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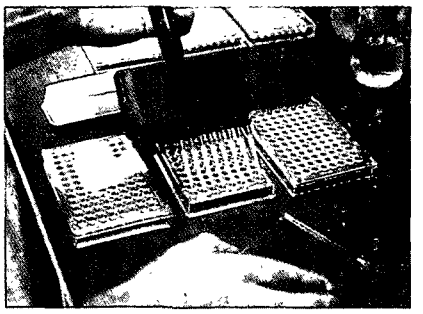

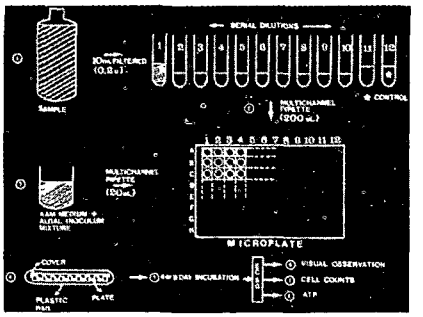

Biological Test Method: Growth Inhibition Test Using the Freshwater Alga *Selenastrum capricornutum*

Report EPS 1/RM/25
November 1992

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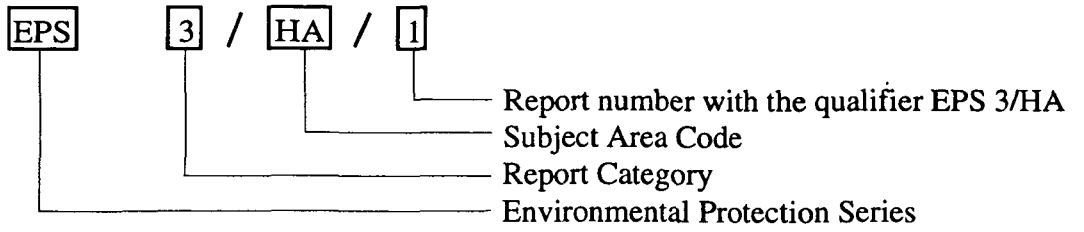

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Abstract

Methods recommended by Environment Canada for determining the chronic toxicity of effluents, elutriates, leachates, receiving waters, or chemicals to the green alga Selenastrum capricornutum, *using the microplate technique, are described in this report.*

General or universal conditions and procedures are outlined for conducting a chronic growth inhibition test using a variety of test materials. 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 rapport décrit les méthodes recommandées par Environnement Canada pour déterminer la toxicité chronique d'effluents, d'élutriats, des lixiviats, de milieux récepteurs ou de produits chimiques chez l'algue verte Selenastrum capricornutum, *au moyen de la microtitration sur plaque.*

Il présente les conditions et méthodes générales ou universelles permettant de réaliser cet essai de toxicité chronique avec diverses substances à expérimenter. Il précise d'autres conditions et méthodes propres à l'évaluation d'échantillons de produits chimiques, d'effluents, d'élutriats, de lixiviats ou de milieux récepteurs. Le lecteur y trouvera des instructions sur les conditions de culture et les exigences de l'espèce soumise à l'essai, la manipulation et le stockage des échantillons, les installations d'essai requises, les méthodes de préparation des solutions d'essai et de mise en route des essais, les conditions prescrites pour les essais, les observations et mesures appropriées, les résultats des essais, les méthodes de calcul et l'utilisation de produits toxiques de référence.

Foreword

This document is one of a series of ***recommended methods*** *for measuring and assessing the aquatic biological effects of toxic materials. Recommended methods are those that have been evaluated by Conservation and Protection (C&P), and are favoured:*

- *for use in C&P aquatic 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 on the basis of their acceptability for the needs of environmental protection and management programs in Environment Canada. These reports are intended to provide guidance and to facilitate the use of consistent, appropriate, and comprehensive procedures for obtaining data on toxic effects of samples of chemical, effluent, elutriate, leachate, receiving water, or, where appropriate, sediment.

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

Table of Contents

Abstract v
Résumé vi
Foreword vii
List of Tables xi
List of Figures xii
List of Abbreviations and Chemical Formulae xiii
Terminology xvi
Acknowledgements xxiii

Section 1
Introduction 1
1.1 Principles of the Test Method 1
1.2 Summary of the Microplate Technique 1
1.3 Application, Advantages, and Limitations of the Microplate System 1
1.4 Conditions for the Validity of the Test 2

Section 2
Test Organism 4
2.1 Species 4
2.2 Source 4
2.3 Culturing 5
2.3.1 Liquid Growth Medium for Stock Algal Culture . . . 5
2.3.2 Solid Growth Medium for Stock Algal Culture 6
2.4 Quality of Test Organisms 7

Section 3
Test System 10
3.1 Facilities 10
3.2 Equipment 10
3.3 Test Conditions 10
3.4 Washing of Glassware 10

Section 4
Universal Test Procedures 14
4.1 Selection of a Microplate Configuration 14
4.2 Preparation of the Enrichment Medium 15
4.3 Beginning the Test 16
4.3.1 Preparation of the Algal Inoculum 16
4.3.2 Preparation of Test Solutions 18
4.3.3 Dispensing Test Solutions, Algal Inoculum, and Nutrient Spike to the Microplate 19
4.3.4 Procedures for Incubation and Measurement of Initial Cell Concentration 19

4.3.5 Quality Control Microplate 19
4.3.6 Test Observations and Measurements 19
4.4 Test Endpoints and Calculations 22
4.5 Reference Toxicant 24

Section 5
Specific Procedures for Testing Chemicals 26
5.1 Properties, Labelling, and Storage of Sample 26
5.2 Control/Dilution Water 26
5.3 Preparing the Test Solutions 26

Section 6
Specific Procedures for Testing Effluent, Elutriate, and Leachate Samples 28
6.1 Sample Collection, Labelling, Transport, and Storage 28
6.2 Control/Dilution Water 28
6.3 Preparation of Test Solutions 28
6.4 Interpretation of Results 29

Section 7
Specific Procedures for Testing Receiving-water Samples 30
7.1 Sample Collection, Labelling, Transport, Storage, and Preparation of Test Solution 30
7.2 Control/Dilution Water 30
7.3 Interpretation of Results 30

Section 8
Reporting Requirements 31
8.1 Test Material 31
8.2 Test Organisms 31
8.3 Test Facilities and Apparatus 31
8.4 Control/Dilution Water 31
8.5 Test Method 32
8.6 Reference Toxicant Testing 32
8.7 Test Results 32

