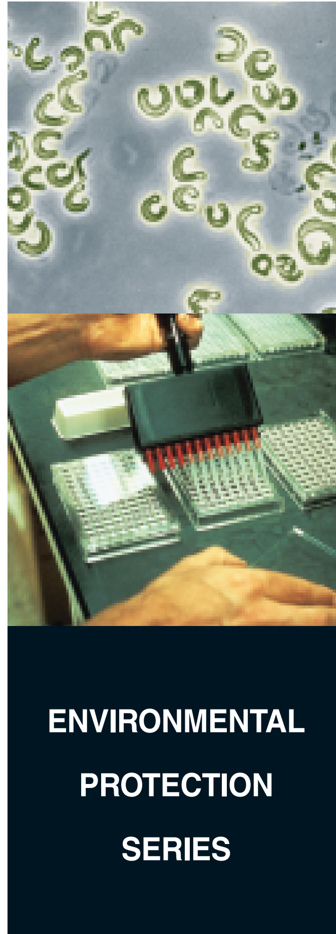
EPS 1/RM/25 Second Edition– March 2007
Environmental Science and Technology Centre
Science and Technology Branch
Environment Canada



Biological Test Method: Growth Inhibition Test Using a Freshwater Alga

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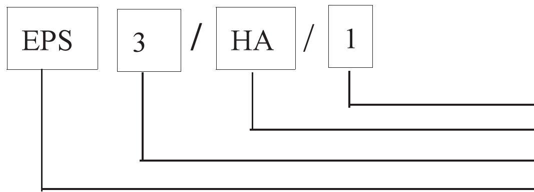

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Biological Test Method: Growth Inhibition Test Using a Freshwater Alga

Method Development and Applications Section
Environmental Science and Technology Centre
Science and Technology Branch
Environment Canada
Ottawa, Ontario

Report EPS 1/RM/25
SECOND EDITION
March 2007

Library and Archives Canada Cataloging in Publication Data

Main entry under title:

Biological test method. Growth inhibition test using a freshwater alga /
Method Development and Applications Section, Environmental Science and
Technology Centre, Environment Canada. -- 2nd ed.

(Report ; EPS 1/RM/25)
Issued also in French under title: Méthode d'essai biologique. Essai d'inhibition
de la croissance d'une algue d'eau douce
Includes bibliographical references.
ISBN 978-0-662-45505-9
DSS cat. no. En49-7/1-25E

1. *Pseudokirchneriella subcapitata*– Toxicity testing.
2. Freshwater algae – Toxicity testing.
3. Aquatic biology – Environmental aspects.

I. Environmental Science and Technology Centre (Canada). II. Canada.
Environment Canada. III. Title: Growth inhibition test using a freshwater alga.
IV. Series: Report (Canada. Environment Canada) ; EPS 1/RM/25.

QK569.P98B56 2007 589.3 C2007-980072-6

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ISBN 978-0-662-45505-9
Cat. no. En49-7/1-25E

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Services des communications
Environnement Canada
Ottawa (Ontario)
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Review Notice

This report has been reviewed by the staff of the Environmental Technology Advancement Directorate, Environment Canada, and approved for publication. Mention of trade names or commercial products does not constitute endorsement by Environment Canada for use. Other products of similar value are available.

Abstract

Methods recommended by Environment Canada for determining the chronic toxicity of effluents, elutriates, leachates, receiving waters, or chemicals to the green alga Pseudokirchneriella subcapitata, *using the microplate technique, are described in this report. This second edition of EPS 1/RM/25, published in 2007, supersedes the first edition that was published in 1992 (amended in 1997). It includes numerous procedural modifications as well as updated guidance and instructions to assist in performing the biological test method.*

General or universal conditions and procedures are outlined for conducting a chronic growth inhibition test using a variety of test materials or substances. Additional conditions and procedures are stipulated that are specific for assessing samples of chemicals, effluents, elutriates, leachates, or receiving waters. Included are instructions on culturing conditions and requirements for the test species, sample handling and storage, test facility requirements, procedures for preparing test solutions and test initiation, specified test conditions, appropriate observations and measurements, endpoints, methods of calculation, and the use of reference toxicants.

Résumé

Le présent document expose les méthodes recommandées par Environnement Canada pour déterminer la toxicité chronique d'effluents, d'élutriats, de lixiviats, d'eaux réceptrices ou de substances chimiques chez l'algue verte Pseudokirchneriella subcapitata*, au moyen de la microtitration sur plaque. Cette deuxième édition du document SPE 1/RM/25, publiée en 2007, remplace la première édition, parue en 1992 (et modifiée en 1997). Elle comporte de nombreuses modifications procédurales, de même que des indications et des instructions à jour qui faciliteront l'exécution de la méthode d'essai biologique.*

Les conditions et méthodes générales ou universelles décrites ici permettent de réaliser des essais d'inhibition chronique de la croissance avec diverses matières ou substances d'essai. Le document précise d'autres conditions et procédures propres à l'évaluation d'échantillons de substances chimiques, d'effluents, d'élutriats, de lixiviats ou d'eaux réceptrices. Il renferme aussi des instructions sur les conditions et règles de culture de l'espèce d'essai, la manipulation et l'entreposage des échantillons, les exigences en matière d'installations d'essai, les procédures entourant la préparation des solutions expérimentales et la mise en route des essais, les conditions prescrites pour les essais, les observations et les mesures pertinentes, les paramètres, les méthodes de calcul et l'utilisation de toxiques de référence.

Foreword

This is one of a series of ***recommended methods*** *for measuring and assessing the toxic effect(s) on single species of aquatic or terrestrial organisms, caused by their exposure to samples of toxic or potentially toxic substances or materials under controlled and defined laboratory conditions. Recommended methods are those that have been evaluated by Environment Canada (EC), and are favoured:*

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

The different types of tests included in this series were selected because of their acceptability for the needs of programs for environmental protection and management carried out by Environment Canada. These reports are intended to provide guidance and to facilitate the use of consistent, appropriate, and comprehensive procedures for obtaining data on the toxicity to aquatic or terrestrial life of samples of specific test substances or materials destined for or within the environment. Depending on the biological test method(s) chosen and the environmental compartment of concern, substances or materials to be tested for toxicity could include samples of chemical or chemical product, effluent, elutriate, leachate, receiving water, sediment or similar particulate material, or soil or similar particulate material. Appendix A provides a listing of the biological test methods and supporting guidance documents published to date by Environment Canada as part of this series.

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

°C . . . . degree(s) Celsius
$CaCl_2$ . . . . calcium chloride
cm . . . . centimetre
$CoCl_2$ . . . . cobalt chloride
$CuCl_2$ . . . . copper chloride
$CuSO_4$ . . . . copper sulphate
CV . . . . coefficient of variation
d . . . . day (s)
EC50 . . . . median effective concentration
EDTA . . . . ethylenediamine tetraacetate ($C_{10}H_{14}O_8N_2$)
$FeCl_3$ . . . . ferric chloride
g . . . . gram(s)
g/kg . . . . gram(s) per kilogram
h . . . . hour(s)
H_3BO_3 . . . . boric acid
HCl . . . . hydrochloric acid
ICp . . . . inhibiting concentration for a (specified) percent effect
KCl . . . . potassium chloride
K_2HPO_4 . . . . potassium phosphate
kPa . . . . kilopascal
L . . . . litre(s)
LC50 . . . . median lethal concentration
LOEC . . . . lowest-observed-effect-concentration
mg . . . . milligram(s)
$MgCl_2$ . . . . magnesium chloride
$MgSO_4$ . . . . magnesium sulphate
min . . . . minute(s)
mL . . . . millilitre(s)
mm . . . . millimetre(s)
mS . . . . millisiemen(s)
$MnCl_2$ . . . . manganous chloride
N . . . . Normal
NaCl . . . . sodium chloride
Na_2EDTA . . . . disodium ethlenediamine tetraacetate
$NaHCO_3$ . . . . sodium bicarbonate
Na_2MoO_4 . . . . sodium molybdenate
NaOH . . . . sodium hydroxide
$NaNO_3$ . . . . sodium nitrate
nm . . . . nanometre(s)
nmol . . . . nanomole(s)
NOEC . . . . no-observed-effect-concentration
p . . . . probability level (for statistical use)
rpm . . . . rotations per minute

SD . . . . standard deviation
T . . . . time
TM (™) . . . . Trade Mark
µg . . . . microgram(s)
µL . . . . microlitre(s)
µmhos/cm . . . . micromhos per centimetre
µmol/($m^2 \cdot s$) . . . . micromole per metre squared per second
UTCC . . . . University of Toronto Culture Collection
v/v . . . . volume to volume
$ZnCl_2$ . . . . zinc chloride
$ZnSO_4$ . . . . zinc sulphate
± . . . . plus or minus
> . . . . greater than
< . . . . less than
≥ . . . . greater than or equal to
≤ . . . . less than or equal to
≫ . . . . much greater than
~ . . . . approximately
≅ . . . . approximately equal to
% . . . . parts per hundred (percentage)
‰ . . . . parts per thousand

Terminology

Note: All definitions are given in the context of the procedures in this report, and may 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 expresses a possibility that something could exist or happen.

General Technical Terms

Algal inoculum is comprised of cells harvested from a liquid stock algal culture that is 3 to 7 days old and in logarithmic phase of growth; in the context of test set up, the algal inoculum is a suspension of 220 000 cells/mL.

Absorbance refers to the amount of light absorbed by algal cells. When measured at a particular wavelength, it is used as an indirect measure of algal biomass.

Axenic cultures contain organisms of a single species, in the absence of cells or living organisms of any other species.

Compliance means in accordance with governmental permitting or regulatory requirements.

Conductivity is a numerical expression of the ability of an aqueous solution to carry an electric current. This ability depends on the concentrations of ions in solution, their valance and mobility, and the temperature of the solution. Conductivity is normally reported in the SI unit of millisiemens/metre, or as micromhos/cm (1 mS/m = 10 µmhos/cm).

Culture, as a noun, means the stock of organisms raised under defined and controlled conditions to produce healthy test organisms. As a verb, it means to carry out the procedure of raising organisms.

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

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

Fluorescence refers to the re-emission of light absorbed by chlorophyll *a* pigments in algal cells. When quantified, it may be used as an indirect measure of algal biomass.

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

Initial (algal) cell density refers to the concentration of algal cells in the microplate wells at the start of the test; in the context of this method, the initial cell density is 10 000 ± 1000 cells/mL. Also referred to as initial cell concentration.

Lux is a unit of illumination based on units per square metre. One lux = 0.0929 foot-candles and one foot-candle = 10.76 lux. For conversion of lux to quantal flux [μmol/($m^2 \cdot s$)], the spectral quality for the light source must be known. Light conditions or irradiance are properly described in terms of quantal flux (photon fluence rate) in the photosynthetically effective wavelength range of approximately 400 to 700 nm. The relationship between quantal flux and lux or foot-candle is highly variable and depends on the light source, the light meter used, the geometrical arrangement, and the possibilities of reflections. Approximate conversion between quantal flux and lux, however, for "cool white" fluorescent light, is 1 lux ≅ 0.014 μmol/($m^2 \cdot s$) (Deitzer, 1994; Sager and McFarlane, 1997).

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

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

pH is the negative logarithm of the concentration of hydrogen ions in grams equilvalents 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 signifying increasingly greater acidic reactions, and numbers greater than 7 indicating increasingly more basic or alkaline reactions.

Photoperiod is the duration of illumination and darkness within a 24-h day.

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

Pretreatment means treatment or dilution of a sample prior to exposure of algae.

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

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

Terms for Test Materials or Substances

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

Control/dilution water is the water used for the sample control and for dilution of the test substance to prepare different concentrations for the various treatments included in the growth inhibitions test using the freshwater alga, *Pseudokirchneriella subcapitata*. It may be reagent water, uncontaminated receiving water, upstream water, uncontaminated groundwater, surface water (from a river or a lake), dechlorinated municipal water, or reconstituted water.

Dechlorinated water is a chlorinated water (usually municipal drinking water) that has been treated to remove chlorine and chlorinated compounds from the solution.

Deionized water is water that has been purified by passing it through resin columns or a reverse osmosis system.

Dilution water is the water used to dilute a test material or substance to prepare different concentrations for the various toxicity test treatments.

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

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

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

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

Quality Control Microplate refers to a microplate that in each experimental well contains only 200 µL of reagent water, 10 µL of enrichment medium, and 10 µL of the algal inoculum. It represents optimal algal growth for a given set of experimental conditions and exposure period.

Reagent water refers to Millipore Super Q™ water or equivalent water that is free of particles, ions, organic molecules, and microorganisms >0.45 µm in diameter.

Receiving water is surface water (e.g., in a stream, river, or lake) that has received a discharged waste, or else is about to receive such a waste (e.g., it is just upstream from the discharge point). Further description must be provided to indicate the intended meaning.

Reconstituted water is deionized or glass-distilled water to which reagent-grade chemicals have been added. The resultant synthetic fresh water is free from contaminants and has the desired pH and hardness characteristics.

Reference toxicant is a standard chemical 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 of results obtained by the laboratory for that chemical.

Sample 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. The sample control must duplicate all the conditions of the exposure treatment(s), but must contain no test material or substance. In this biological test method, it refers to the group of wells in a test microplate containing 200 µL of control/dilution water, 10 µL of enrichment medium, and 10 µL of the algal inoculum. (See also *standard control*).

Standard control is a control treatment used to determine the absence of measurable toxicity due to basic test conditions (e.g., the quality of the control/dilution water, or the health or handling of test organisms). In this biological test method, it refers to the group of wells in a test microplate containing 200 µL of reagent water, 10 µL of enrichment medium, and 10 µL of the algal inoculum. Also referred to as standard reagent control. (See also *sample control*).

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

Test sample refers to the aqueous sample that is to be tested. It might be derived from chemical stock solutions or collected from effluents, elutriates, leachates, or receiving waters.

Test solution refers to an aqueous solution that consists of a prepared test sample, with or without the addition of the enrichment medium and algal inoculum.

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

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

Statistical and Toxicological Terms

Cell yield refers to the change in concentration of algae at the end of the exposure period, relative to the initial cell density.

Chronic toxicity implies long-term effects that are related to changes in metabolism, growth, reproduction, or ability to survive. In this test, chronic toxicity is a discernible adverse effect (lethal or sublethal) induced in the test organisms within a 72 h exposure to a test material or substance.

Coefficient of variation (CV) is the standard deviation divided by the mean, usually expressed as a percentage.

Control limit is plus or minus three standard deviations, calculated on a logarithmic basis, from the historic geometric mean of the endpoints from toxicity tests with a reference toxicant.

EC50 is the median effective concentration. That is the concentration of material in water (e.g., mg/L) that is estimated to cause a discernible sublethal toxic effect to 50% of the test organisms. In most instances the EC50 (together with its 95% confidence limits) is statistically derived by analysis of an observed response for various test concentrations, after a fixed period of exposure. The duration of exposure must be specified (e.g., 72 h). Although it is frequently used to express an estimate of toxicity in algal tests, it is not appropriate.

Endpoint means the variables (i.e., time, reaction of the organisms, etc.) that indicate the termination of a test, and also means the measurements(s) or value(s) that are derived and characterize the results of the test (e.g., NOEC, ICp).

Homoscedasticity refers herein to data showing homogeneity of the residuals within a scatter plot. This term applies when the variability of the residuals does not change significantly with that of the independent variable (i.e., the test concentrations or treatment levels). When performing statistical analyses and assessing residuals (e.g., using Levene's test), for test data demonstrating homoscedasticity (i.e., homogeneity of residuals), there is no significant difference in the variance of residuals across concentrations or treatment levels.

Hormesis is an effect in which low concentrations of the test material or substance act as a stimulant for performance of the test organisms compared to that for the control organisms (i.e., performance in one or more low concentrations is enhanced and "better" than that in the control treatment). This stimulation must be accompanied by inhibition at higher test concentrations to be defined as *hormesis*. *Hormesis* is a specific subset of a *stimulatory effect*. (See also *stimulatory effect*).

ICp is the inhibiting concentration for a (specified) percent effect. It represents a point estimate of a concentration of test material or substance that causes a designated percent reduction in a quantitative biological measurement such as algal-cell yield.

IC50 is the median inhibition concentration, i.e., the concentration estimated to cause a 50% reduction in growth compared to a control. The exposure time must be specified, e.g., "IC50 (72 h)", for growth rate derived IC50s and a test duration of 72 h.

