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Acute Lethal Toxicity of the Organophosphorus Pesticide Chlorpyrifos to Different Species and Strains of *Artemia*

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Presently, organophosphorus pesticides (OPs) have largely replaced organochlorine compounds in the agricultural activities. Total turnover of OPs has increased around the world. Their residues have been detected in ground and drinking waters (Barceló, 1993), natural surface waters (Hernández et al., 1996), marine organisms (Barceló et al., 1990) and food products (Gunderson, 1995; Dejonkeheer et al., 1996). Chlorpyrifos[O,O-diethyl-O-(3,5,6-trichloro-2-pyridil) phosphorothiate, CAS RN 2921-88-2, Dursban, Lorsban, Spannit] is a widely used organophosphorus pesticide in the countries of the European Union (>50000 kg per year) (UNEP 1991). It is classified as probable or transient leacher (Barceló, 1993) according to its half-life time in soil and its adsorption coefficient (Gustafson, 1989).

LC50 values for a number of OPs in molluscs, crustaceans and fish have been reported (Persoone et al., 1985; Serrano et al., 1995), showing that, in general, organophosphorus pesticides present a high variation in both the toxicity of the different pesticides, and in the effect of a given pesticide on different species. Many assays involving several species and effects have been proposed as indicators of environmental damage. Among these, the brine shrimp *Artemia* has been recommended to test the acute lethal toxicity of different toxicants in sea waters due to its availability as cysts, and the development of a standardized short term bioassay with nauplii (Vanhaecke et al., 1980; Vanhaecke and Persoone, 1984; Persoone and Wells, 1987). However, the use of *Artemia* as a test organism in aquatic toxicology is quite recent, and despite the fact that the number of bioassays for general toxicity of organic compounds involving *Artemia,* is now increasing widely (Blizzard et al, 1989, Barahona and Sánchez-Fotún, 1996, Hlywka et al, 1997), there are still relatively few studies. The majority of these studies regard to *Artemia franciscana,* autochthonous from the American continent which, in many cases has been improperly named *A. salina* (Vanhaecke et al., 1980; Persoone and Wells, 1987; Verriopoulos et al., 1986; Crisine et al., 1994; Barahona and Sanchez-Fortún, 1994, 1996), inducing a high degree of confusion.

The genus *Artemia* is composed of a number of sibling species. At least five bisexual species and several parthenogenetic populations have been identified (Browne and McDonald, 1982). Since differences in biology and physiology have been found among species or strains of the genus, it is postulated that these different forms may have different sensitivity to the same toxicants. Comparative studies of this kind are very scare for OPs compounds, so this investigation was undertaken in an attempt to calculate the LC50-24h values of chlorpyrifos for nauplii (Instar II-III, Vanhaecke and Persoone, 1984) of 16

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strains of *Artemia* belonging to 4 species, by means of static acute toxicity tests. The sensitivity of their nauplii was compared in order to know the variability of different species and strains of *Artemia* as response to toxicant exposure and as a first step to discern the evolution of sensitivity of different *Artemia* species and strains to several chemicals in their life history. At the same time, it warns about the indiscriminate use of any source of *Artemia.*

MATERIALS AND METHODS

Artemia nauplii hatched from cysts of the following species were used:
- Bisexual; *A. salina* (= *A. tunisiana*), *A. persimilis* and *A. franciscana.*
- Parthenogenetic: *A. parthenogenetica* diploid (PD) and *A. parthenogenetica tetraploid* (PT). Full details are given in Table 1.

Cysts were hatched in sea-water (38 g/L) at 28 °C under conditions of continuous illumination and aeration. After hatching, the nauplii were separated from their shells, and any remaining unhatched cysts were discarded. Each batch of newly hatched nauplii was washed thoroughly in filtered (0.2pm Whatman WCN/filters) sea-water, transferred to clean filtered sea-water and then acclimated to 20°C for 24h in an incubator under conditions of continuous illumination and aeration before the acute toxicity test was performed.

The 24h lethality bioassay was performed using the procedure described in the ARC-Test (Vanhaecke and Persoone, 1984) Coming 24 well plates were used for the assay. Chlorpyrifos (purity 99%) was purchased from Dr. Ehrenstorfer Reference Materials (Germany). Because of the low solubility of chlorpyrifos in sea-water a stock solution of pesticide (10,000 mg/L) in acetone was prepared. After preliminary tests, the nominal concentrations chosen were: 0.1, 0.56, 1, 5.6, 10 and 18 mg/L. The different concentrations of chlorpyrifos were prepared in volumetric flasks using filtered sea-water and an appropriate quantity of the stock solution, except for the 0.1 and 0.56 mg/L concentrations for which a 10 mg/L stock solution prepared in acetone was used. Actual concentrations were checked (Table 2).