References 35

Appendix A
Members of the Inter-Governmental Aquatic Toxicity Group 39

Appendix B
Conservation & Protection, Regional and Headquarters Offices 41

List of Tables

1	Stock Nutrient Solutions for the Growth Medium of the Stock Algal Culture	6
2	Final Concentration of Nutrients in the Liquid Growth Medium of the Stock Algal Culture	7
3	The Nonconsumable Equipment Required to Execute a Microplate Algal Toxicity Test	11
4	Consumable Equipment Required to Execute a Microplate Algal Toxicity Test	12
5	Reagents Required to Execute the Algal Microplate Toxicity Test	13
6	Test Conditions for the Algal Microplate Toxicity Test	13
7	Control/dilution Water Recommended for the Algal Growth Inhibition Test with the Various Types of Aqueous Samples	14
8	A Summary of the Universal Test Procedures	15
9	Final Concentration of Nutrients in the Microtest Medium	17

List of Figures

1 Laboratory Monitoring of Performance and Culture Health 8

2 Warning/Control Chart for Reference Toxicants 9

3 Standard Microplate Configuration 16

4 Algal Cell Concentration Regressed with Absorbance 21

5 Graphical Estimation of the IC50 23

List of Abbreviations and Chemical Formulae

°C . . . . . degree(s) Celsius

$CaCl_2$ . . . . . calcium chloride

$CaCO_3$ . . . . . calcium carbonate

$CaSO_4$ . . . . . calcium sulphate

$CoCl_2$ . . . . . cobalt chloride

$CuCl_2$ . . . . . copper chloride

d. . . . . day(s)

DO. . . . . dissolved oxygen (concentration)

EC_{50} . . . . . 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

H_2O . . . . . water

ICp. . . . . inhibiting concentration for a (specified) percent effect

I. . . . . percent growth inhibition

KCl . . . . . potassium chloride

K_2HPO_4 . . . . . potassium phosphate

klx . . . . . kilolux

L . . . . . litre(s)

LC_{50} . . . . . median lethal concentration

LT_{50} . . . . . median lethal time

M . . . . . Molar

mg . . . . . milligram(s)

$MgSO_4$ . . . . . magnesium sulphate

min . . . . . minute(s)

mL . . . . . millilitre(s)

mm . . . . . millimetre(s)

$MnCl_2$ . . . . . manganous chloride

N . . . . . Normal

Na_2EDTA . . . . . disodium ethylenediamine tetraacetate

$NaHCO_3$ . . . . . sodium bicarbonate

Na_2MoO_4 . . . . . sodium molybdenate

NaOH . . . . . sodium hydroxide

$NaNO_3$ . . . . . sodium nitrate

nm . . . . . nanometre(s)

OD . . . . . outside diameter

PCBs . . . . . polychlorinated biphenyls

R_c . . . . . mean cell yield in the control

R . . . . . cell yield in a test concentration replicate

SD . . . . . standard deviation

SI . . . . . Système international d'unités

TIE . . . . . Toxicity Identification Evaluation

TM (™) . . . . . Trade Mark

µg . . . . . microgram(s)

µL . . . . . microlitre(s)

v/v . . . . . volume to volume

$ZnCl_2$ . . . . . zinc chloride

> . . . . . greater than

< . . . . . less than

≥ . . . . . greater than or equal to

≤ . . . . . less than or equal to

≃ . . . . . approximately equal to

‰ . . . . . parts per thousand

% . . . . . parts per hundred (percentage)

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

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.

Acclimation means to become physiologically adapted to a particular level of one or more environmental variables such as temperature. The term usually refers to controlled laboratory conditions.

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 valence 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.

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

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.

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. One lux is also approximately equal to 0.015 $\mu E/(m^2 \cdot s)$ or klx $\simeq$ 15 $\mu E/(m^2 \cdot s)$. Light conditions or irradiance are properly described in terms of quantal flux 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.

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 in the final solution.

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

Salinity is the total amount of solid material, in grams, dissolved in 1 kg of seawater. It is determined after all carbonates have been converted to oxides, all bromides and iodides have been replaced by chloride, and all organic matter has been oxidized. Salinity can also be measured directly using a salinity/conductivity meter or other means (see APHA *et al.*, 1989). It is usually reported in grams per kilogram (g/kg) or parts per thousand (‰).

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

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, *Selenastrum capricornutum*. 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 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 Plate 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. 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 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. 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.

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.

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, by virtue of being removed from it in a direction against the current or sufficiently far across the current.

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

Toxicity Terms

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 3- to 4-day exposure to a test material.

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

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 EC_{50} (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 measurement(s) or value(s) that are derived and characterize the results of the test (e.g., NOEC, ICp).

ICp is the inhibiting concentration for a (specified) percent effect. It represents a point estimate of a concentration of test material 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 to which organisms are exposed and for which a statistically significant effect was observed relative to the control.

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

Overt means obviously discernible under the test conditions employed.

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

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

Sublethal concentration means a concentration of test material that does not cause death under the defined test conditions.

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

Toxicity Identification Evaluation is a systematic sample pretreatment (e.g., pH adjustment, filtration, aeration) followed by tests for toxicity. This evaluation is used to identify the causative agent(s) that are primarily responsible for toxicity in a complex mixture.

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.

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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 Aquatic Toxicity Group (IGATG) (Appendix A), 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 *Selenastrum capricornutum* 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, 1989; U.S. EPA, 1989). However, more recently the microplate technique (Blaise, 1984, 1986; Blaise *et al.*, 1986, 1988), which confers a number of advantages over the bottle test (Blanck, 1987; Blaise *et al.*, 1988; Blaise, 1991), has been used to assess algal toxicity (Thellen *et al.*, 1989; Warner, 1990; Blaise and Harwood, 1991; St-Laurent *et al.*, 1992). The methodology presented in this report details standardized procedures for performing algal growth inhibition tests using the microplate technique.

1.1 Principles of the Test Method

Exponentially growing *S. capricornutum* 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 U.S. EPA algal bottle test (Miller *et al.*, 1978; U.S. EPA, 1989). 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 algae (number of cells per mL) is measured with an electronic particle counter or a hemacytometer. 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. Absorbance and fluorescence measurements may also be used as a surrogate for algal biomass after it is established that there is a consistent and quantifiable relationship with cell yield.

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.
- 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 growth of algae in other wells in the microplate. Therefore, experimental design is critical and where volatility is a factor, test concentrations should be isolated from one another, i.e., 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 of a fixed duration, at least 72 h and ≤96 h, to standardize for possible inorganic carbon limitation due to the small volumes of test solutions in these static assays.
- 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). Wherever possible, suggestions and recommendations are included to minimize the effects of the inherent limitations.

1.4 Conditions for the Validity of the Test

For the results of a growth inhibition test to be acceptable and the test to be considered valid, the following conditions must be satisfied:

- Algal growth in the standard controls must not differ significantly ($p > 0.05$) from that in the quality control microplate.
- The number of algal cells in the standard controls must have increased by a factor of more than 16 in 72 h.

- The pH in the controls must not vary by more than 1.5 pH units.
- Culture health assessment with a reference toxicant must satisfy the criteria for acceptability.
- Homogeneity among cell yield estimates in the standard control wells must be demonstrated. An acceptable coefficient of variation is within 20% (U.S. EPA, 1989).

