highest concentration tested, and their LC50 is reported as >18 mg/L. Sensitivity to chlorpyrifos varied as follows: *A. iberus* > *Artemia* adults > *G. affinis* > *Artemia* juveniles > *Artemia* nauplii.

Studies on toxicity of OPs in aquatic fauna show great variability in both toxicity of different pesticides and sensitivity of different species (Persoone et al. 1985; UNEP, 1991). Resistance to chlorpyrifos in *Artemia* was age dependent. *Artemia* adults were the most sensitive to chlorpyrifos in contrasts to what has been reported (Vanhaecke et al. 1980) for inorganic toxicants. However, our results are in agreement with those found by others authors (Barahona and Sanchéz-Fortún 1996), who have shown differences in sensitivity to several compounds for different ages of *Artemia* larvae.

Table 1. Actual chlorpyrifos concentrations in the sea water in the experimental aquaria measured after liquid-liquid extraction with dichloromethane and analysis by GC(NPD).

Nominal µg/L	Actual	Nominal mg/L	Actual
1	1	0.32	0.31
3.2	3.4	0.56	0.50
5.6	5.5	1	0.9
10	11	3.2	3.5
32	32	5.6	5.4
56	51	10	8
100	100	18	16

Our results show that the toxicity of the pesticide varied significantly between the two fish species (see Table 2). *A. iberus* (family Cyprynodonthidae) was 30 times more sensitive to chlorpyrifos than *G. affinis* (family Poecillae). These differences have been reported for fish from different families (Walton et al. 1997). Although the toxicity of chlorpyrifos to *A. iberus* has not been previously investigated, a few chlorpyrifos and others OPs LC50 data are available for *G. affinis*. Boone and Chambers (1996) determined a chlorpyrifos 96h-LC50 value of 0.15 mg/L for mature females of *G. affinis*, which is quite different from that obtained in the present study. This may be attributable to the fact that we used both male and female mosquitofish. Walton et al. (1997) working also with mature female mosquitofish found a 96h-LC50 of 2041 µg/L for the OPs pesticide parathion, whereas Pickering et al. (1962), working on fathead minnows (*Pimephales promelas*) and goldfish (*Carassius aurata*), determined LC50 values for the same pesticide of 1300 µg/L and 2700 µg/L, respectively. Ferrando et al. (1991) found LC50 values of 590 and 540 at 72 and 96h, respectively for *Anguilla anguilla* exposed to chlorpyrifos. These results are very close to those found in the present study for mosquitofish (*G. affinis*).

There were no significant differences ($p \leq 0.05$) for LC50 values determined for mosquitofish (*G. affinis*) at 48, 72, and 96h, respectively. This is consistent with the results reported by Ferrando et al. (1991), who showed that several organochlorine and organophosphorus pesticides, including chlorpyrifos, did not produce differences in LC50 values at different times in the fish *Anguilla anguilla*.

Our results show that *A. iberus* presents a lower tolerance to this pesticide than G. *affinis* (mosquitofish), although they are in the lower range of values reported for OPs in general for fishes. The great range of acute toxicity levels among OP insecticides for any species or for one compound among species may be the result of the differences in inhibitory potency for the target and non target enzymes and/or metabolism (Boone and Chambers 1997).

Table 3 shows the amount of pesticide accumulated in the tissue of *Artemia*

juveniles during 24 and 48 h at different concentrations of pesticide in water. Chlorpyrifos was bioconcentrated at high levels after 24 h exposure at 100, 10 and 1 µg/L. At 48 h of exposure to 1 µg/L, the amount of chlorpyrifos in the organisms increased quickly (6.08 µg/L). Higher concentrations than 1 µg/L produced complete mortality in *Artemia* juveniles, after 24 h of exposure. However, it was noted that *Artemia* juveniles became nearly inactive from 3 until 24 h of pesticide exposure. Wang and Simpson (1996) demonstrated that persistent chlorinated compounds (DDTs) can be concentrated in high levels in *Artemia* nauplii from water, after 24 h pesticide contamination. These authors reported that the concentration in nauplii exposed to 1 µg/L was about twice that organisms exposed to 0.5 µg/L of pesticide.