Triplicate liquid-liquid extractions with dichloromethane (100+50+50 mL) were carried out to extract chlorpyrifos from sea water. Water residues in the extract were eliminated with anhydrous sodium sulfate (pesticide residue analysis quality, Baker). After concentrating with Kuderna Danish, the extract was dried under a gentle nitrogen stream and then dissolved in n-hexane for injection in a Gas Chromatograph (GC) (Hernández et al., 1993). Retention time of chloryrifos was 14.548 min at indicated chromatographic conditions. The recoveries of chlorpyrifos from spiked sea water at 1 mg/L was 90 ± 6 %. The limit of detection was less than 0.1 µg/L. GC analysis were performed on a Hewlett Packard 5890 series II (Avondale, USA) with nitrogen-phosphorus detector, equipped with an HP 7673 autosampler. On column injections of 2µL 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 0.25 mm ID and a film thickness of 0.33 mm. Helium was used as carrier gas at a flow of 0.5 mL/min as well 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 by means of external standard method, by comparison of area units of chlorpyrifos standard (known

Table 1. Species name, ploidy, code, geographic origin and habitat of the different populations of *Artemia* studied.

Species name	Ploidy	Code	Geographic Origin	Habitat
Bisexual populations				
A. salina (*A. tunisiana*)	diploid	LMT(B)	Torrevieja (Alicante), Spain	Coastal saline
A. salina (*A. tunisiana*)	diploid	FP	Málaga, Spain	Inland saline
A. salina (*A. tunisiana*)	diploid	AT	Megrine, Tunissia	Coastal saline
A. salina (*A. tunisiana*)	diploid	ARG	Chott Garaet et Tarf, Argelia	Inland saline
A. persimilis	diploid	A	Hidalgo, Argentina	Inland saline
A. franciscana	diploid	A.F	San Francisco, CA, USA	Coastal saline
A. franciscana	diploid	GSL	Utah, USA	salt lake
A. franciscana	diploid	B	Mossoró, Brasil	coastal saline
Parthenogenetic populations				
A. parthenogenetica	diploid	LMT(PD)	Torrevieja (Alicante), Spain	coastal saline
A. parthenogenetica	diploid	G	Gerri (Lérida), Spain	inland saline
A. parthenogenetica	diploid	BMT	Bonmatí (Alicante), Spain	coastal saline
A. parthenogenetica	diploid	K	Bjurliu Lake, Kazakstan	salt lake
A. parthenogenetica	diploid	CH	Xiao Tan (Shandong), China	coastal saline
A. parthenogenetica	tetraploid	D	Delta del Ebro (Tarragona), Spain	coastal saline
A. parthenogenetica	tetraploid	O	Olmeda (Guadalajara), Spain	inland saline
A. parthenogenetica	tetraploid	P	Pétrola (Albacete), Spain	salt lake

concentration similar to that in the sample) and chorpyrifos in sample.

Table 2. Nominal and actual concentration of chlorpyrifos used in the acute toxicity tests

Chlorpyrifos concentration in water (mg/L)	
Nominal	Actual
0.1	0.09
0.56	0.54
1	0.8
5.6	5.5
10	7

Each acute test was carried out with six different concentrations of the pesticide plus two controls, one control with filtered sea-water and another with acetone at the highest pesticide concentration tested (18 mg/L, chlorpyrifos precipitates in seawater above this concentration). Each multiwell plate was filled with 1 mL of the appropriate concentration of toxicant. A total of 30 nauplii (three replicates of 10 nauplii per well) were used for each concentration of the pesticide. The last row was left as intermediate transfer wells to avoid the dilution of the toxicant solutions when nauplii were introduced. At least four multiwell plates were used for each *Artemia* strain. Once filled, the plates were covered with parafilm and left in an incubator at 20 ± 0.5°C for 24h in darkness. Death of nauplii was used as the test end point. This was established by the total lack of movements during 10 seconds of observation under a dissection microscope (Vanhaecke and Persoone, 1984; Persoone and Wells, 1987).

Percentages of mortality at 24-h were converted to probits. The 24-h LC50 values were calculated by means of the regression probit module of SPSS Systems (SPSS Inc, 1989-1992). The data were log-transformed to eliminate the heterogeneity of variance among species. Mean LC50 of the log-transformed values were compared using analysis of variance (ANOVA) and Tukey's HSD test ($p \leq 0.05$) to determine whether significant differences existed among the different *Artemia* strains studied.

