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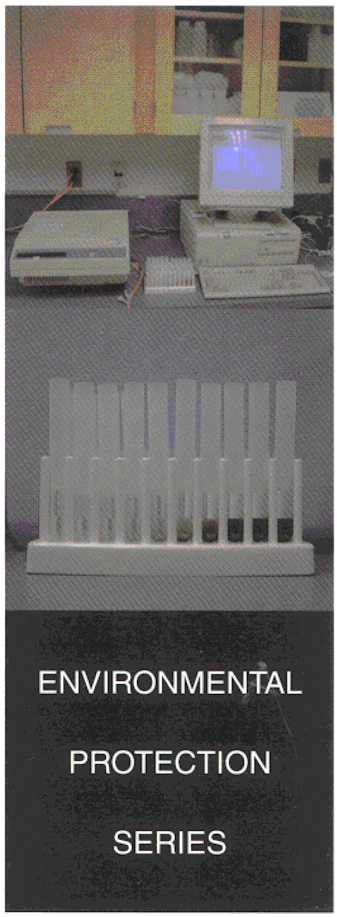

Biological Test Method: Reference Method for Determining the Toxicity of Sediment Using Luminescent Bacteria in a Solid-Phase Test

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Biological Test Method: Reference Method for Determining the Toxicity of Sediment Using Luminescent Bacteria in a Solid-Phase Test

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Abstract

A reference method for measuring the toxicity of samples of whole sediment under controlled and defined laboratory conditions is described in this report. It uses luminescent bacteria (Vibrio fischeri) *as the test organism and inhibition of light production by the bacteria in a solid-phase test as the biological endpoint. The test involves the preparation of a series of concentrations of the sample by serial dilution in water, their mixing with an inoculum of test organisms* (V. fischeri) *and incubation for 20 minutes in test tubes held in a water bath at 15 ± 0.5°C, the filtration of the contents of each test tube, the subsequent stabilization of the filtrate at 15 ± 0.5°C for 10 minutes in a series of glass cuvettes held within wells of a photometer, and thereafter the photometric reading of light produced by the luminescent bacteria remaining in the filtrate. The statistical 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 reference method follows and is built upon the generic (multipurpose) biological test method "Toxicity Test Using Luminescent Bacteria (Photobacterium phosphoreum)*" published previously by Environment Canada (1992; EPS 1/RM/24). It is intended for use with samples of contaminated or potentially contaminated sediment.*

Specific conditions and procedures are stipulated that include instructions on obtaining, shipping, holding, and storing test organisms (Bacterial Reagent)*; acceptable procedures and conditions for transporting, storing, and manipulating samples of sediment to be used in the test; required physicochemical analyses of sediment; procedures and conditions to be followed in preparing for and conducting the test; criteria for acceptable performance and valid test results; measurements and observations to be made; required or recommended data analyses; guidance for interpreting test results; and minimum reporting requirements. Instructions on the use of reference toxicity tests are also provided.*

Résumé

Ce rapport contient une description d'une méthode de référence *permettant de mesurer la toxicité d'échantillons de sédiments entiers analysés dans des conditions de laboratoire contrôlées et définies. Cette méthode fait appel à une bactérie luminescente,* Vibrio fisheri, *comme organisme soumis à l'essai et à l'inhibition de la luminescence comme paramètre de mesure biologique dans un test en phase solide. Pour faire le test, il faut préparer une série de concentrations à partir de l'échantillon grâce à une dilution en série dans l'eau. Ces concentrations sont mélangées à des inoculums d'organismes soumis à l'essai (*V. Fisheri*) et incubées in vitro pendant 20 minutes un bain-marie à 15 ± 0,5 °C. Le contenu de chaque tube est filtré et le filtrat est subséquemment stabilisé à 15 ± 0,5 °C pendant 10 minutes dans une série de cuvettes de verre placées dans les puits d'un photomètre. Une lecture photométrique du filtrat est faite pour mesurer ce qui reste de l'émission de lumière produite par la bactérie luminescente. Le paramètre statistique du test est la concentration estimée provoquant une inhibition de 50 % de la luminescence produite par la bactérie (c'est-à-dire le IC50).*

Cette méthode de référence a été élaborée à partir de la méthode d'essai biologique générique (universelle) appelée Essai de toxicité sur la bactérie luminescente Photobacterium phosphoreum *publiée par Environnement Canada (1992, SPE 1/RM/24). Elle est destinée à être utilisée sur des échantillons composés de sédiments contaminés ou potentiellement contaminés.*

La présente méthode contient des renseignements sur les conditions particulières et les procédures s'y rattachant, y compris des directives sur la façon d'obtenir, d'envoyer, de conserver et d'entreposer les organismes soumis aux essais (réactif bactérien)*; des procédures acceptables et une liste des conditions permettant le transport, l'entreposage et la manipulation des échantillons de sédiments utilisés dans le cadre du test; une liste des analyses physicochimiques requises pour étudier les sédiments; des procédures à suivre et des conditions à respecter pour préparer et exécuter le test; des critères à remplir pour atteindre un rendement acceptable et les résultats valides visés; le mesurage et les observations à consigner; les données d'analyse requises ou recommandées; les conseils à suivre quant à l'interprétation des résultats du test et les exigeances minimales relatives à la préparation d'un rapport. Cette méthode contient également des instructions sur la façon d'utiliser des tests de référence sur la toxicité.*

Foreword

This is one of a series of **reference methods** *for measuring and assessing the toxic effect(s) on single species of aquatic or terrestrial organisms, caused by their exposure to samples of test materials or substances under controlled and defined laboratory conditions.*

A **reference method** *is defined herein as a specific biological test method for performing a toxicity test, i.e., a toxicity test method with an explicit set of test instructions and conditions which are described precisely in a written document. Unlike other multi-purpose (generic) biological test methods published by Environment Canada, the use of a* **reference method** *is frequently restricted to testing requirements associated with specific regulations (e.g., Disposal at Sea Regulations under the Canadian Environmental Protection Act; CEPA, 1999; Government of Canada, 2001).*

Reference methods *are those that have been developed and published by Environment Canada (EC), and are favoured:*

- *for regulatory use in the environmental toxicity laboratories of federal and provincial agencies;*

- *for regulatory testing which is contracted out by Environment Canada or requested from outside agencies or industry;*

- *for incorporation in federal, provincial, or municipal environmental regulations or permits, as a regulatory monitoring requirement; and*

- *as a foundation for the provision of very explicit instructions.*

Appendix A lists those **Reference Methods** *prepared for publication by Environment Canada's Method Development and Applications Section in Ottawa, ON, along with other generic (more widely applicable) biological test methods and supporting guidance documents.*

Words defined in the Terminology section of this document are italicized when first used in the body of the report according to the definition. Italics are also used as emphasis for these and other words, throughout the report.

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List of Abbreviations and Chemical Formulae

cm	centimetre(s)
CV	coefficient of variation
g	gram(s)
h	hour(s)
HCl	hydrochloric acid
HNO_3	nitric acid
ICp	inhibiting concentration for a (specified) percent effect
IC50	50% inhibiting concentration
L	litre(s)
mg	milligram(s)
min	minute(s)
mL	millilitre(s)
mm	millimetre(s)
NaCl	sodium chloride
$NaNO_3$	sodium nitrate
nm	nanometre(s)
NRCC	National Research Council of Canada
p	probability
SD	standard deviation
SOP	standard operating procedure
SPT	solid-phase test
TM (^{TM})	Trade Mark
v:v	volume-to-volume
°C	degree(s) Celsius
µL	microlitre(s)
µm	micrometre(s)
wt:v	weight-to-volume
>	greater than
<	less than
≥	greater than or equal to
≤	less than or equal to
±	plus or minus
%	percentage or percent

Terminology

Words defined herein are italicized when first used in the body of the report according to the definition. Italics are also used as emphasis for these and other words, throughout the report.

Note: all definitions are 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

Compliance means in accordance with governmental regulations or requirements for issuing a permit.

Estuarine (water) is from a coastal body of ocean water that is measurably diluted with fresh water derived from land drainage.

Fines refers to (sediment) particles which are $\leq$ 0.063 mm in size. Measurements of % fines include all particles defined as silt (i.e., particles $\leq$ 0.063 mm but $\geq$ 0.004 mm) or clay (i.e., particles <0.004 mm).

Marine (water) is from or within the ocean, sea, or inshore location where there is no appreciable dilution of water by natural fresh water derived from land drainage.

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 sediment for toxicity.

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 than 7 indicating increasingly greater acidic reactions, and numbers greater than 7 indicating increasingly basic or alkaline reactions.

Reference method refers to a specific protocol for performing a toxicity test, i.e., a biological test method with an explicit set of test procedures and conditions, formally agreed upon by the parties involved and described precisely in a written document. Unlike other multi-purpose (generic) biological test methods published by Environment Canada, the use of a *reference method* is frequently restricted to testing requirements associated with specific regulations (e.g., Government of Canada, 2001).

Salinity is the total amount of solid substance, in grams, dissolved in 1 litre of (sea)water; and is traditionally expressed as parts per thousand (‰). It 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.*, 1995).

Terms for Test Materials or Substances

Artificial sediment refers to a synthetic sediment, prepared in the laboratory using a specific formulation of clay, silt, and/or sand intended to simulate a natural sediment. A mixture of appropriate quantities of *clean* clay, silt, or sand-sized particles with the desired percentages of fine and coarse-grained material is mixed in the laboratory to prepare *(clean) artificial negative control sediment*. A specific formulation of clay, silt, and/or sand is mixed (spiked) together with a toxic chemical (or, in some instances, a highly toxic contaminated sample of field-collected sediment) to prepare one or more concentrations of *artificial positive control sediment*. See also "*clean sediment*", "*negative control sediment*", and "*positive control sediment*".

Bacterial Reagent is a standard culture of a specific strain of freeze-dried (lyophilized) *Vibrio fischeri*, stored in small, sealed vials which each contain about 100 million organisms.

Chemical is, in this report, any element, compound, formulation, or mixture of a substance that might be mixed with, deposited in, or found in association with sediment or water.

Clean sediment is sediment that does not contain concentrations of any contaminant(s) which would reduce the light produced by *V. fischeri* during the test.

Contaminated sediment is sediment containing chemical substances at concentrations that pose a known or potential threat to environmental or human health.

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 added test material or substance. The control is used to determine the absence of measurable toxicity due to basic test conditions (e.g., temperature, health of test organisms, or effects due to their handling or manipulation).

Deionized water is water that has been purified by passing it through resin columns or a reverse osmosis system, for the purpose of removing ions such as Ca^{++}, Mg^{++}, etc.

Diluent is a solution of 3.5% sodium chloride in *distilled* or *deionized* water, which is prepared using reagent-grade salt. *Diluent* comprised of 3.5% NaCl may be used with samples of marine, estuarine, or freshwater sediment. See also "*distilled water"* and "*deionized water".*

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

Liquid-phase (toxicity test) means that which does not include any added sediment particulate (i.e., the test material and concentrations thereof represent an aqueous solution). See also "*solid-phase (toxicity test)"*.

Material is the *substance* or substances from which a thing is made. A *material* might have heterogeneous characteristics, even after mixing. Soil, sediment, or surface water are considered herein as materials. Usually, the *material* would contain several or many substances. See also "*substance*".

Negative control sediment means uncontaminated (*clean*) sediment which does not contain concentrations of one or more contaminants that could affect the performance (in this instance, light production) of the test organisms. This sediment may be natural, field-collected sediment from an uncontaminated site, or *artificial sediment* formulated in the laboratory using an appropriate mixture of uncontaminated ("*clean*") sand, silt, and/or clay. This sediment must contain no added test material or substance, and must enable an acceptable rate of light production by *V. fischeri* according to the test conditions and procedures. The use of *negative control sediment* provides a basis for judging the toxicity of coarse-grained (<20% fines) test sediment. See also "*artificial control sediment"* and "*clean sediment*".

Pore water (also called "interstitial" water) is the water occupying space between sediment particles.

Positive control sediment means sediment which is known to be contaminated with one or more toxic chemicals, and which causes a predictable toxic response (in this instance, inhibition of light production) with the test organisms according to the procedures and conditions of the

test. This sediment might be one of the following: a *standard contaminated sediment*; *artificial sediment* or *reference sediment* that has been spiked experimentally with a toxic chemical; or a highly contaminated sample of field-collected sediment, shown previously to be toxic to *V. fischeri* and for which its physicochemical characteristics are known. The use of *positive control sediment* assists in interpreting data derived from toxicity tests using test sediment. For this *reference method, positive control sediment* must be used as a *reference toxicant* when appraising the sensitivity of the test organisms and the precision and reliability of results obtained by the laboratory for that material. See also "*standard contaminated sediment*", *"artificial sediment", "reference sediment", and "reference toxicant"*.

Product is a commercial formulation of one or more chemicals. See also "*chemical"*.

Reconstitution Solution is non-toxic distilled or deionized water that is used to activate a vial of *Bacterial Reagent*.

Reference sediment is a field-collected sample of presumably *clean* (uncontaminated) *sediment*, selected for properties (e.g., particle size, compactness, total organic content) representing sediment conditions that closely match those of the sample(s) of test sediment except for the degree of chemical contaminants. It is often selected from a site that is uninfluenced or minimally influenced by the source(s) of anthropogenic contamination but within the general vicinity of the site(s) where samples of test sediment are collected. A sample of *reference sediment* should be included in each series of toxicity tests with *test sediment(s)*. This reference sediment might or might not prove toxic due to the presence of naturally occurring chemicals such as hydrogen sulphide or ammonia, or the unanticipated presence of contaminants from human influence at harmful-effect concentrations. The use of such (toxic) sediment as *reference sediment* in future toxicity tests should be avoided, unless the experimental design is cognizant of this and the investigator(s) wish to compare test results for this material with those for one or more samples of test sediment. See also "*clean sediment"* and "*test sediment*".

Reference toxicant is a standard *positive control sediment* used to measure the sensitivity of the test organisms to establish confidence in the toxicity data obtained for a test material or 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 material or substance is evaluated, and the precision and reliability of results obtained by the laboratory for that *reference toxicant*. See also "*positive control sediment".*

Reference toxicity test is a test conducted using a *reference toxicant* in conjunction with a sediment toxicity test, to appraise the sensitivity of the organisms and the precision and reliability of results obtained by the laboratory at the time the test material is evaluated. Deviations outside an established normal range indicate that the sensitivity of the test organisms, and the performance and precision of the test, are suspect. For this *reference method*, a reference toxicity test should be performed using a *positive control sediment* which

can be either a subsample of a reference material comprised of *standard contaminated sediment* with known levels of contaminants (such as is available from the National Research Council of Canada), or a *spiked control sediment* test. See also "*reference toxicant", "positive control sediment", "standard contaminated sediment",* and *"spiked control sediment"*.

Sampling station means a specific location, within a *site* or sampling unit (depending on the study design), where the sample(s) of field-collected sediment are obtained for toxicity tests and associated physicochemical analyses. See also "*site*".

Sediment is natural particulate material, which has been transported and deposited in water, and which usually lies below water. The term can also describe a material that has been experimentally prepared (formulated) using selected particulate material (e.g., sand of particular grain size, bentonite clay, etc.).

Site means a delineated tract of sediment that is being used or considered as a study area, usually from the perspective of it being contaminated or potentially contaminated by human activity.

Soil is whole, intact material representative of the terrestrial environment, that has had minimal manipulation following collection. It is formed by the physical and chemical disintegration of rocks and the deposition of leaf litter and/or decomposition and recycling of nutrients from plant and animal life. Its physicochemical characteristics are influenced by microbial and invertebrate activities therein, and by anthropogenic activities.

Solid-phase (toxicity test) means that which includes a series of test concentrations prepared using an aliquot of the whole sediment (i.e., sample particulate plus pore water, added as a homogeneous mixture). See also "*liquid-phase (toxicity test)"*.

