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Chronic Toxicity of 4-Nitrophenol to *Daphnia magna* Straus Under Statis-Renewal and Flow-Through Conditions

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Data from chronic toxicity tests with Daphnia are frequently required to support environmental hazard assessments of pesticides and other chemicals. These chronic tests are generally conducted in static-renewal systems (Gersich 1984; Lewis and Wee 1983), although flow-through procedures have been employed to help maintain stable exposure conditions when testing volatile or labile compounds (LeBlanc and Suprenant 1983; Biesinger et al. 1982). The relative merits of these two test systems have been discussed by LeBlanc et al. (1983).

The objectives of the present study were to (1) develop a flow-through system for chronic testing with Daphnia magna, (2) compare the reproduction and growth of D. magna under flow-through and static-renewal conditions, and (3) evaluate the chronic toxicity of 4-nitrophenol (4-NP) in the two test systems. This compound, classified as a priority pollutant by the U.S. EPA (Keith and Telliard 1979), has a high water solubility and is relatively stable in aqueous solution.

MATERIALS AND METHODS

Organisms used in the static-renewal and flow-through tests were obtained from a brood stock of D. magna Straus maintained at the Environmental Toxicology facility of Lilly Research Laboratories. The brood stock was held in 600-ml beakers at a density of one animal per 50 ml of conditioned well water. The chemical characteristics of the well water were similar to those shown in Table 1. All organisms were transferred to fresh water three times per week and fed a suspension of green algae (Selenastrum capricornutum Printz) each day.

The S. capricornutum, which served as the food source for the brood stock and test organisms, was cultured in 20-L borosilicate glass carboys containing 16 L of nutrient media supplemented with vitamins (Goulden et al. 1982). The algal cultures were held at 20°C and received ≧ 400 ft-c of fluorescent lighting. The algal suspension used to feed the daphnids was prepared by separating the algal cells from the nutrient media by centrifugation, decanting the supernatant, and resuspending the cells in deionized water. Only algal cultures less than seven days old were used as a food source.

Table 1. Water quality characteristics of exposure solutions in the static-renewal and flow-through tests (mean ± sd).

Water Quality Characteristic	Static-Renewal Test	Flow-Through Test
Temperature (°C)	20.1 ± 0.6	21.0 ± 0.3
Dissolved Oxygen (mg/L)	8.7 ± 0.9	8.9 ± 0.3
pH (Range)	7.7 - 8.8	8.1 - 8.9
Hardness (mg/L as $CaCO_3$)	128.4 ± 9.7	128.4 ± 9.7
Alkalinity (mg/L as $CaCO_3$)	148.3 ± 8.3	154.3 ± 1.5
Conductivity (μS/cm)	200 - 300	250 - 300
Unionized Ammonia (mg/L)	< 0.01	< 0.01

For the static-renewal system, animal exposure chambers consisted of 250-ml borosilicate glass beakers that contained 200 ml of test solution. The nominal 4-NP exposure concentrations were 0.0 (control), 1.25, 2.5, 5.0, 10.0, and 20.0 mg/L. This concentration range was based on results from a 48-h acute test showing the EC_{50} to be 25.8 mg/L. Conditioned well water was used as the diluent for all solutions. Five daphnids less than 24 h old were randomly assigned to each of four replicate test vessels at each exposure level, resulting in a total of 20 organisms per treatment. The test solutions in all exposure chambers were renewed three times per week. Each day the test animals were fed 1.0 to 1.5 ml of a suspension of S. capricornutum. This produced an initial algal cell concentration in each exposure chamber of 3 x 10^5 cells/ml, equivalent to about 6 mg/L (dry weight). The initial cell concentration in one replicate from each treatment was determined every two or three days using a hemocytometer. As measured with a radiometer, light intensity at the surface of the test solutions was 74 ft-c and the photoperiod was 16 h light/8 h dark. The test vessels were covered with a glass plate to retard evaporation.

