Bull. Environm. Contam. Toxicol. 26, 433-439 (1981)

A Rapid Toxicity Test Using *Pseudomonas fluorescens*

J. T. Trevors, C. I. Mayfield, and W. E. Inniss*

Department of Biology, University of Waterloo, Waterloo, Ontario, Canada, N2L 3G1

Rapid methods for assessing toxicological effects of chemicals are needed to simplify the task of screening large numbers of potentially toxic chemicals. Bacteria are particularly suitable for this task because of their rapid growth rates and ubiquitous distribution. Early attempts concentrated on application of chemicals to growing cultures monitored by various means to elucidate growth rate changes or differences in biomass yields. In such studies, especially those in batch cultures, the criticism has been that the exposure period encompasses the complete growth cycle of the organism, so that a particular dose of chemical has been applied to an increasing biomass of cells. Attempts to circumvent these problems have typically used continuous culture methods (MAYFIELD et al. 1980; HEALEY 1979) or exposure of non-proliferating cells. Detection of cells surviving the exposure to the toxicant has been hampered by the presence of residual toxicant in the exposure medium and subsequent transfer of toxicant to the system used to detect viability.

Bacteria in the environment are often growing slowly on low nutrient concentrations (ALEXANDER 1961; JANNASCH 1969,1974). They respond to nutrient input by rapid growth and proliferation. Exposure to toxicants, even briefly, during these inactive periods may affect subsequent growth and activity.

In the present study, we have developed a rapid bacterial toxicity test procedure which exposes the test bacteria to toxicants for short periods under nutrient-limited conditions. Exposure is followed by substantive removal of toxicant from the medium before determination of the percentage of surviving cells. The proposed toxicity test was tested with pentachlorophenol (PCP) as the toxicant because it is a widely used herbicide and fungicide and many of its physical, chemical and biological properties are known (RAO 1978).

MATERIALS AND METHODS

Initial tests on the reactions of the test bacteria to PCP during the growth cycle and on the response of the cells to different temperatures during exposure were performed. The effects of various exposure times were also investigated. From these results a toxicity test procedure was developed which used stan-

* Corresponding author: W. E. Inniss

0007-4861/81/0026-0433 $01.40

dardized inocula of bacteria at different phases of growth and exposed them to the toxicant under limited nutrient conditions. A procedure for removal of any residual toxicant in the exposure medium before the viability of treated cells was determined was also developed and incorporated into the toxicity test procedure.

1). Preparation of standardized inocula.

Pseudomonas fluorescens (ATCC 11250) was cultured for 18 h in nutrient broth (Difco Ltd., concentration 4 g L^{-1}) at 20°C and 120 rpm on a rotary shaker. After suitable times, the cells were aseptically harvested by centrifugation at 8,000 X g for 15 min, washed in sterile 150 mM phosphate buffer (pH 7.0), and resuspended in the same buffer to provide a final cell density of 4 X 10^9 cells mL^{-1}.

2). Effects of PCP on growth of cells.

To determine the effects of PCP on the growth rate of P. fluorescens, 75-mL quantities of nutrient broth were inoculated with the standardized bacterial suspension. Triplicate flasks contained various concentrations of PCP previously filter-sterilized and dissolved in 0.1 mL of 95% ethanol. Control flasks received only ethanol. The flasks were incubated at 20°C at 120 rpm on a rotary shaker and growth was monitored spectrophotometrically at 650 nm.

3). Effects of exposure time on PCP toxicity.

The standardized inoculum (1 mL in 75 mL of nutrient broth) was exposed to PCP at various concentrations (0 - 75 $\mu g\ mL^{-1}$) for times ranging from 1 min to 60 min. After exposure, 1 mL of the broth culture was removed and transferred to 75 mL of fresh nutrient broth. All flasks were then incubated for 24 h at which time the OD_{650} of each flask was measured spectrophotometrically.

4). Effect of temperature on PCP toxicity.

