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# Acute Sensitivity of Three Age Classes of *Artemia salina* Larvae to Seven Chlorinated Solvents

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Chlorinated organic solvents are used widely in industry and research laboratories for such purposes as degreasing operations, dry cleaning, paint stripping and extraction solvents as well as intermediate chemicals for the synthesis of other chemicals. The major releases of chlorinated solvents to the environment are during their production and use in the manufacture of polymers. Due to their volatile nature, the majority of releases are expected to be to the air. Small amounts of chlorinated solvents may be released to water and land in industrial effluents and during disposal to solid wastes. Moreover these chlorinated solvents have been found to occur in ground water. Acute toxicity tests with single species are still the first line of attack when assessing the toxicity of chemicals and they remain the primary means of estimating potential damage or impact to the environment (Cairns 1983). It is recognized that many factors, including age and/or stage of test organism may critically affect the behavior of a chemical and thus affect the outcome of toxicity tests ( Canton and Adema 1978; Berglind and Dave 1984; Persoone *et al.* 1989; Stephenson *et al.* 1991). Previous investigations indicate that earlier life stages of invertebrates are more sensitive than later ones (Epifanio 1971; Conklin and Rao 1978; Bodar *et al.* 1989). *Artemia salina* has gained popularity as a test organism for short-term toxicity testing because of its ease of culture, short generation time, cosmopolitan distribution and the commercial availability of its dormant eggs (cysts). These cysts were suggested as an attractive alternative to standard invertebrate stock cultures since test animals can be hatched synchronously and the resulting cohort of neonates emerges in a uniform physiological condition (Persoone *et al.* 1989).

The present study measures the sensitivity of *Artemia salina* to seven chlorinated solvents (methylene chloride or dichloromethane, 1,2-dichloroethane, 1,2-dichloroethylene, l,l,l-trichloroethylene, 1,1,1-trichloroethane, 1,1,2,2-tetrachloroethylene or perchloroethylene and 1,1,2,2-tetrachloroethane) at specific ages. Three age groups of larvae, 24-, 48- and 72-hr old, were tested to determine if changes in sensitivity occur during the first days after emergence. It is part of a continuing series of investigations conducted by this laboratory on toxicological hazard of selected pollutants to the marine crustacean *Artemia salina.*

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## 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.

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. They 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 aspired with Pasteur pipettes and transferred to two glass flasks containing 200 mL of the synthetic seawater, and maintained for another 24- or 48-hr.

Seven chlorinated organic solvents, including methylene chloride (dichloromethane) 1,2-dichloroethane, 1,2-dichloroethylene, 1,1,1-trichloroethylene, l,l,l-trichloroethane, 1,1,2,2-tetrachloroethylene (perchloroethylene) and 1,1,2,2-tetrachloroethane were tested for their toxic effects on *Artemia salina.* Chlorinated solvents were dissolved in ethanol and appropriate stock solutions were prepared for each test chlorinated solvent. Ethanol and analysis grade chlorinated solvents 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 chlorinated solvent. Each test consisted of exposing groups of 10 *Artemia salina* aged 24-, 48- and 72-hr to various concentrations of organic solvent tested, and the test was replicated four times. Each age group was exposed to the solvent during 24-hr period. Each solvent concentration was set in sextuplicate, and the range of chlorinated solvent concentration tested is expressed in mmol/L. Exposure concentrations were nominal values. Appropriate control systems were included in each experiment. One of the untreated controls was exposed to ethanol which was used to solubilize the chlorinated solvents; in all cases the concentration of ethanol never exceeded a final concentration of 1 ‰, which was non-toxic. The other control consisted of a single synthetic seawater. The plates were then incubated at 25°C in the dark.

The acute toxicity of the seven chlorinated solvents to *Artemia salina,* acting individually, was estimated by determination of the 24 hr-$LC_{50}$ (concentration of the

chlorinated solvent which kills 50 % of the test animals after 24-hr exposure). Larvae were considered dead if they did not exhibit any internal or external movement during 10 sec of observation.

The 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 24 hr-$LC_{50}$ values for three age classes of *Artemia salina* for all tested chlorinated solvents are listed in Table 1.

