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Effect of Dust on the Viability of *Vibrio fischeri* in the Microtox Test

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The standard Microtox test involving the bioluminescent bacterium *Vibrio fischeri* is a frequently used ecotoxicological bioassay whose EC$_{50}$ values have been correlated to acute toxicity parameters of vertebrates, to irritancy measures, and to cytotoxicity indices. The aims were to explore the dependence of light output on viable cell number, with the latter estimated with the naked eye using a colorimetric tetrazolium salt method, the effects of dust on the bioluminescence and cell viability, how the viability of the cells is affected after spills, and how spills can be sampled. The lower limit of the linear dynamic range of the light-emitting bacterium was first defined to be 3.7×10^7 cells/mL, compared with 37×10^7 cells/mL in the Microtox assay. The effects of dust were then explored in the working range by the method of standard additions by adding 5-, 10-, and 20-mg amounts of Standard Reference Material Urban Dust 1649a. This simulated dust samples collected by a cordless vacuum technique involving a filter cassette. A mass of 20 mg dust totally inhibited the Microtox test at all times (5, 15, and 30 min). Masses of 5 and 10 mg dust lowered the luminescence significantly by 20 and 64%, respectively, after 30 min. However, the viability test was totally inhibited by 5 mg of dust. A spectrophotometric modification of the viability test using a wavelength of 508 nm was developed that was twice as sensitive as the naked eye test, and was as sensitive as the Microtox test. Mechanical shock involved with spilling and sampling bacterial reagent on hard surfaces killed the luminescent bacteria as shown by inhibition of luminescence. The optimum filter cassette for Microtox reagent collection was a 25-mm 1.00-μm PTFE filter in a 25-mm Delrin holder operated at 4.0 L/min, with a Tygon sampling probe. © 2001 Elsevier Science

INTRODUCTION

The Microtox test uses the luminescent marine bacterium *Vibrio fischeri* (formerly *Photobacterium phosphoreum*). It is a standard government agency ecotoxicological bioassay in Canada, The Netherlands, France, Germany, Spain, and Sweden; it is also an alternative test of acute toxicity, cytotoxicity, and irritation (Ronnpagel *et al*., 1995; Boyd *et al*., 1997; Pardos *et al*., 1999; Tchounwou and Reed, 1999; Domart-Coulon *et al*., 2000). In the United States, it is involved in American Society for Testing and Materials (ASTM) Method D-5660 (ASTM, 1996), and in the *Standard Methods for the Examination of Water and Wastewater* as Part 8050 (Clesceri *et al*., 1998a). It is sensitive to heavy metals, organics, and their mixtures (Kaiser and Devillers, 1994; Kong *et al*., 1995). The original test is supplemented by chronic, genotoxic, and acute solid-phase bioassays, using the same instrumentation (Azur Environmental, 2000; Strategic Diagnostics Inc., 2001).

The major application of the Microtox test has been to aqueous media and to detect substances that are sufficiently water soluble or toxic to lower luminescence relative to an unexposed positive control blank at a specific time, usually by defining the effective concentration that decreases light emission by 50%, the EC$_{50}$. The bacterium is standardized by storing and transporting it as a freeze-dried powder that is reconstituted into a saline growth medium at 15°C at the time of the analysis. Our research group has published on the variables that affect the test (Chou and Que Hee, 1992, 1993; Chen and Que Hee, 1995) and on some applications (Chou and Que Hee, 1992, 1993, 1994a, b; Chen and Que Hee, 1995; Que Hee, 1997). The present study explores the dependence of light output on viable cell number, the effects of dust on the Microtox test and cell viability, how the viability of the cells is affected after spills, and how spills can be sampled.

MATERIALS AND METHODS

Reagents

The following were from Azur International (Carlsbad, CA): Microtox bacterial reagent stored at −20°C, Microtox diluent, and Microtox reconstitution solution. Total Microbe Hunter to measure bacteria viability contained < 5% (w/w) 2,3,5-triphenyltetrazolium chloride in tryptic soy broth (Crescent Chemical Co., Hauppauge, NY). Standard Reference Material (SRM) 1649a for Urban Dust was

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0147-6513/01 $35.00

from the National Institute for Standards and Technology, (Gaithersburg, MD). Phenol to ensure Microtox data was from Fisher Scientific (Tustin, CA).