LOEC is the lowest-observed-effect concentration. This represents the lowest concentration of a test material or substance to which organisms are exposed and for which a statistically significant effect was observed relative to the control.

Mann-Kendall test is a statistical test used to detect trends in data (e.g., trends in algal growth across standard control wells). It is a non-parametric test, and hence the data do not need to be normally distributed. The test uses only the relative magnitudes of the data rather than their measured values (Gilbert, 1987).

Mann-Whitney U test is a statistical test used to determine whether two samples are different from each other (specifically, whether there are differences in dispersion between two independent samples). It is a non-parametric test, and hence the data do not need to be normally distributed. The raw data is converted into ranks before the test is carried out. It is considered a non-parametric equivalent of the t-test. Also known as the Wilcoxon-Mann-Whitney test, the Wilcoxon Rank-Sum test or simply the U test (Zar, 1999; EC, 2005).

NOEC is the no-observed-effect concentration. This represents the highest concentration of a test material or substance to which organisms are exposed and in which no significant change in algal growth is apparent relative to the control.

Normality (or *normal distribution*) refers to a symmetric, bell-shaped array of observations. The array relates frequency of occurrence to the magnitude of the item being measured. In a *normal distribution*, most observations will cluster near the mean value, with progressively fewer observations toward the extremes of the range of values. The normal distribution plays a central role in statistical theory because of its mathematical properties. It is also central in biological sciences because many biological phenomena follow the same pattern. Many statistical tests assume that data are normally distributed, and therefore it can be necessary to test whether that is true for a given set of data.

Quantitative is an adjective, as in quantitative data, quantitative test, etc. A quantitative effect is one in which the measured effect can take any whole or fractional value on a numerical scale. An example would be the weight attained by individual organisms, or the number of progeny produced at the end of a test.

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

Stimulatory effect refers to enhanced performance (i.e., "stimulation") that is observed in one or more test concentrations relative to that for the control treatment. In this document, *stimulatory effect* refers specifically to enhanced performance (i) at one or more of the highest concentrations tested or (ii) across all concentrations tested. *Hormesis* is a specific subset of a stimulatory effect. (See also *hormesis*).

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

Toxicity is the inherent potential or capacity of a material to cause adverse effects on living organisms.

Toxicity test is a method to determine the effect of a material on a group of selected organisms under defined conditions. An aquatic toxicity test usually measures either (a) the proportions of organisms affected (*quantal*), or (b) the degree of effect shown *(graded or quantitative)* after exposure to specific concentrations of chemical, effluent, elutriate, leachate, or receiving water.

Warning chart is a graph used to follow changes over time in the endpoints for a reference toxicant. The 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 on a logarithmic basis, from the historic geometric mean of the endpoints from toxicity tests with a reference toxicant.

Acknowledgements

The first edition of this report was co-authored by D. St.-Laurent (Centre Saint-Laurent, Longueuil, Quebec), G.L. Stephenson (Ecological Services for Planning Ltd., Guelph, Ontario), and K.E. Day (National Water Research Institute, Burlington, Ontario). G. Sergy and R. Scroggins (Environmental Protection, C & P, Environment Canada) acted as Scientific Authorities and provided guidance throughout the work.

Members of the Inter-Governmental Environmental Toxicity Group (IGETG) (Appendix B) actively participated in the development and review of this report and are thanked accordingly. The laboratory testing support of Centre Saint-Laurent, Environment Canada (Appendix C) is also acknowledged. Special acknowledgement is made of the technical contributions provided by C. Blaise, M. Harwood, G. Costan, R. Legault, and J. Warner.

Many useful comments and suggestions were volunteered by the following persons, who reviewed the initial and final draft documents of the first edition: J.-F. Férard (Centre des sciences de l'environnement, Metz, France), E.T. Howell (Water Resources Branch, Ontario Ministry of the Environment, Toronto, Ontario), H. Peterson (Saskatchewan Research Council, Saskatoon, Saskatchewan), C. Boutin (Canadian Wildlife Service, Gatineau, Quebec), D. McLeay (McLeay Associates Ltd., West Vancouver, British Columbia), C. Bastien (Quebec Ministry of the Environment, Sainte-Foy, Quebec), G. Joubert (Quebec Ministry of the Environment, Sainte-Foy, Quebec), J. Greene (Oregon State University, Corvallis, Oregon), P. Couture (Université du Québec à Rimouski, Rimouski, Quebec), Y. Roy (Analex Inc., Laval, Quebec), B. Dutka and A. Kwan (National Water Research Institute, Environment Canada, Burlington, Ontario), J. Warner (Eco-North Laboratories, Parry Sound, Ontario), J. Sprague (Sprague Associates Ltd., Guelph, Ontario), and G.F. Atkinson (Applied Statistics Div., Environment Canada, Ottawa).

The technical assistance of M. Harwood (Centre Saint-Laurent, Environment Canada) and D. Rodrigue (Method Development and Applications Section, Environment Canada) in preparing the 1997 Amendments to this report is acknowledged with thanks.

This (second) edition was prepared by L. Van der Vliet (Biological Methods Division, Environment Canada), with assistance and guidance from L. Taylor (Manager, Method Development and Applications Section) and R. Scroggins (Chief, Biological Methods Division) of the Environmental Science and Technology Centre, Environment Canada, Ottawa, Ontario, as well as M. Harwood (Environment Canada, Montreal, Quebec). The assistance of J. Michaud (Environment Canada, Montreal, Quebec) and J. Acreman (UTCC, University of Toronto, Toronto, ON) is also acknowledged. The second edition includes numerous updates such as modified test media and the use of regression analyses for quantitative endpoint data. Numerous comments and suggestions for change to the first edition, which were forwarded to Environment Canada's Method Development and Applications Section by Canadian laboratory personnel performing this toxicity test method, were considered when preparing this second edition of Report EPS 1/RM/25.

Photographs for the cover were supplied by Dr. Christian Blaise, Ecotoxicology and Ecosystems Branch, Centre Saint-Laurent, Environment Canada.

Section 1

Introduction

No single test method or test organism can be expected to satisfy a comprehensive approach to environmental conservation and protection. Delivery of the preventative and remedial measures necessary to manage the environment requires the effective use of a selected battery of well-defined aquatic *toxicity tests*. Sergy (1987), in consultation with the Inter-Governmental Environmental Toxicity Group (IGETG) (Appendix B), proposed a set of tests that would be broadly acceptable, and would measure different types of toxic effects in different organisms. The algal growth inhibition test using the microplate technique and the freshwater alga *Pseudokirchneriella subcapitata*[1] was one of several aquatic toxicity tests selected to be standardized sufficiently to help meet Environment Canada's testing requirements.

Traditionally, phytotoxicity of substances has been assessed with standardized algal bottle tests (EEC, 1988; ISO, 1989; OECD, 1984; USEPA, 2002). Recently, the Centre d'expertise en analyse environnementale du Québec published a modified algal method based on the USEPA (2002) procedure; this modified procedure requires smaller test volumes and no EDTA in the test media (CEAEQ, 2005). However, the microplate technique (Blaise, 1984, 1986; Blaise *et al.,* 1986, 1988) confers a number of advantages over the bottle test (Blanck, 1987; Blaise *et al.,* 1988; Blaise, 1991), and has been used to assess algal *toxicity* of individual substances and industrial *effluents* (Thellen *et al*., 1989; Warner, 1990; Blaise and Harwood, 1991; St-Laurent *et al.*, 1992; Scroggins *et al.*, 2002). The methodology presented in this report details standardized procedures for performing algal growth inhibition tests using the microplate technique.

[1] Formerly known as *Selenastrum capricornutum*.

1.1 Principles of the Test Method

Exponentially growing *P. subcapitata* are exposed in a *static*, microtitre system to various concentrations of a test substance, or a dilution series of an effluent or mixture, over several generations, under defined conditions. The growth of the algae exposed to the test substance is compared with the growth of the algae in an appropriate control over a fixed period of time. A test substance is considered toxic when a statistically significant, dose-dependent inhibition of algal growth occurs.

1.2 Summary of the Microplate Technique

The microplate technique is a scaled-down version of the standard USEPA algal bottle test (Miller *et al*., 1978; USEPA, 2002). It involves the use of microlitre volumes of *test solutions* contained on 96-well microplates, as opposed to millilitre volumes of test solutions individually contained in flasks. The test solutions are prepared and dispensed in a predetermined pattern to a microplate. Each well receives 200 µL of test solution, 10 µL of nutrient spike, and 10 µL of *algal inoculum*. The microplate is incubated at a constant temperature with continuous light for 72 h, at which time the concentration of organisms (i.e., number of cells per mL) is measured with an electronic particle counter or a hemocytometer. *Cell yield* is used as a surrogate for algal biomass. The number of algal cells in the test concentrations is then compared with the number in the control solutions.

Measurements of *absorbance* or *fluorescence* are alternate techniques. However, before making such measurements, it must be demonstrated that the technique chosen provides a consistent, quantifiable, and reliable

relationship with cell yield. Additionally, absorbance or fluorescence measurements may only be performed with samples if the test solutions containing algal inocula in microplates have been centrifuged using a special centrifuge head capable of accommodating the microplates, and if the algal cells are then resuspended in a clear solution before deriving the *endpoints* (see Subsection 4.5 for details of application).

1.3 Application, Advantages, and Limitations of the Microplate System

The algal growth inhibition test using the microplate technique is a screening test for phytotoxicity that is used to increase the efficiency in the processing of samples, as compared to the classic algal bottle test. The advantages of the microplate technique have been discussed extensively elsewhere (Blaise, 1986, 1991; Thellen *et al*., 1989) and are briefly summarized as follows:

- The test requires a small sample volume, a small volume of algae, and less space for incubation than bottle assays.
- Microplates and pipette tips are disposable, which eliminates the potential for contamination from the reuse of glassware and minimizes the time required for postexperimental washing of glassware[2].
- The test can easily accommodate a number of replicates per test concentration and a larger number of samples can be processed in a given time.
- The potential for test automation exists.

A concerted effort has been made to minimize the disadvantages and limitations of the microplate technique; however, as with any standardized toxicity test, there are inherent limitations that might or might not be unique to the microplate technique. These limitations are:

- Volatile substances might inhibit algal growth in other wells in the microplate. Where volatility is a factor, test concentrations must be isolated from one another by using separate plates or polyester seals.
- Filtering the sample prior to the test might significantly reduce toxicity of the effluent or mixture.
- High concentrations of dissolved organic material might confound test results.
- pH shifts in test solutions in the wells might be concentration dependent and affect toxicity of the test substance.
- Enhanced growth of algae might occur relative to the growth in the controls if excess nutrients are present in the *test sample*.
- Adsorption of the test substance to the microplate might mask toxicity by reducing the bioavailability of the compound to the algae.
- *Culture* health is critical and algae must be uncontaminated with other species of algae or micro-organisms and be in an exponential growth phase.
- The test period must be 72 h.
- Electronic particle counters do not differentiate between live and dead algal cells.

Despite these disadvantages or inherent limitations, the algal growth inhibition test using the microplate technique has been used effectively to screen toxicity of chemicals and chemical mixtures (Thellen *et al.,* 1989; St-Laurent *et al*., 1992). Whenever possible, suggestions and recommendations are included to minimize the effects of the inherent limitations.

[2] Note that for *chemical* testing only, glass microplates are required (unless the laboratory has conducted side-by-side tests validating the use of the polystyrene microplates, see Section 3.2).

Test Organism

2.1 Species

Pseudokirchneriella subcapitata is a nonmotile, unicellular, crescent-shaped (40 to 60 µm^3) green alga (Chlorophyceae) that is ubiquitous in most fresh waters in North America. This alga can be easily cultured in the laboratory and is readily available from reliable suppliers. Its uniform morphology makes it ideal for enumeration with an electronic particle counter. Clumping seldom occurs in *P. subcapitata* because it is free of complex structures and does not form chains. Growth is sufficiently rapid to accurately measure cell yield after 72 h, and the species is moderately sensitive to toxic substances.

The test species recommended for this test is *P. subcapitata* strains ATCC 22662, UTEX 1648, or UTCC 37. Some alga have very complex structures designed to form colonies or chains; this species does not. Although this test method has been developed specifically for *P. subcapitata*, it can also be used with other test species of algae (Blanck and Björnsäter, 1989; Warner, 1990; Day, personal communication; Peterson, unpublished); however, further research on test conditions (e.g., light intensity, test duration) is necessary with additional species of algae before the test can be standardized.

2.2 Source

A reliable Canadian source of *P. subcapitata* is:

University of Toronto Culture Collection (UTCC)
Department of Ecology & Evolutionary Biology
University of Toronto
Toronto, Ontario
Canada, M5S 3B2
Telephone: (416) 978-3641
Facsimile: (416) 978-5878
email: jacreman@eeb.utoronto.ca

Web site: http://www.botany.utoronto.ca/utcc
Pseudokirchneriella subcapitata : UTCC 37

Two reliable American sources of *P. subcapitata* are:

(a) American Type Culture Collection (ATCC)
P.O. Box 1549
Manassas, Virginia U.S.A. 20108
Telephone: 1-800-638-6597
Facsimile: (703) 365-2750
email: “contact us” form on website
Web site: http://www.atcc.org/
Pseudokirchneriella subcapitata : ATCC 22662

(b) Culture Collection of Algae (UTEX)
The University of Texas at Austin
1 University Station A6700
Austin, Texas U.S.A. 78712-0183
Telephone: (512) 471-4019
Facsimile: (512) 471-0354
e-mail: utalgae@uts.cc.utexas.edu
Web site: http://www.bio.utexas.edu/research/utex
Pseudokirchneriella subcapitata (listed as *Selenastrum capricornutum)* : UTEX 1648

The algae are available from the UTCC as axenic liquid cultures or agar slants. The ATCC delivers a frozen liquid culture in an ampoule that is transported in dry ice and must be resuspended in growth media. The UTEX culture is available in 10 mL agar slants.

The origin of the algal species from the sources previously listed was the Norwegian Institute of Water Research (NIVA). The alga was isolated

in 1948 by Olav Skulberg and since then has been renamed and modified to *Raphidocelis subcapitata* (Nygaard *et al*., 1986). Closely related ecophenes are *Ankistrodesmus bibraianus, Monoraphidium capricornutum*, and *Selenastrum minutum*. The species is commonly known in the literature as *P. subcapitata*.

2.3 Culturing

Algae should be cultured and maintained in a laboratory where temperature and lighting can be controlled. The culture should be isolated from the test chamber where the toxicity test occurs to minimize the risk of culture contamination by volatiles released from sample test solutions.

The "starter" culture of *P. subcapitata* might be on an agar slant, in liquid culture, or frozen in an ampoule as a dried pellet. The "starter" culture may be stored in the dark at 4 °C, and remain viable for at least 6 months. Every 12 months, a new culture must be purchased. The "starter" culture must be aseptically[3] transferred to, and resuspended in, a defined growth medium to maintain a stock culture of organisms as a source for the toxicity test. A new algal culture for toxicity testing must be set up from that "starter" culture every 2 months.

2.3.1 Liquid Growth Medium for Stock Algal Culture

The growth medium for the stock algal culture consists of five stock nutrient solutions and *reagent water*. Prepare the stock nutrient solutions in volumetric flasks using reagent grade chemicals and reagent water (see Table 1). These five preparations are all 1000 times the final concentration of the algal growth medium.

To prepare the liquid growth medium for the stock algal cultures, add 1 mL of each stock nutrient solution in order (1, 2, 3, 4, 5) to approximately 900 mL of reagent water and then complete to 1 L with reagent water in a 1000 mL volumetric flask. Mix well between each addition. Adjust final pH to 7.5 ± 0.1 with 1*N* HCl or NaOH. The final concentrations of the nutrients in the liquid growth medium for the stock algal culture are given in Table 2. The medium minimizes changes in pH over 72 h.

The growth medium should be filter-sterilized at a vacuum not exceeding 50.7 kPa (380 mm Hg), using a sterile apparatus and a prewashed 0.2 µm membrane. Sterilization of the liquid growth medium by autoclaving is not recommended, because this process reduces algal growth.