The survival and reproduction of the organisms were recorded each day the exposure solutions were renewed. Animals showing no movement were counted as mortalities and removed from the test vessels. Reproduction was quantified by transferring the original test animals in each chamber to a clean beaker containing fresh solution, placing the neonates from that replicate onto filter paper (11-cm diameter), and counting the number of young animals with an Artek (Model 880) electronic counter. Using the reproduction and survival data from each replicate, the instantaneous rate of population growth (r) was calculated by the method used by Winner and Farrell (1976). Growth of surviving adults was determined after 21 d of exposure. Each daphnid was anesthetized

with carbon dioxide and measured to the nearest 0.01 mm using a Whipple eye-piece micrometer fitted on a compound microscope. The length of each animal was measured from the apex of the helmet to the base of the tail spine.

To conduct the flow-through test, a proportional diluter intermittently delivered test solutions to four replicate vessels at each treatment. Each animal exposure chamber used with the flow-through system consisted of a 200-ml Berzelius beaker custom-fitted with a vulcanized silicone rubber stopper that was molded to accept both a delivery tube and an outlet port (Fig. 1). The delivery tube (glass, 3.7 mm ID) was connected to the diluter via polyethylene tubing (2 mm ID). The outlet port (glass, 22 mm ID) was approximately 4 cm long and was fitted with a No. 3 silicone rubber stopper which supported an overflow tube (glass, 5 mm ID). A nylon mesh screen was cemented to the bottom of the outlet port to prevent escape of the test organisms. A piece of polyethylene tubing (2 mm ID) inserted into the terminal end of the delivery tube was used to prevent small daphnids from swimming up the inlet line. The exposure chamber was designed to eliminate the air/water interface and produce a completely enclosed vessel that held approximately 200 ml. Because the chamber was sealed and devoid of an air/water interface, it was not possible for young daphnids to be trapped by the surface tension. The presence of "floaters" in flow-through tests with cladocerans has been a frequently encountered problem (Novak et al. 1982). Every 1.5 h the diluter delivered 125 ml of solution to each vessel. The maximum flow rate through an exposure chamber was 11 ml/min.

The diluter was equipped with an automatic feeding system which mixed 5 ml of an algal suspension with the 125 ml of test solution delivered to each chamber every 1.5 h. A reservoir used to supply algae to the diluter was replenished with 3.0 L of fresh algal suspension each day. The cell concentration of the algal suspension was adjusted such that each exposure solution had a nominal algal concentration of 2×10^5 cells/ml. The algal density in one replicate from each treatment was measured every two or three days. The first-instar daphnids used in this test were taken from the same population as those used in the static-renewal system. Reproduction, survival, and growth were determined as described for the static-renewal test.

On days 0, 5, 8, 13, and 16, a 20-mg/L stock solution of reagent-grade 4-NP was prepared in a 210-L stainless steel barrel using conditioned well water. This solution served as the toxicant source for the diluter and was also used to prepare a stock solution for each treatment in the static-renewal test. All stock solutions were aerated during the 21-d study period to maintain dissolved oxygen levels near saturation.

Composite samples from the four replicates at each treatment level were collected on days 0, 2, 5, 8, 12, 15, 19, and 21, and analyzed for 4-NP by the spectrophotometric technique of Sutherland

