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Influence of Production, Processing, and Storage Conditions of Resting Eggs of *Streptocephalus proboscideus* (Crustacea: Branchiopoda: Anostraca) on the Sensitivity of Larvae to Selected Reference Toxicants

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The availability of healthy test animals in sufficient numbers is of major importance in aquatic toxicology. However, the necessity for standardized culturing and maintenance of live stock has become the main obstacle in routine tests owing to the costs of personnel, equipment, and time (Persoone 1991). Moreover, continuous culturing is also associated with variability of results in toxicity tests. For the cladoceran *Daphnia magna*, for instance, different culture conditions of the live stock have been repeatedly implicated as causing variation in acute toxicity test results (Adema 1978, Taylor 1985, Cowgill 1987, Naylor *et al.* 1992). In response to this problem, a number of resting stage-based toxicity tests have been developed with different organisms (Vanhaecke *et al.* 1980, Snell and Persoone 1989 a, b; Snell *et al.* 1991, Persoone 1991, Centeno *et al.* in press). Resting eggs (cysts) can be dry-stored and hatched only as the need for test organisms arises, thus eliminating the necessity of continuous culturing. One of the species in this set of tests is the freshwater anostracan *Streptocephalus proboscideus* (Centeno *et al.* in press) with biological features suitable for use in toxicity tests (for a review of this matter, see Brendonck and Persoone, 1993).

A semi-automatic laboratory culture device has been designed by Brendonck *et al.* (1990) for the production of *S. proboscideus* cysts which optimizes water quality, maintains constant food concentration, and facilitates regular monitoring of the organisms and the subsequent harvest of their cysts. So far, all toxicity results with *S. proboscideus* (Centeno *et al.* 1993) were obtained with cysts produced using the micro-alga *Selenastrum capricornutum* as the sole food. Although this procedure proved to be successful, the high cost of maintaining live algal cultures led to the survey of alternative diets. However, as has been shown for *Daphnia magna*, maternal nutrition may affect the sensitivity of the brood (Cowgill 1987, Baird *et al.* 1989, Enserink *et al.* 1990, Naylor *et al.* 1992). Thus, it will here be tested if different diets for culturing *S. proboscideus* likely alters the sensitivity of its offspring. Another drawback in the full standardization of the *S. proboscideus* test is the low and variable cyst hatchability. To improve hatching success, Brendonck (1992) and Centeno (*unpubl.*) investigated several cyst treatments. It will also be studied here if different cyst treatments such as decapsulation and defatting, drying under different techniques, humidity levels during storage, and storage duration affect larval sensitivity.

MATERIALS AND METHODS

If not stated otherwise, all eggs used for toxicity tests were produced in closed recirculation units as described by Brendonck *et al.* (1990) at a temperature of 25 ± 1°C and a

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photoperiod of 12 L/12 D. Water quality was monitored twice a week. Water was (partly) renewed and/or filters were (partly) cleaned to maintain low metabolite concentrations (maximum NH_3-N =0.42 mg/L; max. NO_3-N = 29 mg/L). Under the standard conditions, parental organisms (at a density of 50 animals/L) were fed semi-automatically at timed intervals with the micro-alga *S. capricornutum* to maintain densities ranging from 8×10^4 to 2×10^5 cells/mL.

To minimize the influence of uncontrolled variables in the collection, processing, and storage of eggs, a standard method was adopted and strictly followed in all experiments (except when stated otherwise). Resting eggs were harvested weekly as in Brendonck *et al.* (1990). Eggs with debris were subsequently washed over a series of sieves with mesh sizes of 600, 200 and 112 µm, respectively. Eggs retained on the 200- µm sieve were further purified by repeated decantations using tapwater. At the end, clean resting eggs (with some mandible-exuvii of similar or higher density) were filtered under vacuum. The above cyst preparation will be referred to as the standard processing procedure (SPP) hereafter. Filtered cysts were dry-stored in a 25°C-temperature controlled room (dimmed light, 40% relative humidity (r.h.)) for at least 2 wk prior to use in toxicity tests. This drying/storage condition will be referred to as the standard drying procedure (SDP) hereafter.

