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Acute Toxicity and Inhibition of Phototaxis Induced by Benzalkonium Chloride in *Artemia franciscana* Larvae

M. C. Bartolomé, S. Sánchez-Fortún

Departamento de Toxicología y Farmacología, Facultad de Veterinaria, Universidad Complutense de Madrid, 28048 Madrid, Spain

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The brine shrimp *Artemia* is one of the most commonly used test organisms in toxicity studies. There are several reasons for this, including its continuous availability in the form of dry cysts, low cost, ease of use, and the fact that large numbers can be kept in small beakers (MacRae and Pandey 1991). Although there are many studies on the effects of toxic substances on *Artemia* (Persoone and Castritsi-Catharios 1989; Migliore et al. 1997), little information is available on their sublethal effects. A great many bioassay techniques have been developed in recent decades in response to a growing concern that too little is known about the effects of chemicals and wastewaters before they are released into natural aquatic systems. The task of assessing the effects of toxic substances involves the monitoring of complex biological systems at the individual and community level. The realization that no single method can fully evaluate how a substance affects the survival and normal functioning of aquatic biota resulted in the development of a series of protocols designed to provide a more systematic solution. At the level of the organism, toxicity testing has developed along two major lines: the lethal or acute bioassay, and the sublethal or chronic bioassay. While the methodology associated with the former is now highly standardized and widely used, chronic bioassays are still in the developmental stages. Sherer (1977) recommended the use of locomotory behavioural patterns for assaying toxicological effects. The circadian patterns of zooplankton migrations are particularly well suited to laboratory investigations: since these vertical movements are cued primarily by light, they are easy to stimulate. The response obtained is measured in terms of the distance travelled in a set time. The quaternary ammonium compound benzalkonium chloride (BKC) has been used as a broad-spectrum antimicrobial agent for disinfecting and preserving pharmaceutical and cosmetic products since the 1930s. BKC is widely used in aquaculture as a disinfectant and as a very effective herbicide. It has been reported to inhibit the growth of several species of microalgae, such as those of the genera *Chlorella*, *Tetraselmis*, *Chaetoceros* and *Isochrysis*, without significantly changing the concentrations of orthophosphate, ammonia or nitrite in the culture water (Lee et al. 1995). The concentration of BKC added varies from 0.5 to 2.0 mg/L (Lee et al. 1994). However, prawn farmers often use excessive amounts, and the possible effect of this on immune function and disease resistance is of some concern.

Correspondence to: S. Sánchez-Fortún

This paper reports the acute toxicity of BKC on *Artemia franciscana* at 24, 48 and 72 hr of age, and establishes the sensitivity ranking of these three stages. The inhibition of phototaxis induced by this compound in 24 hr-old brine shrimp is also examined.

MATERIALS AND METHODS

Benzalkonium chloride (>99% purity) was purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). *Artemia franciscana* cysts were purchased from Argent Chemical Laboratories, Washington, USA (Argentemia Silver Grade). The method of Persoone et al. (1989), modified according to Barahona et al. (1994), was used to obtain live *Artemia*. Briefly, encysted *Artemia* were hydrated in distilled water at 4 °C for 12 h, followed by washing to separate floating from sinking cysts. "Sinkers" were collected in a Buchner funnel and washed with cold distilled water followed by synthetic seawater. The latter was prepared by mixing 35 ‰ Synthetica Sea Salts (Waterlife Research Ltd., England) with distilled, deionised water (Milli-Q Corp., Bedford, MA, USA), stirring for 24 hr with suitable aeration, and filtering through 30 µm Millipore cellulose filters (Milli-Q Corp., Bedford, MA, USA).

Cysts were incubated in a graduated glass cylinder in 100 mL of seawater (25 °C, pH 8.6, light intensity 1000 lux). Slight aeration was maintained via a small tube in contact with the bottom of the cylinder. Under these conditions, the time required for the cysts to hatch was approximately 24 hr. To investigate possible age-dependent sensitivity of *Artemia*, the nauplii were transferred by Pasteur pipettes to two glass flasks containing 200 mL of the seawater medium, and maintained for another 24 or 48 hr.

Benzalkonium chloride was dissolved in distilled water, and stock solutions were kept in the dark at 4 °C. Prior to each experiment, these were equilibrated to room temperature and used to prepare the final test concentrations in 500 mL of air-saturated, filtered (pore size 30 µm) well water.

The standard environmental conditions for all acute toxicity bioassays were: temperature 25 °C, salinity 35 ‰, and pH 8.6. All assays were performed in darkness and conducted in sterile 24-well polystyrene tissue culture plates.

Preliminary 24 h static toxicity tests were performed to define the range of BKC concentrations covering 0 % to 100 % mortality. The test concentrations used, chosen on the basis of preliminary range-finding tests, were 0.0001-100 µg/L. Each test at each concentration was replicated four times and involved 10 organisms per well. Each test series consisted of 4-5 dilutions of biocide and a seawater control. At least four replicate test series were established for each concentration. All plates were placed in an incubator under standard conditions for 24 hr. Larvae were considered dead if they showed neither internal nor external movement over a 10 s observation period.

The standard environmental conditions for the phototaxis inhibition bioassays were the same as those outlined above. All assays were conducted in dark polystyrene chambers (dimensions 10x1x1 cm). These chambers had a window at either end, one for introducing the organisms (which is closed when the assay begins), the other for the light (1000 Lux) to shine through and attract the larvae.

