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A Simple Toxicity Apparatus for Continuous Flow with Small Volumes: Demonstration with Mysids and Naphthalene

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Toxicity tests with small invertebrates afford the advantage of small scale in both cost and effort (MACIOROWSKI & CLARKE 1980). However, many investigators performing continuous-flow toxicity tests with small invertebrates (e.g., HEINLE & BEAVEN 1980; ROBERTS 1980; NIMMO et al. 1978) have adapted complex exposure systems pioneered by MOUNT & BRUNGS (1967) for testing fish. These systems require a nearby natural source of dilution water, are vulnerable to mechanical breakdown and power failure, and are difficult to clean. If the test substance is listed as a priority pollutant under the Clean Water Act, proper disposal of the large volume of wastewater generated by a typical toxicant exposure system may be difficult. Some investigators have recently used innovative small continuous-flow exposure systems, but these are either not suitable for volatile toxicants (e.g., BENOIT et al. 1982; BUCHANAN et al. 1975) or are as complex as the Mount and Brungs system (e.g., RILEY et al. 1981; BIRGE et al. 1979; CAPUZZO et al. 1976).

To determine the toxicity of naphthalene to the mysid crustacean Neomysis americana, we built a small continuous-flow toxicant exposure system using design features of Mariotte bottle chemostats. Chemostats maintain cultures at constant cell concentrations by providing constant nutrient inputs (NOVICK & SZILARD 1950). Such cultures remain well-mixed (HERBERT et al. 1956), and have minimal cell contact with the container (KUBITSCHEK 1970). The same characteristics are useful for exposing small invertebrates to volatile toxicants.

The Mariotte bottles in our exposure system were modified by addition of air inlet restrictors. This modification provided stable flow at low pressure heads; we discovered that flow was unstable under such conditions with standard Mariotte bottle designs (e.g., KUBITSCHEK 1970). Our apparatus provided good water quality and physical environment for 10 mysids per chamber, and a more constant concentration of naphthalene than did static chambers replenished daily.

MATERIALS AND METHODS

Solutions of naphthalene in artificial seawater were prepared with 0.5% naphthalene in 80% ethanol and placed in one-liter glass modified Mariotte bottles (Figure 1) with teflon^{tm} lined lids. The bottles were based on Mariotte bottles described by KUBITSCHEK

(1970) and widely used by physiologists. Two 3.2 mm holes were drilled in each cap and filled with silicone cement. An air inlet tube and a mixture outlet tube were forced through the cement for a positive seal. Resistance to flow was provided by a long piece of thin-wall teflon tubing (after KOCH 1971). A short piece of thin-wall teflon tubing was inserted in the upper end of the air inlet tube to prevent pressure surges as air bubbles were released.

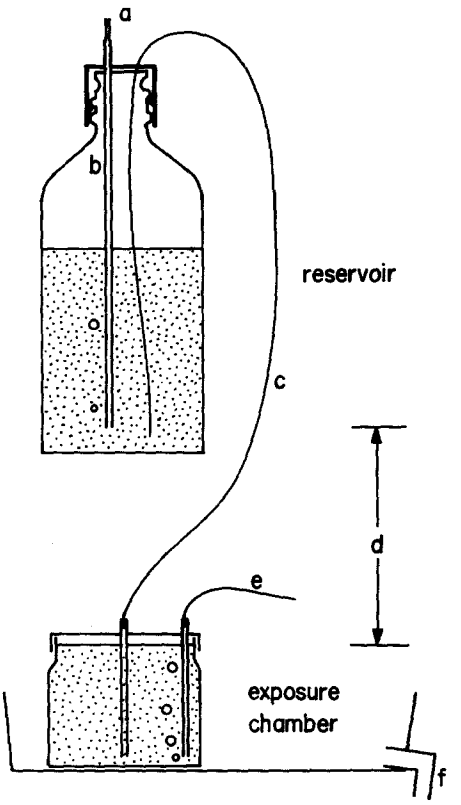

Figure 1. Continuous-flow toxicity apparatus. a = air inlet restriction (15 mm length of 0.562 mm i.d. thin-wall teflon tube), b = air inlet tube (3.2 mm o.d., 1.6 mm i.d. teflon), c = mixture outlet tube (0.562 mm i.d. thin-wall teflon), d = effective pressure head, e = aeration tube (0.305 mm i.d. thin-wall teflon), f = drain to collection jug.