To obtain larvae for toxicity testing, cysts (approx. 3,000) were incubated in Petri dishes containing 10 mL of a moderately hard (80-100 mg $CaCO_3$/L) EPA water (USEPA 1985) at 25 + 1°C, under continuous illumination (1000-2000 lux; provided by white fluorescent lamps) 24 hr prior to the start of tests. This cyst-incubation procedure will be referred to as the standard incubation procedure (SIP) hereafter. All static 24-hr toxicity tests hereunder were conducted in polystyrene multiwell plates (6 columns X 4 rows) with at least 5 nominal toxicant concentrations and a control using instar II-III larvae as test organisms. The details of the standard procedure for conducting the tests are described in Centeno *et al.* (1993).

In a first series of experiments to determine the influence of parental feeding conditions on larval sensitivity, eggs were harvested from cultures fed with "YM20" (a pea/corn mixture), "POME" (palm oil mill effluent), and algae (standard conditions), respectively. Both YM20 and POME suspensions were freshly prepared every 2 days by homogenizing 5 g of the dry product in 1 L of tapwater for 2 min, and were kept in a refrigerator at 4°C where suspensions were continuously aerated. Food suspensions were distributed semi-automatically to the respective tanks by peristaltic pumps activated at defined time intervals (see Brendonck *et al.* 1990). For both algae and inert diets, two different feeding regimes (low/high) were performed; under the high-food regime, food quantity was doubled for the inert food (i.e., 30 mL of the suspension/hour), while for the algae-fed cultures, algal cell density increased to 6 to 7 x 10^5 cells/mL (at least 4 times higher than the low-food regime). Eggs produced under the above conditions were processed separately according to the SPP. After 2 wk (SDP), cyst-rehydration was initiated 24 hr before start of toxicity tests (SIP). The sensitivity of instar II-III larvae were determined using three selected reference toxicants: potassium dichromate (99% $K_2Cr_2O_7$, Fluka), copper sulfate ($CuSO_4.5H_2O$, AG, UCB) and sodium pentachlorophenate (98% NaPCP, Merck). The tests were repeated in time for at least 4 times for each food condition ($n \geq 4$).

In a second series of tests, the effects of cyst decapsulation and cyst defatting on larval sensitivity were determined. From one batch of "SPP + SDP" cysts produced from algae-fed cultures, one part was decapsulated, another defatted, and a third part used as control. Cysts were decapsulated by soaking during 7.5 min in a solution of 7.5 mL NaOCl in 92.5 mL deionized water which was stirred by air-bubbling. Subsequently, eggs were washed for 5 min with tapwater on a 112-µm sieve. The activity of pure NaOCl determined using the HACH^R method was about 7.5%. Defatting was done by immersion in 5 mL of pure acetone for 2 min. Cysts were then washed on a 112-µm sieve with tapwater for 5 min.

The sensitivity of hatched instar II-III nauplii was compared with that of larvae from untreated eggs (control) in simultaneous toxicity tests using potassium dichromate as toxicant.

To investigate the effect of drying conditions on larval sensitivity, a batch of freshly harvested cysts produced from algae-fed cultures was divided into several parts, and the following treatments were done: a) storage in 30 mL of the original culture medium (25 ± 1°C) until total evaporation (after 24-48 h), and cysts were subsequently dry-stored for 2 wk (SDP); b) drying for one wk in a whirl cylinder (25 ± 1°C) with continuous air stream (40 to 50% r.h.)(fluidized-bed drying); c) drying for one wk in an incubator (35 ± 1°C), at 40% r.h.. After drying, eggs were incubated as above (SIP) to obtain instar II-III larvae for toxicity testing using copper sulfate.

In a fourth series of experiments, the effect of cyst storage conditions on larval sensitivity was assessed. Several parts of a batch of freshly harvested cysts from algae-fed cultures were transferred into closed bottles, each containing a different saturated salt solution creating a vapour of specific and constant relative humidity. The different vapours were prepared with solutions of P_2O_5, $ZnCl_2$, $MgCl_2.6H_2O$, NaCl and $Na_2C_4H_4O_6.2H_2O$ to obtain relative humidities of 0, 10, 34, 76 and 92%, respectively (see Winston and Bates 1960). In this procedure, contact of cysts and solution was avoided. The same batch of cysts stored similarly in a bottle without any solution (40% r.h.) was used as the control. After 2 mon of storage, cyst rehydration was performed as above (SIP) and the sensitivity of hatched larvae (instar II-III) were assessed using potassium dichromate.