Place the filter-sterilized medium into sterile Erlenmeyer flasks with sterile stoppers. The sterile liquid growth medium can be stored in the dark at 4 °C for up to 6 months. The volume of growth medium will be determined by the total quantity of algal cells required for a toxicity test. Refer to Section 4.3 for calculation of this volume. A volume-to-flask ratio of 20% for the growth medium is recommended to avoid growth inhibition due to carbon monoxide limitation. For example: 25 mL medium in 125 mL flask; 50 mL medium in 250 mL flask; 100 mL medium in 500 mL flask.

Aseptically transfer either 1 mL of the "starter" algal culture using a disposable sterile pipette or a group of cells using a sterile loop to the liquid growth medium in the Erlenmeyer flask. Incubate the algal stock cultures at 24 ± 2 °C under continuous "cool white" fluorescent light with an intensity of 4000 *lux* at the surface of the flask [light quantal flux should approximate 56 µmol/($m^2 \cdot s$)]. The flask with the algae should be placed on a continuous shaker at 100 rpm or shaken manually twice daily. The algal

[3]See Appendix D for general guidance on maintaining sterile conditions.

Table 1 Stock Nutrient Solutions for the Growth Medium of the Stock Algal Culture

Stock Nutrient Solution	Compound	Quantity per 500 mL of Reagent Water
1	$NaNO_3$	12.75 g
2	$MgCl_2 \cdot 6H_2O$	5.0 g
	$CaCl_2 \cdot 2H_2O$	2.21 g
	H_3BO_3	92.76 mg
	$MnCl_2 \cdot 4H_2O$	207.81 mg
	$ZnCl_2$	1.64 mg[a]
	$CoCl_2 \cdot 6H_2O$	0.714[b]
	$CuCl_2 \cdot 2H_2O$	0.006 mg[c]
	$Na_2MoO_4 \cdot 2H_2O$	3.63 mg[d]
	$FeCl_3 \cdot 6H_2O$	80.0 mg
	$Na_2EDTA \cdot 2H_2O$	150.0 mg[e]
3	$MgSO_4 \cdot 7H_2O$	7.35 g
4	K_2HPO_4	0.522g
5	$NaHCO_3$	7.5 g

[a] Weigh out 164 mg of $ZnCl_2$ and dilute to 100 mL. Add 1 mL of this solution to Stock Nutrient Solution 2.
[b] Weigh out 71.4 mg of $CoCl_2 \cdot 6H_2O$ and dilute to 100 mL. Add 1 mL of this solution to Stock Nutrient Solution 2.
[c] Weigh out 60.0 mg of $CuCl_2 \cdot 2H_2O$ and dilute to 1000 mL. Dilute 1 mL of this solution to 10 mL. Add 1 mL of this second solution to Stock Nutrient Solution 2.
[d] Weigh out 363 mg of $Na_2MoO_4 \cdot 2H_2O$ and dilute to 100 mL. Add 1 mL of this solution to Stock Nutrient Solution 2.
[e] If this *stock solution* is being used for testing of metal substances in water or metal mining effluents, a separate stock must be prepared and used which will yield a final concentration of 25% of the full amount of $Na_2EDTA \cdot 2H_2O$. If prepared as described in the above table for testing, this amount would be 37.5 mg/500 mL. The original amount (150.0 mg) is always used for culturing.

Table 2 Final Concentrations of Nutrients in the Liquid Growth Medium of the Stock Algal Culture

Macronutrient	Concentration (mg/L)	Element	Concentration (mg/L)
$NaNO_3$	25.5	N	4.20
$MgCl_2 \cdot 6H_2O$	10.0	Mg	2.65
$CaCl_2 \cdot 2H_2O$	4.42	Ca	1.20
$MgSO_4 \cdot 7H_2O$	14.7	S	1.91
K_2HPO_4	1.04	P	0.186
		K	0.469
$NaHCO_3$	15.0	Na	11.0
		C	2.14

Micronutrient	Concentration (µg/L)	Element	Concentration (µg/L)
H_3BO_3	185.52	B	32.44
$MnCl_2 \cdot 4H_2O$	415.62	Mn	115.38
$ZnCl_2$	3.28	Zn	1.57
$CoCl_2 \cdot 6H_2O$	1.43	Co	0.35
$CuCl_2 \cdot 2H_2O$	0.012	Cu	0.004
$Na_2MoO_4 \cdot 2H_2O$	7.26	Mo	2.88
$FeCl_3 \cdot 6H_2O$	160	Fe	33.1
$Na_2EDTA \cdot 2H_2O$	300	-	-

culture may take 7 to 14 days to reach the exponential growth phase. When this occurs, the culture is very green in colour and the cell concentration is approximately 2×10^6 to 3×10^6 cells/mL.

The culture should be renewed on a weekly basis to ensure a regular supply of exponentially growing algal cells. This can be accomplished easily by aseptically transferring 1.0 mL of a stock algal culture that is, on average, between 3 and 7 days postinoculation, to a flask containing fresh liquid growth medium. Purity of the stock culture must be verified at each transfer by examining a subsample under a microscope for contamination by micro-organisms and by transferring 1 mL of algal stock culture to Petri dishes containing solid bacterial nutrient medium (e.g., Standard Plate Count Agar), and incubating at 24 °C for 48 h. This procedure should reveal the presence of contaminating bacteria that cannot be detected microscopically, even at high magnifications.

2.3.2 Solid Growth Medium for Stock Algal Culture

To ensure culture purity, periodically streak plate algal cells from a liquid culture onto sterile solid growth medium. The solid growth medium can then be used to isolate colonies of *P. subcapitata* to generate pure liquid stock algal cultures.

To prepare the solid growth medium, prepare the liquid growth medium described in Subsection 2.3.1. Add 1% agar and heat to dissolve. Sterilize by autoclaving at 98 kPa (1.1 kg/cm^2) and 121 °C for 30 min or 10 min/L, whichever is longer. Aseptically pour into Petri plates, cover, and leave to cool. Petri plates with solid growth medium can be stored upside down, in the dark, and at 4 °C, for up to 3 months.

Under aseptic conditions, and using streak-plate procedures, transfer algal cells from a liquid culture onto sterile solid growth medium. Incubate the plates upside down under conditions which match culturing conditions (i.e., 24 °C ± 2 °C under continuous “cool white” fluorescent light with an intensity of 4000 lux; no agitation necessary) until colonies are visible (approximately 2 weeks). A fresh liquid stock algal culture should be started every two months, using an algal colony isolated from the solid growth medium. Cells will remain viable for up to three months if the colonized Petri plates are stored in the dark at 4 °C. Algal cells used for testing should not be obtained from the first stock culture derived from a solid-phase (agar slant) “starter” culture.

2.4 Quality of Test Organisms

The test alga must be identified to species by microscopic examination, and the identification confirmed by an algal taxonomist. Routine microscopic examination of the stock algal culture also presents the opportunity to evaluate culture health in terms of cell morphology and colour, clumping, and contamination of the culture by micro-organisms. Culture health and performance must be evaluated by periodically measuring rate of growth (see Figure 1a) and the relative sensitivity of the algal culture to a *reference toxicant* (see Figure 2 and Section 4.7).

It is important to assess, on a routine basis, the performance and health of the algal species to be used for this biological test method. Therefore, an algal growth curve, starting with an inoculum from the algal stock culture, must be determined over an 8- to 10-day period using an Erlenmeyer flask (Figure 1a). It is recommended that an algal growth curve be performed ≥4 times/year, but must be performed at least 2 times/year. If the laboratory performs algal testing throughout the year, preparation of growth curves should be separated by 5-6 months; if the laboratory only performs algal testing seasonally (e.g., during summer months), these growth curves should be

conducted at the beginning and end of the testing period for that year[4].

From an algal stock culture, that is, on average, between 3 and 7 days post-inoculation, an inoculum of algal cells is aseptically transferred to an Erlenmeyer flask which contains fresh liquid growth medium, and incubated under the conditions recommended in Section 2.3.1. At t = 0 d and each subsequent day, an aliquot is aseptically taken from the Erlenmeyer flask and enumerated. This procedure is ended after the plateau phase is reached, which is usually between 8 and 10 days (the exponential phase is normally between 3 and 7 days). Culture conditions should be reviewed if exponential growth is not achieved in 3 to 7 days and/or the plateau phase is not reached in 8 to 10 days.

Although their use in optional, *quality control microplates* can be used to assess algal growth and to monitor any pH drift under the test conditions. To do this, three separate microplates, each containing 220 µL of reagent water in the peripheral wells and 200 µL of reagent water plus 10 µL of *algal inoculum* and 10 µL of enrichment media in each of the interior wells, are sealed and incubated under the same conditions as a test microplate.

At t = 0 h, cell counts are made randomly in at least six wells (peripheral wells excluded) using the first microplate, and the pH measured in six other wells. Reagent water (220 µL) is placed into each used well and the microplate returned to the incubator. Using this same microplate but different wells, cell counts and pH are also measured at t = 24 h. At t = 48 h, cell counts are made randomly in at least six wells (excluding peripheral wells) and pH measured in six other wells using the second microplate. The process is repeated at t = 72 h using a third microplate. Changes in pH (between t = 0 h and t = 72 h) should be less than 1.5 pH units. If this is not the case, culture conditions should be reviewed.

The results allow the generation of a growth curve over 72 h (Figure 1b). The average of the algal growth at t = 72 h gives a standard of performance to which the control wells of a test microplate can be compared. This type of growth curve has a different purpose than that generated using an Erlenmeyer flask according to the procedure described for Figure 1a. The benefit of using quality control microplates for generating a growth curve is that the user will have an indication of algal health under normal test conditions over a 72-h period.

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[4]In both of these scenarios, growth curves are assumed to be conducted twice yearly. If the laboratory conducts growth curves more frequently, these likewise should be spaced out to capture the testing period.

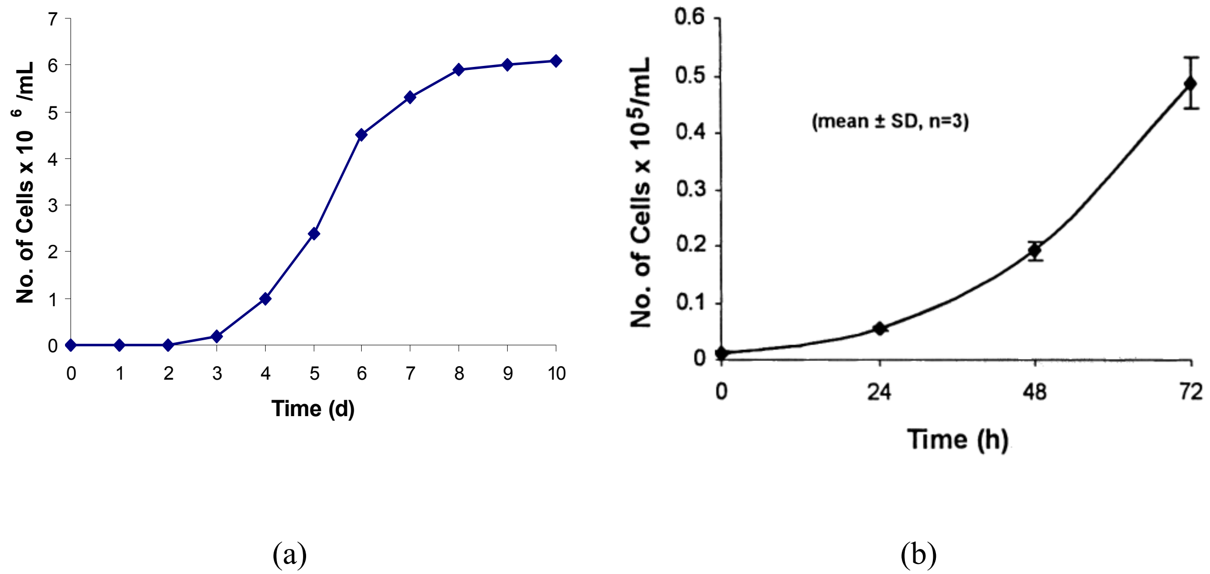

Figure 1 Growth Curves for *P. subcapitata*
(a) 8 to 10-day growth curve for *P. subcapitata* using an Erlenmeyer flask. (b) 72 h growth curve for *P. subcapitata* using quality control microplates

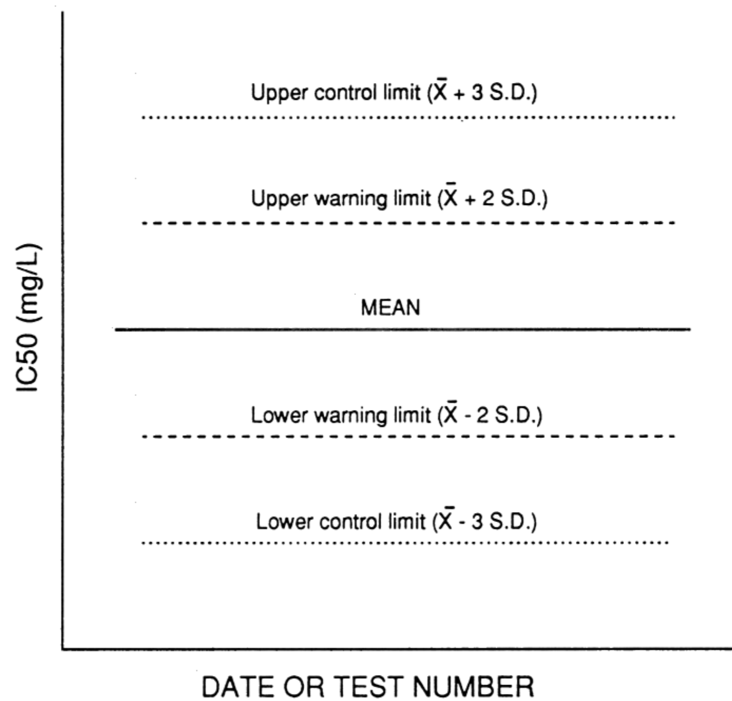

Figure 2 Warning Chart for Reference Toxicants
Adapted from Environment Canada (1990). Procedures for use are described in Section 4.7.

Test System

3.1 Facilities

The microplate algal growth inhibition test should be conducted in a facility where the temperature and lighting can be controlled and monitored continuously. An environmental chamber or incubator isolated from the algal culturing facility is recommended. The environmental chamber must meet the specifications for test type, temperature, and light quality and intensity (Section 3.3). The test chamber should be well-ventilated, free of toxic dust and vapours, and protected from unnecessary external perturbations. Test conditions should be uniform throughout the environmental chamber and identical to those in the culturing facility.

3.2 Equipment

All instruments for routine measurements of the basic chemical, physical, and biological variables must be maintained properly and calibrated regularly. Any equipment that contacts the test organisms, reagent water, nutrient solutions, growth media, enrichment medium, or test solutions must be made of chemically inert material (e.g., glass, stainless steel, plastic, porcelain) and be clean and free of substances that might interfere with the test (Section 3.4). Equipment must not be made of copper, zinc, brass, galvanized metal, lead, or natural rubber. Equipment not previously used in tests should be pre-rinsed in *dilution water* and tested for cytotoxicity prior to its use. Tables 3, 4, and 5 list the equipment, the disposable materials, and the reagents required to execute the algal growth inhibition test using the microplate technique. Test vessels used for this test are sterile disposable, rigid, polystyrene, 96-well microplates. Untreated microplates are recommended. If an electronic particle reader or manual enumeration is used, U-shaped microplates are required. If the photometric method is used, flat bottom plates are required. For chemical testing, glass microplates must be used to limit sorption. If laboratories can demonstrate, with side-by-side comparisons, that the test chemical does not sorb to polystyrene more than it does to the glass, the disposal polystyrene microplates may be used.

3.3 Lighting Conditions

Light conditions to which the algae are subjected should be the same as those defined in Section 2.3.1. The required light source is “cool white” fluorescent and the required intensity is 4 000 ± 400 lux (light quantal flux should approximate 56 ± 6 µmol/($m^2 \cdot s$)) at the surface of the test container.

3.4 Control/Dilution Water

For a given test, the same water must be used to prepare sample dilutions and controls. The choice of *control/dilution* water will depend on the objectives of the study, the test material or substance, the logistics of sample collection, handling, and transportation, practicality, and costs. Accordingly, these factors could lead to the selection of a specific type of control/dilution water best suited for a particular situation. The control/dilution water may be reagent water, uncontaminated *receiving water*, *upstream water*, uncontaminated groundwater, surface water (river, lake, or *dechlorinated* municipal water), or *reconstituted water*. Except when reagent water is used as control/dilution water, *standard controls* with reagent water must be included in the test as well as *sample controls*. This systematic incorporation of reagent water as a standard

control allows assessment of the toxicity of the selected diluent itself.

