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Importance of Bioassay Volume in Toxicity Tests Using Algae and Aquatic Invertebrates

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Toxicity bioassays are routinely employed to predict the environmental impact of xenobiotics towards aquatic ecosystems. Many of these tests are static in nature and involve incubating test organisms in water treated with the compound of interest. One problem with the toxicity data elicited from these bioassays is the lack of uniformity between results obtained from different laboratories. This may be due, in part, to a lack of standardization in bioassay conditions, particularly the choice of total test volume and the number of test subjects (Smith and Stratton 1986). These two factors determine the amount of toxicant that each cell or organism is exposed to at any given toxicant concentration and, therefore, have a profound influence on LC_{50} and EC_{50} values (Smith and Stratton 1986). As a result, it has recently been suggested that these values be replaced in aquatic bioassays by ones based upon toxicant exposure; ie. LE_{50} and EE_{50}, which are the exposures required to kill 50% of the test subjects or cause a 50% inhibition of the test criterion, respectively (Smith and Stratton 1986). These can be calculated mathematically from LC_{50} and EC_{50} data, if both the total test volume and the number of test subjects are known. This often improves the uniformity among toxicity data obtained from different sources (Smith and Stratton 1986). The purpose of the present study was to further investigate the effect of bioassay volume on the toxicity of several pesticides towards an aquatic invertebrate and an alga.

MATERIALS AND METHODS

The unicellular green alga Chlorella pyrenoidosa and the freshwater cladoceran Daphnia magna were used as test organisms. C. pyrenoidosa was obtained from the Department of Botany and Genetics, University of Guelph, Guelph, Ontario, Canada. Axenic stock cultures were grown in 250-mL flasks sealed with cotton bungs and containing 150 mL of an inorganic nitrogen-free medium (Stratton and Corke 1979) supplemented with 1.5 g/L of $NaNO_3$. Flasks were incubated for seven days at 25±1°C and a light intensity of 7000 lux on a 12-hr light-dark cycle. Cell concentrations were determined microscopically using a haemocytometer and standardized to $1x10^6$/mL prior to use. D. magna were reared in 10-L glass aquaria containing filtered pond water. They were fed daily with C. pyrenoidosa cells (10^8 cells per aquarium) supplemented with several grams of baker's yeast. Aquaria were incubated under the conditions outlined above.

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Several pesticides were used as test chemicals. These included the *s*-triazine herbicide atrazine (technical grade; Ciba-Geigy Canada Ltd., Cambridge, Ontario, Canada; 95% pure), the pyrethroid insecticide permethrin (technical grade; Chipman Chemical Ltd., Stoney Creek, Ontario, Canada; 86.6% pure; 40:60 mixture of cis and trans isomers), and p,p' DDT (technical grade; Chem Service Inc., West Chester, Pennsylvania, U.S.A.; 99% pure). Pesticide stock solutions were prepared in pesticide-grade acetone (Caledon Laboratories, Georgetown, Ontario, Canada). The maximum solvent levels employed (1.0% with C. pyrenoidosa and 0.01% with D. magna) did not interfere with the bioassay results, as determined using the solvent-pesticide interaction analysis method of Stratton et al. (1982). Pesticide concentrations refer to the active ingredient.

Photosynthesis was used as the bioassay criterion with C. pyrenoidosa and was quantitated by following the uptake of $^{14}CO_2$ from ^{14}C-sodium bicarbonate (Amersham Corp., Oakville, Ontario, Canada; containing 3.72 MBq of radioactivity per mL; 1 MBq=27 μCi), as outlined previously (Stratton and Corke 1979). The required number of cells were incubated in the appropriate volume of culture medium, containing 0.1 μCi of radioactivity per mL, and treated with either atrazine dissolved in acetone, or acetone alone. The background solvent concentration was 1.0% v/v. Each test system had its own solvent control. All treatments were replicated ten times. Half of these were incubated at 25±1°C for 24 hr under a continuous light intensity of 7000 lux, while the other half were incubated in the dark. Radioactivity incorporated into the cells was determined using the method of Stratton (1989). This involved harvesting the cells by filtration through membrane filters, solubilization of the pads, and counting radioactivity using a liquid scintillation technique. Corrected counts were adjusted for dark uptake and recorded as cpm/10^5 cells. Cell concentrations were determined in unfiltered aliquots as outlined above. Percent inhibition was calculated for each test system using appropriate controls.

