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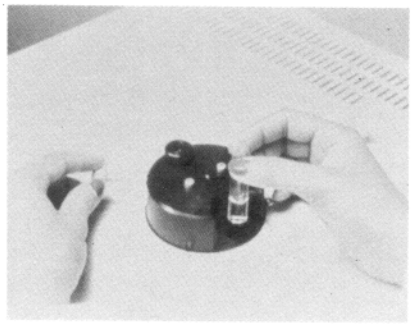

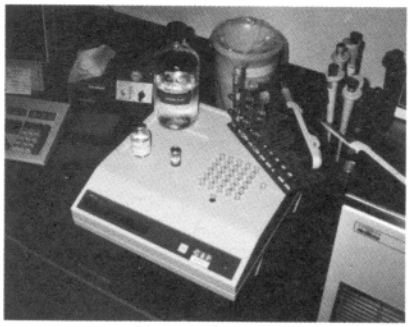

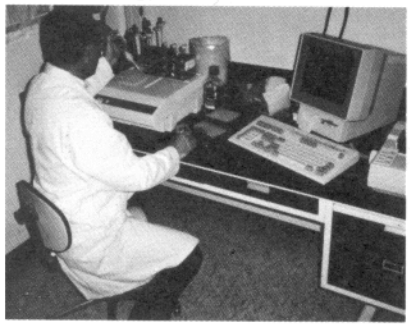

Biological Test Method: Toxicity Test Using Luminescent Bacteria

Report EPS 1/RM/24
November 1992

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Toxicity Test Using Luminescent Bacteria

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Abstract

Methods recommended by Environment Canada for performing toxicity tests with the luminescent bacterium Vibrio fischeri, *are described in this report.*

General or universal conditions and procedures are outlined for testing a variety of substances. Additional conditions and procedures are stipulated that are specific for assessing samples of chemical, effluent, leachate, elutriate, receiving water, and sediment or other solids such as soil. Included are instructions on sample handling and storage, test facility requirements, procedures for preparing test solutions and initiating tests, specified test conditions, appropriate observations and measurements, endpoints, methods of calculation, and the use of reference toxicants.

The endpoint of the test is the concentration of sample which is estimated to cause 50% inhibition of light production by the bacteria (i.e., the IC50). This could be estimated after exposures of 5, 15, or 30 minutes.

Résumé

Le présent document expose les méthodes recommandées par Environnement Canada pour l'exécution d'essais de toxicité sur la bactérie luminescente Vibrio fischeri.

Il présente les conditions et méthodes générales ou universelles permettant de réaliser des essais sur un large éventail de substances. On y précise aussi d'autres conditions et méthodes propres à l'évaluation d'échantillons de produits chimiques, d'effluents, de lixiviats, d'élutriats, de milieux récepteurs et de sédiments iy d'autres solides tel que des sols. Le lecteur y trouvera des instructions concernant la manipulation et le stockage des échantillons, les installations d'essai, la préparation des solutions d'essai et la mise en route des essai, les conditions prescrites pour les essais, les observations et mesures appropriées, les résultats des essais, les méthodes de calcul et l'utilisation de produits toxiques de référence.

Le résultat de l'essai est la concentration de l'échantillon qu'on estime qui cause une inhibition de 50% de la production de lumière par la bactérie (c'est-à-dire la CI50). L'estimation de cette valeur peut se faire après 5, 15, ou 30 minutes d'exposition.

Foreword

This is one of a series of recommended methods for measuring and assessing the aquatic biological effects of toxic substances. Recommended methods are those which have been evaluated by the Environmental Protection Service (EPS), and are favoured:

- *for use in Environment Canada and provincial aquatic toxicity laboratories;*
- *for testing which is contracted out by Environment Canada or requested from outside agencies or industry;*
- *in the absence of more specific instructions, such as are contained in regulations; and*
- *as a foundation for the provision of very explicit instructions as may be required in a regulatory program or standard reference method.*

The different types of tests included in this series were selected on the basis of their acceptability for the needs of programs for environmental protection and conservation carried out by Environment Canada. These documents are intended to provide guidance and to facilitate the use of consistent, appropriate, and comprehensive procedures for obtaining data on toxic effects of samples of chemical, effluent, leachate, elutriate, receiving water, and sediment or similar solid.

Mention of trade names in this document does not constitute endorsement by Environment Canada; other products with similar value are available.

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List of Abbreviations and Chemical Formulae

ATP . . . . . adenosine triphosphate
°C . . . . . degree(s) Celsius
cm . . . . . centimetre(s)
d . . . . . day
DO . . . . . dissolved oxygen (concentration)
EC50 . . . . . median effective concentration
g . . . . . gram
g/kg . . . . . grams per kilogram
h . . . . . hour(s)
HCl . . . . . hydrochloric acid
H_2O . . . . . water
ICp . . . . . inhibiting concentration for a (specified) percent effect
IC50 . . . . . 50% inhibiting concentration
L . . . . . litre(s)
LC50 . . . . . median lethal concentration
mg . . . . . milligram(s)
min. . . . . minute(s)
mL . . . . . millilitre(s)
mm . . . . . millimetre(s)
MOAS . . . . . Microtox Osmotic Adjustment Solution
mS . . . . . millisiemen(s)
N . . . . . normal
NaCl . . . . . sodium chloride
NaOH . . . . . sodium hydroxide
Recon . . . . . Reconstitution Solution
SD . . . . . standard deviation
sp. . . . . species
TIE . . . . . toxicity identification evaluation
TM (™) . . . . . trade mark
µg . . . . . microgram
µL . . . . . microlitre
> . . . . . greater than
< . . . . . less than
≥ . . . . . greater than or equal to
≤ . . . . . less than or equal to
± . . . . . plus or minus
% . . . . . percentage or percent

Terminology

Note: all definitions have been given in the context of the procedures in this report, and might not be appropriate in another context.

Grammatical Terms

Must is used to express an absolute requirement.

Should is used to state that the specified condition or procedure is recommended and ought to be met if possible.

May is used to mean "is (are) allowed to".

Can is used to mean "is (are) able to".

Might is used to express the possibility that something could exist or happen.

General Technical Terms

Bioluminescence is a phenomenon of light emitted from living organisms as a result of their biochemical activities, usually enzymatic.

Compliance means in accordance with governmental permitting or regulatory requirements.

Conductivity is a numerical expression of the ability of an aqueous solution to carry an electric current. This ability depends on the concentrations of ions in a solution, their valence and mobility, and on the solution's temperature. Conductivity in fresh waters is normally reported in the SI unit of millisiemens/metre, or as micromhos/centimetre (1 mS/m = 10 μmhos/cm). Conductivity is a standard method for measuring salinity *(q.v.)*, with a result which is usually read off as g/kg or "parts per thousand".

Dispersant is a chemical substance which reduces the surface tension between water and a hydrophobic substance (e.g., oil), thereby facilitating the dispersal of the hydrophobic material throughout the water as an emulsion.

Emulsifier means a substance that aids the fine mixing (in the form of small droplets) within water, of an otherwise hydrophobic substance.

Flocculation is the formation of a light, loose agglomeration of particles (i.e., a floc) from a solution.

Hardness is the concentration of cations in water that will react with a sodium soap to precipitate an insoluble residue. In general, hardness is a measure of the concentration of calcium and magnesium ions in water, and is expressed as mg/L calcium carbonate or equivalent.

Luminescent means emitting light, from a cause other than high temperature.

Lyophilized means freeze-dried under a vacuum, and is applied to the bacteria used in the Microtox test, as received from the supplier.

Monitoring is the routine (e.g., daily, weekly, monthly, quarterly) checking of quality or collection and reporting of information. In the context of this report, it means either the periodic (routine) checking and measurement of certain biological or water-quality variables, or the collection and testing of samples of effluent, leachate, elutriate, or marine/estuarine receiving water for toxicity.

Percentage (%) is a concentration expressed in parts per hundred parts. One percent represents one unit or part of material (e.g., effluent, elutriate, leachate, or receiving water) diluted with water to a total of 100 parts. Concentrations can be prepared on a volume-to-volume or weight-to-weight basis, and are expressed as the percentage of test substance in the final solution.

pH is the negative logarithm of the activity of hydrogen ions in gram equivalents per litre. The pH value expresses the degree or intensity of both acidic and alkaline reactions on a scale from 0 to 14, with 7 representing neutrality, numbers less then 7 signifying increasingly greater acidic reactions, and numbers greater than 7 indicating increasingly basic or alkaline reactions.

Precipitation means the formation of a solid (i.e., precipitate) which comes from a solution.

Pre-treatment means, in this report, treatment of a sample or diluting it, prior to testing its toxicity.

Salinity is the total amount of solid material, in grams, dissolved in 1 kg of aqueous solution. For seawater, salinity is determined after all carbonates have been converted to oxides, all bromide and iodide have been replaced by chloride, and all organic matter has been oxidized. Salinity can also be measured directly using a salinity/conductivity meter or other means (see APHA *et al.*, 1989). Salinity is reported as a percentage, to agree with Microtox manuals. The normal unit would be g/kg, or the approximate equivalent of that, parts per thousand (‰).

Surfactant is a surface-active substance (e.g., detergent) which, when added to a non-aqueous liquid, decreases surface tension and facilitates dispersion of materials in water.

Turbidity is the extent to which the clarity of water has been reduced by the presence of suspended or other matter that causes light to be scattered and absorbed rather than transmitted in straight lines through the sample. It is generally expressed in terms of Nephelometric Turbidity Units.

Terms for Test Substances

Blank is used interchangeably with the term control *(q.v.)*, in this document.

Chemical is, in this report, any element, compound, formulation, or mixture of a substance that may enter the environment through spillage, application, or discharge. Examples of chemicals that are applied to the aquatic environment are insecticides, herbicides, fungicides, sea lamprey larvicides, and agents for treating oil spills.

Control is a treatment in an investigation or study that duplicates all the conditions and factors that might affect the results of the investigation, except the specific condition that is being studied. In an aquatic toxicity test, the control must duplicate all the conditions of the exposure treatment(s), but must contain no test substance. The control is used to determine the absence of measurable toxicity due to basic test conditions (e.g., quality of the dilution water, health of test organisms, or effects due to their handling).

Deionized water is water that has been purified to remove ions from solution by passing it through resin columns or a reverse osmosis system.

Diluent is the standard water used for dilution of test substance in the Microtox test; see also *dilution water*.

Dilution water is the water used to dilute a test substance in order to prepare different concentrations for a toxicity test. The standard dilution water used in the Microtox test is a specific formulation of saline water called *Diluent*.

Distilled water is water that has been passed through a distillation apparatus of borosilicate glass or other material, to remove impurities.

Effluent is any liquid waste (e.g., industrial, municipal) discharged to the aquatic environment.

Elutriate is an aqueous solution obtained after adding water to a solid substance (e.g., sediment, tailings, drilling mud, dredge spoil), shaking the mixture, then centrifuging or filtering it or decanting the supernatant.

Estuarine water is brackish seawater, residing in a coastal body of ocean water that is measurably diluted with fresh water derived from land drainage.

Pore water is the water occupying space between sediment particles. The amount of pore water is expressed as a percentage of the wet sediment, by weight.

Leachate is water or wastewater that has percolated through a column of soil or solid waste within the environment.

Marine water is seawater residing in or obtained from the ocean, sea, or inshore location where there is no appreciable dilution by natural fresh water derived from land drainage.

Receiving water is surface water (e.g., in a river) that has received a discharged waste, or else is about to receive such a waste (e.g., it is just “upstream” or up-current from the discharge point). Further descriptive information must be provided to indicate which meaning is intended.

Reference toxicant is a standard chemical used to measure the sensitivity of the test organisms in order to establish confidence in the toxicity data obtained for a test substance. In most instances a toxicity test with a reference toxicant is performed to assess the sensitivity of the organisms at the time the test substance is evaluated, and the precision of results obtained by the laboratory for that chemical.

Sediment is natural particulate substance that has been transported to, and deposited at the bottom of, a body of water. [In certain sections of this report, "sediment" is designated as a term of convenience which includes similar substances such as industrial and municipal sludges, and soils.]

Stock solution is a concentrated aqueous solution of the substance to be tested. Measured volumes of a stock solution are added to dilution water in order to prepare the required strengths of test solutions.

Substance is a particular kind of material having more or less uniform properties.

Upstream water is surface water (e.g., in a stream, river, lake, estuary, or marine water body) that is not influenced by the effluent (or other test substance), by virtue of being removed from it in a direction against the current or sufficiently far across the current.

Wastewater is a general term that includes effluents, leachates, and elutriates.

Toxicity Terms

Acute means within a short period in relation to the life span of the organism, and would be of the order of some minutes for bacteria.

EC50 is the median effective concentration, i.e., the concentration of substance in water that is estimated to cause a specified effect in 50% of the individuals exposed to that concentration. The effect could be lethal but is usually sublethal. EC50, like LC50, refers to a quantal effect since each exposed individual must be categorized as either showing the effect or not showing it. The effect must be specified, and often also the exposure time, for example "the two-month EC50 for reproductive failure" or "the EC50 for avoidance reactions". The term does not apply to a percent reduction in some rate of process in an organism or a group of organisms; accordingly, this terminology should not be used in the luminescent bacteria inhibition test (see ICp).

Endpoint means the variables (i.e., time, reaction of the organisms, etc.) that indicate the termination of a test, and also means the measurement(s) or value(s) derived, that characterize the results of the test (lethal concentration, IC50, etc.).

ICp is the inhibiting concentration for a (specified) percent effect. It represents a point estimate of the concentration of test substance that would cause a designated percent impairment in a quantitative biological function such as light production by bacteria or growth of fish, relative to the control. This term should be used for any toxicological test which measures a change in rate, such as reproduction, growth or respiration. (The term median effective concentration (EC50) is not appropriate in tests of this kind because it is limited to quantal measurements, i.e., an estimate that 50% of the individual organisms which were exposed to that concentration would show a particular effect, while the other 50% would not show the effect.)

LC50 is the medial lethal concentration, i.e., the concentration of substance in water that is estimated to be lethal to 50% of the test organisms exposed to that concentration. The LC50 and its 95% confidence limits are usually derived by statistical analysis of mortalities in several test

concentrations, after a fixed period of exposure. The duration of exposure must be specified (e.g., 7-d LC50). This endpoint cannot be used in the luminescent bacteria inhibition test.

Lethal means causing death by direct action. Death is usually defined as the cessation of all visible signs of movement or other activity.

Static describes toxicity tests in which test solutions are not renewed during the test.

Sublethal means detrimental to a living organism, but below the level which directly causes death within the test period.

Toxicity is the inherent potential or capacity of a substance to cause adverse effects on living organisms. The effect could be lethal or sublethal.

Toxicity Identification Evaluation describes a systematic sample pre-treatment (e.g., pH adjustment, filtration, aeration) followed by tests for toxicity. This evaluation is used to identify the causative agent(s) which are primarily responsible for toxicity in a complex mixture. The toxicity test can be lethal or sublethal.

Toxicity test is a determination of the effect of a substance on a group of selected organisms, under defined conditions. As aquatic toxicity test usually measures either (a) the proportions of organisms affected *(quantal)*, or (b) the degree of effect shown *(graded or quantitative)*, after exposure to specific concentrations of chemical, wastewater, receiving water, or liquid derived from sediment or similar solid substance. The assay with luminescent bacteria must be considered a graded toxicity test since there is no measurement of the proportions of individual bacteria that are directly affected, but instead, overall measurements of the degrees of reduction in a physiological function, shown by groups of bacteria.

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Members of the Inter-Governmental Aquatic Toxicity Group (IGATG, Appendix A) are thanked for their active participation in developing and reviewing this report. Special acknowledgement is made of the technical contributions from the IGATG subcommittee members responsible for initial and final review: G. Joubert (Québec Ministère de l'Environnement, Ste-Foy, Que.); M. Korchinski (Alberta Energy Resources Conservation Board, Calgary, Alta.); A.A. Qureshi (Alberta Environmental Centre, Vegreville, Alta.); and IGATG members C. Blaise, B.J. Dutka, G. Elliot, and G.C. van Aggelen.. The laboratory testing support of Environment Canada (Appendix B) is also acknowledged. Mr. P.C. Thomas of the former Microbics Enterprises (Forest, Ont.) generously provided technical literature and loan of equipment for trial.

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Introduction

1.1 Background

Aquatic toxicity tests are used within Canada and elsewhere to measure, predict, and control the discharge of substances that might be harmful to aquatic life. Recognizing that no single test method or test organism can be expected to satisfy a comprehensive approach to environmental conservation and protection, the Inter-Governmental Aquatic Toxicity Group (members in Appendix A) proposed the development and standardization of a set of single species aquatic toxicity tests that would be broadly acceptable, and would measure different toxic effects using organisms representing different trophic levels and taxonomic groups (Sergy, 1987). A toxicity test with luminescent bacteria was one of several "core" aquatic toxicity tests that were selected to help meet Environment Canada's testing requirements.[1]

Universal procedures for an assay with luminescent bacteria are described in this report. Also presented are specific sets of test conditions and procedures, required or recommended when using the test for evaluating different types of substances (namely samples of chemical, effluent, leachate, elutriate, receiving water, sediment, or other solid substance) (see Figure 1). Those specific procedures and conditions, relevant to conducting the test and standardizing it, are delineated and, as appropriate, discussed in explanatory footnotes.

Although this assay can be generically described as a test of light production by a strain of bacteria, methods described herein are for the only test system commercially available within Canada at the time of this writing[2], the *Microtox*™ test. The test method described here is the exclusive property of Strategic Diagnostics Inc. (formerly *Microbics* Corp. and AZURE Environmental Corp.) of Carlsbad, California *(SDI)*.

