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METHOD FOR MEASURING TOXICITY OF SUSPENDED PARTICULATES IN WATERS

by

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MANAGEMENT PERSPECTIVE

A protocol for testing toxicant activity of different fractions of suspended particulates in waters is outlined.

The importance of this procedure is that it provides information on toxicant activity of a specific particulate fraction which modellers can use to develop toxicant transport models.

PERSPECTIVE GESTION

L'auteur présente un protocole d'essai visant à déterminer l'activité des agents toxiques dans diverses fractions de matières particulaires en suspension dans l'eau.

L'essai est d'un grand intérêt, car il renseigne sur l'activité des agents toxiques dans une fraction précise de matières particulaires, données pouvant servir à la modélisation du transport des produits toxiques.

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1.0 OBJECTIVE

This procedure is intended to be used for determining the presence of toxicity in the suspended particulates in waters and effluents.

2.0 SCOPE

This procedure is applicable to the following types of samples containing suspended particulates:
(i) river and lake waters;
(ii) surface and ground waters; and
(iii) industrial waste waters.

3.0 PRINCIPLE

This method employs the cascade filtration technique to separate various particle size ranges from a sample. The toxicity of each fraction is determined using the Microtox toxicity test procedure.

To obtain the different particle size fractions, the sample is filtered using cascade filtration procedure (Fig. 1) in sequence through as many filter sizes (μm) as necessary. Each of these fractions are resuspended in a known volume of sterile milli-Q-reagent grade water or distilled water. A specific volume of resuspended fraction is concentrated onto a 0.2 μm nuclepore filter and the fraction is extracted using a suitable organic solvent such as dimethyl sulfoxide (DMSO). The extract is tested for the presence of toxicity using the Microtox™ Bioluminescence Bioassay Technique.

In the Microtox test, a luminescent bacteria (Photobacterium phosphoreum) is used to assess the level of toxicant activity in waters or sediments. This test is functional because the metabolism of the luminescent bacteria is influenced by low levels of toxicants and, occasionally, stimulants. Any alteration of metabolism affects the intensity of the organism's light output. By sensing these changes in light output, the presence and relative concentration of toxicants can be obtained by establishing the EC50 levels from graphed data: EC50 being that concentration of toxicant causing a 50% reduction in light from the baseline level.