Section 2

Test Organism

2.1 Species

Selenastrum capricornutum is a nonmotile, unicellular, crescent-shaped (40 to 60 μm^3) green alga (Chlorophyceae) that is ubiquitous in most freshwaters 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 *S. capricornutum* because it is free of complex structures and does not form chains. Growth is sufficiently rapid to accurately measure cell yield after 72 and 96 h, and the species is moderately sensitive to toxic substances.

The test species recommended for this test is *S. capricornutum* 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 *S. capricornutum*, 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

Two reliable Canadian sources of *S. capricornutum* are:

(a) University of Toronto Culture Collection (UTCC)
Department of Botany
University of Toronto
Toronto, Ontario
Canada, M5S 3B2
Telephone: (416) 978-3641
Facsimile: (416) 978-5878
Selenastrum capricornutum: UTCC 37

(b) Environment Canada
St. Lawrence Centre
Ecotoxicology and Ecosystems Branch
Microbioassay Laboratory
1001 Pierre Dupuy
Longueuil, Quebec
Canada, J4K 1A1
Telephone: (514) 928-4200
Facsimile: (514) 928-4264
Selenastrum capricornutum: ATCC 22662

Two reliable American sources of *S. capricornutum* are:

(a) American Type Culture Collection (ATCC)
12301 Parklawn Drive
Rockville, Maryland
U.S.A. 20852
Telephone: (301) 881-2600
Facsimile: (301) 231-5826
Selenastrum capricornutum: ATCC 2266

(b) Culture Collection of Algae (UTEX)
Botany Department
University of Texas
Austin, Texas
U.S.A. 78712
Telephone: (512) 471-4019
Facsimile: (512) 471-3878
Selenastrum capricornutum: UTEX 1648

The algae are available from the UTCC as axenic liquid cultures or agar slants. Using a courier service, the ATCC delivers a frozen liquid culture in an ampule that is transported in dry ice and must be resuspended in growth

media. The UTEX culture is available in 10 mL agar slants and can be delivered either by surface mail or courier.

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 *S. capricornutum*.

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 *S. capricornutum*, depending on its source, may be on an agar slant, in liquid culture, or frozen in an ampule as a dried pellet. The "starter" culture must be aseptically transferred to and resuspended in a defined growth medium to maintain a stock culture of algae as a source for the toxicity tests. The "starter" algae can also be stored in the dark at 4°C and remain viable for up to 6 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 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 macronutrients and micronutrients in the liquid growth medium for the stock algal culture are given in Table 2. The medium minimizes changes in pH over 72 h (Blaise, personal communication; Day, unpublished data).

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 dioxide 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 one colony 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 4.0 klux at the surface of the flask (light quantal flux should approximate 60 to 80 µE/($m^2 \cdot s$)). The flask with the algae should be placed on a continuous shaker at 100 rpm or shaken manually twice daily. The algal culture may take 7 to 14 days to reach the exponential growth phase. When this

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 mg[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
	$Na_2EDTA \cdot 7H_2O$	150.0 mg
3	$MgSO_4 \cdot 7H_2O$	7.35 g
4	K_2HPO_4	0.522 g
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 36.6 mg of $Na_2MoO_4 \cdot 2H_2O$ and dilute to 100 mL. Add 1 mL of this solution to Stock Nutrient Solution 2.

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 4 and 7 days postinoculation, to a flask containing fresh liquid growth medium. Purity of the stock culture should 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 37.5°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 *S. capricornutum* 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

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	-	-

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 until colonies are visible (approximately 2 weeks). Store at 4°C in the dark for future use. A fresh liquid stock algal culture should be started each month from 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.

2.4 Quality of Test Organisms

The test algae should 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. Performance and culture health must be evaluated by routinely measuring rate of growth and the

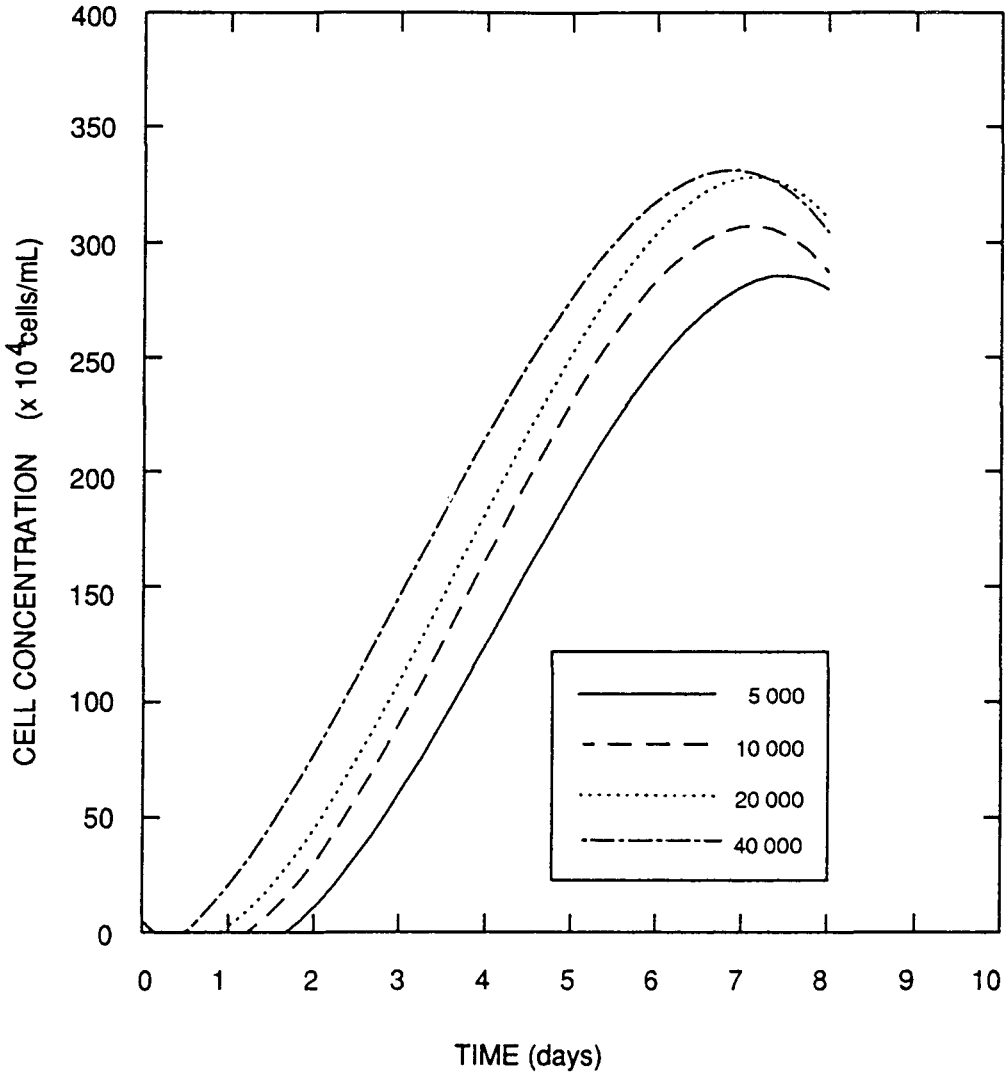

Figure 1 Laboratory Monitoring of Performance and Culture Health*

* To monitor performance and culture health, algal growth curves at various initial inoculum concentrations should be performed monthly (see Section 2.4).