Toxicant mixture was delivered to the exposure chamber by gravity. Flow was controlled by the constant pressure head of the Mariotte bottle and the constant resistance of the teflon tube, predicted by the Poiseuille-Hagen equation (WILSON 1979):

$$\dot{V} = \frac{P \pi r^4}{8 \eta L} \quad (1)$$

$\dot{V}$ = flow ($cm^3\ s^{-1}$)
P = pressure ($dyn\ cm^{-2}$)
r = tube radius (cm)
η = viscosity ($dyn\ s\ cm^{-2}$ = poise)
L = tube length (cm)

The exposure chamber was a 500 ml Pyrex^{tm} culture dish with a petri dish lid. The toxicant-water mixture entered the chamber through the center of the lid; effluent left the chamber by overflowing the sides. This left little air space between the water surface and the chamber lid, which decreased evaporation of naphthalene and prevented mysids from jumping from the water and sticking to the lid. A teflon air tube supplied clean compressed air at a rate of several bubbles per second. Aeration aided the animals' orientation (NIMMO et al. 1978), promoted mixing, and maintained adequate oxygen levels.

The exposure chambers were grouped in polyethylene trays with drain tubes leading to a collection jug. Twelve complete toxicant exposure systems fit inside a Precision^{tm} refrigerated incubator which allowed short-term temperature control within 0.2°C. With modifications, 24 or more reservoirs and chambers could fit in the incubator.

Predictability of flow was determined using seven combinations of tube length and pressure head; constancy of flow was determined by measuring output of deionized water from two reservoirs in a 20°C incubator for 53 h. Flushing time was calculated using the decaying exponential equation:

$$A_t = A_o e^{-kt} \quad (2)$$

A_t = absorbance at time t (absorbance units)
A_o = absorbance at time 0
k = theoretical rate constant (chamber volumes per hour)
t = time (hours)

Flushing time was measured experimentally by introducing clear deionized water at 461 ul day^{-1} to a chamber filled with water containing green dye with a peak absorbance at 629 nm. Decay of the dye concentration was measured for 22 h with a Perkin-Elmer Lambda 3 dual beam spectrophotometer with a 200 µl flow cell.

Rate of naphthalene loss from the reservoirs was determined by continuously measuring naphthalene concentration from a reservoir for 77 h. Naphthalene concentration was calculated from differential absorbance at 219 nm (HARGREAVES et al. in press). Concentration of naphthalene in seawater in the continuous-flow apparatus was compared to that in static chambers changed daily, simulating the modified 96 h static toxicity test used by HARGREAVES

et al. (in press) and THOMPSON & HARGREAVES (1981). In the continuous flow apparatus, 600 $\mu g\ l^{-1}$ naphthalene in the reservoir resulted in a steady state concentration of 360 $\mu g\ l^{-1}$ in the exposure chamber. A naphthalene concentration of 300 $\mu g\ l^{-1}$ was used in the static chamber.

Preliminary 96 h toxicity tests with N. americana were performed at 15°C and 25°C at concentrations of 900 and 1800 $\mu g\ l^{-1}$ naphthalene in artificial seawater, for comparison with similar tests performed under static conditions by HARGREAVES et al. (in press).

RESULTS AND DISCUSSION

Pressure head, tubing length, and tubing radius combinations produced flow rates within 14% of those predicted by equation (1). Discrepancies between observed and predicted flows were assumed to be the result of irregularities in the tubing. Flow could be reproduced by using the same ratio of tube length to pressure head. Very low flows were not attempted, but constant flows of 1 ml h^{-1} should be possible.

Flow remained constant within 1.3% for 53 h, regardless of volume remaining in the reservoir. KUBITSCHEK (1970) used flared ends on air intake tubes of Mariotte bottles to stabilize flow rate. The increased volume at the flare prevented liquid from rising in the air inlets during rapid decreases in atmospheric pressure or increases in temperature. Such changes in liquid level in the air tube would raise the pressure head and result in unstable flow rates. We found flared intake tubes unsuitable at low pressure heads because they released large bubbles that caused flow surges. We tried capillary air inlet tubes, but found that liquid levels oscillated in capillary tubes, causing pressure variations. Oscillations were less severe with tubes of intermediate size, and were successfully damped by air inlet restrictors (Figure 1). Air inlet filters, used in chemostats to maintain sterile media (e.g., SHAMAT & MAIER 1980), may also damp oscillations if they provide sufficient resistance to air flow. The oscillations would not be significant if a large pressure head were used, but this would require substantially more space and tubing.