Apparatus

The Microtox Model 500 data collection/data system was from Microbics (Carlsbad, CA) (Microbics Corp., 1992). A Hewlett–Packard 8451A diode array spectrophotometer (Palo Alto, CA) and 1-cm Suprasil cells were used for ultraviolet/visible spectrophotometry for viability measurements. A 10 (×) 10-cm Plexiglas template (SKC Inc., Eighty Four, PA) defined the sampling area for spilled materials. Sampling cassettes used were: three-piece clear polystyrene sampling cassette with preweighed 37-mm 0.80-μm mixed cellulose ester filter (Omega Specialty Instrument Co., Chelmsford, MA); 25-mm 0.45-μm Teflon membrane filter in a polypropylene Swinnex-25 holder (Gelman Sciences from Fisher Scientific), and 25-mm 1.00-μm PTFE filter in a 25-mm Delrin holder (Gelman Sciences from Fisher Scientific). The sampling probe to the filter holder was a 3.5-cm-long 0.60-cm-i.d. tubing with the sampling end cut at a 45° angle with a scalpel. The cordless portable sampling pump that provided the collection vacuum was an Airchek Model 224-PCXR4 from SKC Inc. Pump calibrations before and after each sampling were performed with a mini Buck Model M-5 calibrator (A. P. Buck, Orlando, FL). An inclinometer (Edmund Scientific Co., Lev-O-Gage) measured the angle to the sampling surface and the sampling probe.

A refrigerated water bath allowed preequilibration of samples at 15°C. A calibrated 10-μL gas chromatography syringe facilitated accurate dispensing of volumes between 0.1 and 10 μL.

Microtox Bioassay

The techniques for Microtox assays and quality control/assurance with phenol are described elsewhere (Chou and Que Hee, 1992, 1993). Phenol EC_{50} values at 5 min always fell between 13 and 26 mg/L as required by Microbics Inc. and Azur International Inc. Instrument equilibration time was set at 30 min; the equilibration time for reconstitution and diluent solutions was 5 min at 15°C; and the equilibration time for the reconstituted bacterial reagent was 15 min at 15°C. Additions were always followed by mixing through filling and dispensing the solution with a pipettor. Light levels were read relative to the positive control blank 0, 5, 15, and 30 min after the target solution was mixed with the diluted solution containing 10.0 μL reconstituted bacteria.

To assess the basic sensitivity of the procedure, reconstituted Microtox reagent was added in triplicate from the original 1.0 mL reconstituted solution at 15°C in aliquots of 10, 7, 5, 3, 1, 0.7, 0.5, 0.3, 0.1, and 0 μL with the Microtox diluent volume adjusted correspondingly so as to maintain constant total volume as for the standard 10-μL method.

The same procedure in triplicate was repeated with 5, 10, and 20 mg of SRM 1649a mixed in with the lyophilized bacterial reagent for 30 s by manual shaking before addition of 1 mL of Microtox reconstitution solution, except that centrifugation at 3400 rpm (900 *g*) for 2 min occurred before the 15-min stabilization period before dispensing 10-μL aliquots for analysis.

Assessment of Viability of Vibrio fischeri

A 4-mL volume of reconstituted Microtox reagent (four vials) was added to 4 mL of Microtox diluent in the sampling cup of the Total Microbe Hunter, and then mixed with a 1-mL pipettor by filling and dispensing five times before stabilizing at 15°C for 15 min. A Total Microbe Hunter ampoule was then inserted into one of the slots in the bottom of the sampling cup, and the ampoule allowed to fill by breaking the tip. The ampoule powder was mixed by hand rotation to dissolve all powder as directed by the kit directions. The solution was placed in an incubator at 35.0 ± 0.2°C. The color was monitored every 5 min, and the time noted when an orange color was evident. The ampoule was then removed. The number of cells was then read from the Total Microbe Hunter plot of cell number versus time to change color. Similar procedures were used for dilutions of 3 ×, 5 ×, 7.5 ×, and 10 × the original cell number, all done in triplicate. Ultraviolet/visible scanning from 190 to 820 nm of the final colored solution relative to the original solution occurred in a 1-cm Suprasil cell. The maximum wavelengths were identified with a view toward enhancing sensitivity relative to the naked eye, and to define its Beer's law region.