The effect of temperature during exposure to PCP on subsequent growth was investigated by adding PCP in 1 mL of 95% ethanol to the standardized inoculum in phosphate buffer (pH 7.0). The flasks were incubated at 4, 20 or 30°C for 120 min while shaking at 120 rpm. The flasks and contents equilibrated after a maximum time of 10 min. At zero time and at periodic intervals samples were removed and the viable cells were counted using a standard plate count technique with nutrient agar (4 g L^{-1} nutrient broth solidified with 15 g L^{-1} agar).

5). Toxicity test procedure.

Three 250-mL Erlenmeyer flasks each containing 75 mL of nutrient broth were inoculated with 1 mL of standardized inoculum and grown for 12, 18 or 32 h at 20°C at 120 rpm on a rotary shaker. Each of these cultures was then diluted to a final OD_{650}

of 0.2 with sterile nutrient broth. Eighteen-mL quantities of these suspensions were transferred to 50-mL sterile centrifuge tubes, harvested at 8,000 x g for 15 min, washed 3 times in sterile phosphate buffer (pH 7.0) and resuspended in 17 mL of buffer. The cell concentrations of the suspensions were determined using standard plate counts using nutrient agar.

Pentachlorophenol was dissolved in 95% ethanol, filter sterilized (0.22 μm mean pore size), and 0.1 mL was added to the centrifuge tubes to provide the required final concentrations. The tubes were shaken at 120 rpm for 1 h at 20°C. The same tubes were then centrifuged at 8,000 x g for 15 min at 20°C. Twelve mL of the supernatant was carefully removed with a sterile pipette without disturbing the cells at the bottom of the tube. Twelve mL of sterile buffer was added to replace that removed. The cells were resuspended and the process of centrifugation and removal was repeated twice. After the last centrifugation, 11.9 mL of buffer was added instead of the previous 12 mL quantities to compensate for the addition of the 0.1 mL of PCP solution.

Initial trials showed that these procedures did not change the viable cell numbers in the suspension; essentially all viable cells survived the treatments. The procedure also effectively diluted out any residual PCP in the exposure medium, thus preventing its transfer to the dilution plates.

After the final wash, viable cells were counted and the remaining cell suspension was then incubated for a further 16 h at 20°C before the final viable cell count. Probit analysis (SAS INSTITUTE INC. 1979) was used to calculate the LC_{50} values for PCP under the various conditions.

RESULTS & DISCUSSION

Biological toxicity assays have been designed to fulfill many different functions in toxicological testing , ranging from acute lethality studies to whole life cycle exposure studies. All have a role in risk assessment but rapid screening procedures for large numbers of potential toxicants are urgently required. Toxicity assays using bacteria have the advantages of rapidity and less expense than tests using animals and plants. Bacteria are also widely distributed with a marked lack of local geographic distribution patterns.

In the development of the present toxicity test procedure, it became apparent from growth studies in the presence of PCP (Fig. 1) that the onset of exponential growth was progressively delayed by increasing concentrations of PCP. At the highest dose (75 $\mu g\ mL^{-1}$) no growth was observed during the experimental period. The final biomass of the cells was decreased with increased PCP concentration but growth rates (when growth occurred) were not significantly affected. It is difficult to assess such studies,

since the initial concentration of PCP is progressively diluted over a larger biomass as growth occurs. There may also be selection of resistant cells or mutants during the growth cycle or of cells which can detoxify or degrade the toxicant.