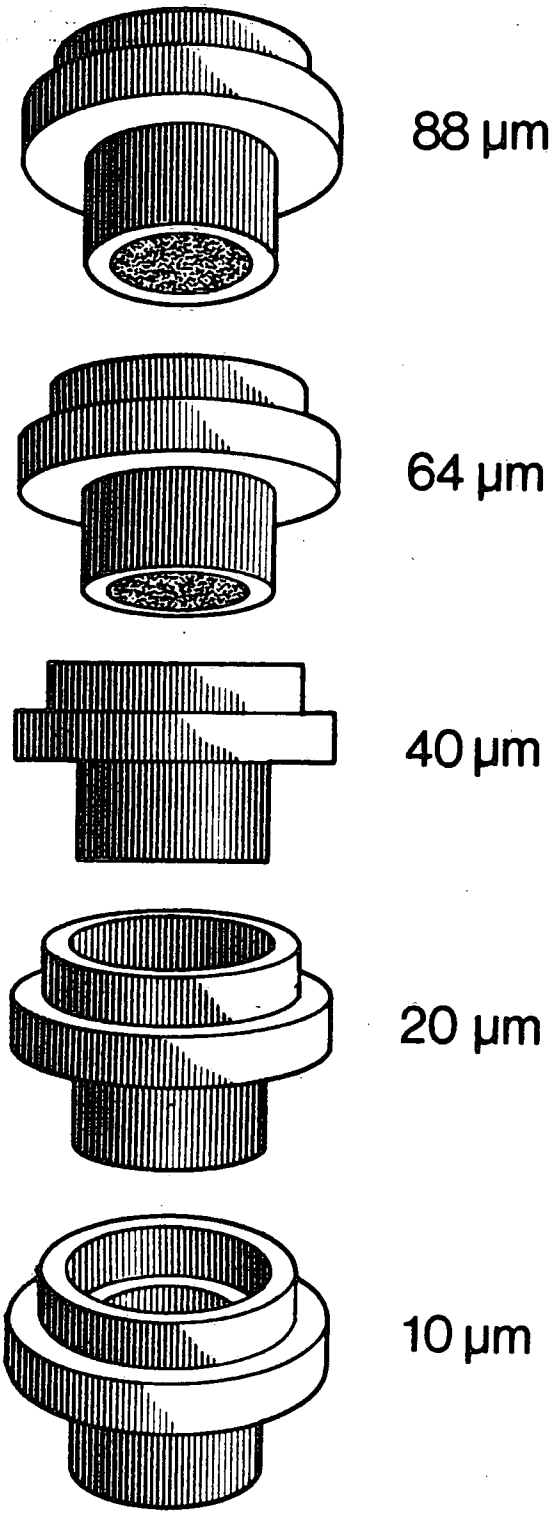

Figure 1 Illustration of a Cascade Filtration System.

4.0 REAGENTS, MATERIALS AND APPARATUS

4.1 Reagents

4.1.1 Lyophilized bacteria (Microbics Enterprises[1])

4.1.2 Diluent[1] (prepared from 3% analytical grade NaCl, 4 µg/mL yeast extract and 135 µg/mL dibasic sodium phosphate (Na_2HPO_4) at pH Of 7.0).

4.1.3 Osmotic Adjusting Solution (prepared by adding analytical grade sodium chloride to Milli-Q reagent grade water to obtain a 22% solution).

4.1.4 Reconstitution Solution[1]

4.1.5 Milli-Q-reagent grade water and glass distilled water

4.1.6 DMSO (Dimethyl sulfoxide)

4.2 Materials

4.2.1 Disposable glass cuvettes[1] (11.75 mm x 50 mm)

4.2.2 0.2 µm nuclepore polycarbonate filter

4.2.3 Eppendorf pipettes (10, 100, 500, 1000 µL)

4.2.4 Eppendorf pipette tips (100, 1000 µL)

4.2.5 Forceps

4.3 Apparatus

4.3.1 Microtox™ Toxicity Analyzer[1] (Model 2055)

4.3.2 Beckman Recorder (Model 2055) or equivalent

4.3.3 Recorder Chart Paper

4.3.4 IBM PC or Sharp Model EL-5100 Programmable Calculator or equivalent

4.3.5 Microtox Data Analysis Program for IBM Computer or Sharp Programmable Calculator

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[1]Microbics Corporation, 2232 Rutherford Road, Carlsbad, CA 92008

4.3.6 Ultrasonic Dismembrator[2] or equivalent

4.3.7 Filter unit with filter funnel base and flask.

4.3.8 Vacuum pump with 15 lbs pressure kilopascals or equivalent

4.3.9 Cascade filtration system or equivalent with nylon mesh[3]

4.4 Reagent Storage, Temperature and Estimated Shelf Life

(i) Lyophilized Bacteria at -40°C to -60°C (2-3 years)

(ii) Diluent at 4°C (2-3 years)

(iii) Osmotic Adjusting Solution at 4°C (2-3 years)

(iv) Reconstitution Solution at 4°C (2-3 years)

5.0 SAMPLE COLLECTION AND SAMPLE TRANSPORTATION

5.1 Collect approximatly 5 gal of a sample in a container not allowing any air space that would allow vigorous agitation and break up of the particles during transportation.

5.2 Keep the sample in a cool spot (≈18-20°C) after collection and during transportation (refrigeration is not necessary).

6.0 FILTRATION PROCEDURE FOR THE SEPARATION OF THE SUSPENDED PARTICULATES

6.1 Mix gently by swirling a 5 L volume of the sample to minimize particle breakup.

6.2 Filter the entire volume first through the largest pore size sieve.

6.3 Immerse the bottom of the filter (sieve surface) below the surface of the filtrate (2-3 times) to open the clogged filter pores and allow the smaller sized particles to pass through the filter while retaining the targetted particle size on the filter.