Spiked control sediment is *clean artificial sediment* or *clean* field-collected *reference sediment* to which a test substance or material such as a chemical, a mixture of chemicals, drilling mud, contaminated dredge spoil, sludge, or *contaminated sediment* has been added experimentally, and mixed thoroughly to evenly distribute the substance or material throughout the control sediment. See also "*clean sediment", "artificial sediment", "reference sediment", contaminated sediment",* and "*positive control sediment*".

Standard contaminated sediment is a field-collected sediment for which contaminant concentrations are known, documented, and available (e.g., from the National Research Council of Canada); and one which has proven to be toxic to *V. fischeri* using the *reference method* described herein.

Substance is a particular kind of material having more or less uniform properties. This term includes any distinguishable kind of organic or inorganic matter, whether inanimate or animate.

Test sediment is a field-collected sample of whole sediment, taken from a marine, estuarine, or freshwater site thought to be contaminated (or potentially so) with one or more chemicals, and intended for use in the *solid-phase toxicity test* with luminescent bacteria. In some instances, the term also applies to any solid-phase sample (including *reference sediment*, *artificial sediment, negative control sediment, positive control sediment*, or dredged material) used in the test. See also "*solid-phase toxicity test", "reference sediment", "artificial sediment", "negative control sediment",* and "*positive control sediment".*

Water-only (toxicity test) refers to a (toxicity) test which does not include any sediment or other solid-phase material (e.g., a test using an aqueous solution of a *reference toxicant*). The term *water-only (toxicity test)* is synonymous with *liquid-phase (toxicity test).* This test is used to confirm that the *Reconstitution Solution* does not decrease light production by *V. fischeri* (see Section 3.2.4). See also "*liquid-phase (toxicity test)"* and "*solid-phase (toxicity test)".*

Statistical and Toxicological Terms

Acute means within a short period in relation to the life span of the test organism (of the order of some minutes for bacteria).

Acute toxicity is an adverse effect (lethal or sublethal) induced in the test organisms within a short period (for purposes of this document, within a few minutes) of exposure to *test sediment*(s).

Battery of toxicity tests is a combination of several toxicity tests, normally using different species of test organisms (e.g., a series of sediment toxicity tests using *V. fischeri*, one or more species of marine or estuarine amphipods, and a polychaete worm).

Coefficient of Variation (CV) is the standard deviation (SD) of a set of data divided by the mean, expressed as a percentage. It is calculated as: CV (%) = 100 SD ÷ mean.

Endpoint means the measurement(s) or value(s) that characterize the results of a test (e.g., ICp). This term might also mean the reaction of the test organisms to show the effect which is measured upon completion of the test (e.g., inhibition of light production).

Gamma is the measure of light loss used in calculating the ICp. It is calculated individually for each cuvette containing a filtrate of a particular test concentration. Gamma (Γ) is calculated based on the ratio between the amount of light emitted by a test filtrate and that emitted by the control solutions, as follows: $\Gamma = (I_c/I_t) - 1$, where I_c = the average light reading of filtrates of the control solutions, and I_t = the light reading of a filtrate of a particular concentration of the test material (Section 6.1). When Gamma equals unity ($\Gamma = 1$), half of the light production has been lost as a result of toxicity or other confounding factors (Section 6.2).

Geometric mean is the mean of repeated measurements, calculated logarithmically. It is advantageous, in that extreme values do not influence the mean as is the case for an arithmetic mean. The *geometric mean* can be calculated as the n^{th} root of the product of the "n" values, and it can also be calculated as the antilogarithm of the mean of the logarithms of the "n" values.

ICp is the inhibiting concentration for a (specified) percentage effect. It represents a point estimate of the concentration of test substance or material in sediment that causes a designated percent inhibition (*p*) compared to the control, in a quantitative biological function such as light production by bacteria or growth of fish, relative to the control. For example, an IC50 could be the concentration estimated to cause a 50% reduction in the quantity of light emitted at the end of the test by the test organisms, relative to that in the control. This term should be used for any toxicological test which measures a continuously variable effect, such as light production, reproduction, respiration, or dry weight at test end.

Precision refers to the closeness of repeated measurements of the same quantity to each other, i.e., the degree to which data generated from replicate measurements differ. It describes the degree of certainty around a result, or the tightness of a statistically derived endpoint such as an ICp.

Replicate refers to a single test chamber containing a prescribed inoculum of organisms in either one concentration of the test material or substance, or in the control or reference treatment(s). A replicate is an independent test unit; therefore, any transfer of organisms or test material from one replicate to another would invalidate the test. The term is also used to refer to more than one sample of test material taken at one time from a particular location and depth (i.e., *field replicates*), or for subsamples of a particular test material taken for multiple (duplicate or more) toxicity tests using identical procedures and conditions (i.e., *laboratory replicates*).

Replicate samples are field-replicated samples of sediment collected from the same sampling station, to provide an estimate of the sampling error or to improve the precision of estimation. A single sediment sample from a sampling station is treated as one *replicate*. Additional samples are considered to be additional *replicate samples* when they are treated identically but stored in separate sample containers (i.e., not composited).

Toxic means poisonous. A toxic chemical or material can cause adverse effects on living organisms, if present in sufficient amount at the right location. *Toxic* is an adjective or adverb, and should not be used as a noun; whereas *toxicant* is a legitimate noun.

Toxicant is a toxic substance or material.

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

Toxicity test is a determination of the effect of a material or substance on the test organism (e.g., *Vibrio fischeri*), under defined conditions. An aquatic toxicity test usually measures either (a) the proportions of organisms affected (*quantal*); or (b) the degree of effect shown (*quantitative* or *graded*), after exposure to a specific test material (e.g., a sample of sediment) or mixture (e.g., a chemical/sediment mixture). A *solid-phase* toxicity test with luminescent bacteria must be considered a graded (*quantitative*) 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 (i.e., light emission), shown by groups of bacteria.

Treatment refers to a specific *test sediment* (e.g., *site sediment* or *reference sediment* from a particular sampling station and depth), or a concentration thereof. Samples or subsamples of test sediment representing a particular *treatment* are typically replicated in a toxicity test. See also *"replicate"*.

Warning chart is a graph used to follow changes over time, in the endpoints for a reference toxicant. Date of the test is on the horizontal axis and the effect-concentration is plotted on the vertical logarithmic scale.

Warning limit is plus or minus two standard deviations, calculated logarithmically, from a historic geometric mean of the endpoints from tests with a reference toxicant.

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Photos for the cover were supplied by Paula Jackman, Troy Steeves, and Dale Hughes (Environmental Science Centre, Environment Canada, Moncton, NB).

Introduction

This *reference method* specifies the procedures and conditions to be used when preparing for and undertaking a *solid-phase* test for measuring the *toxicity* of samples of *test sediment*, using luminescent bacteria (*Vibrio fischeri*). The *reference method* herein represents one of the biological test methods to be used as part of *sediment* assessments consistent with the Federal regulations on disposal at sea under the *Canadian Environmental Protection Act* (EC, 1997a; CEPA, 1999; Government of Canada, 2001). It *can* also be used to measure the toxicity of samples of sediment being considered for disposal on land or at any freshwater, *estuarine*, or *marine site*s where regulatory appraisals or stringent testing procedures apply. Another reference method, intended for applications with sediment samples, has been published by Environment Canada (EC, 1998) and other Federal (Environment Canada) biological test methods for measuring sediment toxicity are available (see Appendix A).

Solid-phase tests for measuring sediment toxicity, using luminescent bacteria (*V. fischeri*, formerly identified as *Photobacterium phosphoreum*) have been used by Canadian, the US, and other researchers and regulators since the introduction of this test procedure by Canadian researchers for evaluating the toxicity of test sediments from Hamilton Harbour (Brouwer *et al.*, 1990). An *acute* solid-phase test for sediment (or soil) toxicity was subsequently adopted and standardized by Microbics Corporation (Carlsbad, CA), as one of several Microtox™ test methods (Microbics, 1992). At that time, Environment Canada (1992) recommended the use of the Microtox™ *solid-phase* test method for evaluating the toxicity of samples of sediment or similar solids, while recognizing that the standardization of the test method was in its infancy. Notwithstanding, Environment Canada (1992) continues to serve as a useful reference source and companion to the present document, when performing acute *solid-phase* (or, using EC 1992 guidance, *liquid-phase*) *toxicity test*s with luminescent bacteria.

Many components of the procedures and conditions specified herein are consistent with the guidelines and approaches for measuring sediment toxicity using the solid-phase test option and *V. fischeri* as test organisms, which are described in various methodology documents or laboratory standard operating procedures (SOPs) including: EC (1992), Microbics (1992, 1995a), ASTM (1995), EC (1996a), AZUR (1997), AZUR (1998a, b), EC (1999a), and NICMM (1999). Appendix E provides a review of the similarities and differences associated with various procedures and conditions specified in those documents. The contribution of those methods and SOPs to all parts of this *reference method* is acknowledged, and they are recommended as sources of supporting rationale. Procedures and conditions stipulated in this report *should*, however, be taken as the definitive ones when planning and undertaking *solid-phase* sediment toxicity tests with luminescent bacteria (*V. fischeri)* for regulatory purposes in Canada.

Besides the existing methodology guideline documents or SOPs for which pre-test and test conditions and procedures are summarized in Appendix E, a number of reports are now available in the scientific literature that provide further useful details regarding the performance of solid-phase sediment toxicity tests using luminescent bacteria. These include but are not restricted to the following: studies of the influence of sediment composition on apparent toxicity (Benton *et al.*, 1995; Ringwood *et al.*, 1997; Tay *et al.*, 1998); an investigation into the role of sulphide toxicity in reduced sediments as a factor in (freshwater) sediment toxicity (Brouwer and Murphy, 1995); a study of correlations between a number of solid-phase sediment toxicity tests and *in-situ* benthic community structure (Day *et al.*, 1995); input to test design and data interpretation using the *V. fischeri* solid-phase test for sediment toxicity (Ross and Leitman, 1995); a survey of the toxicity of Halifax Harbour sediments (Cook and Wells, 1996); the role of this and other sediment assays in assessing the toxicity of test sediments (Ross, 1998; Bombardier and Birmingham, 1999); and the interlaboratory *precision* of a solid-phase test for sediment toxicity using *V. fischeri* (Ross *et al.*, 1999).

Before finalizing this *reference method*, two series of interlaboratory studies with samples of reference and contaminated sediment were performed to determine intralaboratory and interlaboratory precision, and to validate the test method. Each series involved the same six testing laboratories, with varied ($\leq$1 to 8 years) experience in performing sediment toxicity tests using luminescent bacteria in a solid-phase test. The first series of tests was conducted by each laboratory using an identical set of aliquots (subsamples) of eight dry test materials identified only as samples numbered 1 to 8. Seven of those aliquots were dry, certified standard sediments (reference or contaminated) provided by the National Research Council of Canada (NRCC), and the eighth aliquot was 100% dried kaolin clay. Although not identified as such, four of the eight aliquots submitted to each laboratory during the first series of tests were subsamples taken from the same batch of a single composite sample of a standard NRCC contaminated sediment. For the second series of tests, an identical set of eleven subsamples of field-collected "wet" sediment, obtained from a number of contaminated or reference sampling stations within Canadian coastal waters, was sent to each laboratory for testing. Although not identified, three of those eleven test materials were from the same batch of a composite sample of a single reference sediment. Findings from these studies, which are detailed in a technical report available from Environment Canada's Method Development and Applications Section (McLeay *et al.*, 2001), indicated very good intralaboratory and interlaboratory precision for each series of tests performed using the pre-test and test conditions detailed in this *reference method*.

Test Organisms

2.1 Species

Organisms used in this test come from a standardized culture (strain NNRL B-11177; see Table 10, Appendix E), and belong to a particular species of luminescent marine bacteria (i.e., *Vibrio fischeri*; formerly classified as *Photobacterium phosphoreum*). This is a bacterium which normally lives in the ocean, and produces a continuous blue-green light by enzymatic reactions if sufficient oxygen is available (EC, 1992).

2.2 Source and Holding

Standard cultures of *V. fischeri* can be purchased from Strategic Diagnostics Inc.[1] Bacteria are marketed as a uniform strain of lyophilized (i.e., freeze-dried under vacuum) bacteria ("*Bacterial Reagent*"), harvested during the exponential phase of growth. Production lots are sold in packages which each contain ≥10 sealed vials. Each vial contains about 100 million (1×10^6) lyophilized organisms.

Containers of lyophilized *Bacterial Reagent* are reportedly stable for up to one year when kept in a freezer at -20°C (EC, 1992).[2]

Storage temperature should be constant and range within -20 to -25°C; "frost-free" freezers which warm up during the defrosting phase should not be used. The viability of new lots of bacteria, or of individual lots used over an extended period, should be determined by a *solid-phase* reference toxicity test performed with one or more chemicals using the procedures and conditions described in Section 5. Each lot is suitable for at least two hours (EC, 1992) and up to three hours (Gaudet, 1998) of testing, after they have been reconstituted to an active state.

The number and expiry date of the lot of bacteria used in each toxicity test *must* be recorded; this information must be included in the test-specific report together with the species and strain of the test organism (see Section 7.1.2). Other data specific to the test organisms, including their source, date of receipt, and temperature during storage or holding, must either be included in the test-specific report or the general report, or held on file for a minimum of five years (see Section 7.2.2).

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[1] This and related products and disposal supplies for performing solid-phase toxicity tests using *V. fischeri* were formerly marketed by AZUR Envrionmental Ltd. (Carlsbad, CA). Marketing rights for Microtox™ products and reagents have now been acquired by Strategic Diagnostics Inc. in Newark, DE. For contact information, see their Web site at www.sdix.com, or phone 1-800-544-8881.

[2] Labels on boxes and vials supplied by Strategic Diagnostics Inc. recommend storage between -25 and -20°C.

Facilities, Equipment, and Supplies

3.1 Facilities

The test 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 that were to be tested, and by the risk of sample and apparatus contamination. Facilities must be well ventilated, free of fumes, and isolated from physical disturbances or airborne contaminants that *might* affect the test organisms. The testing facilities should also be isolated from areas where test sediments are prepared, and removed from areas where equipment is cleaned.

3.2 Apparatus

3.2.1 Cleaning Procedure

All equipment and supplies that might contact test sediment or water must be clean and dry. All nondisposable materials should be washed after use. The following cleaning procedure (EC, 1997b, c) is recommended.

1. Soak in tap water for 15 minutes, then scrub with detergent or clean in an automatic dishwasher.

2. Rinse twice with tap water.

3. Rinse carefully with fresh, dilute (10%, v:v[3]) nitric (HNO_3) or hydrochloric acid (HCl) to remove scale, metals, and bases.

4. Rinse twice with deionized water.

5. Rinse once with full-strength, pesticide-grade acetone to remove organic compounds (use a fume hood or canopy). Use hexane for oily residues.

6. Rinse three times with high-quality deionized water.

3.2.2 Outline of Test and Associated Apparatus

This solid-phase test for measuring the toxicity of samples of whole sediment involves the following steps:

- preparation of a series of concentrations of the sample by serial dilutions in water;

- mixing this series with an inoculum of test organisms (reconstituted *V. fischeri*) and incubation for 20 minutes in test tubes held in a water bath at 15 ± 0.5°C;

- immediately thereafter, filtration of the contents of each test tube;

- the subsequent stabilization of the filtrate at 15 ± 0.5°C for 10 minutes in a series of cuvettes held within wells of a photometer; followed by

- the photometric reading of light produced by the luminescent bacteria remaining in the filtrate.

3.2.3 Equipment

The equipment and supplies required to achieve these steps are described briefly here. The supplier of specialty items should be

[3] To prepare a 10% solution of acid, carefully add 10 mL of concentrated acid to 90 mL of deionized water.

consulted for further details. Equipment and supplies which contact sediments or water must not contain *substance*s that can be leached or dissolved in amounts that adversely affect the test organisms, and should be chosen carefully to minimize sorption of *material*s from water.