**Table 1.-** 24 hr-$LC_{50}$ values (95 % CL, n=6 bioassays) for seven chlorinated solvents tested against three age classes of *Artemia salina*, expressed in mmol/L.

| | | $LC_{50}$ (mmol/L) | | |
|---|---|---|---|---|
| | | Age Class of *Artemia salina* | | |
| | molecular weight | 24 hr | 48hr | 72hr |
| Methylene chloride | 84.94 | 1.44 (1.18-1.65) | 1.14 (0.92-1.38) | 1.03 (0.73-1.26) |
| 1,2-dichloroethane | 98.96 | 0.34 (0.29-0.39) | 0.13[a] (0.1-0.16) | 0.07[a] (0.05-0.11) |
| 1,1,1-trichloroethane | 133.42 | 0.85 (0.71-1.05) | 0.33[a] (0.3-0.36) | 0.06[ab] (0.04-0.09) |
| 1,1,2,2-tetrachloroethane | 167.85 | 0.07 (0.06-0.08) | 0.03[a] (0.02-0.06) | 0.005[ab] (0.004-0.006) |
| 1,2-dichloroethylene | 96.94 | 0.22 (0.2-0.23) | 0.1[a] (0.09-0.1) | 0.07[a] (0.03-0.14) |
| 1,1,1-trichloroethylene | 131.39 | 0.23 (0.19-0.27) | 0.04[a] (0.02-0.06) | 0.007[ab] (0.006-0.008) |
| 1,1,2,2-tetrachloroethylene | 165.85 | 0.14 (0.11-0.17) | 0.04[a] (0.02-0.07) | 0.002[ab] (0.002-0.003) |

[a]: Significantly different ($p<0.05$) from 24-hr $LC_{50}$ for *Artemia salina* 24-hr old.
[b]: Significantly different ($p<0.05$) from 24-hr $LC_{50}$ for *Artemia salina* 48-hr old.

There are considerable variations among the three age classes in relative order of

toxicity for chlorinated solvents. Comparisons of the $LC_{50}$ values indicate that 1,1,2,2-tetrachloroethane was the most toxic of the solvents tested to both *Artemia salina* 24- and 48-hr old, although 1,1,1-trichloroethylene, 1,1,2,2-tetrachloroethylene and 1,1,2,2-tetrachloroethane have similar $LC_{50}$ values to *Artemia salina* 48-hr old. However, 1,1,2,2-tetrachloroethylene replaced 1,1,2,2-tetrachloroethane as the most toxic of these solvents to *Artemia salina* 72-hr old.

On the other hand, methylene chloride, 1,2-dichloroethane and l,l,l-trichloroethane are the three least toxic solvents for all age groups of *Artemia salina.* Calleja *et al.* (1994) also tested l,l,l-trichloroethane and dichloromethane with the standard operational procedure of the Artoxkit M *(Artemia salina)* and they found a 24 hr-$LC_{50}$ of 30,600 and 12,300 µmol/L respectively. Their reported 24-hr $LC_{50}$ values for both chlorinated solvents are higher to those exhibited for three age classes of *Artemia salina* in the present study, which may be due to differences in bioassay techniques and purity of solvents. In comparison with other test species used in aquatic toxicology, *Artemia salina* is in some cases more sensitive and in other cases less sensitive, depending on the compound and species compared. *Artemia salina* appears to have similar sensitivity to methylene chloride when compared to other saltwater organisms such as the decapod crustacean, *Palaemonetes pugio* and the estuarine fish *Fundulus heteroclitus.* The 48 hr-$LC_{50}$ for methylene chloride was 108.5 (92.37-130.90) mg/L for juvenile grass shrimp and 97.0 (89.44-105.14) mg/L for juvenile killifish (Burton and Fisher 1990). Although, this chlorinated solvent was more toxic to *Artemia salina* than other test organisms such as *Mysidopsis bahia, Pimephales promelas* and *Cyprinodon variegatus.* A 96 hr-$LC_{50}$ of 256 mg/L and 310 mg/L has been reported for the mysid shrimp and the fathead minnow respectively (LeBlanc 1984; Alexander *et al.* 1978). Heitmuller *et al.* (1981) obtained 24-, 48- and 96-hr $LC_{50}$ of 370, 360 and 330 mg/L respectively for the sheephead minnow. With respect to the freshwater crustacean *Daphnia magna,* the sensitivity of three ages of *Artemia salina* larvae is higher. Lilius *et al.* (1994) determined a 24 hr-$EC_{50}$ of 22.86±0.85 mM for Daphnia magna exposed to methylene chloride.

