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Environmental Contamination and Toxicology

Improved Bacterial Growth Test for Rapid Water Toxicity Screening

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Bacteria have several attributes which make them attractive as test organisms for the rapid screening of chemical pollution in natural waters. They have relatively short life cycles and, therefore, respond rapidly to environmental change. They are easily handled and inexpensively maintained. Their rate of multiplication is such that a large number of homogeneous individuals are available for utilization in toxicity test procedures (Bitton 1983).

The degree of toxicity of chemicals to bacteria is normally established by measuring viability or growth. Techniques involving assessments of toxic effects upon bacterial metabolic parameters such as respiration, enzyme activity, nitrification and luminescence have also been employed (Bitton 1983). A variety of assays involving the inhibitory effects of toxicants upon growth have been described. Bacterial suspensions are usually incubated for 16 to 24 h in the presence of nutrients and toxicants, growth being measured turbidimetrically (Alsop et al. 1980; Dutka and Kwan 1981; Trevors et al. 1981).

Bringmann and Kühn (1980) described a very sensitive test measuring cell multiplication inhibition of *Pseudomonas putida*, results being obtained after a 16 h incubation period. Because of their short generation time it is possible, however, that bacteria are capable of manifesting measurable growth within a shorter incubation period. In the present study *P. putida* was cultured under modified test conditions aiming at an equally sensitive but more rapid growth test. Subsequent to initial tests, using different growth media, a toxicity test procedure was developed which uses a medium with low complexing capacity, a standardized inoculum and a 6 h incubation period.

MATERIALS AND METHODS

Pseudomonas putida strain Berlin 33/2 was obtained from Prof. H. Stolp, Deutsche Sammlung von Mikroorganismen, Teilsammlung, Bayreuth. Stock cultures were made on nutrient agar slants in McCartney bottles. Cultures were kept at 4°C after incubation at 27°C for 16 to 18 h.

Copper ($CuSO_4$), cadmium ($CdCl_2$), zinc ($ZnSO_4.7H_2O$), mercury ($HgCl_2$), cyanide (NaCN), phenol and acetone were tested. Test

solutions spanned a 2 to 3 log concentration gradient. Deionized distilled water was used for the preparation of chemical solutions and for control tests. Test solutions were distributed in 45 mℓ quantities in 100 mℓ medical flats and autoclaved at 121°C for 15 min. Phenol and acetone solutions were filter sterilized. Chemical solutions spanned a minimum 3 log concentration gradient.

The minimal growth medium was a dilution (10x) of that of Grabow and Smit (1976) and comprised: 1.05 g K_2HPO_4, 0.45 g KH_2PO_4, 0.047 g Na_3-citrate.$2H_2O$, 0.1 g $(NH_4)_2$ SO_4, 0.01 g $MgSO_4.7H_2O$ and 0.25 g glucose per litre of deionized water (pH:7.15). Glucose was autoclaved separately. The medium was prepared 12.5 times concentrated to have the desired nutrient concentrations after addition to test solutions.

From stock cultures subcultures were made in 50 mℓ growth medium in 100 mℓ medical flats. Cultures were incubated at 27°C for 16 to 18 h. Cells were diluted with fresh medium to a density with an absorption of 0.8 at 600 nm, 30 min before being used for inoculation. Density measurements were carried out using a spectrophotometer (PMQ II, Carl Zeiss, FDR).

Four millilitres of the concentrated growth medium were dispersed into test solutions (45 mℓ). One millilitre of the cell suspension was used for inoculation. Flats were incubated at 27°C for 6 h at an angle of 10° with screwcaps slightly unscrewed. Density measurements were conducted immediately after growth had been terminated using 0.1 mℓ of a 37% formaline solution.

Uninoculated test medium was used as reference sample. Six replicate tests were carried out. Results were expressed as percentage inhibition compared with controls. EC10 and EC50 values (concentrations exhibiting a 10 and 50% inhibition in growth respectively) were calculated using regression analysis (log concentration versus % inhibition).