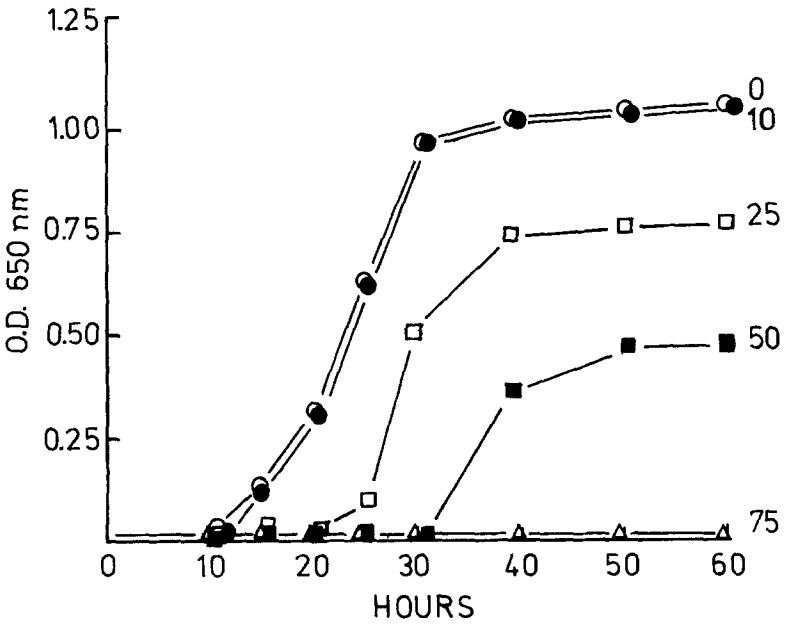

Figure 1. Effect of PCP ($\mu g\ mL^{-1}$) on the growth of P. fluorescens.

When the cultures were exposed to PCP for various times and then transferred to fresh medium (Fig.2), there was a correlation between the eventual OD_{650} and the initial period of exposure at all dose levels tested. Pentachlorophenol produced a decreased OD_{650} even after exposure periods as low as 1 min at concentrations of 50 and 75 $\mu g\ mL^{-1}$. For the toxicity test, a standard exposure time of 1 h was chosen.

Temperature during exposure also had a marked effect on PCP toxicity (Fig. 3). In general, increasing temperature of incubation decreased the survival of the bacteria exposed to PCP, such that survival at 30°C with 10 $\mu g\ mL^{-1}$ was essentially the same as that at 4°C with 50 $\mu g\ mL^{-1}$. For the toxicity test, all operations were performed at 20°C.

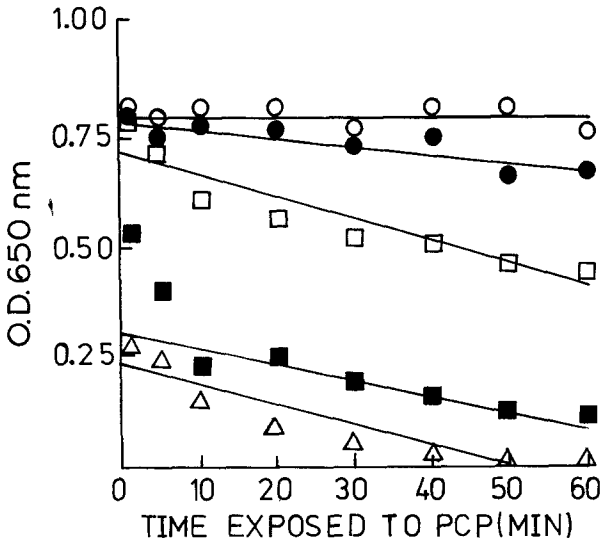

Figure 2. The effect of previous exposure time to PCP (μg mL^{-1}) on the growth of P. fluorescens measured after a 24-h incubation. 0 (○), 10 (●), 25 (□), 50 (■) and 75 μg mL^{-1} PCP (△).