C. pyrenoidosa cells were treated with atrazine in two series of experiments. In the first, the cell numbers were held constant while the total bioassay volume varied. Cells were exposed to 0.4 ppm (mg/L) of atrazine in volumes of 10, 25, 50, 100, and 150 mL contained in 200-mL glass beakers. Each system contained a total of $1x10^6$ cells. In the second series of experiments, the bioassay volume was held constant while the cell numbers varied. Cells were exposed to 0.75 ppm atrazine in a volume of 10 mL contained in 74-mL tissue culture flasks. Total cell numbers varied from $1x10^5$ to $1x10^7$ per flask.

Immobilization was used as the toxicity criterion with D. magna. This was defined as the inability to show any motion when prodded with a glass rod. Juvenile (1-2 mm long, <24 hr old) or adult (3-5 mm long) D. magna were harvested (Stratton and Corke 1981) and exposed to permethrin or DDT in glass vessels containing an appropriate volume of filtered pond water. Background levels of acetone were ≤0.01% v/v. Ten D. magna were added to each container. Each test system had its own solvent control and all treatments were replicated five to ten times. Experiments were held at 25±1°C under the light conditions used for rearing, and percent immobilization was determined after 48 hr. D. magna remained unfed during the bioassays. Data from treated systems were corrected for toxic effects in solvent controls using Abbott's formula (Finney 1971). Where

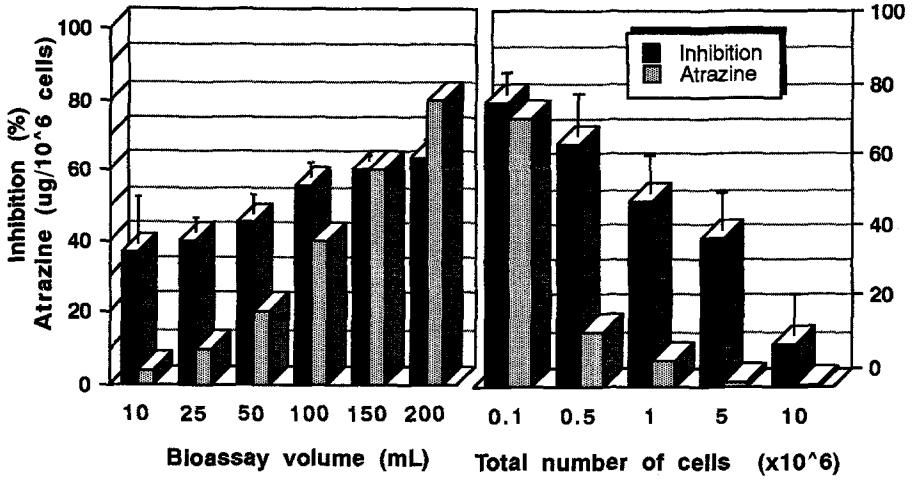

Figure 1. Effect of bioassay volume and cell numbers on the toxicity of atrazine towards photosynthesis in C. pyrenoidosa.

applicable, LC_{50} values were calculated from dosage-toxicity curves using probit analysis (Finney 1971; SAS statistics software, SAS Institute Inc., Cary, NC, U.S.A.).

D. magna was treated with permethrin or DDT in several series of experiments in which the bioassay volume varied. With permethrin, D. magna was exposed to a concentration of 0.5 ppb (μg/L) in volumes of 50, 100, 150, 200, 250, 300, 350, and 400 mL contained in either 400-mL beakers (all volumes except 400 mL) or 500-mL flasks. Separate dosage-toxicity experiments were also performed using permethrin levels of 0.05, 0.1, 0.5, 1.0, 5.0, 10, and 25 ppb at bioassay volumes of 150 and 300 mL. With DDT, complete dosage-toxicity curves were prepared using DDT concentrations of 0.25, 0.5, 1.0, 1.5, and 2.0 ppb at each of the following bioassay volumes: 1000, 750, 500, 300, and 100 mL. Several types of containers were used in these experiments in order to maintain a constant ratio between the total bioassay volume and the surface area of glass exposed to the test solution. This ratio was 2.10±0.06 mL/cm^2. The surface area of the glass was calculated mathematically using formulae that best approximated the shape of the containers used.

