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Comparative Sensitivity of Three Age Classes of *Artemia salina* Larvae to Several Phenolic Compounds

M. V. Barahona, S. Sánchez-Fortún

Department of Toxicology and Pharmacology, Faculty of Veterinary Medicine, Complutense University of Madrid, 28040 Madrid, Spain

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Phenolic compounds have been used because they are among the most toxic and ubiquitous environmental contaminants present in many industrial wastes. It is recognized that factors such as water hardness, pH, temperature, chemical formulation, species, age and/or stage of development of test organisms, to mention only a few, may critically affect the behavior of a chemical and thus effect the outcome of toxicity tests (Canton and Adema 1978; Berglind and Dave 1984; Persoone et al. 1989). Several studies dealing with the susceptibility of early life stages of invertebrates to pollutants have been reported in the literature (Middaugh and Dean 1977; Conklin and Rao 1978; Kaur and Dhawan 1993). An extensive literature review revealed information on the toxicity of different phenolic compounds to some species of fish and invertebrates. However, there is little information on acute toxicity of these compounds to different age classes of *Artemia salina* larvae. *Artemia salina* are of major importance in the aquaculture industry and they have been proposed as a uniform world-wide test system for toxicity of chemical substances (Vanhaeke et al. 1981) and for studies in developmental toxicology (Sleet and Brendel 1985). Previous investigations in this laboratory have shown that *Artemia salina* larvae exhibit increased sensitivity to certain chemicals in relation to aging.

The present study was conducted to determine the acute toxicity of some phenolic compounds (pentachlorophenol (PCP), 2,6-dichloroindophenol (2,6-DCIP), 2,4-dinitrophenol (2,4-DNP), o-nitrophenol (o-NP), p-nitrophenol (p-NP), diamidophenol, diaminophenol and 2,6-dimethylphenol(2,6-DMP)) on *Artemia salina* 24-, 48- and 168-hr old to determine if changes in sensitivity occur during the first week after emergence.

MATERIALS AND METHODS

A strain of *Artemia salina* provided in encysted eggs in dry state by San Francisco Bay Brand, Inc. (Division of Metaframe Co., Menlo Park, CA, USA) was used as the test animals.

Correspondence to: M. V. Barahona

The method of Persoone et al. (1989), to obtain *Artemia salina* for the test was applied and modified according to the following procedure. For this purpose, the encysted brine shrimp of species *Artemia salina* were obtained from 100 mg of eggs. They were hydrated in distilled water at 4°C for 12 hr, followed by washing to separate the cysts that sink from those that float. The cysts that sank were collected on a Büchner funnel and washed with cold distilled water, followed by synthetic seawater. Synthetic seawater was prepared by mixing 35 ‰ of Synthetica sea salt (Waterlife Research Ltd., England), with distilled and deionized (Milli-Q) water, stirring for 24 hr with suitable aeration and successive filtration with thick cellulose filters. The cysts were then incubated in a graduated glass cylinder for about 24 hr at 25°C with continuous side illumination (15-W fluorescent lamp), in 100 mL of synthetic seawater, at pH 8.6, and with a slight aeration maintained by a small tube in contact with the botton of the cylinder. The nauplii produced were aspirated with Pasteur pipets and transferred to two glass flasks containing 200 mL of the synthetic seawater, and maintained for another 24- or 144- hr.

Eight phenolic compounds including pentachlorophenol (PCP), 2,6-dichloroindophenol (2,6-DCIP), 2,4-dinitrophenol (2,4-DNP), o-nitrophenol (o-NP), p-nitrophenol (p-NP), diamidophenol, diaminophenol and 2,6-dimethylphenol(2,6-DMP) were tested for their toxic effects on *Artemia salina*. Phenolic compounds were dissolved in DMSO and appropriate stock solutions were prepared for each test phenolic compounds. DMSO and phenolic compounds analytical grade were obtained from Sigma Chemical Company (St. Louis, USA).