To assess the influence of SRM Urban Dust on the viability test, 5, 10, and 20 mg of dust were evaluated. The specific weight of dust was added to each of three vials containing the freeze-dried bacteria and mixed by manual shaking for 30 s; then 1.0 mL of reconstitution solution was added to each, followed by mixing. The contents of these three vials were then added to 6.0 mL of diluent in the sampling cup of the Total Microbe Hunter, mixed again, and allowed to stabilize at 15°C for 15 min. The Total Microbe Hunter ampoule was then inserted, filled, its contents mixed, and incubated at 35°C as indicated above. Color changes were monitored every 5 min for 8 h at the various dust masses and cell numbers examined above in triplicate. Absorbance measurements were also performed at maximum and other specific wavelengths.

Surface Sampling for Microtox Reagent Spills

The sampling method was based on a previously published technique developed by one of the present authors

(Kim *et al.*, 2000), modified from an earlier one (Que Hee *et al.*, 1985).

The technique consists of applying a known weight of a solid over a hard smooth surface defined by a template 10×10 cm square, and then vacuuming up the particles using multiple sampling passes. A portable cordless pump is connected to a filter sampling cassette with Tygon tubing of 0.60-cm i.d. The sampling cassette also has a Tygon tubing sampling probe 3.5 cm long and 0.60 cm in i.d., with the sampling end cut at an angle of 45°. The sampling probe was held with the thumb and index finger at a 45° angle and a flow rate of 4.0 L/min. The difference in mass of the probe and cassette after sampling relative to that before is the mass collected, and is compared with the mass applied to define sampling efficiency per sampling pass or over a number of sampling passes (Kim *et al.*, 2000). One sample pass is from along the inner edge of the template area to its center in patterns of squares of progressively decreasing area. The weighed Microtox reagent bottle was opened, and the solid quickly poured onto the surface of the template. The empty bottle was reweighed to define the mass applied by difference. The particles were crushed quickly with the bottom of the Microtox reagent bottle. The solid was dispersed physically with a spatula. The optimum geometry of the collection cassette was evaluated from the following choices: a three-piece clear polystyrene sampling cassette with preweighed 37-mm 0.80-μm mixed cellulose ester filter; 0.45-m Teflon membrane filter in a polypropylene Swinnex-25 holder; and a 25-mm 1.00-μm PTFE filter in a 25-mm Delrin holder. After the final weighing, 1.0 mL of reconstitution solution was applied slowly along the inner walls of the filter cassette, and the solution shaken for 15 s. The solution was then transferred with a Pasteur pipet to a clean Microtox cuvet, and equilibrated for 15 min in the Model 500 at 15°C before a 10.0-μL aliquot was taken for evaluation as described above. The contents of a new Microtox reagent vial in 1.0 mL of reconstitution solution served as positive control.

RESULTS

Dependence of Light Intensity in the Microtox Test on Inoculation Volume and Time

As expected, light intensity relative to positive control at zero time decreased with increasing time after sample inoculation and with decreasing inoculation volume at a specific time. For the latter, the inoculation volume linear dynamic range was 1–10 μL for 5, 15, and 30 min. The slopes at 15 and 30 min were 73 ± 2 and 52.5 ± 0.1% of those at 5 min, the value for the latter being 7.0 ± 0.1 light intensity units relative to the positive control at zero time per microliter of inoculation volume. The zero time value is 10 light intensity units per microliter of inoculation volume. The least quantifiable limit (LQL) for <10% coefficient of variation (CV) was about 0.70 μL inoculation volume. The detection limit for signal/noise of 3.0 was < 0.1 μL. Thus reliable readings of luminescence were possible for a volume 0.10th that used for inoculation in the standard assay, with a detection limit of about 0.01th that volume.