SDI provides explicit instructions for conducting the test, and this report does not replace those instructions to facilitate orientation and guidance, and provide a supplement. The test procedures available from *SDI* and from governmental or international agencies do not necessarily address all issues that are important in Canadian government aquatic toxicity laboratories. They do not necessarily cover methods for handling different kinds of samples, the question of pH adjustment, variations in methods associated with differing test objectives or types of samples, or how to deal with samples which contain appreciable solids or floating material. Existing methodology documents usually include procedures for testing samples of effluent or chemical, but sometimes provide only minimal guidance for testing samples of leachate, elutriate, receiving water and sediment or similar solid.

In describing these procedures, an attempt was made to balance scientific, practical, and financial considerations, and to ensure that the results would be accurate and precise enough for the majority of situations in which they will be applied. The authors assume that the user has a certain degree of familiarity with aquatic toxicity tests. Explicit instructions on every detail are not provided in this report, since those can be obtained from the Microtox manuals.

[1] Methods for five tests with fish and crustaceans have already been published as the result of the IGATG proposal (Environment Canada, 1990a; 1990b; 1990c; 1992a; 1992b).

[2] A similar, competitive method *(Lumistox™)* and products are now being marketed in Europe.

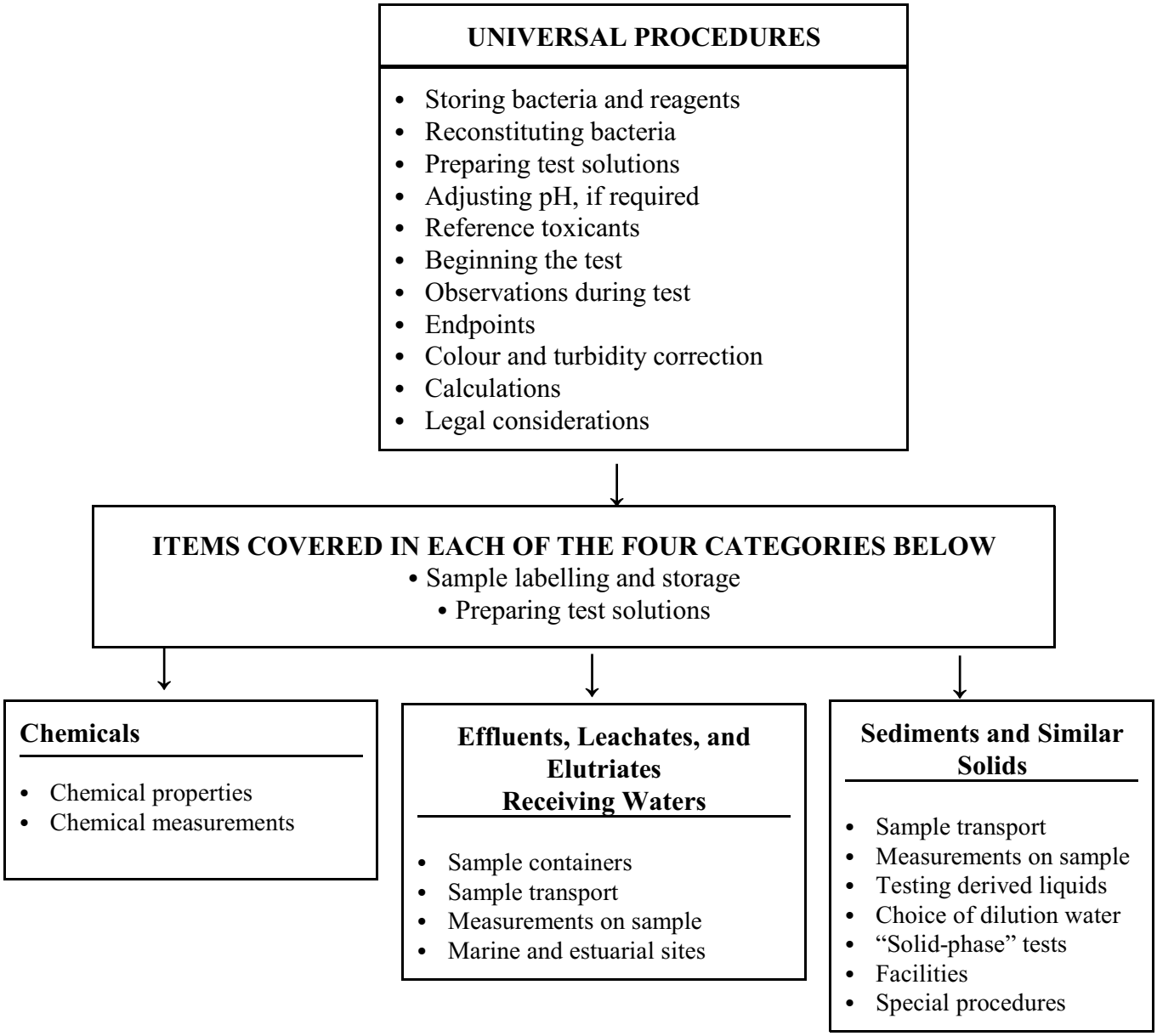

Figure 1 Diagram of Approach Taken in Delineating Test Conditions and Procedures Appropriate to Various Types of Substances

1.2 The Test and Test Species

A specific strain of the marine bacterium *Vibrio fischeri*[3] is used in this test to determine the toxicity of samples. This bacterium emits light as the result of normal metabolic processes, and the light is measured with a standard photodetection device under specific conditions. Reduction of light at 5, 15, or 30 minutes is taken as a measure of toxicity.

The Microtox toxicity test was developed commercially and first offered for sale in 1978. It is now marketed and used around the world, and there is extensive scientific literature on the test and the results of using it (Bulich, 1986; and Microbics, 1989a). A toxicological data bank of appreciable magnitude has been assembled for the Microtox tests by Canadian scientists Kaiser and Ribo (1988). The bacterial bioluminescence test is not limited to aquatic pollutants. For example, it is being considered by the United States Pharmacopeia for testing materials used as containers for drugs and medical devices (USP, 1989).

The background of previous toxicity data for this test, its demonstrated sensitivity to aquatic contaminants, and its widespread availability as a standardized procedure make it a logical choice as one tool for tests in Canadian laboratories.[4] It has been compared with 50 other microscale tests (Munkittrick and Power, 1989), and described as "very useful as field microassay for screening purposes", with a high rating for "environmental relevance" (defined as similarity to whole-organism effects on fish and crustaceans). At least one province has adopted a standard Microtox procedure (BNQ, 1987), and other provinces have informal documents outlining procedures (Subsection 1.2.1). Microtox has been specified for three-times-a-week effluent monitoring at certain pulp mills in British Columbia.

The test is particularly useful for exploration or monitoring because it is rapid, simple, uses small samples, and is inexpensive once the photometer *(Analyzer)*[5] has been purchased. It is suitable for assessing the short-term toxicity of industrial or municipal effluents, leachates, elutriates, mixing zones of surface waters, chemicals, toxicants released from sediments or soils, and in fact, materials that enter water from a variety of sources. Microtox can also directly test samples of sediment or other semi-solid substance such as municipal or industrial sludge, and has been recommended for testing soils at contaminated waste sites (USEPA, 1987; 1989c). It could be used to assess the progress of detoxification or biodegradation of toxic substances.

The test is convenient since there is no need to maintain a living culture; bacteria are purchased in a freeze-dried state and can be stored for months as if they were a chemical reagent. This characteristic makes Microtox convenient for on-site comparisons of toxicity over time for a given industry or location. The rapidity of testing and ability to carry out many assays with little additional expense facilitates broad screening programs for toxicity of effluents or chemicals, toxic components in a complex waste, or other sizeable investigations. For example, it has been

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[3] There are many strains of this bacterium, and at least some of them are known to differ in their patterns of sensitivity to toxicants. The strain designated by *SDI* for use in their test is "NRRL B-11177" deposited with the Northern Regional Research Laboratory in Peoria, Illinois, USA.

[4] Use of the test does not indicate endorsement by Environment Canada or any of its laboratories.

[5] This report has adopted most of the terminology used by *SDI* for reagents, apparatus, and processes, to allows the user to work more easily between this report and the *SDI* manuals. Some terms can have different connotations in ordinary usage. To facilitate use in this report, specific terminology of *SDI* is printed in italics with the initial letter(s) in upper case.

used for surveys of large sections of the St. Lawrence River (Kaiser *et al.,* 1988a).

The sensitivity of this test is, in general, similar to that of acute lethality tests using fish (reviewed by Munkittrick and Power, 1989; Munkittrick *et al.,* 1991). Compared to lethality tests that used fathead minnows, trout, and *Daphnia*, Microtox was about as sensitive to pure organic compounds, municipal wastes, and the more toxic industrial effluents, but was often less sensitive to inorganic toxicants and pesticides (Munkittrick and Power, 1989; Munkittrick *et al.,* 1991).

Microtox can be appreciably more or less sensitive than a particular species of fish or other multi-cellular organism to any given toxic substance, with hundred-fold differences one way or the other in some cases (Munkittrick *et al.,* 1991). This is not necessarily a disadvantage since micro-organisms are sometimes among the most sensitive species in aquatic ecosystems, and it has been recommended that they should always be included in toxicity evaluations (Sloof *et al.,* 1983). This recommendation is in keeping with the excellent principle of using a battery of toxicity tests. For some toxicants, an invertebrate or micro-organism might be most sensitive, and for other toxicants a fish or plant might be most sensitive. Therefore, to protect an aquatic ecosystem, toxicity information should be available for a variety of organisms. The corollary is that one type of organism should not be expected to predict sensitivity of another type of organism. It should not be expected that Microtox will necessarily predict the results of a test with trout, nor that the trout test will predict the result from Microtox. Rather, any such lack of correlation should be regarded as further evidence of the desirability of testing a variety of organisms.

Vibrio fischeri is a marine organism, and the Microtox test is normally carried out a 2% salinity by adding a salinity-adjusting solution (or NaCl) to the sample and using a dilution water *(Diluent)* that has a salinity of 2%. Light production of this bacterium is as high at 2% salinity as in full-strength seawater within that range, with a peak at 2.7 % (Krebs, 1983). Accordingly, samples of full-strength seawater may be tested if desired, but it is important to carry out the test at $\geq$2% salinity.

It is conceivable that the toxicity of a given substance might be different in fresh and salt water, and that Microtox results might therefore be less suitable for protecting fresh waters. Most of the common toxicants, however, are of similar toxicity to marine and freshwater organisms when each is tested in its own medium (Sprague, 1985). Experience has shown that Microtox generally gives toxicity results similar to those for acute lethality to freshwater organisms (Blaise *et al.,* 1987; Munkittrick *et al.,* 1991), and the test is accordingly suitable as one tool for investigating freshwater situations. An option for adjusting osmotic strength of solutions with sucrose instead of salts might have relevance to freshwater situations (see Subsection 4.8.4).

Within Canada, the source of procedural manuals as well as the specific strain of bacterium, equipment, and supplies for the Microtox test is Strategic Diagnostics Inc.[6] Earlier manuals prepared by *Microbics* are included in the reference list (Microbics, 1988a; 1988b; 1989b). *Microbics* produced a comprehensive and up-to-date manual (Microbics, 1992). An older "operating manual" (Beckman, 1982) provides useful detail on some aspects of testing. Various "Microtox application notes" are listed in the reference section under *Microbics* (1983), and

[6] The toll-free phone and fax numbers to reach *SDI* from Canada are: 1-800-544-8881 and 1-302-456-6782, respectively. Strategic Diagnostics Inc., 2232 Rutherford Road, Carlsbad, California 92008-8883, USA. Mention of commercial products and their suppliers does not imply endorsement by Environment Canada or any of its laboratories. This information is provided for the convenience of the reader.

some particular applications are included in general manuals (Microbics, 1989b). These deal with specific topics such as a rapid screening method, complex effluents, and groundwater. A training video is available from *SDI*, and provides useful orientation for conducting the assay. A bibliography lists scientific projects that have used or evaluated the Microtox test (Microbics, 1989a).

Quebec has an official Microtox method (BNQ, 1987). Methods guides have been produced by two branches of the Alberta government, i.e., the Energy Resource Conservation Board (Alberta, 1986) , and Alberta Environment (Alberta, 1987). In Alberta, Microtox is routinely used to test extracts of hydrocarbons, drilling fluids, soils, and sediments, as well as chemicals and water. An informal "Western Canada Microtox Users Committee" has conducted round-robin testing and facilitated standard approaches. The B.C. Ministry of Environment has an in-house guidance document for tests. A procedural guide has also been compiled by Environment Canada (Dutka, 1988). A brief description of a standard operating procedure has been prepared by a testing centre of the U.S. Environmental Protection Agency (USEPA, 1989b). In Germany, a draft standard method (DIN, 1989) gives generic instructions for a photometer and for making up solutions. The German instructions parallel the Microtox method without naming it or Strategic Diagnostics Inc., but mention that the freeze-dried bacteria are commercially available. All of these documents have been used in preparing this report and, in general, all of them describe the same method.

Test Organisms

2.1 Species

Organisms used in this test come from a standardized culture, and belong to a particular strain of *Vibrio fischeri* (a strain designated *NRRL B-11177*). This is a bacterium which normally lives in the oceans, and produces blue-green light by enzymatic reactions, on a continual basis if sufficient oxygen is available.

2.2 Source and Holding

The standard culture of organisms is obtained from *SDI*. According to *SDI*, the bacterium is cultured as a genetically uniform strain, then harvested during the exponential phase of growth and lyophilized (i.e., freeze-dried under vacuum). Bacteria are purchased in that state, in small lots in sealed containers, each lot suitable for at least two hours of testing after they have been reconstituted to an active state.[7]

The container of lyophilized *Bacterial Reagent* is said to be stable for one year when kept in a freezer at -20 °C (Microbics, 1989b). Storage can be "extended somewhat" at -40 °C, while storage in a refrigerator at 4 °C "greatly shortens shelf-life" (Microbics, 1992). Storage temperature should be constant, and therefore self-defrosting or "frost-free" refrigerators and freezers should not be used.

The bacteria are brought back to an active, living state *(Reconstituted Reagent)* by adding liquid *(Reconstitution Solution or Recon)* and bringing them to a temperature of 5 °C.

[7] See Subsection 4.3.1 for further information on useful working time for the *Reconstituted Bacteria*.

Test System

3.1 Principles of Test and Variability

Subsamples of *Reconstituted Bacteria* are exposed to concentrations of the sample. About a million bacteria are introduced into each test vessel. Any toxic action of substances in the sample is presumed to affect metabolic processes of the bacteria, and bioluminescence is inhibited in proportion to the metabolic effect. That inhibition is measured and expressed as the IC50 (concentration causing 50% inhibition[8]) after specific periods of exposure (some or all of 5, 15, and 30 minutes).

3.1.1 Limits and Reproducibility

All manipulations of the sample and the bacteria are manual, and depend on the skill of the operator in reconstituting the bacteria, handling the micropipettor, mixing solutions, etc. Variability in volumes transferred by a good operator might contribute about 1% uncertainty in light readings. Geometrical variations in cuvettes contribute another 1% (Beckman, 1982).

Tests by the Quebec Ministry of Environment (BNQ, 1987) involving 236 results indicated that 17% and 83% inhibition of light production represented the minimum and maximum values that could be quantified with statistical significance. The detection limit in the same work was 12% inhibition.

Variability of Microtox is low compared to other tests of aquatic toxicity. In a series of 81 tests with a reference toxicant (sodium lauryl sulphate), the overall coefficient of variation was 18% (Bulich *et al.,* 1981).[9] Within the three lots of bacteria used in the tests, the individual coefficients of variation were from 6 to 10%. Although three people carried out the tests on three different *Analyzers*, this did not lead to significant differences in results. An overall mean deviation of replicates from the mean IC50 for eight organic chemicals was 10% (Curtis *et al.*, 1982). Tests done for the Canadian Petroleum Association by three laboratories showed an average coefficient of variation of 11 % (Strosher, 1984). Munkittrick and Powers (1989) summarize these comparisons and others by listing average coefficients of variation that range from 2 to 30%, except for metal tests which had an average coefficient of 60%.

3.1.2 Interference and Other Limitations

High colour of a sample, particularly red or brown, can interfere with light transmission and therefore with toxicity measurements. This could be important for some effluents such as those from pulp mills. A correction for this may be made by calculation after performing an additional test using a *Colour Correction Cuvette*, as described in Section 4.9.

Turbidity resulting from suspended solids can also reduce light transmission and increase the toxicity estimate. The colour-correction method previously mentioned may be used with dark, optically absorbing particles, but is not effective if used with white, optically reflecting particles. Centrifugation will sometimes remedy the turbidity problem (see Subsection 4.1.1).

The highest concentration of sample tested in the

[8] Throughout the literature from *SDI*, generally in U.S. scientific literature, and often in Canadian work, the IC50 has been wrongly called the EC50 (see Terminology). This will no doubt be corrected in the future, as the correct terminology has been recognized by ASTM and is being promulgated by that organization for appropriate tests.

[9] C. Bastien of the Quebec Ministry of Environment reports similar coefficients of variation of 15 to 20% during four years of testing with this same reference toxicant.

standard Microtox technique is 45% (see Subsection 4.2.2). If the sample is weakly toxic, an investigator might wish to test a nearly full-strength sample. This can be done by using the special technique described in Subsection 4.8.3.