RESULTS AND DISCUSSION

Preliminary growth tests were carried out to select a suitable growth medium, inoculum concentration and incubation period. Minimal growth medium (1x and 10x concentrated) and nutrient broth (0.8 and 4 g/ℓ) were used for culturing. The growth curve of *P. putida* cultured in minimal medium (1x concentrated) is shown in Figure 1a. An inoculum with an absorption of 0.8 produced an easily measurable cell density (absorption:0.5) after 6 h of incubation. Cells were in the late logarithmic phase at this time. The same growth rate was obtained when the 10x concentrated minimal medium was used, the generation time (G) being 1.2 h. The final density was, however, slightly higher. By increasing or decreasing the inoculum concentration a higher or lower cell density was obtained within the 6 h incubation period. Growth rate (G=0.84) of *P. putida* in nutrient broth was higher than in minimal medium (Fig. 1b). A low cell density was obtained when culturing in 0.8 g/ℓ nutrient broth (absorption: ∿0.5 after 16 h incubation).

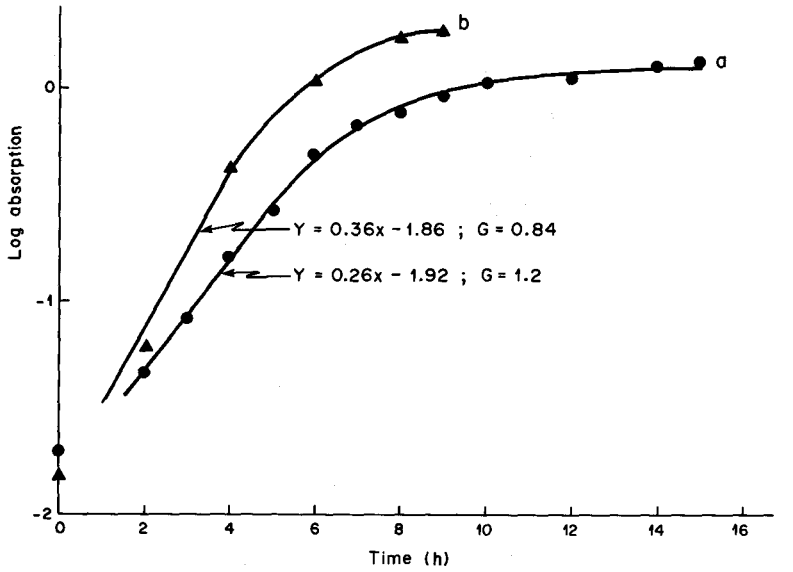

Figure 1. Growth of *Pseudomonas putida* cultured in (a) minimal medium and (b) nutrient broth (4 g/ℓ).

Initial toxicity tests were conducted using cadmium as the toxicant and employing different growth media for culturing (Fig. 2). Cadmium was detected at a lower concentration (EC10: 0.08 mg/ℓ) when using the 1x concentrated minimal medium than with any other media, comparable EC10 values being 0.09 mg/ℓ for 0.8 g/ℓ nutrient broth, and 0.45 mg/ℓ for 10x concentrated minimal medium and 4 g/ℓ nutrient broth. The lowest EC50 value for cadmium toxicity was obtained when using 0.8 g/ℓ nutrient broth and 1x concentrated minimal medium. In practice the 1x concentrated minimal medium was preferred to the 0.8 g/ℓ nutrient broth for toxicity tests due to the better growth obtained in the former.

The results of the toxicity tests are presented in Table 1. A high correlation was observed between toxicant concentration and growth inhibition for all chemicals tested. The growth response curve for Hg^{2+} was, however, non-linear. Concentrations of $Hg^{2+} \leq 0.025$ mg/ℓ had no effect, while concentrations ≥ 0.05 mg/ℓ exhibited total inhibition. Minimum effect concentrations being represented by the EC10, the results indicate *P. putida* to be more sensitive to heavy metals and cyanide than to organic chemicals.