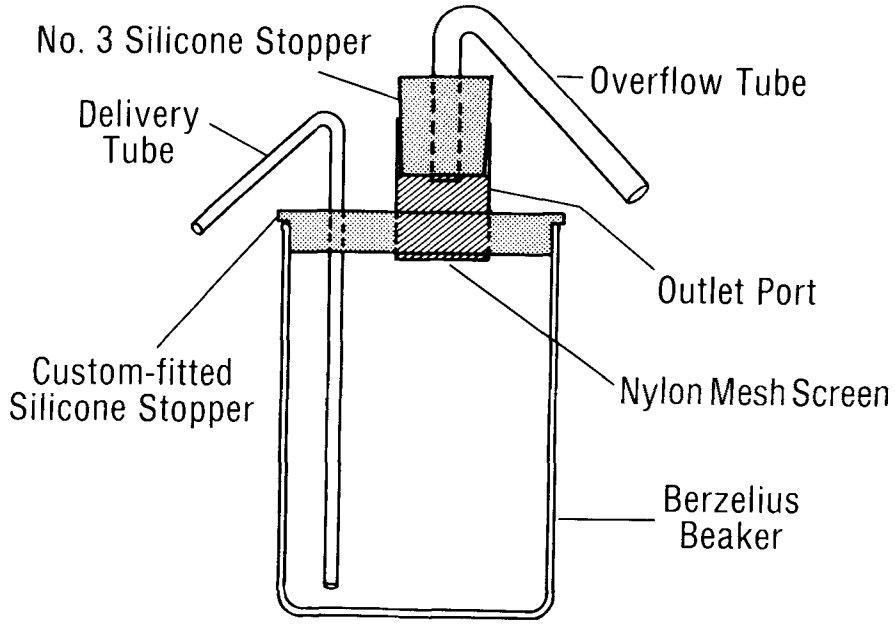

FIGURE 1. EXPOSURE CHAMBER USED WITH THE FLOW-THROUGH SYSTEM.

and Miskus (1964). Prior to analysis, each sample was passed through a 0.45-μm membrane filter to remove algae and other suspended material.

RESULTS AND DISCUSSION

Survival frequencies in the static-renewal test ranged from 70% at 20 mg/L to 100% in the control (Table 2). At 4-NP concentrations ≦ 5.0 mg/L, average neonate production per female ranged from 111.7 to 124.6. Significant decreases in reproduction were detected at 10.0 and 20.0 mg/L where mean neonate production per female dropped to 88.4 and 11.0, and r was reduced by 8% and 57%, respectively. Although the exact number of broods per daphnid could not be determined, it was estimated that five or six broods were produced between days 8 and 21. The only significant ($p \leqq 0.05$) reduction in growth occurred at 20.0 mg/L where body lengths averaged 3.87 mm compared to mean lengths of 4.19 to 4.27 mm at concentrations ≦ 10.0 mg/L. The algal cell concentrations observed in the test solutions immediately after the animals were fed averaged 2.7×10^5 cells/ml (5.4 mg/L, dry weight) with a coefficient of variation (CV) of 2.6%. However, measurements taken during the third week of the study showed that cell densities generally dropped by a factor of ten within 24 h after feeding. The concentrations of 4-NP in the exposure solutions

Table 2. Chronic toxicity data for Daphnia magna exposed to 4-nitrophenol for 21 days in a static-renewal test.

Nominal Level (mg/L)	Survival (%)	Neonates[a] per Female	Population Growth Rate (r)	Body[a] Length (mm)
Control	100	114.7 ± 7.3	0.362	4.27 ± 0.14
1.25	80	124.6 ± 16.4	0.352*	4.25 ± 0.13
2.5	75	120.9 ± 6.7	0.356	4.23 ± 0.09
5.0	100	111.7 ± 12.1	0.355	4.19 ± 0.09
10.0	85	88.4 ± 7.1*	0.333*	4.19 ± 0.19
20.0	70	11.0 ± 3.9*	0.156*	3.87 ± 0.25*

[a]Mean ± SD

*Significantly different ($p \leq 0.05$) from the control value using Dunnett's t-test. The natural log transformation, e^r, was used in the statistical test on r.

Table 3. Chronic toxicity data for Daphnia magna exposed to 4-nitrophenol for 21 days in a flow-through test.

Nominal Level (mg/L)	Survival (%)	Neonates[a] per Female	Population Growth Rate (r)	Body[a] Length (mm)
Control	90	169.9 ± 10.5	0.385	4.48 ± 0.16
1.25	90	167.6 ± 9.7	0.376	4.44 ± 0.12
2.5	70	159.6 ± 15.5	0.373	4.49 ± 0.16
5.0	85	155.6 ± 13.1	0.391	4.49 ± 0.17
10.0	65	110.9 ± 8.6*	0.322*	4.13 ± 0.21*
20.0	0	-	-	-

[a]Mean ± SD

*Significantly different ($p \leq 0.05$) from the control value using Dunnett's t-test. The natural log transformation, e^r, was used in the statistical test on r.