Exposure times are somewhat arbitrary as in all toxicity tests, and appropriate ones must be adopted by judgement. Most Microtox tests are completed in 15 minutes. Phenol completes its action within five minutes, so the best measure of its toxicity is the 5-min IC50. For bivalent metals, the light output might still be decreasing after 15 minutes, and the IC50 at 30 minutes or longer will be most sensitive. For unknown samples, therefore, readings should be taken at several standard times, and the most appropriate one(s) chosen. The best exposure would be the one when light inhibition levelled off, or just after that, i.e., allowing just enough time for the toxicant to exert its maximum action.

3.2 Facilities

The tests can be conducted in a normal, clean laboratory with standard lighting. The need for any special facilities would be governed by the degree of hazard associated with the samples or chemicals that were to be tested, and by the risk of sample contamination.

3.2.1 Photometer

Light production could be measured using a variety of photometers, such as a standard photometer connected to a water bath (Dearborn, 1986), or an adenosine triphosphate (ATP) photometer as evaluated by Awong *et al.* (1989). The *Microbics Toxicity Analyzers* Model 2055 and 500 are, however, conveniently designed to carry out the test with dispatch under controlled conditions. Use of those machines is described herein, and their general appearance is shown in Figure 2 (Model 2055 Analyzer) and Figure 4 in Section 5 (Model 500 Analyzer). Methods in general, as well as particular methods for the Model 2055 Analyzer are described in Section 4. Because many of the operations are performed manually with the Model 2055, Section 4 provides an awareness of the steps in an analysis. For the newer Model 500, many of the steps are carried out automatically. The methods for this machine are described in Section 5.

Using the Toxicity Analyzer Model 2055, light output of the bacteria is measured by a photomultiplier tube which has two ranges, 1X (no enhancement) or 10X (tenfold enhancement). The *Analyzer* is more than a photometer since it holds all the cuvettes for a test and keeps the contents at a pre-selected temperature. Circuits are built in to allow temperature readout from areas that contain test solutions and bacteria.

The layout of the top working surface of the Model 2055 *Toxicity Analyzer*, with which an operator must be familiar, is shown in the lower part of Figure 2. The fifteen numbered circles A1 to C5 are the incubator wells which can incubate cuvettes of sample and control solutions at 15 ± 0.3 °C. The *Precooling Well* holds the *Reconstitution Solution* and bacteria at 3 to 5 °C. The *Turret Well* is where the reading of light production takes place; cuvettes must be cycled in turn to that well.

3.2.2 Other Equipment

Other equipment necessary to perform these tests include:

(a) **Freezer or refrigerator** for storage of reagents. A freezer should maintain its contents at -20 °C. A refrigerator should be capable of regulation between 2 °C and 8 °C. Neither machine should be self-defrosting.

(b) **pH meter.**

(c) **Count-down timer or stopwatch.** (Alternatively, the chart recorder may be used as a timer – an easier and more fail-safe procedure.)

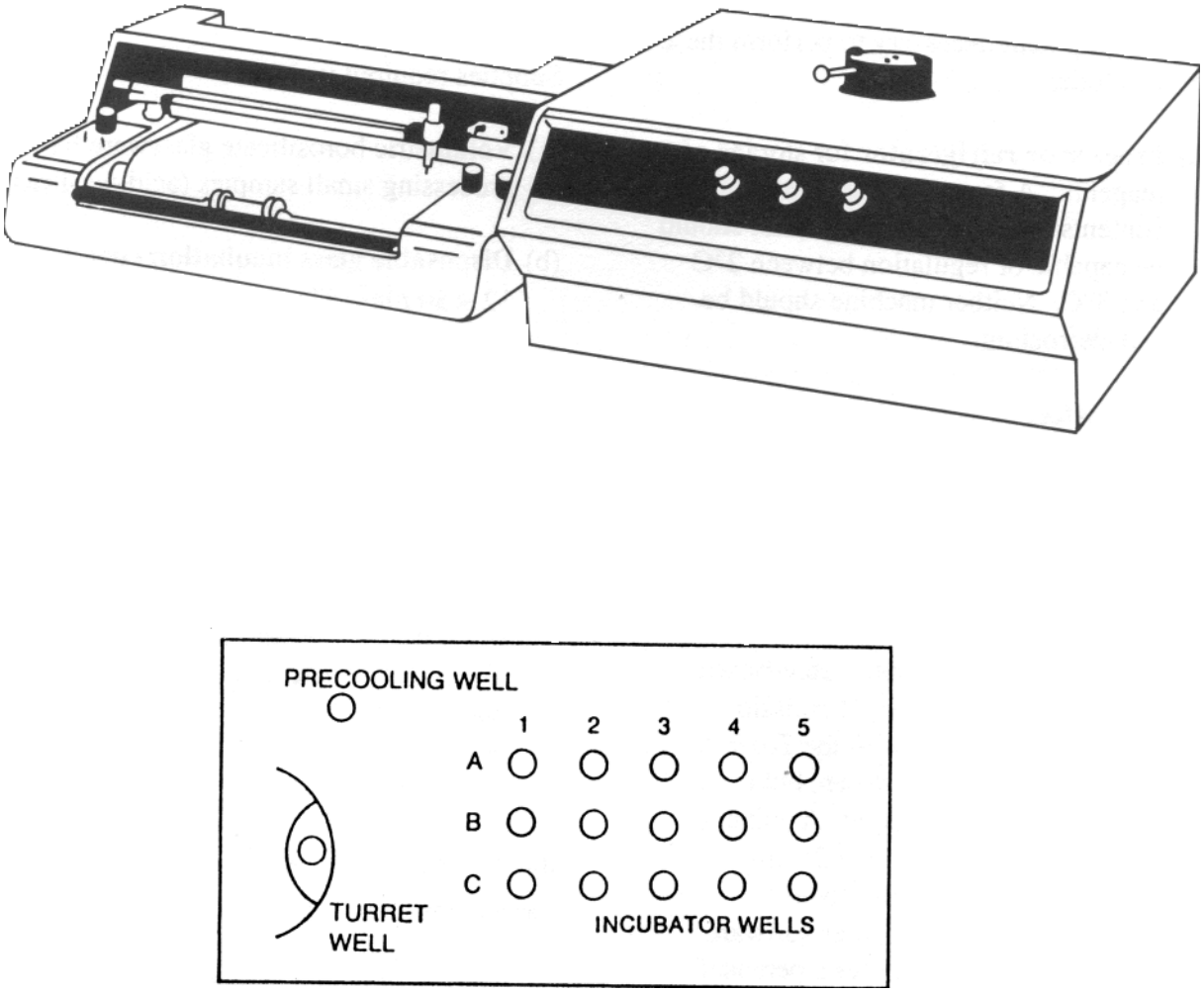

Figure 2 **Appearance of the *Microbics Toxicity Analyzer* Model 2055 and Chart Recorder.** The upper part of the figure shows the *Toxicity Analyzer* on the right, with the "turret" protruding from the top, and a chart recorder on the left. The lower part of the figure shows the wells used to contain the test cuvettes, on the top surface of the *Toxicity Analyzer.*

(d) **Chart recorder or computer-based recording system**. Light emission values may be read from the *Toxicity Analyzer* and written down, but a recorder is advantageous. A suitable chart recorder would be a 25-cm, single-pen, manual-lift model.[10] *SDI* offers computer software, which allows a personal computer to collect data directly. The software and computer replace the recorder with the newer Model 500 *Analyzer* (see Section 5), and with Model 2055 *Analyzers* which are equipped with the necessary interface.

(e) **Calculator or computer.** Results from the test can be conveniently processed in BASIC on an IBM-compatible personal computer. The software is available from *SDI.* Data can also be processed with a Sharp Model EL-5150 programmable calculator, a device which is no longer sold.

3.2.3 Supplies

Supplies required include:

(a) **Volumetric borosilicate glassware** for processing small samples (acid-washed).

(b) **Disposable glass incubation cuvettes**, 12 × 50 mm. These are the containers used as test vessels for the various dilutions in the *Analyzer*.

(c) ***Colour Correction Cuvette***. A reusable double cuvette used for correcting readings with coloured or turbid samples.

(d) **Micropipettors** of 10, 200, or 250, 500, and if desired, 50 and 1000 µL, with disposable plastic tips. The micropipettors are also used for mixing liquids in cuvettes since they aspirate as well as dispense accurately.

[10] *SDI* offers a compatible chart recorder, Part No. 686008, and previously recommended a Beckman Model 2055 as a match for their Model 2055 *Analyzer.*

3.2.4 Organisms and Reagents

Organisms and reagents required include:

(a) **Supply of bacteria** (*Microtox Reagent* or more recently *Bacterial Reagent*). *Vibrio fisheri* are obtained from *SDI* as small, sealed containers of about 100 million lyophilized organisms. Storage should be in a freezer or a refrigerator, the former being suitable for at least a year of storage, the latter for an unspecified but much shorter time. After reactivation of the bacteria, *SDI* used the name *Reconstituted Reagent*.

(b) ***Reconstitution Solution (Recon)*** is distilled water, free of toxic material (Microbics, 1989b); used to reconstitute the bacteria to their active living state at the start of a test. It is supplied in sealed containers by *SDI*, must not be frozen, and may be stored for a year at a temperature between 2 and 8 °C, or at room temperature for an unspecified time period.

(c) ***Diluent*** is used to dilute the samples to desired concentrations; it is supplied by *SDI,* and contains 2 % sodium chloride in purified water; storage requirements are the same as for *Recon.* A special Solid-Phase diluent is used in the "solid-phase" test (see Subsection 9.3.1).

(d) ***Microtox Osmotic Adjustment Solution (MOAS).*** A 22% solution of sodium chloride for increasing salinity of samples to the desired level. The assay is normally run at 2 % salinity, so adjusting a freshwater sample is done by adding one part of *MOAS* to 10 parts of *sample*. Storage requirements are the same as for *Recon*.

(e) **Sodium chloride** (NaCl, analytical grade, 99%). This may be used, if desired, for adjusting a sample to the desired salinity for testing.

Universal Test Procedures

Procedures described in this section are for use with the Microtox *Analyzer* Model 2055. Many of the concepts and general procedures for the Model 2055 also apply to tests with the newer Model 500 *Analyzer*, made available by *SDI* in early 1989. Details of procedures vary, however, and many of the steps are automated in the newer Model 500 (see Section 5). The procedures described in this section apply to all the tests described in Sections 6, 7, 8, and 9. All aspects of the test system described in the preceding section must be incorporated into these universal test procedures. The summary checklist of recommended conditions and procedures in Table 1 includes not only universal procedures but also those for specific types of test materials.

A brief summary of the procedure follows. A series of dilutions of the sample plus control(s) is tested. Light outputs of the cuvettes of *Reconstituted Bacteria* are measured before the sample (test solution) is added, then again after 5 and 15 minutes of exposure, and perhaps after 30 minutes or longer if there are slow-acting toxic agents. The readings are corrected according to the change in the control(s) (nontoxic *Diluent* only), to allow for drifts in light output over time, and small effects from dilution of the bacteria when sample is added. A dose-effect curve is analyzed, and the concentration causing 50% inhibition of light production is estimated mathematically.

The following outline is organized by category, and does not necessarily represent the most effective chronological sequence for testing. *SDI* instructions give guidance on sequence.

4.1 Interfering Characteristics of Sample

4.1.1 Colour, Turbidity, and Floatables

Check for colour or turbidity of the sample to be tested, as either of these could affect light measurements. It is difficult to specify numerical guidelines for troublesome levels of such variable characteristics as colour or turbidity, but the optional Microtox procedure to correct for these qualities is simple enough to carry out (see Section 4.9) and should be used if the investigator has any doubts about light transmission.

If the turbidity is light-reflecting, the correction procedure given in Section 4.9 is not suitable. The preferred option is to remove the suspended matter causing the turbidity, and test for the residual toxicity in the liquid part of the sample. Filtration might be satisfactory but the particular kind of filters to be used should be checked by a Microtox test on *Diluent* run through the filter. Some filter papers and apparatus can add measurable toxicity, according to Microbics (1988b), sometimes because of wetting agents added to the filter. A filter paper might also sorb toxic substances and remove then from the sample filtrate. Suspended matter can be reduced by centrifugation, or by a few hours of settling.

There is no standard recommendation for dealing with the problem of material floating on top of the sample. It is not possible to put any mixing device into or onto the cuvettes during the test. Attempts to homogenize floating liquids, to last for the duration of the test, could cause suspended droplets that would interfere with light transmission. The matter is left to the judgement of the investigator, and depends on the type of material. Options would be a test of the underlying liquid or an attempt at homogenization, and the former is recommended.

Table 1 Checklist of Recommended Test Conditions and Procedures

Universal		
Test type	–	static, 5 and 15 min duration, to 30 or 60 min if necessary
Species	–	*Vibrio fischeri*, strain NRRL B-11177
Facilities	–	*SDI* photometer, either Model 2055 or automated Model 500
Control/dilution water	–	standard pure water with 2% NaCl *(Diluent)*; optional: sucrose instead of NaCl for increased sensitivity to ammonia and some metals
Temperature	–	15 ± 0.3° C
pH	–	no adjustment if pH of sample is within the range 6.0 to 8.5; adjustment optional outside that range, depending on purpose
Colours, solids, or floatables	–	use colour-correction technique for high colour and/or dark solids; remove light-coloured solids; for floatables, test underlying liquid
Oxygen/aeration	–	normally no aeration of sample or prepared test concentrations; if dissolved oxygen is <40% or >100% saturation, it is optional to pre-aerate the sample or all test solutions for ≤20 min
Preparing test solutions	–	highest concentration normally 45% with lower concentrations in two-fold dilutions; option for testing nearly full-strength sample, or other concentrations in logarithmic series
Observations	–	light production initially, and at 5 and 15 min after introduction of sample; sometimes after 30 min or longer exposures
Endpoints	–	IC50s at 5 and 15 min, and any other appropriate times
Reference toxicant	–	choice of toxicants, tested at monthly intervals and with each new batch of test organisms; phenol, zinc, potassium dichromate, or sodium lauryl sulphate recommended
Test validity	–	valid numerical estimate of ICp should be based on concentrations showing light inhibition both greater than, and less than, the inhibition at the ICp

Chemicals

Solvents	–	these or other solubilizing aids (e.g., dispersants) used only in special cases
Concentration	–	measure concentration in stock solution (desirable, not mandatory)

Effluents, Leachates, and Elutriates

Transport and storage	–	if >7° C, cool to 1 to 7° C (ice or frozen gel packs); transport and storage in dark at 1 to 7° C (preferably 4 ± 2° C); store at 4 ± 2° C in the dark; sample must not freeze; test should start within 24 h, and must start within 72 h of sampling/extraction
Control/dilution water	–	normal *Diluent* as in **Universal**; clean seawater is option for effluents that will discharge to marine location, or for elutriate that utilized seawater

Receiving Waters

Transport and storage	–	as for effluents and leachates
Control/dilution water	–	for fresh surface waters, as in **Universal**; if sample is marine, adjust *Diluent* salinity or use clean seawater; if sample is estuarial, adjust *Diluent* to sample salinity if above 2%

Sediments or Other Solids

Transport and storage	–	temperatures as for effluents and leachates; test should start within two weeks and must start within six weeks
Preparing test substance	–	centrifuge to separate pore water from solids; if desired, test pore water or aqueous extract as for effluent
Reference sediment	–	parallel test with clean sediment of similar physicochemical properties
Facilities	–	Model 500 photometer, or Model 2055 with computer interface
Control/dilution water	–	standard *Solid-Phase Diluent* to test the sediment or other solid substance
Observations	–	as **Universal**, except that exposure is for 25 minutes and light emission of control also serves as “initial value” for all concentrations
Endpoints	–	as **Universal**, IC50 calculated by the solid-phase program of *SDI*

4.1.2 pH

Toxicity tests should normally be carried out without adjustment of pH.[11] The pH of the sample should, however, be measured before testing it. If the sample is likely to cause the pH of any test solution to be outside of the range 6.0 to 8.5[12], and/or it is desired to assess toxic chemicals, rather than the effect of pH itself or the modifying effect of pH on toxicity of substances in the sample, then the pH of an aliquot of the sample should be adjusted before using it.[13] Alternatively, a second (pH-adjusted) test could be conducted concurrently with the unadjusted one.

For an adjusted test, the initial pH of the sample may, depending on objectives, be adjusted to within ± 0.5 pH units of that of *Diluent*, or to within 0.5 units of the pH of natural seawater. Another acceptable approach for an adjusted test is to change the pH of the sample upwards to the range pH 6.0 to 6.5 (if the sample has pH <6.0), or downwards to pH 8.0 to 8.5 (if the sample has pH >8.5).[14] Solutions of HCl or NaOH at strengths ≤ 1 *N* should normally be used for all pH adjustments. Some situations (e.g., effluents with highly buffered pH) might require higher strengths of acid or base, to avoid large changes in volume of the adjusted sample.

Adjustment of pH can cause precipitation of dissolved solids which could influence the readings of light in the tests; such precipitation should be watched for, and attempts made to deal with it if necessary (see Subsection 4.1.1).

Abernethy and Westlake (1989) provide useful guidelines for adjusting pH. Aliquots of samples

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[11] A justification for not changing the pH of the sample or solution is that pH can have a strong influence on the toxicity of a substance being tested, judging by results for testing fish and invertebrates. Toxicity might decrease (e.g., for ammonia if pH moves from alkaline to neutral), or might increase (e.g., for zinc moving from either acid or alkaline water to neutral). For the (generally) low concentrations of waste found in receiving water after dilution, any change in pH caused by the test substance, with concomitant modification of toxicity, could be accepted as "part of the pollution package". That leads to the rational that the pH should not be adjusted in tests.