Effect of Dust on Microtox Luminescence

When the bacterial reagent was ground in a mortar and pestle in the absence of soil, no luminescence was detected. Thus mortar and pestle could not be used to mix dust and bacteria. Therefore the addition was done by adding the dust sample to the bacteria reagent in solution, and manually shaking the bacteria and dust within the bottle. No detectable luminescence was observed after adding 20 mg dust, but light emission occurred after the 5-mg and 10-mg additions. Addition of 5 mg dust over the inoculation volume range 0.1–7.0 μL at 5, 15, and 30 min caused sensitivity to decrease to 46, 60, and 80% of the corresponding values without dust, respectively. Similarly, addition of 10 mg decreased the luminescence to 18, 26, and 36%, respectively. For both 5- and 10-mg-additions there was no dependence on reading time, the respective arithmetic mean slopes and standard deviations being 3.1 ± 0.2 (CV = 6.0%) and 1.29 ± 0.02 (CV = 2.0%) light units/μL inoculation volume, respectively. This behavior was also observed in the absence of dust at low light intensities for inoculation volumes between 0.1 and 0.5 μL, where CV values also varied between 6.0 and 80%. Both the 5- and 10-mg dust additions produced luminescence above the LQL of the no-dust response curve, however. CV values for individual inoculation volumes varied between 17 and 43%, supportive of a dust matrix effect.

Viability and the Microtox Test, and the Influence of Dust

The first task was to determine the original number of viable bacteria in the Microtox reagent. The Total Microbe Hunter color change chart data for log of colony-forming unit (CFU) per milliliter (y) versus color change time (t) was

$$\log y = -0.5968t + 8.3417, \quad r^2 = 0.9982, \quad P < 0.05. \qquad (1)$$

The times to change color for different dilutions of the Microtox reagent are provided in Table 1 for the Total Microbe Hunter test. The CV for twofold dilution is too high (45%), with acceptable precision (<10% CV) occurring at greater dilutions up to fivefold. Beyond fivefold dilutions, the viability test is too insensitive. The grand mean and standard deviation for the original cell number in the Microtox reagent was $(3.68 \pm 0.21) \times 10^8$ CFU, CV = 5.6% (Table 1). Thus the Total Microbe Hunter test detected only between 0.74×10^8 and 3.68×10^8 cells/mL *V. fischeri.*

Spectrophotometry showed that the Total Microbe Hunter blank had maxima at about 240 and 290 nm. After the color change, the maxima beyond 300 nm were 370 and 508 nm. Table 1 also presents the absorbance data at these absorption maxima and at 490 nm on the short-wavelength side of the long-wavelength band. Only the 508-nm wavelength is suitable for spectrophotometric assay for cell number since its absorbances decreased consistently with increasing dilution. The spectrophotometric method is more sensitive and convenient than the color change time method because absorbance values at 508 nm still changed at 7.5- and 10-fold dilutions. Thus spectrophotometry at 508 nm is about double the sensitivity of the naked eye. For 5-mg dust additions of the 3-fold diluted Microtox reagent, the dust precipitated after 30 min incubation at 35°C in the viability test. However, there was no color change even after 8 h of incubation. Thus dust is a negative interference in the Total Microbe Hunter test.

Sampling Microtox Reagent Spills

The Microtox reagent consists of 4% (w/w) *V. fischeri*, 3% sodium chloride, 92% skim milk solids, and 1% water, according to Azur Environmental (2000). When the reagent was weighed directly on a balance, its weight continually increased, and the original powder became sticky and pebbly. The bacterial reagent was therefore deliquescent.

The standard sampling technique for dry solids on hard smooth surfaces (Kim *et al.*, 2000) did not work, because the 3-mm inlet of the filter cassette became blocked by a wet Microtox reagent pebble. The cassette was also too large to allow 1.0 mL of reconstitution solution to dissolve all the solid on the filter surface, but 2.0 mL did. The inlet diameter did not allow a pipet tip to be inserted to allow the usual mixing. After 2 mL reconstitution solution was placed inside the cassette and then shaken manually, Microtox analysis of a 20-μL aliquot led to no light emission.

Obstruction also occurred for the 4-mm inlet of the 0.45-μm Teflon filter in its 25-mm filter cassette. The inlet diameter still did not allow the usual mixing. The cassette volume was too small to contain 1.0 mL of reconstitution solution.