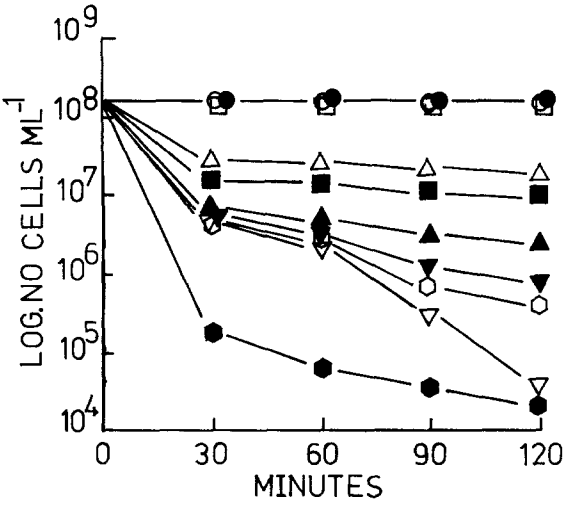

Figure 3. The effect of temperature on the toxicity of PCP (μg mL^{-1}) to P. fluorescens. 0 at 4, 20 and 30°C (○), 10 at 4°C (●), 10 at 20°C (□), 10 at 30°C (■), 50 at 4°C (△), 50 at 20°C (▲), 50 at 30°C (▽), 75 at 4°C (▼), 75 at 20°C (⬡) and 75 μg mL^{-1} at 30°C (⬢).

Using the toxicity test procedure, the observed percent mortality of bacteria exposed to different levels of PCP (Table 1) showed that a 1-h exposure to PCP killed an increasing percentage of cells in proportion to increased PCP concentration. Cells which survived those applications exhibited no further mortality after a 16-h recovery period in medium containing no PCP. The calculated LC_{50} was 29 $\mu g\ mL^{-1}$ for an exposure time of 1 h for cells grown for 18 h prior to exposure. When the age of the cells exposed was considered, it was clear that the age of the exposed cells had a marked effect on the observed mortality. Early log phase cells (12 h) and maximum stationary phase cells (32 h) were more sensitive to PCP than were cells grown for 18 h (mid-log phase). The LC_{50} values for 12, 18, and 32-h cells were ($\pm$ standard error of the mean) 18 $\pm$ 0.6, 29 $\pm$ 0.4, and 8.6 $\pm$ 1.0 respectively.

The proposed toxicity test procedure provided results quickly with only small amounts of variation. It is not complicated by growth and proliferation of cells during exposure, nor by transfer of toxicant after treatment. The organism chosen is a type common to many soil and water habitats and may be considered representative.

TABLE 1

Percent mortality of test organism after exposure to pentachlorophenol for 1 h, and after a subsequent recovery period of 16 h.

PCP treatment ($\mu g\ mL^{-1}$)	Percent mortality after 1 h ($\pm$ S.E.M)	Percent mortality after 16-h recovery period.
0	0	0
10	0	0
25	63.5 $\pm$ 0.46	0
50	84.4 $\pm$ 0.06	0
75	99.9 $\pm$ 0.06	0

The test may be modified for many other applications. The basic procedure of dosage, dilution and centrifugation may remain the same but the exposure times, bacterial isolate, and recovery times may be varied. Multiple application of toxicants, in sequence or together, may be made. Repair or recovery can be more closely monitored during the recovery period. Cultures can be initially grown under different nutritional and physiological conditions before application of the test so that the effects of these conditions on susceptibility can be assessed. Uptake and pharmacokinetic measurements may be facilitated by this testing procedure since the biomass can be standardized and analysis of cells at intervals during dosing and during recovery may be more easily accomplished.

REFERENCES

ALEXANDER, M.: An Introduction to Soil Microbiology. New York-London-Sydney: John Wiley & Sons, Inc. 1961.

HEALEY, F.P.: Canadian Special Publication of Fisheries and Aquatic Sciences Ed. E. Scherer. 44, 57 (1979).

JANNASCH, H.W.: J. Bacteriol. 99, 156 (1969).

JANNASCH, H.W.: Archiv fur Mikrobiol. 59, 165 (1974).

MAYFIELD, C.I., W.E. INNISS and P. SAIN: Water, Air and Soil Poll. 13, 335 (1980).

RAO, K.R.: Pentachlorophenol: Chemistry, Pharmacology and Environmental Toxicology. New York-London: Plenum Press 1978.

SAS INSTITUTE INC.: SAS User's Guide, 1979 Edition. Raleigh, North Carolina; SAS Institute Inc. 1979.