[12] Microbics (1988a) indicated no loss of light production caused by pH in the range 6.3 to 7.8. That range has been widened in this report for two reasons. First, Krebs (1983) shows a graph with light production affected no more than ± 5% over the pH range 5.5 to 9.0. Second, *Vibrio fischeri* is a marine organism, and the pH of the oceans has an average close to 8.1 (Thurman, 1975), frequently reaches pH 8.3, and has a normal range of 7.5 to 8.5, whether it is brackish or full strength. Since Environment Canada laboratories undoubtedly test samples of seawater that have received wastes, it is necessary to have an upper limit for testing that is pH 8.3 or higher, since that might be natural in the samples. The upper limit has been set slightly higher than the pH 8.3 in this report, with a recommended range of 6.0 to 8.5, selected on the basis of being 0.5 units within the range of little effect found by Krebs (1983). There might sometimes be reasons for adjusting pH within the recommended range for toxicological reasons described in this section.

[13] The rationale for making these adjustments does not contradict the previous rationale of accepting divergent pH as "part of the pollution package", but depends on the purpose of the test. Some chemicals and wastewaters will create levels of pH that have direct sublethal or lethal effects, especially in monitoring or compliance tests with full-strength effluent. An investigator might not be primarily interested in whether extreme pH is toxic, because such a pH might be unlikely after even moderate dilution in receiving water. If pH *per se* were of primary interest, it could be economically assessed by physicochemical measurements. An investigator would often wish to know if toxic substances were present in a wastewater, and detecting them would require elimination of any masking by toxic action of pH. The rationale leads to the use of pH-adjusted samples, in a parallel manner to the standardization of temperature and salinity at favourable levels when testing for toxic substances.

[14] Addition of acid or base to an undiluted sample of effluent, leachate, or elutriate can significantly alter the ionized/non-ionized form of some toxicants (e.g., ammonia, resin acids, zinc) and can destroy the integrity of the test sample. If it is desired to adjust pH, it should be carefully done to just inside the limit specified, in this case 6.0 to 8.5, or within 0.5 units of the pH of *Diluent* or seawater. Overshoot should be avoided, especially "titrating through" pH 7.0 from either direction.

receiving pH-adjustment should be allowed to equilibrate after each incremental addition of acid or base. The amount of time required for equilibration will depend on the buffering capacity of the sample. For effluent samples, a period of 30 to 60 minutes is recommended for pH adjustment (Abernethy and Westlake, 1989).

If the purpose of the toxicity test is to gain an understanding of the nature of the toxicants in an effluent, leachate, elutriate, or receiving-water sample, then pH adjustment is frequently used as one of several techniques (e.g., oxidation, filtration, air stripping, addition of chelating agent, etc.) for characterizing sample toxicity. Mount and Anderson-Carnahan (1988) list pH adjustment as one of nine Toxicity Identification Evaluation (TIE) techniques which, when performed with an acutely toxic aqueous sample, provide the investigator with a useful method for assessing the physicochemical nature of the toxicant(s) and susceptibility to detoxification.

4.1.3 Dissolved Oxygen and Aeration

Normally there is no aeration of samples or of a prepared test solution. This is not considered necessary for the Microtox test.[15] Aeration is an option, however.[16] If (and only if) the measured dissolved oxygen is <40% or >100% of air saturation in the sample or in an aliquot of a test solution when it has been made up to start the test, the sample of all solutions[17] could be aerated before starting ("pre-aeration"). For this purpose, oil-free compressed air should be dispensed through a disposable glass pipette, at a minimal rate for effective aeration of the particular vessel and volume of fluid being used. Duration of pre-aeration should be the lesser of 20 minutes and attaining 40% saturation (or 100% saturation, if supersaturation is evident) in the sample or highest test concentration. Any pre-aeration of test solutions or sample must be reported (Section 10).

4.2 Preparing for the Test

4.2.1 Setting up the Toxicity Analyzer

(a) Check that the temperatures of the *Turret Well* and *Incubator* region are at 15 ± 0.3° C. If not, adjust with the "TEMP SET" dial.

(b) Set the photodetector in the 10X (enhancement) range. Set the *SPAN* (sensitivity) dial to maximum.

(c) Confirm that the high voltage (HV) switch is on. It should be on for a few minutes before step (d).

(d) Confirm that the photodetector output is 000, with the minus sign flashing. If not, set it at 000 with the *ZERO* dial.

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[15] Several Canadian laboratories and *SDI* report that low oxygen, at least any levels as low as 1mg/L, do not appear to affect the results of Microtox tests. Normal handling of samples, and standard dilution by one half with aerated solutions, is generally considered to provide adequate oxygen for the test, even it the sample is deficient.

[16] Many of the biological test methods published by Environment Canada (e.g., 1990a; 1990b; 1990c; 1992a; 1992b) require aeration if there is low oxygen or supersaturation, and the option shown here is similar to the procedure that is a requirement in those tests. The option allows a laboratory to carry out the Microtox test with procedures that are as similar as possible to those which are used in other tests, if that is desired. For example, aeration can strip volatile chemicals from solution, or increase their rate of oxidation and degradation, changing the toxicity. If that were being done in a test with say, rainbow trout, an investigator might wish to carry out the Microtox test in parallel fashion. Alternatively, two Microtox tests might be performed, with and without pre-aeration, to determine the effect of that treatment.

[17] In the Microtox test, there would usually only be one test solution which would be aerated, and that would be the highest concentration intended for addition to a cuvette, or the sample itself. Lower concentrations would be obtained by dilutions in the cuvettes.

(e) Perform a calibration check. Set the *SPAN* at 4, then press the *CAL CHECK* button which turns on a tiny standard light in the *Turret Well*. The display should be in the range 80 to 120, and if so, testing can begin with the dials remaining in their existing positions.

(f) Turn on the recorder with chart speed of 1 cm/min. Adjust pen to zero, and check that the photomultiplier reading of the *Toxicity Analyzer* remains at 000. The recorder can be turned off until the appropriate time.

(g) Put new cuvettes in each of the wells that will be used. All wells should contain cuvettes; those from a previous test can be left in wells not required for the current test.

(h) If any of the above readings fall outside the specified range, consult the Microtox manual for remedial action.

4.2.2 Preparing the Test Solutions

Most of the vessels and measuring tools used in the test are disposable and are assumed to be clean and ready for use when obtained (e.g., cuvettes and tips for pipettors). Some additional measurement devices, stirring equipment, etc. might be required for handling the sample, and they must be thoroughly cleaned and rinsed in accordance with standard operational procedures. Washing of glassware has been known to add toxicity in the Microtox test, so rinsing must be thorough and it is recommended that a direct check be made for effect of any washing technique on results of the test.

For test intended to estimate an IC50, at least four test concentrations plus a control solution (100% *Diluent*) are to be prepared. Four concentrations must be considered a minimum with which to generate an adequate dose-response relationship, especially if the general degree of toxicity of the sample is unknown. BNQ (1987) calls for five concentrations. Six concentrations in a suitable series would usually be advantageous in routine use, and could easily be accommodated in the cuvette wells of the standard *Analyzer*. Use of six concentrations and a control is common practice at Canadian laboratories, and is recommended. As in other toxicity tests, the standard series of concentrations is in a geometric or logarithmic series, in this case with each successive concentration being 50% of the previous one, and a high concentration of 45%, the highest that can be obtained by means of the basic technique. The description which follows uses a standard *SDI* procedure with four concentrations.

Other series of concentrations might be more suitable for some kinds of samples, and are encouraged despite the standard instructions given below for four concentrations. As the simplest variation, lower concentrations could easily be added in the same series of 50% dilutions, by continuing exactly the same procedures using extra cuvettes in the wells in row C. For samples of low to moderate toxicity, a more closely spaced series of six concentrations from about 18% to 45% has been found useful for routine testing in some laboratories.[18]

Test solutions are readied by the following steps.

(a) Add 1000 µL (1 mL) of *Reconstitution Solution* to the cuvette in the *Precooling*

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[18] The suggestion is from Mr. R. Salahub of Edmonton, Alberta. For example, an approximate logarithmic series of concentrations of 18, 22.5, 27, 31.5, 38.25, and 45% might be obtained by (a) bringing an aliquot of sample to proper salinity with *MOAS*, (b) adding to the cuvettes of row A, that adjusted sample in volumes of 0.80, 1.0, 1.2, 1.4, 1.7, and 2.0 mL, (c) making up volumes in those cuvettes to 2.0 mL with *Diluent*, and (d) continuing with the regular procedure of transfer to cuvettes in row B. Note that older versions of the computer program for the Model 500 *Analyzer* were limited to two-fold dilutions, but that restriction does not exist for recent software, Version 6.0 (Section 5.2).

Well, using a micropipettor. (This prepares for a subsequent reconstitution of a vial of *Bacterial Reagent* (Subsection 4.3.1) by allowing the *Recon* to come to temperature during the following steps.)

(b) Add *Diluent*. Pipette 1000 µL of *Diluent* into each cuvette in wells A1 (to become the control), A2, A3, and A4 (to become dilutions of the sample). Pipetted 500 µL of *Diluent* to each of the five cuvettes in Row B.

(c) Prepare the (eventual[19]) 45% sample in cuvette A5 by adding 200 µL of *MOAS*, then 2000 µL (2.0 mL) of sample. Mix five times by aspirating liquid back into the 500 µL micropipettor used to add the sample, and then dispensing it again into the cuvette. Discard 200 µL.

(d) Prepare the dilutions in cuvettes A2 to A4. Transfer 1000 µL from cuvette A5 to cuvette A4, and mix with pipettor. Transfer 1000 µL from A4 to A3, then from A3 to A2 in the same way. After the mixing of A2, discard 1000 µL to bring it to the same volume (1.0 mL) as A1, and A3 to A5, to achieve parallel treatment of the concentrations including temperature equilibrium in the well. Allow another five minutes for the contents of the cuvettes to come to the correct temperature.

4.3 Beginning the Test

4.3.1 Reconstituting Bacteria[20]

(a) Obtain a vial of *Bacterial Reagent* from the freezer. Reconstitute it immediately by thoroughly mixing the lyophilized bacteria with the *Recon* from the *Precooling Well*, then returning the cuvette to that well. This must be done by quickly inverting the cuvette of *Recon* and rapidly pouring the *Recon* into the vial of *Reagent*. (Slow addition can result in lysing of the bacteria.) Quickly swirl the vial three or four times, then pour the mixture back into the cuvette that had contained the *Recon* and place it in the cooling well. The contents should be further mixed by drawing them into a clean 500 µL pipette and expelling them back, 20 times. The *Reconstituted Reagent* now in the *Precooling Well* is theoretically enough for 20 standard tests. Light emission should remain strong for two hours. If the Reagent is to be used for longer than that, it is advisable to include a reference toxicant in the test, and to watch for other indications of a problem of reduced light emission.[21]

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[19] Statements can be found in the literature or in older manuals that the top concentration is 45.45%, but that is not correct for the standard procedure. The sample is originally 90.91% in cuvette A5. That is diluted half and half in cuvette A4, achieving 45.45% sample, but that is not used for testing, Using the highest concentration as an example of calculations, 0.5 mL of 90.91% sample from cuvette A5 is added to 0.5 mL of *Diluent* and 0.01 mL of *Reagent* in cuvette B5. Upon that addition, the toxicity test begins, and the concentration in cuvette B5 is:

90.91% * 0.5/(0.5 + 0.5 + 0.01) = 45.004%.

Successively lower concentrations are dilutions of this by a factor of two, and those are the concentrations used in this report.

[20] It is usually a more efficient use of time to reconstitute the bacteria near the start of preparations, and, while waiting 15 minutes (step 4.3.1 c), prepare the test solutions as indicated in Subsection 4.2.2.

[21] The *Reconstituted Bacteria* continue to emit light for some hours. The maximum length of time for use of the bacteria will vary. Beckman (1982) states that "the light level and stability of the Microtox *Reagent* will remain satisfactory for several hours after reconstitution". He also states that mixing bacteria into cuvettes (Subsection 4.3.1, step (b)) "should begin within five minutes of reconstitution", because sensitivity to some toxicants might change with time after reconstitution. Recent documents

(b) Set up diluted *Bacterial Reagent* in the cuvettes of Row B. Add 10 µL of the *Reconstituted Reagent* (about 1 million bacteria) to each of the five cuvettes in Row B, which already contain *Diluent*. (Addition of the bacteria must be made below the surface of the *Diluent* as described in the major Microtox manuals.)

(c) Wait 15 minutes for the light production to reach a relatively steady state (slowly decreasing).

4.3.2 Time-Zero Readings

The time-zero levels of light from the cuvettes of Row B must be measured before the sample is added. Then the sample and bacteria are mixed, and the toxicity test starts.

(a) Close the turret and turn on the chart recorder. Move cuvette B1 to the *Turret Well* and read the light production, adjusting the sensitivity of the *Toxicity Analyzer* so that a reading between 80 and 90 is obtained.

(b) Make a time-zero mark on the chart by pressing the *CAL CHECK* button of the *Toxicity Analyzer*. Without delay, read the time-zero light levels from cuvettes B1 to B5 by moving them into the *Turret Well* in turn. These observations are called by *SDI* "I_0", signifying "light production at time zero". The values should be in the range 80 to 100.

(c) Immediately mix 500 µL from each cuvette of Row A to the corresponding cuvette of Row B, i.e., from A1 to B1, A2 to B2, ...A5 to B5. (This is the formal beginning of the toxicity test. The cuvettes are allowed to sit until their light levels are checked at 5 min, 15 min, etc.) Follow the order of increasing concentration indicated above, so that the same tip can be used for the micropipettor. The actual sample concentrations in the cuvettes of Row B are now as given below, and should be recorded on the report sheet.

B1 = 0.0 % (Control or Blank)
B2 = 5.63 %
B3 = 11.3 %
B4 = 22.5 %
B5 = 45.0 %

4.4 Test Observations and Measurements

The reading of light emission after exposure should be taken for each of the cuvettes B1 to B5 at 5.0 min after the fluid from the corresponding A tube was added. Read the B cuvettes in the same manner as was done for the time-zero readings, and in the same order as was used for mixing A and B cuvettes (Subsection 4.3.2). Care should be taken that each cuvette is read at

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state that the "sensitivity of the reagent is essentially unchanged for 2 hours after reconstitution" (Microbics, 1989b), or "for 1 to 2 hours after reconstitution", with sensitivity monitored in any longer use, by conducting a standard phenol test (Microbics, 1991b). Qureshi *et al.* (1983) state that tests should begin within one hour of reconstitution; they found that sensitivity increased after that, as light emission declined.

Using the Model 2055 *Toxicity Analyzer*, viability of newly reconstituted *Reagent* can be judged by light output ten minutes after reconstitution; light output should read 80 or more with sensitivity set at 1X and *SPAN* turned up full (Microbics, 1988a). Manuals do not indicate whether the same criterion can be used to judge when *Reconstituted Bacteria* are past use.

Microtox users have indicated that *Reagent* can be used for up to 4 to 6 hours provided that suitable (time-related) checks are made on performance and results. Some users test samples in duplicate, or include a reference toxicant with each test, or both. Investigators using Model 2055 *Toxicity Analyzer* should also watch for changes in span setting at start of test, amount of span adjustment needed between tests, and blank ratios. Use of these precautions is recommended, especially the tests with reference toxicant, if *Reconstituted Bacteria* are used after two hours.

exactly five minutes after it received the fluid from cuvette A. There must not be any sequential error in exposure times caused by faster or slower operations at 5 min than at the time of mixing. The amounts of light being emitted at five minutes are called the "I_5" readings by *SDI*.

Repeat the readings after fifteen minutes of total exposure.

Slow-acting toxicants such as bivalent metals might be acting in samples of unknown toxicity. Another reading at 30 min is frequently desirable, and one or more additional reading(s) at 45 and/or 60 min might sometimes be useful. By following the series of readings, the investigator can determine whether (a) the light emission is still decreasing appreciably (more than a 10% change since the previous reading), in which case exposure should be continued for another reading, or (b) the decline has levelled out, in which case the test can be ended. The objective is to allow enough time for the sample to show its maximal inhibition, but not to proceed any further than necessary into the "plateau", beyond which the sample is not causing appreciable additional inhibition. Since the readings of the control (and the test concentrations) will gradually decline with time that must be taken into consideration when evaluating the declines in the sample cuvettes, and the best way is to use the "Gamma" statistic described in Section 4.5. In any case, it is not desirable to use an unnecessarily long exposure, and the *15-min IC50 should be regarded as the usual standard*. In unusual cases, bacteria might recover and light production could increase at a later time; in those cases, the earlier data should be used.

If the sample is more toxic or less toxic than expected, all the concentrations might diminish the light production by more than 50%, or by less than 50%. In either case, the test should be repeated with a more suitable range of concentrations.

4.5 Test Endpoints and Calculations

The readings for light emission are to be recorded. If a chart recorder is used for this, the values may be read from the chart at a later time. One suitable format for recording data is shown in Appendix C, and a list of items to report is given in Section 10.

The endpoint is the concentration causing 50% inhibition of light, i.e., the IC50. The calculations to estimate the 5-min IC50 are those given in the following steps. The 15-min and 30-min calculations would follow the same steps.