Equipment for performing the solid-phase test for sediment toxicity includes:

- a Microtox™ Model 500 *Analyzer* or equivalent temperature-controlled photometer (15 ± 0.5°C for ≥15 cuvettes with test solutions; 5.5 ± 1°C for single cuvette holding reconstituted bacteria in *Reagent* well) capable of reading light output at a wavelength of 490 ± 100 nm (ASTM, 1995)
- refrigerated water bath with temperature controlled at 15 ± 0.5°C
- test tube rack or incubator block for incubating test tubes containing concentrations of test material and *V. fischeri* in the water bath
- freezer (not self-defrosting or "frost free" type) for storing lyophilized bacteria (*Bacterial Reagent*)
- pipettors for delivering volumes of 20, 500, 1000, and 1500 µL, with disposable plastic tips[4]
- disposable polystyrene *SPT* tubes (15.5 × 56 mm, 7.5-mL capacity, hemispherical bottom) or equivalent test tubes
- disposable glass cuvettes (borosilicate, 3-mL capacity, 50 mm length × 12 mm diameter, flat bottom)
- disposable filter columns for *SPT* or equivalent test tubes[5]
- volumetric borosilicate glassware (acid washed as per Section 3.2.1) for processing small aliquots of samples
- countdown timer or stopwatch
- magnetic plate mixer with Teflon™ stir bar
- a balance, accurate to 0.01 g
- a drying oven (100 ± 5°C)
- weighing vessels for dry weight determination
- metal spoon or spatula for sample homogenization

3.2.4 Supplies

The supply of test organisms is purchased as a standardized culture of freeze-dried bacteria ("*Bacterial Reagent*", see Section 2). Storage should be in a freezer at -20°C (EC 1992; ASTM 1995).

Non-toxic distilled or deionized water is used

[4] Precision and accuracy should be high (e.g., for 20-µL volumes, 2% precision and 10% accuracy or better; for 500-µL volumes, 1% precision and 5% accuracy or better; and for 1500-µL volumes, 2% precision and 5% accuracy or better. Disposable tips for the pipettors dispensing 1500-µL volumes should have a tip aperture >1.5-mm diameter to minimize clogging by solid particles (ASTM, 1995; EC, 1996a).

[5] These are polyethylene serum separation columns, 13-mm OD, 11-mm ID, 67-mm high, with a 15-mm diameter flexible seal on the lower end where the filter mates with the *SPT* tube. The filter itself, located at the lower end, has a 6-mm diameter and is 4-mm thick. Pore size is 15–45 µm according to the supplier [Evergreen Scientific, Los Angeles, CA, telephone (323) 583-1331, part number 208-3193-020].

to activate a vial of *Bacterial Reagent* (see Section 4.6). This water is frequently referred to as *Reconstitution Solution*, and can either be purchased as such (see Table 11; Appendix E), or taken from a laboratory supply and used after testing to confirm that it does not decrease light production by *V. fischeri*. This testing is accomplished by using a portion of the water to be used as *Reconstitution Solution* as the *control* water and for preparing test concentrations of the *reference toxicant*(s) used routinely at the laboratory for evaluating test performance with *V. fischeri*. Accordingly, the light production of *V. fischeri* achieved using the intended *Reconstitution Solution* should be evaluated in a *water-only test* with one or more reference *toxicant*s, according to the procedures and conditions given in Environment Canada (1990) as well as those provided in Section 4 "*Universal Procedures"* of Environment Canada's biological test method for performing *liquid-phase* toxicity tests using *V. fischeri* (EC, 1992).

A supply of *Solid-Phase Diluent* (see Table 5; Appendix E), comprised of 3.5% sodium chloride (NaCl), is required for diluting each sample of test sediment (see Section 4.6). This *Diluent* can either be purchased or prepared by dissolving 35.0 g NaCl (reagent grade) in 1000 mL of the *Reconstitution Solution* (i.e., non-toxic distilled or deionized water).

Procedure for Testing Sediment

4.1 Sample Collection

Environment Canada (1994) provides guidance on field sampling designs and appropriate techniques for sample collection; this guidance document should be followed when collecting samples of sediment to be tested for toxicity using this *reference method*.

Procedures and equipment used for sample collection (i.e., core, grab, dredge, or composite) will depend on the study objectives or regulatory requirements, and on the nature of the material being sampled. Samples of dredged sediment should be taken at all depths of interest.

Each series of toxicity tests performed using this *reference method* should include one or more samples of *reference sediment.* Sites for collecting samples of *reference sediment* should be sought where the geochemical properties of the sediment, including grain size characteristics, are similar to those at the site(s) where samples of *test* (contaminated or potentially contaminated) sediment are collected. Ideally, reference sediment should be collected from a site uninfluenced by the source(s) of contamination but within the general vicinity of the site(s) where samples of test sediment are taken. It is recommended that reference sediment from more than one site be collected to increase the likelihood of a good match with grain size and other physicochemical characteristics of the test sediments.

Any toxicity test involving one or more samples of coarse-grained test sediment with less than 20% fines must include a sample of *clean reference sediment* or *negative control sediment* as part of the testing regime, for comparative purposes in judging sample toxicity (see Section 6.2). This reference or negative control sediment must have a percent fines content that does not differ by more than 30% from that of the sample(s) of test sediment against which it is compared (Section 6.2). Field-collected samples of clean reference sediment or negative control sediment may be used for this purpose if their percent fines meets this matching requirement, in which instance the guidance here for sample collection applies. Alternatively, the investigator may choose to formulate one or more samples of *artificial negative control sediment* for this purpose, to enable the close matching of its/their percent fines with that of the sample(s) of coarse-grained test sediment. Guidance on preparing artificial negative control sediment is provided in Sections 4.3 and 4.4.

The number of stations to be sampled at a study site and the number of *replicate samples* per station will be specific to each study. This will involve, in most cases, a compromise between logistical and practical constraints (e.g., time and cost), and statistical considerations. Environment Canada (1994) should be consulted for guidance with respect to the sampling design, including the recommended minimum number of field replicates. Additional guidance on sampling for disposal-at-sea applications is found in Environment Canada (1995a; 2002a). Applicants are encouraged to consult with their regional Environment Canada Ocean Disposal Office (see Appendix C for contact information), before sampling and testing.

Where practical and consistent with the study design and objectives, a minimum of five samples of sediment should be taken from each discrete *sampling station* and depth of interest. Where practical and appropriate (see Section 6), sample collection should also include ≥5 samples from each of one or more reference stations (i.e., sites where uncontaminated sediment, having physicochemical properties similar to that of the test sediments, can be found) within the vicinity. The objective of collecting replicate samples at each station (*field replicates*) is to allow for quantitative statistical comparisons within and among different stations (EC, 1994; 1998; 2002b). Accordingly, each of these "true replicate" samples of sediment should be tested for its *acute toxicity* to *V. fischeri*. *Laboratory replicates*, using subsamples of each field-collected sample of test and reference sediment after mixing and other manipulation (see Section 4.3), might also be included in a study in instances where sample homogeneity or precision of test results are in question.

A benthic grab (i.e., Smith-MacIntyre, Van Veen, PONAR) or core sampler should be used to sample sediment rather than a dredge, to minimize disruption of the sample. Care must be taken during sampling to minimize loss of fines. The same collection procedure should be used for all field sites sampled.

The volume of sample required to perform a multi-concentration test for sediment toxicity using *V. fischeri* is small (see Section 4.6). A sample volume of ~100 mL should be submitted specifically for the performance of this test.[6] A per-sample volume of at least 5 to 7 L of whole sediment is frequently required (EC, 1994), although this will depend on the study objectives/design and on the nature of the associated physicochemical analyses and the *battery of toxicity tests* to be performed. To obtain the required sample volume for a battery of toxicity tests, it is frequently necessary to combine subsamples retrieved using the sampling device. Guidance provided in Environment Canada (1994) for compositing subsamples in the field should be followed.

4.2 Sample Labelling, Transport, and Storage

In addition to the following, more detailed and useful guidance pertaining to sample labelling, transport, and storage is found in Environment Canada (1994). Persons undertaking these procedures should be familiar with this guidance document.

Containers for transporting and storing samples must be new or thoroughly cleaned, and rinsed with clean water. Environment Canada (1994) should be consulted for guidance in selecting suitable containers. Each sample container should be filled completely, to exclude air. Immediately after filling, each sample container must be sealed, and labelled or coded. Labelling and accompanying records made at this time must include at least a code which can be used to identify the sample or subsample. A cross-referenced record, which might or might not accompany the sample or subsample, must be made by the field personnel identifying the sample type (e.g., grab, core, composite), source, precise location (e.g., water body, latitude, longitude, depth), *replicate* number, and date of collection. This record should also include the name and signature of the sampler(s). Sediment sample collectors should also keep records describing:

[6] This volume is sufficient to provide >100 g wet weight of sediment, and is enough to allow ten or more tests to be run on the sample.

- the nature, appearance, volume and/or weight of each sample;
- the sampling procedure and apparatus;
- any procedure used to composite or subsample grabs or cores in the field;
- the number of replicate samples taken at each *sampling station*;
- the sampling schedule;
- the types and numbers of containers used for transporting the samples;
- any field measurements (e.g., temperature, *salinity*, *pH*, dissolved oxygen) of the overlying water or sediment at the collection site; and
- procedures and conditions for cooling and transporting the samples.

Upon collection, warm (>7°C) samples should be cooled to between 1 and 7°C with regular ice or frozen gel packs, and kept cool (4 ± 3°C) in darkness throughout transport (EC, 1994; 1998). As necessary, gel packs, regular ice, coolers, or other means of refrigeration should be used to assure that sample temperatures range within 1 to 7°C during transit. Samples must not freeze or partially freeze during transport or storage, and must not be allowed to dry (EC, 1994).

Upon arrival at the laboratory, the sample temperature and date of receipt must be recorded on a bench sheet (see example, Appendix F). Samples to be stored for future use must be held in airtight containers and in darkness at 4 ± 2°C (EC, 1994; 1998). It is recommended that samples of sediment or similar particulate material be tested as soon as possible after collection. The sediment toxicity test should begin within two weeks of sampling, and preferably within one week; the test must start no later than six weeks after sample collection (EC, 1994; 1997b; 1997c; 1998).

4.3 Sample Manipulation and Characterization

Samples of field-collected *test sediment* and *reference sediment* must not be wet-sieved. Particles ≥2 mm should be removed along with large debris or large indigenous macro-organisms. Depending on the sample, this *may* be accomplished by using forceps or a gloved hand. Forceps or gloves contacting each sample should be rinsed or replaced thereafter, to prevent cross-contamination. If a sample contains a large number of particles ≥2 mm and/or a large number of indigenous macro-organisms which cannot be removed using forceps or a gloved hand, the sample may be press-sieved (not washed) through one or more suitably sized (e.g., ≥2 mm) mesh stainless steel screens. Such manipulation should include all portions of the sample used for physicochemical (including grain size) analyses as well as those used for solid-phase sediment toxicity tests with *V. fischeri*. Procedures used to manipulate each sample must be recorded on the bench sheet (see "Notes" in example, Appendix F).

Any *pore water* that has separated from the sample during shipment and storage must be mixed back into the sediment. To achieve a homogeneous sample, either mix it in its transfer/storage container, or transfer it to a clean mixing container. The sample should normally be stirred using a nontoxic device (e.g., stainless steel spoon or spatula), until its texture and colour are homogeneous. Alternatively, a mechanical method (EC,

1994; 1998) may be used to homogenize the sample. For each sample included in a test, mixing conditions including duration and temperature must be as similar as possible. If there is concern about the effectiveness of sample mixing, subsamples of the sediment should be taken after mixing, and analyzed separately to determine homogeneity.

Immediately following sample mixing, subsamples of test material required for this and other (e.g., EC, 1998) toxicity tests and for physicochemical analyses must be removed and placed in labelled test chambers, and in the labelled containers required for storage of samples for subsequent physicochemical analyses. Any remaining portions of the homogenized sample that might be required for additional toxicity tests using amphipods (EC, 1998) or other test organisms should also be transferred at this time to labelled containers. All subsamples to be stored should be held in sealed containers with no air space, and must be stored in darkness at 4 ± 2°C until used or analyzed. Just before it is analyzed or used in the toxicity test, each subsample must be thoroughly re-mixed to ensure that it is homogeneous.

Each sample (including all samples of *reference sediment, negative control sediment,* and *positive control sediment*) must be characterized by analyzing subsamples for at least the following (EC, 1998): for whole sediment—percent very coarse-grained sediment (i.e., particles >1.0 mm), percent sand (i.e., particles >0.063 to 2.0 mm), percent *fines* (i.e., particles ≤0.063 mm), percent water content, and total organic carbon content; for pore water—salinity and pH. Other analyses could include: total inorganic carbon, total volatile solids, biochemical oxygen demand, chemical oxygen demand, cation exchange capacity, acid volatile sulphides, metals, synthetic organic compounds, oil and grease, petroleum hydrocarbons, and porewater analyses for various physicochemical characteristics such as ammonia (total and un-ionized) or hydrogen sulphide. Recommended procedures for collecting pore water are described in Environment Canada (1994) and should be followed here. For disposal-at-sea applications, minimum information requirements are explained in two guideline documents by Environment Canada (i.e., EC, 1995a; 2002a).

Analyses for particle size distribution must be undertaken as soon as possible after sample collection, to enable the selection of the appropriate sample(s) of *reference sediment* and, if used, *negative control sediment* (see Sections 4.4 and 6.2).

Previous studies have found that *V. fischeri* is quite tolerant of elevated concentrations of ammonia in water (Qureshi *et al*., 1982) or pore water of sediment samples collected from the marine or estuarine environment (McLeay *et al.*, 2001). These limited data indicate that elevated levels of ammonia in sediment pore water are not a major confounding factor influencing test results. However, investigators might wish to investigate the extent to which porewater ammonia might contribute to sample toxicity as determined using this *reference method*. In this instance, analyses for porewater pH, salinity, and ammonia should be undertaken within 24 h of the solid-phase test for sediment toxicity using *V. fischeri*, to enable determinations of the concentrations of total and un-ionized ammonia to which test organisms were exposed and the possible influence of this on test results (Section 6.2). Ammonia analyses must be conducted using

a recognized and standardized procedure (for example, APHA *et al.*, 1995; Standard Methods). Calculations of concentrations of un-ionized ammonia must be based on the test temperature and on the porewater pH and salinity of the sample (Trussell, 1972; Bower and Bidwell, 1978).

4.4 Negative and Positive Control Sediment

Toxicity tests restricted to one or more samples of fine-grained sediment (i.e., samples with ≥20% fines) need not include a sample of negative control sediment or *clean* reference sediment, since the toxicity of such samples is not judged by comparison of test results with those for a sample of uncontaminated sediment with similar grain-size characteristics. However, any toxicity test involving one or more samples of test sediment with <20% fines must include a sample of *clean sediment* with a percent fines content that does not differ by more than 30% from that of the sample(s) of test sediment against which it is compared when judging toxicity (see Section 6.2). Field-collected *clean* sediment from an uncontaminated site may be used for this purpose. However, the use of laboratory-formulated ("*artificial"*) negative control sediment is recommended, since it can be prepared to closely match the percent fines of the test sediment(s). Artificial negative control sediment should be prepared in the laboratory using an appropriate mixture of commercially available kaolin clay and/or washed silica sand with grain sizes matching those of the test sediment(s). These ingredients should be mixed thoroughly in proportions similar to those of the test sediment(s). Preparation procedures and results for *V. fischeri* solid-phase toxicity tests using artificial negative control sediment are reported in Ringwood *et al.* (1997), Tay *et al.* (1998), and McLeay *et al.* (2001).

The percentage of fines (particles ≤0.063 mm) for the sample(s) of negative control sediment included in a particular toxicity test should be matched as closely as possible to that of the test sediment(s). If a series of test sediments with a wide range of percent fines is being measured consecutively for toxicity as part of a study, more than one negative control sediment, with percent fines matched as closely as possible to the range of percent fines for the test sediments, might be included in the study.