Relationships between bioassay conditions and toxicity effects were presented graphically (means with standard deviation bars). Equations of the lines were obtained using microcomputer software (Cricket Graph, Cricket Software, Malvern, PA, U.S.A.). Significant differences were determined using an analysis of variance followed by a Duncan's multiple range test at P=0.05 (SAS Institute Inc., 1985).

RESULTS AND DISCUSSION

The effects of varying the bioassay volume and total cell numbers on the toxicity of atrazine towards photosynthesis in C. pyrenoidosa are summarized in Figure 1. Atrazine exposure can be increased, either by increasing the bioassay volume or by decreasing the total number of cells. Increased atrazine exposure was reflected by an increase in toxicity, since

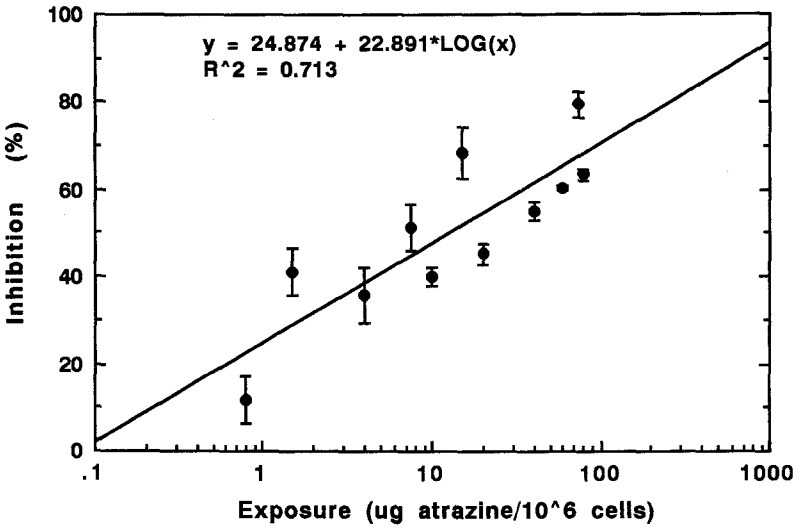

Figure 2. Relationship between atrazine exposure and the inhibition of photosynthesis in C. pyrenoidosa.

more atrazine was available to interact with the individual cells. When the exposure values from these experiments were combined and plotted against the percent inhibition, a logarithmic relationship was obtained (Figure 2). Although the correlation coefficient was somewhat low, it is apparent that exposure data from various experiments can be pooled. Using the equation in Figure 2, the exposure level that induces a 50% inhibition of photosynthesis in C. pyrenoidosa (EE_{50}) was calculated as 12.5 μg of atrazine per 10^6 cells. A range was estimated for this by calculating EE_{50} values from data obtained in the individual experiments outlined in Figure 1. The correlation coefficients were >0.90 (data not shown) and the calculated EE_{50} values ranged from 6.1 to 23.1 μg of atrazine per 10^6 cells.

Numerous data are available on the toxicity of atrazine towards algae. In general, EC_{50} values for growth and photosynthesis range from 0.04 to 1.0 ppm (reviewed in Stratton 1987). However, it is difficult to compare these data with the results obtained in the present study. This is because both bioassay volume and cell numbers are needed to calculate toxicant exposure. Most researchers provide details regarding the bioassay volume used, but fail to indicate the numbers of microorganisms employed in their experiments, even though it has been known for some time that cell numbers have a significant effect on the toxicity of pesticides towards microorganisms (Kar and Singh 1978). Few reports are available that provide the required details to calculate atrazine exposure towards Chlorella. Larsen et al. (1986) reported that the EC_{50} of atrazine towards photosynthesis in C. vulgaris was 0.29 to 0.33 ppm. This can be recalculated as an EE_{50} of 29 to 33 μg of atrazine per 10^6 cells. Stratton (1984) reported an EC_{50} of 0.5 to 1.0 ppm for the effects of atrazine towards growth and photosynthesis in C. pyrenoidosa. This can be recalculated as an EE_{50} of 5 to 10 μg of atrazine per 10^6 cells. These are both close to the exposure range obtained in the present study. The problem with microbial bioassays is the difficulty in accurately determining cell numbers. Counting errors can lead to significant discrepancies in exposure results.