6.4 Repeat the above steps, 6.1, 6.2 and 6.3 until the sample has been filtered through all sizes of filter sieves in succession to obtain all the desired fractions.

6.5 Carefully resuspend each of the fractions on the filters in 200 ml distilled water.

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[2]Branson Smith Kline Co., Shelton, Conn., U.S.A.
[3]B&S.H. Thompson & Co. Ltd., 140 Midwest Road, Scarborough, Ontario, Canada H4P 2K6

7.0 TEST PROCEDURE

7.1 Extraction of Toxicants from the Particulates

7.1.1 Filter 50 mL of each of the fractions (from Step 6.5) through a 0.2 µm nucleopore polycarbon filter.

7.1.2 Rinse the filter funnel using 10 ml milli-Q-reagent grade water.

7.1.3 Transfer the membrane filter into a scintillation vial containing 500 µl dimethyl sulfoxide (DMSO) using clean forceps.

7.1.4 Cover the vial with screw cap.

7.1.5 Place the vial containing the suspended particles in an ultrasonic dismembrator for 30 min.

7.1.6 After sonication, add 49.5 ml milli-Q-reagent grade water to the vial to obtain a final concentration of 1% DMSO.

7.1.7 Repeat steps 7.1.1 to 7.1.6 for each of the suspended particulate fractions.

7.2 Microtox Toxicity Test

7.2.1 Set up Analyzer and Recorder

7.2.2 Check temperatures of the TURRET (15.0 ± 0.2°C), INCUBATOR (15.0 ± 0.2°C) and AIR (3.0 ± 1.0°C).

7.2.3 Check the HIGH VOLTAGE (HV) reading and confirm that it reads between -500 to -900.

7.2.4 Check DPM (Digital Panel Meter) and confirm it is set at 000.

7.2.5 Turn recorder on and set chart speed to 1 cm per minute.

7.2.6 Using recorder ZERO ADJUSTMENT dial adjust pen to zero (or 0%) and the analyzer DPM should continue to record 000.

7.3 Reconstitution of Lyophilized Bacteria

7.3.1 Remove a vial of lyophilized bacteria from the -60°C freezer to a refrigerator (4°C) for 20-30 minutes to allow temperature to stabilize.

7.3.2 Pipette 1000 µL of the Reconstitution Solution into the PRE-COOLING WELL CUVETTE (Fig. 2) and allow to stand for 20-30 minutes for temperature stabilization.

7.3.3 Take lyophilized bacteria vial from refrigerator, remove the seal and rubber stopped (make sure there is a vacuum releasing sound, if not, repeat Step. 7.3.1 with a new vial).

7.3.4 Pour the pre-cooled Reconstitution Solution into the lyophilized bacteria vial and mix by swirling for 2-3 seconds.

7.3.5 Pour the Reconstitution Solution plus the lyophilized bacteria back in the cuvette and replace the cuvette into the PRE-COOLING WELL.

7.4 Sample Preparation, Two-fold Serial Dilution Method

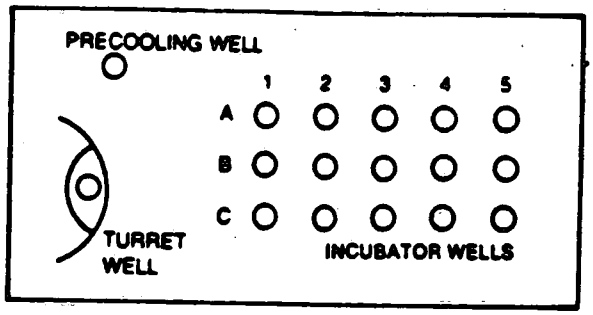

Figure 2. Arrangement of incubator wells in the Microtox Toxicity Analyzer

7.4.1 Place cuvettes into all 15 incubator wells in the Microtox Toxicity Analyzer.

7.4.2 Transfer 200 µL (0.2 mL) of osmostic adjustment solution to cuvette in well A1.

7.4.3 Add 2000 µL (2.0 mL) of sample to A1 using the same 1000 µL micropipettor, mixing the solution by aspirating and dispensing the solution 3 to 4 times.

