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Use of the Bioluminescent Bacterium *Photobacterium phosphoreum* To Detect Potentially Biohazardous Materials in Water

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Rapid detection of biohazardous materials that result from industrial or domestic operations is an important criterion when evaluating bioassays. However, it is not only important to detect these materials but also to predict their potential toxicity (Thomulka et al. 1993). Although analytical methods can detect low concentrations of many compounds, they are not predictive of toxicity or synergistic effects. Thus, a multiple bioassay system could be used as an initial screening tool (Reteuna et al. 1989). If toxicity is not indicated, then further and more expensive chemical analyses could be minimized.

The bioassay described in this paper, which had been previously described by Thomulka et al. (1992), is a direct assay measuring bioluminescence reduction in living cell suspensions of *Photobacterium phosphoreum*. The process of bioluminescence involves luciferase and several other related enzymes (e.g. electron transport). These enzyme systems are inhibited by one, or both, of two possible mechanisms: the direct inhibition of bioluminescence activity or the indirect inhibition of bioluminescence by general cytoplasmic poisons which interfere with essential and related metabolic processes. The *Photobacterium* bioassay described in this paper differs from the commercially available MICROTOX$^{(R)}$ bioassay in that the MICROTOX$^{(R)}$ system employs the use of lyophilized cells (Reteuna et al. 1989; Vasseur et al. 1984; Ribo and Rogers 1990).

Since there is no practical test that can equally detect all potentially toxic materials (Ribo and Rogers 1990) and predict their toxicity, the use of a battery of biological tests is advocated (Ribo and Rogers 1990; Brouwer et al. 1990; Paran et al. 1987; Ribo and Kaiser 1987: Bulich et al. 1990)

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for evaluating the toxicity of aquatic environments. Our two-assay system could be an integral component, or, the initial screening test for toxicity, or changes in toxicity, of effluents. Detection of environmental pollution is often limited due to analytical methods being unavailable to incorporate into the test system synergistic effects, costs associated with analysis, reproducibility of the assay and rapidness of the test (Dutka and Kwan 1981). It appears that these criteria can be achieved with a battery of biological screening assays. Combination of this and other previously described assays (Kamlet et al. 1986) may in the future be the cornerstone of a biohazardous assay system (Lange et al. 1993).

This study has expanded our original investigations of *V. harveyi* and *P. phosphoreum* bioassays (Thomulka et al. 1992; Thomulka et al. 1993). Estimated median effective concentration (EC_{50}) values for bioluminescence reduction are reported for various categories of chemicals that were evaluated using a *P. phosphoreum* bioassay.

MATERIALS AND METHODS

The procedures for *P. phosphoreum* were essentially the same as the previously published (Thomulka et al. 1992; Thomulka et al. 1993) direct assay except as noted.

P. phosphoreum strain A-13 was obtained from John Lee. It was transferred weekly on artificial seawater agar (ASW) which contained 30 g NaCl, 3.6 g NaH_2PO_4, 1.2 g KH_2PO_4, 0.5 g $(NH_4)HPO_4$, 0.1 g $MgSO_4$, 10 g Bacto-peptone, 3.0 ml glycerol, 15 g agar and 1000 ml H_2O. NaOH was used to adjust the pH to 7.4. Liquid medium was similar but lacked agar.

Overnight cultures (ONC) were obtained by inoculating about 25 ml of ASW broth in a 125-ml Ehrlenmeyer flask. This flask was capped loosely since oxygen is required for luminescence. The flask was shaken at about 100 times a minute in a reciprocating water bath for 24 hr.

The ONC was refrigerated until use. The absorbence at 600 nm of the ONC was taken using ASW broth as a blank. The absorbence of an aliquot was adjusted to 0.5 using phosphate-buffered saline containing sucrose and nutrient broth (PBS-SNB) and stored in an ice bath until use. Immediately before use the 0.5 suspension was diluted 1:150 with cold PBS-SNB and stored in an ice bath during use.