(a) *Calculate the "Blank Ratio"* which is the proportion of the initial light which remains at a given time, in the blank. This is measured in, and calculated for, the control or blank cuvette only, i.e., cuvette B1.

There is a slight natural decline in light production during the test. This must be allowed for when evaluating the loss of light caused by toxicity. The Blank Ratio provides a means for making this allowance or correction.

Blank Ration $R_5 = I_5/I_0$

where: I_0 = light emitted by the blank at time zero

I_5 = light emitted by the blank at 5 minutes

(b) *Calculate "Gamma"*. Gamma is the measure of light loss used in calculating the IC50. It is calculated individually for each cuvette which contains some of the sample being tested.

Specifically, Gamma for a given cuvette at a given exposure-time, is the ratio between the

amount of light lost to toxicity and the remaining light at that time.[22]

Gamma = (light lost)/(light remaining)
$= [(R_5 \cdot I_0) - I_5]/I_5 = [(R_5 \cdot I_0)/I_5] - 1$

where: I_0 = light emitted at time zero
I_5 = light emitted at 5 minutes
R_5 = Blank Ratio

When Gamma equals unity, half of the light production has been lost to toxicity, and that is the IC50.

4.5.1 Graphic Estimate of IC50

Values of Gamma are plotted on the vertical scale of logarithmic paper, against the corresponding sample concentrations on the horizontal logarithmic axis (Microbics, 1992). A line is fitted by eye, then the IC50 is read off as the concentration that corresponds to a Gamma of 1.0 (Figure 3).

The IC50 should be interpolated, that is, there should be at least one observation of a concentration causing more than 50% reduction of light (concentration(s) higher than the IC50), and at least on observation of a concentration causing less than 50% reduction of light. Subsection 4.8.5 provides further discussion of the need for interpolation, and of endpoints such as IC20 instead of IC50.

Accuracy of this method depends on the skill of the investigator in fitting a line by eye. For this reason, it is usually desirable to use a computer- or calculator-based method as the primary means of estimating the IC50 (see Subsection 4.5.2).

Nevertheless, a graphic analysis should be considered a routine part of each test, as a check on the reasonableness of the calculated IC50. A plot of the data is particularly useful for detecting anomalies in the test results, which might suggest further investigation or might reduce confidence in the results, any major disparity between calculated and graphically-derived IC50s should be resolved.

Hand plotting is also useful for detecting incorrect entries, if observations are being manually entered into a computer. Any error might not otherwise be detected. The *SDI* software program will provide readouts of intermediate data and a useful plot of the results, on demand. If manually entered results are used for a computer-generated graph, the plotted points should be checked against the observed readings to guard against errors in entry and anomalous estimates of IC50. Such error might be detected by the hand-plotted check.

4.5.2 IC50 Estimates Using a Calculator or Computer

An investigator could fit a straight line to the relation of log Gamma to log concentration by standard mathematical methods, although such steps are not covered in detail in Microtox manuals. Least-square regressions are apparently used in the calculation by the *SDI* computer program, so any statistics textbook would provide the methods for hand calculations. Gamma and the test concentrations should first be transformed to either natural or base-10 logarithms. Note that the widely available computer programs for calculating median lethal concentrations (LC50) or effective concentrations (EC50) are for quantal data and are *not suitable* for the Microtox data.

Computer software from *SDI* is recommended as the preferred method of analysis, in view of the wide availability of personal computers in Canadian laboratories. *SDI* supplies software in BASIC that is compatible with IBM personal computers.

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[22] Gamma is the ratio of light lost to the amount of light remaining. A simpler measure might be used, such as the absolute amount of light lost because of toxicity, but a better linear relation is generally obtained by plotting the logarithm of Gamma against the logarithm of concentration. Gamma is therefore preferred as a method of describing the dose-effect curve and estimating IC50 and confidence limits. An example comparing the two approaches is given by Beckman (1982).

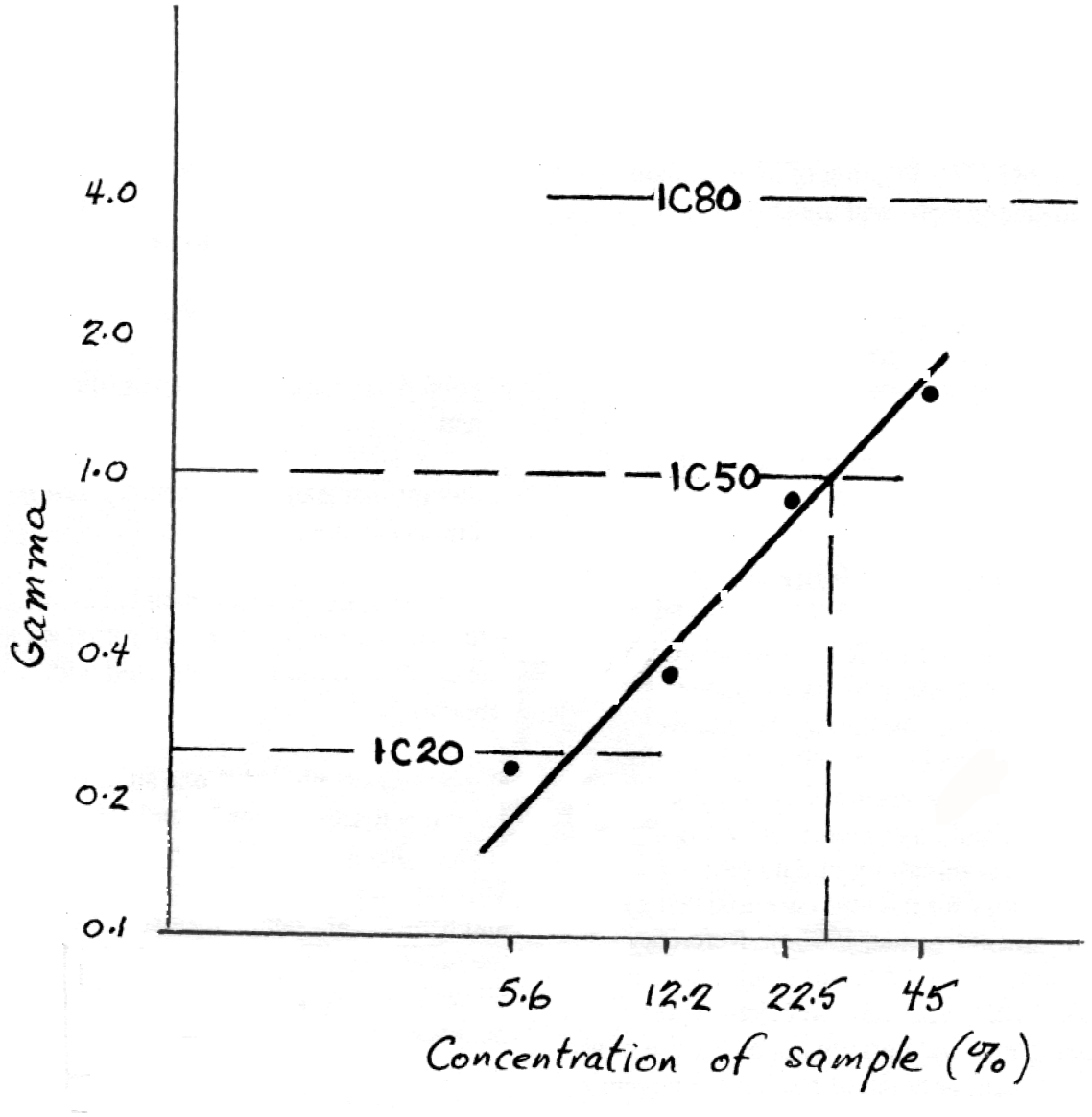

Figure 3 **Example of Estimating the IC50 with a Hand-drawn Graph on Logarithmic Paper.** The concentration causing 50% inhibition of light production by *Vibrio fischeri* can be read from the graph. In this hypothetical example there were four concentrations. The concentration expected to cause 50% inhibition can be read by following across from a Gamma value of 1.0 (broken line in graph) which represents 50% decrease, to the intersection with fitted line, then down to the horizontal axis, for an estimated IC50 of about 28%. The IC20 could be estimated in a similar way if desired, but higher concentrations should be tested before estimating the IC80 (see Subsection 4.8.5).

SDI's software is designed for the newer Model 500 Microtox *Analyzer* (see Section 5), but can also be used with the Model 2055. The software automatically transfers light readings from the test to a computer, with the Model 500 *Analyzer*, and also with the Model 2055 if it is fitted with an adapter for that purpose.

The computer program handles a variety of procedures including various concentration ranges and, in fact, all the types of tests covered herein. There are options for printing various intermediate steps in calculations, but usually the printed output includes details of the material tested, a description of the basic test variables, concentrations and Gamma values, estimate of IC50 and confidence limits, and a plot indicating the observations and fitted line. When using the computer program, it is imperative to check for reasonableness of the measurements of light reduction and estimate of IC50. Printing of intermediate steps in calculations will assist in such a check.[23]

The desirability of basing calculations on measured values higher and lower than 50% light reduction is discussed in Subsection 4.8.5. Other endpoints which might be useful, such as IC20 are also discussed in this subsection.

4.6 Reference Toxicant

The routine use of a reference toxicant or toxicants is necessary to assess, under standardized test conditions, the relative sensitivity of the bacteria after reconstitution, the accuracy of dilution techniques, and other factors affecting the precision and reliability of data produced by the laboratory for the reference toxicant(s) (Environment Canada, 1990d). Reference toxicant(s) should be tested at least once a month during periods when Microtox tests are being run, and initially upon first use of a new shipment or batch of *Bacterial Reagent.* Test conditions and procedures should be the routine ones outlined herein and in *SDI* literature.

Criteria considered in recommending appropriate reference toxicants for this test include:

- a chemical, readily available in pure form;
- stable (long) shelf life;
- highly soluble in water;
- stable in aqueous solution;
- minimal hazard posed to user;
- easily analyzed with precision;
- good dose-response curve for Microtox; and
- any influence of pH on toxicity, if it is known.

Four chemicals are recommended as suitable reference toxicants for the Microtox test and one or more of them may be used within a laboratory.

Reagent-grade phenol is one suitable reference toxicant. For phenol, *SDI* advises that a 5-min IC50 in the range 13 to 26 mg/L should be expected at 15 °C. Kaiser and Ribo (1988) tabulate eleven results from the literature showing a mean 5-min IC50 of 30 mg/L. A stock solution of phenol should be made up on the day of use.

Zinc sulphate or potassium dichromate may also be used as the reference toxicant, and both have been found satisfactory in Canadian laboratories. Zinc is recommended by *SDI*, with an estimated 5-min IC50 in the range 1.4 to 2.7 mg Zn/L at 15° C. The chromium in potassium dichromate is carcinogenic and should be handled with suitable precautions (Environment Canada, 1990d). Sodium lauryl sulphate is

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[23] The Western Canada Microtox Users Committee has analyzed data using different computer programs and found that they yield very similar results. For extrapolated data, however, different programs show more variability in results. It is appropriate for investigators to carry out within-laboratory checks by different methods, and to compare techniques and results with other laboratories, through informal groups such as the Users Committee.

another possible choice. It is recommended by BNQ (1987) with suggested test concentrations spanning the range 0.5 to 25 mg/L; Kaiser and Ribo (1988) list seven IC50s with a geometric mean value of 1.3 mg/L. This substance is not included in the list of reference toxicants recommended by Environment Canada (1990d).

Concentration of reference toxicant in a stock solution should be measured by appropriate chemical methods (e.g., APHA *et al.,* 1989). Calculation of IC50 should be based on the measured concentration if it is appreciably different (i.e., ≥20%) from the nominal one, and if the accuracy of the chemical analyses is satisfactory.

A warning chart (Environment Canada, 1990d) should be prepared and updated for each reference toxicant used. Successive IC50s should be plotted on this chart, and examined to determine whether the results are within ± 2 SD of IC50s obtained in previous tests. The geometric mean IC50 with its upper and lower warning limits (± 2 SD calculated on a geometric [logarithmic] basis)[24] are recalculated with each successive IC50 until the statistics stabilize (USEPA, 1989a; Environment Canada, 1990d).

If a particular IC50 falls outside the warning limits, the reagents, test system, or technique are suspect. Since this might occur 5% of the time due to chance alone, and outlying IC50 does not necessarily mean that the sensitivity of the *Reagent* or the precision of toxicity data produced by the laboratory are in question. Rather, it provides a warning that this might be the case. It would be wise to carry out a thorough check of all test conditions, including use of a new supply of *Bacterial Reagent*.

Use of warning limits does not necessarily indicate that a laboratory is generating consistent results. A laboratory that produced extremely variable data for a reference toxicant would have wide warning limits; a new data point could be within the warning limits but still represent undesirable variation in results obtained in tests. A coefficient of variation of 20% or 30% is tentatively suggested as a limit by Environment Canada (1990d). Although that seems a reasonable range, establishing firm limits for allowable variation would require evaluation of the reproducibility that Canadian laboratories can achieve in testing various reference toxicants with Microtox, under normal operating conditions.

4.7 Legal Considerations

Bacterial toxicity tests have been specified for an industry in at least two Canadian provinces, at the date of compiling this report. Considerations on regulatory use of Microtox are therefore in order. Specification of endpoints of the test, for legal purposes, is beyond the scope of this report; however, certain useful generic practices are addressed.

If test data are likely to be used for prosecution, care must be taken to ensure that samples are collected and tested in ways that are admissible in court. For this purpose, legal samples must be: representative of the substance being sampled; uncontaminated by foreign substances; identifiable as to date, time, and location of origin; clearly documented as to the chain of custody; and analyzed as soon as possible after collection. Persons responsible for conducting the test and reporting the findings must maintain continuity of evidence for court proceedings (McCaffery, 1979), and ensure the integrity of the test results.

4.8 Variations of Basic Procedure

4.8.1 Range-Finding Tests

With a sample of known toxicity, it will usually be helpful to obtain an approximate estimate of

[24] If the IC50s fail to show a lognormal distribution, an arithmetic mean and SD might prove more suitable.

toxic strength in a preliminary test. Results might show that the final tests should be run with concentrations that are lower than the standard ones, or in a narrower range (smaller ratio between the concentrations, see Subsection 4.2.2).

Such a preliminary range-finding test (Microbics, 1983 No. M101; 1988a) can be done quickly by adding 10 µL of sample to a standard cuvette which contains the usual 500 µL of *Diluent* and 10 µL of *Reconstituted Reagent*, producing a sample concentration of about 2%. A 60% reduction of light in five minutes would indicate an extremely toxic sample. A ten-to-one dilution of the original sample could be used in a final test.

In general, a light reduction of 60% in five minutes in the preliminary tests, would suggest that a ten-to-one dilution is necessary before standard Microtox preparations (Section 4.2) are carried out. A light reduction >20% and <60% in the range-finder would suggest an initial 5-to-1 dilution. The range-finding test is fast enough that it could be done during the waiting period for light stabilization, using some extra cuvettes of *Diluent* and *Reconstituted Reagent*, made up in Row C of the *Toxicity Analyzer*.

4.8.2 Replicates

A replicate of the control is recommended as a useful safeguard, since an anomalous reading from a single control cuvette would bias the overall result.

If desired, replicates of the test concentrations may be run, although general experience in Canadian laboratories does not suggest that this is necessary (see Subsection 3.1.1). Replicates could be run in the wells of Row C, using the same techniques as used for the cuvettes in Row B. In such a case, there should be increases in the amounts of liquid added to cuvettes of Row A. In Subsection 4.2.2, the following changes would be made to the standard procedure. In step (b), 1500 µL of *Diluent* would be added to cuvettes A1 to A4, instead of 1000 µL. In step (c), cuvette A5 would receive 3000 µL of sample and 300 µL of *Microtox Osmotic Adjustment Solution (MOAS)*. Transfers in step (d) would be 1500 µL instead of 1000 µL. Subsequently in Subsection 4.3.2 (c), transfers to Row C would be made from Row A in the same way and with the same volumes of fluid, as was specified for transfers to Row B.

4.8.3 Full-Strength Sample

The highest concentration in the standard procedure is 45% (Section 4.2), but sometimes an investigator would wish to test a sample at full strength or nearly so. It is possible to test 90% sample by omitting *Diluent* and adjusting salinity of the sample with *MOAS*. Alternatively, a sample can be tested at almost full strength by omitting both *Diluent* and *MOAS*, and using analytical grade, crystalline sodium chloride to bring the sample to 2% salinity. Light production at time zero cannot be measured in this case, since *Reagent* is added directly to the sample. The decrease in light production in each dilution of sample is estimated by comparing with a blank of *Diluent*.

Microbics (1989b) suggests that testing at full strength should not be used for samples having appreciable toxicity (i.e., toxicity that is still measurable in dilutions); there could be loss of precision because the full-strength tests are very sensitive to the technique used by the operator.

The procedure for testing a sample at full strength is actually very similar to that given in steps (a) to (d) in Subsection 4.2.2, and steps (a) to (c) in Subsection 4.3.1, but is repeated below, essentially as given by Microbics (1989b). One difference is that only one set of cuvettes (A1 to A5) is used in the following method. A similar method for several replicates of 99 % sample is outlined by Dutka (1988).

(a) Add 1000 µL *Recon* to a cuvette in the *Precooling Well*.

(b) Add 1000 µL of *Diluent* to A1, A2, A3, and A4.

(c) Add 200 µL *MOAS* to A5, for the 90% sample.

(d) Add 2000 µL of sample to A5, mix, then discard 200 µL.

(e) Transfer 1000 µL from A5 to A4, and mix A4.