The test concentrations, chosen on the basis of preliminary acute toxicity tests, covered the range of 1/100-1/10 of the LC_{50} values obtained for BKC. Twelve replicates were used at each test concentration, and with twenty 24 hr-old *Artemia* larvae per replicate chamber. Each test series consisted of 4 dilutions of the test biocide (1/10, 1/25, 1/50 and 1/100 LC_{50}) and a seawater control. The bioassay holders were placed in an incubator under standard conditions for 120 min. At the end of each assay, the number of larvae that completely migrated from the inclusion window to the light source window were counted.

Lethality and phototactic inhibition were expressed as the median lethal concentration (LC_{50}) and the median inhibitory concentration (IC_{50}) respectively; all estimates were made within 95 % confidence limits and determined according to the method of Litchfield and Wilcoxon (1949) using Pharmacological Calculation System software (PCS version 4.0, NY, USA). The LC_{50} and IC_{50} estimates were subjected to two-way analysis of variance (ANOVA) with replication within the subgroups, followed by *post hoc* analysis with the Newman-Keuls test. Significance was set at $p<0.05$.

RESULTS AND DISCUSSION

The acute toxicity of BKC was influenced by the age of the test organisms. Table 1 shows that 24 hr- and 48 hr-old *Artemia* were equally sensitive but that the 72 hr-old shrimp were significantly less tolerant to this disinfectant. The 24 hr LC_{50} values showed the sensitivity of the three age classes to be 24 h ≈ 48h < 72-hr.

Table 1. 24 hr LC_{50} values (95 % CL, n=16 bioassays) for *Artemia franciscana* larvae of different ages exposed to benzalkonium chloride.

A. franciscana **Age Classes**	**n**	**LC_{50} (µg/L)**	**CL (95 %) (µg/L)**
24 h	16	32.67	20.76-51.41
48 h	16	9.14[a]	4.10-13.00
72 h	16	0.0007[ab]	0.0002-0.002

[a, ab] Significant differences ($p<0.05$) from 24- and 48-hr LC_{50} respectively.

The results show that *A. franciscana* larvae are very sensitive to BKC. The LC_{50} values obtained were lower than those recorded by other authors for different seawater organisms. For example, the value of 32.67 µg/L obtained in this work is lower than that obtained in static assays with *Penaeus* spp, *Metapenaeus ensis* and *Alburus alburus* (Liao and Guo 1990), whose LC_{50} values were established in the mg/L range.

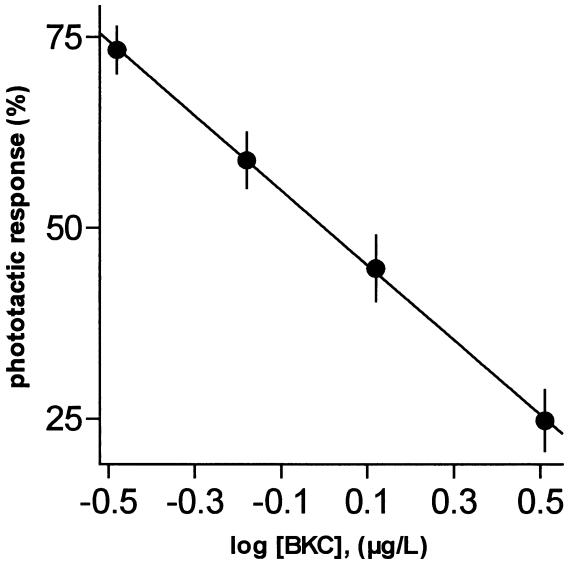

Figure 1. Linear regression analysis of the phototaxis response induced by benzalkonium chloride (●) in 24 hr-old *Artemia franciscana* larvae. Points represent the mean; vertical lines represent the standard error.

It is well known that the different stages of *Artemia* are sensitive to different pollutants (Sorgeloos et al. 1978), but although the 72 hr stage seems generally to be the most sensitive (Barahona et al. 1994; Sánchez-Fortún et al. 1997) some authors have found that there were not significant differences between 48- and 72-hr stages when selected pollutants were tested by means acute toxicity assays (Sánchez-Fortún and Barahona 1996). In similar studies, great sensitivity to environmental pollutants was seen in nauplii of 48 and 72 hr of age. (Lewis 1995).

The IC_{50} value obtained in the phototactic inhibition assays with larvae of 24 hr of age was 0.96 (0.85-1.13) µg/L. These results indicate that BKC inhibits the phototactic capacity of larvae at concentrations below the LC_{50} values. Thus BKC inhibits phototaxis at concentrations 34 times lower than the 24 hr LC_{50} (Figure 1). This large difference between the IC_{50} and LC_{50} values contrasts with results obtained by other authors who worked with different aquatic organisms. For example, when *Daphnia magna* was exposed to different pollutants, LeBlanc (1980) found the IC_{50} values to be 17-20 times lower than the 24 hr LC_{50}. A similar relation (IC_{50} = 1/26 LC_{50} value) was obtained by Whitman and Miller (1982) in phototaxis assays when these same organisms were exposed to naphthalene. However, when *Daphnia pulex* was exposed to this pollutant, IC_{50} values just 1/3 of the LC_{50} values were obtained (Southwork et al. 1978). The difference in salinity conditions between the present work and that performed

with *Daphnia* might explain some of these variations. Although the relationship between salinity and the toxicity of xenobiotic agents remains somewhat unclear, several contaminants seem to show increased toxicity at high salinity (Hall et al. 1994).

In summary, BKC is highly toxic to *A. franciscana*, but to a degree influenced by the age of the organism: significant differences were seen among the LC_{50} values obtained at the 24, 48 and 72 hr larval stages. Phototaxis bioassays offers several advantages over more conventional life-cycle and acutely lethal bioassays. Complications due to food requirements, which are particularly troublesome in life-cycle bioassays and acute bioassays lasting longer that 24 hr, are virtually eliminated.

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