A 1.0-μm 25-mm-diameter Teflon filter in a 25-mm Delrin filter cassette of inlet i.d. 10 mm successfully collected the pebbles, permitted mixing by the standard technique after adding 1 mL of reconstitution solution, and allowed the taking of a 10-μL aliquot for inoculation. The measured sampling efficiency for the filter cassette on a mass basis was at least 81% at 4.0 L/min flow rate, with the rest of the collected mass being on the sampling probe wall. There was a large imprecision in recovered mass for triplicates because of the deliquescent nature of the Microtox reagent. On Microtox testing, the light intensity for each triplicate was below the LQL of the positive control, but decreased with incubation time as a valid Microtox test should. In addition, the CV for the light intensity at 5, 15, and 30 min varied between 0.70 and 5.0%, also acceptable behavior. Thus, sufficient bacteria were recovered in the cassette to be detected with precision in the Microtox test, but the viability of the cells was still affected.

DISCUSSION

The viable cell number dependence of the Microtox test was examined with a view toward sampling low numbers of luminescent and nonluminescent bacteria in dust samples. The intrinsic sensitivity to viable bacteria ranged down to about an order of magnitude below the original cell number for both the standard Microtox test and spectrophotometric analysis at 508 nm for the Total Microbe Hunter test. The

TABLE 1
Triplicate Determination of Number of Bacteria in the Microtox Reagent by the Total Microbe Hunter Kit by the New Spectrophotometric Method at 508 nm and by Color Change Time

	Dilution factor					
Parameter	2	3	4	5	7.5	10
Color change time ± SD (h)	0.11 ± 0.05	0.39 ± 0.05	0.60 ± 0.05	0.79 ± 0.04	>6	>6
Number of bacteria $\times 10^{-7}$	18.9	12.9	8.46	7.43	NA	NA
Original number ± SD $\times 10^{-8}$	3.77 ± 1.71	3.86 ± 0.49	3.38 ± 0.24	3.71 ± 0.19	NA	NA
Grand mean ± SD $\times 10^{-8}$	3.68 ± 0.21					
Mean absorbance						
370 nm	0.830	0.593	0.278	0.406	0.170	0.135
490 nm	0.860	0.574	0.200	0.224	0.106	0.070
508 nm	0.877	0.580	0.240	0.218	0.100	0.066

Note. The Original number accounts for the dilution factor; the Number of bacteria data were calculated from Eq. (1); the Mean absorbance is the mean of triplicate readings, all with CVs <10%. NA, not applicable.

color change viability test for the naked eye was only half as sensitive as the spectrophotometric method. The actual detection limit of the Microtox test was equivalent to about $< 4 \times 10^4$ viable cells. The LQL was about 3.7×10^5 viable cells compared with 3.7×10^6 viable cells used in the standard test. The Microtox manual provides a figure of about 10^8 CFU in the storage vial (Microbics Corp., 1992). The present investigation determined the number to be actually $(3.7 \pm 0.2) \times 10^8$ CFU.

The Total Microbe Hunter test uses a tetrazolium salt that is reduced to a colored formazan product when electrons are accepted from oxidized substrates or appropriate reducing agent cofactors like reduced nicotinamides and flavins produced during growth and division, but which are not produced by dead cells (Vistica *et al*., 1991; Bitton and Koopman, 1992). Johnson *et al.* (1985) incorporated tetrazolium dye reduction into portable kits that shortened the time of analysis for viable gram-negative bacteria from 15–18 h for the standard laboratory test to 4–6 h, with 93% correlation. Both methods are still much shorter than the 3–4 days required for the standard agar plate dilution/colony counting bioassay for bacteria in aqueous media (Clesceri *et al*., 1998b) or collected on a filter (Clesceri *et al*., 1998c). Hjertstedt *et al.* (1998) showed that sporulating *Candida albicans* yeast contributed to the tetrazolium bioassay response, but did not form colonies. This is not a factor in bacterial bioassays. A factor in the insensitivity of the Total Microbe Hunter test might be the short lifetime of *V. fischeri.*

Mechanical shock intrinsic to the sampling process lowered the viability of the luminescent bacteria of the Microtox test, whether from its original storage bottle or on a surface. Since only viable bacteria luminesce in the Microtox test, bioluminescence diminution could be interpreted as chemical toxicity, if the standard reconstitution technique is not followed exactly for the case of the original storage bottle. Such effects might also be observed for dropped or roughly handled vials, and may account for some of the known intershipment variability.