7.4.4 Add 1000 µL (1.0 mL) of Microtox diluent to cuvettes in wells A2 to A5 and B1.

7.4.5 Transfer 1000 µL (1.0 mL) of sample from A1 to A2 and mix the contents as Step 7.4.3.

7.4.6 Transfer 1000 µL (1.0 mL) of sample from A2 to A3 and mix the contents as in Step 7.4.3

7.4.7 Transfer 1000 µL (1.0 mL) of sample from A3 to A4 and mix the contents of A4 as in Step 7.4.3.

7.4.8 Transfer 1000 µL (1.0 mL) of sample from A4 to A5 and mix the contents as in Step 7.4.3.

7.4.9 Allow 10-15 minutes for temperature equilibration. The sample concentrations at this point are:

Cuvette	Sample Concentration (%)
A1	90.9
A2	45.4
A3	22.7
A4	11.4
A5	5.7
B1 (Control)	0.0 (Diluent)

7.5 50% Assay with 15 Minute Test Time

7.5.1 Pipette 500 µL (0.5 mL) of Microtox Diluent into cuvette B2 through B5 and C1 through C5.

7.5.2 Allow 10-15 minutes for temperature equilibration.

7.5.3 Transfer 10 µL (0.01 mL) of the reconstituted lyophilized bacteria (9.0) to cuvettes B2 through B5 and C1 through C5 using a new micropipette tip each time and mix the contents by flicking the cuvettes 2-3 times with a finger.

7.5.4 Allow 10-15 minutes for temperature equilibration which can be observed either from the RECORDER or the DPM.

7.5.5 After the equilibration period, transfer cuvette B2 into the TURRET and turn on the RECORDER..

7.5.6 Close the TURRET and (by adjusting the SPAN (100% ADJ) dial) situate the recorder pen so that cuvette B2 produces a light output reading of approximately 70-80% on the chart paper.

7.5.7 Plot the light level until a constant slope (stable light output) is obtained.

7.5.8 Adjust the SPAN (100%) ADJ dial for either a DPM or recorder reading of approximately 080 or 80% respectively.

7.5.9 Open the TURRET and return cuvette B2 back to its incubator well.

7.5.10 Cycle the cuvettes through TURRET in the order C1, C2, C3, C4, C5, B5, B4, B3, and B2 to check for variability in light levels.

7.5.11 Discard the three (3) most deviant cuvettes (an acceptable light level is 80-100%) leaving B2, B3 and B4 wells vacant.

7.5.12 Rearrange cuvettes in either ascending or descending order according to the light levels (this is an option).

7.5.13 Cycle the cuvettes C1, C2, C3, C4, C5, and B1 in order to obtain initial light levels (Io).

7.5.14 Immediately, and without removing cuvettes from the INCUBATOR wells, transfer 500 µL (0.5 mL) of DILUENT (cuvette B1) and samples dilutions (cuvettes A1 to A5) into cuvettes B5 and C1 to C5 as follows:

B1 to B5
A1 to C1
A2 to C2
A3 to C3
A4 to C4
A5 to C5

7.5.15 Fifteen minutes later, recycle cuvettes B5, C1, C2, C3, C4, and C5 through TURRET to obtain the 15 minutes light levels (I_{15}).

7.6 Calculation of Final Test Sample Concentrations (%)

7.6.1 Following Step 7.4.3, 500 µL (0.5 mL) of sample is mixed with 500 µL (0.5 mL) of DILUENT. Therefore, the final test concentrations in the test cuvettes for the designated serial dilutions are:

Cuvette	Final Assay Concentration (%)
C1	45.4
C2	22.7
C3	11.4
C4	5.7
C5	2.8
B5 (Control)	0.0

7.7 Data Recording and Reduction

7.7.1 Data Recording

Record the initial (I_0) and the final (I_{15}) light output readings for control (B5) and each assay sample dilution (C1, C2, C3, C4, and C5) from the recorder chart or from the DPM if RECORDER is not available, in a DATA REDUCTION FORM or equivalent.