The control/dilution water recommended for the various types of aqueous samples is presented in Table 6. All control/dilution waters that have been field-collected must be filtered through a 0.45 µm filter before use to reduce the possibility of native algal contamination.

3.5 Washing of Glassware

All reusable glassware (Erlenmeyer and volumetric flasks, graduated cylinders, beakers, etc.) must be cleaned and treated to remove all trace metals and organics. The following method is recommended:

- wash with nonphosphate detergent solution;
- using a stiff-bristle brush, loosen any material attached to the inside wall of the glassware;
- rinse three times with tap water;
- rinse with cleaning solution (chromic-sulphuric acid or equivalent);
- for large containers, fill partially and swirl so that the entire inner wall is bathed;
- rinse three times with tap water;
- rinse with 50% HCl (v/v); (for large containers, fill partially and swirl so that the entire inner wall is bathed);
- rinse three times with *deionized water*;
- place in an oven at 105 °C until dry; and
- cover the opening of each container with aluminum foil or other cap, as appropriate, and store.

Reusable equipment made of any material other than glass must also be washed by this recommended method, if it can withstand the treatment.

Table 3 The Equipment Required to Execute a Microplate Algal Toxicity Test

- hemacytometer or electronic particle counter for enumerating algal cells
- environmental chamber or incubator
- Millipore Super - Q™ water purification system (or equivalent)
- refrigerator

- microscope with phase contrast providing 100 to 400 × magnification
- centrifuge 4 × 15 mL capacity; 2000 × g [1.33×10^{-7} $m^3/(kg \cdot s^2)$]; swing-out cups or brackets for centrifuge tubes and/or microplates
- calculator
- reading mirror

- burner and gas source
- adjustable digital microlitre multichannel pipettes: one with 10 to 100 µL capacity; one with 100 to 1000 µL capacity
- tube racks: one for 20-mm tubes; one for 40-mm tubes
- inoculating loop and holder

- analytical balance and weighing spatula
- wash bottle
- volumetric flasks: 100, 500, and 1000 mL capacities
- pH meter or pH paper
- glass microplates (for chemical testing only)

- filter apparatus: 47-mm stainless steel filter holder; 1-L filtering flask; a vacuum pump and tubing
- magnetic stirrer and stirring bars
- glass Erlenmeyer flasks of 125 mL to 4 L capacity, depending on the number of exponentially growing organisms required for inoculation (see Section 2.3)

- glass graduated cylinders: 25, 50, 100, 500, and 1000 mL capacity
- 1-L glass beaker
- heat sealer
- photometer 0 to 10 000 lux

Optional:

- microplate fluorometer
- microplate photometer

Table 4 Disposable Materials Required to Execute a Microplate Algal Toxicity Test

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- microplates: sterile disposable, rigid, polystyrene, 96-well (capacity approximately 0.25 mL); untreated microplates recommended; U-shaped microplates are required for electronic particle reader (Coulter counter) and manual enumeration (hemacytometer), flat bottom plates are required for photometric method (microplate reader)
- sterile disposable serological, 1 and 10 mL pipettes
- sterile disposable microlitre pipette tips for the microlitre and multichannel pipette
- sterile disposable plastic reservoirs
- sterile disposable glass test tubes (16 × 150 mm)
- sterile disposable centrifuge tubes with screw caps (15 and 50 mL capacity)
- sealable transparent plastic bags (approximately 16 × 20 cm)
- transparent plastic cups (20 mL)
- sterile disposable 100 × 15 mm Petri dishes
- filtration membrane (0.20 and 0.45 µm porosity)
- aluminum foil
- weighing dishes
- glass Pasteur pipettes
- hemacytometer cover glasses
- calibration microspheres for electronic particle counter:
  8.7 µm diameter polystyrene divinyl benzene latex particles are recommended
- polyester adhesive microplate film

Table 5 Reagents Required to Execute the Algal Microplate Toxicity Test

- reagent grade chemicals are to be used in all tests
- reagent water: Millipore Super Q™ water or equivalent water (e.g., must be free of ions, organic molecules, and particles, and micro-organisms greater than 0.45 µm diameter)
- reference toxicant(s)
- cleaning reagents: commercially available nonphosphate detergent and chromic-sulphuric and hydrochloric acids
- isotonic diluent composed of: 0.15 mol/L NaC1, 3.0 nmol/L KC1, 15 nmol/L phosphate buffer (pH 7.5)
- certified buffer solutions of pH 4, 7, and 10 for calibration of pH meter
- stock nutrient solutions (see Subsection 2.3.1; Table 1)
- liquid growth medium (see Subsection 2.3.1)
- solid growth medium (see Subsection 2.3.2)
- microtest enrichment medium (see Section 4.2)
- water-bicarbonate solution
- NaOH and HC1 solutions, ≤ 1 *N*
- *algal inoculum* of *P. subcapitata* from stock algal culture that is 3 to 7 days old and in logarithmic growth phase

Table 6 Control/dilution Water Recommended for the Algal Growth Inhibition Test with the Various Types of Aqueous Samples

Effluents, *elutriates*, *leachates*	Reagent water or receiving water
Receiving water	Reagent water or upstream water
Reference toxicants	Reagent water
Chemicals	Reagent water or receiving water

Universal Test Procedures

Procedures described in this section apply to all types of aqueous samples and all test chemicals. All aspects of the test system described in Section 3 must be incorporated into these universal test procedures. Additional test procedures for samples of chemicals with or without volatile constituents, effluents, elutriates, leachates, and receiving waters, are delineated in Sections 5, 6, and 7.

The procedures and conditions that are fundamental to all algal microplate growth inhibition tests are summarized in Table 7 and described in detail in the following sections.

4.1 Selection of a Microplate Configuration

Microplate configurations recommended for toxicity tests performed with or without reagent water as the control/dilution water[5] are illustrated in Figure 3.

Peripheral wells on the microplate are excluded from the test because of an "edge-effect" phenomenon associated with microplates. Evaporative loss in these wells is greater and introduces unnecessary variability among replicates. Nevertheless, the peripheral wells are filled with reagent water to saturate head space with humidity in these wells, which will, in turn, minimize evaporative losses from the inner wells. The evaporative loss from test solutions in the wells during incubation should not exceed 10%. The insertion of a central row of control replicates (e.g., row D, Figure 3), parallel to the gradient of test concentrations, identifies potential contamination due to toxic volatile substances from adjacent test wells. In the case where the control/dilution water used was not reagent water, an additional row of controls (e.g., row E, Figure 3) must also be included. Section 4.6 provides guidance for statistical procedures which deal with heterogeneity among cell yield estimates due to volatile substances or other causes.

4.2 Preparation of the Enrichment Medium and Test Solutions

Inhibition of algal growth in the test can be attributed to toxicity and/or nutrient deficiency inherent to the test solution. The addition of a 10 µL nutrient spike (i.e., enrichment medium) to each treatment well permits the elimination of false negative results due to nutrient deficiencies. The addition also ensures that algal cells will reproduce to an acceptable, measurable level over the incubation period.

The nutrient spike is derived from an enrichment medium that is prepared by adding 13.75 mL of each of the five nutrient stock solutions in Table 1 to approximately 800 mL of reagent water, and then diluting to 1 L with reagent water[6]. The final concentration of each nutrient in the treatment wells on the microplates is presented in Table 8. The pH is then adjusted and the medium is filter-sterilized as described in Subsection 2.3.1.

The uninoculated, sterile enrichment medium must be stored in the dark, at 4 °C, in a closed inert container. This solution can be stored under these conditions for up to 6 months.

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[5]See Section 3.4 for options and recommendations for choice of control/dilution water.

[6]If testing metal substances in water or metal mining effluent, the stock solutions and the volumes used must produce a final concentration which is 25% of the full amount. Given the stock solutions used in Table 1 and the volumes given here, stock 2 would need to contain 37.5 mg/500 mL $Na_2EDTA \cdot 2H_2O$.

Table 7 Checklist of Recommended Test Conditions and Procedures for Conducting Toxicity Tests Using the Microplate and algae *Pseudokirchneriella subcapitata*

Universal	
Test type	– static, 72-h duration
Test container	– 96-well polystyrene microplate, U-shaped or flat-bottomed; glass microplate (chemical testing only)
Control/dilution water	– reagent water, uncontaminated receiving water, "upstream" water, uncontaminated groundwater, surface water or reconstituted water
Test organism	– *Pseudokirchneriella subcapitata* a culture that is between 3 and 7 days old and in exponential phase of growth; initial cell density 10 000 ± 1000 cells/mL
Number of concentrations	– minimum of 7, plus standard reagent control (and additional dilution water, if applicable); recommended 10, plus control(s)
Number of replicates	– minimum of 3 enumerated for each test concentration; recommended 4 (two controls) or 5 (one control); 10 replicates for control(s) with 8 enumerated
Temperature	– 24 ± 2 °C
Filtration	– test solutions filtered through 0.45 µm filter
Nutrient spiking	– test samples are spiked with the same nutrients, at the same concentrations as those used in the control/dilution water
Aeration	– none
pH	– no adjustment if pH of test solution is in the range 6.5 to 8.5; a second (pH-adjusted) test is recommended for pH outside this range
Lighting	– continuous overhead "cool-white" fluorescent illumination with 4000 ± 400 lux at the surface of the test container, and a quantal flux between 50 to 62 µmol/($m^2 \cdot s$)
Observations	– cell concentration (or absorbance at 430 nm) at 72 h
Measurements	– pH, temperature of incubator or chamber
Endpoint	– cell yield (*ICp*)
Reference toxicant	– phenol, $ZnSO_4$, or $CuSO_4$
Test validity	– valid if: *coefficient of variation* in the standard control wells is ≤ 20%; coefficient of variation in standard control wells is ≥ 10% but ≤ 20% and no trend or gradient present in standard control treatment (*Mann-Kendall test*); the number of algal cells measured or estimated (if photometry used) for standard controls increases by a factor greater than 16

Chemicals

Test container	– glass microplate
Solvents	–only in special circumstances; additional solvent control required;
Control/dilution water	– reagent water; receiving water if the objective is to assess local toxic effects
Test validity	– as in universal; if solvent is used, *Mann-Whitney U test*

Effluents, Elutriates and Leachates

Sample requirement	– single 1 L sample
Transport and storage	– if warm (> 7° C), must be cooled to 1 to 7° C with regular ice (not dry ice) or frozen gel packs upon collection; sample must not freeze during transit or storage; store in the dark at 4 ± 2° C; use in testing should begin as soon as possible after collection and must start within 3 days of sample collection or elutriate extraction
Control/dilution water	– reagent water if *monitoring* regulatory *compliance*; receiving water if assessing local effects

Receiving Water

Sample requirement	– as for effluents, leachates and elutriates
Transport and storage	– as for effluents, leachates and elutriates
Control/dilution water	– “upstream” water if assessing local effects (separate reagent water controls are also included in the test); reagent water in special circumstances

Figure 3A

	1	2	3	4	5	6	7	8	9	10	11	12
A	Reagent Water only											
B		T_1	T_2	T_3	T_4	T_5	T_6	T_7	T_8	T_9	T_{10}	
C		T_1	T_2	T_3	T_4	T_5	T_6	T_7	T_8	T_9	T_{10}	
D		S	S	S	S	S	S	S	S	S	S	
E		T_1	T_2	T_3	T_4	T_5	T_6	T_7	T_8	T_9	T_{10}	
F		T_1	T_2	T_3	T_4	T_5	T_6	T_7	T_8	T_9	T_{10}	
G		T_1	T_2	T_3	T_4	T_5	T_6	T_7	T_8	T_9	T_{10}	
H	Reagent Water only											

Figure 3B

	1	2	3	4	5	6	7	8	9	10	11	12
A	Reagent Water only											
B		T_1	T_2	T_3	T_4	T_5	T_6	T_7	T_8	T_9	T_{10}	
C		T_1	T_2	T_3	T_4	T_5	T_6	T_7	T_8	T_9	T_{10}	
D		S	S	S	S	S	S	S	S	S	S	
E		C	C	C	C	C	C	C	C	C	C	
F		T_1	T_2	T_3	T_4	T_5	T_6	T_7	T_8	T_9	T_{10}	
G		T_1	T_2	T_3	T_4	T_5	T_6	T_7	T_8	T_9	T_{10}	
H	Reagent Water only											

Figure 3 **Standard Microplate Configuration**
The set-up shown in 3A is to be used for tests where reagent water is used for dilution and as the control water; the set-up shown in 3B is to be used for tests where reagent water is not used as the diluent (e.g., receiving water used to perform dilutions). Peripheral wells are filled with 220 µL of reagent water. Test concentrations are designated by T, with T_1 and T_{10} as the highest and lowest test concentrations, respectively. Standard reagent controls are designated by S, and sample controls, composed of dilution water other than reagent water, are denoted by C.

Table 8 Final Concentration of Nutrients in the Test Medium

Macronutrient	Concentration (mg/L)	Element	Concentration (mg/L)
$NaNO_3$	15.94	N	2.63
$MgCl_2 \cdot 6H_2O$	6.25	Mg	1.65
$CaCl_2 \cdot 2H_2O$	2.76	Ca	0.75
$MgSO_4 \cdot 7H_2O$	9.19	S	1.20
K_2HPO_4	0.65	P	0.12
		K	0.293
$NaHCO_3$	9.38	Na	6.88
		C	1.34

Micronutrient	Concentration (µg/L)	Element	Concentration (µg/L)
H_3BO_3	115.95	B	20.27
$MnCl_2 \cdot 4H_2O$	259.76	Mn	72.11
$ZnCl_2$	2.05	Zn	0.98
$CoCl_2 \cdot 6H_2O$	0.89	Co	0.22
$CuCl_2 \cdot 2H_2O$	0.008	Cu	0.003
$Na_2MoO_4 \cdot 2H_2O$	4.54	Mo	1.8
$FeCl_3 \cdot 6H_2O$	100	Fe	20.7
$Na_2EDTA \cdot 2H_2O$	187.5	-	-
	46.9[a]		

[a] Use of this lower concentration of $Na_2EDTA \cdot 2H_2O$ is required when testing metal substances in water or metal mining effluent.

4.3 *Beginning the Test*

4.3.1 *Preparation of the Algal Inoculum*

The *algal inoculum* must be prepared no more than 2 or 3 hours before incubation of the microplate. The inoculum is composed of *P. subcapitata* cells harvested from a liquid stock algal culture that is 3 to 7 days old and must be in a logarithmic phase of growth (as demonstrated from growth curves). Algal cells should not be obtained from the first stock culture derived from the starter culture. The *initial cell density* for the microplate algal growth inhibition test must be 10 000 ± 1 000 cells/mL. Because the final test volume per well is 220 µL, an absolute number of 2200 cells (10 000 cells/mL × 0.220 mL) is required for each well.

Estimate[7] the volume of liquid stock algal

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[7]For example, to get the appropriate algal cell concentration, the following steps may be taken:

(1) Multiply the total number of wells to be inoculated by 2200 to obtain the total number of cells required for inoculation.
(2) At the time of the actual inoculation, approximately 1 mL of *algal inoculum* is not available for use. To account for this loss, add 500 000 to the total number of cells determined.
(3) Use an automatic particle counter or hemacytometer of cells in the algal stock culture.
(4) Divide the value calculated in (2) by the cell density obtained from (3).
(5) An inevitable loss of cells must be accounted for in the subsequent centrifugation and resuspension phase. To compensate for this loss, multiply the value from (4) by 1.5 to give the volume of the stock algal culture to harvest.

For example, with one microplate to inoculate and a stock

culture needed to complete the test, and withdraw this volume.

The harvested cells must be centrifuged at 2000 g for 15 min, the supernatant discarded, and the cells resuspended in a few millilitres (e.g., 5 to 10 mL) of a bicarbonate solution ($NaHCO_3$ at 15 mg/L). The bicarbonate solution used in this procedure may be prepared by diluting stock nutrient solution 5. Determine the concentration of cells (cells per mL) with an electronic particle counter or hemocytometer. If necessary, dilute the algal suspension to 220 000 cells/mL with the solution of $NaHCO_3$, so that adding 10 µL of *algal inoculum* to each microplate well will give the required *initial cell density* of 10 000 ± 1000 cells/mL. The final volume in the well must be 220 µL (e.g.[8] 200 µL of test sample, 10 µL of *algal inoculum*, and 10 µL of enrichment medium).

