



Marine Algae *(Dunaliella tertiolecta)*

Chronic Toxicity Test Protocol

Appendix to:
Hall, J.A.; Golding, L.A. (1998). Standard methods for whole effluent toxicity testing: development and application. Report no. MFE80205. NIWA report for the Ministry for the Environment, Wellington, New Zealand.

Reproduction, adaptation, or issuing of this publication for educational or other non-commercial purposes is authorised without prior permission of the copyright holder(s). Reproduction, adaptation, or issuing of this publication for resale or other commercial purposes is prohibited without the prior permission of the copyright holder(s).

Marine algae

(Dunaliella tertiolecta)

**Chronic Toxicity
Test Protocol**

Abstract

The method prepared by the National Institute of Water and Atmospheric Research (NIWA) for determining the chronic toxicity of whole effluents to the marine alga *Dunaliella tertiolecta*, using the microplate technique, is described. Included are details on culture 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 data analyses, including statistical procedures, and the use of reference toxicants.

Table of Contents

Abstract	iii
Table of Contents	iv
List of Tables	vi
List of Figures	vi
List of Abbreviations and Chemical Formulae	vii
Terminology	ix
Acknowledgements	xiii
1.0 Introduction	1
1.1 Principles of the Test Method	1
1.2 Historical Use of the Test.....	1
1.3 Summary of the Microplate Technique.....	1
1.4 Application, Advantages, and Limitations of the Microplate System	1
2.0 Test Organism	3
2.1 Species.....	3
2.2 Source.....	3
2.3 Culturing.....	3
2.3.1 Starter Culture	3
2.3.2 Stock Culture.....	4
2.3.3 Liquid Growth Medium for Stock Algae Culture	4
2.4 Quality of Organism.....	5
3.0 Test System	6
3.1 Summary of test system	6
3.2 Facilities	6
3.3 Equipment	6
3.3.1 Selection of a Microplate Configuration.....	8
3.4 Test Conditions	9
3.5 Washing of Glassware.....	9
3.6 Preparation of Reagents	9
3.6.1 Preparation of Oceanic Sea Water and Diluent Water.....	9
3.6.1.2 Sterile Oceanic Seawater (26 ppt).....	10
3.6.1.3 Sterile Diluent Water (35 ppt).....	10
3.6.1.4 Sterile Diluent Water (26 ppt)	10
3.6.2 Preparation of Reference Toxicant	10
3.6.2.1 Preparation of ZnSO ₄ (1 mg/L) Solution	11
3.6.2.2 Preparation of Reference Toxicant Dilution Series	11
3.6.3 Preparation of Hypernutrient Solution (HPN)	11
3.7 Preparation of Test Solutions.....	12
3.7.1 Collection and Handling of Effluent and Leachates	12
3.7.2 Preparation of Effluent Test Solution	12
3.7.3 Preparation of Test Solution Dilution Series	12
3.8 Preparation of Algal Inoculum.....	13
4.0 Test Procedures	15
4.1 Summary of Test Procedure	15
4.2 Preparation for the test	15
4.3 Beginning the test.....	16
4.3.1 Inoculating control plate	16
4.3.2 Inoculating reference toxicant plate.....	16
4.3.3 Inoculating the test plate	16
4.4 During the Test.....	17
4.5 Ending the Test and Recording Data and Observations.....	17

4.5.1	Measuring cell concentration in the wells.....	17
5.0	Acceptability of Test Data	18
6.0	Data Analysis	19
6.1	Test Endpoints and calculations.....	19
7.0	Reporting of Results.....	22
7.1	Test Material	22
7.2	Test Organisms.....	22
7.3	Test Facilities and Apparatus.....	22
7.4	Control/Dilution Water	22
7.5	Test Method	22
7.6	Test Conditions	23
7.7	Test Results	23
8.0	References	24
9.0	Appendices	26
	Appendix 9.1 - Preparation of Bacterial Nutrient Agar.....	26
	Appendix 9.2 - Report Forms.....	27
	Appendix 9.3 - Example of Toxcalc ^{TN} results	30

List of Tables

Table 1:	Preparation of Working Stock Nutrient Solutions for F/2 Liquid Growth Medium	5
Table 2:	Equipment and reagents required to perform 72 h <i>D. tertiolecta</i> microplate toxicity test.....	7
Table 3:	Test Conditions for the Algal Microplate Toxicity Test	9
Table 4:	Summary of Recommended Test Conditions for the 72 h <i>D. tertiolecta</i> Toxicity Test.....	15

List of Figures

Figure 1:	Layout of dilution series used in microplates. C = concentration; Cont = control (sterile Oceanic water (26ppt)); Dil = control in diluent water if applicable. SW = sterile sea water only.....	8
Figure 2:	Analytical quality control chart with mean \pm 2 standard deviations (taken from Environment Canada, 1990).....	11
Figure 3:	Flow diagram of USEPA approved statistical methods performed by Toxcalc TM . (Tidepool, 1994)......	21