Bioconcentration tests with *G. affinis* and *A. iberus* were performed at a concentration of 100 and 3.2 µg/L, respectively in water since this was found to be the highest concentration that did not produce significant mortality. The results are reported in Figure 2. The concentration of chlorpyrifos accumulated were higher than the pesticide concentration in water. *G. affinis* exposed to 100 µg/L accumulated more than 50 µg/g chlorpyrifos in 96 h, and *A. iberus* exposed 72h at 3.2 µg/L accumulated 0.01 µg/g; which is a sign of high bioconcentration ability. An increase of pesticide concentration can be observed with increasing time of pesticide exposure. A similar effect has been recorded in fish, in whole body as well as in different tissues after exposure to a toxicant (Sancho et al. 1994).

As can be deduced from Table 3 and Figure 2, the ratio between pesticide concentration in *Artemia* and fish and the nominal concentration in water increased with time. However, an inverse relationship in Artemia was found. This fact may be attributable to the toxic effect on the metabolism (respiration) of *Artemia* juveniles, at the highest pesticide concentrations tested. In our experiments the organisms were not fed, so the uptake of chlorpyrifos from the water to the tissue took place mainly by diffusion through the pleopodes (respiratory and swimming system). On the other hand, *G. affinis* showed a higher ratio values between the concentration of pesticide in fish and the concentration of pesticide in water than *A. iberus*, at all times of exposure. It is known that the bioconcentration of the pesticides in aquatic animals can be affected by different factors apart from lipid and water solubility, such as species, body size and environmental conditions. Our results are in agreement with those reported by Davis and Dobbs (in Barron 1990), who showed that the partitioning of many hydrophobic organic chemicals seem to be species dependent. The partition coefficient n-octanol-water (Kow) simulates the fat-solubility of the compounds. Toxicants with a log Kow> 4 are considered as fat-soluble. This is the case of chlorpyrifos (log Kow: 5.11 (Noble 1993)).

Therefore, it can be concluded that chlorpyrifos presents a potential risk to aquatic fauna, that can reach to humans through the food chain. Therefore, more studies on analytical methodology, physiology and ecotoxicology are needed in

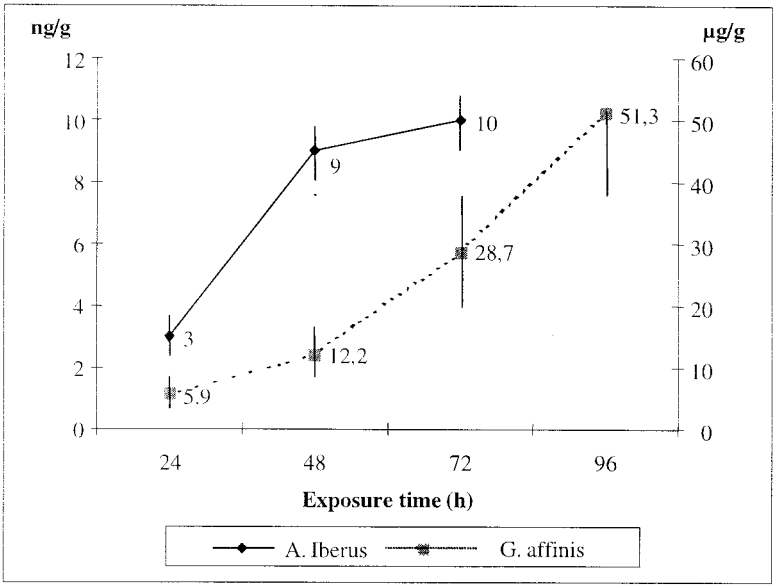

Figure 2. Bioconcentration of chlorpyrifos in *Gambusia affinis* and *Aphanius iberus* after exposure to 100 and 3.2 µg/L pesticide, respectively, at different times. Values are mean ± standard deviations, n=3.

Table 2. Acute toxicity of chlorpyrifos for organisms studied. Mean ± standard deviations, n=3

Organism	exposure time (h)	LC50 values (mg/L)
A. parthenogenetica –nauplii	24	>18
A. parthenogenetica -juveniles	24	3.9±0.9
A. parthenogenetica -adults	24	0.08±0.01
		LC50 values (µg/L)
G. affinis	48	520±60
	72	540±50
	96	520±50
A. iberus	48	38.6±7.2
	72	18.01±0.02

order to know the potential risk of these chemicals to the aquatic ecosystems, as nowadays these compounds represent a threat to the aquatic environments.