Effects of atmospheric pressure and temperature on flow stability were prevented by careful selection of flow rate and reservoir size. With a one liter reservoir, a flow of 144 ml day^{-1} is sufficient to prevent liquid from rising in the air inlet tube during pressure decreases of up to 4.24 torr h^{-1}, twice the maximum rate of decrease recorded on our barometer during several years. A comparable pressure change would occur in the reservoir during a temperature increase of 17.4°C h^{-1}, which we avoided by placing the apparatus in an incubator. Stabilization of flows lower than 144 ml day^{-1} could be achieved by using a smaller reservoir.

The theoretical rate constant k (equation 2), determined by influent rate, was 0.0402 h^{-1} at the test flow of 461 ml day^{-1} and chamber volume of 470 ml. Observed k (the negative slope of the

line $\ln A_t = \ln A_o - kt$) was 0.0433 h^{-1} ($r^2 = 0.9994$). Observed k exceeded theoretical k by 8%, indicating a slight tendency for newly introduced water to stratify at the bottom of the chamber. The observed time needed to renew 90% of the chamber's contents was 51.6 h; theoretical time for 90% renewal was 56.4 h. Flows of 1080 and 540 ml day^{-1} would result in theoretical 90% renewal times of 24 and 48 h, respectively.

Naphthalene concentration in the reservoir declined at 2.3 µg l^{-1} h^{-1} over a 77 h period ($r^2 = 0.977$). The final concentration was 325 µg l^{-1}. Naphthalene disappearance probably resulted from evaporation into the reservoir head space, which could be reduced in future tests by passing intake air through a bottle containing naphthalene test solution before introducing it into the reservoir.

Naphthalene concentrations declined significantly between daily static chamber refills, but remained constant in the continuous-flow chambers after 36 h (Figure 2). The rate of toxicant loss from the static system appeared to become greater each day, possibly the result of naphthalene-utilizing bacteria growing in the exposure chamber. Toxicant degradation by bacteria may have to be controlled for long term continuous-flow tests by adding an antibiotic to the toxicant mixture.

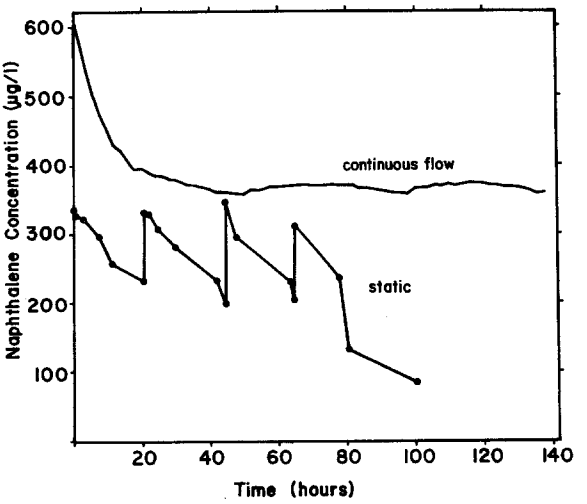

Figure 2. Concentration of naphthalene in artificial seawater in continuous-flow exposure chambers and in static chambers replenished daily.

Estimated continuous-flow 96 h LC50s were 1280 µg l^{-1} at 15°C and 850 µg l^{-1} at 25°C. Naphthalene-induced mortality was significantly greater at 25°C than at 15°C for both concentrations ($p<0.025$, Wilcoxon's signed rank test, SOKOL & ROHLF 1969). These results agree closely with the static test results of 1420 µg l^{-1} at 15°C and 800 µg l^{-1} at 25°C reported by HARGREAVES et al.

The exposure system offered several advantages. Low flow allowed use of artificial seawater in small amounts, making it possible to perform tests at an inland location and with standard dilution water. The materials were easily obtained, inexpensive, and chemically inert. Completed systems fit in a small space, were easy to operate and clean, and required no electricity to maintain toxicant concentration. Used toxicant could be collected for proper disposal.

The apparatus should prove useful for tests similar to ours, but it is not universally applicable. It was not suited for large organisms or high flow rates. Considerable toxicant volatilization occurred in the exposure chambers, requiring allowance for toxicant loss and time to reach steady state concentration. Dilution water had to be equilibrated to the test temperature, or bubbles formed in the feed tubes and blocked flow. Filtration may be advisable for removing stable microbubbles (JOHNSON & COOKE 1982) that may act as nuclei for formation of larger bubbles.

We believe this apparatus fills a gap in the techniques available to aquatic toxicologists, particularly those working with small test organisms. The apparatus could be modified to serve as a miniature flow respirometer for individual mysids. A proportional dilutor could be built based on different flows from one reservoir of stock solution. Off-site wastewater toxicity tests could be performed with samples small enough to be carried by hand and in small vehicles. Perhaps most important, this method allows high quality toxicity testing to be done at low cost.

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