There could be as many as six aqueous solutions used in this microplate test:

(1) the test sample;

(2) the control/dilution water;

(3) the reagent water;

(4) a bicarbonate solution ($NaHCO_3$ at 15 mg/L);

(5) an enrichment medium or nutrient spike; and

(6) solutions for pH adjustment.

All aqueous solutions should be at room temperature prior to testing.

4.3.2 Preparation of Test Solutions

Test samples should be shaken vigorously to ensure homogeneity and to resuspend particulate. A subsample sufficient to complete the test (e.g. 5 to 10 mL) must be filtered[9] through a preconditioned[10] membrane of 0.45 µm pore diameter, and then placed in a test tube.

The pH of any aqueous sample must be measured just before it is used to prepare test solutions. The toxicity test should normally be carried out without adjustment of pH. However, if the pH of a sample is outside the range 6.5 to 8.5, and it is desired to assess toxic chemicals rather than the deleterious or modifying effects of pH, then the pH of the sample should be adjusted to 6.5 or 8.5 (whichever is closest to the initial pH of the sample) before the start of the test. HCl or NaOH (≤ 1 *N*) should normally be used for all pH adjustments. A recommended procedure for distinguishing the influence of sample pH on toxicity is to conduct two tests concurrently, with and without pH adjustment.

Ideally, a test should include a concentration that has no effect on algal-cell yield, a concentration that completely inhibits algal growth, and two concentrations each above and below the *IC50* value. If the toxicity of a substance to *P. subcapitata* is known, prepare concentrations of the test solutions to encompass a response range that includes no inhibition of growth and no growth. These test

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algal culture density of 1 000 000 cells/mL, the total volume to withdraw from the culture flask would be:
(1) 60 wells × 2200 cells/well = 132 000 cells
(2) + 500 000 cells = 632 000 cells
(3) 1 000 000 cells/mL
(4) 632 000 cells ÷ 1 000 000 cells/mL = 0.632 mL
(5) 0.632 mL × 1.5 = 0.948 mL.

[8] The ratios of test sample, *algal inoculum* and enrichment medium may be altered, for example, to test a higher concentration of effluent. However, the results must be reported based on the dilution-corrected concentrations, and the final volume in the microplate must be 220 µL.

[9] The requirement for filtering does not apply for chemical testing; stock solutions used in chemical testing are not to be filtered. Reagent water may be filtered prior to preparation of the stock solution of the test chemical.

[10] Filter is rinsed with a small volume of test solution, and this rinse water is discarded.

concentrations can be prepared with reagent water, or with dilution of a test sample with other control/dilution water (see Section 3.4). If the toxicity of a sample is unknown, a preliminary range-finding test should be used. The purpose of this test is to define a response range of concentrations that encompasses the IC50, and to determine if there are volatile substances in the sample.

For any test that is intended to estimate the ICp, at least seven concentrations plus the control/dilution water must be prepared, and more (10 plus a control) are recommended to improve the likelihood of bracketing the endpoint. An appropriate geometric series may be used in which each successive concentration is about a factor of 0.5 of the previous one (e.g., 100, 50, 25, 12.5, 6.3, 3.1, 1.6, or in the case of *wastewater* and receiving-water samples, 91, 46, 23, 11, 5.7, 2.8, 1.4). In the case of wastewater and receiving water samples, the actual concentrations are slightly lower because the test concentrations are diluted[11] with the addition of the nutrient spike and *algal inoculum*; these actual (calculated) concentrations must be used in endpoint calculations and in reporting.

Test concentrations may be selected from other appropriate dilution series (e.g., 100, 75, 56, 42, 32, 24, 18, 13, 10, 7.5; see column 7 in Appendix E). If the sample is suspected to be highly toxic then a range of lower concentrations should be included as well (e.g., 11, 3.7, 1.2, 0.41, 0.14, 0.05, 0.02% v/v).

For each test solution, 4 replicates (for two controls) or 5 replicates (for one control) must be set-up, and a minimum of 3 replicates must be enumerated. Ten replicates must be used for the standard control and any additional dilution water (if used); 2 of the 10 standard control wells are used to measure pH, and the remaining 8 must be used for cell enumeration.

The total volume of a sample required for a test is approximately 5 mL, with a dilution factor of 0.33. Dilute the sample in test tubes of the appropriate size with the selected dilution water. While performing the dilutions, the sample aliquot should be injected below the surface of the dilution water at the bottom of the test tube to minimize volatilization. After each transfer, the solutions should be well mixed in the test tube. Once the dilutions are completed, the solution volume for each sample concentration must be at least 3 mL. If chemical analysis of the test concentrations is desired, a higher volume of each test solution must be prepared.

4.3.3 Dispensing Test Solutions, Algal Inoculum, and Nutrient Spike to the Microplate

The microplates and lids should be sterilized with ultraviolet light for 15 to 20 min prior to use. A multichannel pipette is used to dispense 220 µL of reagent water from a plastic reagent reservoir to each of the 36 peripheral wells of a microplate. The microplate should be labelled to identify test substance, concentration, date, and time of test. Pipette 200 µL of reagent water into each of the 10 wells that will serve as the standard reagent controls (i.e., wells D2 to D11). With the multichannel pipette dispenser, starting with the lowest concentration of test solution (i.e., the highest dilution) and ending with the highest concentration of test solution (i.e., the lowest dilution), add the test solutions to the appropriate microplate wells. Take care not to contaminate the reagent water, control/dilution water, and test solutions. Use a separate plastic reservoir for each test concentration. A single reservoir may be used with impunity for all test concentrations provided that the lowest concentration is dispersed first with a progression toward the highest concentration, and the reservoir is adequately emptied between each addition.

[11]Using the standard 10 µL of algal inoculum, 10 µL of enrichment medium and 200 µL of test sample, this dilution would be 0.9091.

Combine equal volumes of *algal inoculum* and enrichment medium. A check of the cell concentration of this mixture should be made with an electronic particle counter or hemacytometer. The cell concentration must be such that the *initial cell density* in each microplate well is 10 000 ± 1000 cells/mL. If the initial density is appropriate, then with a multichannel pipette, add 20 µL of this mixture to each well of the microplate; omit the peripheral walls. An initial cell concentration outside of the acceptable range implies that the mixture should repeated.

The mixture should be kept homogeneous during the inoculation step. The test begins as soon as the *algal inoculum* and nutrients are added to the wells. A lid or polyester film is added to cover each microplate.

If volatility of toxic substances in test solutions is known or anticipated, either a separate microplate must be used for each test concentration or polyester film which seals the individual wells must be used.

4.3.4 Procedures for Incubation and Measurement of Initial Cell Concentration

Place the microplates in transparent plastic bags and seal to minimize evaporation during the exposure period. After each microplate is sealed in a plastic bag, all microplates must be placed in an incubator or environmental chamber. Microplates should be distributed randomly throughout the incubator.

4.3.5 Quality Control Microplate

Although their use is optional, quality control microplates could be used to provide a worthwhile standard for appraising algal growth under the test conditions and also to monitor the pH inside the wells. The details on how to prepare and employ a growth curve for *P. subcapitata*, using quality control microplates, are provided in Section 2.4 (see Figure 1b). It should be noted that a growth curve derived using quality control microplates does not replace the obligatory requirement for the generation of a growth curve from the algal stock culture in an Erlenmeyer flask (see Figure 1a). In the same way, pH measurements using quality control microplates do not replace the requirement for pH determinations in the two median control wells of each microplate that contains test solutions (see Section 4.5).

4.4 Test Conditions

The duration of the *P. subcapitata* test is 72 hours. The test is a static test with no solution renewal.

The test must be conducted at a temperature of 24 ± 2 °C. Light conditions must be as described in Section 3.3. Test solutions must not be aerated during the test.

The test must be considered invalid if the conditions described in Section 4.6.1 are not met.

4.5 Test Observations and Measurements

After incubation, remove the microplates from their plastic bags. Record whether condensation is present on the lid or in the bag, and describe the location of this condensation. Place the microplate on a white background and visually examine the plate for algal growth in the test treatments. The U-shaped or round-bottomed wells tend to concentrate the organisms into the centre, and in clear solutions it is relatively easy to distinguish wells with algal growth (green) from those with no growth (white). The presence of a white growth could indicate bacterial growth during incubation. Record these observations.

Measure the pH of one standard control well per microplate at the start (t = 0 h) and end (t = 72 h) of the test (e.g., median wells D6 and D7) , using a microprobe or pH measurement paper. The measurement at the end of the test should be made before the algal cells are resuspended. Algal counts should not be made for these two wells. The pH difference between these two readings should not differ by more than 1.5 pH units; if it does, the test should be repeated.

Enumerate the cells in the remaining 8 standard control wells (D2 to D5 and D8 to D11) and at least three wells containing each test concentration, and also, if it applies, in each of the sample control wells (see Figure 3). If enumeration results from the three replicates of test concentrations are inconsistent (e.g, high variation), counts must be completed of additional replicates.

In certain circumstances, the enumeration of less than 7 concentrations is permissible. These circumstances are limited to the following situations: (i) if the cell counts of the lower test concentrations show that a large effect (e.g., ⋙ IC50) has been reached, counts at test concentrations which are higher than this are not required ; (ii) if the cell counts show that there is no effect, only 6 concentrations are required for enumeration; the highest test concentration must be enumerated.

Algal-cell concentration may be measured by direct enumeration with an electronic particle counter or a hemacytometer, or indirectly by measuring absorbance with a microplate photometer.

Electronic Cell Enumeration. The use of an electronic particle counter[12] allows a rapid determination of the cell concentration (cells/mL). Use of U-shaped or round-bottomed plates are required for this method. The counter must be calibrated according to standard operating procedures. The operative aperture diameter recommended for *P. subcapitata* is 70 µm.

Algal cells, which in all likelihood have settled to the bottom of the wells, must be resuspended so that the contents are homogenous within each well. To resuspend the cells, carefully draw the contents into the multichannel micropipette and expel the contents back into the wells. Repeat at least 10 times; then, with the micropipette, withdraw 170 µL from each well and dispense into individual plastic cups.

Fill each cup to 10 mL with isotonic diluent (dilution factor of 10:0.170), and enumerate algae with the particle counter. Each sample should be counted one to three times. Ideally, cells should be counted immediately after the isotonic solution is added. Alternatively, cover cups with an appropriate cap and store in the dark at 4 °C. Resuspend and count the cells within 24 h of adding the isotonic solution. Tabulate the results according to the microplate configuration used in the toxicity test. With a microscope, check the replicates for microbial contamination.

Manual Cell Enumeration. Algal cells may be counted using a microscope and a hemacytometer. Refer to APHA *et al.* (2005) for details on the microscope counting method. Although this method is less precise than the electronic counting method, it permits the direct examination of the condition of the cells and cell debris. The cells must be resuspended prior to subsampling. Record the results according to the microplate configuration used in the toxicity test (i.e., eight horizontal alphabetical rows from A to H and 12 vertical numerical columns from 1 to 12). Consider the dilution factor, and determine the cell concentration for each treatment replicate, and tabulate accordingly.

[12] Other automatic counters, such as optical particle counters, may also be used for rapid cell enumeration.

If manual enumeration is used, enumeration must be performed on the same day the test is terminated; microplates must not be stored at 4°C.

Microplate Photometry. An indirect method of estimating cell concentration[13] is to measure light absorbance of the resuspended algal cells in each well. This can be done quickly and easily using flat-bottomed microplates. If photometry is to be used, concurrent cell counts must be made using an electronic particle counter or a hemocytometer, for at least 3 wells containing test solutions and representing high, medium, and low cell densities. The results for these three direct counts must be compared to the estimates of cell density obtained by the photometric method for the same wells. The direct counts of cell density should be within the expected variation (i.e., ± 2 SD) for the respective points on the standard calibration curve representing absorbance versus cell concentration (Figure 4). If this is not the case, algal counts for each of the wells on the microplate should be determined only by direct enumeration, using an electronic particle counter or a hemocytometer.

For photometric determinations, the algal cells in each well must be resuspended in reagent water before measuring absorbance. To do so, place the microplate in a temperature-controlled centrifuge equipped with a head appropriate for centrifuging microplates. Centrifuging for 5 to 10 min at 2000 g [1.33×10^{-7} m^3 /(kg · s^2]. After centrifugation, remove the microplate lid and/or polyester seal, and carefully decant the supernatant with the multichannel pipette.

[13] Other indirect determinations of culture density (e.g., microturbidimetry, microfluorometry) may be used if sufficiently sensitive, and the endpoint is correlated with the algal cell concentration. All indirect measurements of algal concentration require an *a priori* demonstration of the relationship with cell concentration. This is obtained from standard curves or regression analysis (see Figure 4).

Ensure that the algal plug at the bottom of the wells is not disturbed. Add to each well 200 µL of reagent water, and resuspend the organisms using a multichannel pipette as described previously.

Thereafter, place the uncovered microplate in a microplate photometer, with the wavelength set to 430 nm. Follow the standard operating procedures for calibrating the machine. The reagent water should be used as a blank. Following calibration, measure the absorbance in each well of the microplate.

4.6 Test Endpoints and Calculations

The endpoint of this test is based on growth inhibition of the algae exposed to the test materials or substances. The variable used to determine the endpoint is the algal cell yield, which is defined as the change in cell concentration of the algal population over the incubation period of 72 h.

To determine the cell yield in each well, subtract the *initial cell density* (~10 000 cells/mL; see Section 4.3) from the final measured concentration (or estimated concentration if using photometry). The cell yield in the standard control wells of each sample microplate (i.e., wells D2 to D5 and D8 to D11) must have a coefficient of variation of ≤20%. If the coefficient of variation is ≥10% but ≤20%, the results for these same wells must also be compared statistically using trend analysis by the *Mann-Kendall test* (Gilbert, 1987) to check that there is no gradient of effect in the control treatment (i.e., $p > 0.05$, indicating no positive or negative trend in algal cell concentration).

Any trend indicates that volatile contaminants in the sample have affected the controls and other treatments, in which instance the test must be repeated using a multiple microplate approach (i.e., one microplate per test concentration) or a polyester film which seals the individual wells.

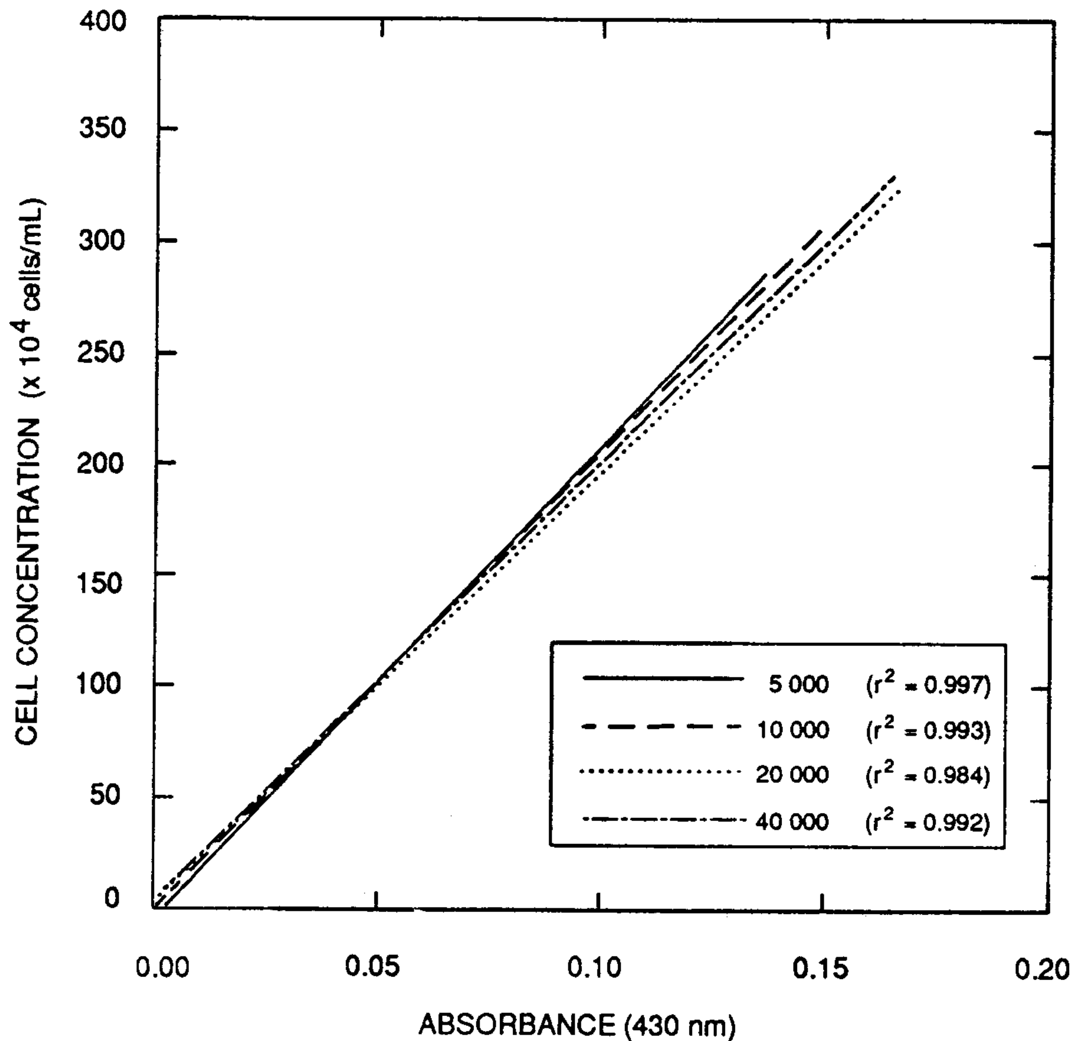

Figure 4 **Algal Cell Concentration Regressed with Absorbance**
A first-order linear regression was performed on cell concentration versus absorbance, at 430 nm, for four initial cell concentrations.