The use of one or more samples of *positive control sediment* is recommended for inclusion in each series of toxicity tests, to assist in interpreting test results (Section 6.2). The *positive control sediment* might be a *standard contaminated sediment* such as one available through the National Research Council of Canada's Marine Analytical Chemistry Standards Program, Ottawa, ON (e.g., HS-3, Cook and Wells, 1996; HS-6, Tay *et al.*, 1998). A second approach is to use a sample of *clean sediment* (e.g., *artificial negative control sediment* or field-collected *clean reference sediment*) that has been spiked experimentally with a *toxic chemical* (EC, 1995b). A third option is to use a highly contaminated sample of field-collected sediment shown previously to be toxic to *V. fischeri* in solid-phase testing; this option is not recommended unless the characteristics (including performance in a *solid-phase* test using *V. fischeri*) of this sediment are well known beforehand. *Positive control sediment* must be used as a *reference toxicant* when appraising the sensitivity of the test organisms and the precision and reliability of results obtained by the laboratory for that reference material (Section 5).

4.5 Test Conditions

4.5.1 Outline of Test

This solid-phase test for measuring the toxicity of samples of whole sediment involves the steps and associated apparatus outlined in Section 3.2.2.

Table 1 provides a checklist of the conditions that are required or recommended for this *reference method*. Further details are given in Sections 4.5.2 to 4.5.5.

4.5.2 Manipulations, Adjustments, and Corrections

- Test sediments, including the sample(s) of *reference sediment* recommended for inclusion in each test series, must not be wet-sieved and no adjustments of porewater salinity are permitted.[7]

- Sample pH must not be adjusted.

- No aeration of samples, test concentrations, or filtrates should be performed.

- Light-emission readings for concentrations of test sediments must not be adjusted or corrected using readings for concentrations of reference or other sediments.[8]

- The statistical *endpoint* for the test (i.e., IC50; see Section 6.1) must be normalized for the moisture content of the sample.

4.5.3 Temperature

- The test is conducted using the luminescent bacterial species, *Vibrio fischeri*, strain NRRL B-11177, which is reconstituted to an active state in pure non-toxic water and held at 5.5 ± 1.0°C until aliquots are transferred to each test concentration. Normally this temperature is met by placing the cuvette with the reconstituted bacterial solution in the specified well of the photometer if a Microtox™ Model 500 *Analyzer* is used. Otherwise a temperature-controlled incubator must be used for this purpose.

- All concentrations of each sample of test sediment (including reference sediment) inoculated with bacteria must be incubated for 20 minutes at 15 ± 0.5°C. A temperature-controlled water bath or room would serve this purpose.

- Following incubation and filtration, all test solutions transferred to cuvettes must be held at 15 ± 0.5°C during the subsequent 10-minute period for stabilization of the filtrates. This temperature control is normally achieved within the wells of the photometer. Alternatively, the cuvettes containing test filtrates can be held within this temperature range in a cuvette holder placed in a temperature-controlled incubator or room.

4.5.4 Timing of Events

- The lyophilized bacteria should be reconstituted immediately before inoculating the test concentrations. This bacterial solution should be used within 2 h, and must be used within 3 h of

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[7] The use of *Solid-Phase Diluent* comprised of 3.5% NaCl, when preparing the series of test concentrations (see Section 4.6.4), provides effective osmotic adjustment for samples of marine, estuarine, or freshwater sediment.

[8] A number of current or older solid-phase sediment toxicity tests using luminescent bacteria correct for colour and/or turbidity of test sediments, by adjusting light-emission values against corresponding ones for the reference sediment (see Appendix E, Tables 14 and 16). Such an approach assumes a close match between test and reference sediments for these variables, which might not be the case.

Table 1 Checklist of Required or Recommended Test Conditions

Facilities and equipment	– photometer (e.g., Microtox™ Model 500 *Analyzer*) reading light output at 490 ± 100 nm; incubator for single cuvette holding reconstituted bacteria at 5.5 ± 1°C; for ≥15 cuvettes, at the test temperature (15 ± 0.5°C), in an incubator or controlled-temperature room
Reconstitution Solution	– pure, non-toxic water
Control/dilution water (*"Diluent"*)	– use 3.5% NaCl solution, purchased (e.g., from Strategic Diagnostics Inc.) or made using *Reconstitution Solution*.
Test temperature	– 15 ± 0.5°C
Sample pH, salinity	– no adjustment
Colour, turbidity	– no correction
Aeration	– none required
Subsamples for moisture content	– 3 replicates of 5.0 ± 0.2 g (precision, ± 0.01 g) dried at 100 ± 5°C for 24 h
Primary dilution	– 7.00 ± 0.05 g whole, homogenized sediment in 35.0 mL dilution water, glass or disposable plastic beaker, mixed for 10 min on a magnetic stirrer with Teflon stir bar, at a rate such that the vortex depth is half the height of the liquid level
Test concentrations	– maximum test concentration normally 197 000 mg/L (19.7%, wet wt:vol) on wet-weight basis with two-fold dilutions, for a total of 12 test concentrations in disposable polystyrene tubes; three *control* solutions (*Diluent* only); left for 10 min to equilibrate to the test temperature
Test species	– *Vibrio fischeri*, strain NRRL B-11177, reconstituted by swirling vial three to four times, emptied into disposable glass cuvette, mixed 10 times with 0.5 mL pipette and held at 5.5 ± 1°C
Inoculum	– 20 µL into each test concentration, mixed three times with 1.5 mL pipette
Incubation	– 20 min at test temperature, filter columns inserted into tops of *SPT* tubes above surface of test concentration
Filtrate transfer	– 500 µL into disposable glass cuvettes at test temperature, left 10 min
Observations	– cuvettes inserted into photometer read well, light levels of all test filtrates and controls measured
Endpoint	– IC50 (mg/L), calculated by software or manually; normalized for moisture content of sediment (i.e., calculated on dry-weight basis)
Reference sediment	– should be included in the test series, using the same procedures applied to the sample(s) of *test* (contaminated or potentially contaminated) *sediment*
Test with *reference toxicant*	– performed within one month of each solid-phase sediment toxicity test with *V. fischeri*, using a suitable *positive control sediment* and the procedures and conditions herein for measuring the toxicity of test sediment

reconstitution. The time of reconstitution should be logged on a bench sheet (see example, Appendix F).

- Test concentrations must be allowed to equilibrate to 15 ± 0.5°C for a minimum of 10 minutes before inoculation with bacterial solution. Inoculation should proceed as quickly as possible; all test concentrations should be inoculated within a total time span of ≤4 minutes. Record the time of the first inoculation as the start of the test, on the bench sheet (Appendix F).

- All test concentrations must be incubated for 20 minutes. Once the test concentrations are filtered and transferred to cuvettes, the filtrates must be incubated in cuvettes for 10 minutes before their light output is measured.[9]

- Total lapsed time for the transfer of filtrates to cuvettes and for reading the luminescence of the test filtrates should be similar to that (≤4 min) spent inoculating the test concentrations with bacteria.

4.5.5 Test Array

- The test array consists of three controls (comprised of dilution water only) and twelve test concentrations.

- The maximum test concentration is normally 197 000 mg/L (19.7%, wet wt:v), with each successive concentration normally being 50% of the previous one.

[9] This is to allow the temperature of the test concentrations and the luminescent bacteria to stabilize following their filtration, before reading the bacterial light emission.

4.6 Test Procedures

This biological test method involves the simultaneous incubation of a minimum of three control solutions (comprised of an inoculum of reconstituted *V. fischeri* in *Diluent*) together with 12 differing concentrations of test material in *Diluent*.[10] After a prescribed incubation period, the incubated solutions (held in test tubes at a controlled temperature) and test concentrations are filtered, and the resulting filtrates are transferred to cuvettes. After a brief period for stabilization of holding conditions for the filtrates, the light production by the test organisms remaining in the filtrate is measured by a photometer.

In this section, the procedures applied to a photometer assume the use of a Microtox™ Model 500 *Analyzer* or another photometer with similar features. Since the Microtox™ Model 500 *Analyzer* has 30 wells for holding cuvettes containing filtrates of test concentrations, the laboratory technician using this photometer has the option of duplicating a test, or performing two tests simultaneously on different samples. The following procedural description follows a single sample of sediment through the various steps. Depending on the study design and the nature and source of the test sediments, one or more samples of field-collected *reference sediment* should also be included in each study (see Sections 4.1 and 6.1). Toxicity tests involving one or more

[10] The Model 500 *Analyzer* has 30 wells for incubating cuvettes at 15 ± 0.1°C. Using this or an equivalent temperature-controlled photometer with ≥30 incubation wells, two samples can be tested at the same time. This approach has been recommended by McLeay *et al.* (2001) as a more efficient way of testing multiple samples and as a means of minimizing the amount of *Bacterial Reagent* required.

samples of coarse-grained (i.e.,<20% fines) sediment must include a *negative control sediment* (artificial or natural) or a *reference sediment*, with a percent fines content that does not differ by more than 30% from that of the test sediment(s) (see Section 6.2). The use of a *positive control sediment* (Section 4.4) as part of each series of toxicity tests is also recommended. In order to include one or more samples of *test sediment* along with *reference sediment, negative control sediment*, and/or *positive control sediment* in a single study, it is necessary to run each sample consecutively.

4.6.1 Photometer, Water Bath, and Bench Sheet

- Switch on the computer, photometer, and balance.
- For the Model 500 *Analyzer,* ensure that the temperature selector switch at the back is set to "Microtox Acute".
- Place 15 cuvettes in the first three rows (A–C) of wells. These will be maintained at 15 ± 0.5˚C. The incubated wells are arrayed in a grid of rows labelled A to C and columns numbered 1 to 5. They are referenced as A1 to C5.
- Place one cuvette in the reagent well, and pipette 1.0 mL of *Reconstitution Solution* into it. This will be maintained at 5.5 ± 1˚C.
- Switch on the water bath incubator. Allow the water temperature to stabilize at 15 ± 0.5˚C.
- Stir the sample to homogenize it, using a stainless steel spoon.
- Fill out a bench sheet (see Section 7.2.7 and Appendix F). Place 15 *SPT* tubes (see Appendix E, Table 5) into a rack.

4.6.2 Subsamples for Moisture

- For each test sediment, label and weigh three empty vials (e.g., 20-mL glass scintillation vials or other suitable containers for drying and weighing), and record the weights to the nearest 0.01 g. Enter the data, if a spreadsheet is used when converting the IC50 to a value based on dry weight (Section 6.1).
- Add 5.0 ± 0.2 g of sediment to the vials and record the weights to the nearest 0.01 g (or enter them into the spreadsheet).
- Dry the subsamples by putting the vials into an oven at 100 ± 5°C for 24 h. Record the oven temperature.
- Record the dry weights to the nearest 0.01 g.

4.6.3 Primary Dilution

- Weigh 7.00 ± 0.05 g of homogenized sample into a glass or disposable 50-mL plastic beaker.
- Add a 2.5-cm Teflon-coated magnetic stir bar and 35.0 mL of *Solid-Phase Diluent* to the beaker.
- Stir for 10 min on a magnetic stirrer at a rate sufficient to create a vortex that reaches 1/2 the height of the liquid level.

4.6.4 Test Concentrations

- Dispense 1.50 mL *Solid-Phase Diluent* (Section 3) into the first 14 tubes in the rack (Section 4.6.1). Tube 15 will be the highest concentration, taken from the primary dilution.
- Following the 10-minute stirring of the primary dilution, use a large-bore macropipette tip to transfer 1.50 mL of sample suspension from the 50-mL

beaker, while it is still stirring, to each of *SPT* tubes 15 and 14. To do this, insert the pipette tip near the side of the beaker, at about half the depth of the stirring sample. Avoid plugging the tip while aspirating the sample.

- Beginning at *SPT* tube 14, which now has 3.00 mL of solution, make 1:2 serial dilutions as follows. Mix the contents three times with the macropipette, and then quickly draw up a volume of 1.50 mL from about one third depth (to help draw some of the heavier sand grains). Transfer this aliquot to tube 13. Repeat this mixing and transferring from tube 13 to tube 12, and continue consecutively thereafter from tube 12 to tube 11, tube 11 to tube 10, etc. until 1.5 mL of the 1:2 serial dilutions is transferred into tube 4. Finally, discard 1.5 mL from tube 4. Tubes 1–3 contain *Diluent* only, and serve as the controls.

- Place the rack with *SPT* tubes containing all of the test concentrations (including controls) into the water bath at 15 ± 0.5°C. Leave it for 10 min to allow the temperature to equilibrate. The water level of the bath should be just above the liquid level in the *SPT* tubes.

4.6.5 Reconstitution of Bacterial Reagent

- Take a vial of freeze-dried bacteria (Microtox *Acute Reagent*) from storage.

- Open the vial and reconstitute its contents by quickly pouring the *Reconstitution Solution* from the cuvette in the reagent well of the Model 500 *Analyzer* (or other photometer) into the vial, swirling three times and pouring the rehydrated bacteria into the same cuvette.

- Replace the cuvette in the reagent well.

- Using a 500-µL pipette, aspirate any remaining *Reconstituted Bacterial Reagent* from the vial, add it to the cuvette, and mix 10 times using the same pipette and tip.

- Record the reagent lot number, expiry date, and time of reconstitution on the bench sheet (see Section 7.2.7 and Appendix F).

4.6.6 Inoculation and Incubation

- Prepare the following three pipettes: (a) a repeat pipette (such as an Eppendorf or Oxford Nichiryo) with a 0.5-mL syringe fitted with an ultramicro tip; (b) a macropipette (e.g., Oxford 1-5 mL); and (c) a 500-µL pipette.

- Following the 10-min temperature equilibration of the *SPT* tubes containing the test concentrations (Section 4.6.4), set a timer for 20 min but do not start it.

- Make sure the repeat pipette is set to dispense 20 µL per ejection. Place the tip below the surface of the reconstituted bacteria (*Reconstituted Bacterial Reagent)*, and draw up sufficient *Reagent* for at least 18 ejections.

- Holding the tip above the *Reagent* and against the cuvette wall, eject two times. Carefully wipe the tip with a clean wiper (e.g., using a KimWipe™) after drawing up the *Reagent*. Then, immerse the tip in a tube or beaker containing *Solid-Phase Diluent* and make another ejection (to rinse the outside of the tip). Discard the contents of this tube or beaker.

- Start the 20-min timer, record the time as the "Start" of the test on the bench sheet, and immediately eject 20 µL of *Reagent* into each of the *SPT* tubes, starting with tube 1 (first control solution) and

continuing consecutively to and including tube 15. Resting the collar of the ultramicro tip on the top edge of each tube will assure the tip will be at the surface of the sample and not on the bottom of the tube.

- Remove and discard the ultramicro tip. Eject the remaining *Reagent* from the syringe into the holding cuvette in the reagent well.

- If a second test (i.e., a test with either a second sample or a duplicate test with the same sample) is to be performed concurrently using the remaining 15 wells of the *Analyzer,* refit the syringe with a clean tip, refill it with *Reagent*, and inoculate the next series of test concentrations. This second (concurrent) test may be performed using another sample of *test* (*contaminated* or potentially contaminated) *sediment*, *reference sediment*, *negative control sediment,* or *positive control sediment*.

- With the macropipette set at 1.5 mL, mix each tube twice, beginning with tube 1 (first control) and proceeding consecutively through to and including tube 15.

- Insert a filter column (see footnote 5 in Section 3.2.3) in each tube, with its lower end positioned ~1 cm above the surface of the liquid. Do not wet the filter, since this might adversely affect filtration of the sample.

4.6.7 Preparing the Computer

- Prepare the computer to receive data from the photometer.

- Start the appropriate software program and menus for the *solid phase* test.

- Follow the on-screen instructions.

- Refer to the user's manual for additional information.