Table 3. Bioconcentration of chlorpyrifos in *Artemia* juveniles exposed at different times and concentrations (mean ± std, n=3)

Concentration in seawater (μg/L)	Time of exposure (h)	Concentration in organisms (μg/g)
100	24	6.40 ± 1.2
10	24	1.50 ± 0.50
1	24	0.26 ± 0.09
1	48	6.08 ± 0.48
0.5	24	nd
0.5	48	0.48 ± 0.10

nd: not detected

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REFERENCES

Barahona MV, Sánchez-Fortún S (1996) Comparative sensitivity of three age classes of Artemia salina to several phenolic compounds. Bull Environ Contam Toxicol 56: 271-278

Barron MG (1990) Bioconcentration: Will water-borne chemicals accumulate in aquatic animals. Environ Sci Technol 24: 1612-1618

Boone JS, Chambers JE (1996) Time course of inhibition cholinesterase and aliesterase activities, and non-protective sulfhydryl levels following exposure to organohosphorus insecticides in mosquitofish (Gambusia affinis). Fund Appl Toxicol 29: 202-207

Boone JS, Chambers JE (1997) Biochemical factors contributing toxicity differences among chlorpyrios, parathion, and methyl parathion in mosquito fish (Gambusia affinis). Aquatic Toxicol 39: 333-343

Ferrando MD, Sancho E, Andreu-Moliner E (1991) Comparative acute toxicities of selected pesticides to Anguilla anguilla. J Environ Sci Health 26: 491-498

Hernández F, Serrano R, Pitarch E, López FJ (1998) Automated sample clean-up procedure for organophosphorus pesticides in several aquatic organisms using normal phase liquid chromatography. Anal Chim Acta 374, 215-229

Noble A (1993) Partition coefficcients (n-octanol-water) for pesticides: A review. J Chromatogr 642: 3-14

Persoone G, Vanhaecke P, Gobbers E (1985) Evaluation of the impact of parathion, methyl-parathion, fenitrothion and fenthion on the aquatic environment. Report of the ECC XI/785/83

Pickering QH, Henderson C, Lemke AE (1962) The toxicity of organic phosphorus inseticides to different species of warmwater fishes. Trans American Fish Soc 91: 175-184

Sancho E, Ferrando MD, Gamon M, Andreu E (1994) Uptake and clearence of diazinon in different tissues of the european Eel (Anguilla anguilla). Biomonitor Environ Sci 7: 41-49

Serrano R, Hernández F, López, FJ, Peña JB (1997) Bioconcentration, depuration and chronic toxicity of the organophosphorus pesticide chlorpyrifos in the marine mollusc Mytilus edulis. Arch Environ Contam Toxicol 33: 47-52

UNEP/FAO/WHO/IAEA (1991) Assessment of the state of pollution of the Mediterranean sea by organophosphorus compounds. MAP Technical Reports Series No 58 UNEP (Athens)

Vanhaecke P, Persoone G, Claus C, Sorgeloos P (1980) Research on the development of a short-term standard toxicity test with the Artemia nauplii. Vol 1. The brine shrimp Artemia: Morphology, Genetics, Radiobiology, Toxicology. Persoone G, Sorgeloos P, Roels O, Jaspers E (eds.) Universa Press, Wetteren (Belgium) 263-285

Varó I, Serrano R, Navarro JC, López FJ, Amat F (1998) Acute lethal toxicity of the organophosphorus pesticide chlorpyrifos to different species and strains of Artemia. Bull Environ ContamToxicol 61: 778-785

Walton WJ, Brown KL, Lydy MJ (1997) Diurnal fluctuations in toxicity in two fish species: Gambusia affinis and Notropis ludibundis Bull Environ Contam Toxicol 59: 414-421

Wang JS, Simpson KL (1996) Accumulation and depuration of DDts in the food chain from Artemia to Brook trout (Salvelinus fontinalis). Bull Environ Contam Toxicol 56: 888-895