For toxicity testing, samples of 10 larvae each were added to 1 mL of synthetic seawater in plastic 16-mm petri dishes containing the appropriate volume of phenolic compound. Each test consisted of exposing groups of 10 *Artemia salina* aged 24-, 48- and 168- hr to various concentrations of phenolic compound tested, and the test was replicated four times. Each age group was exposed to the phenolic compound during 12- or 24- hr period. Each phenolic compound concentration was set in sextuplicate, and the range of phenolic compound concentration tested are expressed in mg/L. Refer to the nominal concentrations at the onset of the experiments. Appropriate controls were included in each experiment; one of the untreated controls was exposed to the solvent used to solubilize the phenolic compounds; in all cases the concentration of DMSO never exceeded a final concentration of 1 ‰ , which was non-toxic. The other control consisted of a single synthetic seawater. The larvae were then incubated at 25°C in the dark.

The acute toxicity of the eight phenolic compounds to *Artemia salina*, acting individually, was estimated by determination of the 12- or 24 hr-LC_{50} (concentration of the phenolic compound which kills 50 % of the test animals after 12- or 24-hr exposure). Larvae were considered dead if they did not exhibit any internal or external movement during 10 sec of observation.

The 12- or 24 hr-LC_{50} values, with 95 % confidence limits, were calculated according to Litchfield and Wilcoxon method (1949) implemented in the

Pharmacologic Calculation System (PCS version 4.0, New York). These values were subjected to a two-way analysis of variance with replication within the subgroups (ANOVA), followed by post hoc contrast with Newman-Keuls Test.

RESULTS AND DISCUSSION

The acute toxicity of the eight phenolic compounds tested to *Artemia salina* larvae was influenced by duration of exposure (Table 1).

Table 1. LC_{50} values, in mg/L (95% CL, n=6 bioassays) for *Artemia salina* 24-hr old and phenolic compounds at different times of exposure.

The comparison of LC_{50} values in relation to time of exposure showed significant differences between 12- and 24-hr. PCP, o-NP, p-NP, 2,6-DMP, diamidophenol and diaminophenol are 1-3 times more toxic to *Artemia salina* 24-hr old for 24-hr exposure than for 12-hr, while 2,6-DCIP and 2,4-DNP are 6-7 times more toxic

	LC_{50}, mg/L Exposure duration (hr)	
	12	24
PCP	12.1 (8.3-17.8)	3.6[a] (2.6-5)
2,4-DNP	24 (20-28.9)	3.4[a] (1.6-7)
o-NP	11 (9.4-12.8)	6.5[a] (5.2-8)
p-NP	27 (23.5-30.9)	22.1[a] (18.6-26.4)
2,6-DCIP	20.1 (15.3-26.5)	3.3[a] (2-5.6)
diaminophenol	20.2 (16.1-25.3)	14.7[a] (11.4-19.1)
diamidophenol	15.8 (13.2-38.2)	10.4[a] (8.6-12.5)
2,6-DMP	15.3 (13.2-17.6)	11.2[a] (10.3-12.1)

PCP=pentachlorophenol; 2,4-DNP=2,4-dinitrophenol; o-NP=o-nitrophenol; p-NP=p-nitrophenol; 2,6-DCIP=2,6-dichloroindophenol; 2,6-DNP=2,6-dimethylphenol.

[a] Significantly different ($p<0.01$) from 12-hr LC_{50} for *Artemia salina* 24-hr old.

to this organism for 24-hr exposure than for 12-hr. Most investigators agree that there is a tendency for the LC_{50} to decrease with longer exposures (Stephenson et al. 1991).

The influence of age of test organism on the toxicity of each phenolic compound was evaluated (Table 2). *Artemia salina* 48-hr old is significantly less tolerant to these compounds than *Artemia salina* 24-hr old. There is one exception where 2,6-DCIP is equally toxic to three age classes of *Artemia salina* tested at exposure duration of 24-hr. *Artemia salina* 48-hr old is 10-15 times less resistent to PCP, 2,4-DNP, p-NP and diamidophenol than was *Artemia salina* 24-hr old. On the other hand, *Artemia salina 48-hr old is 3-6 times less tolerant to o-NP and 2,6-DMP than is Artemia salina 24-hr old.*

Table 2. 24 hr-LC_{50} values in mg/L (95% CL, n=6 bioassays) for eight phenolic compounds tested against *Artemia salina* expressed in mg/L.