Alexander *et al.* ( 1978) determined a 96 hr-$LC_{50}$ of 105 mg 1,1,1-trichloroethane/L, 21.4 mg 1,1,2,2-tetrachloroethylene/L and 66.8 mg l,l,l-trichloroethylene/L for *Pimephales promelas.* These results suggest that *Artemia salina* 24-h old was more resistent to both 1,1,1-trichloroethane and 1,1,2,2-tetrachloroethylene than *Pimephales promelas,* whereas 48- and 72-hr old larvae presented higher sensitivity than this fish. However, 1,1,1-trichloroethylene was found to be less toxic to *Pimephales promelas* than to all age classes tested of *Artemia salina.* Thompson and Carmichael (1989) found that the survival and reproduction of daphnids over a test period of 17 days were not affected at a measured concentration for l,l,l-trichloroethane of 1.3 mg/L. Thus, this chlorinated solvent was found to be less toxic to *Artemia salina* than to *Daphnia magna.*

Calleja *et al.* (1994) compared the sensitivity of the aquatic invertebrates used in the Toxkits *(Artemia salina, Streptocephalus proboscideus, Brachionus calyciflorus)* with the conventional acute *Daphnia magna* and Microtox™ *(Photobacterium*

*phosphoreum)* tests for 50 chemicals including chlorine-substituted aliphatic compounds. Their mean acute toxicity data indicated that there were some differences among the test organisms in the relative order of sensitivity to chemicals tested. The rank order of sensitivity to 1,1,1-trichloroethane was: *Photobacterium phosphoreum* (marine bacteria) > *Daphnia magna* (freshwater crustacean) > *Streptocephalus proboscideus* (freshwater crustacean) > *Artemia salina* (marine crustacean) > *Brachionus calyciflorus* (freshwater rotifer), while to methylene chloride it was *Daphnia magna* > *Artemia salina* > *Streptocephalus proboscideus* > *Brachionus calyciflorus* > *Photobacterium phosphoreum.*

The influence of age of test organism on the toxicity of each chlorinated solvent was evaluated. *Artemia salina* 48- and 72-hr old are significantly more sensitive than *Artemia salina* 24-hr old to these compounds. There is one exception where all three age classes tested were similar in their sensitivity to methylene chloride.

*Artemia salina* 48-hr old is 2-6 times less resistent to these chlorinated solvents than was *Artemia salina* 24-h old, while *Artemia salina* 72-hr old is 2-70 times more sensitive than *Artemia salina* 24-hr old.

On the other hand, *Artemia salina* 48- and 72-hr old were equally sensitive to 1,2-dichloroethane and 1,2-dichloroethylene, while *Artemia salina* aged 72-hr is 5-20 times more susceptible to 1,1,1-trichloroethane, l,l,1-trichloroethylene, 1,1,2,2-tetrachloroethane and 1,1,2,2-tetrachloroethylene than 48-hr old larvae.

Analysis of 24 hr-$LC_{50}$ values revealed that the sensitivity of three age classes of *Artemia salina* to 1,1,l-trichloroethane, 1,1,1-trichloroethylene, 1,1,2,2-tetrachloroethane and 1,1,2,2-tetrachloroethylene is in the order of 72-hr > 48-hr > 24-hr old. In the case of 1,2-dichloroethane and 1,2-dichloroethylene it was 72-hr = 48-hr > 24-hr old, and for methylene chloride it was 72-hr = 48-hr = 24-hr old.

Toxicity data for three age groups of *Artemia salina* larvae indicate that sensitivity to several chlorinated solvents differed with age. Previous studies have reported that *Artemia salina* larvae aged 48-hr appear to be one of the more sensitive ages when tested to several organophosphorous insecticides such as dichlorvos and coumaphos, organochlorine insecticides such as dieldrin and *p,p'*-DDT, organic solvents such as ethanol and phenolic compounds as pentachlorophenol, 2,4-dinitrophenol, *o*-nitrophenol, *p*-nitrophenol, diaminophenol, diamidophenol and 2,6-dimethylphenol (Barahona *et al.* 1994, Sánchez-Fortún *et al.* 1995; Barahona & Sánchez-Fortún 1996). 72-hr old *Artemia salina* larvae exhibited higher sensitivity than 48-hr old *Artemia salina* larvae for lindane and 2,6-dimethylphenol only. Otherwise, there was similar sensitivity for the 48- and 72-hr old *Artemia*. In the present study, the significantly greater sensitivity of *Artemia salina* 72-hr old to four of the seven solvents tested suggests that this age is sometimes a more sensitive indicator of several chlorinated solvents stress than *Artemia salina* 48-hr old. The influence of age of *Artemia salina* on the toxicity of different chemicals may be related to the fact that larval development is rapid and the three age classes of *Artemia salina* studied

correspond with different stages of development where remarkable changes in both morphological and functional characters are produced. These data reinforce the observation that different ages and/or stages of test organisms are required in order to evaluate in a satisfactory manner potentially hazardous compounds during acute and chronic exposures. Our results were in agreement with the findings of other investigators. Fischer and Hall (1992) determined that larval and immature stages of aquatic organisms were most susceptible to several contaminants due to the frequency of molting cycles. Their results from chronic field studies indicate that the susceptibility to different chemicals is also dependent on the timing of compound application in relation to an organism's molt cycle.

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