(f) Transfer 1000 µL from A4 to A3, and mix A3.

(g) Transfer 1000 µL from A3 to A2, mix A2 then discard 1000 µL.

(h) Wait five minutes for temperature equilibration.

(i) Reconstitute a vial of bacteria in the usual way (Subsection 4.3.1 (a)).

(j) Start a timer. Transfer 10 µL of the *Reconstituted Reagent* to each of the five cuvettes A1 to A5[25], and mix in the same order with a 500 µL pipettor.

(k) After 5 minutes and 15 minutes, read the light emissions from each cuvette, and make tabulations and calculations by comparing A2 to A5 with the blank A1. The concentrations from high to low are 90.0%, 45.0%, 22.5%, 11.3%, and 0%.

To test a "full-strength" sample, do not add *MOAS* to A5 in step (c). Instead, add 40 mg of dry sodium chloride to A5, and dissolve it in the added sample. Do not discard fluid from A5 in step (d). The test concentration in cuvette A5 is close to full strength. Allowing for the addition of *Reagent*, the sample concentration can be considered to be 99%.[26]

4.8.4 Sucrose as the Osmotic Adjusting Agent

The sensitivity of the Microtox system to certain metals and ammonia can be increased by using sucrose instead of sodium chloride, as the base of the *Diluent*. *SDI* indicates (Microbics, 1988c) that the IC50 decreases by a factor of 34 for a salt of cadmium, a factor of 36 for a salt of ammonia, 15, 10, and 10-fold for salts of nickel, copper, and zinc, but with little change for salts of arsenic, chromium, and mercury. At the same time, the *Sucrose Diluent* did not adversely affect sensitivity of Microtox to other kinds of toxicants tested (phenol, octanol, xylene, and potassium cyanide). Tests by Ankley *et al.* (1990) confirmed major increases in sensitivity to zinc and nickel when using sucrose, but little difference for copper, and similar results for most of 44 whole effluents.

This adaptation of the Microtox procedure would obviously be suitable for testing metals and ammonia as pure chemicals. It could also be useful as an addition to the standard procedure, for effluents and other environmental samples suspected of having certain metals or ammonia as major contributors of toxicity.

The procedure involves little change from the standard one. Instead of *MOAS*, 2 g of solid sucrose (ACS grade) is added to 10 mL of sample; 2 mL of that modified sample is put into cuvette A5. Instead of the usual *Diluent*, 1000 µL of *Sucrose Diluent* (20% sucrose) is added to each of cuvettes A1 to A4, and 500 µL to each of cuvettes B1 to B5. Serial dilution, mixing with *Reagent*, and reading of results then

[25] It is important that the stock of *Reconstituted Reagent* should not be contaminated by toxic sample carried back by the pipette. The tip must be changed after each transfer.

[26] If calculated on the basis of weight, with allowance for the salt added, the concentration tested would be 97.1%.

proceed as with the standard test. The percent concentrations tested in cuvettes B2 to B5 are respectively 6.19, 12.4, 24.8, and 49.5.

4.8.5 Alternate Endpoints

With any method of estimating the IC50, whether graphic or fitting as line by calculator or computer, it is possible to estimate other percent effects, such as IC50 or IC80. Although IC50 is recommended here as a standard measure of effect, estimates of other percent effects might be appropriate for particular purposes and could be used. The IC50 should always be included in the report if it can be estimated. The 95% confidence limits should also be calculated for any estimates of other percent effects. The procedure should not be taken to extremes; it would be illusionary to attempt to calculate some sort of "threshold of toxic effect" as the IC1 or IC0.1, etc. The conclusion of Quebec Environment from a large set of tests, reviewed in Subsection 3.1.1, was that a 17% inhibition of light was the minimum that could be quantified with statistical significance (BNQ, 1987). A 20% effect corresponds to a Gamma of 0.25, and IC80 corresponds to a Gamma of 4.0, and other values are given in Beckman (1982).

Estimates of a particular "inhibiting concentration" (IC50, IC80, etc.) should properly be made only when percent effects were obtained in the test at higher and lower concentrations. For any toxicity test, the most reliable endpoint of effect will be within the range of concentrations that was tested and provided useful information. In particular, it is not advisable to extrapolate in order to estimate an IC50 form data that show only less than 50% effect. Accordingly, it is recommended here, that to be considered valid for purposes of reporting to Environment Canada, an ICp should have at least one data point that is >p.[27]

Some information can be gleaned from extrapolations, particularly since the highest valid IC50 should be about 45%, in the standard procedure which has 45% as the highest concentrations tested. Obviously it could be said that "the IC50 was greater than 45%". Alternatively, it might be possible and useful to estimate the IC20 rather than the IC50. An ICp obtained by extrapolation might be used for general comparative purposes, such as the relative degree of toxicity of different effluents. Any such values should be clearly labelled as "predicted IC50", "extrapolated IC50", or other suitable qualifier.

4.9 Procedure for Coloured or Turbid Samples

Various concentrations of sample should be tested for toxicity in the usual way, then corrected by the following method. The procedure uses a double cuvette, i.e., a narrow tube fixed within a normal one, the *Colour Correction Cuvette (CCC)*. It is obtainable from *SDI* and is reusable. An aspirator that comes with the tube, or other long-tipped pipette, is used to manipulate the liquids without generation of air bubbles. This procedure is not suitable for light-reflecting samples, not for the Model 500 *Analyzer* which requires different volumes.

After the regular test with the sample, the following steps are carried out.

(a) Add 1500 µL of *Diluent* into the outer chamber of the *CCC* and install it in C1. Add 1000 µL of *Diluent* to a standard cuvette in well C2, and 1500 µL to C4.

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[27] *SDI* has argued (1983, No. M110) that extrapolation can be done with relative safety for the Microtox test because it measures a rate of biological activity rather than a quantal effect such as mortality of fish in a lethal test. There is some truth in the assertion, but it might not always be correct to assume that a linear relation in low concentration will extend with unchanged slope into high concentrations, or vice versa. For example, Beckman (1982) shows some non-linear logarithmic plots of Gamma versus concentration. Canadian investigators report some such irregular curves.

(b) Add 1500 µL of a selected concentration of sample into a standard cuvette in well C3. Normally, the concentration would be the highest one that yielded usable results in the test just completed, or the concentration closest to the IC50 just determined. Wait at least five minutes for thermal equilibrium.

(c) Add 50 µL of *Reconstituted Reagent* to C2 and mix. Wait at least 10 minutes for stabilization of light emission.

(d) Using aspirator, add liquid from C2 to inner chamber of *CCC* until it equals the height of liquid in the outer chamber.

(e) Move *CCC* to the *Turret Well*. Adjust photocell to read about 90% and record until a stable rate of decrease is evident, then use the last four minutes for calculations.

(f) Without moving the *CCC*, remove *Diluent* from the outer chamber with the aspirator, then pipet 1500 µL of the test concentration from C3 to the outer chamber. Record light emission until a stable rate of decrease is evident, again using the last four minutes for calculations.

(g) Without moving the *CCC*, remove sample from the outer chamber with the aspirator, then refill with *Diluent* from C4. Record light level for at least three minutes.

(h) On the chart record, measure loss of light transmission caused by the sample, by using the graphic method in Microbics (1988b). The reading with the sample is called I_f, and is simply the top right (final) value of the reading recorded in step (f). The light transmission with *Diluent* is called I_p, and is interpolated to the time of reading the sample by drawing straight lines on the three readings which were traced on the chart in (e), (f), and (g). One line is drawn to join the top right-hand (final) ends of reading(s) (e) and (g), and another straight line is drawn vertically up from the top right of reading (f). The intersection of those lines yields I_p, the light transmission with *Diluent*.

If there are several samples to be run through the colour-correction procedure, they may all be done sequentially before taking the second reading of *Diluent* in step (g). In that step, after removing the sample from the outer chamber, replace it with the next sample, take a reading as before, and continue through all the samples. Finally, complete step (g) by reading with *Diluent* in the outer chamber. The graphic estimates in step (h) will use a series of vertical lines drawn up from the top right of sample readings, to determine a series of *Diluent* interpolations (I_p) to be used in corrections of the respective samples.

The correction of the IC50 involves constants derived from the geometric proportion of the *CCC*. *SDI* recommends using calculator or computer programs to do the arithmetic (Subsection 4.5.2). The following formulae for doing the calculations by hand are given by BNQ (1987), and by Microbics (1988a) in a slightly modified format.

Calculate A_c:

$$A_c = 3.1 \ln (I_p/I_f)$$

For any concentration C_x used in a test, the absorbance due to that concentration of sample is called A_x and should be calculated as:

$$A_x = A_c (C_x/C)$$

Convert that absorbance into the transmittance expected at the test concentration T_x, by calculating:

$$T_x = (1 - e^{-A_x})/A_x$$

Calculate a corrected value for Gamma, G_c, from the Gamma originally observed for the test concentration, G_x, as:

$$G_c = T_x (1 + G_x) - 1$$

where: I_0 = light production at time zero
I_5 = light production at five minutes
I_f = light production by the sample (c) used in a colour-correction test (obtained from the chart)
I_p = light production for the diluent in a colour-correction test (obtained by interpolation on the chart)
C = concentration of sample used for the colour correction procedure
A = absorbance
A_c = absorbance from the correction procedure at concentration C
C_x = any (specified) concentration used in a test
A_x = absorbance at concentration C_x
T_x = calculated transmittance at concentration C_x
e = base of natural logarithms
G = Gamma
G_x = the value of Gamma originally observed for the test concentration C_x
G_c = the corrected value of Gamma

The corrected Gammas for the test concentrations are then used to calculate a corrected IC50. This assumes linearity of colour interference at all concentrations, which occasionally might not be true. Absorbance could be measured at each concentration, but that is seldom warranted; at most, a second check might be run at a high concentration of sample, as discussed by Beckman (1982).

Procedures for Model 500 Analyzer

All the procedures in Section 4 apply to the Microtox Model 2055 *Analyzer*, which has been the most common model used until recently in Canadian laboratories. Many of the concepts and general methods described in Section 4 also apply to the newer Model 500 Analyzer (1989), but some differences exist. These are listed in the following text and are shown in Figure 4 and Table 2.

5.1 General Changes

The major differences in the Model 500 *Analyzer* (Table 2) are:

(a) The Model 500 automates most of the functions in the *Analyzer*, including lowering a given cuvette into position for reading light emission, at the touch of a button. This helps to standardize the timing and pace of readings. However, the new model could also be operated manually, if desired, for any procedure that is used on the Model 2055.

(b) The Model 500 accommodates 30 cuvettes instead of 15. This allows three samples to be tested simultaneously if desired.

(c) The new *Analyzer* is designed to send data to a computer and/or chart recorder. The computer software available from *SDI* is flexible, with many options for running different series of concentrations, duplicate, manual or automatic input of data, etc. This software can also be used on the Model 2055 Analyzer if it has the computer interface.

The *Analyzer* is similar to the Model 2055 (Figure 2), but the Model 500 has wells for 30 cuvettes, has no projecting turret, and lacks adjustment knobs. A computer would likely receive data from the test, rather than a chart recorder.

5.2 Specific Procedural Changes for Model 500 Analyzer

Procedures are detailed in a recent manual from Microbics (1992). In general, the kinds and columns of fluids manipulated, and the timing, remain the same as given in the preceding sections. Some steps of the manual procedure are no longer required, and those items are given below, with the initial codes referring to preceding sections of this document. Some general annotations and recommendations are given with the descriptions of changes.

3.2.2 (d). The chart recorder will not likely be selected as an option by investigators, since it has been rendered rather obsolete for the new *Analyzer*. Model 500 will send readings to a micro-computer (Subsection 3.2.2 (e)) for recording and later processing. Alternatively, the light readings can be read from a display on the front of the *Analyzer* and recorded manually. The Model 500 adjusts readings to relative values from 0 to 199. During initial standardization, a value of zero is assigned to a reading in darkness, and a value close to 95 is assigned to the reading of the Reagent Blank.

4.2.1 (a) to (e). The *Analyzer* checks temperatures automatically and signals the operator with a warning light if there is deviation from desired temperature. The steps for calibrating the *Analyzer* are carried out by the machine when the SET button is pressed.

4.2.2 The *SDI* computer software allows for diverse series of concentrations. Older versions of the computer program required two-fold

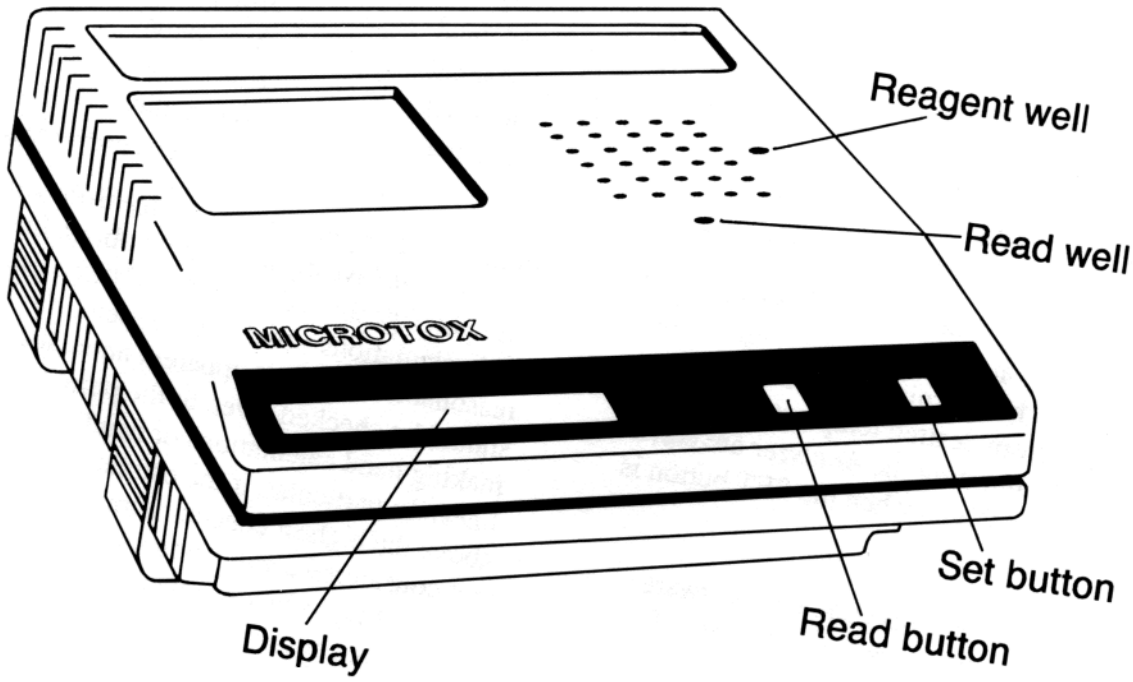

Figure 4 General Appearance of the Model 500 *Analyzer* for the Microtox Test
This *Analyzer* is similar to Model 2055 (Figure 2), but Model 500 has wells for 30 cuvettes, has no projecting turret, and lacks adjustment knobs. A computer would likely receive data from the test, rather than a chart recorder.

Table 2 Comparison of Models 2055 and 500 Microtox *Analyzers*

Item	Model No.	
	2055	500
Number of incubator wells	15	30
Span control	Manual	Automatic
Temperature control	Manual	Automatic
Zero adjustment	Manual	Automatic
HV (high voltage) check	Manual	Automatic
CAL check	Manual	Automatic
Cuvette feed	Manual	Automatic
Computer port	Optional	Yes
Recorder port	Yes	Yes
Turret check	Manual	Automatic
Incubator check	Manual	Automatic
Air check	Manual	Automatic
Sensitivity setting	Manual	Automatic

dilutions, and users of those early versions would be well advised to obtain recent software.

4.3.2 (b). It is unlikely that timing will be marked on a chart record. If direct computer input is used, the program signals the appropriate times for light readings. Otherwise, a timer is used.

4.5 (a) and (b). Calculations of the "blank ratio" and "Gamma" will most likely be done by a computer program (see Subsection 4.5.2). The investigator should obtain the computer-generated plot. All of these data and calculations should be inspected for reasonableness. Any apparent anomalies should be checked, even to the point of making hand calculations or hand-fitted lines. In particular, if the estimated ICp is above the highest concentration tested, it is not considered a valid estimate for purposes of reporting to Environment Canada. Such an ICp can, however, be labelled as "tentative", "extrapolated", or other similar qualifying term, and used for general comparative purposes (see Subsection 4.8.5).

4.9. For a colour-correction procedure, the general approach, manipulations, and readings are similar to those for the Model 2055 but differ in detail. No graphic analysis is done by measuring on a chart, but a corrected value for I_0 is calculated arithmetically and used in the programs to estimate an IC50 or ICp.

Specific Procedures for Testing Chemicals

This section gives particular instructions for testing chemicals, in addition to the procedures listed in Sections 4 and 5.

6.1 Properties, Labelling, and Storage of Sample

Information should be obtained on properties of the chemical to be tested, including water solubility, vapour pressure, chemical stability, dissociation constants, and biodegradability. Data sheets on safety aspects of the test substance should be consulted, if available. Other available information such as structural formula, degree of purity, nature and percentage of significant impurities, amounts of additives, and n-octanol:water partition coefficient should be obtained and recorded.[28] An acceptable analytical method should also be known for the chemical in water at concentrations intended for the test, together with data on precision and accuracy. If the chemical's aqueous solubility is in doubt or problematic, acceptable procedures used previously for preparing aqueous solutions should be reported.

