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Evaluating Median Effective Concentrations of Chemicals with Bioluminescent Bacteria

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Numerous methods have been described for calculation of estimated median effect concentration values (Litchfield and Wilcoxon 1949; Deichmann and Le Blanc 1943; Harris 1959). These methods were developed primarily for the pharmacological and toxicological sciences, and involved the use of discrete organisms. As the use of animals has decreased in scientific investigations, a greater emphasis is being applied to the use of single celled organisms such as bacteria (Thomulka et al. 1992) for investigative purposes. This study evaluated six different bioluminescent bacteria with three closely related organophosphorous compounds using a method designed to rapidly estimate the effective median concentration (EC_{50}). The method of calculating EC values was modified from a previously published procedure (Frumin et al. 1992) that employed fewer organisms than other commonly used methods in determining estimated median concentration values (LD_{50}, LC_{50}) (Thomulka et al. 1993a; 1993b). Frumin's method was adapted for bacteria by using the number of replicates in place of the number of test animals in the experimental group (Thomulka et al. 1993a). To evaluate EC_{50} values with this modified Frumin method, a comparison is made using the probit method. A comparison of this method is similar to that performed in an initial study of this methodology (Frumin et al. 1992).

The organisms used in this study, *Vibrio harveyi, V. fischeri, Photobacterium phosphoreum,* and *P. leiognathi,* are luminescent only under aerobic conditions. Exposure of bioluminescent bacteria to a toxicant typically reduces luminescence in proportion to toxicity. Data resulting from these occurrences are

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represented as the median effective concentration (EC_{50}) in ppm at which luminescence (counts) is reduced.

MATERIALS AND METHODS

The method of calculating EC and confidence interval (CI) values is identical to that described by Frumin et al. (1992), except that determination of the descriptor "n" is representative of the number of bacterial tubes counted (replicates) (Thomulka et al. 1993a; 1993b; 1993c).

Calculation of EC_{50} and CI values by the probit analysis method was performed using a computer program (US Environmental Protection Agency, 1988). Program parameters included the number of organisms entered which was always 100, representing 100% luminescence, and the number responding which was the percent reduction in luminescence by the individual concentration of chemical. Estimated median effective concentration and corresponding CI are in ppm (Somasundaram et al. 1990).

Bacteria used in this comparison evaluation study were: *Vibrio harveyi,* obtained from D. Lapota, Naval Oceans Systems Center, San Diego, CA; *V. harveyi* strain 392 and *V. fischeri,* obtained from M. Haygood, Scripps Inst. of Oceanography, Univ. of CA, La Jolla, CA; *Photobacterium phosphoreum* strain A-13 and *P. leiognathi* strain S-l, obtained from J. Lee, Univ. of GA, Athens, GA; *P. phosphoreum* strain MTX was obtained by rehydrating a vial of lyophilized bacteria supplied by Microtox and cultured on nutrient agar containing 3% NaCl (NA). *Vibrio* and *Photobacterium* cultures were maintained by weekly transfer on NA or artificial sea water agar, respectively. Chemicals used in this evaluation were diethyl methanephosphonate (DEMP), diisopropyl methanephosphonate (DIMP) and dimethyl methanephosphonate (DMMP). Two methods were performed using these bacteria, a direct toxicity test, which has been previously described (Thomulka et al. 1992) and an aerobic growth toxicity test, which is described in this manuscript.

For the aerobic growth toxicity test an overnight culture (ONC) was made using a routine air-accessible culture. Nutrient broth (NB), 5 ml, containing 3% NaCl was added to a 16 x 125 mm sterile glass culture tube with a Morton cap. Analysis was begun by adding a toxicant or its dilutant in a volume of 100 ul or less to 5 ml of NB. Control tubes received 5 ml NB and no chemical. These tubes were inoculated with 10 ul of an ONC and incubated for 24 hr at 23°C. The luminescence

present in these tubes was assayed by adding 950 ul of 0.2 M imidazole buffered saline, pH 7.2, containing 0.4% sucrose and NB (1.0 ml per 100) to each 12 x 50 mm glass cuvette followed by 50 ul of control or test culture. These tubes were counted immediately. Controls and tests were counted in sets of ten or four cuvettes, respectively. One cycle to load, count, print and unload a tube took 20 sec. The photometer used was a Picolite Model 6200 Luminometer. The number of replicates (n) analyzed ranged from 11 to 34.

Results for the modified Frumin and probit analysis methods were considered to be statistically similar if the confidence intervals overlapped (Greenberg et al.

Table 1. Comparison of the modified Frumin and probit analysis with a growth toxicity test.