- Information requested by the software might include the number of controls (3), number of dilutions (12), initial concentration (197 000 mg/L), dilution factor (2), and the test time (30 min).

4.6.8 Filtration

- When the 20-min timer sounds, respond to the computer software as appropriate.

- Reset the timer for 10 min and start it. If using Strategic Diagnostics Inc. software (Omni™ Version 1.18 or equivalent), this period is automatically initiated. Otherwise, program the computer software to initiate this 10-min period automatically at the touch of a key.

- Gently push the filter in tube 1 (first control) down far enough to obtain slightly greater than 500 µL of filtrate in the tube. Then, using the 500-µL pipette, transfer 500 µL of filtrate to the cuvette in well A1 of the photometer.

- Repeat this step for all 15 tubes using the same pipette tip, ending with 500 µL of filtrate being transferred from tube 15 to cuvette C5.

- Take note of the time required to complete all the transfers.

- Often with sediments having a high proportion of *fines*, the filter in the highest test concentration (tube 15) will become plugged. Obtain what can be recovered, and transfer it to the designated cuvette (C5). Usually the luminescence from these samples will

have decreased to zero before you test the highest concentration. Make a note of such problems on the bench sheet (see Section 7.2.7 and Appendix F).

- At the end of all transfers, respond to the computer if required. Usually this will involve reading light production (Section 4.8), taking approximately the same amount of time as was taken to filter and transfer the filtrate to the cuvettes.

- The data is often sent from the photometer to the computer, and received by the software which builds a data file.

4.7 Test Measurements and Observations

Section 4.3 should be consulted for requirements and recommendations related to sample characterization (e.g., measurements of grain size and other physicochemical characteristics of the test material).

The procedure to measure light production of the bacteria in the test concentrations will vary depending on the photometer and software used. For the Model 500 *Analyzer*, place the first control (cuvette A1) into the read well and press the "set" button. The instrument lowers the cuvette into the well (sometimes 2 or 3 times) to set the zero (dark) and control reading at about 95 and thereby establish the appropriate sensitivity range for light measurements. After the green "ready" light appears, press the "read" button. Read the cuvette, then remove it from the read well and replace it in the incubator block. If the software program does not collect the light measurement data, record it on the bench sheet. Proceed to read and record the light emission from all the cuvettes, taking approximately the same average time per cuvette as was taken to do the filtering and transferring (see Section 4.6.8). If using Strategic Diagnostics Inc. software (Omni^{TM} Version 1.18 or equivalent), this timing is performed by the computer and prompts occur indicating when each cuvette should be read.

If a problem arises, refer to the user manuals for whatever photometer and software you are using.

4.8 Criterion for a Valid Test

The following criterion must be met for a valid sediment toxicity test, performed using this *reference method*:[11]

> The *coefficient of variation* representing the mean light reading measured for the filtrates of the three control solutions included in the test must be $\leq 12\%$.

[11] This criterion is derived from ASTM (1995) (see Appendix E, Table 17).

Procedure for Testing a Reference Toxicant

The routine use of a reference toxicant 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 laboratory personnel using the Solid Phase Reference Method (EC, 1992). Reference toxicant(s) must be tested at least once a month during periods when solid-phase tests for sediment toxicity using *V. fischeri* are being run, and initially upon first use of a new shipment or batch of *Bacterial Reagent*.

One or more samples of *positive control sediment* (see Section 4.4) must be used as the reference toxicant(s) for this *reference method*. When performing each *reference toxicity test*, the test procedures and conditions defined in Sections 4.5 and 4.6 must be followed.

For performing a reference toxicity test according to the test procedures and conditions defined herein, use of a *standard contaminated sediment* such as a NRCC Harbour Marine Reference Material (e.g., HS-5 or HS-6), which can be purchased from the National Research Council of Canada, is recommended.[12] Alternatively, a *spiked control sediment* may be used as a reference toxicant, after suitable spiking procedures are standardized. In this regard, Environment Canada's guidance document on using control sediment spiked with a reference toxicant should be consulted (EC, 1995b).

When undertaking a reference toxicity test, a series of concentrations should be chosen which, based on preliminary and/or previous tests performed using the same conditions and procedures, will enable calculation of an IC50 for light emission by *V. fischeri* (see Section 6.1), with acceptably narrow 95% confidence limits. The selected test concentrations should bracket the predicted IC50.

It is the responsibility of laboratory personnel to demonstrate their ability to obtain consistent, precise results with the reference toxicant before definitive sediment assays are conducted using this *reference method*. To meet this responsibility, the laboratory personnel should initially determine their intralaboratory precision, expressed as percent coefficient of variation (% CV), by performing five or more reference toxicity tests with different lots of *Bacterial Reagent* (Section 2.1), using the same reference toxicant and the procedures and conditions defined herein. This should be conducted to gain experience with the test procedure, and as a point of reference for future tests (EC, 1998). While routinely performing this reference toxicity test with each lot of *Bacterial Reagent*, laboratory personnel should continue to follow this same procedure. Once sufficient data are available (EC, 1995b), IC50s derived from these tests must be plotted successively on a reference toxicant-specific *warning chart*, and examined to determine whether the results

[12] National Research Council of Canada, Marine Analytical Chemistry Standards Program, Institute for Marine Biosciences, 1411 Oxford Street, Halifax, NS; phone: 902-426-8280; fax: 902-426-9413; e-mail: crm.imb@nrc.ca.

are within ± 2 SD of values obtained in previous tests using the same reference toxicant and test procedure. A separate warning chart must be prepared and updated for each reference toxicant used as part of this *reference method*. The warning chart should plot logarithm of concentration on the vertical axis against date of the test or test number on the horizontal axis. Each new IC50 for the reference toxicant must be compared with the established limits of the chart; the IC50 is acceptable if it falls within the *warning limits*. All calculations of mean and standard deviation should be made based on log(IC50).

The logarithm of concentration (including IC50) should be used in all calculations of mean and standard deviation, and in all plotting procedures. This simply represents continued adherence to the assumption by which each IC50 was estimated based on logarithms of concentrations. The warning chart may be constructed by plotting the logarithmic values of the mean and ±2 SD on arithmetic paper, or by converting them to arithmetic values and plotting those on the logarithmic scale of semi-log paper. If it were demonstrated that the IC50s failed to fit a log-normal distribution, an arithmetic mean and SD might prove more suitable. The mean of the available values of log(IC50), together with the upper and lower warning limits (±2 SD), should be recalculated with each successive IC50 until the statistics stabilize (EC, 1995b; 2002b).

If a particular IC50 fell outside the warning limits, the sensitivity of the test organisms and the performance and precision of the test would be suspect. Since this might occur 5% of the time due to chance alone, an outlying IC50 would not necessarily indicate abnormal sensitivity of the lot of *Bacterial Reagent* or unsatisfactory precision of toxicity data. Rather, it would provide a warning that there might be a problem. A thorough check of all test conditions and procedures should be carried out. Depending on the findings, it might be necessary to repeat the reference toxicity test, or to obtain a new lot of *Bacterial Reagent* for evaluating the toxicity of the samples of test material (together with a new reference toxicity test using the new lot of test organisms).

Results that remained within the warning limits might not necessarily indicate that a laboratory was generating consistent results. Extremely variable data for a reference toxicant would produce wide warning limits; a new data point could be within the warning limits but still represent undesirable variation in test results. A coefficient of variation of no more than 30%, and preferably 20% or less, is suggested as a reasonable limit by Environment Canada (1995b). For this *reference method*, the coefficient of variation for mean historic data derived for the reference toxicity tests performed at a testing facility should not exceed 30%.

Data Analysis and Interpretation

6.1Data Analysis

The mean and standard deviation of the light readings for the control solutions used in the study (see Sections 4.6.4, 4.7, and 4.8) must be calculated. These values are used to calculate the coefficient of variation of the mean for the control solutions, which is used as the criterion described herein for judging if the test results are valid (Section 4.8).

A study performed according to this *reference method* should include one or more samples of *test* (contaminated or potentially contaminated) *sediment* together with one or more samples of *reference sediment*. Additionally, the inclusion of one or more samples of *positive control sediment* (see Sections 4.4 and 5) is recommended. Tests involving one or more samples of coarse-grained sediment (i.e., sediment with <20% fines) must also include one or more samples of *negative control sediment* (artificial or natural) or *clean reference sediment* with a percent fines content that does not differ by more than 30% from that of the coarse-grained test sediment(s) (see Section 6.2). In each instance, the statistical endpoint to be calculated for each of these test materials is the *ICp* (*inhibiting concentration for a specified percent effect*).

Unless specified otherwise by regulatory requirements or by design, the endpoint for this *reference method* is the concentration causing 50% inhibition of light, i.e., the IC50. The calculations to estimate the IC50 and its 95% confidence limit are included in the most recent (1999 or later) Omni™ software packages (Version 1.18 or equivalent) formerly marketed by AZUR Environmental Ltd. and now available from Strategic Diagnostics Inc. (see Section 2.2 for contact information).[13] Alternatively, guidance for estimating IC50 (together with its 95% confidence limit) is provided in EC (1992), and other statistical software packages are available which enable this calculation (EC, 1997b; 1997c; 2002b).

In the absence of a computer with appropriate software, the IC50 can be calculated using one of the following two equations and approaches.

For each test concentration, *Gamma* (Γ; see definition in Terminology) is calculated (ASTM, 1995) as:

$$\Gamma = (I_c/I_t) - 1$$

where: I_c = the average light reading of filtrates of the control solutions, and

I_t = the light reading of a filtrate of a particular concentration of the test material.

The Gamma values for each test filtrate falling within 0.02 < Gamma < 200 are

[13] In 1999, AZUR released an operating software termed "Microtox Omni". This software includes a calculation option called "*AutoCalc*". The "*AutoCalc On*" option must be selected if this software is applied; otherwise, erroneous results might occur. *AutoCalc* selects only the data points around the IC50 that have *Gamma* in the range of 0.02 < Gamma < 200. Using these data points, all the various contiguous series are examined by an iterative convergence regression analysis to determine the tightest 95% confidence limits.

plotted manually, and a line is fitted by eye. Then, the IC50 is read off as the concentration that corresponds to a Gamma of 1.0. The manually plotted points should be checked against the observed readings to guard against errors in entry and anomalous estimates of IC50. The manual plot and its estimated IC50 should also be checked against any computer-generated graph and the computer calculation of the IC50 (EC, 1992).

Alternatively, a linear regression of logC (concentration, on the ordinate) vs. logΓ (on the abscissa) is computed according to ASTM (1995):

$$\log\Gamma = b(\log C) + \log(a)$$

In this equation, 'b' is the slope and 'log(a)' is the intercept of the regression line with the ordinate (y-axis) at logΓ = 0, corresponding to Γ = 1. Therefore, 'a' obtained as $10^{\log(a)}$ is the IC50.

The IC50 and its associated values for the 95% confidence limits must be converted to and expressed as mg/L on a dry-weight basis. This is achieved using the dry-weight data (see Section 4.6.2) (ASTM, 1995). The IC50 (as well as the upper and lower value of the confidence limits) of the wet sediment is multiplied by the average of the ratios of the dry-to-wet subsample weights:

$$IC50 = IC50_w \times [(S1_d/S1_w) + (S2_d/S2_w) + (S3_d/S3_w)]/3$$

where: $IC50_w$ is the calculated IC50 (or its 95% confidence limits) of the wet sediment sample,

$S1_d$ through $S3_d$ are the dry weights of the sediment subsamples from Section 4.6.2, and

$S1_w$ through $S3_w$ are the corresponding wet weights.

These calculations can be expedited by entering the weights and IC50 values into a spreadsheet and using the necessary formulae.

Investigators should consult Environment Canada (2002b) for detailed guidance regarding appropriate statistical endpoints and their calculation. The objectives of the data analysis are: to quantify contaminant effects on test organisms exposed to various samples of test (contaminated or potentially contaminated) sediment; to determine if these effects are statistically different from those occurring in a *reference* sediment; and to reach a decision as to sample toxicity (Section 6.2). Initially, *ICp* (normally, IC50) is calculated for each of the samples (including those representing the field-collected *reference* sediment).

Depending on the study design and objectives, an appropriate number (typically, $\geq$5/station, each depth of interest) of *replicate* samples of field-collected *test* and *reference* sediments should be collected and tested (see Section 4.1). Each series of toxicity tests must include a minimum of three replicate control solutions (see Sections 4.6 and 4.8), and one or more *test sediments*. Test sediment might be represented by replicate samples of dredged material from a particular depth or locale (sampling station) of interest, or replicate samples of field-collected sediment from a particular station within or adjacent to an ocean disposal site. Alternatively, test sediment might be represented by one or more subsamples (i.e., laboratory replicates) of a single (non-replicated) sample of sediment from a particular sampling station or site-specific depth (see Section 4.1). The same number of

replicates per *treatment* (i.e., test sediment from a particular sampling station and site-specific depth) and per sample should be used in the test wherever possible, to maximize statistical power and robustness (EC, 2002b).

6.2 Interpretation of Results

Interpretation of results is not necessarily the sole responsibility of the laboratory personnel undertaking the test; this might be a shared task which includes an environmental consultant or other qualified persons responsible for reviewing and interpreting the findings.

Environment Canada (1999b) provides useful advice for interpreting and applying the results of toxicity tests with environmental samples, and should be referred to for guidance in these respects. Initially, the investigator should examine the results and determine if they are valid. In this regard, the criterion for a valid test (see Section 4.8) must be met. Additionally, it is recommended that the dose-response curve for each sample of test sediment be examined to confirm that light loss decreases as test concentration decreases, in an approximately monotonic manner. If not (e.g., if one or more data points appear to be "out of place" with respect to the others), consideration should be given to repeating the test for that sample.

The findings of the reference toxicity test which was initiated with the same lot of *Bacterial Reagent* as that used in the sediment toxicity test (see Section 5) should be considered during the interpretive phase of the investigation. These results, when compared with historic test results derived by the testing facility using the same reference toxicant and test procedure (i.e., by comparison against the laboratory's warning chart for this reference toxicity test), will provide insight into the sensitivity of the test organisms as well as the laboratory's testing precision and performance for a reference toxicity test with *V. fischeri*.

All data representing the known physicochemical characteristics of each sample of test material (including that for any samples of *reference sediment* or *negative control sediment* included in the study) should be reviewed and considered when interpreting the results. The analytical data determined for whole sediment and pore water (see Section 4.3) should be compared with the known influence of these variables on light production by *V. fischeri*.

Concentrations of porewater ammonia and/or hydrogen sulphide can be elevated in samples of dredged material or field-collected sediment. The elevated levels might be due to organic enrichment from natural and/or anthropogenic (man-made) sources. The known influence of ammonia (see, for example, Tay *et al.*, 1998 and McLeay *et al.,* 2001) and hydrogen sulphide (Brouwer and Murphy, 1995; Tay *et al.*, 1998) on the inhibition of light production by *V. fischeri* should be considered together with measured concentrations of these variables in the pore water of test samples, when considering and interpreting results for field-collected samples of test and reference sediments.

Observations of turbid or highly coloured filtrates analyzed for light emission by *V. fischeri* (see Section 4.8) should be considered when reviewing and interpreting the test results.

A number of variables besides toxicity can interfere with readings of light production by *V. fischeri* surviving in the filtrate of each test

concentration, and thus can confound the interpretation of the test results. Investigators performing this *reference method*, as well as those interpreting the findings, should be aware of these confounding factors and their implications in terms of judging if test materials are toxic or not. Variables which can interfere with the light production of *V. fischeri* in test filtrates include (ASTM, 1995; Ringwood *et al.*, 1997; Tay *et al.*, 1998):

- sorption of *V. fischeri* to sediment particles (especially fine-grained ones) retained on the filter; and the resulting loss of transfer of these luminescent bacteria to the filtrate;

- sorption of *V. fischeri* to the filter, and the resulting loss of transfer of these luminescent bacteria to the filtrate;

- optical interference of the filtrate, due to colour (light absorption) and/or turbidity (light scatter); and

- loss of metabolic activity of *V. fischeri* transferred to and surviving in the filtrate, due to toxic and/or handling and mechanical stress.

