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New Continuous-flow Bioassay Technique Using Small Crustaceans[1]

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The increasing number of chemicals being detected in the environment or proposed for manufacture and use has stimulated an increased demand for toxicological information sufficient to enable evaluation of the potential harm posed by these chemicals to aquatic resources. A fundamental need for evaluating the large number and variety of chemicals involved is the availability of rapid and reliable bioassay techniques.

In this paper we describe an improved bioassay system for use in determining the toxicity of chemicals to small crustaceans. Although our system has several similarities to currently accepted standard methods (AMERICAN PUBLIC HEALTH ASSOCIATION [APHA] et al. 1980, AMERICAN SOCIETY FOR TESTING AND MATERIALS [ASTM] 1980), it offers three advantages: (1) it is an easily built and operated, compact flow-through system, (2) it incorporates improved invertebrate handling techniques, and (3) it enables the use of a native North American cladoceran, Bosmina longirostris, as a test organism. The method in brief is as follows: The animals are exposed in a full, stoppered test tube, which is continuously emptied (as opposed to filled) by a peristaltic pump. The liquid exchange takes place through two glass capillaries protruding through the stopper. A pair of flexible hoses connect one capillary to the diluent source and the other to the peristaltic pump. Cladocerans are introduced into the test tube automatically through the intake capillary, and are counted as they pass a particle counter.

To compare this technique with standard methods currently in use we conducted 48- and 96-h continuous-flow bioassays, using Daphnia magna, D. pulex, and B. longirostris in uncontaminated dilution water (i.e., the equivalent of controls in a toxicity study).

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MATERIALS AND METHODS

The test animals were the offspring (<24 h old) of parthenogenetically reproducing females of D. magna, D. pulex, and B. longirostris reared according to standard requirements (ASTM 1980). The animals in all of the 96-h tests and some of the 48-h tests received a constant amount of food. Characteristics of diluent water, flow rates, food supply, and other variables (Table 1) were within guidelines outlined for flow-through bioassays (ASTM 1980).

TABLE 1. Chemical, physical, and dietary conditions during the 48- and 96-h flow-through control tests.

Condition, variable, and unit	D. pulex B. longirostris	D. magna[1/]
Water		
Hardness (as $CaCO_3$), mg/L	120	160
Alkalinity (as $CaCO_3$), mg/L	320	115
Dissolved oxygen, mg/L	9.5	9.5
pH	6.8	7.3
Temperature, °C	17	17
Food (cells/mL)		
Chlorella vulgaris	32×10^3	none
Chlorella pyrenoidosa	63×10^3	none
Chlamydomonas epiphytica	6×10^3	none
Volumes[2/]		
Test tube (exchangeable), mL	13	21
Pump tubes (test tubes), mL/min	0.05	0.16
Pump tubes (air), mL/min	0.015	0.015
Flow rate[3/], VA/24-h	5.3	5.1

1/ Reconstituted water (ASTM 1980).
2/ Pump tubes used to withdraw excess volume from debubbler (Figs. 4 and 5) are not shown; volumes withdrawn were variable.
3/ Volume addition (VA) is the placement into the test chamber of a volume of test solution equal to the volume of solution in the chamber.

The system can be divided schematically into three procedures (Fig. 1): the harvesting and loading of neonates; flow-through testing; and delivery of diluent, toxicant, and food.

1. To obtain a ready supply of test organisms, we used the classical methods of separation by size and phototaxis (CLARKE 1932, DEWEY and PARKER 1964). First, gravid female B. longirostris were placed in a brood chamber with a screened bottom (Fig. 2).