RESULTS AND DISCUSSION

The mean 24h-LC50 values obtained for the different *Artemia* species and populations studied are shown in Fig. 1, Some bisexual and parthenogenetic populations did not show a concentration/response relationship and did not attain 50% mortality at the highest concentration tested. Their LC50 are thus reported as > 18 mg/L. The mean LC50 values for the bisexual populations were: 0.95, 1.86, 3.19, 5.12 and 8.45 mg/L for AT, FP, LMT(B), ARG and A, respectively. For the parthenogenetic populations BMT, CH and P the mean LC50 were 1.99, 4.51 and 10.95 mg/L, respectively. Significant differences (ANOVA, $p \leq 0.05$) were detected in the LC50 means of the different strains of *Artemia*. Tukey's HSD test (*a posteriori*, $p \leq 0.05$) indicated that the bisexual strain AT was the most sensitive to chlorpyrifos and significantly different from the other strains. The sensitivity to chlorpyrifos varied in the order AT > FP = BMT > LMT(B) = CH = ARG > A = P

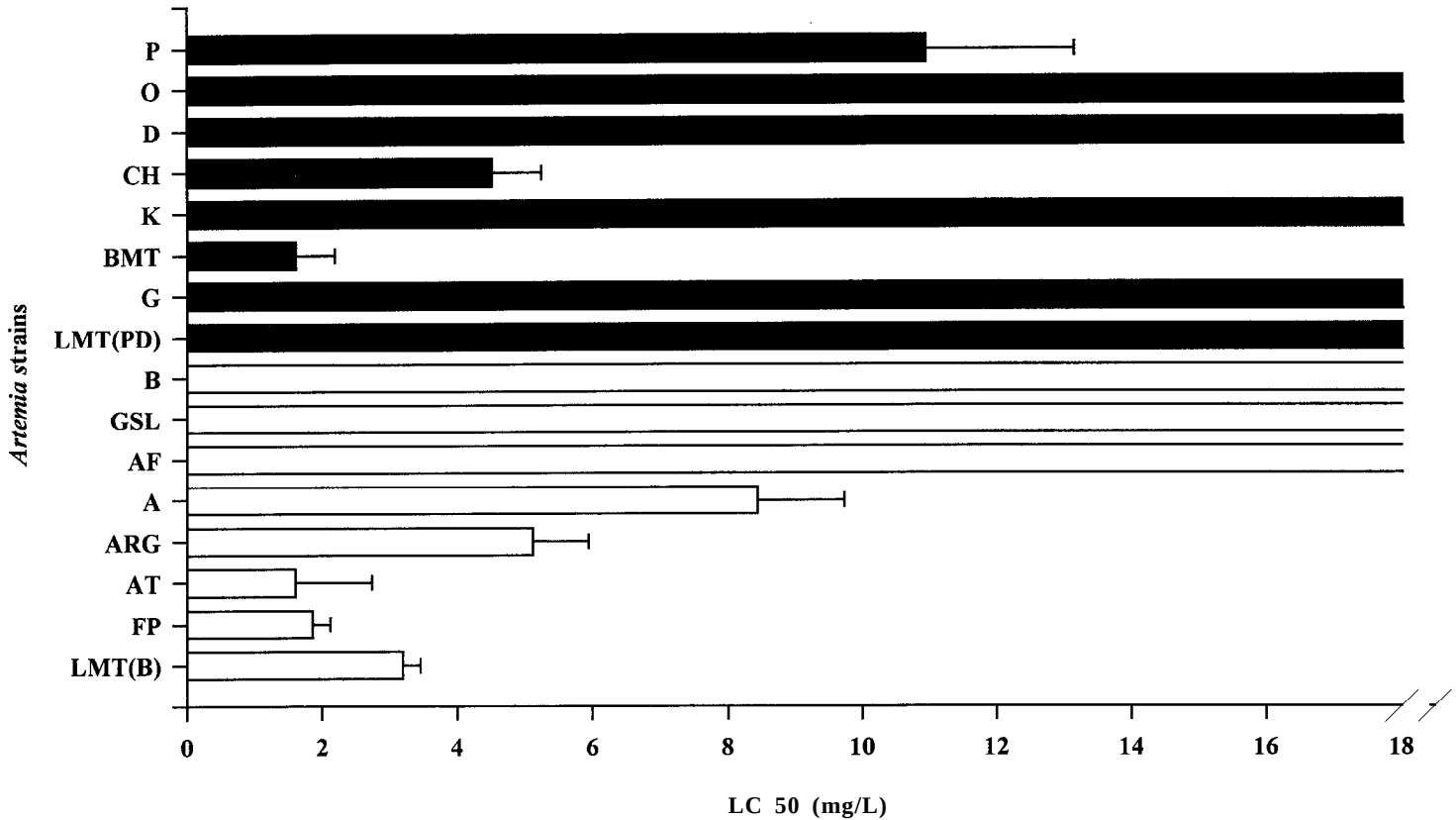

Figure1. LC50-24h values obtained for different *Artemia* strains . Black bars: parthenogenetic strains. White bars: bisexual strains. The broken axis represents that maximum values were over 18 mg/L