The grain size of test sediments can be a significant confounding factor, since an increasing percentage of clay in the test material has been demonstrated to cause a proportionate decrease in resulting IC50s determined for *V. fischeri* recovered in filtrates of uncontaminated sediment. Samples of uncontaminated sediment comprised primarily of sand-sized particles (e.g., 0–5% fines) characteristically yield an IC50 of 28 000 to >100 000 mg/L in a *V. fischeri* solid-phase assay (Cook and Wells, 1996; Ringwood *et al.*, 1997; Tay *et al.*, 1998). IC50s show a "precipitous drop" (Benton *et al.*, 1995; Ringwood *et al.*, 1997) when the percentage of fines in uncontaminated sediment increases from 5 to ~20%, whereupon the IC50 might range from, say, 5000 to 15 000 mg/L depending on the nature of the fines (e.g., percent clay and percent silt) (Ringwood *et al.*, 1997; Tay *et al.*, 1998; McLeay *et al.,* 2001). Higher percentages of fines in uncontaminated sediment typically show a "leveling off" of further declines in IC50s associated with increasing sediment fines. *V. fischeri* solid-phase tests with 100% kaolin clay have reported IC50s ranging from 1373 to 2450 mg/L (Ringwood *et al.*, 1997; Tay *et al.*, 1998). In an interlaboratory study to validate this *reference method*, IC50s for a sample of 100% kaolin clay ranged from 1765 to 2450 mg/L (McLeay *et al.*, 2001). Together, these findings support the following new interim guidelines for judging samples as toxic or not, according to the *V. fischeri* solid-phase assay. These new guidelines take into account the percentage of fines in the test sediment and the known sharp inflection of values when their fines content reaches or exceeds 20% (Ringwood *et al.*, 1997), as well as the ability of a sample of test material comprised of 100% clay to reduce the IC50 to as low as 1765 mg/L using this *reference method* (McLeay *et al.*, 2001).

Two interim guidelines for judging the toxicity of samples of test sediment using this *reference method* are discussed in the following paragraphs. The first one, which has been recommended and applied by Environment Canada in the past (EC, 1996b; Porebski and Osborne, 1998), is based on the premise that all samples are toxic according to this biological test method if their IC50 is <1000 mg/L, regardless of grain size characteristics. The second guideline is based on the premise that samples with <20% fines might be toxic at an IC50 ≥1000 mg/L, since confounding grain size effects are appreciably less in coarse-grained sediment.

The first interim guideline should be applied to all samples of test sediment with ≥20% fines, as well as to any sample with <20% fines which has an IC50 <1000 mg/L. The second interim guideline should be applied to all samples of test sediment with <20% fines that have an IC50 ≥1000 mg/L. Applying the second interim guideline to samples of sediment with <20% fines and IC50s ≥1000 mg/L enables toxic coarse-grained sediments to be identified as such when their IC50 is appreciably higher than 1000 mg/L. It is recommended that the second interim guideline be applied to each sample of test sediment with <20% fines, except in the instance where the IC50 is <1000 mg/L in which case the sample should be judged as toxic and the second guideline does not apply.

Guideline 1

Any test sediment from a particular sampling station and depth is judged to have failed this sediment toxicity test if its IC50 is <1000 mg/L, regardless of grain size characteristics.

Guideline 2

For any test sediment from a particular sampling station and depth which is comprised of <20% fines and has an IC50 of ≥1000 mg/L, the IC50 of this sediment must be compared against a sample of "clean" reference sediment or negative control sediment (artificial or natural) with a percent fines content that does not differ by more than 30% from that of the test sediment.[14] *Based on this comparison, the test sediment is judged to have failed the sediment toxicity test if, and only if, each of the following two conditions apply:*

(1) its IC50 is more than 50% lower than that determined for the sample of reference sediment or negative control sediment[15]*; and*

(2) the IC50s for the test sediment and the reference sediment or negative control sediment differ significantly.

For Guideline 2, and throughout this *reference method*, "*fines*" refers to (sediment) particles which are ≤0.063 mm in size. Measurements of percent fines include all particles defined as silt (i.e., particles ≤0.063 mm but ≥0.004 mm) or clay (i.e., particles <0.004 mm).

The first condition for Guideline 2 is tested to see if it is met using the following examples for calculations as a guide: If the sample of

[14] The following two examples are provided to illustrate how this "must" criterion is to be applied when choosing a negative control sediment or reference sediment with a percent fines that does not differ by more than 30% from that of the test sediment. If the test sediment has a fines content of 10%, the percent fines of the reference sediment or negative control sediment must range within 7 to 13%. Similarly, if the test sediment has a fines content of 5%, the percent fines of the reference or negative control sediment must range within 3.5 to 6.5%.

[15] This condition for judging sample toxicity was derived in light of the findings of two series of interlaboratory studies performed to validate this *reference method* (McLeay *et al.*, 2001). In one series of tests with four identical subsamples of a contaminated sediment tested in separate assays by each of six participating laboratories, the lowest laboratory-specific IC50 was 14 to 48% lower (mean intralaboratory difference, 31%; n = 6) than its highest IC50. Given this degree of intralaboratory variability in IC50s for the same test sediment, as determined within individual laboratories, it is considered prudent to require that the IC50 for a sample of coarse-grained test sediment, if ≥1000 mg/L, must be more than 50% lower than that for the negative control sediment or reference sediment against which it is compared, as one of the conditions for judging the sample as toxic.

reference or negative control sediment used to judge the toxicity of the course-grained test sediment has an IC50 of 20 000 mg/L, the IC50 of the test sediment must be <10 000 mg/L. Similarly, if the sample of reference or negative control sediment used to judge the toxicity of the course-grained test sediment has an IC50 of 5050 mg/L, the IC50 of the test sediment must be <2025 mg/L.

The second condition for Guideline 2 must be tested to see if it is met using the pairwise comparison of the values for the two IC50s and their 95% confidence limits, which is described in Sprague and Fogels (1977) as a means of comparing two LC50s.[16] When testing for a significant difference, $p<0.05$ is to be used as the distinguishing effect level.

[16] This pairwise comparison test delineated in Sprague and Fogels (1977) is also thought to be suitable for comparing two IC50s (J.B. Sprague, pers. com., September 2001, Sprague Associates Ltd., Saltspring Island, BC). An electronic spreadsheet developed by Environment Canada's Pacific Environmental Science Centre (PESC; North Vancouver, BC) enables this statistical comparison to be easily performed. A copy of this spreadsheet can be obtained from either this Centre or Environment Canada's Atlantic Environmental Science Centre (AESC) in Moncton NB, by contacting the appropriate person at either of these Centres using the contact information for C. Buday (PESC) or K. Doe (AESC) included in Appendix D.

Reporting Requirements

Each test-specific report must indicate if there has been any deviation from any of the "must" requirements delineated in Sections 2 to 6 of this *reference method*, and, if so, provide details of the deviation. The reader must be able to establish from the test-specific report whether the conditions and procedures preceding and during the test rendered the results valid and acceptable for the use intended.

Section 7.1 provides a list of the items that must be included in each test-specific report. A list of items that must either be included in the test-specific report, provided separately in a general report, or held on file for a minimum of five years, is found in Section 7.2. Specific *monitoring* programs or regulations might require selected test-specific items listed in Section 7.2 (e.g., details regarding the test material and/or explicit procedures and conditions during sample collection, handling, transport, and storage) to be included in the test-specific report, or might relegate certain test-specific information as *data to be held on file*.

Procedures and conditions common to a series of ongoing tests (e.g., routine toxicity tests for monitoring or *compliance* purposes) and consistent with specifications herein, may be referred to by citation or by attachment of a general report outlining standard laboratory practice.

Details on the conduct and findings of the test, which are not conveyed by the test-specific report or general report, should be kept on file by the laboratory for a minimum of five years so that the appropriate information can be provided if an audit of the test is required. Filed information might include:

- a record of the chain-of-continuity for field-collected or other samples tested for regulatory or monitoring purposes;
- a copy of the record of acquisition for the sample(s);
- chemical analytical data on the sample(s) which are not included in the test-specific report;
- bench sheets for the observations and measurements recorded during the test;
- bench sheets and warning chart(s) for the reference toxicity tests;
- detailed records of the source of the test organisms, their lot number, expiry date, factory reference toxicity test(s), date of receipt, and temperature during storage or holding; and
- information on the calibration of equipment and instruments.

Original data sheets must be signed or initialed, and dated by the laboratory personnel conducting the tests.

7.1 Minimum Requirements for a Test-Specific Report

The following sections list items that must be included in each test-specific report.

7.1.1 Test Material

- brief description of sample type (e.g., dredged material, *reference sediment*, contaminated or potentially contaminated field-collected sediment, *negative control sediment,* or *positive control sediment*) or coding, as provided to the laboratory personnel;
- information on labelling or coding of each sample; and
- date of sample collection; date sample(s) received at test facility.

7.1.2 Test Organisms

- species, strain, lot number and expiry date.

7.1.3 Test Facilities and Apparatus

- name and address of test laboratory;
- name of person(s) performing the test; and
- name and model number of *Analyzer* (photometer) used for measuring light emissions.

7.1.4 Reconstitution Solution and Solid-Phase Diluent

- type and source.

7.1.5 Test Method

- citation of biological test method used (i.e., as per this report); and
- name and citation of program(s) and methods used for calculating statistical endpoints.

7.1.6 Test Conditions and Procedures

- design and description if any deviation from or exclusion of any of the procedures and conditions specified in this report;
- number of discrete samples per treatment; number of replicates (if any) for each treatment; number and description of treatments in each test including the control solution(s), *positive control sediment*(s), and field-collected *reference sediment*(s);
- date when test was performed;
- for each sample—percent very coarse-grained sediment (i.e., particles >1.0 mm), percent sand, percent fines, percent water content, total organic carbon; porewater salinity, porewater pH, and porewater ammonia;
- indicate if any samples of test sediment (including reference sediment) were press-sieved to remove large particles and/or detritus or indigenous organisms, including the procedure and mesh size used if applied; and
- appearance (colour, turbidity) of filtrates in cuvettes, for each treatment.

7.1.7 Test Results

- light readings (mean, SD, CV) for replicate control solutions;

- any IC50s and their 95% confidence limits, with method of calculation and units (mg/L), expressed to three significant figures;[17]

- all statistical results for "pairwise" or other comparisons of endpoint values;

- a statement as to whether or not a test sediment is judged to be toxic, including a description of the guidelines used to reach that judgement;

- results for each IC50 (including its 95% confidence limits) with the reference toxicant(s) determined using the same lot of *Bacterial Reagent* as that used in the sediment toxicity test, determined within one month of the test and when the lot was first tested; together with the *geometric mean* value (± 2 SD) for the reference toxicant(s) as derived previously at the laboratory; and

- anything unusual about the test, any deviation from these procedures and conditions, any problems encountered, any remedial measures taken.

7.2 Additional Reporting Requirements

The following sections list items that must be either included in the test-specific report or the general report, or held on file for a minimum of five years.

7.2.1 Test Material

- identification of person(s) who collected and/or provided the sample(s);

- records of sample chain-of-continuity and log-entry sheets; and

- conditions (e.g., temperature, in darkness, in sealed container) of sample(s) upon receipt and during storage.

7.2.2 Test Organisms

- source, lot number, expiry date, factory reference toxicity test(s), date of receipt, temperature during storage or holding.

7.2.3 Test Facilities and Apparatus

- description of laboratory's previous experience with this *reference method*;

- description of systems for providing lighting and regulating temperature during the incubation of test concentrations;

- description of test chambers during incubation phase (i.e., test tubes) and during the stabilization and reading phase of the test (i.e., cuvettes);

- description of pipettes and disposable tips used to prepare and transfer test concentrations;

- description and calibration record of balance used for weighing sediments;

- temperature in oven used for drying sediment subsamples;

[17] IC50s that are less than the lowest test concentration (i.e., <96.3 mg/L) are to be reported as "<96.3 mg/L", and those that are greater than the highest test concentration (i.e., >197 000 mg/L) are to be reported as ">197 000 mg/L".

- description of disposable filters; and
- description of procedures used to clean and rinse test apparatus.

7.2.4 Reconstitution Solution and Solid-Phase Diluent

- type and quantity of any chemical(s) added to *Reconstitution Solution* or *Diluent*;
- results of tests to confirm that either solution does not inhibit light production by *V. fischeri*; and
- storage conditions and expiry date (or date prepared).

7.2.5 Test Method

- methods used (with citations) for chemical analyses of test material (sediment and pore water); including details concerning aliquot sampling, preparation, and storage before analysis.

7.2.6 Test Conditions and Procedures

- temperature as monitored in the water bath during incubation of test concentrations;
- temperature as monitored in the *Analyzer* (photometer) during holding of *Reconstituted Reagent* and test filtrates; and
- measurements of concentrations of chemicals in test materials and test concentrations, other than those included in the test-specific report.

7.2.7 Test Results

- records of the presence of indigenous organisms in each sample of field-collected reference and test sediment, their removal manually or by press sieving, and their description including type (family, genus, species?) and approximate size and numbers per unit volume of sample if known;
- warning chart showing the most recent and historic results for toxicity tests with the reference toxicant(s); and
- original bench sheets (see example, Appendix F) and other data sheets, signed and dated by the laboratory personnel performing the test and related analyses.

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Appendix A

Biological Test Methods and Supporting Guidance Documents Published by Environment Canada's Method Development and Applications Section[1]

Title of Biological Test Method or Guidance Document	Report Number	Publication Date	Applicable Amendments
A. Generic (Universal) Biological Test Methods			
Acute Lethality Test Using Rainbow Trout	EPS 1/RM/9	July 1990	May 1996
Acute Lethality Test Using Threespine Stickleback (*Gasterosteus aculeatus)*	EPS 1/RM/10	July 1990	March 2000
Acute Lethality Test Using *Daphnia* spp.	EPS 1/RM/11	July 1990	May 1996
Test of Reproduction and Survival Using the Cladoceran *Ceriodaphnia dubia*	EPS 1/RM/21	February 1992	November 1997
Test of Larval Growth and Survival Using Fathead Minnows	EPS 1/RM/22	February 1992	November 1997
Toxicity Test Using Luminescent Bacteria (*Photobacterium phosphoreum*)	EPS 1/RM/24	November 1992	—
Growth Inhibition Test Using the Freshwater Alga *Selenastrum capricornutum*	EPS 1/RM/25	November 1992	November 1997
Acute Test for Sediment Toxicity Using Marine or Estuarine Amphipods	EPS 1/RM/26	December 1992	October 1998
Fertilization Assay Using Echinoids (Sea Urchins and Sand Dollars)	EPS 1/RM/27	December 1992	November 1997
Toxicity Tests Using Early Life Stages of Salmonid Fish (Rainbow Trout, Coho Salmon, or Atlantic Salmon)	EPS 1/RM/28 1st Edition	December 1992	January 1995
Toxicity Tests Using Early Life Stages of Salmonid Fish (Rainbow Trout)	EPS 1/RM/28 2nd Edition	July 1998	—
Test for Survival and Growth in Sediment Using the Larvae of Freshwater Midges *(Chironomus tentans* or *Chironomus riparius)*	EPS 1/RM/32	December 1997	—
Test for Survival and Growth in Sediment Using the Freshwater Amphipod *Hyalella azteca*	EPS 1/RM/33	December 1997	—
Test for Measuring the Inhibition of Growth Using the Freshwater Macrophyte, *Lemna minor*	EPS 1/RM/37	March 1999	—

[1] These documents are available for purchase from Environmental Protection Publications, Environmental Protection Service, Environment Canada, Ottawa, Ontario, K1A 0H3, Canada. For further information or comments, contact the Manager, Method Development and Applications Section, Environmental Technology Centre, Environment Canada, Ottawa, Ontario K1A 0H3.

