World Journal of Microbiology and Biotechnology, 8, 316–318

Development of a bioassay reagent using *Photobacterium phosphoreum* as a test for the detection of aquatic toxicants

A.E. Ronco

Lyophilized cells of *Photobacterium phosphoreum*, rehydrated in 2% (w/v) NaCl in 0.022 M KH_2PO_4 at pH 7.0, were used for developing an assay to test the acute toxicity of organic and inorganic compounds. The standardized assay gave good reproducibility of results with 11 organic and four inorganic compounds. Results were compared with reported data obtained with other test organisms and are within their sensitivity ranges. Environmental screening of wastes from oil and petrochemical industries is discussed.

Key words: Acute toxicity, bioassay reagent, *Photobacterium phosphoreum*, production

Luminous bacteria have long been used to study the effects of drugs by measuring the intensity of light they emit (Taylor 1932). The effects of inhibitors, temperature and pressure on the light emission process have subsequently been investigated (Johnson 1947).

Development of bacterial bioluminescent monitoring systems for water and waste-water (Jeffers & Taylor 1977; Taylor & Jeffers 1977) preceded the introduction of the Microtox Toxicity Analyzer by Beckman Instruments (Bulich 1984, Qureshi *et al.* 1984; Vasseur *et al.* 1984; Indorato *et al.* 1984). More recent reports (Novikova *et al.* 1986) describe use of the bioluminescent bacterium, *Beneckea harveyi*, for assaying the toxicity of effluent from the sulphate-cellulose industry.

Most of the assays reported in the literature are based on fresh cultures of bacteria. The possibility of working with lyophilized batches of bacteria, ready at any time, in addition to its convenience, reduces the costs of biomass production, which seems to be one of the more expensive factors when using bioassay monitoring (Persoone & van de Vel 1987).

A diagnostic reagent kit using lyophilized *Photobacterium phosphoreum* was produced to test the toxicity of industrial wastes. This alternative test uses a readily available type strain, eliminating dependence on a commercial supply, and can be produced on a laboratory scale at low cost.

A.E. Ronco is with the Centro de Investigaciones del Medio Ambiente (CIMA) and Centro de Investigación y Desarrollo de Fermentaciones Industriales (CINDEFI), Facultad de Ciencias Exactas, Universidad Nacional de La Plata, 47 esq. 115, 1900-La Plata, Argentina.

Materials and Methods

Cell Growth and Lyophilization Conditions

Photobacterium phosphoreum ATCC 11040 was cultured in a modified 101 ATCC formula medium using 0.3% glycerol as carbon source. Cells were grown at 17°C in shake flasks at 170 rev/min to an absorbancy (A_{650}) of 4.0 to 4.5 (10^9 cells/ml) at maximum luminescence.

Cultures were harvested by centrifugation at 8000 × ***g*** for 10 min and the cells were resuspended in a 75% (v/v) sterile horse serum, 7.5% (w/v) glucose and 0.3% (w/v) nutrient broth (Difco) mixture and frozen on dry ice/ethanol. Lyophilization of the resuspended cells at 10^{10} cells/ml was performed at approximately 0.01 Pa for 15 h. Lyophilized cultures were stored at −20°C or below.

Luminescence spectra of cultures ranged from 430 to 600 nm and had maxima at 474 nm, values similar to those reported by McElroy (1965). Light intensity from 4.2×10^9 cells/ml (dry wt 3.5 mg/ml) was 3×10^{-3} $\mu W/cm^2$.

The Test System

Rehydrated lyophilized bacteria, in 2% (w/v) NaCl in 0.022 M KH_2PO_4 at pH 7.0, were used at 10^7 cells/ml for each toxicant concentration and controls. All solutions were osmotically adjusted with NaCl at 2% (w/v).

Light emission was measured with a spectrofluorimeter using instrumental conditions for total emission.

Bioassays used six dilutions of each chemical and two controls without toxin. Assays were carried out at 16°C for 5, 10 and

15 min. All measurements were done within 30 min of reconstituting the reagent.

Liquid environmental samples from oil and petrochemical industries were directly diluted and osmotically adjusted. Oily, non-aqueous and solid samples were extracted in water (1 part of sample plus 5 parts of water) with agitation for 24 h and tested for toxicity on dilutions of the aqueous extracts.

Toxicity Calculations

Data were fitted by regression analysis. The EC_{50} values (concentration of test compound causing a 50% reduction in the assay response) were calculated from the dose-response plots using 95% confidence limits of at least three replications for each dilution of the toxic chemical. Best fit was assessed by its correlation coefficient.