In a last series of tests, the effect of the duration of cyst storage on sensitivity of larvae was studied. Eggs were harvested at different times but were all produced from algae-fed cultures, and processed (SPP) and dried (SDP) under the standard conditions. After 0.5, 1, 3, 6, 9, 12, 15, and 19 mon of storage, cyst rehydration was performed as above (SIP) to obtain instar II-III populations for toxicity testing with potassium dichromate and copper sulfate.

All 24-hr LC50's were calculated using the Trimmed Spearman-Karber method (Hamilton *et al.* 1977). The LC50 values were tested for normality using the Kolmogorov-Smirnov goodness of fit test prior to further analysis. The differences in larval response using cysts either produced, processed, or stored differently were analyzed using one- or two-way analysis of variance (ANOVA) as appropriate. A one-way ANOVA extended with the Multiple Range Duncan's Procedure was used to evaluate LC50 values obtained using eggs stored under different humidity levels, and eggs stored for a different duration (Sokal and Rohlf 1981).

RESULTS AND DISCUSSION

Although changes in the nutritional status of an organism may eventually lead to a change in its biological and biochemical characteristics (see Richman 1958), manipulation of parental food quality and quantity in culturing *S. proboscideus* for resting egg production did not significantly influence the sensitivity of the offspring. At the low food regime, 24-hr LC50 values of $K_2Cr_2O_7$, $CuSO_4.5H_2O$, and NaPCP were not significantly affected by the different diets of the parental organisms ($p>0.05$)(Fig. 1). At increased food levels, only LC50 values of NaPCP with larvae obtained from algae-fed cultures (24-hr LC50 = 0.82 ± 0.04 mg/L) were significantly different ($p<0.05$) from those from YM20-fed cultures (24-hr LC50 = 0.69 ± 0.10 mg/L). However, these values were still in the same order of magnitude, which from the ecotoxicological point of view, are not significantly different from each other. Statistical analysis revealed no overall significant effect ($p>0.05$) of the interactions of food quality and quantity on the resulting LC50 values. Mean LC50 values for all food types in both feeding regimes

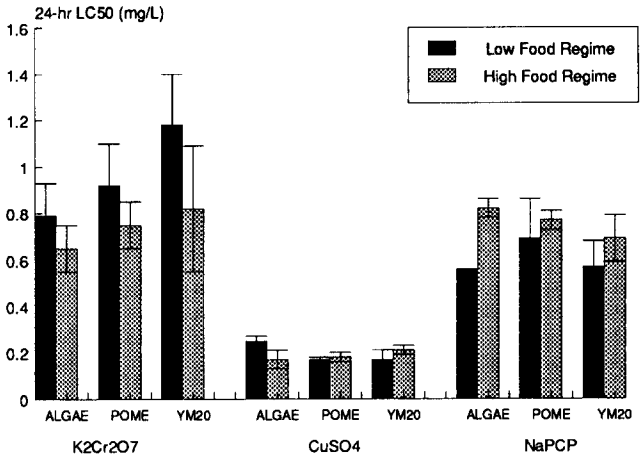

Figure 1. Effect of different food type on larval sensitivity to $K_2Cr_2O_7$, $CuSO_4.5H_2O$ and NaPCP. Error bars indicate the 95% confidence limits.

range from: a) 0.56 to 0.82 mg/L for NaPCP; b) 0.17 to 0.25 mg/L for $CuSO_4.5H_2O$; and, c) 0.65 to 1.18 mg/L for $K_2Cr_2O_7$. The species may thus be successfully cultured with dry and rather cheap food, and the produced cysts may be used for toxicity studies without causing extra variability.