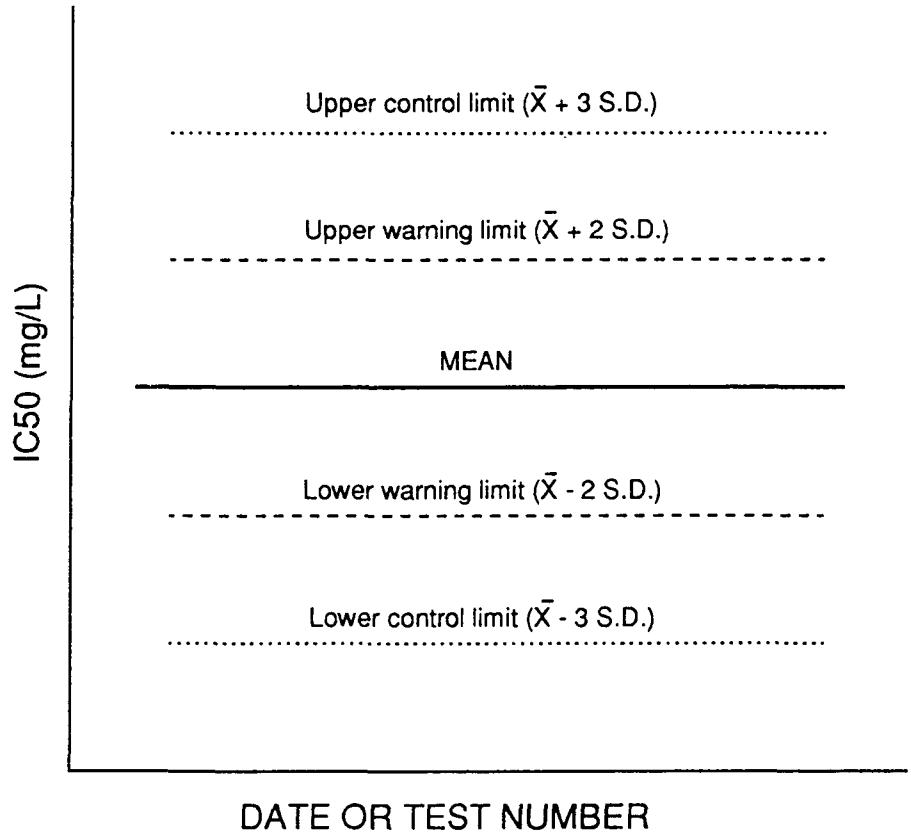

Figure 2 Warning/Control Chart for Reference Toxicants*

* Adapted from Environment Canada (1990). Procedures for use are described in Section 4.5.

Test System

relative sensitivity of the algae to a reference toxicant (Figures 1 and 2; Section 4.5).

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 recommended specifications for agitation, 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 preconditioned and tested for cytotoxicity prior to its use. Tables 3, 4, and 5 list the nonconsumable equipment, the consumable equipment, and the reagents required to execute the algal growth inhibition test using the microplate technique.

3.3 Test Conditions

Test conditions (Table 6) must be constant and monitored continuously throughout the test.

3.4 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 according to the following method:

- 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 aluminium foil or other cap, as appropriate, and store.

Equipment made of any material other than glass, and which can withstand the recommended washing treatment, must be washed using this method.

Table 3 The Nonconsumable 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 X magnification
- centrifuge: 4 x 15 mL capacity
  2000 x g (1.33×10^{-7} $m^3/(kg \cdot s^2)$)
  swing-out cups or buckets 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
- 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 kilolux (klx)

Optional:

- microplate fluorometer
- microplate photometer

Table 4 Consumable Equipment Required to Execute a Microplate Algal Toxicity Test

- microplates: sterile disposable, rigid, polystyrene, 96-well (capacity approximately 0.25 mL), U-shaped or flat bottom, microplates for Coulter counter or microplate reader, respectively, with cover
- 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 x 150 mm)
- sterile disposable centrifuge tubes with screw caps (15 and 50 mL capacity)
- sealable transparent plastic bags (approximately 16 x 20 cm)
- transparent plastic cups (20 mL)
- sterile disposable 100 x 15 mm Petri dishes
- filtration membrane (0.20 and 0.45 µm porosity)
- aluminium 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 *M* NaCl, 3.0 n*M* KCl, 15 n*M* 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 HCl solutions, ≤1 *N*
- inoculum of *S. capricornutum* from stock algal culture that is 4 to 7 days old and in logarithmic growth phase

Table 6 Test Conditions for the Algal Microplate Toxicity Test

Temperature:	• 24 ± 2°C
Lighting:	• continuous "cool-white" fluorescent
	• 4 ± 10% klx
	• quantal flux should be approximately 60 to 80 μE/(m^2 • s)
Agitation:	• static

Section 4

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.

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, 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 7.

The procedures and conditions that are fundamental to all algal microplate growth inhibition tests are summarized in Table 8 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 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

Table 7 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

Table 8 A Summary of the Universal Test Procedures

Test type	• static, 72-h duration
Test container	• 96-well polystyrene microplate, round- or flat-bottomed
Test water	• reagent or reconstituted water, filtered through 0.45 µm filter
Lighting	• continuous overhead "cool-white" fluorescent illumination with 4.0 klx at the surface of the test container, and a quantal flux between 60 to 80 µE/(m^2 • s)
Temperature	• 24 ± 2°C
Test organism	• *S. capricornutum* from a culture that is between 4 and 7 days old and in exponential phase of growth
Algal inoculum	• 10 000 cells/mL
Number of replicates	• minimum of three
Observations	• cell concentration at 72 h or absorbance at 430 nm
Measurements	• pH, temperature of incubator or chamber
Endpoint	• cell yield (IC50, NOEC/LOEC)
Reference toxicant	• phenol, $ZnSO_4$, or NaCl

evaporative losses from the inner wells. The evaporative loss from test solutions in the wells during incubation should not exceed 10%. The central insertion of a row of control replicates (e.g., row D), parallel to the gradient of test concentrations, identifies potential contamination due to toxic volatile substances from adjacent test wells. Heterogeneity among cell yield estimates in the control wells suggests the potential occurrence of volatile effects.

4.2 Preparation of the Enrichment Medium

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 a 13.75 x enrichment medium, 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. The final concentration of each nutrient in the treatment wells on the microplates is presented in Table 9. The pH is then adjusted and the medium is filter-sterilized as described in Subsection 2.3.1.