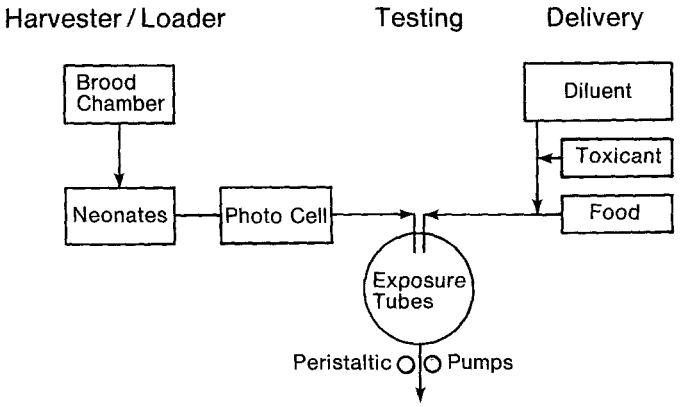

FIGURE 1. Schematic of the major components of a system that enables the use of small crustaceans in bioassays.

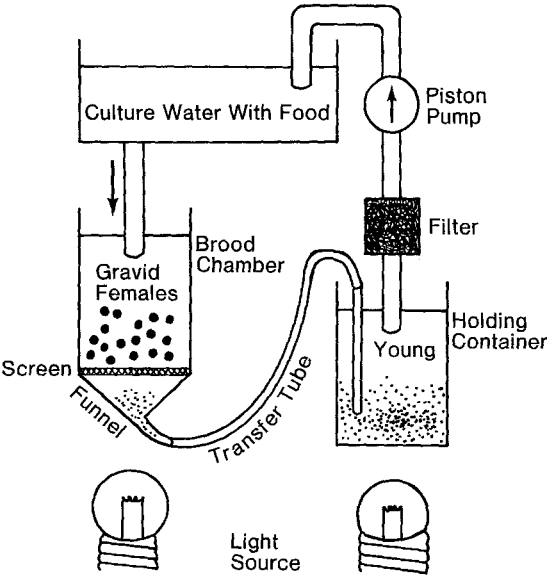

FIGURE 2. Schematic of a system continuously providing neonates of small crustaceans.

After release from the clutch, the neonates, attracted to the light, actively swam through the screen to the intake of the transfer tube, where they were swept by the current into the holding container. The effluent from the holding container was filtered and recirculated. The age limit of the neonates could be set at will, inasmuch as the age difference among the neonates was inversely proportional to the emptying frequency of the holding container of all young.

To count and introduce B. longirostris into an exposure vessel without inadvertently harming them, we used a simple colorimeter (a converted Technicon[4/] Auto Analyzer I colorimeter with handmade flow-cuvette--light path, 1.0 mm; ID of cuvette, 1.0 mm; slit diameter, 0.7 mm; light source, visible, red region) and a strip chart recorder, which enabled us to distinguish between individual neonates (length ca. 200 µm) in the tubing as they passed the photocell at intervals as short as 0.1 s. Accuracy in counting was increased by a flushing of the dead volume between the three-way valve and the test tube with purging water (Fig. 3), and by capping the outlet capillary of the test tube with sintered glass to prevent accidental losses (insert of Fig. 4). For D. magna and D. pulex, the gravid females were held in beakers, and the neonates were removed and transferred with a pipette.

2. The full and sealed exposure vessel (Fig. 4), with its own peristaltic pump tube, formed one bioassay unit; it could be independently regulated, clamped off, held in any position, or submerged. To avoid absorption and leaching of substances from and onto the tygon pump hose, we positioned the peristaltic pump (Technicon Autoanalyzer Proportionating Pump) at the outlet of the test tube. To obtain any desired exchange rate, we increased or decreased the renewal at a preset pump speed by substituting peristaltic hoses of different delivery volumes or test tubes of different sizes. Measurements of the 48-h delivery volume of 27 randomly selected Technicon pump tubes of 0.05 mL/min nominal size yielded a coefficient of variation of 7.8% ($\bar{x}$ = 0.0468 mL/min, SE = ± 0.0007). In constructing exposure vessels, teflon stoppers of standard taper, with interchangeable matching glass centrifuge tubes proved to be most convenient.