	LC_{50}, mg/L		
	24 hr	48 hr	168 hr
PCP	3.6 (2.6-5)	0.3[Ó] (0.2-0.5)	0.3[Ó] (0.1-0.8)
2,4-DNP	3.4 (1.6-7.1)	0.2[Ó] (0.2-0.3)	0.1[Ó] (0.1-0.2)
o-NP	6.5 (5.2-8)	2.1[a] (1.3-3.3)	0.9[a] (0.3-2.3)
p-NP	22.1 (18.5-26.3)	2.4[a] (1.9-2.9)	0.6[a] (0.5-0.8)
2,6-DCIP	3.3 (2-5.6)	2.3 (2.1-2.5)	0.5 (0.4-0.6)
diaminophenol	14.7 (11.3-19)	2.2[a] (1-4.8)	0.7[a] (0.5-1)
diamidophenol	10.4 (8.6-12.5)	0.7[a] (0.2-2)	0.5[a] (0.2-1.6)
2,6-DMP	11.2 (10.3-12.1)	2.2[a] (0.9-5.2)	0.5[aß] (0.4-0.6)

PCP=pentachlorophenol; 2,4-DNP=2,4-dinitrophenol; o-NP=o-nitrophenol; p-NP=p-nitrophenol; 2,6-DCIP=2,6-dichloroindophenol; 2,6-DNP=2,6-dimethylphenol.

[a] Significantly different ($p<0.01$) from 24-hr LC_{50} for *Artemia salina* 24-hr old.

[Ó,ß] Significantly different ($p<0.05$) from 24-hr LC_{50} for *Artemia salina* 24- and 48-hr old, respectively.

There are no statistically significant differences between the toxicities of selected phenolic compounds to *Artemia salina* 48- and 168-hr old. The sole exception to this general result is observed in the toxicity of 2,6-DMP. There are statistically significant differences among the toxicity of 2,6-DMP to different ages of *Artemia salina* larvae. *Artemia salina* larvae aged 168-hr are 4.76 and 24.30 times more sensitive for this compound than *Artemia salina* larvae 24- and 48-hr old, respectively. Adema (1978) concluded from the experiments with *Daphnia magna* and pure PCP that 7-day daphnids were as sensitive or slightly less sensitive as 1-day old daphnids. These data are comparable to those found in this work with *Artemia salina* larvae.

Analysis of 24-hr LC_{50} values revealed that the sensitivity of three age classes of *Artemia salina* to PCP, 2,4-DNP, o-NP, p-NP, diaminophenol and diamidophenol is in the order of 168-hr = 48-hr > 24-hr old. In the case of 2,6-DCIP it was 168-hr = 48-hr = 24-hr and in the case of 2,6-DMP it was 168-hr > 48-hr > 24-hr old.

Other authors have studied the relative sensitivity to several compounds of different stages of the life cycle in crustaceans. Sleet and Brendel (1985) reported that with cadmium and mercury, LC_{50}s decreased as *Artemia* nauplii aged and developed; whereas with azide LC_{50}s did not vary. Bookhout and Monroe (1977) have reported a higher susceptibility of the early zoea stages in *Callinectes sapidus* exposed to malathion. Epifanio (1971) encountered the same relative sensitivity among larvae stages of *Leptodius floridanus* exposed to dieldrin. Buchanan et al. (1970) showed that the early juvenile stages of *Cancer magister* were more sensitive to Sevin than the more advanced ones, the latter being very similar in their resistence when compared with adults.