The uninoculated, 13.75 x sterile enrichment medium must be stored in the dark, at 4°C, in

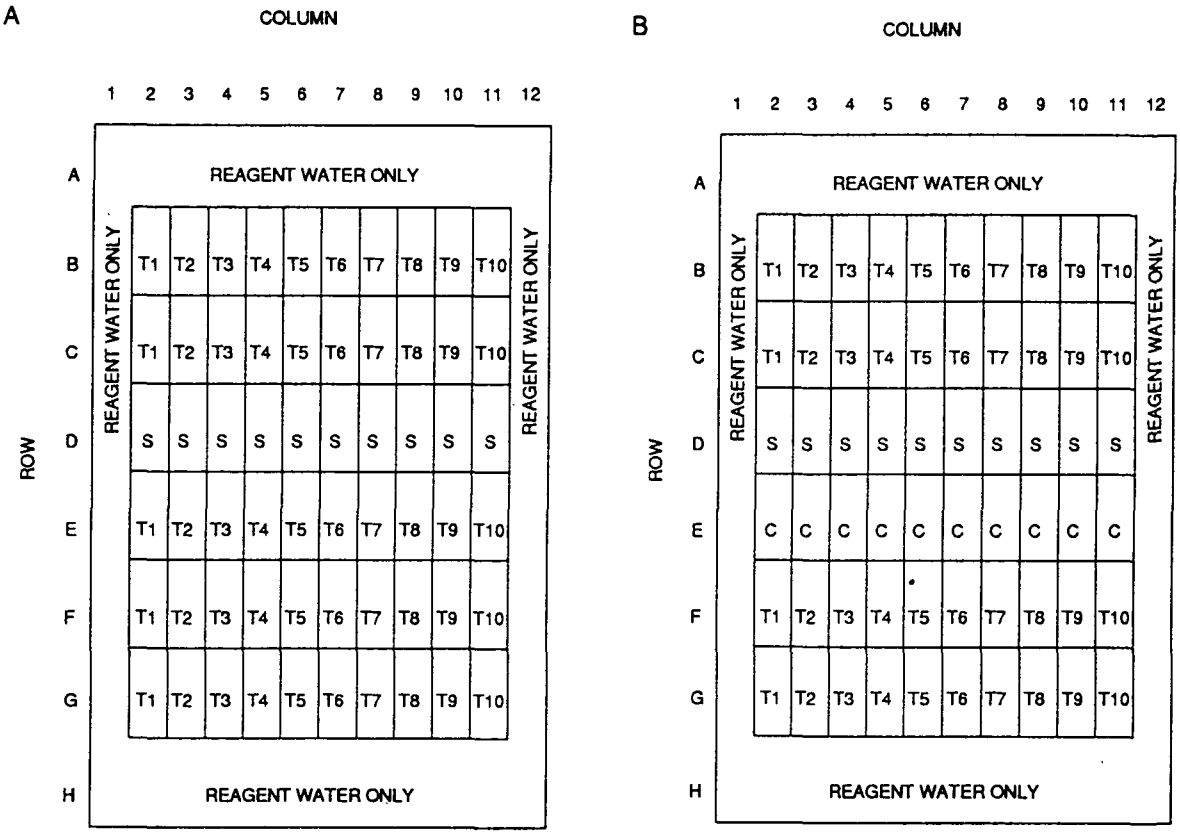

Figure 3 Standard Microplate Configuration*

* To be used for tests where reagent water is used for dilution and as the control water (A); and for tests where reagent water is not used as the diluent (B). Peripheral wells are filled with 220 µL of reagent water. T1 and T10 are 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.

a closed inert container. This solution can be stored under these conditions for up to 6 months.

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 *S. capricornutum* cells harvested from a liquid stock algal culture that is 4 to 7 days old and in a logarithmic phase of growth. The initial cell density for the microplate algal growth inhibition test is 10 000 cells/mL. Because the final test volume per well is 220 µL, an absolute number of 2200 cells (10 000 cells/mL x 0.220 mL) is required for each well.

To get the appropriate algal cell concentration, the following steps must be taken:

(1) Multiply the total number of wells to be inoculated by 2200 to obtain the total number of cells required for inoculation.

Table 9 Final Concentration of Nutrients in the Microtest 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	-	-

(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 to estimate the concentration 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 algal culture density of 1 000 000 cells/mL, the total volume to withdraw from the culture flask would be:

(1) 60 wells x 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 x 1.5 = 0.948 mL

The harvested cells must be centrifuged at 2000 g for 15 min, the supernatant discarded, and the cells resuspended in a small volume (5 to 10 mL) of $NaHCO_3$ at 15 mg/L. Centrifuge and decant again, and resuspend

cells in a few millilitres of the water–bicarbonate solution. Determine the concentration of cells (cells per mL) with an electronic particle counter or hemacytometer. If necessary, dilute the algal suspension with $NaHCO_3$ to 220 000 cells/mL, so that adding 10 µL of inoculum to each microplate well will give an initial algal concentration of 10 000 cells/mL. The final volume in the well is 220 µL with 200 µL of test sample, 10 µL of algal inoculum, and 10 µL of enrichment medium.

There are potentially six aqueous solutions used in a microtest:

(1) the test sample;

(2) the control/dilution water;

(3) the reagent water;

(4) a bicarbonate solution;

(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 resuspend particulate. A subsample between 5 and 10 mL must be filtered through a preconditioned 0.45 µm pore diameter membrane, and placed in a test tube. Because pH may influence the toxicity of a substance, the pH of test samples must be measured prior to testing. If the pH of the test sample is between 6 and 9, the microtest may be carried out without pH adjustment. If, however, the pH of the sample is <6 or >9 and the objective is to assess toxicity independent of pH effects, adjust the pH of the samples with either the NaOH or HCl solutions, to 6.5 or 8.5, whichever is closest to the initial pH of the sample. For these samples, it is recommended that toxicity of the pH-adjusted sample and the sample at the initial pH be evaluated concurrently.

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 *S. capricornutum* is known, prepare concentrations of the test solutions to encompass a response range that includes no inhibition of growth (NOEC) and no growth (IC90-100). These test concentrations of chemicals can be prepared from stock solutions with reagent water, or with dilution of a test sample with dilution water prior to testing, and can be either an arithmetic progression or a geometric series. A geometric series is recommended. 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.

To prepare a geometric series of test concentrations of an aqueous sample with unknown toxicity, dilute by a factor of 0.33 to give concentrations of 100, 33, 11, 3.7, 1.2, 0.41, 0.14, 0.05, 0.02, and 0.01 %v/v. 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, 0.01, 0.0017, and 0.0006 %v/v). The actual concentrations are slightly lower because the test concentrations are diluted by a factor of 0.9091 with the addition of the nutrient spike and algal inoculum.

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 that the reservoir is adequately emptied between each addition.