Results and Discussion

The results of acute toxicity tests, using pure toxins and the new lyophilized bacteria reagent were compared with those of various other bioassays (Table 1).

Although most of the EC_{50} values obtained with *P. phosphoreum* are within the sensitivity ranges reported for other organisms, this bioassay is not as sensitive as the Microtox assay system. Even so, on the basis of reproducibility, low cost and use of already-available equipment, it could be a satisfactory alternative for laboratories needing to develop their own diagnostic reagent. Some of the assayed compounds, such as Aroclor 1242 (polychlorinated biphenyl with 42% Cl), benzene, anthracene, chrysene and Pb(II) were not soluble under the experimental conditions and their EC_{50} are not included in Table 1. Dimethyl sulphoxide showed no toxic effect at concentrations up to 1% (v/v).

Good reproducibility of the bioassays was observed when using different lots of lyophilized cultures. The coefficient of variation of ethanol EC_{50} from ten replications conducted with three lots of bacteria was 0.196.

From the statistical analysis of results we can conclude that the method shows a coefficient of variation between 0.063 and 0.213 for all tested (Table 1). These values are within the range expected for this type of test.

The performance of the test with environmental samples was assessed using industrial by-products and wastes from two petrochemical industries and an oil refinery (Table 2). Final petrochemical effluents and intermediate effluents from different industrial production stages were tested after various treatments. The order in the table corresponds to the waste-treatment flow-sheet for each type until liquid effluents are dumped and solid wastes stored.

The efficiency of the effluent treatments can be clearly quantified using the results of acute toxicity assays of the solids or floccules in equilibrium with aqueous phases and the input and output of the aeration lagoon.

This acute toxicity test seems suitable for screening field samples and most useful when monitoring fluctuations in routine plant effluents, as also reported for Microtox assays (Vasseur *et al.* 1984).

Although the role of toxicity bioassays using bacteria as test organisms is still unclear, new approaches towards multi-level testing schemes are being accepted. Cost

Table 1. EC_{50} values, as mg/l, of various compounds in toxicity tests with *Photobacterium phosphoreum* compared with other test systems.

Test compound	*P. phosphoreum*		Microtox‡		Tchan assay‡		*Spirillum volutans*	Fish assays‡	*Aeromonas hydrophila*‡
	EC_{50}* (5 min)	C.V.†	EC_{50}‡ (5 min)	C.V.	EC_{50} (1 h)	C.V.	EC_{50} (120 min)	LD_{50}	EC_{50} (18 h)
Ethanol	15500	0.20	31000	0.15	19400	0.27	–	13500	–
Methanol	30000	0.17	56700	0.02	–	–	–	>1.0	–
Sodium dodecyl sulphate	28	0.09	2.5–6.2	0.33	–	–	60	5–46	3700
Phenol	92	0.12	25–41	0.24	880	0.46	300	9–66	1600
Formaldehyde	47	0.16	3.0	1.67	3150	0.24	–	18–185	–
Urea	32000	0.21	24000	–	–	–	–	12000	–
Cu(II)	17	–	8.0	0.57	69	0.29	10	0.1–10.7	21
Hg(II)	0.8	0.07	0.065	0.20	1.07	0.36	0.2	0.01–0.9	0.049
Zn(II)	6.0	–	3.5	0.58	30.9	0.29	12	0.24–7.2	500
NaCN	67	0.06	16	–	–	–	83	0.1–0.44	25

* Concentration of test compound (in mg/l) causing a 50% reduction in the assay response. Time units indicates exposure duration to toxin.

† Coefficient of variation (standard deviation/mean). Microtox and Tchan assay coefficients of variation were taken from McFeters *et al.* (1983).

‡ Results obtained from McFeters *et al.* (1983) and Dutka & Kwan (1981).

Table 2. EC_{50} values, as % (w/v) or (v/v), of environmental samples (liquid and semi-solid by-products and wastes from oil and petrochemical industries), determined by using the *P. phosphoreum* assay.