Chemical containers should be sealed and coded or labelled (chemical name, supplier, date received, person responsible for testing, etc.) upon receipt. Storage conditions (e.g., temperature, protection from light) are frequently dictated by the nature of the chemical. Standard operating procedures for chemical handling and storage must be followed.

[28] Knowledge of the properties of the chemical will aid in determining any special precautions to be taken while handling and testing it (e.g., testing in a well-ventilated facility). Information on chemical solubility and stability will also be useful in interpreting test results.

6.2 Preparing Test Solutions

If a new pesticide of similar category of chemical were being tests under a formal program of regulations for registering the chemical, those regulations might require the use of replicate test concentrations.

The "sample" to be tested will be a stock solution, or diluted from a stock solution that was prepared by dissolving the chemical to be tested in deionized water. The concentration of the test chemical in the solution should be measured (Section 6.3), and stability of the chemical determined, before the test. Stock solutions subject to photolysis should be shielded from light, and unstable solutions must be prepared at a suitably short time before use. The sample should be adjusted to 2% salinity [Subsection 4.2.2 (c)] at the beginning of a test. The sample should meet the requirements for colour turbidity, pH, and dissolved oxygen content specified in Section 4.1. The Microtox assay can then proceed as in the Universal method of Section 4.

For chemicals that do not dissolve readily in water, stock solutions may be prepared using the generator column technique (Billington *et al.*, 1988; Shiu *et al.*, 1988) or, less desirably, by ultrasonic dispersion. User of ultrasonics can produce droplets that differ in size and uniformity, some of which could migrate towards the surface of the liquid, or vary in biological availability creating variations in toxicity. There could also be effects on light transmission, which is of major importance in the Microtox test. Organic solvents, emulsifiers, or dispersants should not be used to increase chemical solubility except in instances where they might be formulated with the test chemical for its normal commercial purposes. If used, an additional

control solution should be prepared containing the same concentration of solubilizing agent as in the most concentrated solution of the test chemical. Such agents should be used sparingly, and should not exceed 0.1 mL/L in any test solution. If solvents are used, the following are preferred: dimethyl formamide; dimethyl sulphoxide (DMSO);[29] triethylene glycol; methanol; ethanol; and acetone (USEPA, 1985).

6.3 Observations and Measurements on Sample

During preparation, each stock solution should be examined for evidence of chemical presence and change (e.g., solution colour and opacity, precipitation or flocculation). Any observations should be recorded.

It is desirable to measure the concentration of the chemical in the stock solution.[30] Aliquots should be preserved, stored (if necessary), an analyzed chemically according to best proven methodologies available. If a measured concentration is available, it should be used to calculate and express the toxicity, unless there is good reason to believe that the measurement is not accurate.

[29] Keep DMSO in its liquid form at 19°C or higher. If repeatedly frozen and thawed its toxicity might increase.

[30] Such analyses need not be undertaken in all instances, due to cost, analytical limitations, or previous technical data indicating chemical stability in solution under conditions similar to those used in preparation and in the test. Chemical analyses are particularly advisable if the test material is volatile, of low solubility, precipitates, or sorbs to the container.

Specific Procedures for Testing Samples of Effluent, Leachate, and Elutriate

Particular instructions for testing samples of effluent, leachate, and elutriate, in addition to the procedures listed in Sections 4 and 5, are given in this section. Note that some tests of sediments can involve separate tests of interstitial water following the procedures used for an elutriate.

7.1 Sample Labelling, Transport, and Storage

Containers for transportation and storage of samples of effluent, leachate, or elutriate must be made of nontoxic material. For large samples intended for tests with larger organisms as well as Microtox, glass or Teflon™-coated containers are preferred, as they are inert and reduce sorption of chemicals. Polyethylene or polypropylene containers manufactured for transporting drinking water are less desirable but may also be used. For smaller samples intended only for the Microtox test, borosilicate glass containers are recommended, with screw-caps lined with Teflon or other closure known to be nontoxic.

Samples of 500 mL to 1 L are recommended for the Microtox test, to allow portions to be used for measuring initial pH, dissolved oxygen, and other characteristics (Subsections 4.1.2, 4.1.3, and Section 7.4). All containers must either be new or thoroughly cleaned, and rinsed with uncontaminated water. They should also be rinsed with the sample to be collected. Containers should be filled completely to minimize air space.

Upon collection, each sample container must be sealed and labelled or coded. Labelling must include at least sample type, source, date and time of collection, and name of sampler(s).

Unlabelled or uncoded containers arriving at the laboratory should not be tested. Nor should samples arriving in partially filled containers be routinely tested, since volatile toxicants escape into the air space. However, if it is known that volatility is not a factor, such samples might be tested at the discretion of the investigator.

Testing of effluent and leachate sample should commence as soon as possible after collection. Whenever possible, testing should begin within 24 h, and must commence no later than 72 h after sampling. Samples of sediment or soil collected for extraction and subsequent testing of the elutriate should also be tested as soon as possible; extraction procedures should begin within two weeks of sampling (preferably within one week), and testing must start no later than six weeks after collection (Environment Canada, 1994). Procedures given in Environment Canada (1994) should be followed. Testing of such elutriates must commence within 72 h of their preparation, or as specified in a regulation or protocol.

All samples of effluent of leachate should be kept cool (1 to 7° C), preferably, 4 ± 2° C) throughout their period of transport. Upon collection, warm (>7° C) samples should be cooled to 1 to 7° C with ice or frozen gel packs. As necessary, gel packs or other means of refrigeration should be used to assure that sample temperature remains within 1 to 7° C during transit. Samples must not freeze during transport. Samples should be stored in darkness in sealed containers at 4 ± 2° C. Sub-samples for testing should be brought to 15° C immediately before the test.

Temperature conditions should also be as indicated for transportation and storage of

elutriate, as well as for samples intended for aqueous extraction and subsequent testing of elutriate, unless otherwise specified.

7.2 Preparing Test Solutions

Samples in the collection containers must be agitated thoroughly just prior to pouring to ensure the re-suspension of any settleable solids. Sub-samples (i.e., a sample divided between two or more containers) must be mixed together to ensure their homogeneity. If further sample storage is required, the composited sample (or a portion of it) should be returned to the sub-sample containers and stored (Section 7.1) until used.

The pH and dissolved oxygen content of the sample should be checked with regard to the limits in Subsections 4.1.2 and 4.1.3.

Tests for monitoring and for compliance with regulatory requirements should normally include an undiluted portion of the sample (Subsection 4.8.3) and a control solution. Regulations might require replicates of the control and some or all of the test concentrations. Toxicity tests conducted for other purposes (e.g., determination of in-plant sources of toxicity, treatment effectiveness, effects of process changes on toxicity) may, depending on the study objectives, be single- or multi-concentration tests. Single-concentration tests are often cost-effective for determining the presence or absence of toxicity or as a method for screening many samples for relative toxicity.

7.3 Dilution Water

For freshwater discharges of effluents, and freshwater leachates and elutriates, the *Diluent* described in the Universal procedure should normally be used in tests. Surface water from lakes, rivers, and streams is normally unsuitable for use as dilution water in the test, in an attempt to simulate a local situation.[31]

There are additional options for effluents being discharged to marine situations, and for elutriates which have been obtained using seawater, as in an evaluation of marine sediments. The choice amount the options depends on the purpose for doing the test. If a "standardized" toxicity rating is desired, the Universal procedure should be followed, using the normal Microtox *Diluent* and the standard salinity of 2%. If it is desired to simulate the local situation, salinity of the sample and *Diluent* should be that of the receiving water. The *Vibrio fischeri* used in the tests has maximal light production in the salinity range 2 to 3.7%.

Use of clean seawater in place of the Microtox *Diluent* is the preferred option if the sample itself were essentially seawater, such as a seawater elutriate, or a saline leachate or effluent. Use of clean seawater as diluent is preferred since the bacteria generally produce significantly more light in natural seawater than in *Diluent*, which uses only sodium chloride, even if salinity of the *Diluent* is adjusted upwards to that of seawater.

It would be a much less desirable option to use Microtox *Diluent* for testing a sample that was essentially or predominantly seawater, such as a seawater elutriate. The greater light production in seawater compared to NaCl solution, would mean an imbalance in the test with regard to the "background" production of light in the various

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[31] The Microtox test does not simulate the precise conditions in local bodies of fresh water, because the test is run at a fixed salinity of 2%. The estimates of toxicity obtained by using clean *Diluent*, therefore, are more suitable as "standardized" toxicity values for a particular chemical, wastewater, sediment, or similar solid material.

Pollutants already in the receiving water can add toxicity to that of the chemical or wastewater under investigation. In such instances, the standard uncontaminated *Diluent* would give an accurate estimate of the toxicity of an effluent, leachate, or elutriate, but not necessarily of the total impact in the site of interest.

dilutions of sample. That problem would not be solved by raising the salinity of the *Diluent*, using *MOAS* or sodium chloride, upwards from 2% salinity to a standard seawater value such as 3.5%, or to the salinity of the sample. Such adjustment of salinity should be done, if this option were selected for some reason, but it would be preferable to use the option described in the previous paragraph, of replacing *Diluent* with seawater.

If the effluent, leachate, or elutriate were saline but below the salinity of seawater, the Microtox test should not be run at less than 2% salinity (an exception could be rough range-finding tests). Light emission drops off appreciably as salinity declines below 2% (Krebs, 1983). All test salinities could, however, be adjusted upwards to above 2% but below the salinity of full seawater, preferably by using appropriate amounts of clean seawater. The guiding principle in any such test is that the strength of "natural" seawater should be constant in all cuvettes.

7.4 Observations and Measurements on Sample

Colour, turbidity, odour, and homogeneity (i.e., presence of floatable material or settleable solids) of the effluent, leachate, or elutriate sample should be observed at the time of preparing test solutions. Samples with an appreciable solids content should be analyzed for total suspended solids (APHA *et al.,* 1989). Precipitation, flocculation, colour change, odour, or other reactions upon dilution with water should be recorded.

If there is concern about interference with light transmission by elevated colour, solids, floating or emulsified materials, the test should use the colour-corrected technique outlined in Subsection 4.1.1 and Section 4.9.

7.5 Endpoints and Calculations

Tests for monitoring and for compliance with regulatory requirements might be single concentrations with a control (Section 7.2). Endpoints for these tests would depend on the objectives of the undertaking, but could include arbitrary "pass" or "fail" ratings based on a selected percent reduction in light emission at a given concentration. Multi-concentration tests would have the normal endpoint of IC50 as in Section 4.5.

Specific Procedures for Testing Samples of Receiving Water

Instructions for testing samples of receiving waters, in addition to those provided in Sections 4 and 5, are given here.

8.1 Sample Labelling, Transport, and Storage

Procedures for the labelling, transportation, and storage of samples should be as described in Section 7.1. Testing of samples should start as soon as possible after collection, preferably within 24 h, and no later than 72 h after sampling.

8.2 Preparing Test Solutions

Samples in the collection containers should be agitated before pouring to ensure their homogeneity. Compositing of sub-samples should be as described in Section 7.2. For samples with high suspended solids, the cautions of Subsection 4.1.1 should be observed. The pH and dissolved oxygen content of the sample should be checked with regard to the limits in Subsections 4.1.2 and 4.1.3.

8.3 Dilution Water

For fresh surface waters, the standard (Universal) procedure should be used. For samples of marine or estuarine surface waters, there are additional options.

If the sample of receiving water is itself seawater, *MOAS* would not be added to the sample. In such as case, the preferred option is to use clean "natural" seawater as the diluent, i.e., for the controls and for diluting the samples. The rationale and approach is described further in Section 7.3.

If the receiving water is estuarine, its salinity must be measured and adjusted upwards, if necessary, to achieve at least 2% salinity for the test. This could be done with *MOAS* or crystalline sodium chloride (NaCl), if desired, but it would be highly desirable for the diluent to contain clean seawater adjusted to the same strength (salinity) that was in the sample. If the sample of receiving water had a salinity greater then 2% but less than that of full-strength seawater, the best option would be to use a diluent of clean seawater diluted to the same salinity as that of the sample. Again, the guiding principle in any such manipulation would be that each cuvette in the test should end up with the same amount of seawater, and the same amount of added NaCl if that is used.

8.4 Observations and Measurements on Sample

Observations of sample colour, turbidity, foaming, precipitation, etc. should be made as described in Section 7.4, during preparation of test solutions.

8.5 Endpoints and Calculations

Endpoints for tests with samples of receiving water should be consistent with the options and approaches identified in Sections 4.5 and 7.5.

Tests for monitoring and compliance purposes should normally include, as a minimum, an undiluted portion of the sample (Subsection 4.8.3), and one or more control solutions. In instances where toxicity of receiving-water samples is likely, such as mixing zones, and information is desired about the improvements or further dilution is necessary to permit satisfactory conditions, a test to determine the IC50 should be

conducted as described under Universal tests, Section 4. Often, surface water samples will not be toxic enough to cause an IC50. The Microtox system can be used to categorize surface waters on the basis of light loss caused by 45% sample, the highest concentration used in the standard procedure (Kaiser *et al.,* 1988a; 1988b).

Specific Procedures for Testing Samples of Sediment or Similar Solids

General instructions are given here for testing liquids derived from samples of sediment or similar solids such as soils, and for the "solid-phase" Microtox test. These are in addition to general instructions provided in Sections 4 and 5. In this section, the word "sediment" is used for convenience but should be taken to include other similar solid substances such as soils, and industrial or municipal sludges, which might contribute pollutants to surface waters or require testing for other reasons.

9.1 General Aspects of Procedure

General guidance is given here on application of Microtox for testing sediments and the water derived from sediments. It is not the purpose of this report to provide instructions for carrying out a field survey of sediments, sampling them, or obtaining liquids or extracting materials from them. Detailed guidance for the collection, handling, transport, storage, and manipulation of sediments is provided in Environment Canada (1994). This guidance document should be consulted and followed when collecting samples of sediment and preparing them for Microtox tests. Additional guidance on these topics is provided in recent books (Murdoch and Macknight, 1991), reviews (Geisy and Hoke, 1989; McLeay and Sprague, 1991), and United States standard methods (ASTM, 1991a; 1991b).

Microtox has generally been judged as a useful and rapid method for comparing toxicity extracts of contaminated sediments (Schiewe *et al.,* 1985). It has been recommended as one of four toxicity tests to include in a battery of sediment tests, on the basis of an extensive review of available tests (Geisy and Hoke, 1989). A survey of 50 sediments from the coast of Washington State showed that Microtox, oyster embryo, and amphipod tests yielded good agreement, although there was benefit in using a diversity of tests. Microtox detected toxicity in a greater number of sediments than did either of the other tests (Williams *et al.,* 1986; Becker et al., 1990). Other sediment tests showed that Microtox and a toxicity test with echinoderm embryos were the most sensitive of seven sediment tests evaluated (Pastorak and Becker, 1989). Strosher (1984) tested 48 liquids overlying waste drilling muds, and concluded that bacterial luminescence tests were more sensitive, reproducible, quicker, and more economical for evaluating toxicity than were two kinds of tests with fish and one with germination of seeds.

Brouwer *et al.* (1990) used a solid-phase Microtox test to characterize sediments in the harbour of Hamilton, Ontario. They found that method to be more sensitive to hydrophobic toxicants such as polychlorinated biphenyl (PCB) than another Microtox method based on a liquid elutriate. Similarly, the solid-phase test proved more sensitive than the liquid-extraction test, to sediments from Halifax Harbour (Tay *et al.,* 1991), and several other industrialized Canadian sites (van der Geest, 1991).

9.1.1 Sample Labelling, Transport, and Storage

General procedures for the labelling, transportation, and storage of sediment samples should be as described in Section 7.1. Additional guidance is found in Environment Canada (1994). Temperature limits are those described in Section 7.1, and in particular, samples must not freeze or partially freeze, and must not be allowed to dry (Environment Canada, 1994; ASTM, 1991b).

For the liquids derived from sediments, containers and handling procedures should be the

same as those given in Section 7.1 for elutriates. If a non-aqueous solvent has been used to extract substances, a glass container should be used to store the liquid, so that it will not be affected by the solvent, nor leach materials into the sample.

Testing of samples should start as soon as possible after collection. Solid-phase tests, or extraction procedures, should begin within two weeks of sampling, and preferably within one week. Testing must start no later than six weeks after sample collection.[32] Testing of the liquid obtained from sediments must begin within 72 h of making such preparations, or as specified in a regulation or other designated procedure.

9.1.2 Preparing Sample

Depending on the nature of the sample and the objectives of the test, homogenization of samples might or might not require mixing before testing. If mixing is carried out, it must be thorough. Sub-samples (i.e., a sample divided between two or more containers) must be mixed together. If further sample storage is required, the composited sample, or a portion of it, should be returned to the sub-sample containers and stored.

9.1.3 Observations and Measurements on Sample

Observations of the colour, turbidity, foaming, precipitation, etc. should be made on both the sediment and any liquid derived from it, as described in Section 7.4, during preparation of test solutions.

9.1.4 Control or Reference Sediments

One or more samples of control or reference (unpolluted) sediment must be assessed in the same manner as the sediment under investigation. Although the test procedures include a blank or control which does not contain material from the sediment being studied, experience indicates that such a control might not be sufficient for an acceptable evaluation of toxicity. Environment Canada and other laboratories have frequently recorded apparent toxic effects with unpolluted sediments in solid-liquid suspension tests (Section 9.3). Accordingly, one or more control or reference ("clean") sediments should be included as a sample, with each test of a sediment or series of sediments, to help establish a baseline or "normal" level.