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 should be such that the initial cell concentration in each microplate well is $10\,000 \pm 1000$ 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 wells. An initial cell concentration outside of the acceptable range implies that the mixture should be repeated. The mixture should be kept homogenous during the inoculation step. The test begins as soon as the algae and nutrients are added to the wells. Lids are added to cover each microplate. If volatility of toxic substances is expected to occur, seal the individual wells with an adhesive polyester film. If polyester film is used to cover the microplates, the exposure duration should be 72 h. Place the microplate lid over the plates. Alternatively, separate microplates for each test concentration may 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.

4.3.5 Quality Control Microplate

For every algal growth inhibition test that is performed, the following must also be incubated: a microplate containing 220 µL of reagent water in the peripheral wells, and 200 µL of reagent water, 10 µL of algal inoculum, and 10 µL of enrichment media in the other wells. This quality control microplate is a standard for optimum growth of algae under the test conditions. There should be no statistically significant difference in the cell-yield estimates of the standard reagent controls on each test microplate versus the cell-yield estimates in the quality control plate. Significant differences suggest a problem with volatility or with the experimental procedures. If any significant differences occur, the test should be repeated.

4.3.6 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-bottom wells tend to concentrate the algae 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 pH of the test solutions with a microprobe or pH indicator paper prior to resuspension of algal cells. Changes in pH due to CO_2 depletion are minimal over 72 h and algal growth is measurable. Enumerate the cells in at least three wells of each column, in all of the standard control wells, and, if it applies, in each of the sample control wells. Similarly, enumerate the algal cells in the quality control plate wells corresponding to the standard control wells.

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[1] allows a rapid determination of the cell concentration (cells/mL). The counter must be calibrated according to standard operating procedures. The operative aperture diameter recommended for *S. capricornutum* 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.* (1989) 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 the distinction between algal 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.

Microplate Photometry. An indirect method of estimating cell concentration[2] is to measure light absorbance of the resuspended algal cells in each well. This can be done quickly

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

[2] 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).

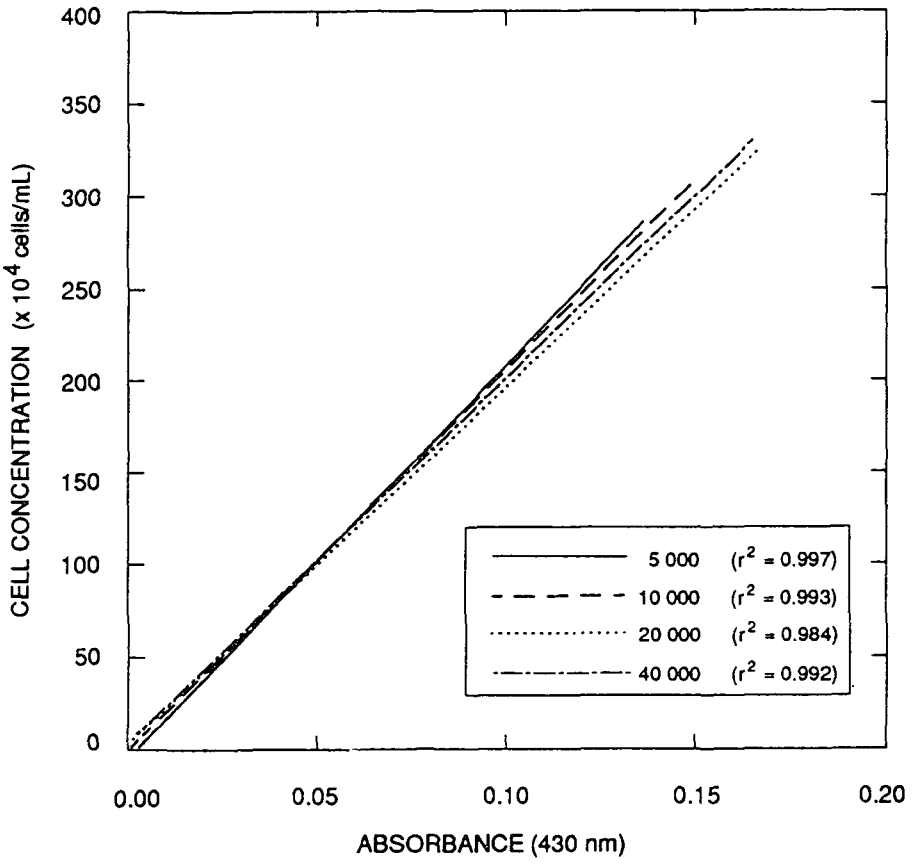

Figure 4 Algal Cell Concentration Regressed with Absorbance*

* A first-order linear regression was performed on cell concentration versus absorbance, at 430 nm, for four initial inoculum concentrations.

and easily if the test solutions are colourless. If photometry is to be used, flat-bottomed microplates are recommended. Resuspend the algal cells in the flat-bottomed wells of the microplate with the multichannel pipette as described previously; then, place the uncovered microplate in a microplate photometer with the wavelength set to 430 nm, for optimal light absorbance by the suspension of *S. capricornutum*. Follow the standard operating procedures for calibrating the machine. Generally, the reagent water serves as a blank for standardizing the machine. Measure the absorbance in each well of the microplate.

If the test samples are coloured, as is the case for many samples of elutriate, effluent, or leachate, the algal cells in each well must be resuspended in reagent water prior to measuring absorbance. To do this, remove the microplate from the plastic bags. Place the microplates securely in a temperature-controlled centrifuge equipped with a head appropriate for centrifuging microplates, and centrifuge for 5 to 10 min at 2000 g [1.33×10^{-7} $m^3/(kg \cdot s^2)$].

Remove the microplate from the centrifuge and remove the microplate lid and/or polyester seal. Decant the supernatant very carefully, with the multichannel pipette. Be

sure not to disturb the algal plug at the bottom of the wells. Combine the supernatant of the replicates within a treatment, and measure pH with a microprobe. Add to each well 200 µL of reagent water, and resuspend the algae using a multichannel pipette as described previously. Measure the absorbance in each well of the microplate.

4.4 Test Endpoints and Calculations

The endpoints of the microplate algal growth inhibition test with *S. capricornutum* are an IC50 and/or NOEC/LOEC. The IC50 is an estimate of the sample concentration causing a 50% reduction in the growth of the algal population compared to a control. The variable used to determine the IC50 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 algal cell concentration (~10 000; see Subsection 4.3) from the final measured algal cell concentration. The cell yield in the standard control wells of the sample microplate must be statistically compared with the cell yield in the same wells on the quality control microplate, using nonparametric Mann-Whitney U-test procedures (Zar, 1984; p. 138) or the parametric equivalent (t-test) after formally testing for normality (Shapiro–Wilks test) and homogeneity of variances (Hartley test). The cell yield in the D2 to D11 standard control wells of the sample microplate should also be statistically compared using trend analysis (Kendall test) to ensure homogeneity within the control wells on the sample microplate. If the microplate configuration includes both a standard reagent control and a sample control, both should be used in the trend test.