Type of industry	Type of industrial step	Type of sample	EC_{50} (5 min)	Toxicity rank*
Oil refinery	API-Pool (coke production sector)†	Sludge	6.8%(w/v)	3
		Aqueous effluent	71%(v/v)	5
	API-Pool (lubricating oils sector)	Sludge	8.6%(w/v)	3
Petrochemical (polyethylenes)	Gravity decanter	Light phase	33%(v/v)	5
	Flocculation (Treatment I)	Aqueous phase	11%(v/v)	4
	(Treatment II)	Aqueous phase	19%(v/v)	4
	Aeration pool	Aqueous input	17%(v/v)	4
		Aqueous effluent	59%(v/v)	5
	Flocculation (Treatment I)	Flocculesǂ	0.1%(w/v)	1
	(Treatment II)	Flocculesǂ	1.1%(w/v)	2
	Gravity decanter	Heavy phase	0.4(w/v)	1
Petrochemical (aromatics)	API-Pool	Aqueous effluent	49%(v/v)	5

* The log rank toxicity classification system from class 1 (most toxic) to 6 (non toxic) described by Kenaga (1978) was used to categorize EC_{50} data.
† American Petroleum Institute standardized pool.
ǂ Floccules added to gravity decanter's heavy phase and kept stored in an isolated lagoon.

efficiency, rapid response (the toxicity level of an effluent can be determined in less than 30 min), small laboratory and space requirements and the advantages of using freeze-drying technology, should be weighed against the greater sensitivity of the traditional fish assays.

Acknowledgements

The author wishes to thank Professor J. A. Catoggio, at the Centro de Investigaciones del Medio Ambiente, Universidad Nacional de La Plata, for reading the manuscript and helpful comments and Lic. Cecilia Damonte and Dr R. Maronna, at the Laboratory of Applied Mathematics, Facultad de Ciencias Exactas, Universidad Nacional de La Plata, for helping in the statistical analysis of the results.

References

Bulich, A.A. 1984 Microtox-A bacterial toxicity test with several environmental applications. In *Toxicity Screening Procedures Using Bacterial Systems*, eds Liu, D. & Dutka, B.J., pp. 55–64. New York and Basel: Marcel Dekker.

Dutka, B.J. & Kwan, K.K. 1981 Comparison of three microbial toxicity screening tests with the Microtox Test. *Bulletin of Environmental Contamination Toxicology* **27**, 753–757.

Indorato, A.M., Snyder, K.B. & Usinowicz, P.J. 1984 Toxicity screening using Microtox analyzer. In *Toxicity Screening Procedures Using Bacterial Systems*, eds. Liu, D. & Dutka, B.J., pp. 37–53. New York and Basel: Marcel Dekker.

Jeffers, E. & Taylor, R.E. 1977 NASA on-line microbiological detection systems for water/wastewater. *Progress in Water Technology* **9**, 109–110.

Johnson, F.H. 1947 Bacterial luminescence. *Advances in Enzymology* **7**, 215–264.

Kenaga, E.E. 1978 Test organisms and methods useful for early assessment of acute toxicity of chemicals. *Environmental Science and Technology* **12**, 1322–1329.

McElroy, W.D. 1965 Bacterial luminescence. In *The Bacteria*, Vol. 2, *Metabolism*, eds Gunsalus, I.C. & Stainer, R.Y., pp. 479–509. New York and London: Academic Press.

McFeters, G.A., Bond, P.J., Olson, S.B. & Tchan, Y.T. 1983 A comparison of microbial bioassays for the detection of aquatic toxicants. *Water Research* **17**, 1757–1762.

Novikova, L.N., Gil, T.A., Rudykh, A.R. & Stom, D.I. 1986 A bioluminescence technique for assaying the toxicity of coloured compounds in the sewage of the sulfate-cellulose industry. *Mikrobiologiya* **55**, 152–154.

Persoone, G. & Van de Vel, A. 1987 Cost-analysis of 5 current aquatic ecotoxicological tests. In *Environment and Quality of Life*. Report EUR 1134EN. DGXI-Environment, Consumer Protection and Nuclear Safety. Brussels: Commission of the European Communities.

Qureshi, A.A., Coleman, R.N. & Paran, J.H. 1984 Evaluation and refinement of the Microtox test for use in toxicity screening. In *Toxicity Screening Procedures Using Bacterial Systems*, eds Liu, D. & Dutka, B.J., pp. 1–22. New York and Basel: Marcel Dekker.

Taylor, G.W. 1932 The effects of hormones and certain other substances on cell [luminous bacteria] respiration. *Journal of Cellular and Comparative Physiology* **1**, 297.

Taylor, R.E. & Jeffers, E. 1977 NASA water monitoring system for Community application. *Progress in Water Technology* **9**, 121–129.

Vasseur, P., Ferard, J.F., Rast, C. & Larbaight, G. 1984 Luminescent marine bacteria in ecotoxicity screening tests of complex effluents. In *Toxicity Screening Procedures Using Bacterial Systems*, eds Liu, D. & Dutka, B.J., pp. 23–35. New York and Basel: Marcel Dekker.

(Received in revised form 9 January 1992; accepted 25 January 1992)