Toxic effects of the chemicals tested were reported by their estimated median effective concentration, in ppm, to reduce bioluminescence by 50% (EC_{50}) which was determined graphically (Dutka and Kwan 1981).

Confidence intervals (CI) at 95% were determined as described by Frumin et al. 1992. Numerical values (n) used in calculating CI were determined by the number of replicates analyzed for each chemical which ranged from 4 to 23.

RESULTS AND DISCUSSION

Salts of the metals cadmium, cobalt, mercury, silver and dibutyl and tributyl tin reduced luminescence (Table 1.). Mercuric chloride was the most potent. The next was silver nitrate which was about ten times more toxic than the other metalic compounds. Bioluminescence was not affected in *Photobacterium* by zinc chloride and copper sulfate. Although direct and growth assays were performed using *Vibrio* in previous studies (Thomulka et al. 1992; Thomulka et al. 1993), growth assays were not possible for *Photobacterium* due to its slow generation time in the ASW medium.

Strong oxidizing agents were very effective in reducing bioluminescence. Sodium hypochlorite was the most potent, followed by hydrogen peroxide. Calcium hypochlorite was about 100 times less toxic than sodium hypochlorite but about 10 times more toxic than potassium dichromate.

Sodium azide, potassium nitrate and sodium fluoride did not inhibit bioluminescence. Formalin, sodium dodecyl sulfate (SDS) and phenol were toxic, while triton was not toxic.

The alcohols tested had no effect until a concentration of about 30,000 ppm or 3% was reached. It is moot to evaluate the impact of alcohol concentrations in this range on environmental toxicity due to the improbability of finding alcohols at this level. Therefore, for all practical purposes alcohols were not detected.

Several compounds, including antibiotics, were not effective in reducing bioluminescence. At concentrations much higher than that which were required to inhibit growth, none of the antibiotics had an effect on bioluminescence.

This paper has extended our investigation of a bioassay method for screening toxic pollutants found not only in the marine and aquatic environments but also in agricultural, domestic or industrial effluents (Thomulka et al. 1992; Thomulka et al. 1993). The types of specimens used or potential toxic materials detected are only limited by the requirement that the compound be soluble and stable in 3% saline. Work in progress suggests that this assay is adaptable to terrestrial

Table 1. EC_{50} values and confidence intervals for various chemicals reported as ppm using a *Photobacterium phosphoreum* bioluminescence bioassay.

Compounds Evaluated	EC_{50} values	
Mercuric chloride	.002	(.0014 - .0026)
Silver nitrate	.038	(.033 - .043)
Cobaltous chloride	3.8	(3.4 - 4.2)
Zinc chloride	>100	(+)
Tributyl tin	.3	(.23 - .37)
Cadmium chloride	.38	(.34 -.42)
Dibutyl tin	.24	(.19 - .29)
Cupric sulfate	>100	(+)
Sodium hypochlorite	.061	(.050 - .062)
Calcium hypochlorite	6.1	(4.2 - 8.0)
Hydrogen peroxide	1.8	(1.5 - 2.1)
Potassium dichromate	60	(48 -72)
Sodium azide	>100	(+)
Potassium nitrate	>100	(+)
Sodium fluoride	>100	(+)
Methanol	25000	(21000 - 29000)
Ethanol	9000	(7300 - 10700)
2-Propanol	7700	(7350 -8150)
Phenol	12.5	(11 - 14)
Sodium dodecylsulfate	37	(28 - 46)
Triton-X-100	>100	(+)
Formalin	25	(22 - 28)
Novobiocin	>100	(+)
Tetracycline	>100	(+)
Chloramphenicol	>100	(+)
Nalidixic acid	>100	(+)
Streptomycin	>100	(+)
Ampicillin	>100	(+)

( ) Confidence interval range
(+) Confidence interval cannot be calculated

samples (i.e., aqueous soil suspensions). For practical purposes, an EC_{50} value equal to or greater than 100 ppm was defined as non-toxic to these bacterial assays due to the low probability of finding toxic

pollutants at such an elevated level in most normal effluents. Although use of a definitive EC_{50} value for classifying a substance as toxic or non-toxic is arbitrary, and may ignore categorical degrees of toxicity, this value does provide a reference point for descriptively evaluating the data (Table 1.). The assay described suggests the ability to detect many different toxic materials that directly interfere with luminescence.