It is also recommended that the average cell yield in the standard control wells on each microplate (i.e., D2 to D5 and D8 to D11) be compared with that obtained for the standard control wells in another test (e.g., a reference toxicity test) using identical conditions and procedures. If these data do not correspond (within 2 SD, calculated for the reference toxicant), then the test should be repeated using one test concentration per microplate.

Calculate the mean cell yield for the standard and/or sample controls. If the microplate configuration includes both a standard reagent control and a sample control (Figure 3), a statistical comparison for significant differences of means must be performed using a paired t-test or the Wilcoxon signed rank test. If no significant difference exists, then the options available are to pool them to form one control or use the sample control and exclude the standard reagent control.

4.6.1 Validity of Test

For a valid test, each of the following must be met:

- Homogeneity must be demonstrated for the standard control wells, among the measurements or photometric estimates of cell yield. For a valid test, the coefficient of variation must not exceed 20% (i.e., coefficient of variation must be less than or equal to 20%);

- Where the coefficient of variation in the standard control wells is greater than or equal to 10% but less than or equal to 20%, a trend analysis (*Mann-Kendall test*; see Gilbert, 1987) must be applied to estimates of cell yield in the standard control wells and must indicate that there is no trend or gradient in algal cell concentration across the control treatment ($p > 0.05$)[14]; and

- The number of algal cells measured or estimated (if photometry used) for the standard controls must have increased by a factor of greater than 16 in 72 h.

4.6.2 Multi-Concentration Tests

The required statistical endpoint for the microplate alga test is an ICp[15,16] and its 95% confidence limits; cell yield is used in all calculations. Environment Canada (2005) provides direction and advice for calculating the ICp, including decision flowcharts to guide the selection of appropriate statistical tests. All statistical tests used to derive an endpoint require that concentrations be entered as logarithms. Concentrations used in calculations and in reporting must be corrected for the volume of enrichment media and algal inoculum[17], if applicable.

An initial plot of the data (cell yield) against the logarithm of concentration is highly recommended, both for a visual representation of the data, and to check for reasonable results by comparison with later statistical computations.[18] Any major disparity between the approximate graphic ICp and the subsequent computer-derived ICp must be resolved. The graph would also show whether a logical relationship was obtained between log concentration (or, in certain instances, concentration) and effect, a desirable feature of a valid test (EC, 2005).

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[14] If the coefficient of variation in the control treatment is less than 10%, testing for a trend in standard control wells does not apply.

[15] Historically, investigators have frequently analyzed *quantitative* sublethal endpoints from multi-concentration tests by calculating the *no-observed-effect concentration (NOEC)* and the *lowest-observed-effect-concentration (LOEC)*. Disadvantages of these statistical endpoints include their dependence on the test concentrations chosen and the inability to provide any indication of *precision* (i.e., no 95% or other confidence limits can be derived) (Section 7.1 in EC, 2005). Given these disadvantages, ICp is the required statistical endpoint for growth data derived from a multi-concentration test using *Pseudokirchneriella subcapitata*.

[16] The *ICp* is the *inhibiting concentration* for a specified *percent* effect. The "p" represents a fixed percentage of reduction, and is chosen by the investigator. Typically, its value is chosen as 25% or 20%.

[17] For example, in the case of the typical preparation of 200 µL test solution, 10 µL enrichment media and 10 µL algal inoculum, this would result in a 91% dilution of full-strength effluent. If different ratios of these components are used, the appropriate dilution correction must be applied.

[18] As an alternative to plotting cell yield directly, investigators might choose to calculate and plot the percent inhibition for each test concentration. This is calculated from the equation:

$$I = \frac{R_c - R}{R_c} \times 100$$

where:

I is the percentage inhibition of algal growth for each test-concentration replicate;
R_c is the mean cell yield for the control; and
R is the cell yield for each test-concentration replicate.

When displayed graphically, all values above the x-axis will represent growth inhibition and all values below the x-axis will represent enhanced growth. The I will be a negative value if growth in the wells with a test concentration is greater than that in the control wells (i.e., enhancement).

Regression analysis is the principal statistical technique to be applied here and must be used for calculation of the ICp, provided that the assumptions below are met. A number of models are available to assess growth data (using a quantitative statistical test) via regression analysis. Use of regression techniques requires that the data meet assumptions of *normality* and *homoscedasticity*. Weighting techniques may be applied to achieve the assumption of *homoscedasticity*. The data are also assessed for outliers using one of the recommended techniques (see Section 10.2 in EC, 2005). Any outliers and the justification for their removal must be reported. An attempt must be made to fit more than one model to the data. Finally, the model with the best fit[19] must be chosen as the most appropriate for generation of the ICp and associated 95% confidence limits. The lowest residual mean square error is recommended to determine best fit; this statistic is available in the ANOVA table for any of the models. Endpoints generated by regression analysis must be bracketed by test concentrations; extrapolation of endpoints beyond the highest test concentration is not an acceptable practice.

The ICp results must always be reported with the exposure duration (72 h) and expressed in percent v/v for wastewater samples (corrected for the volume of enrichment media and algal inoculum) and in appropriate units of concentration (µg/L or mg/L) for chemicals. The 72-h ICp for a nontoxic wastewater is reported as ND (nondeterminable) or >100% v/v. When the ICp is below the lowest concentration tested, report the ICp as less than the lowest concentration, or rerun the assay at more dilute concentrations.

If absorbance is used to estimate cell concentrations, correct the absorbance values in the standard controls by subtracting the absorbance of the control blank of 200 µL of reagent water and 20 µL of equal part $NaHCO_3$ plus nutrient spike. Calculate the mean light absorbance in the standard reagent controls.

Subtract the measured light absorbance in each well for each treatment from the mean light absorbance in the standard reagent controls. The corrected light absorbance values are converted to final cell concentrations by an equation predetermined from regressing absorbance against cell concentration, or directly from a standard curve (Figure 4). The concentration resulting in a specified percent growth inhibition (e.g., IC50, IC25, or IC20) is then determined.

Computer software has been developed specifically for microplate photometers, and programs are available that will do the absorbance corrections automatically according to any microplate configuration. The generated data files can easily be imported into standard analytical programs for further data manipulations, statistical analysis, and graphic displays.

The ability to mathematically describe *hormesis* (i.e., a stimulatory or “better than control” response occurring only at low exposure concentrations) in the dose-response curve has been incorporated into recent regression models for quantitative data (see Section 10.3 in EC, 2005). Data exhibiting hormesis can be entered directly, as the model can accommodate and incorporate all data points; there is no trimming of data points which show a hormetic response.

In the event that the data do not lend themselves to regression analysis (i.e., assumptions of

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[19] As described in Section 6.5.8 of EC (2005), Environment Canada’s current guidance on statistical methods for environmental toxicity tests specifies the use of the following five models for regression analysis, when estimating the ICp: linear, logistic, Gompertz, exponential and *hormesis* (logistic adapted for hormetic effect at low doses). Specific mathematical expressions of the model, including worked examples for a common statistics package, are also provided in that guidance document (Section 6.5.8 and Appendix O in EC, 2005).

normality and/or *homoscedasticity* cannot be met), linear interpolation (e.g., ICPIN; see Section 6.4.3 in EC, 2005) can be used to derive an ICp. If the data exhibited *hormesis* and ICPIN is used, control responses must be entered for those concentrations which demonstrated hormesis (Option 4, Section 10.3.3 in EC, 2005).

For each test concentration including the control treatment(s), the mean (± SD) cell yield and the corresponding *coefficient of variation* must be calculated.

4.6.3 Stimulatory Effects

A *stimulatory effect* (increased response at all concentrations or at high concentrations) must be reported for all concentrations in which significant stimulation was observed. If a stimulatory effect was observed, statistical comparison with controls is performed using ANOVA analysis, followed by appropriate pairwise comparisons with control (see Section 3.3 and 7.5 of EC, 2005). This analysis will identify which concentrations show a stimulatory effect that is significantly different from controls. The percent stimulation for these concentrations must be reported as a test endpoint, using the following calculation[20]:

$$S(\%) = \frac{T - C}{C} \times 100$$

Where:

S(%) = percent stimulation
T = average cell yield at test end in test solutions
C = average cell yield in the controls

[20] In the context of effluent testing, T = mean effluent or surface water response and C = mean control response (USEPA, 2002). However, these abbreviations have been more broadly defined to cover all applications of this method in the percentage stimulation equation. A similar calculation is used by the Centre d'expertise en analyse environnementale du Québec (CEAEQ, 2005).

4.6.4 Other Test Designs

The method described in this document uses biomass, expressed in terms of cell density and algal cell yield, in the calculation of the growth inhibition endpoint(s). Other published methods may use different expressions of biomass[21], such as dry weight[22], or the average specific growth rate[23] in endpoint calculations. Estimates of toxicity expressed in terms of final biomass are generally more sensitive than those based on average specific growth rate (Weyers and Vollmer, 2000; ISO 2006). The average specific growth rate is useful for comparing data from tests having different test conditions (e.g., nutrient, light, test duration), and tests using different algal strains (Weyers and Vollmer, 2000; Eberius et al., 2002; ISO, 2006).

4.7 Reference Toxicant

Reference toxicants are used to assess the reproducibility and reliability (as precision and consistency) of results using a given test organism, test procedure, and/or laboratory, over a specific period of time. Results for a reference toxicant are compared with historical test results to identify whether they fall within an acceptable range of variability. Results that do not fall within the acceptable range indicate a

[21] Some previous editions of published methods also use the biomass integral, area under the growth curve, but this does not appear in any current editions (ISO, 2002; ISO 2006). For a comparison of area under the growth curve with growth rate, see ISO 2006.

[22] Dry weight may be determined gravimetrically, or may be calculated indirectly using conversion factors (ASTM, 2006).

[23] The average specific growth rate for a specified period is calculated as the logarithmic increase in biomass for each single vessel of controls and treatments as follows (OECD, 2004):

$$\mu_{i\text{-}j} = \frac{\ln X_j - \ln X_i}{t_j - t_i} \quad (\text{day}^{-1})$$

where: $\mu_{i\text{-}j}$ is the average specific growth rate from time *i* to *j*;
X_i is the biomass at time *i*;
X_j is the biomass at time *j*

change in test organism health or genetic sensitivity, a procedural inconsistency, or a combination of these factors. Therefore, a reference toxicant can be used to confirm the acceptability of concurrent test results and demonstrate satisfactory laboratory performance.

One or more of the following three[24] chemicals (reagent grade) should be used as reference toxicant(s) for this test: copper sulphate ($CuSO_4$), zinc sulphate ($ZnSO_4$), or phenol. Each of these chemicals is easy to measure analytically and poses a minimal hazard to the user. *P. subcapitata* is sensitive to chemicals including these which have a low potency (e.g., the slope of the dose-response curve is low), and the toxicity of these chemicals to algae is not affected significantly by changes in water quality. The source and purity of the reference toxicant must be reported.

The toxicity test with the reference toxicant is performed according to the universal test procedures outlined in this Section. The methods for preparation of the test concentrations are described in Appendix E of Environment Canada (1990). The reagent water routinely used in the algal toxicity tests should be used as the control/dilution water in tests with the reference toxicant.

Toxicity testing with one or more reference toxicants must occur within 14 days before or after the toxicity test (i.e., the reference toxicity test must be started within 14 days of the period over which the test was conducted). The same batch of organisms should be used for tests on both the reference toxicant and the sample. The test with reference toxicant(s) must be performed under the same experimental conditions as those used with the test sample(s).

A *warning chart* (EC, 1990, 2005) must be prepared and updated for each reference toxicant used. Successive ICps are plotted on this chart and examined to determine whether the results are within ± 2 SD (= *warning limits*) or ± 3 SD (= *control limits*) of values obtained in previous tests using the same reference toxicant and test procedure. The mean and standard deviation of available log ICps is recalculated with each successive test until the statistic stabilizes (EC, 1990, 2005). The warning chart should plot logarithm of ICp on the vertical axis against date of the test (or test number) on the horizontal axis (see Figure 2).

The logarithm of concentration (log ICp) must 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 ICp was estimated on the basis of logarithms of concentrations. The warning chart may be constructed by plotting the logarithms of the mean and its limits on arithmetic paper, or by plotting arithmetic values on the logarithmic scale of semi-log paper. If it were definitely shown that the ICps failed to fit a log-normal distribution, an arithmetic mean and limits might prove more suitable.

Each new ICp for the reference toxicant should be compared with the established warning limits of the chart; it is considered acceptable if it falls within the warning limits.

If a particular ICp falls outside the warning limits, the sensitivity of the algal culture and the performance and precision of the test are suspect. Since this might occur 5% of the time due to chance alone, an outlying ICp does not necessarily mean that the sensitivity of the culture or the precision of the data are in question. Rather, it provides a warning that this might be the case. A thorough check by

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[24]Sodium chloride (NaCl) is no longer deemed acceptable for reference toxicant use; recent laboratory data have shown that reference toxicant ICp values derived using NaCl are insensitive to contamination and culture health (Harwood, 2006).

laboratory personnel of all culturing and test conditions and procedures is required at this time. Depending on the findings, it might be necessary to prepare a new algal culture for use with the test material or substance and reference toxicant(s).

Results that remained within the warning limits would 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. For guidance on reasonable variation among reference toxicant data, see Section 2.8.1 and Appendix F in EC, 2005.

If an ICp fell outside the control limits (mean ± 3 SD), it would be highly probable that the test was unacceptable and should be repeated, with all aspects of the test being carefully scrutinized. If endpoints fell between the control and warning limits more than 5% of the time, a deterioration in precision would indicated, and again the most recent test should be repeated with careful scrutiny of procedures, conditions, and calculations.

Specific Procedures for Testing Chemicals

This section gives specific instructions for chemicals. They are in addition to the procedures in Section 4.

5.1 Properties, Labelling, and Storage of Sample

Physical and chemical properties of the chemical being tested should be obtained. Material safety data sheets (MSDs) should be consulted, if available. Information critical to procedures and data interpretation includes water solubility, vapour pressure, dissociation constants, structural formula, n-octanol:water partition coefficient, degree of purity, and the nature and amounts of impurities or additives. The relative stability of the chemical and its persistence in fresh water is also useful information.

Chemical containers must be sealed, and labelled with the chemical name, the supplier, the date received, and grade or purity. The chemical should be stored according to the instructions on the label or according to the MSDS.