3. The schematic diagram in Fig. 4 shows the premixing of the test solutions with minimum contact time between food (algae) and toxicant. The two fluids--i.e., water containing food and water containing toxicant or solvent, or both, from the syringe pumps--combined with metered air from the peristaltic pump, entered the mixing coil. The 14-turn mixing coil (Technicon) ensured homogeneity of the two liquids and uniform aeration. To prevent air accumulation inside the exposure tube and resulting capture of cladocerans on the surface film, we interposed a debubbler (Figs. 4 and 5) between the mixing coil and test chamber and adjusted the

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4/ Use of trade names or manufacturers' names does not imply U.S. Government endorsement of any commercial product.

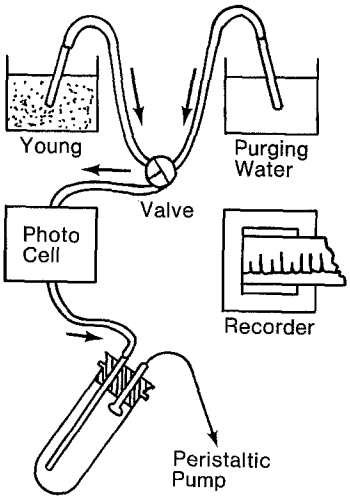

FIGURE 3. Schematic of the transfer system showing position of particle counter for counting neonate crustaceans.

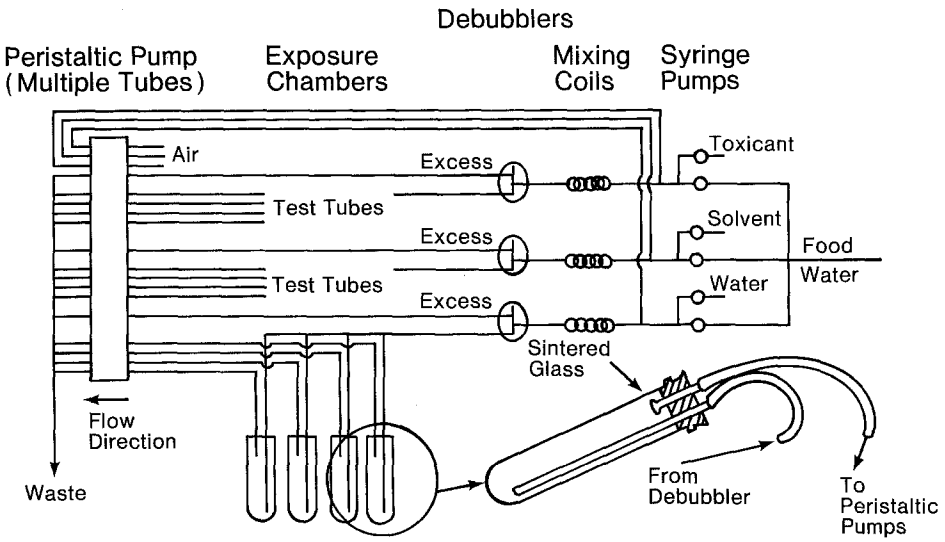

FIGURE 4. Schematic of continuous-flow system that provides one organic toxicant concentration, one solvent control, and one control with diluent water only. (For tube sizes, see Table 1.)

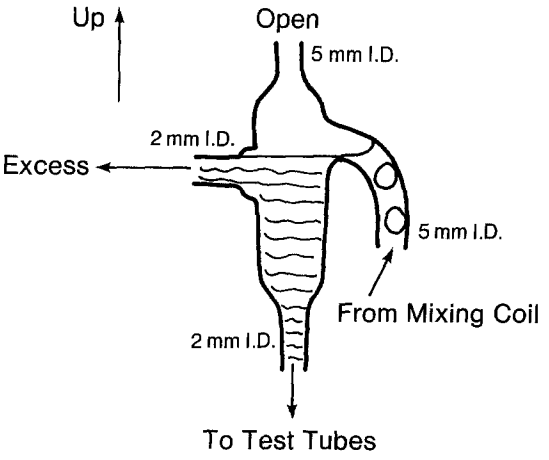

FIGURE 5. Freehand drawing of Technicon Company's C 6 "T" connector with bubbler. Numbers indicate inside diameter in mm.

amount of water entering the debubbler to be in excess of the volume drawn into the exposure tubes, thus ensuring the exposure tubes would constantly be full.