Our results indicate that *Artemia salina* larvae aged 48-hr appear to be one of the more sensitive ages to these phenolic compounds. This is consistent with the finding that *Artemia salina* aged 48-hr is more sensitive to some organic solvents and pesticides than other ages of this crustacean (Barahona and Sánchez-Fortún 1994; Sánchez-Fortún and Barahona 1995). These data indicate that 48-hr of age would be a suitable choice for bioassays involving *Artemia salina* larvae. Stephenson et al. (1991) conducted tests on three age classes (young, juvenile and adult) of *Daphnia magna* and found that pure pentachlorophenol was equally toxic to all age classes of *Daphnia magna* but susceptibility to technical pentachlorophenol decreased with maturation.

There are considerable variations among the three age classes in relative order of toxicity for phenolic compounds. However 2,4-DNP and PCP are the two most toxic compounds for the three age classes of *Artemia salina*. p-NP is the least toxic to *Artemia salina* aged 24- and 48-hr old, while that for *Artemia salina* 168-hr old is o-NP.

In comparison with other test species currently used in aquatic toxicology, *Artemia salina* is in some cases more sensitive and in other less sensitive, depending on

the compound and species compared. *Artemia salina* 48- and 168-hr old are more sensitive to PCP than rotifers like *Brachionus calyciflorus* with a 24-hr LC_{50} of 2.16 mg/L (Ferrando et al. 1992). With respect to fishes, the resistence of both ages of *Artemia salina* is higher. The 96-hr LC_{50} was 0.083 mg/L for *Notopterus notopterus*, 115 µg/L for *Oncorhynchus mykiss*, 68 µg/L for *Oncorhynchus tshawytscha* and 54 µg/L for *Salmo trutta* (Verma et al. 1981). The generalization that crustaceans are less sensitive than fish to PCP is supported by this work. However, we find that *Artemia salina* 48- and 168-hr are more sensitive to 2,4-DNP than are fishes. The 96-hr LC_{50} for this toxicant was 1.34 mg/L for *Notopterus notopterus*, 0.70 mg/L for juvenile Atlantic salmon and 18 mg/L for rainbow trout eggs (Verma et al. 1981).

Ferrando et al. (1992) determined a 24-hr LC_{50} of 0.39 mg/L for *Daphnia magna* exposed to PCP and Keller (1993) found a 48-hr LC_{50} of 0.33 mg/L for this species. Thus *Artemia salina* and *Daphnia magna* appaer to have similar sensitivity to PCP. The 48-hr LC_{50} estimates for adult daphnids of three species (*D. magna*, *D. pulex* and *D. galeata*) exposed to pure PCP were 1.78, 4.59 and 0.51 mg/L respectively (Stephenson et al. 1991); these results suggest that *Artemia salina* 48- and 168-hr old are 6-15 times less resistent to this compound than *Daphnia magna* and *Daphnia pulex* while a minor difference was encountered between *Artemia salina* and *Daphnia galeata*.

The median lethal concentration (LC_{50}) for PCP to *Artemia salina* are much higher than those registered in the literature for sodium pentachlorophenate (Na-PCP) in other species of aquatic crustaceans like *Crangon crangon, Palaemons varians* and *Pdaemonetes pugio*. According to Van Dijk (1979) the lethal concentrations of Na-PCP for three species of decapod crustaceans and their larvae vary between 1.79 and 10.39 ppm and 0.084-0.363 ppm, respectively. Conklin and Rao (1978) found that the sensitivity of adult *Palaemonetes pugio* varied with the stage in the molt cycle and at the most sensitive stage the 96-hr LC_{50} value of Na-PCP was 0.436 ppm.

The present study emphasizes the need for utilization of differing developmental stages and/or age of test species and many species of taxonomically and ecologically divergent groups in order to evaluate potentially hazardous compounds. There are many types of toxicity tests, a basic toxicity evaluation should include both acute and chronic studies. Standarized acute toxicity tests are often the first step in an evaluation program. Obviously, chronic tests are needed in order to examine the effects of longer exposures to sub-acute concentrations of the organism test on maturation, growth, reproduction and survival, and often have more ecological relevance than acute toxicity data.

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