5.2 Control/Dilution Water and Test Vessels

Reagent water is recommended as the control/dilution water for tests designed to assess toxicity of a chemical to *P. subcapitata*. However, if the objective is to assess the toxic impact of a chemical on a particular receiving water, then the recommended control/dilution water is the receiving water itself. Appraisals of the impact of chemical spills or intentional application of a pesticide to a water body would warrant use of receiving water as the control/dilution water. The objective of the test must be decided *a priori*, because the toxicity results could differ for two sources of water.

Microplates used for chemical testing must be made of glass. If laboratories can demonstrate, with side-by-side comparisons, that the test chemical does not sorb to polystyrene more than it does to the glass, the disposal polystyrene microplates may be used.

5.3 Preparing the Test Solutions

Test solutions of the chemical should be prepared by diluting measured volumes of fresh stock solutions with reagent water. Stock solutions used in chemical testing are not to be filtered. Reagent water may be filtered prior to preparation of the chemical stock solution. Volumetric flasks should be used for the preparation of stock and test solutions. For chemicals that do not readily dissolve in water, stock solutions may be prepared using the generator column technique (Billington *et al.*, 1988; Shiu *et al.*, 1988) or, less desirably, by ultrasonic dispersion[25]. Solubility of the test chemical might also be enhanced by the use of organic solvents, *emulsifiers*, or *surfactants*. The use of such solubilizing agents should be restricted to those agents or carriers formulated with the chemical for normal commercial purposes. If used, an additional carrier control solution must be prepared that contains the highest concentration of the solubilizing agent used in the test. It should be placed in wells E2 to E11, adjacent to the regular control replicates (i.e., wells D2 to D11)(see Figure 3B).

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[25] Ultrasonic dispersion is not a preferred technique, since the ultrasonics can produce droplets that differ in size and uniformity, some of which might migrate towards the surface of the liquid, or vary in biological availability, creating variations in toxicity.

Procedures for preparation of the test solutions (test sample, algal inoculum and nutrient spike) are described in Section 4; if a metal substance in water or metal mixture is being tested, the final amount of $Na_2EDTA \cdot 2H_2O$ must be reduced by 25% for a final concentration of 46.9 µg/L.

The highest concentration of solubilizing agent in any test solution should be less than that solvent's NOEC in control/dilution water. If the NOEC is unknown, it can be determined by conducting the algal growth inhibition test with different concentrations of the agent, following the standard test procedures. The NOEC is calculated according to recognized procedures (EC, 2005). Recommended organic solvents are acetone and methanol (St-Laurent *et al*., 1992), which have NOECs >0.91% v/v (Stratton and Smith, 1988).

A test involving the use of a solubilizing agent is considered valid if the cell yield in the carrier solvents is not significantly different from that of the standard controls as determined by a non-parametric *Mann-Whitney U test* (Zar, 1999). Controls must be compared statistically using trend analysis (*Mann-Kendall test*—Gilbert, 1987) to detect any effect of volatiles (if present) in the sample (see Section 4.4 for details).

Specific Procedures for Testing Effluent, Elutriate, and Leachate Samples

This section gives specific instructions for testing effluent, elutriate, and leachate samples. They are in addition to the procedures in Section 4.

6.1 Sample Collection, Labelling, Transport, and Storage

Generally, a l-L sample of effluent or leachate is sufficient for conducting a microplate test for algal growth inhibition. Each sample must be collected and placed in a labelled or coded container of inert material. Labelling or coding with related record keeping should identify sample type, source and/or location of collection point, date and time of collection, and name of sampler(s). The container must be new or thoroughly cleaned, and rinsed with uncontaminated water. It should also be rinsed with the sample to be collected, and then filled to the brim and sealed. The chain of custody during sample collection, transport, and storage should be recorded.

An effort must be made to keep samples of effluent or leachate cool (1 to 7 °C, preferably 4 ± 2 °C) throughout the period of transport. Upon collection, warm (>7 °C) samples must be cooled to 1 to 7 °C with regular ice (not dry ice) or frozen gel packs. As necessary, ample quantities of regular ice, gel packs, or other means of refrigeration must be included in the transport container in an attempt to maintain sample temperature within 1 to 7 °C during transit.

Samples must not freeze during transit or storage. Upon arrival at the laboratory, the temperature of the sample must be recorded. An aliquot of effluent or leachate required at that time may be adjusted immediately or overnight to the test temperature, and used in the test. Samples or portions of samples to be stored for subsequent use must be held in sealed containers without air headspace, in the dark at 4 ± 2 °C.

Effluents and leachate samples should be tested as soon as possible and must be tested within three days after collection. Extraction of samples for elutriates should occur within 10 days of sample receipt, and the elutriate must be tested within three days thereafter.

If the water used in the preparation of the test solutions (i.e., control/dilution water) is not reagent water (see Section 4), then sufficient control/dilution water must also be collected, transported, and stored in a manner identical to that of the aqueous samples. The control/dilution water should not be stored longer than 14 days because of the problem associated with slime growth (USEPA, 2002). Ideally, samples should be transported at temperatures between 1 and 7 °C. Samples must not freeze during transport.

6.2 Control/Dilution Water

Tests conducted with samples of effluent, elutriate, or leachate should use reagent water as the control/dilution water if the objective is to monitor for regulatory compliance. If the objective is to assess the potential impact of a sample on a particular receiving water, then the receiving water should be used as the dilution and control water. A standard control (i.e. with reagent water) must also be included in the test.

If a high degree of standardization is required, reagent water should be used for all dilutions and as the control water, because use of reagent water increases the probability of reducing the modifying influences attributable to different chemical compositions of dilution water. For example, such use would be appropriate in studies intended to compare toxicity data for various effluent, leachate, or elutriate types and sources, derived from a number of test facilities.

The objective of the test must be decided before a choice is made because the toxicity results could be quite different for the two sources of water.

6.3 Preparation of Test Solutions

Procedures for preparation of test sample and test solutions are described in Section 4; if metal mining effluent is being tested, the final amount of $Na_2EDTA \cdot 2H_2O$ must be reduced by 25% for a final concentration of 46.9 µg/L. It is recommended that these aqueous samples be tested in duplicate (e.g., two microplates). Because of the addition of the *algal inoculum* and the enrichment medium, the highest test concentration of effluent, elutriate or leachate is normally 91%. Endpoint calculations must reflect this dilution (see Section 4.3.2).

Colour, *turbidity*, odour, and the presence of floating or settled solids should be recorded before and after the sample is filtered. Any changes that occur during preparation of the test sample should also be recorded (e.g., precipitation, *flocculation*, change in colour or odour, release of volatiles).

It might be desirable to measure total suspended solids and total settled solids (APHA *et al*., 2005) in effluents characterized with appreciable amounts. Removal of these fractions of the effluent could influence the results of the toxicity tests.

6.4 Interpretation of Results

For any test which uses a water source other than reagent water as the control/dilution water, particular attention should be given to a comparison of algal growth in the control/dilution water with that in the standard controls using reagent water. This comparison is necessary to determine whether the control/dilution water is phytotoxic. Also, controls must be compared statistically using trend analysis (*Mann-Kendall test* Gilbert, 1987) to detect any effect of volatiles (if present) in the sample (see Section 4.4 for details). Any enhanced growth in test solutions, relative to that in the control solutions, must be considered when interpreting the findings, and reported.

Specific Procedures for Testing Receiving-water Samples

This section presents specific procedures for testing samples of receiving water. They are in addition to the procedures in Section 4.

7.1 Sample Collection, Labelling, Transport, Storage, and Preparation of Test Solutions

Procedures specific to receiving-water samples are the same as those described in Sections 6.1 and 6.3.

7.2 Control/Dilution Water

To assess the toxicity of receiving-water samples collected in the area of a point source of possible contamination (e.g., wastewater discharge or chemical spill), "upstream" water should normally be sampled. The upstream water should be used as control water and as the diluent for any diluted downstream water samples. This control/dilution water should be collected at a point as close as possible to the contaminant source(s) of concern, but upstream from or outside of the zone influenced by the source. If upstream water is used as control/dilution water, a separate set of replicate control solutions must be prepared using reagent water, and these "reagent water controls" must be included in the test.

If growth inhibition effects are revealed for control algae exposed to the upstream water, a separate test should be conducted on a set of concentrations of downstream water prepared with reagent water. For such an eventuality, sufficient volumes of downstream water should be collected to allow the preparation of these sample dilutions.

If standardization is a high priority objective of the test, or if interlaboratory comparison of results is desirable, then reagent water should be used as the control/dilution water. The objective(s) of the test must be decided *a priori*.

7.3 Interpretation of Results

In addition to the procedures described in Section 4, the items raised in Section 6.4 should also be addressed.

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 7 of this biological test method, and, if so, provide details as to 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 8.1 provides a list of items which must be included in each test-specific report. Section 8.2 gives a list of those items which 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. Specific monitoring programs or related test protocols might require selected test-specific items listed in Section 8.2 to be included in the test-specific report, or might relegate certain test-specific information as "data to be held on file" (e.g., details regarding the test material or substance and/or explicit procedures and conditions during sample collection, handling, transport, and storage).

Procedures and conditions that are common to a series of ongoing tests (e.g., routine toxicity tests for monitoring or compliance purposes) and consistent with specifications in this document, may be referred to by citation or by attachment of a general report which outlines standard laboratory practice.

Details pertinent to the conduct and findings of the test, which are not conveyed by the test-specific report or general report, must 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 samples tested for regulatory or monitoring purposes;
- a copy of the record of acquisition for the sample(s);
- certain chemical analytical data on the sample(s);
- 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 and health of the breeding stock; and
- information on the calibration of equipment and instruments.

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

8.1 Minimum Requirements for Test-specific Report

Following is a list of items that must be included in each test-specific report.

8.1.1 Test Substance or Material

- brief description of sample type (e.g., chemical or chemical substance, effluent, elutriate, leachate, or receiving water), if and as provided to the laboratory personnel;
- information on labelling or coding of each sample;

- date of sample collection; date and time sample received at test facility;
- measurement of pH of aqueous sample, just before its preparation and use in toxicity test;
- for effluent or leachate, measurement of temperature of sample upon receipt at test facility; and
- for a test with elutriate, dates for sample generation and use.

8.1.2 Test Organisms

- species, strain number, and origin of culture;
- age (i.e., 3 to 7 days) of culture used to provide inocula of test organisms, at the start of the test; and
- any unusual appearance or treatment of known-age culture, before its use in the test.

8.1.3 Test Facilities

- name and address of test laboratory; and
- name of person(s) performing the test.

8.1.4 Control/Dilution Water

- type(s) and source(s) of water used as control and dilution water; and
- type and quantity of any chemical(s) added to control or dilution water.

8.1.5 Test Method

- citation of biological test method used (i.e., as per this document);
- design if specialized procedure (e.g., test performed with and without filtration of sample; test performed with and without adjustment of sample pH; preparation and use of elutriate; preparation and use of solvent and, if so, solvent control); and
- name and citation of program(s) and methods used for calculating statistical endpoints.

8.1.6 Test Conditions and Procedures

- design and description if any deviation from or exclusion of any of the procedures and conditions specified in this document;
- mean test temperature;
- number and concentration of test solutions;
- number of replicate test wells per treatment (including controls);
- initial cell density in the microplate wells at the start of the test;
- statement and description (i.e., procedure, rate, and duration) if any aeration of sample or test solutions before starting the test;
- description of procedure for sample filtration;
- brief description of any sample or test solutions receiving pH adjustment including procedure(s);
- measurement of pH of sample before any dilution, at the start of the test;
- measurements of pH of the two median control wells at the start and end of the test; and
- dates when test was started and ended; duration of test.

8.1.7 Test Results

- cell concentration in each replicate (including controls) at end of test;

- if absorbance is used, cell concentration (direct count) in the three wells containing high/medium/low test concentrations, and their corresponding values estimated using the absorbance method;
- mean (±SD) cell yield at 72 h for each treatment (including controls), with corresponding coefficient of variation (CV = 100 × standard deviation/mean);
- results of the Mann-Kendall test (if applicable);
- ICp for percent inhibition of cell yield (together with its 95% confidence limits) using concentrations corrected for the volume of algal inoculum and enrichment media; details regarding any weighting techniques applied to the data; and indication of quantitative method used;
- any outliers and the justification for their removal
- details regarding any statistical transformation of data that was required;
- ICp and 95% confidence limits for any toxicity tests with the reference toxicant(s) started within 14 days of the test, together with the geometric mean value (± 2 SD) for the same reference toxicant(s) as derived at the test facility in previous tests;
- any findings of growth stimulation, at any concentration(s); and
- anything unusual about the test, any problems encountered, any remedial measures taken.

8.2 Additional Reporting Requirements

Following is a list of 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.

8.2.1 Test Substance or Material

- identification of person(s) who collected and/or provided the sample;
- records of sample chain-of-continuity and log-entry sheets; and
- conditions (e.g., temperature, in darkness, in sealed container of sample upon receipt and during storage).

8.2.2 Test Organisms

- description of culture conditions and procedures, including: lighting (intensity and quality) and temperature conditions; composition of growth medium; and procedures and conditions for preparation and storage of growth medium;
- frequency of renewal of cultures;
- procedures, observations, and records related to the purity of stock cultures;
- confirmation that the algal cells for the algal inoculum were not obtained from the first stock culture derived from the starter culture (see Section 4.3.1); and
- records of algal growth curves performed to monitor culture health and performance.

8.2.3 Test Facilities and Apparatus

- description of culture and test incubators and apparatus;
- description of systems for regulating light and temperature within the culturing and test facilities; and
- description of procedures used to clean or rinse test apparatus.

8.2.4 Control/Dilution Water

- sampling and storage details if the control/dilution water was "upstream" receiving water;
- details regarding any water pretreatment (i.e., procedures and conditions for filtration, sterilization, temperature adjustment, de-gassing, aeration, adjustment of pH); and
- measured water quality variables before and/or at time of starting the test.

8.2.5 Test Method

- description of laboratory's previous experience with this biological test method for measuring toxicity using *P. subcapitata*;
- details of date and time algal inoculum was prepared, relative to start of test;
- type of microplate used (disposable round-bottomed, disposable flat-bottomed, or glass);
- for chemical testing only, side-by-side comparison of chemical sorption to polystyrene microplate and glass microplates (if applicable)
- procedure used in preparing and storing stock and/or test solutions of chemicals; description and concentration(s) of any solvent used;
- methods used (with citations) for chemical analyses of sample or test solutions; details concerning sampling, sample/solution preparation and storage, before chemical analyses; and
- use and description of preliminary or range-finding test.

8.2.6 Test Conditions and Procedures

- *photoperiod*, light source, and intensity adjacent to the surface of test solutions;
- appearance of sample and test solutions before and after sample filtration;
- procedures for measuring cell concentrations and calculating cell yields;
- water quality measurements for control/dilution water;
- any other physical or chemical measurements on sample, stock solutions (e.g., concentrations of one or more specific chemicals before and/or at time of the test); and
- conditions, procedures, frequency, dates and times for toxicity tests with reference toxicant(s).

8.2.7 Test Results

- results of the Mann-Whitney U-test (if applicable);
- results for range-finding test (if conducted);
- graphical presentation of dose-response data;
- warning chart showing the most recent and historic results for toxicity tests with the reference toxicant(s);
- any other observed effects; and
- original bench sheets 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 & Applications Section [a]

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 2nd Edition	February 2007	—
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 a Freshwater Alga	EPS 1/RM/25 2nd Edition	March 2007	—
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	—

[a] These documents are available for purchase from Communications Services, Environment Canada, Ottawa, Ontario, K1A 0H3, Canada. Printed copies can also be requested by e-mail at: epspubs@ec.gc.ca. These documents are freely available in PDF at the following website: http://www.etc-cte.ec.gc.ca/organization/bmd/bmd_publist_e.html. For further information or comments, contact the Chief, Biological Methods Division, Environmental Science and Technology Centre, Environment Canada, Ottawa, Ontario K1A 0H3.

Title of Biological Test Method or Guidance Document	Report Number	Publication Date	Applicable Amendments
A. Generic (Universal) Biological Test Methods (cont'd.)			
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 2nd Edition	January 2007	—
Test for Survival and Growth in Sediment Using Spionid Polychaete Worms (*Polydora cornuta*)	EPS 1/RM/41	December 2001	—
Tests for Toxicity of Contaminated Soil to Earthworms (*Eisenia andrei, Eisenia fetida*, or *Lumbricus terrestris*)	EPS 1/RM/43	June 2004	—
Tests for Measuring Emergence and Growth of Terrestrial Plants Exposed to Contaminants in Soil	EPS 1/RM/45	February 2005	—
Test for Measuring Survival and Reproduction of Springtails Exposed to Contaminants in Soil	EPS 1/RM/47	December 2006	—
B. Reference Methods[b]			
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	—
Reference Method for Determining the Toxicity of Sediment Using Luminescent Bacteria in a Solid-Phase Test	EPS 1/RM/42	April 2002	—

[b] 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.