Example: Data Reduction Form

LABORATORY INFORMATION	SAMPLE INFORMATION
LABORATORY NO. _____	DATE RECEIVED _____
ASSAYED BY _____	DATE ANALYSIS _____
	SAMPLE NO. _____
	SAMPLE TYPE _____
	VISUAL COLOR _____
	INITIAL pH _____

	LIGHT LEVELS ZERO MINUTE	15 MINUTES
BLANK READING (B5)		
CUVETTE C1 (45.4%)		
CUVETTE C2 (22.7%)		
CUVETTE C3 (11.4%)		
CUVETTE C4 (5.7%)		
CUVETTE C5 (2.8%)		

7.7.2 Data Reduction

7.7.2.1 Blank Ratio Calculation

The Blank Ratio allows compensation of toxicity data for the changes in light output by the bacteria during their exposure to a non-toxic sample (DILUENT). The Blank Ratio is calculated as:

$$R_{15} = \frac{I_{15}}{I_0}$$

where

R_{15} = Blank Ratio for 15 minutes

I_0 = Initial light level of blank cuvette (B5) at zero minute

I_{15} = Final light level for corresponding blank cuvette (B5) at 15 minutes

7.7.2.2 Normalized Ligh Loss (Γ Effect)

The responses observed for all test cuvettes are normalized against the blank response by muliplying the initial light output of each cuvette by the blank ratio for 15 minutes (R_{15}). This product is the light output expected at 15 minutes for any particular cuvette if it had been used as a

blank. The toxic response is then calculated using the actual final light reading and the light reading to be expected for a non-toxic sample, both at 15 minutes. This scheme corrects for the effects of both light drift and the off-set in light output due to the dilution which occurs when the organisms are challenged. The normalized Γ effect may be calculated for each cuvette using the following formula:

$$\Gamma_{15} = \frac{\text{Light Lost}}{\text{Light Remaining}}$$

$$= \frac{(R_{15})\ (I_0) - (I_{15})}{I_{15}}$$

$$= \frac{(R_{15})\ (I_0)}{(I_{15})} - 1$$

where

I_0 = the initial light reading for any given cuvette at zero minute, just before challenging the organisms

I_{15} = the final light reading for the corresponding test cuvette at 15 minutes

where

R_{15} = blank ratio at 15 minute time

Γ_{15} = the Γ effect calculated for exposure time of 15 minutes

7.8 Determination of EC 50 Value

7.8.1 Graphical Method

7.8.1.1 Plot the concentrations of sample (X-axis) against effects (Y-axis) at 15 minutes on a log-log graph paper.

7.8.1.1 Draw a best-fit regression line through points.

7.8.1.2 A line is drawn from Γ = 1.00 (50% light loss, Refer to Beckman Microtox™ System Operating Manual Section 3) parallel to the concentrations (X-axis). The EC_{50} concentration is the point where this line intersects the slope.

7.8.2 Calculator Method

Sharp Model EL-5100 Programmable Calculator Method or equivalent (Refer to Beckman Microtox™ System Operating Manual Section 11).

7.9 Personal Remarks

7.9.1 Precision of results is directly dependent on the precision of the operator.

7.9.2 Toxicity effect is TEMPERATURE and TIME dependent. If one wishes to extend the contact time, a thirty minute test time is sufficient for most chemical and environmental samples.

7.9.3 A setting of less than 5.0 turns of SPAN dial indicates healthy and relatively stable bacteria, whereas a setting of greater than 7.0 indicates unstable and unhealthy bacteria. Repeat Step 7.3.1 with a new vial.

7.9.4 It is critical that no longer than 15-20 seconds is taken between transfer in Step 7.5.10 and 7.5.15.

7.9.5 If more than one blank is used in Step 7.7.2.2 the MEAN of each BLANK RATIO is used for data reduction.

7.9.6 DO NOT store lyophilized bacteria at the temperature as recommended on the bottle. Our research indicates that the light level of lyophilized bacteria is more stable when preserved at a lower temperature (-60°C).

7.9.7 All glassware used for toxicity tests should be free from all contaminants. The use of acid washed glassware is preferred.

7.9.8 If organic solvent is used for extraction, a solvent blank must be used to establish the lowest concentration that has no effect on the test organism before the initial concentration is chosen for the test.