Contrary to the present findings with *S. proboscideus*, several authors have found that maternal diet implicated variation in neonate sensitivity. For the widely applied test organism *Daphnia*, for example, diet plays an important role in determining its survival, growth, reproductive performance and sensitivity to toxicants (Richman 1958, Smith 1963, Winner *et al.* 1977, Stephenson and Watts 1984, Taylor 1985, Cowgill 1987) and consequently affects the body size as well as the sensitivity of their offspring (Cowgill 1987, Elendt 1990, Enserink *et al.* 1990, Cox *et al* 1992, Naylor *et al.* 1992). On the other hand, other results obtained with *Daphnia*, live neonates as well as those hatched from ephippia, agree with our findings. For instance, Stephenson and Watts (1984) did not detect any effects of maternal nutrition on *D. magna* neonate sensitivity to chromium. Likewise, no differences in sensitivity to potassium dichromate were found for *D. magna* neonates produced under different culture conditions (Univ. Sheffield workshop, 1991). Similarly, *D. pulex* neonates obtained from differently produced ephippia revealed no differences in sensitivity to potassium dichromate (Cotou 1993).

In contrast to the unaltered sensitivity of *S. proboscideus* larvae, Brendonck (1992) and Centeno (*unpubl.*) found different hatching responses between eggs produced with different diets. This finding may be related with the variable hatching of *S. proboscideus* cysts between batches produced under identical culture conditions and even within the same batch (Brendonck 1992; *Centeno, unpubl.*). It remains to be tested if it is not a safety mechanism that even under sub-optimal food and other environmental conditions, cysts maintain approximately the same quality to ensure survival of a next generation.

Despite the chemical treatments in decapsulation and defatting of resting eggs prior

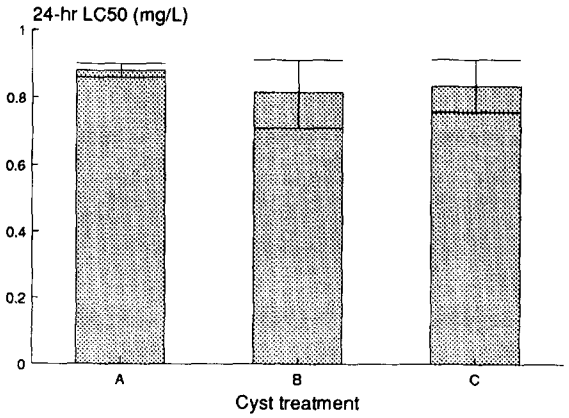

Figure 2. Effect of different cyst treatment on the larval sensitivity to $K_2Cr_2O_7$; A = Standard method; B = Cyst decapsulation; C = Cyst defatting. Error bars indicate the 95% confidence limits.

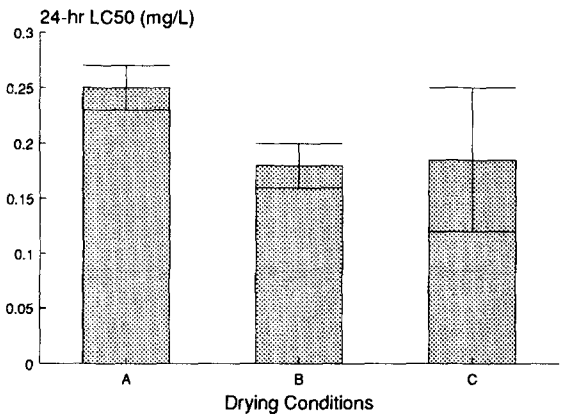

Figure 3. Effect of cyst drying conditions on larval sensitivity to $CuSO_4.5H_2O$; A= drying in standard conditions (air-exposure at 25°C); B= drying by air-blowing at 25°C; C= drying by exposure in incubator at 35°C. Error bars indicate the 95% confidence limits.

to incubation to facilitate the hatching process, the larvae exhibited the same sensitivity as those hatched from cysts which did not undergo pre-treatment in SPP (Fig. 2). While decapsulation generally had a positive effect on cyst hatching (Brendonck 1992; *Centeno, unpubl.*) as probably explained by the (partial) removal of the tertiary egg envelope, both decapsulating and defatting did not significantly ($p>0.05$) affect larval sensitivity to potassium dichromate. It was expected that by removing the shell, larvae would have more energy left after hatching, thus, be more tolerant to toxicants. Nauplii obtained from decapsulated *Artemia franciscana* cysts, for instance, showed less susceptibility than those hatched from untreated cysts (Landau and Sanchez 1991).