The nauplii from the different populations analysed showed different responses to the toxicant and a high degree of inter- and intra-specific variability. The three strains of *A. franciscana* and the five *A. parthenogenetica* ones did not show 50 % mortality below the maximum concentration tested (18 mg/L). Analogous response was found in nauplii of *A. franciscana* exposed to some antibiotics (Migliore et al,1997). In general, with the exception of *A. persimilis* (A), partehenogenetic strains as well as North American bisexuals seem to be more resistant.

Resistance to chlorpyrifos in *Artemia* does not seem to be species-specific. The same *A. salina* from different Spanish and Mediterranean basin biotopes showed different LC50 values (LMT (B) and FP). The same was true for *A. salina* from Tunissia and Argelia, and for some parthenogenetic forms (LMT (PD) and BMT). This indicates that the degree of resistance to the toxicant may not be only genetic. On the other hand, different species inhabiting the same biotope show different resistance (LMT (B) and LMT (PD)).

No correlation was found between the coastal-inland nature of the waters and brines in which the cysts were produced and the resistance to the toxicant. A high range of LC50 values for the strains tested was observed. Vanhaecke et al. (1980) using other toxicants (sodium lauryl sulphate and potassium dichromate) reported less variability for 7 different populations belonging to three species of *Artemia.* However, these authors also found that the parthenogenetic strains were more resistant than the bisexuals. Working on some of the *same* species of *Artemia* used in this study *Sarabia et al.* (1997) have also reported that parthenogenetic strains are more tolerant to cadmium than the bisexuals ones, with the exception of the bisexual *A. persimilis*. In contrast, Browne (1980) found that parthenogenetic strains were less tolerant to copper sulphate. In partial agreement with the results reported here, Varó et al. (1997) have shown high differences in the LC50 of three species of *Artemia* tested for resistance to the organochlorine pesticide endosulfan, with a parthenogenetic diploid strain being less sensitive than the other two, tetraploid and bisexual, respectively.

The 24 hr toxicity tests of the different *Artemia* species and strains showed some variability. The coefficient of variation in the bioassays using *Artemia* ranged from 8.5% (LMT(B)) to 20% (P). Variability due to differences in sensitivity between sexual and asexual species, as well as among intra-strains and clones of the same species has been reported for other aquatic invertebrates used in ecotoxicological studies (Baird et al, 1990; Moller et al, 1996). Interlaboratory coefficients of variation around 35 % have been obtained for *Daphnia magna* and *D. Pulex* exposed to reference toxicants in acute tests (U.S. EPA, 1985). Similar variations were found by Pakhurtst et al (1992) for a variety of species. These authors reported coefficients of variation with mean values of 33% and 46% in intra- and inter-laboratory acute tests of single chemicals respectively.

The differences in tolerance to chlorpyrifos found in this study among species and within strains of the same specie is similar to those obtained in previous work with *Artemia* (Vanhaecke et al.,1980, Sarabia et al. 1997, Varó et al. 1997). This results are in agreement with the observations of Moller et al (1996) who found differences between closely related sexual and asexual species of estuarine gastropods in response to acute exposure to cadmium. Our data are also consistent with the general purpose genotype hypothesis (Lynch, 1984) in that, parthenogenetic species have been considered to be better adapted to environmental stress, with broad tolerance range to environmental perturbations

and a higher generalized genotype than their closely related bisexuals (Lynch, 1984; Bierzychudek, 1989).

Although at present Artemia is not widely used in applied aquatic toxicology, its utilisation as a test organism could be very important based on the commercial availability of cysts. The cysts are an off-the-shelf product from which it is easy to obtain test organisms, i.e. the nauplii, offering a broad response to toxicants. From another point of view, and in parallel with their use as live preys in aquaculture, well characterized nauplii and adults exposed to toxicants could be used as live toxic vehicles in an artifical (experimental) trophic chain

Our results show that all the *Artemia* strains and populations do not show the same sensitivity to the same toxicant. This fact offers the possibility of using different *Artemia* strains, with different degrees of sensitivity before the same toxicant, in aquatic ecotoxicological tests.This variability may be important to characterize the different species and strains of *Artemia* and potentially useful in the study of the mechanism of response to toxicant exposure. On the other hand, the use of different *Artemia* strains with different sensitivity could be an advantage in the evaluation of the toxicity of hazardous compounds in marine and saline environments.

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