Title of Biological Test Method or Guidance Document	Report Number	Publication Date	Applicable Amendments
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	—
Guidance Document for Testing the Pathogenicity and Toxicity of New Microbial Substances to Aquatic and Terrestrial Organisms	EPS 1/RM/44	March 2004	—
Guidance Document on Statistical Methods for Environmental Toxicity Tests	EPS 1/RM/46	March 2005	—

Appendix B

Members of the Inter-Governmental Environmental Toxicity Group[a] (as of December 2006)

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[a] Previously named the "Inter-Governmental Aquatic Toxicity Group" (IGATG).

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Appendix C

Environment Canada Regional and Headquarters' Office Addresses

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General Procedures for Maintaining Sterile Conditions – *Pseudokirchneriella subcapitata* (modified from Acreman, 2006)

Axenic algal cultures are free from microbial contaminants. They are grown under sterile conditions in liquid media or on nutrient agar using methods similar to those used for microbiology and plant tissue culture. Good sterile technique is essential for axenic culturing of *P. subcapitata*. Careful monitoring of the cultures and regular testing for microbial contamination is crucial. A basic rule when working with all axenic cultures is to treat the workspace for manipulation of the cultures as you would a surgical operating area. An axenic culture is valuable and if it becomes contaminated, the contamination is not always easy to eliminate. Always make multiple subcultures of the species to help ensure that at least one or more of them will remain sterile. The following guidelines should help to reduce the potential for microbial contamination of the cultures.

Maintaining a Clean Laboratory

The areas where the cultures will be stored or incubated should be cleaned every 2-3 months with 1% sodium hypochlorite (bleach) solution to keep down the levels of dust mites, bacteria and fungal spores. Vacuum the area before applying the solution to reduce any organic contaminants present as they will reduce the effectiveness of the treatment. The bleach solution should be freshly prepared each time and allowed to remain on the surfaces for at least 20-30 minutes. The shelf life of concentrated bleach solution is about 4-6 months once opened, depending on the exposure to light and high temperature. As an alternate solution, granular calcium hypochlorite may be mixed with water at approximately 10 g/L providing 70% available chlorine. The dry powder has the added benefit of extended shelf life; if it is kept dry, cool and in an airtight container, it may be stored up to 10 years with minimal degradation. See "Solutions for Disinfecting Surfaces" for details of preparation of these solutions.

Laminar Flow Hood: Operation and Maintenance

The use of a laminar flow hood is the best method for manipulation of axenic cultures. A regular maintenance schedule should be followed to ensure the hood is functioning properly. Inexpensive hoods are available at a cost range of $1000-$3000 (Envirco, USA, Tel 1-800-645-1610).

The most important part of a laminar flow hood is a High Efficiency Particulate Air (HEPA) filter. Room air is taken into the unit and passed through a pre-filter to remove gross contaminants (lint, dust etc). The air is then compressed and channeled up behind and through the HEPA filter in a laminar flow fashion. The purified air flows out over the entire work surface in parallel lines at a uniform velocity. The HEPA filter is about 99% efficient in removing bacteria and fungal spores of > 0.22 microns from the air. HEPA filters should be replaced approximately every 7 years for best performance. Routinely check the filter for cracks or damage by sharp instruments. The flow velocity patterns should also be checked annually by a filter service company professional (e.g. H.E.P.A. Filter Services Inc. Tel: 1(800) 669-0037) for any blocked or damaged areas.

If no testing service is available or your budget cannot accommodate the cost of testing, the hood can also be checked for efficiency by using sterility test agar plates. These plates are prepared from growth medium supplemented with organic components such as glucose (1 g/L), peptone (1 g/L) and yeast extract (0.3 g/L). It is good practice to periodically check the hood efficiency using this method in between checks by a filter specialist. Spread the plates across the center of the bench and leave them open for at least 24 hours with

the hood running. Note the position of each numbered plate. Close the plates, seal them with a double layer of Parafilm and leave in a warm dark location for at least 5 days to monitor for bacterial or fungal growth. If your test indicates that some areas of the HEPA filter are defective, it is possible to repair the filter by injecting silicone sealant if the damaged areas are small. Large patches will cause some air turbulence in the workspace. If possible, the repairs should be done by a company that specializes in HEPA filtered equipment.

Laminar flow hoods are best left on at all times. If this is not practical, an ultra-violet germicidal light should be installed to sterilize all surfaces, and the fan blower for the hood should then be turned on at least 30 minutes prior to using it, to ensure that all the air in the hood will be sterile.

Ideally, the ultra-violet lamp should be left on when the hood is not in use. If this not practical it should at least be left on for 30 minutes prior to using the hood. UV light can cause skin and eye burn hazards if used improperly. For safe and reliable use of germicidal lamps follow these recommendations:

- Post warning signs near the lamp.
- Clean the bulb at least every 2 weeks; turn off power and wipe with an alcohol-moistened cloth.
- Factors such as lamp age and poor maintenance can reduce performance. Measure radiation output of the bulb at least twice yearly with a UV meter or replace the bulb when emission declines to 70% of its rated output (after about 1 year of normal use). If no UV meter is available replace the bulb once a year.

The working area of the hood, including the bench top and sides should be cleaned with a surface cleaner such as Bio-Clean, Cidex, Sporocidin (VWR) or Viralex (Canadawide). Ethanol is adequate as a disinfectant to reduce microbes but is not recommended as a sterilizing agent since it is not effective as a fungicide or virucide and will not kill bacterial spores. Alcohol (e.g. ethanol) used in concentrations of less than 90% is more effective than pure alcohol because the water added to dilute the alcohol allows better penetration of the bacterial cell walls. Optimal concentration range is between 70% and 80%; contact time should be at least 10 minutes. The cleaning agents are sprayed on the surface and left for the appropriate length of time before being wiped clean with paper towels or lint-free tissues. Clean the working area before and after each use.

Keep the hood free of clutter. A direct, unobstructed path must be maintained between the HEPA filter and the area inside the hood where the culture manipulations are being performed. The air downstream from non-sterile objects (such as solution containers, hands etc.) becomes contaminated from particles blown off these objects. Avoid keeping any large containers in the hood.

Pre-filters should be monitored for dust build-up and washed every 2-3 months, depending on how dusty the work area is. They should be thoroughly dry before re-installation. Some pre-filters are not washable and should be discarded when dusty.

Maintaining Cultures on the Lab Bench

If a laminar flow hood is not available, manipulation of cultures on the lab bench is possible provided a Bunsen burner is available and there is a clean area free of drafts. A room used exclusively for cell culture with minimal foot traffic would be best. The room may be equipped with an ultra-violet lamp to sterilize the area. The workspace is cleaned with a surface cleaner such as Bio-Clean, Cidex, Sporocidin (VWR) or Viralex (Canadawide), followed by cleaning with 70% ethanol. See guidelines above for cleaning the working surfaces. Clean the working area before and after each use. After the area is dry, a Bunsen burner is lit and subculturing is performed close to the flame. The heat and convection of the Bunsen burner

provides a sterile environment around the work area. Caution should also be used with using any flammable liquid, such as ethanol, around the Bunsen burner. Work quickly to avoid entry of contaminants.

Sterilization of Loops and Other Instruments
Bunsen burners and other continuous flame gas burners are effective for sterilizing loops and other instruments in the open bench method, but can produce turbulence in a laminar flow cabinet, disturbing the protective airflow patterns of the laminar flow cabinet. Additionally, the heat produced by the continuous flame may damage the HEPA filter. If a gas burner must be used in a hood, one with a pilot light should be selected and the burner should not be closer than 20 cm from the HEPA filter. Electric sterilizers may also be considered for sterilization of loops. Alternatively, disposable plastic loops and needles may be used for culture work where electric incinerators or gas flames are not available.

Hand Cleaning and Personal Protective Equipment
Before performing any manipulations or subculturing, remove any rings or jewelry and wash hands thoroughly with an antibacterial soap followed by cleaning with a waterless, alcohol-based cleaner such as Endure, One-Step, etc. Pay attention particularly to the areas of your hands that may come in contact with the culture vessels or transfer loops. Examination gloves (e.g. Nitrile or Microflex) are recommended for handling cultures. The gloves may also be cleaned with ethanol or waterless cleanser. Once the gloves are removed and discarded, wash hands again using the antibacterial soap. A laboratory coat and safety glasses are also recommended. The laboratory coat will protect the users clothes from accidental spills or mishaps. Safety glasses will protect the users eyes from infection or loss of sight caused by accidental spills, splashes and mishaps; simple preventative measures make for a safe and healthy environment.

Preparation and Sterilization of Media
Autoclaving is the most widely used technique for sterilizing culture media, and is the ultimate guarantee of sterility (including the destruction of viruses). Note that for liquid media used for *P. subcapitata* culture, filter sterilization is recommended (see Section 2.3.1). A commercial autoclave is best, but pressure cookers of various sizes are also suitable. Sterility requires 15 minutes at a pressure of 15 psi and a temperature of 121°C in the entire volume of the liquid (i.e. longer times for larger volumes of liquid; approximately 25 min for 100- 200 mL, 30 min for > 200-1000 mL, 45 min for 1-2 L and 60 min for > 2 L). It is best to autoclave the medium in small batches to minimize the time for effective autoclaving and avoid chemical changes in the medium due to long exposure to high temperatures. Large loads in the autoclave should be avoided, as they will require more time to reach the sterilization temperature and there is the risk that the media may not be properly sterilized.

Heat sensitive indicator tape that changes colour should be used on the outside of media vessels and packages of material for sterilization to indicate that the appropriate temperature has been reached. They are NOT a guarantee of sterility and only indicate that the material has been through the sterilization process. It is important to ensure that large volumes of media or large loads in the autoclave have reached the appropriate temperature for sterilization. Commercially available biological indicators in sealed ampoules (e.g. Raven Biological Laboratories) or chemical integrator strips (e.g. STEAMPlus Steam Sterilization Integrator strips from SPS Medical) may be used. A simple, alternate method is to put a small piece of autoclave tape into a Pasteur pipette, heat-seal the tip and cotton-plug the other end. Attach a cotton string to the pipette and lower it into the medium, keeping the plugged end about 10-15 cm above the liquid surface. Tape the other end of the string to the outside of the flask so that you can easily pull the indicator out. Recover the indicator after the run and confirm that it has changed colour. The latter method is not as reliable as using biological or chemical integrator strips.

Autoclave efficiency should also be regularly checked with biological indicator tests containing bacterial spores. There are commercially available test indicator kits (e.g. VWR Cat #55710-014) that use spores of *Bacillus stearothermophilus* that are rendered unviable at 250 °F or 121 °C. For the test, spore strips or ampoules of *B. stearothermophilus* are autoclaved, incubated for 48 hours in Tryptic Soy broth, and then observed for any sign of growth, which would indicate that the autoclave is not sterilizing properly.

Agar plates are convenient for long-term maintenance of *P. subcapitata*. They are usually prepared at least 2 days before use and allowed to dry in the laminar flow hood before double sealing with Parafilm (VWR) or Duraseal (VWR or Sigma). If plates are not to be used in a week or so after preparation they should be wrapped in plastic film, inverted, placed in sealed plastic bags and stored at room temperature for a few days to monitor for contamination before storing in the refrigerator. For slants, place the filled tubes on a 45 ° angle and allow agar to gel with the caps slightly unscrewed to prevent excessive condensation build-up. After they are dry, tighten the caps securely and refrigerate after monitoring for contamination at room temperature. Slants and agar plates may be stored for several months at 4 °C.

Transfer Techniques

The following procedures should always be used when transferring cultures:

- All culture vessels, transfer tools, cotton-plugged pipettes and media must be sterilized and ready for use. Media should be at room temperature.
- Wash hands thoroughly and put on laboratory coat, nitrile glove, and safety glasses.
- Loops should be flame or electric sterilized for 15 seconds until they are red-hot before use. Cool the loop by touching it to sterile agar or liquid before use
- Clear the laminar flow hood or bench area so that nothing is between the path of the airflow coming from the HEPA filter and the area or near the flame where the subculture is being done. Do not allow anything to come in contact with the HEPA filter.
- Clean the work surface of the laminar flow or bench just before use but avoid spraying any solutions on the HEPA filter. Repeat the cleaning when the transfers are completed.
- To minimize contamination, always carry out the transfers at least 6 inches (15 cm) from the front of the hood or Bunsen burner flame to ensure that the area is not contaminated by room air. Where possible, perform the operation at eye level.
- Don't touch anything that will come in contact with the culture and if you do touch it, sterilize it again before using it. Avoid pouring sterile solutions from flasks or tubes. Use sterile serological or Pasteur pipettes where possible. If pouring cannot be avoided, ensure that the openings of the vessels are appropriately flamed for about 10 second prior to transfer.
- Avoid talking, singing, whistling, coughing or sneezing in the direction of things that should be sterile. Long hair should be tied back for safety reasons and may be a source of contamination.
- Work quickly to minimize the time that the culture vessels are open.
- Try not to touch the edges of the Petri plate covers. Hold the cover by the top.
- Seal all Petri plates with a double layer of Parafilm or Duraseal. Monitor carefully for cracks. (Dust mites are attracted to the smell of the media and may crawl into the sterile plates.)
- Monitor plates every 2-3 days for presence of contaminants.
- Transfer the cultures every 2-3 weeks for best results.

Solutions for Disinfecting Surfaces

The following may be used in maintaining sterile conditions on surface in the laboratory.

1 % sodium hypochlorite solution (0.5 L)

1. Commercially prepared bleach is normally a 5% sodium hypochlorite solution. Prepare the dilution just before use.
2. Use a 500 mL graduated cylinder to measure 100 mL of commercial bleach. Add 400 mL of *distilled* or deionized water to dilute the bleach in the graduated cylinder to a volume of 500 mL.

Chlorinated solution from powder

1. Add 10 g of granular calcium hypochlorite to 1 liter of distilled water.
2. Stir vigorously and allow the mixture stand for 6 hours or overnight. Wear gloves and mask as chlorine gas is corrosive. If possible, make the solution in a fume hood.
3. Filter the supernatant into a clean plastic jug and stopper tightly. If storing in glass the solution should be kept in the dark.

70% ethanol (used to wipe down laminar flow hood surfaces and to spray gloves)

1. Use a 500 mL graduated cylinder to measure 370 mL of 95% ethanol.
2. Add distilled water to bring the volume of liquid in the cylinder to 500 mL.
3. Keep in a tightly capped container.

Appendix E

Logarithmic Series of Concentrations Suitable for Toxicity Tests[a]

Column (Number of concentrations between 100 and 10, or between 10 and 1)[b]						
1	2	3	4	5	6	7
100	100	100	100	100	100	100
32	46	56	63	68	72	75
10	22	32	40	46	52	56
3.2	10	18	25	32	37	42
1.0	4.6	10	16	22	27	32
	2.2	5.6	10	15	19	24
	1.0	3.2	6.3	10	14	18
		1.8	4.0	6.8	10	13
		1.0	2.5	4.6	7.2	10
			1.6	3.2	5.2	7.5
			1.0	2.2	3.7	5.6
				1.5	2.7	4.2
				1.0	1.9	3.2
					1.4	2.4
					1.0	1.8
						1.3
						1.0

[a] Modified from Rocchini *et al.* (1982).

[b] A series of seven (or more) successive concentrations may be chosen from a column. Midpoints between concentrations in column (x) are found in column (2x + 1). The values listed can represent concentrations expressed as percentage by volume or weight (e.g, mg/L or µg/L). As necessary, values can be multiplied or divided by any power of 10. Column 1 might be used if there was considerable uncertainty about the degree of toxicity. More widely spaced concentrations should not be used. For effluent testing, there is seldom much gain in precision by selecting concentrations from a column to the right of column 3; the finer gradations of columns 4 to 7 might occasionally be useful for testing chemicals that have an abrupt threshold effect. If testing effluent, elutriate or leachate samples, the highest test concentration is normally 91%, and the reported dilution series should reflect this.