Brendonck (1992) and Centeno (*unpubl.*) demonstrated that different drying conditions had no influence on the cumulative hatching results. Likewise, the application of other

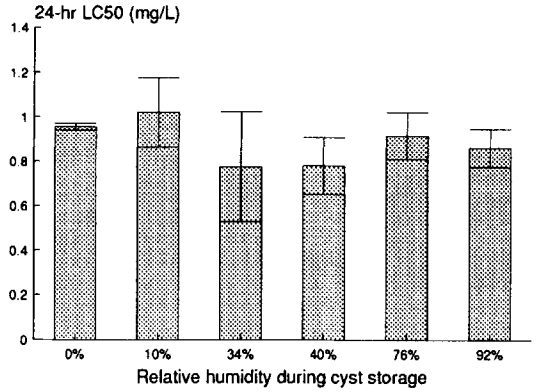

Figure 4. Effect of humidity levels during cyst storage on larval sensitivity to $K_2Cr_2O_7$. Error bars indicate the 95% confidence limits.

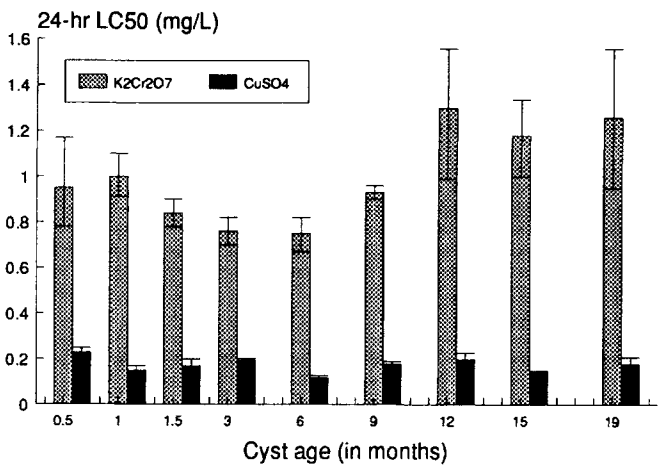

Figure 5. Influence of storage duration (cyst age) on larval sensitivity to $K_2Cr_2O_7$ and $CuSO_4.5H_2O$. Error bars indicate the 95% confidence limit.

cyst-drying techniques showed no change in larval response when compared to the standard drying techniques previously applied. The mean 24-hr LC50 values of $CuSO_4.5H_2O$ obtained using cysts dried in the whirl cylinder (25°C) and those dried in the incubator (35°C) are comparable. The LC50 values obtained from cysts dried under standard conditions (SDP) are significantly ($p>0.05$) higher (Fig. 3), however, all LC50 values were still in the same order of magnitude (means range from 0.18 to 0.25 mg/L).

Although the humidity levels during storage affected the onset of hatching of *S.*

proboscideus cysts (*Centeno, unpubl.*), they did not have a significant ($p>0.05$) effect on *S. proboscideus* larval sensitivity to $K_2Cr_2O_7$ (Fig. 4). No two groups of treatments gave significantly ($p>0.05$) different results (mean 24-hr LC50 values range from 0.78 to 1.02 mg/L). Vanhaecke *et al.* (1980) also found no effect of storage methods on sensitivity of *Artemia* nauplii.

Cysts stored from 0.5 to 19 mon did not also show marked differences in the final LC50 values of potassium dichromate and copper sulfate. The ratio of highest (1.30 mg/L) to lowest (0.75 mg/L) mean 24-hr LC50 values of $K_2Cr_2O_7$ obtained with cysts stored for different duration is 1.73 (Fig. 5). For $CuSO_4.5H_2O$, the highest to lowest LC50 ratio is 1.92 (values range from 0.12 - 0.23 mg/L) (see Fig. 5). Although LC50 values differed significantly ($p>0.05$), they did not differ by more than an order of magnitude. Likewise, similar results of acute tests were found with neonates from *Brachionus calyciflorus* cysts that were stored even after a period of 2 yr (Snell *et al.* 1991).

The suitability of *S. proboscideus* resting eggs for routine toxicological testing is supported here since the sensitivity of the nauplii is not significantly altered by either changing the parental diets, cyst pre-treatment, cyst storage under different humidity conditions, or storage for long durations. Care must, however, still be taken to store eggs under dry and room-temperature conditions to ensure their viability.

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