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 reagent control.

Using this value, calculate, for each test concentration replicate, the percentage of growth inhibition 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.

The average I values for each treatment should be displayed graphically, such that 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). These values cannot be included in the calculation of the IC50 but should be reported.

To determine the IC50, graphically plot the percentage inhibition on the y-axis and the test concentrations on the x-axis (logarithmic scale) (Figure 5). To calculate the IC50, first test for homogeneity of variances and transform the data (e.g., logarithmic, Weber *et al.*, 1989) if it is necessary; then, determine the simple linear regression on the data located within the 16 to 84% range (APHA *et al.*, 1989), and perform an inverse prediction to estimate the IC50 value and the 95% confidence interval (Zar, 1984; p. 276). Alternatively, a computer program called BOOTSTRP (Norberg-King, 1988) may be

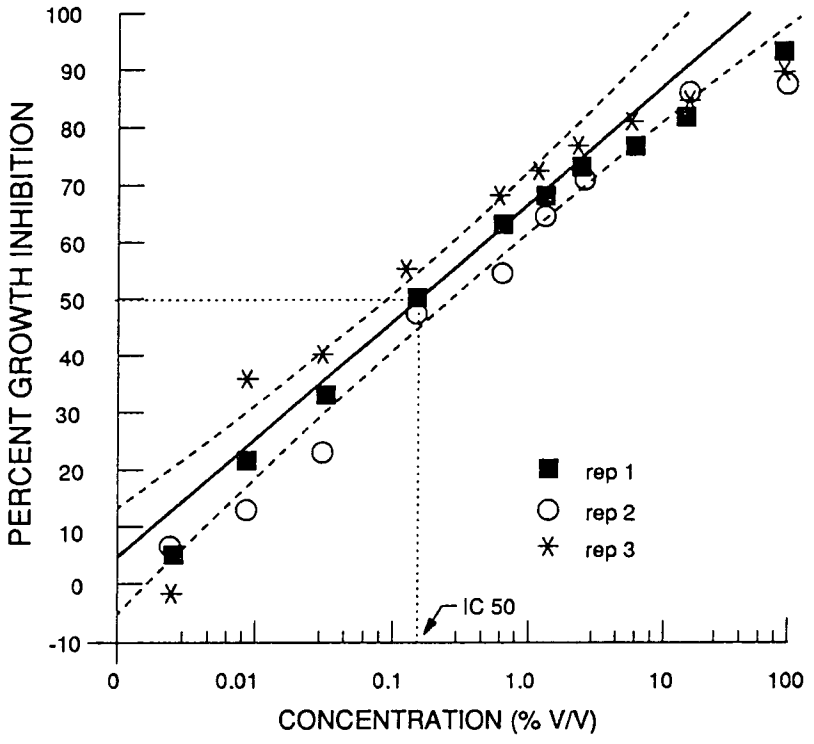

Figure 5 Graphical Estimation of the IC50

used to determine the ICp, an interpolated point estimate of toxicity. This program is available from Environment Canada (see Appendix B).

If the endpoint criterion is absorbance, then the following procedures should be followed. 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.

Substract the measured light absorbance in each well of 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 50% growth inhibition (IC50) 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 IC50 results should always be reported with the exposure duration (72 h) and expressed in percent v/v for wastewater samples and in appropriate units of concentration (µg/L or mg/L) for chemicals. The IC50 (72 h) results for nontoxic wastewater samples are reported as ND (nondeterminable) or >100% v/v.

When the IC50 is below the smallest concentration tested, report the IC50 as smaller than the lowest tested concentration, or rerun the assay at more dilute sample concentrations.

When there are insufficient data to allow determination of the linear regression, repeat the test or report the IC50 as a range. This range, where the upper and lower limits are tested concentrations, is the smallest interval within which the IC50 lies.

The IC50 is an estimate of toxicity derived from a laboratory experiment performed under the standard conditions previously described. The IC50 indicates the potential hazard of a substance, but it cannot be used directly to predict effects in the natural environment. As such, this test can be used effectively as a screening tool. However, a toxicant can influence algal growth in different ways. A single endpoint measurement and subsequent determination of an IC50 can detect an effect, but it does not always reflect the nature of the effect. Adams *et al.* (1985) discuss the merits of the various endpoints for algal toxicity testing, as well as the value of calculating quantitative toxic values. The microplate technique described herein can be modified and adapted, depending on the objective of the test, to assess the growth of algae daily over an exposure period that should not exceed 4 days. As such, the data generated from these tests may also be evaluated using statistical procedures dictated by the objective of the analysis. For example, if the objective of the test were to determine significant differences among treatments or between the treated organisms and those in the controls, the hypothesis-testing approach would be appropriate. Gad and Weil (1988) present decision trees for selecting, statistical hypothesis-testing modelling, and reduction-of-dimensionality procedures. Weber *et al.* (1989) also present the hypothesis-testing procedures that can be used to determine LOEC and NOEC values.

The computer program, "TOXSTAT" (Gulley *et al.*, 1989), which is available from Environment Canada (see Appendix B), provides the statistical procedures necessary for hypothesis testing.

4.5 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 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.

Sodium chloride (NaCl) and zinc sulphate ($ZnSO_4$) are the recommended inorganic reference toxicants, and phenol the recommended organic reference toxicant, for the algal growth inhibition test using the microplate technique. Other reference toxicants are available (Environment Canada, 1990), but the chemicals recommended above are available in a pure form, readily water soluble yet stable in aqueous solutions, and have a stable shelf life. The toxicants are easy to measure analytically and pose a minimal hazard to the user. *S. capricornutum* is sensitive to the chemicals that 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 significantly affected 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). A geometric series of test concentrations is recommended. 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 reference toxicants should occur at least once a month. The test results should be plotted according to a mean chart (Environment Canada, 1990), where the vertical axis represents the endpoint concentration (e.g., IC50 72-h), and the horizontal axis represents the test date or test number (Figure 2). The mean chart can be constructed with either measured or nominal endpoint concentrations, but not with a mixture of both. It is recommended that measured concentrations should be obtained at the beginning of each test, using a standardized method of chemical analyses (e.g., APHA *et al.*, 1989).

Separate control charts should be prepared for each reference toxicant. Each new test result should be compared with the established warning limits of the mean control chart to assess the acceptability of the result (Environment Canada, 1990). The geometric mean ICp together with its upper and lower warning limits (± 2 SD) calculated on a geometric (e.g., logarithmic) basis are recalculated with each successive ICp until the statistics stabilize.

Prepare a mean control chart by plotting the results of successive acceptable tests, with a reference toxicant, on a chart, where the x-axis represents the test date, or number, and the y-axis represents the endpoint concentration (e.g., IC50).