(Mean ± SD, n = 8) were 1.31 ± 0.19, 2.35 ± 0.62, 5.15 ± 0.20, 10.46 ± 0.23, and 19.35 ± 0.87 mg/L at nominal levels of 1.25, 2.5, 5.0, 10.0, and 20.0 mg/L, respectively. The water quality conditions observed during the static-renewal and flow-through tests are presented in Table 1.

Under flow-through conditions, survival frequencies at 4-NP concentrations ≦ 10.0 mg/L ranged from 65% to 90% compared to the control survival of 90% (Table 3). Total mortality occurred at the 20-mg/L treatment by day 7. As in the static-renewal test, 10.0 mg/L was the lowest concentration that caused a significant decrease in reproduction. By pooling the reproduction data from treatments ≦ 5.0 mg/L, it was seen that daphnids in the flow-through system produced an average of 45 more neonates per female compared to organisms tested under static-renewal conditions. The instantaneous rate of population growth (r) ranged from 0.373 to 0.391 at concentrations ≦ 5.0 mg/L but dropped to 0.322 at 10.0 mg/L. The first broods were released on days 7 and 8. An estimated five or six broods were produced over the test period. A significant reduction in growth occurred at 10.0 mg/L where the mean length was 4.13 mm, compared to 4.48 mm for controls. Daphnids exposed to nontoxic concentrations in the flow-through system generally grew larger than those in the static-renewal system. The algal concentrations in the exposure chambers averaged 1.9 x 10^5 cells/ml (3.8 mg/L, dry weight) with a CV of 14.1%. The concentrations of 4-NP in the exposure solutions (Mean ± SD, n = 8) were 1.26 ± 0.28, 2.11 ± 0.59, 4.35 ± 0.78, 9.12 ± 1.56, and 18.28 ± 2.69 mg/L, at respective treatment levels of 1.25, 2.5, 5.0, 10.0, and 20.0 mg/L. The concentrations remained stable over the test period except on day 5 when all exposure levels dropped to approximately 50% of nominal. This condition was immediately remedied by preparing a fresh stock solution. Because 4-NP has been shown to be very susceptible to microbial degradation (Nyholm et al. 1984) and the stock solution appeared cloudy on day 5, it is likely that the reduced concentrations resulted from bacterial decomposition of the compound in the stock solution. Excluding data from day 5, the exposure concentrations averaged 97% of the nominal levels.

Results from these tests indicated that 4-NP concentrations ≧ 10.0 mg/L were chronically toxic to D. magna under both static-renewal and flow-through conditions. Although growth and reproduction of the daphnids were substantially better in the flow-through test, the 4-NP concentrations that caused significant impacts on the populations were the same in both systems. No effects on growth or reproduction were observed at 4-NP concentrations ≦ 5.0 mg/L in either system, and the r -values observed at these treatments were in close agreement with those previously reported for D. magna (Winner and Farrell 1976). However, data from nontoxic treatments showed that daphnids tested under flow-through conditions grew almost 6% larger and produced 38% more neonates per female compared to organisms in the static-renewal system. At the same concentrations, r averaged 7% greater in the flow-through system despite the slight reductions in survival

observed at 2.5 and 5.0 mg/L. At 10.0 mg/L, daphnid reproduction and r were significantly reduced in both test systems, but growth was significantly diminished only under flow-through conditions.