Title of Biological Test Method or Guidance Document	Report Number	Publication Date	Applicable Amendments
B. Reference Methods[2]			
Reference Method for Determining Acute Lethality of Effluents to Rainbow Trout	EPS 1/RM/13 1st Edition	July 1990	May 1996, December 2000
Reference Method for Determining Acute Lethality of Effluents to Rainbow Trout	EPS 1/RM/13 2nd Edition	December 2000	—
Reference Method for Determining Acute Lethality of Effluents to *Daphnia magna*	EPS 1/RM/14 1st Edition	July 1990	May 1996, December 2000
Reference Method for Determining Acute Lethality of Effluents to *Daphnia magna*	EPS 1/RM/14 2nd Edition	December 2000	—
Reference Method for Determining Acute Lethality of Sediment to Marine or Estuarine Amphipods	EPS 1/RM/35	December 1998	—
C. Supporting Guidance Documents			
Guidance Document on Control of Toxicity Test Precision Using Reference Toxicants	EPS 1/RM/12	August 1990	—
Guidance Document on Collection and Preparation of Sediment for Physicochemical Characterization and Biological Testing	EPS 1/RM/29	December 1994	—
Guidance Document on Measurement of Toxicity Test Precision Using Control Sediments Spiked with a Reference Toxicant	EPS 1/RM/30	September 1995	—
Guidance Document on Application and Interpretation of Single-Species Tests in Environmental Toxicology	EPS 1/RM/34	December 1999	—

[2] For this series of documents, a *reference method* is defined as a specific biological test method for performing a toxicity test, i.e., a toxicity test method with an explicit set of test instructions and conditions which are described precisely in a written document. Unlike other generic (multi-purpose or "universal") biological test methods published by Environment Canada, the use of a *reference method* is frequently restricted to testing requirements associated with specific regulations.

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Procedural Variations for Solid-Phase Sediment Toxicity Tests Using Luminescent Bacteria, as Described in Canadian, United States, and Netherlands Methodology Documents

Source documents are listed here chronologically, by originating agency rather than by author(s).

EC 1992 represents EC (Environment Canada), "Biological Test Method: Toxicity Test Using Luminescent Bacteria (*Photobacterium phosphoreum*)", Conservation and Protection, Ottawa, ON, Report EPS 1/RM/24, 61 p. (1992).

MICROBICS 1992 represents Microbics, "Detailed Solid-Phase Test Protocol", p. 153–178, *in: Microtox Manual—A Toxicity Testing Handbook*, Microbics Corporation, Carlsbad, CA (1992).

MICROBICS 1995a represents Microbics, "Microtox Acute Toxicity Solid-Phase Test", Microbics Corporation, Carlsbad, CA, 18 p. (1995a).

ASTM 1995 represents ASTM (American Society for Testing and Materials), "Standard Guide for Conducting Sediment Toxicity Tests with Luminescent Bacteria", Draft No. 8, ASTM, Philadelphia, PA (1995).

EC 1996a represents EC (Environment Canada), "Procedure for Conducting a Microtox Solid-Phase Test", Toxicology Section, Environmental Quality Laboratories, Environmental Conservation Branch, Moncton, NB, Standard Operating Procedure #43, 10 p., Prepared January 1996 by G. Wohlgeschaffen. Atlantic Environmental Science Centre, Moncton, NB (1996a).

AZUR 1997 represents AZUR, "Basic Solid-Phase Test", AZUR Environmental, Carlsbad, CA, Draft 6-11-97, 4 p. (1997).

AZUR 1998a represents AZUR, "Basic Solid-Phase Test (Basic SPT)", AZUR Environmental, Carlsbad, CA, 16 p. (1998).

AZUR 1998b represents AZUR, "Solid-Phase Test (SPT)", AZUR Environmental, Carlsbad, CA, 19 p. (1998).

EC 1999a represents EC (Environment Canada), "Standard Operating Procedure for the Solid-Phase Toxicity Test Using Luminescent Bacteria (*Vibrio fischeri*)", SOP No. IC50MS10.SOP, First Draft, 18 p., prepared October 1999 by C. Buday. Pacific Environmental Science Centre, North Vancouver, BC (1999a).

NICMM 1999 represents NICMM (National Institute for Coastal and Marine Management/RIKZ), "Standard Operating Procedures, Marine: Microtox Solid-Phase *(Vibrio fisheri*) Sediment Toxicity Test", C.A. Schipper, R.M. Burgess, M.E. Schot, B.J. Kater, and J. Stronkhorst, Project SPECIE*BIO, RIKZ/AB-99.107x, Ver.1.1, June 1999, 23 p, NICMM, Middelburg, The Netherlands (1999).

1. Sample Type, Collection, and Transport

Document	Sample Type	Collection Procedure	Transport Temp (°C)	Container	Field Replicates?
EC 1992	soil, sediment, sludge	follow guidance in ASTM (1991) and EC (1994)	1–7	glass or Teflon™-coated are preferred; polyethylene, polypropylene acceptable; 500 mL to 1 L	NI
MICROBICS 1992	soil, sediment	NI [1]	NI	new borosilicate TFE-lined caps	NI
MICROBICS 1995a	soil, sediment	NI	NI	new borosilicate Teflon-lined caps	NI
ASTM 1995	sediment	follow ASTM Standard Guides E 1367, E1383, and E 1391	4 ± 2	new [2] borosilicate TFE-lined caps	yes [3]
EC 1996a	soil, sediment	NI	NI	NI	NI
AZUR 1997	soil, sediment	NI	NI	NI	NI
AZUR 1998a	soil, sediment	NI	NI	NI	NI
AZUR 1998b	soil, sediment	NI	NI	NI	NI
EC 1999a	soil, sediment	NI	NI	50 mL centrifuge tube	NI
NICMM 1999	sediment	NI	NI	NI	NI

[1] NI = not indicated.
[2] If reused, clean with: non-toxic soap, acid wash, distilled water rinse two times.
[3] Replicates samples should be collected from each site to determine variance in sediment characteristics.

2. Sample Storage

Document	Airspace?	Temp (°C)	Light/Dark	Maximum Time
EC 1992	no	4 ± 2	dark	6 weeks
MICROBICS 1992	no	2–8	NI	48 h if possible
MICROBICS 1995a	no	fridge	NI	48 h if possible
ASTM 1995	no	4 ± 2	dark	2–8 weeks
EC 1996a	NI [1]	NI	NI	NI
AZUR 1997	NI	NI	NI	NI
AZUR 1998a	NI	NI	NI	NI
AZUR 1998b	NI	NI	NI	NI
EC 1999a	no	4 ± 2	dark	6 weeks
NICMM 1999	NI	4	dark	< 4 weeks

[1] NI = not indicated.

3. Sample Pre-treatment and Characterization

Document	Sieve?	Remove Large Organisms?	Remove Large Particles?	Homogenize Sample?	Sediment Characterization
EC 1992	NI [1]	NI	NI	mix thoroughly	colour
MICROBICS 1992	NI	NI	yes (≥ 1 mm)	yes	pH
MICROBICS 1995a	NI	NI	NI	yes (1–3 min)	pH
ASTM 1995	no	yes	yes (>5 mm)	yes	organic carbon or total volatile solids, particle size distribution, ammonia, percent water, pH
EC 1996a	NI	NI	NI	yes	NI
AZUR 1997	NI	NI	NI	yes (1–3 min)	NI
AZUR 1998a	NI	NI	NI	mix thoroughly	NI
AZUR 1998b	NI	NI	NI	mix thoroughly	NI
EC 1999a	no	yes	yes	mix thoroughly	NI
NICMM 1999	NI	NI	NI	yes	for porewater:[2] pH, salinity, sulphide, ammonia

[1] NI = not indicated.
[2] Centrifuged sediment 20 min, 2500 × g.

4. Drying Subsample for Determining Water Content

Document	Amount (g)	Replicates	Weighing Precision (g)	Temperature (°C)	Time
EC 1992	NI [1]	NI	NI	NI	NI
MICROBICS 1992	5	NI	NI	NI	NI
MICROBICS 1995a	5 ± 0.2	3	± 0.01	100 ± 5	24 h
ASTM 1995	a few	NI	NI	~100	overnight
EC 1996a	3–5	3	± 0.01	60–100	48 h
AZUR 1997	NI	NI	NI	NI	NI
AZUR 1998a	NI	NI	NI	NI	NI
AZUR 1998b	NI	NI	NI	NI	NI
EC 1999a	5 ± 0.2	3	± 0.01	100 ± 5	24 h
NICMM 1999	~ 5	3	NI	100	24 h

[1] NI = not indicated.

5. Sediment Subsample, Diluent, Container for Primary Dilution and Test Concentrations

Document	Subsample Weight (g)	Discard Pore Water?	Type of Diluent	Volume of Diluent (mL)	Container for Primary Dilution	Container for Test Concentrations
EC 1992	0.4	yes (centrifuge)	*Solid-Phase Diluent*	4.0	NA [4]	*SPT* tube [6]
MICROBICS 1992	0.3	yes (centrifuge)	*Solid-Phase Diluent*	3.0	NA [4]	*SPT* tube [6,7]
MICROBICS 1995a	7.00	optional	NaCl or $NaNO_3$ *Diluent*	35	new 50 mL glass or plastic	*SPT* tube [6,7]
ASTM 1995	7 ± 0.35 [1]	no	3.5% NaCl or 3.02% $NaNO_3$ [3]	35	new, clean borosilicate glass [5]	new, clean borosilicate glass tube [5]
EC 1996a	7 ± 0.05	NI [2]	*Solid-Phase Diluent*	35	new 50 mL disposable polypropylene	*SPT* tube [6,7]
AZUR 1997	7.00	NI	*Diluent* or reference seawater	35	~50 mL beaker	cuvette [8]
AZUR 1998a	7.00	NI	*Solid-Phase Diluent*	35	~50 mL beaker	cuvette [8]
AZUR 1998b	7.00	NI	*Solid-Phase Diluent*	35	~50 mL beaker	*SPT* tube [6,7]
EC 1999a	0.3	yes (centrifuge)	3.5% NaCl	3.0	NA [4]	*SPT* tube [6,9]
NICMM 1999	7.0	NI	*Solid-Phase Diluent*	35	serum tube	*SPT* tube [6,7]

[1] Weight actually used should be known within ± 0.05 g, and must be known within ± 0.35 g.
[2] NI = not indicated.
[3] Use NaCl (3.5%) for marine or estuarine sediments, and $NaNO_3$ (3.02%) for freshwater sediments.
[4] NA = not applicable. The first dilution (maximum test concentration) is made in the *Solid-Phase Test* styrene tube.
[5] 16-mm diameter (15.6 mm nominal), with a 15.3-mm diameter hemispherical bottom. These are placed in a suitable test tube support in an incubator at 15 °C.
[6] *SPT = Solid-Phase Test*. Each tube is polystyrene, 15.5 × 56 mm (7.5-mL capacity), with a hemispherical bottom, that mates with the filter column (Table 12, this appendix). They are supplied by Evergreen Scientific, part number 208-3193-020, Los Angeles, CA, telephone (323) 583-1331.
[7] *SPT* tubes are supported in a test tube rack (incubator block) which is incubated at 15 °C (water bath).
[8] Disposable borosilicate, 50 mm length × 12 mm diameter (11.75 nominal); flat bottom. Cuvettes are placed in incubator wells.
[9] *SPT* tubes are supported in a test tube rack (incubator block) at room temperature.

6. Mixing the Primary Dilution of Sediment

Document	pH Adjustment?	Mixing Device	Vortex Depth	Mixing Time (min)
EC 1992	NI [1]	shake and use pipette	NA [4]	NI (mix well)
MICROBICS 1992	NI [2]	shake and use pipette	NA	NI (completely dissociate and suspend sample)
MICROBICS 1995a	NI	magnetic plate, Teflon™ stir bar	half the height of liquid level	10
ASTM 1995	no [3]	magnetic plate, Teflon stir bar	half the height of liquid level	20 [5]
EC 1996a	NI	magnetic stirrer	⅓ the height of liquid level	10
AZUR 1997	NI	magnetic plate, Teflon stir bar	half the height of liquid level	10
AZUR 1998a	NI	magnetic plate, Teflon stir bar	half the height of liquid level	10
AZUR 1998b	NI	magnetic plate, Teflon stir bar	half the height of liquid level	10
EC 1999a	no	shake and use pipette	NA	NI (completely dissociate and suspend sample)
NICMM 1999	NI	magnetic plate, Teflon stir bar	half the height of liquid level	10, or until suspension is homogeneous

[1] NI = not indicated.
[2] pH of 6 to 8 has minimal effect on *Reconstituted Reagent* (i.e., reconstituted freeze-dried bacteria).
[3] Optimal pH is 7.3; little effect if pH is 6.6 to 7.8.
[4] NA = not applicable.
[5] Check after 5 min, to see if larger particles which settle quickly after stirring, are free of clay.

7. Preparation of Series of Test Concentrations

Document	Dilution Factor	Number of Test Concentrations	Volume Transferred from Primary Dilution to Tube with Maximum Test Concentation (mL)	Number of Times to Mix with Pipette to Resuspend Particles	Volume Transferred Serially from Maximum to Lowest Concentration (mL)
EC 1992	1:2	9	NA [2]	NI [7]	2.0
MICROBICS 1992	1:2	12 [1]	NA	5–6×	1.5
MICROBICS 1995a	1:2	13 [1]	1.5 [3]	NI	1.5
ASTM 1995	1:2 [1]	12 [1]	1.5 [4]	several	1.5
EC 1996a	1:2	12	1.5 [3,4]	3–4×	1.5
AZUR 1997	1:2	9	2.0 [5]	thoroughly	1.0
AZUR 1998a	1:2	9	2.0 [5, 6]	mix	1.0
AZUR 1998b	1:2	13	1.5 [3, 6]	mix	1.5
EC 1999a	1:2	10	1.5 [4]	5–6×	1.5
NICMM 1999	1:2	13	1.5	10×	1.5

[1] Described array, but use of other combinations of control, dilution factor, and dilutions are listed as well.
[2] NA = not applicable.
[3] Tube for maximum test concentration already contains 1.5 mL *Diluent*.
[4] Use large aperture pipette tip to avoid clogging; hold ~5 mm from wall, ~15 mm from bottom.
[5] Transferred directly to the cuvette in the M500 *Analyzer*.
[6] Use large aperture pipette tip to avoid clogging, placed adjacent to wall, ~2 cm from bottom.
[7] NI = not indicated.

8. Maximum Test Concentration, Controls, Duplicates, and Containers Inoculated

Document	Maximum Test Concentration[1]	Number of Control Solutions [3]	Duplicates of Each Test Concentration?	Initial (I_0) Light Readings?	Containers Inoculated with *Reagent* [6]
EC 1992	10%	1	optional	no	*SPT* tubes
MICROBICS 1992	98 684 mg/L or 9.868%	3	optional	no	*SPT* tubes
MICROBICS 1995a	19.737%	4	yes	no	*SPT* tubes
ASTM 1995	10%	3	no	no	glass tubes
EC 1996a	197 368 mg/L	3	no	no	*SPT* tubes
AZUR 1997	9.9% or 99 000 mg/L	1	optional [4]	yes [5]	cuvettes [7]
AZUR 1998a	99 000 mg/L	1	optional [4]	yes [5]	cuvettes [7]
AZUR 1998b	9.9% or 99.01 mg/L [2]	2	no	no	*SPT* tubes
EC 1999a	9.868%	3	no	no	*SPT* tubes
NICMM 1999	19.737%	4	yes	no	*SPT* tubes

[1] Percent values are on a weight-to-volume (wt:v) basis.

[2] Seems to be a mistake. More likely 99 000 mg/L.

[3] These are comprised of diluent only, to which the bacterial inoculum is added.

[4] When duplicate testing is required, perform this procedure twice.

[5] Zero-time (I_0) light levels are measured on 500-µL aliquots of *Diluent plus Reagent* only, in 10 replicate cuvettes.