		Probit analysis			Frumin analysis		
	chemical	EC_{50}	UCI	LCI	EC_{50}	UCI	LCI
P. leiognathi	DEMP	2050	1639	2669	2850	2467	3253
	DIMP	580	100	4658	710	644	766
	DMMP	5821	4042	6859	5950	5194	6706
P. phosphoreum, A-13	DEMP	2154	1756	2577	2500	2142	2858
	DIMP	685	597	772	750	649	851
	DMMP	6045	4697	8556	6800	6015	7585
P. phosphoreum, MTX	DEMP	3729	2673	5175	4850	4198	5502
	DIMP	422	287	593	530	463	597
	DMMP	nd			nd		
V. harveyi, Lapota	DEMP	3424	2779	4170	4400	3809	4991
	DIMP	1020	870	1193	1250	1126	1374
	DMMP	3434	2579	4767	4600	3942	5258
V. fischeri	DEMP	1810	725	4111	2500	2142	2858
	DIMP	198	158	254	295	267	323
	DMMP	3442	2541	4563	4000	3557	4443
V. harveyi, 392	DEMP	2982	2694	3288	3150	2567	3753
	DIMP	493	429	566	580	513	647
	DMMP	4889	4143	5751	5450	4670	6230

LCI = lower confidence interval
UCI = upper confidence interval
nd = not detectable
MTX = Microtox

1991; Lange and Thomulka 1993; Thomulka and Lange 1995). Overlap of the CI with EC_{50} values was also examined for comparison purposes. Evaluation of statistical similarity of EC_{50} values was performed using a two-fold (2 times) method (Thomulka et al. 1993a, 1993b, 1993c).

RESULTS AND DISCUSSION

The comparative EC_{50} data for both growth and direct toxicity tests are shown in tables 1 and 2. Thirty-six comparison data sets comprised this experiment, with six sets having EC results greater than 30,000 ppm which was described as not detectable. These data show that all the CI's overlap except for the growth toxicity test using *V. fischeri* with DIMP. Evaluating the EC_{50} for the probit analysis with Frumin CI and

Table 2. Comparison of the modified Frumin and probit analysis with a direct toxicity test.

		Probit analysis			Frumin analysis		
	chemical	EC_{50}	UCI	LCI	EC_{50}	UCI	LCI
P. leiognathi	DEMP	13308	11018	16911	13500	11425	15575
	DIMP	2189	1800	2590	2500	2082	2918
	DMMP	nd			nd		
P. phosphoreum, A-13	DEMP	12421	10239	15539	12600	10663	14537
	DIMP	2001	1616	2389	2300	1818	2642
	DMMP	nd			nd		
P. phosphoreum, MTX	DEMP	11777	10946	12548	11600	10620	12580
	DIMP	2282	1822	2760	3150	2666	3634
	DMMP	nd			nd		
V. harveyi, Lapota	DEMP	5697	4663	7119	4800	3912	5688
	DIMP	1818	1223	2429	2600	2165	3035
	DMMP	22493	19461	27107	23700	21174	26226
V. fischeri	DEMP	13032	10396	20235	13200	11523	14877
	DIMP	3506	2798	4339	4050	3044	5056
	DMMP	nd			nd		
V. harveyi, 392	DEMP	7769	6333	10068	8200	6683	9717
	DIMP	2461	1872	3100	3500	3093	3904
	DMMP	nd			nd		

LCI = lower confidence interval
UCI = upper confidence interval
nd = not detectable
MTX = Microtox

Frumin EC_{50} with probit CI, three and two values did not overlap, respectively, and eight had no overlap for either comparison. Thirty-three percent (10/30) of the reportable tests had differences of 1 to l0%, 37% (11/30) had differences from 11 to 20%, 27% (8/30) had differences from 21 to 30%, and 3% (l/30) had a difference exceeding 30%. All EC_{50} values were within 2 times (fold) each other. This degree of similarity is narrower than the suggested acceptable variability of 2.5 for static toxicity tests (Frumin et al. 1992).

Data presented here suggest that these EC_{50} values by both statistical methods employed, CI and two fold, are essentially equivalent. This study suggests that the modified Frumin method provides an acceptable estimate of the median effective toxicity. Adaption of this methodology expands the use of various bacterial toxicity tests, as reported in other bacterial studies using *V. harveyi* and *P. phosphoreum* (Thomulka et al. 1993a; Thomulka and Lange 1995; Lange and Thomulka 1993).

Using the growth toxicity test, *V. fischeri* demonstrated a greater sensitivity to DEMP and DIMP, while *V. harveyi,* strain Lapota, was most sensitive to DMMP. The least sensitive Organisms towards DEMP, DIMP and DMMP were *P. phosphoreum,* Microtox strain, *V. harveyi,* strain Lapota, and *P. leiognathi,* respectively. Other strains exhibited intermediate sensitivity.