In this study a total of 28 compounds were tested. The direct treatment of *Photobacterium phosphoreum* cell suspensions failed to detect toxicity of 15 compounds at a descriptive value of 100 ppm or lower, including the alcohols and antibiotics. Descriptively, comparing these results to data previously obtained with *Vibrio harveyi* (Thomulka et al. 1993), one or both of the *Vibrio harveyi* assays (direct or growth) displayed a two fold or greater sensitivity than the direct assay using *P. phosphoreum* for fourteen compounds, while twelve were equally sensitive and two less sensitive.

In a comparison of each of the chemicals tested to our previously published *Vibrio* data (Thomulka et al. 1993), one or both assays were able to detect the toxicity of zinc chloride and copper sulfate which were observed to be non-toxic by *Photobacterium*. In addition, *Vibrio* was able to detect the toxicity of levels of cobaltous chloride about 10 times lower than *Photobacterium*. With respect to oxidizing agents, *Photobacterium* was less sensitive as compared to *Vibrio*. The only oxidizing agent tested that had a similar value was hydrogen peroxide. No electron transport inhibitor reduced bioluminescence in *Photobacterium,* although dinitrophenol and sodium azide inhibited bioluminescence using the growth assay with *Vibrio*. Previous data have shown *Vibrio* to be able to detect about 20 times less formalin as compared to this study using *Photobacterium*, while it was insensitive to phenol and SDS. Both organisms were not affected by Triton. In general, bioluminescence in *Vibrio* was slightly more sensitive to the alcohols. Neither direct bioassay (*Vibrio* or *Photobacterium*) was sensitive to antibiotics, although the *Vibrio* growth assay was sensitive to five out of six antibiotics tested.

A comparison of the data in this study, using *Photobacterium phosphoreum*, with a two fold or greater EC_{50} value to descriptively define a difference (Thomulka et al. 1993) to the published literature values for MICROTOX(R) (Reteuna et al. 1989; Ribo and Rogers 1990; Vasseur et al. 1984; Ribo and Kaiser 1987; Bulich et al. 1990; Kamlet et al. 1986; Schiwew 1985) was performed for compounds tested in common. These results showed that out of the 14 compounds evaluated in common, two values were the same, MICROTOX(R) was more sensitive for six compounds and the *Photobacterium* system was more

sensitive for six. Thus, based on the limited data published in this study, the *Photobacterium* and MICROTOX(R) systems have similar sensitivities while the previously described *Vibrio* assay seems to be more sensitive.

The assay described in this paper studied the effects of several chemicals on living cell suspensions of *Photobacterium phosphoreum* and compared these results to data in our previous publication that employed the use of either living (a direct assay) and/or growing suspensions (a growth assay) of *Vibrio harveyi*. These results, and previously published data (Thomulka et al. 1992; Thomulka et al. 1993), suggest that the combined use of a direct and growth assay using *Vibrio harveyi* is capable of detecting a broader spectrum of toxic agents than a direct assay employing *Vibrio* or *Photobacterium* alone. It appears that the bioassay methods described in this and our previous papers may be useful in the hazardous waste industry as a preliminary screen for the presence of toxic substances. Adaption and refinement of this assay system may prove useful to various industries (e.g. environmental site assessments) for providing a descriptive toxicity value where multiple chemical compounds are present.

Numerous investigations have used *Vibrio harveyi* or *Photobacterium phosphoreum* to determine the presence and relative toxicity of pollution (Brouwer et al. 1990). These studies have evaluated toxicity through *in vitro* bioluminescence methods. This study, and its related publications, use living cell suspensions, which makes use of all the essential cellular functions contributing to bioluminescence.

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