[6] The term "*Reagent*" refers to reconstituted freeze-dried bacteria (see Table 11). See "Container for Test Concentrations" in Table 5, for a description of the containers.

[7] The test concentrations are not inoculated with *Reagent*. Rather, a 500-µL aliquot of each test concentration is transferred to the initial cuvettes after the initial light measurements are taken. See Table 5, for a description of the cuvettes.

9. Equilibration of Test Concentrations Before Inoculation with Bacteria

Document	Equilibration Temperature (°C)	Equilibration Time (min)
EC 1992	15 ± 0.3	NI
MICROBICS 1992	15	10
MICROBICS 1995a	15	10
ASTM 1995	15 ± 0.5	NI
EC 1996a	15	10
AZUR 1997	NI [1]	5
AZUR 1998a	NI [1]	5
AZUR 1998b	15	5
EC 1999a	room temperature (~20)	5
NICMM 1999	15	10

[1] NI = not indicated. Assumed to be 15°C since the test solutions are made up in cuvettes directly in the M500 *Analyzer* (Microbics, 1995b).

10. Test Organisms, Source, and Storage

Document	Species	Strain	Source	Storage Temperature (°C) [5]
EC 1992	*P. phosphoreum* [1]	NRRL B-11177 [3]	Microbics	-20
MICROBICS 1992	*P. phosphoreum* [1]	NI	Microbics	NI
MICROBICS 1995a	NI [2]	NI	Microbics	NI
ASTM 1995	*V. fischeri*	NRRL B-11177 [3]	NI	-20
EC 1996a	*P. phosphoreum* [1]	NI	NI	NI
AZUR 1997	NI	NI	AZUR [4]	NI
AZUR 1998a	NI	NI	AZUR	NI
AZUR 1998b	NI	NI	AZUR	NI
EC 1999a	*V. fischeri*	NRRL B-11177 [3]	AZUR	-20
NICMM 1999	*V. fischeri*	NI	AZUR	NI

[1] Subsequently reclassified as *Vibrio fischeri.*
[2] NI = not indicated.
[3] Archived with Northern Regional Research Laboratory, Preoria, IL, USA.
[4] On August 15, 1996, Microbics Corporation changed their name to AZUR Environmental.
[5] Test organisms are purchased as lyophilized (freeze-dried) bacteria in a vial ("*Bacterial Reagent")*, and can be stored for an extended period at -20°C.

11. Reconstitution of Lyophilized (Freeze-dried) Bacteria

Document	Source of Reconstitution Solution [1]	Temp (°C)	Usable Life	Swirl Vial	Mixing Reconstituted Bacteria
EC 1992	Microbics	3–5	2 h	3– 4×	20× with 0.5 mL pipette
MICROBICS 1992	Microbics	NI [2]	2 h	3–4×	20× with 0.5 mL pipette
MICROBICS 1995a	Microbics	NI [2]	NI	NI	10× with 0.5 mL pipette
ASTM 1995	water	5.5 ± 1	NI	a few times	20× with 0.5 mL pipette
EC 1996a	AZUR	NI [2]	NI	3×	10× with 0.5 mL pipette
AZUR 1997	AZUR	NI [2]	3 h	NI	10× with 0.5 mL pipette
AZUR 1998a	AZUR	NI [2]	3 h	3–4×	≥10× with 0.5 mL pipette
AZUR 1998b	AZUR	NI [2]	3 h	3–4×	≥10× with 0.5 mL pipette
EC 1999a	lab-distilled water	NI [2]	3–4 h	3–4×	20× with 0.5 mL pipette
NICMM 1999	AZUR	NI [2]	4 h	no [3]	20× with 1 mL pipette

[1] Non-toxic, distilled water, verified by acute toxicity test with luminescent bacteria.
[2] NI = not indicated. Temperature assumed to be 5°C, since the M500 *Analyzer* (Microbics, 1995b) is used.
[3] Reconstitution solution from the cuvette in the reagent well is pipetted into the vial, mixed 20× and pipetted into the cuvette in the reagent well and left to equilibrate 30 min.

12. Inoculation, Incubation, and Filtration of Test Concentrations

Document	Volume of Inoculum (µL)	Mixing Procedure	Times Mixed	Incubation Time (min)	Incubation Temperature (°C)	Insert Filter Columns? [4]
EC 1992	NI [1]	NI	NI	20	15 ± 0.3	yes
MICROBICS 1992	20	1.5 mL pipette	5–6×	20	15	yes
MICROBICS 1995a	20	shake, then 1.5 mL pipette	NI	20	15	yes
ASTM 1995	20	shake or vortex	NI	20	15 ± 0.5	yes
EC 1996a	20	1.5 mL pipette	2×	20	15	yes
AZUR 1997	10 [2]	shake	NI	30 [2]	NI [3]	no [5]
AZUR 1998a	10 [2]	shake	NI	30 [2]	NI [3]	no [5]
AZUR 1998b	20	1.5 mL pipette	NI	20	15	yes
EC 1999a	20	1.5 mL pipette	5–6×	20 temperature (~20)	room	yes
NICMM 1999	20	manual	NI	20	15	yes

[1] NI = not indicated.

[2] Initial cuvettes containing 0.5 mL *Diluent* are inoculated, NOT test concentrations, and left 15 min for stabilization after mixing. Then initial readings are taken. A 30-min incubation period begins after 500 µL of each test concentration is transferred to the initial cuvettes and mixed with the pipette.

[3] Assumed to be 15°C, since the cuvettes are in the M500 *Analyzer* (Microbics, 1995b).

[4] Polyethylene serum separation columns, 13-mm OD, 11-mm ID, 67-mm high, with a 15-mm diameter flexible seal on the lower end where the filter that mates with the *SPT* tube (Table 5). The filter itself, located at the lower end, has a 6-mm diameter and is 4-mm thick. Pore size is 15–45 µm according to supplier, Evergreen Scientific, Los Angeles, CA, telephone (323) 583-1331, part number 208-3193-020.

[5] Test method does not employ filtration.

13. Measuring Bacterial Luminescence

Document	Analyzer	Volume Transferred (µL)	Cuvette Type	Stabilization Conditions [7]	Reading Cuvette
EC 1992	Microtox Model 2055, Model 500	500	glass [5] 15 ± 0.3°C	5 min, inserted	manually
MICROBICS 1992	Microtox Model 500	500	glass [5]	5 min [8]	manually inserted
MICROBICS 1995a	Model 500	500	glass [5]	10 min [8]	manually inserted
ASTM 1995	photometer	500	glass [5]	10 min, 15 ± 0.5°C	photometer instruction manual
EC 1996a	Model 500	500	glass [5]	NI [1,8,9]	manually inserted
AZUR 1997	NI [1,2]	500 [3]	NI [1, 6]	NI [1, 8, 10]	manually inserted
AZUR 1998a	Model 500	500 [3,4]	NI [1, 6]	NI [1, 8, 10]	manually inserted
AZUR 1998b	Model 500	500	NI [1, 6]	10 min [8]	manually inserted
EC 1999a	Microtox Model 500	500	glass [5]	5 min [8]	manually inserted
NICMM 1999	Microtox Model 500	500	glass [5]	NI [8]	manually inserted

[1] NI = not indicated.
[2] Assumed to be a Model M500 *Analyzer*.
[3] Transferred to the initial cuvettes, at which time a 30-min incubation period begins. Cuvettes shaken after 15 min. Unnecessary to shake cuvettes before reading light, because whether shaken or not, "the EC50 tends to be similar."
[4] Take readings for cuvettes without shaking at 30-min elapsed time, and again after shaking at 31-min elapsed time, and compare the results of IC50.
[5] Disposable borosilicate, 50 mm length × 12 mm diameter (11.75 nominal); flat bottom.
[6] Assumed to be the usual glass type as in all the other related methodology documents.
[7] After the filtered bacterial solution is transferred from the incubated tube to the cuvette in the *Analyze*r, it is left for the specified time to equilibrate to the specified temperature.
[8] Temperature assumed to be 15 ± 0.5°C, using the Model M500 *Analyzer* (Microbics, 1995b).
[9] Assumed to be 10 min (software data capture program waits 10 min before requesting a reading).
[10] Stabilization time is included in the single 30-min incubation period (see Table 12).

14. Use of Reference Sediment

Document	Use and Properties of Reference Sediment	Use to Correct Sample Values? [2]
EC 1992	must use [1]; parallel test; should be similar in particle sizes and organic/inorganic content	NI [3]
MICROBICS 1992	optional/desirable; "control" samples from area useful in distinguishing toxicity	NI
MICROBICS 1995a	desirable; match with test material for particle size, organic and moisture content	optional; analytical data "reduced" versus reference
ASTM 1995	recommended; important to match withoptional [4]; analytical data test material for particle size	"reduced" versus reference
EC 1996a	optional	NI
AZUR 1997	recommended; match with test material for particle size, organic and moisture content	no [5]
AZUR 1998a	recommended; match with test material for particle size, organic and moisture content	no [5]
AZUR 1998b	recommended; match with test material for particle size, organic and moisture content	NI
EC 1999a	recommended; "control" samples from area with similar particle size useful in distinguishing toxicity	no
NICMM 1999	correction made for the measured silt and clay fraction of sample using an equation derived from own data and from Ringwood *et al.* (1997)	use equation to correct EC50

[1] Test requires the use of one or more samples of reference or control ("clean") sediment.

[2] Corrected light values (Gammas; Γ_T) for each test concentration are computed automatically by the software applying the reference sediment light values (Microbics, 1995) or by using equations 1 to 3, Section 6.1.

[3] NI = not indicated.

[4] Correction against a reference sample can be important for samples with IC50 greater than ~0.25%; correction is seldom justified for very toxic samples with IC50 less than ~0.1%. The decision to use this correction is left to the discretion of the investigator.

[5] Select a reference sample that has similar characteristics (e.g., particle size, organic material, moisture content) as the test material; compare results for each when judging if test material toxic or not (see Table 15).

15. Data Analysis and Guidelines for Judging Sample Toxicity

Document	Data Entry	Method of Analysis	Endpoint [4] (Units)	Guidelines for Judging Toxicity? [5]
EC 1992	software data capture	manual or software graphing [3]	IC50 (% wt:v)	no
MICROBICS 1992	software data capture	manual or software graphing	ECxx (mg/L)	no
MICROBICS 1995a	software data capture	manual or software graphing	ECxx (% wt:v)	no
ASTM 1995	manual	manual calculations and graphs	ICxx (% wt:v)	yes [6]
EC 1996a	software data capture	software graphing	ICxx (mg/L)	no
AZUR 1997	software data capture	software graphing	EC50 (% wt:v) or EC50 (mg/L)	yes [7]
AZUR 1998a	NI [1,2]	software graphing	IC50 (mg/L)	yes [8]
AZUR 1998b	NI [1,2]	NI [1,3]	NI	no
EC 1999a	software data capture	software graphing	IC50 (% wt:v)	yes [9]
NICMM 1999	software data capture	manual or software graphing	ECxx (% wt:v)	no

[1] NI = not indicated.
[2] Assumed to be by software data capture.
[3] Assumed to be by software graphing.
[4] EC = effective concentration, IC = inhibitory concentration.
[5] Specific guidelines, based on endpoint results, for judging if test material is toxic or not.
[6] An apparent IC50 of 0.7% can represent a non-toxic sample with a high percentage of clays, in which case low toxicity for such a sediment would be IC50 >0.1%. An apparent IC50 of ≥10% is common for non-toxic samples containing fewer very fine particles, thus low toxicity would be IC50 >5%.
[7] Test material is toxic when its EC50 is lower than that of the reference sample.
[8] Test material is toxic when its IC50 is lower than that of the reference sample.
[9] Test material is practically non-toxic when its moisture-corrected IC50 is >0.5%, marginally toxic when its moisture-corrected IC50 is within the range of 0.1% to 0.5%, and toxic when its moisture-corrected IC50 is <0.1%.

16. Normalizing and Correcting Data for Moisture, Colour, and Turbidity

Document	Normalize for Moisture?	Correct for Colour?	Correct for Turbidity?
EC 1992	NI [1]	NI	NI
MICROBICS 1992	no	yes [3]	yes [3]
MICROBICS 1995a	yes [2]	yes [4]	yes [4]
ASTM 1995	optional	optional [4, 5]	NI
EC 1996a	yes	yes	yes
AZUR 1997	NI	no [6]	no [7]
AZUR 1998a	NI	no [6]	no [7]
AZUR 1998b	NI	no [6]	no [7]
EC 1999a	yes [2]	no	no
NICMM 1999	yes [2]	NI	NI

[1] NI = not indicated.
[2] Use Microtox™ software to insert wet/dry sediment data, make the correction, and automatically create a new dry-weight corrected file.
[3] Check the sample concentration at the EC50 value for visible colour or visible turbidity; if it contains obvious colour and/or obvious turbidity, perform the *Colour Correction Protocol*.
[4]
[5] Correct using the corresponding value for a reference sample, as per footnote 2, Table 14, this appendix. This correction is not recommended when data from testing a suitable reference sample are available.
[6] Since the colour of the reference sample and the test sample(s) should be similar, the net effect of colour would be negligible.
[7] Since the turbidity of the reference sample and the test sample(s) should be similar, the net effect of turbidity would be negligible.

17. Quality Control

Document	Reference Toxicant	Control Sediment?	Requirement for Valid Sediment Toxicity Test
EC 1992	phenol, zinc, potassium dichromate, sodium lauryl sulphate	yes [2]	valid numerical estimate of ICp based on concentrations showing light inhibition both greater than, and less than, the inhibition at the ICp
MICROBICS 1992	phenol, zinc	NI [1,3]	NI [1]
MICROBICS 1995a	NI [1]	NI [1]	if Gamma for each test concentration is >1, a repeat test is required using <7 g in primary dilution
ASTM 1995	phenol, zinc	yes (may be the reference sediment)	if SD of the light reading for the three control solutions exceeds ~12% of the mean, the test should be considered unacceptable [4]
EC 1996a	NI [1]	NI [1]	NI [1]
AZUR 1997	NI [1]	NI [1]	NI [1,5]
AZUR 1998a	NI [1]	NI [1]	NI [1,5]
AZUR 1998b	NI [1]	NI [1]	NI [1]
EC 1999a	HS-6 standard sediment	Roberts Bank "clean" sediment	IC50s for "clean" sediment and HS-6 within warning limits (± 2 SD) of respective control charts
NICMM 1999	zinc	yes [2]	porewater sulphide, <16.5 mg/L; porewater pH, 6.5–7.5 (± 0.5), porewater salinity, >8‰; EC50 for reference toxicant ($ZnSO_4$) within ± 3 SD of control chart; EC50 for control sediment, <1000 Toxic Units

[1] NI = not indicated.
[2] Test requires the use of one or more samples of control or reference ("clean") sediment.
[3] This procedure refers to "control samples"; however, these are described as "samples from the area" which might have "naturally quite high" toxicity levels (i.e., the description pertains to reference samples).
[4] It is recommended that a reference toxicant be used for the validation of data produced with different lots of *Reagent* (i.e., different lots of freeze-dried bacteria) or for individual lots used over an extended period of time.
[5] If Gamma for each test concentration is >1.0, a repeat test might be required using <7 g in primary dilution (see Table 5, this appendix). Conversely, if the Gamma for each test concentration is <1.0, a repeat test might be required using >7 g in primary dilution.

Bench Sheet for Sediment Toxicity Tests Using *V. fischeri*

Project:							
Test Method:					**Analyst:**		
Reagent Lot:					**Expiry Date:**		
Date of Test	Filename and/or Sample	Date Collected in Field	Date Received in Lab	Temp (°C) on Arrival	*Reagent* Recon Time[1]	Test Start Time	Notes[2]

[1] Time of reconstitution of *Bacterial Reagent*.

[2] Details such as sample colour, odour, and manipulation (e.g., procedures for sorting or sieving sample, species removed, press-sieve size, problems, changes).