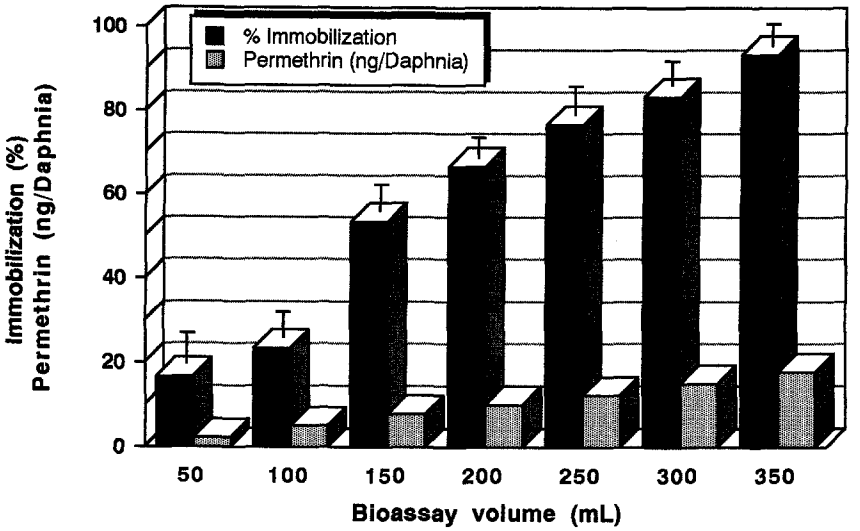

Figure 3. Effect of bioassay volume on the toxicity of permethrin towards D. magna.

The effects of bioassay volume on the toxicity of permethrin towards juvenile D. magna are summarized in Figure 3. Again there was an increase in toxicity as the bioassay volume, and hence the total amount of available pesticide, was increased. When the exposure values for the permethrin experiments were combined and plotted against the percent immobilization, a logarithmic relationship was again obtained (Figure 4). In contrast to the Chlorella data (Figure 2), the Daphnia data yielded a higher correlation coefficient. Using the equation in Figure 4, the exposure level that induces a 50% immobilization (LE_{50}) was calculated as 6.4 ng of permethrin per Daphnia. A range of 2.9 to 8.0 ng/Daphnia was established for the LE_{50} by repeating the bioassays (data not shown). Dosage-toxicity curves were also prepared using adult Daphnia and bioassay volumes of both 150 and 300 mL. In the former, permethrin yielded a 48-hr LC_{50} value of 1.06 ppb, which was recalculated as an LE_{50} of 15.9 ng/Daphnia. When the bioassay volume was 300 mL, the LC_{50} value was 0.43 ppb, while the LE_{50} was 12.9 ng/Daphnia. Although the LC_{50} values differ significantly, the LE_{50} values do not. Therefore, the calculation of exposure values can reduce the variability in data resulting from either differences in bioassay volume or cell numbers. However, when the data for juvenile and adult Daphnia are compared it is apparent that the toxic exposure level is itself dependent upon the size of the organism. Variations in size would not be a major problem with most microorganisms, since the cell sizes are fairly uniform. Many bioassays with aquatic invertebrates already standardize the age, and hence the size, of organisms used.

The LC_{50} values obtained for permethrin in the present study are comparable to those published elsewhere. Pyrethroids are extremely toxic to aquatic invertebrates and most EC_{50} and LC_{50} values are $\leq$1.0 ppb (reviewed in Smith and Stratton 1986). For example, Stratton and Corke (1981) reported that the 48-hr LC_{50} for permethrin towards D. magna was 0.2 to 0.6 ppb, which can be converted into an LE_{50} value of 4 to 12 ng/Daphnia. This is within the exposure range noted in the present study.