Calculate the mean ($\bar{X}$) and standard deviation (SD) of the accumulated IC50 values, and the warning limits ($\bar{X} \pm 2$ SD). With a sufficiently large sample size (e.g., 15 to 20 data points), the chart can be used to assess the validity of results from subsequent tests with that reference toxicant. If an endpoint value falls outside the control limits ($\bar{X} \pm 3$ SD), it is highly probable that the test is unacceptable and should be repeated with all aspects of the test being carefully scrutinized (Figure 2). If the frequency of endpoint values located between the control and the warning limits exceeds 5%, there has been a deterioration in precision and again the test should be repeated with careful scrutiny of test procedures and calculations. The mean control chart should be updated with each additional acceptable endpoint, by recalculating the mean, warning, and control limits. (For further details on establishing and updating the warning chart and data interpretation see Environment Canada, 1990; Section 5).

Section 5

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

Reagent water is recommended as the control/dilution water for tests designed to assess toxicity of a chemical to *S. capricornutum*. 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.

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. 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 by ultrasonic dispersion. Solubility of the test chemical may 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 should 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). This microplate configuration allows the detection of effects from toxic volatile substances (Figure 3B; Section 4.1).

Generally, the highest concentration of the solubilizing agent will be less than its NOEC. 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 the method described in the U.S. EPA (1989). The recommended organic solvents are acetone and methanol (St-Laurent *et al.*, 1992), which have NOEC values >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 controls is not significantly different from that of the standard controls as determined by a nonparametric Mann–Whitney U-test (Zar, 1984; p.138). All controls (carrier and standard) must be statistically compared with the controls of the quality-control microplate.

Section 6

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

Aqueous samples must be collected to ensure that they adequately reflect the true nature of the leachate, elutriate, or effluent. Generally, a sample volume of 1 L is sufficient for testing. Each sample must be collected and placed in a labelled container of inert material and transported in the dark and on ice (1 to 7°C, preferably 4 ± 2°C), to the laboratory. The containers for transport and storage should be new or thoroughly cleaned. Rinse the container with the sample prior to filling. Fill to the brim to minimize headspace and seal the container. Clearly label with the type of sample, source and/or sample location, sample identification, date and time of collection, and name of sampler(s). The chain of custody must be maintained throughout. The sample should be stored in a closed appropriately labelled container, without headspace, in the dark at 4°C.

Effluent and leachate samples should be tested as soon as possible and must be tested within 72 h after collection. Extraction of samples for elutriates should occur within 10 days and the elutriate should be tested within 3 days.

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 (U.S. EPA, 1989). 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 with reagent water must 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. It is recommended that these aqueous samples be tested in duplicate (e.g., two microplates). 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.*, 1989) 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

Particular attention must be given to the comparison of standard algal growth in the reagent control treatment with growth in the control water to determine if the control/ dilution water is phytotoxic. Also, a comparison of both control treatments with algal growth in the quality control microplate might identify procedural inconsistency or the presence of volatiles. Enhanced growth in test solutions, relative to the control solutions, must be reported.

Section 7

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 Section 6.1 and 6.3.

7.2 Control/Dilution Water

To assess the chronic toxicity of receiving-water samples collected in the area of a point source of possible contamination (e.g., wastewater discharge, chemical spill), "upstream" water should normally be sampled and used as control water and diluent for the 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 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. A quality control microplate, using reagent water, should accompany each test (see Subsection 4.3.5).

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.

Section 8

Reporting Requirements

The test report should describe the objective, materials and methods used, as well as the results. Any changes or modifications of the procedures described in this report must be reported. The reader should be able to establish from the report whether the conditions and procedures rendered the results acceptable for the use intended.

The following information should be included in the test report. Additional information relevant to good laboratory practices (GLP) must be kept on file by the testing laboratory.

8.1 Test Material

(a) General

- sample type, source, and description (chemical, effluent, elutriate, leachate, or receiving water);
- specifics regarding nature, appearance, chemical and physical properties, volume and/or weight;
- information on labelling or coding of the test material;
- identification of person(s) collecting and/or providing the sample; and
- dates and times for sample collection/preparation, delivery and receipt at test facility.

(b) Effluents, elutriates, leachates, or receiving waters

- sampling location and method;
- name of receiving water body; and
- details on manner of sample collection, transport, and storage (e.g., batch, grab, or composite sample, description of container, temperature of sample upon receipt, and during storage).

8.2 Test Organisms

- species, strain number, origin, age of culture, and method of cultivation;
- composition of growth medium;
- culturing apparatus (e.g., size, shape, and material of culture vessels) and incubation procedure;
- light intensity and quality; and
- temperature.

8.3 Test Facilities and Apparatus

- name and address of test laboratory;
- name of person(s) performing the test; and
- description of culture and test incubators, including light, aeration, and temperature regulating systems.

8.4 Control/Dilution Water

- type(s) and source(s) of water used as control and dilution water;
- type and quantity of any chemical(s) added to control or dilution water;
- sampling and storage details if the dilution water was "upstream" receiving water;
- water pretreatment (adjustment of temperature, hardness, pH,

degassing, aeration rate and duration, etc.); and

- measured water quality variables before and/or at time of commencement of toxicity test.

8.5 *Test Method*

- if a standard method is used, cite this document;
- describe procedure if modifications or changes to specific experimental design occur;
- date, times, and duration of tests;
- concentrations tested (specify if they were measured or nominal);
- method of preparing and storing stock and test solutions(s);
- vehicle and method used for solubilizing the test substance and concentration of the vehicle in the test solutions, if applicable;
- description of pH adjustment procedure, if applicable;
- any chemical and physical analyses of test solutions and reference to analytical method(s) used;
- number of replicate test wells per treatment and control;
- initial cell density of the inoculum;
- composition of the test medium;
- light intensity and quality at surface of test solutions;
- temperature;
- frequency and type of observations made during test; and
- method for measuring cell concentration and calculating cell yield.

8.6 *Reference Toxicant Testing*

- conditions and procedures of reference toxicant testing;
- standard toxicant(s) used and source(s);
- date and time of most recent test(s);
- dilution water use in test(s);
- mean IC50 (72 h) and 95% confidence limits;
- warning/control chart for each reference toxicant tested, including mean IC50 (72 h) and warning and control limits; and
- methods used for measuring physical and chemical variables.

8.7 *Test Results*

- pH of test solutions at the beginning and at the end of a test;
- appearance of test solutions and changes noted during test;
- at each time of measurement, report cell concentration in control and test concentration replicates, and mean cell concentration of control and individual test concentrations with corresponding coefficient of variation (CV = 100 x standard deviation / mean);
- growth curves (cell concentration versus time) if daily measurements were made;
- graphical representation of the dose–response relationship

(percentage growth inhibition values against concentration);

- numerical or range value of the IC50 and method of determination; 95% confidence limits if IC50 is numerical; and
- other observed effects.

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Appendix B

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* Computer programs "BOOTSTRP" and TOXSTAT" are available for copying onto a formatted 13-cm IBM-compatible floppy disk supplied by the user, by contacting the Laboratory Division at this address.