Food availability has been shown to be one of the most important factors governing growth and reproduction of D. magna (LeBlanc et al. 1983). The availability of algae in the exposure chambers may have been the most important difference between the flow-through and static-renewal systems. In the static-renewal test, the algal concentrations fluctuated from about 3×10^5 cells/ml immediately after feeding to about 2×10^4 cells/ml after 24 h. Previous studies in our laboratory have shown that grazing rates in a similar static-renewal test ranged from about 6×10^3 cells/ml/h during the first week to 1×10^4 cells/ml/h during the third week when the daphnids were larger and their offspring were feeding. In comparison, food availability in the flow-through system remained at a relatively constant level of 2×10^5 cells/ml because fresh test solutions containing algae were delivered to the exposure chambers every 1.5 h. The stable feeding conditions maintained by the diluter appeared to be the primary factor responsible for the greater growth and reproduction observed in this system.

Toxicity tests conducted under flow-through conditions generally have provided a more sensitive measure of toxicant stress than have static or static-renewal tests (LeBlanc and Suprenant 1983). The primary reason for this is the capacity of flow-through systems (continuous or intermittent) to maintain stable exposure concentrations, even with volatile, lipophilic, or readily degradable chemicals. However, the flow-through system in the present study did not show greater sensitivity than the static-renewal test. This apparently resulted from the high water solubility and relative stability of 4-NP which allowed constant exposure conditions to be maintained in both systems. While the sensitivities of the two systems were comparable, the higher reproduction and growth rates seen in the flow-through test did appear to enhance the resolution between the "effect" and "no-effect" levels. At the lowest concentration that caused statistically significant effects (10.0 mg/L), reproduction, r, and growth in the flow-through system were 34%, 16%, and 8%, respectively, below control responses. In comparison, these same parameters were diminished by only 23%, 8%, and 2% at 10 mg/L in the static-renewal test.

REFERENCES

Biesinger KE, Anderson LE, Eaton JG (1982) Chronic effects of inorganic and organic mercury on Daphnia magna: Toxicity, accumulation, and loss. Arch Environ Contam Toxicol 11:769-774

Gersich FM (1984) Evaluation of a static renewal chronic toxicity test method for Daphnia magna Straus using boric acid. Environ Toxicol Chem 3:89-94

Goulden CE, Comotto JA, Hendrickson Jr JA, Hornig LL, Johnson KL (1982) Procedures and recommendations for the culture and use of Daphnia in bioassay studies. In: Pearson JG, Foster RB, Bishop WE (eds) Aquatic toxicology and hazard assessment. American Society for Testing and Materials (STP 766), Philadelphia, PA, p 139

Keith LH, Telliard WA (1979) Priority pollutants, I. A perspective view. Environ Sci Technol 13:416-423

LeBlanc GA, Schoenfeld DA, Surprenant DC (1983) Effects of food concentration, animal interactions, and water volume on survival, growth, and reproduction of Daphnia magna under flow-through conditions. In: Bishop WE, Cardwell RD, Heidolph BB (eds) Aquatic toxicology and hazard assessment. American Society for Testing and Materials (STP 802), Philadelphia, PA, p 494

LeBlanc GA, Suprenant DC (1983) The acute and chronic toxicity of acetone, dimethyl formamide, and triethylene glycol to Daphnia magna (Straus). Arch Environ Contam Toxicol 12:305-310

Lewis MA, Wee VT (1983) Aquatic safety assessment for cationic surfactants. Environ Toxicol Chem 2:105-118

Novak AJ, Berry DF, Walters BS, Passino DRM (1982) New continuous-flow bioassay technique using small crustaceans. Bull Environ Contam Toxicol 29:253-260

Nyholm N, Lindgaard-Jorgensen P, Hansen N (1984) Biodegradation of 4-nitrophenol in standardized aquatic degradation tests. Ecotox Environ Safety 8:451-470

Sutherland GL, Miskus E (1964) Parathion. In: Zweig G (ed) Analytical methods for pesticides, plant growth regulators, and food additives, Vol. II-Insecticides. Academic Press, New York, p 321

Winner RW, Farrell MP (1976) Acute and chronic toxicity of copper to four species of Daphnia. J Fish Res Board Can 33:1685-1691

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