Different results were obtained through the direct toxicity test. *V. harveyi,* strain Lapota, demonstrated the most sensitivity to all three compounds. All *Photobacteria* tested were insensitive to DMMP. *V. harveyi,* strain 392, and *V. fischeri* demonstrated some toxicity with DMMP, but it was insufficient to calculate an EC_{50}. The least sensitive strains to DEMP and DIMP were *P. leiognathi* and V. *fischeri,* respectively.

All strains of bacteria demonstrated greater sensitivity towards all chemicals in the growth assay than the direct assay. The direct assay can only detect toxicity that is directed against the enzymes that are already present. However, in the growth assay, organisms are allowed to grow for 24 hr in the presence of biohazardous substances. The material will be present during all phases of the growth cycle. In general, cytoplasmic poisons such as compounds that stop protein synthesis, DNA replication, and electron transport, have the potential to be detected in the growth toxicity test.

In each assay DIMP displayed greatest toxicity, DMMP displayed the least toxicity and DEMP displayed intermediate toxicity towards the bacteria. This toxicity may be related to the lipid solubility of the organic side groups. A methyl group is the smallest and possibly the least soluble in the cell membrane, while the isopropyl group could be the most lipophilic. In support of this hypothesis, preliminary data suggested that dibutyl methanephosphonate (DBMP) was more toxic than either DIMP, DEMP or DMMP.

These experiments demonstrated a large amount of variation in sensitivity of toxicity of three closely related organophosphorus compounds as related to six test organisms using a growth and direct bioassay. No single organism was uniformly sensitive to all compounds using both assays. The growth assay was able to demonstrate a greater amount of toxicity of these compounds than the direct assay. Additionally, the organisms most sensitive using the growth toxicity test were not the same organisms most sensitive with the direct toxicity. Because of this diversity in toxicity, it is suggested that when conducting an environmental screen for ecotoxicity, a battery of tests and organisms be used (Thomulka et al. 1993a).

REFERENCES

Deichman W, Le Blanc T (1943) The determination of the approximate lethal dose with about six animals. J Industr Hyg 25:415-417

Frumin G, Chuiko G, Pavlov D, Menzkova O (1992) New rapid method to evaluate the median effective concentrations of xenobiotics in hydrobionts. Bull Environ Contam Toxicol 49:361-367

Greenberg AE, Eatum AD, Clesceri LS (eds) (1991) Standard Methods for Examination of Water and Waste Water. Amer Public Health Assoc, Washington DC

Harris EK (1959) Confidence limits for the LD_{50} using the moving angle-average methods. Biometrics 15:422-432

Lange JH, Thomulka KW (1993) Evaluation of aquatic toxicity studies using three different statistical methods: reference to aquatic bacterial bioassays. Fresenius Environ Bull 2:758-763

Litchfield JT, Wilcoxon F (1949) A simplified method of evaluation of dose-effect experiments. J Pharmacol Exp Ther 96:99-113

Somasundaram L, Coats JR, Rache KD, Stahr HM (1990) Application of the Microtox system to assess the toxicity of pesticides and their hydrolysis metabolites. Bull Environ Contam Toxicol 44:254-259

Thomulka KW, Kriebel JA, Schroeder JK, Lange JH (1993c) Toxicity of various chemicals in a sand and water mixture using the marine bacterium *Vibrio harveyi* in a direct bioluminescence reduction bioassay. J Clean Tech and Environ Sci 3:217-226

Thomulka KW, Lange JH (1995) Combined toxicology of ethylamine and methylamine in an aquatic system bioassay using the marine bacterium *Vibrio harveyi.* Chem Ecol (in press)

Thomulka KW, McGee DJ, Lange JH (1992) Evaluation of organic compounds in water using *Photobacterium phosphoreum* and *Vibrio harveyi* bioassays. Fresenius Environ Bull 1:815-820

Thomulka KW, McGee DJ, Lange JH (1993a) Detection of biohazardous materials in water by measuring bioluminescence reduction with the marine organism *Vibrio harveyi.* J Environ Sci Health A28:2153-2166

Thomulka KW, McGee DJ, Lange JH (1993b) Use of the bioluminescent bacterium *Photobacterium phosphoreum* to detect potentially biohazardous materials in water. Bull Environ Contam Toxicol 51:538-544

US Environmental Protection Agency (1988) Probit, US Environ Monitoring and Support Laboratory, US Environ Protection Agency, Cincinnati, OH