Throughout the experiment, neither the test animals nor the adult females were exposed to water that had come in contact with anything but inert and nontoxic material. Accordingly, screens, valves, flexible tubing, syringe pumps, etc., were of glass, teflon, or polyethylene; heat welding and sleeving replaced gluing at every connection.

RESULTS AND DISCUSSION

The usefulness of the method is illustrated by the results of typical control bioassays (Table 2) run according to the conditions stated above. The percentage of immobilized D. pulex or D. magna, 1.7% and 3.0% in 48-h and 1.7% (D. pulex) in 96-h tests, showed that the flow-through apparatus functioned adequately. (Standard methods require that at least 90% of the control animals survive [APHA et al. 1980]). The low immobilization incidence for the Bosmina experiments (Table 2)--4.0% and 4.9% in 48-h and 6.9% in 96-h tests--showed that the system as a whole worked well, since B. longirostris appeared to be more sensitive to manipulation than the other two species.

The bioassay equipment required only 0.5 m^2 of counter top area. The compactness of the system was made possible by loading many neonates (usually 10) into each test tube and placing a large number of these tubes into a test tube rack. To avoid mistakes, we color-coded the pairs of flexible tubing to the exposure vessels

and bundled the double-veined leads in cable fashion (to facilitate unhindered access to the vessels). Although the system was designed for small cladocerans, it could easily be scaled-up to accommodate larger organisms by converting to larger exposure containers and pump tubes.

The system should function well regardless of the choice of components, provided that (1) the particle counter has a light path and slit width commensurate with the size of the organism; (2) the recorder is sensitive enough to distinguish between neonates passing through the flow cell in close succession; (3) the peristaltic pump operates with a flow rate not more than ± 10% tolerance (APHA 1980); and (4) the peristaltic pump accommodates a large number of hoses.

Use of this system lessens or resolves many of the difficulties inherent in bioassays with small crustaceans. Whereas the minute size of B. longirostris would hamper counting and handling when using conventional methods, the smallness of the organisms in the described system greatly facilitates testing. Loading the test containers with an exact number of young bosminids of uniform age and size is now possible. Other advantages include the capability of monitoring flow volume, concentration of food, dissolved oxygen, etc., at the outlet of the peristaltic pump. In addition, at the end of the experiments, fixatives, fluorescent dyes, strong oxidants, or cleansing agents can be pumped through the system to preserve the test organisms, enhance their visibility during counting, or decontaminate the assembly. The system also eliminates the possibility of cladocerans being trapped in the surface film.

TABLE 2. Results of 48- and 96-h flow-through control tests with Bosmina longirostris, Daphnia pulex, and D. magna: Percentage of organisms immobilized during 48- and 96-h test periods ($\bar{x}$ ± SE).

Test animal	Neonates		Test period	
	No./test tube	No. at 0 h	48 h	96 h
Bosmina longirostris				
Fed	10	100	4.9 ± 0.7	6.9 ± 1.2
Unfed	10	50	4.0 ± 1.8	--
Daphnia pulex				
Fed	5	60	1.7 ± 0.5	1.7 ± 0.5
Unfed	5	60	3.3 ± 0.9	--
D. magna				
Unfed	15	300	3.0 ± 0.2	--

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REFERENCES

AMERICAN PUBLIC HEALTH ASSOCIATION, AMERICAN WATER WORKS ASSOCIATION, and WATER POLLUTION CONTROL FEDERATION: Standard methods for the examination of water and wastewater, 15 ed. Washington, DC: Am. Public Health Assoc. 1980.

AMERICAN SOCIETY FOR TESTING AND MATERIALS: Standard practice for conducting acute toxicity tests with fishes, macroinvertebrates, and amphibians. Philadelphia, PA: Am. Soc. Test. Mat. E 729-80 1980.

CLARKE, G. L.: J. Exper. Biol. 9, 180 (1932).

DEWEY, J. E., and B. L. PARKER: J. Econ. Entomol. 6, 821 (1964).

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