List of Abbreviations and Chemical Formulae

°C	degree(s) Celsius
CoCl ₂	cobalt chloride
CuSO ₄	copper sulphate
d	day
DO	dissolved oxygen (concentration)
EC ₅₀	effective concentration
EDTA	ethylenediamine tetraacetate (C ₁₀ H ₁₄ O ₈ N ₂)
FeCl ₃	ferric chloride
FeSO ₄	ferrous sulphate
g	gram
h	hour
HCl	hydrochloric acid
H ₂ O	water
ICp	inhibiting concentration for a (specified) percent effect
L	litre
LC ₅₀	median lethal concentration
LOEC	lowest observed effect concentration
M	Molar
m	metre
mg	milligram
min	minute
mL	millilitre
MnCl ₂	manganous chloride
MSD	minimum significant difference
N	normal
NaNO ₃	sodium nitrate
NaH ₂ PO ₄	sodium dihydrogen phosphate
nm	nanometre(s)
NOEC	no observed effect concentration
ppt	parts per thousand
s	second(s)
SD	standard deviation
^{TN}	Trade Name
ZnSO ₄ ·7H ₂ O	zinc sulphate

μ	micro
$\mu\text{mol m}^{-2} \text{s}^{-1}$	micro moles per metre square per second
UV	ultra violet
v/v	volume to volume
>	greater than
<	less than
\geq	greater than or equal to
\leq	less than or equal to
\cong	approximately equal to

Terminology

Note:

All definitions are given in the context of the procedures in this protocol, 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”.

General Technical Terms

- Culture* as a noun, means the stock of organisms raised under defined and controlled condition to produce healthy test organisms. As a verb, it means to carry out the procedure of raising organisms.
- Monitoring* is the routine (e.g., daily, weekly, monthly, quarterly) checking of quality or collection and reporting of information. In the context of this protocol, 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. Concentrations can be prepared on a volume-to-volume or weight-to-weight basis, and are expressed as the percentage of test material in the final solution.
- pH* is the negative logarithm of the activity of hydrogen ions in gram equivalents per litre. The pH value expresses the degree or intensity of both acidic and alkaline reactions on a scale from 0 to 14, with 7 representing neutrality, numbers less than 7 signifying increasingly greater acidic reactions, and numbers greater than 7 indicating increasingly basic or alkaline reactions.
- Photoperiod* is the duration of illumination and darkness within a 24-h day.
- Photosynthetic active radiation (PAR)* PAR is a unit of illumination based on units per square metre. Light conditions or irradiance are properly described in terms of quantal flux in the photosynthetic wavelength range of approximately 400 to 700 nm.

<i>Precipitation</i>	is the formation of a solid (i.e., precipitate) from a solution.
<i>Pre-treatment</i>	is the treatment of a sample or dilution thereof, prior to exposure of algae.
<i>Salinity</i>	is the total amount of solid material, in grams, dissolved in 1 kg of sea water. 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 <i>et al.</i> , 1989). It is usually reported in grams per kilogram (g/kg) or parts per thousand (ppt).

Terms for Test Materials

<i>Control</i>	is a treatment in an investigation or study that duplicates all the conditions and factors that might affect the results of the investigation, except the specific condition that is being studied. In an aquatic toxicity test, the control must duplicate all the conditions of the exposure treatment(s), but must contain no test material. The control is used to determine the absence of measurable toxicity due to basic test conditions (e.g., quality of the control/dilution water, health or handling of test organisms).
<i>Control Plate</i>	refers to a microplate that in each experimental well contains only 200 μL of sterile oceanic water and 20 μL of algal inoculum. The plate represents optimal algal growth for a given set of experimental conditions and exposure period.
<i>Control/Dilution water</i>	is the water used for diluting a test material, in order to prepare different concentrations for the various toxicity test treatments, or for the control test, or both. In this protocol it is the uncontaminated oceanic water (26 ppt) used for the sample control plate and for dilution of the test substance. It may also be uncontaminated receiving water. Regardless, it must be adjusted to the appropriate salinity and filter sterilized using a 0.2 μm membrane.
<i>Deionised water</i>	is water that has been passed through resin columns to remove ions from solution and thereby purify it.
<i>Distilled water</i>	is water that has been passed through a distillation apparatus of borosilicate glass or other material, to remove impurities.
<i>F/2</i>	is the liquid growth medium used in the culturing of the algae.
<i>Receiving water</i>	is surface water that has received a discharged waste, or else is about to receive such a waste (e.g., it is away from the discharge point). Further descriptors must be provided to indicate which meaning is intended.
<i>Reference toxicant</i>	is a standard chemical used to measure the sensitivity of the test algae in order 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.

- 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 and 20 μL of the algal inoculum .
- Stock solution* is a concentrated aqueous solution that can be stored. Measured volumes of a stock solution are added to dilution water in order to prepare the required strengths of solutions.
- Test plate* is the microplate used to test the test sample. It consists of a dilution series of the test sample as well as a control row using the laboratory control/dilution sea water and an additional control row if a different control/dilution water is used e.g. a receiving water.
- Test sample* refers to the aqueous sample that is to be tested. It might be derived from chemical stock solutions or collected from whole effluents or receiving waters.
- Test solution* refers to an aqueous solution that consists of a prepared test sample, with or without the addition of the algal inoculum.
- Wastewater* is a general term which includes effluents, leachates, and elutriates.
- Whole effluent* is any liquid waste (e.g., industrial, municipal) discharged to the aquatic environment.