While no luminescence was detected for the 20-mg addition, 5- and 10-mg dust additions did allow Microtox test responses, but with lowered light emission relative to no dust present. Masking or luminescence absorption by the dust akin to a color interference may be occurring. No matter the cause, the addition of a dust matrix even at as low a mass as 5 mg causes an apparent chemical toxicity effect that must be accounted for when using the standard Microtox test for turbid liquids, sediments, sludges, or solid samples. Our recommendation for turbid liquid samples is to centrifuge and then filter the supernatant through 0.45-μm and then 0.20-μm filters and dilute appropriately before adding the reconstitution solution containing bacteria to achieve results not dependent on particulate matter. A high apparent background soil toxicity in the standard Microtox test has also been reported (Cassells *et al*., 2000).

Much recent work has occurred with the new Microtox solid-state bioassay (Bitton and Koopman, 1992; Kong *et al*., 1995; Ronnpagel *et al*., 1995; Kwan and Dutka, 1995; Wendt *et al*., 1996; Cheung *et al*., 1997; Harkey and Pradhan, 1998; Lee *et al*., 1999; Dorn and Salanitro, 2000), although the modified test still appears to lack sensitivity for xenobiotics in soils, sludges, and sediments. Microtox results that are precise have been obtained for xenobiotics in extracts from soils, sludges, and sediments (Bitton and Koopman, 1992; Kong *et al*., 1995; Karuppiah *et al*., 1997; Sunahara *et al*., 1998, 1999; Cassells *et al*., 2000; Cook *et al*., 2000).

The surface sampling results show that *V. fischeri* is a delicate bacterium that loses viability quickly on a hard smooth surface, and is susceptible to rough mechanical treatment. The sampling of the Microtox reagent itself had to be done as quickly as possible because of its deliquescence, with subsequent stickiness and pebbling.

Very few methods to sample bacteria on hard surfaces have been reported. Eginton *et al.* (1995) using a tetrazolium salt-based method and colony counting, showed that 15 sampling passes had to be done to obtain a negative tetrazolium salt test for transfer of gram-negative bacteria deposited on hard smooth tiles to agar plates. The authors attributed their results to the strength of the attachment of the bacteria to the tile surface. Decreasing bacteria viability may also have been a factor in light of the results of the present investigation. Bacteria on human skin have been sampled with a pad and rinsing techniques (Hambraeus *et al*., 1990). Bacteria on citrus fruit surfaces were removed better by washing and waxing, rather than by washing alone (Pao and Brown, 1998). Scrubbing allowed removal of bacteria from the skin of horses (Hague *et al*., 1997). Real-time monitoring of *Escherichia coli* adhering to beef carcass surfaces was accomplished by inserting a *lux* reporter gene into the applied *E. coli*, and monitoring the surface bioluminescence to assess surface adherence and decontamination in real time (Sirigusa *et al*., 1999).

The above study focused on the viability and thus potential infectivity of the microorganisms deposited on a surface or in the substrate. Gram-negative bacteria endotoxin also in *V. fischeri* is toxic whether the bacteria are alive or dead. Further studies with markers like 3-hydroxymyristic acid (Kirschner *et al*., 1985; Parenteral Drug Association, 1990) or lipopolysaccharides using the *Limulus* amoebocyte lysate test (Parenteral Drug Association, 1990; Hurley, 1995) are suggested also to screen for public health and environmental safety.

CONCLUSIONS

The sensitivity of the Microtox test to detect viable bacteria was about the same as that of a spectrophotometric method developed for the Total Microbe Hunter test, but was twice that for the color change detected by the naked eye in the Total Microbe Hunter test. Addition of dust decreased viability in both the Microtox test and the Total Microbe Hunter test, the latter being much more sensitive. The sampling of a Microtox reagent spill using a cordless vacuum method was accomplished by optimizing the geometry of the collection cassette, but the viability of the bacteria decreased markedly.

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