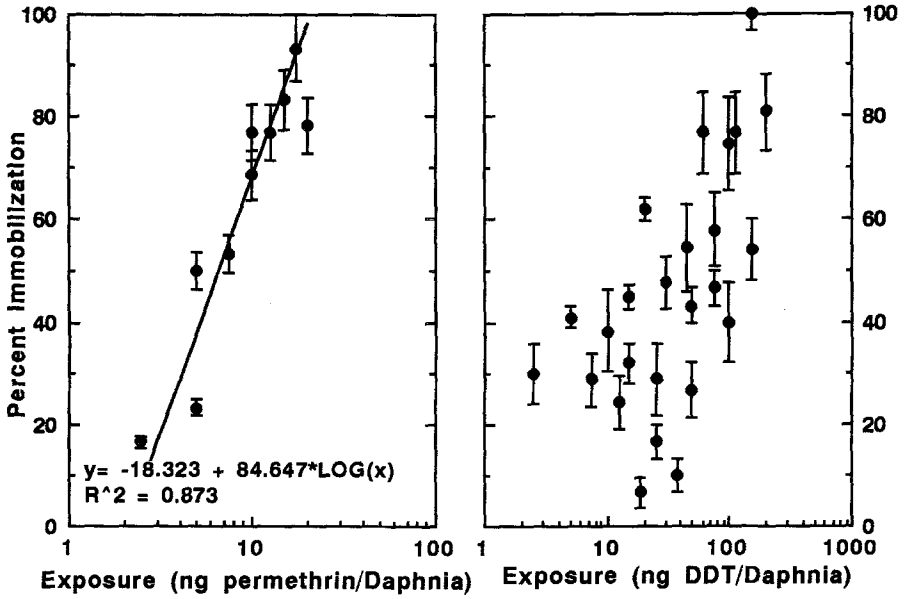

Figure 4. Relationship between pesticide exposure and immobilization in juvenile D. magna.

In contrast to microbial tests, bioassays with aquatic invertebrates usually do provide details on both the bioassay volume used and the number of test organisms employed. The problem is insufficient standardization between laboratories.

The effects of varying the bioassay volume on the toxicity of DDT towards juvenile D. magna were examined in a different manner. Pesticides are known to bind to glass surfaces, so in these experiments the possible influence of total surface area was investigated. Containers were chosen so that various bioassay volumes could be used, but in which the ratio of volume to exposed surface area was the same (2.10±0.06 mL/cm^2). Complete dosage-toxicity curves were prepared at each of five bioassay volumes ranging from 100 to 1000 mL. These data were used to calculate 48-hr LC_{50} values. The correlation coefficients of the resulting probit lines ranged from 0.966 to 0.996. The LC_{50} values were 1.23, 1.08, 1.20, 1.13, and 1.51 ppb at volumes of 1000, 750, 500, 300, and 100 mL, respectively, which are equivalent to LE_{50} values of 123, 81, 60, 34, and 15 ng of DDT/Daphnia. Although the LC_{50} values do not differ significantly, the LE_{50} values do. This is in contrast to data obtained in the other experiments. When the exposure values for the DDT experiments were combined and plotted against the percent immobilization, no clearly defined logarithmic relationship was obtained, although the data still indicate a general upwards trend (Figure 4; correlation coefficient <0.35). These data indicate that surface area, as well as total volume, should be standardized in aquatic bioassays.

Numerous other toxicity studies have been performed using Daphnia and DDT. Most of these report a 48-hr LC_{50} value of <1.0 to 2.0 ppb, which is consistent with the data presented here. For example, Randall et al.

(1979) reported a 48-hr LC_{50} of 1.0 to 1.3 ppb towards juvenile D. magna, but provided no details on bioassay volume. Berglind and Dave (1984) obtained a 48-hr LC_{50} of 1.1 to 1.3 ppb, also using juvenile D. magna. This can be converted into an LE_{50} value of 11 to 13 ng DDT/Daphnia. Sanders and Cope (1966) reported a 48-hr LC_{50} of 0.36 ppb with D. pulex, which yields an LE_{50} of 5.4 ng DDT/Daphnia. LC_{50} values for adult D. magna are somewhat higher, reaching levels up to 6.5 ppb DDT (Rawash et al. 1975).

The data presented here indicate that LC_{50} and EC_{50} values may not be the most suitable method of presenting toxicity data from aquatic bioassays. This is because these values refer to toxicant concentrations, not total toxicant exposure. Exposure is the important criterion in bioassays and is dependent upon both the bioassay volume and the number of test organisms used. The problem is that these two factors are often not standardized in bioassays, particularly when using microorganisms. Based upon the data presented here, it appears as though an exposure index, such as EE_{50} or LE_{50}, can be used to overcome some of these problems. However, exposure can also be significantly affected by the size of the test organism and the surface area of glass exposed to toxicant in the bioassay container. These parameters also need to be standardized. Use of the suggestions outlined above should improve the uniformity in toxicity data obtained from different laboratories.

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