Table 2 shows a comparison with results obtained with other bacterial growth tests. Bringmann and Kühn (1980) measured *P. putida* cell multiplication inhibition after 16 h exposure using a growth medium with low nutrient concentrations. Results represent the chemical concentrations producing a 3% (or more) growth inhibition. A comparison of 6 h growth EC10 values with these data show that the 6 h growth test was less sensitive to CN^- (18x) and Cu^{2+} (3x), equally sensitive to Cd^{2+} and more sensitive to phenol (4x) and acetone (3x). No effect and 100% inhibition levels obtained with

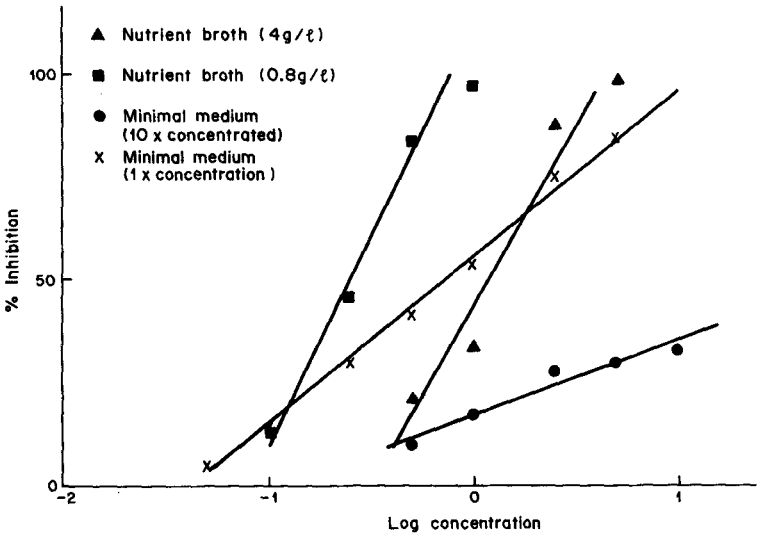

Figure 2. The effect of cadmium on the growth of *Pseudomonas putida* in different growth media.

Table 1. *Pseudomonas putida* growth inhibition results.

Chemical	Growth EC10 (mg/ℓ)	(95% CI)[1]	Growth EC50 (mg/ℓ)	(95% CI)[1]	r^2
Cu^{2+}	0.01	0.07 - 0.15	1.05	0.72- 1.54	0.98
Cd^{2+}	0.08	0.07 - 0.09	0.72	0.67- 0.79	1.0
Zn^{2+}	0.15	0.08 - 0.29	7.15	4.34-11.78	0.97
CN^-	0.018	0.012- 0.026	0.69	0.51- 0.95	0.99
phenol	15.1	0.7 - 33.7	244	98 - 612	0.90
acetone	594	344 - 1028	4 385	3342 - 5754	0.98

[1] 95% confidence intervals of the EC_{10} and EC_{50} values
[2] Correlation coefficient

Hg^{2+} show that the 6 h test was less sensitive (3 to 5x) than the 16 h test. Dutka and Kwan (1981) measured population density inhibition of *P. fluorescens* and *Aeromonas hydrophila* after 18 h incubation using nutrient broth. Both tests were less sensitive than the 6 h growth test to all chemicals except Hg^{2+}. The lower sensitivity of these tests may be attributed to the complexity of the growth medium, the growth phase of the cells and the different bacteria employed.

The advantages of this improved growth inhibition test are that results are produced within 8 h and a sensitivity is exhibited which compares favourably with that of the very sensitive 16 h *P. putida* growth test. The test is simple and inexpensive, and provides reproducible data which are easily interpreted. Being very

Table 2. *Pseudomonas putida*, *Pseudomonas fluorescens* and *Aeromonas hydrophila* growth inhibition results.

Chemical	6 h *P. putida* test EC10	6 h *P. putida* test EC50	16 h *P. putida* test[1] min. EC[2]	18 h *P. fluorescens* test[3] EC50	18 h *A. hydrophila* test[3] EC50
Cu^{2+}	0.01	1.05	0.03	17	710
Cd^{2+}	0.08	0.72	0.08	NA	NA
Zn^{2+}	0.15	7.15	NA	360	500
Hg^{2+}	0.025[4]	0.05[4]	0.01	0.031	0.049
CN^-	0.018	0.69	0.001	14	25
phenol	15.1	244	64	880	1600
acetone	594	4 385	1 700	NA	NA

[1] Bringmann and Kühn (1980)
[2] dilution concentration exhibiting ≧3% inhibition
[3] Dutka and Kwan (1981)
[4] No effect and 100% inhibition levels
NA not available

sensitive to metals its main application may be the toxicity screening of metal containing industrial effluents.

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