It would be desirable to establish a standard, clean "reference sediment" for this purpose, or a series of reference sediments of differing characteristics which could be matched with those of the sediments being tested. It would also be desirable to test for significant differences among results for the reference and test sediments. Testing a control or reference sediment, or liquid extract from such a sediment, is not necessarily required in *SDI* procedures, but is an integral part of the procedure in the present document. The control or reference sediment(s) should be similar in general physical and chemical characteristics to the sediment(s) being investigated. In particular, an attempt should be made to match the distribution of particle sizes and organic/inorganic balance (ASTM, 1991a; 1991b; McLeay and Sprague, 1991; Environment Canada, 1994).

There is no single procedure for making use of the results from the control or reference sediment. If it shows no toxicity, then results for the test sediment (i.e., the one under investigation) are accepted as valid. If the control (reference) sediment does show toxicity, no standard method appears to have been

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[32] The toxicity and geochemistry of contaminated sediments from Hamilton Harbour was reported to change with storage for longer than one week, although the data supporting that statement were not provided (Brouwer *et al.,* 1990). Testing within two weeks conforms with current standardization in U.S. procedures (ASTM, 1991b) and with Environment Canada's (1994) recent guidelines. A maximum permissible storage time of six weeks has been specified (Environment Canada, 1994) in view of practical difficulties for shorter times, including time required if initial chemical analyses are to be performed.

developed, as yet, to adjust the results for the test sediment.[33] Caution should be exercised in interpreting findings for the test substance. Tests of statistically significant differences could be carried out, with guidance from a statistician, if paired observations or replicate tests are available.

9.2 Testing Liquids Extracted from Sediment or Similar Solids

Toxicants from sediments or soils can enter an aqueous phase and affect organisms in surface water, and Microtox can be useful for testing such effects. The aqueous phase might be a liquid derived from a soil or sediment (e.g., pore water), or a liquid used to treat the sample and extract potential toxicants (e.g., an elutriate). The procedures recommended in Environment Canada (1994) for extracting pore water, or for preparing an elutriate, should be followed in deriving these liquids.

A liquid obtained from sediment for toxicity testing would be expected to fall into one of four broad categories.

(1) Pore water, i.e., that which fills the spaces between particles, and could exchange with the overlying water making up the lake, river, estuary, etc. It is normally obtained from a sediment by centrifuging or squeezing it (Subsection 9.3.2).

(2) Water used to obtain an aqueous extract of substances from the sediment (i.e., elutriate), for example by shaking a sample with added clean water.

(3) Microtox *Diluent* or other pure water with adjusted salinity, used to obtain an aqueous extract as in (2).

(4) Solvents other than water (e.g., organic solvents), used to remove substances from the sample of sediment (Schiewe *et al.,* 1985; True and Heyward, 1990).

The water in the first three categories could be tested as a normal liquid sample, following the universal procedures given in Section 4. Particular attention should be required for any problems caused by colour or turbidity (see Subsection 4.1.1 and Section 4.9).

The preferred option for the fourth category, solvents, is to have the same concentration of solvent in each of the curettes. The *Diluent* to be used in the test is brought to the same concentration of solvent as that in the highest concentration of sample that will be tested. The solvent effect, if any, should be the same in all cuvettes. Another option is to include a solvent control at the highest concentration of solvent represented in the test, as well as a control without solvent. It would be desirable to run a separate test to determine the IC50 of the solvent. Specific steps for testing, when organic solvents are involved, are available as a manuscript from Microbics (1990) and might be included in future manuals.

9.2.1 Preparing Test Samples

Compositing of "sub-samples" of liquid obtained from the sediment (e.g., successive extraction) should be a described in Section 7.2 and recommended in Environment Canada (1994). Sub-samples would not be composited if it were desired to ascertain the relative toxicity of successive extractions. For samples with appreciable colour or turbidity, the procedures and cautions of Subsections 4.1.1 and Section 4.9 should be followed. The pH and dissolved oxygen content of the sample should be checked with regard to the limits in Subsections 4.1.2 and 4.1.3.

[33] Brouwer *et al.* (1990) incorporated such a control by expressing the light emission of the test sediment as a percentage of that from the control (reference) sediment. However, only one concentration of each was tested, and the technique does not appear to have been extended to a multi-concentration test.

Once the liquid has been obtained, test concentrations are prepared in the standard manner (Subsection 4.2.2). As in testing effluents, leachates, and elutriates, the test could be a single-concentration (plus control) test for regulatory purposes, or a multi-concentration test to determine the IC50 (Section 7.2). The procedures for obtaining and testing liquid from samples of control or reference sediment should be identical to those for the test sediment.

9.2.2 Dilution Water

For freshwater samples derived from sediment, the standard (Universal) procedure should be used.

It the sediment samples are marine or estuarine, or saline waters are derived from them, there are additional options. If the water obtained from sediment is essentially seawater, *MOAS* would not be added to the sample of water which is to be tested. The preferred option for diluent would be to use clean seawater for the controls and for diluting the samples. A less desirable alternative would be to adjust the salinity of the Microtox *Diluent* with *MOAS* to bring it to the salinity of the sample.

If the water derived from the sediment has a salinity lower than that of full-strength seawater, i.e., similar to estuarine water, its salinity should be measured, and adjusted upwards if necessary, to attain at least 2% salinity. If the sample of water has a salinity greater than 2% but less than that of full-strength seawater, the salinity of the diluent should be raised to the same salinity. The methods adopted to carry out these manipulations would be the same as outlined in Section 8.3.

9.2.3 Endpoints and Calculations

Endpoints for tests with liquids derived from sediment should be consistent with the options and approaches identified in Sections 4.5 and 7.5.

9.3 Solid-Liquid Suspension Test

A particular modification of the Microtox procedure, called the "solid-phase test" has been provided by *SDI* and can be applied to sediment, sludges, soils, and similar substances. Since the procedure allows direct contact of the bacteria with the solids of the sample, it is said to allow the assessment of toxic substances in or on the particles. In fact, liquid is added and the organisms are exposed to a suspension of the sediment; thus the test is also partly comparable to an aqueous extraction of the sample.

An early version of the procedure became available at the beginning of 1991 (Microbics, 1991a), and a more detailed draft was available later that year (Microbics, 1991b), and can be expected to undergo modification in details, as experience with the test accumulates. Therefore, only the general procedure is given here, and investigators are advised to obtain detailed procedures from the most recent instructions of *SDI*.

To summarize the test, the phosphorescent bacteria are left in contact with saline aqueous suspensions of the sediment at various concentrations, for 20 minutes. The solid material is removed by filtration and the liquid is left for a further 5 minutes. Light emission from the liquid is then measured, and the IC50 is estimated.

9.3.1 Facilities

The test is designed to used the Model 500 *Analyzer* with analysis by a computer, so the general methods of Section 5 apply. The test can also be done, however, using the older Model 2055 *Analyzer*.

As well as the usual pipettes and flat-bottomed curettes, some special supplies are required:

Solid-Phase Diluent;
Solid-Phase Tubes; and
Solid-Phase Filter Columns.

9.3.2 Preparing Test Solutions

After mixing, the sample of sediment is centrifuged or squeezed to remove free (interstitial) water[34] (ASTM, 1991b; Environment Canada, 1994). The solids are mixed again before use.

Ten *Solid-Phase Tubes* are readied in a rack and designated A1 to A5, and B1 to B5. Into tube B5, place 400 mg of the sediment which has been prepared by removing the water.

9.3.3 Beginning the Test

The steps are described precisely by Microbics (1991a) for a simple test with a series of nine concentrations and a control, and a synopsis is given here. Procedures using several controls, duplicate concentrations, etc, are given in Microbics (1991b).

(a) Add 1.0 mL *Recon* to a cuvette in the *Reagent Well*.

(b) Place cuvettes in wells A1 to A5, and B1 to B5.

(c) Reconstitute a vial of bacteria in the usual way (Subsection 4.3.1 (a)), using the *Recon* from the *Reagent Well*, and return the *Reconstituted Bacteria* to that well.

(d) Pour the *Reconstituted Bacteria* into a bottle of *Solid-Phase Diluent*, and mix well.

(e) Add 2.0 mL of the mixture from (d) to each of the *Solid-Phase Tubes* in a rack (Subsection 9.3.2) except the tube which contains sediment.

(f) Set a timer for 20 minutes, and add 4.0 mL of the mixture containing bacteria from (d) to the *Solid-Phase Tube* B5 which contains sample, and mix well by shaking and pipetting.

(g) Transfer 2.0 mL from Tube B5 to B4, then 2.0 mL from B4 to B3, and similarly in succession to B2, B1, A5, A4, A3, and A2, but not to A1. Each tube is mixed thoroughly before transferring it. Discard 2.0 mL from Tube A2, so that each tube contains 2.0 mL. The highest concentration of solid substance is 10% in Tube B5, running downwards in two-fold dilutions to 0.039% in Tube A2, and zero in Tube A1.

(h) At the 20-minute signal, filter contents of each tube in turn by pushing a filter column down in the tube.[35] Start at zero concentration (A1) and move upwards in concentration (ending at B5). Each time, transfer 0.5 mL of the liquid filtrate to the cuvette with the corresponding number.

(i) Allow the liquid to stand for a further five minutes. Set the *Analyzer* ("Set"button) on the liquid in cuvette A1, the control, and read the light emission. Read the emission from liquid in other cuvettes from low concentration (A2) to high (B5). Record the readings or have them recorded by computer.

(j) Repeat the procedure with the sample(s) of control or reference ("clean") sediment.

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[34] This water could be tested as described in Section 9.2. Some trials by Canadian laboratories indicate that the solid-phase Microtox tests show greater toxicity than is obtained with Microtox tests on the pore water obtained by centrifuging (Tay *et al.,* 1991; van der Geest, 1991).

[35] *SDI* standard filtration method will probably be generally adopted. This technique is recommended to allow comparison of results from different laboratories using a standard method. It is reported by Brouwer *et al.* (1990), however, that centrifugation gave at least an order of magnitude more toxicity in a solid-phase test than did filtration at any pore size from 0.1 to 8 µm.

9.3.4 Endpoints and Calculations

Calculation of the IC50 is carried out in the general fashion outlined in Section 4.5. This would normally be done by the *SDI* computer program, and a manual check made for absence of errors and for reasonable calculations.

Certain features of the test should be noted. There is no "time-zero reading" (I_0) taken for each cuvette. The reading for the control (blank) is taken as the time-zero reading for each concentration as well as being the control reading. Therefore, the "blank ratio" (Section 4.5 (a)) is always 1.0 and nee not be estimated, if the calculations are being done by hand. Some earlier versions of the *SDI* computer program were limited to a two-fold factor of dilution for concentration, and an investigator is advised to use Version 6.0 of the program, or a later version.

Reporting Requirements

The test report should describe the materials and methods used, as well as the test results. The reader should be able to determine from the report whether the conditions and procedures make the results acceptable for the use intended.

Procedures and conditions that are common to a series of ongoing tests (e.g., routine toxicity tests for monitoring or compliance purposes), and consistent with specifications in this document may be referred to by citation or by attachment of a general report which outlines standard laboratory practice. Where choices exist, the approach selected should be specified. The general report should convey the procedural information indicated in Sections 10.2 to 10.4. An individual test report giving the findings should contain the information indicated in Sections 10.1 and 10.5. Specific monitoring programs might require selected items (e.g., procedures and results for tests requiring salinity adjustment) in the test report. Other details pertinent to the conduct and findings of the test, which are not conveyed by the reports, should be kept on file by the laboratory, so that the appropriate information can be provided if an audit of the test is required.

10.1 Test Substance

(a) Sample type, source and description (chemical, effluent, leachate, elutriate, receiving water, sediment, or other similar solid substance; sampling location and method; specifics of the nature, appearance and properties, volume and/or weight).

(b) Information on labelling or coding of the test substance.

(c) Details on manner of sample collection, transport and storage (e.g., batch, grab or composite sample, description of container, temperature of sample upon receipt and during storage).

(d) Identification of person(s) collecting and/or providing the sample.

(e) Dates and times for sample collection and start of definitive test.

(f) Any physicochemical measurements on sample (e.g., chemical concentration, colour, suspended solids content).

10.2 Test Organisms

(a) Batch or lot number of organisms used.

(b) Date obtained and temperature of holding.

10.3 Test Facilities and Apparatus

(a) Name and address of test laboratory.

(b) Name of person(s) performing the test.

(c) Model number of *Analyzer* and description of any non-standard items of apparatus.

10.4 Test Methods and Conditions

(a) Brief mention of method used if standard [e.g., this document or Microbics (1988a; 1988b; 1989b)].

(b) Dissolved oxygen of sample used for testing, before and after initial aeration if any. Statement concerning aeration of solutions before the test (if any, give rate, duration, manner of application).

(c) Any other chemical analyses of stock or test solution, and analytical procedure(s) used.

(d) Procedure used in preparing stock and/or test solutions of chemicals.

(e) Details of any salinity adjustment of samples.

(f) Sampling and storage details if clean seawater was used for dilution of samples.

(g) Concentrations tested including controls, and number of cuvettes at each concentration.

(h) Appearance of test solutions and any changes during test.

(i) Temperature as monitored in the incubation section of *Toxicity Analyzer*.

(j) The pH of sample and description of any pH adjustment.

(k) Observations times during test.

10.5 Test Results

(a) Results for range-finding test (if conducted).

(b) Code used for identifying chart recording, if used.

(c) Time-zero readings of light emissions.

(d) Percent light inhibition in each test solution (including control) at each observation time.

(e) Results of "colour-correction" test (loss of light transmission caused by colour and other attributes of sample).

(f) Any IC50s and their 95% confidence limits, with method of calculation and values for important intermediate variables.

(g) The IC50 and 95% confidence limits for the reference toxicant(s) determined within one month of the test, or when a new batch of *Bacterial Reagent* was first used, together with the geometric mean value (± 2 SD) for the reference toxicant as derived previously at the laboratory.

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Appendix C

Sample of Possible Format for Recording Results of Microtox Tests

This sample is taken from Beckman (1982).

SAMPLE ID NO.: N/A COLLECTED BY: K.W.

SOURCE: Baker Reagent $ZnSO_4 \cdot 7H_2O$ DATE: 4-22-81

TIME: 1000

REPORTED RESULTS: TOXIC ☐ NON-TOXIC ☐ MAX Γ = N/A FOR CONCENTRATION = N/A

GRAPH ☒

CALC ☐ EC_____ (_____ MIN. _____ °C) = ☐ % SAMPLE ☒ mg/L

CONFIDENCE FACTOR = - _____ ☐ OTHER _____

95% CONFIDENCE INTERVAL _____ TO _____

REMARKS: _____

SAMPLE DATA: SAMPLE TYPE (EFFLUENT, LEACHATE, ETC.): purified chemical

DATE ASSAYED: 4 - 22 - 81 TIME: _____

VISUAL COLOR: none CORRECTION REQUIRED: YES ☐ NO ☒

VISUAL TURBIDITY: none SEPARATION REQUIRED: YES ☐ NO ☒

INITIAL pH: 5.6 for 1% pH AJUSTED TO: N/A WITH N/A

OSMOTICALLY ADJUSTED WITH: MOAS ☐ DRY NaCl ☒ OTHER ☐ _____

PRIMARY DILUTION OF SAMPLE: 1% diluted 50 to 1

OPERATOR: K.W. REAGENT VIAL LOT NO. M- 101289

REMARKS: For $mgZn^{++}/L$, multiply EC value by 65.4 ÷ 287.4

TABLE OF OBSERVED AND CALCULATED RESULTS

BLANK CUVETTE	FINAL ASSAY CONCENTRATION (SPECIFY UNITS)	INITIAL READING I (0)b	FINAL READINGS 5-MIN I (5)b	15-MIN I (15)b	30-MIN I (30)b
B1	0 (BLANK)	90.3	79.2	72.3	61
C1	0 (BLANK)	88.5	77.9	71.5	60.3
SUMS OF READINGS		178.8 [A]	157.1 [B]	143.8 [B]	121.3 [B]
MEAN BLANK RATIOS [B] ÷ [A] = R̄ (t) =			R̄ (5) = .8786	R̄ (15) = .8043	R̄ (_) = .6784

BLANK RATIO CHECK
B1 : R (5) = .8771
C1 : R (5) = .8802

ABSORBANCE (COLOR) CORRECTION DATA
I_o = N/A
I_t = N/A
C_o = N/A

SAMPLE CUVETTE	FINAL ASSAY CONCENTRATION (SPECIFY UNITS)	INITIAL READING I (0) [C]	FINAL READINGS 5-MIN I (5) [D]	15-MIN I (15) [D]	30-MIN I (30) [D]	Γ (t,T) EFFECTS Γ (5)	Γ (15)	Γ (30)	Γ (_)
B2	3.7 mg/L	90	68.7	57.1	46.7	.1511	.2676	.3074	
C2	3.7	87.1	66.8	54.9	43.4	.1456	.2760	.3615	
B3	11.1	90.2	63.3	36.4	15.7	.2520	.9930	2.898	
C3	11.1	85.8	61.1	34.4	15.1	.2338	1.006	2.855	
B4	33.3	85.9	46.5	13.6	4.2	.6231	4.080	12.88	
C4	33.3	90	47	14.0	5.0	.6825	4.170	11.21	
B5	100	81.4	24.8	4.8	1.6	1.884	12.64	33.51	
C5	100	85.1	24.9	5.0	1.8	2.003	12.69	31.07	
EC 50 (t-MIN, 15 °C)			BY GRAPH			50.0	11.0	6.4	
			BY CALCULATOR						