Toxicity Terms

<i>Chronic toxicity</i>	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 organism during a significant and sensitive part of the life-cycle.
<i>EC₅₀</i>	is the effective concentration (i.e., the concentration of material in water that is estimated to produce a specifically quantified effect to 50% of the test organisms). The EC ₅₀ and its 95% confidence limits are usually derived by statistical analysis of a quantal, “all or nothing”, response (such as death, fertilization, germination, or development) in several test concentrations, after a fixed period of exposure. The duration of exposure must be specified (e.g., 72 h EC ₅₀).
<i>End point</i>	means the variables (i.e., time, reaction of the organisms) that indicate the termination of a test, and also means the measurement(s) or value(s) derived, that characterize the results of the test (IC ₅₀).
<i>IC₅₀</i>	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., “IC ₅₀ (72 h)”, for a growth rate derived IC ₅₀ and a test duration of 72 h.
<i>LOEC</i>	lowest observed effect concentration. The lowest concentration tested causing a statistically measurable effect to the test system.
<i>NOEC</i>	no observed effect concentration. The highest concentration tested causing no statistically measurable effect to the test system.
<i>MSD</i>	minimum significant difference. The difference between values for individual treatments that would have to exist before it could be concluded that there was a significant difference between the groups. MSD is provided by certain statistical tests including <i>Dunnnett’s multiple-range test</i> , a standard statistical procedure.
<i>Overt</i>	means obviously discernible under the test conditions employed.
<i>Static</i>	describes toxicity tests in which test solutions are not renewed during the test.
<i>Toxicity</i>	is the inherent potential or capacity of a material to cause adverse effects on living organisms.
<i>Toxicity test</i>	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) as measured by EC ₅₀ , or (b) the degree of effect shown (graded or quantitative) after exposure to specific concentrations of whole effluents or receiving water as measured by an IC ₅₀ .

Acknowledgements

This document was prepared with the assistance of Tracey Edwards, Lisa Golding and Dr Julie Hall (NIWA). It was reviewed by Dr Chris Hickey (NIWA) and Dr Marion Nipper (Texas A&M University).

1.0 Introduction

No single test method or test organism can be expected to satisfy a comprehensive approach to environmental conservation and protection (Environment Canada, 1990). When used as a component in a suite of well-defined toxicity tests where a variety of endpoints are measured and species are tested, the results can contribute to an holistic interpretation of toxic impacts. Whole effluent toxicity testing as opposed to single chemical testing enables a greater correlation between the toxicity test results and the likely impacts in the actual environment. The relevance of laboratory toxicity testing to the environment is also enhanced through the use of native species such as the marine alga *Dunaliella tertiolecta*. The growth inhibition test using the marine alga is one of several “core” aquatic toxicity tests selected for standardization.

1.1 Principles of the Test Method

Exponentially growing *D. tertiolecta* are exposed in a static, microplate system to a dilution series of a test substance, an effluent or zinc reference toxicant, 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 algae growth occurs.

1.2 Historical Use of the Test

Traditionally, phytotoxicity has been assessed with standardized algal bottle tests (USEPA, 1987). However, more recently the microplate technique has been used to assess algal toxicity using a variety of marine and freshwater species (Environment Canada, 1992).

1.3 Summary of the Microplate Technique

The microplate technique is a scaled-down version of the standard USEPA algal bottle test (Miller *et al.*, 1978; USEPA, 1989). It

involves the use of microlitre volumes of test solutions in 96-well microplates, as opposed to milliliter volumes of test solutions individually contained in flasks. The test solutions are prepared and dispensed in a predetermined pattern into a microplate. Each well receives 200 μL of test solution, and 20 μL of algal inoculum and nutrient solution. 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. Cell numbers are 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.

1.4 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 post-experimental 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 substance 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.
- Absorption 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 of 72 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.

2.0 Test Organism

2.1 Species

Dunaliella tertiolecta is a motile, unicellular rod shaped (9 - 11µm) green alga (Chlorophyceae) that is common in saline waters. This alga can be easily cultured in the laboratory and is available from suppliers. It's morphology makes it ideal for enumeration with an electronic particle counter as *D. tertiolecta* does not clump or form chains. Growth is sufficiently rapid to accurately measure cell numbers after 72 h, and the species is moderately sensitive to toxic substances.

2.2 Source

Cultures of *D. tertiolecta* (CSIRO culture strain CS-175) can be ordered from:

CSIRO Marine Laboratory
GPO Box 1538
Hobart
Tasmania 7001
Australia
Telephone: 61 (02) 325 316
Facsimile: 61 (02) 325 000
email: microalgae@ml.csiro.au

The algae are available from the CSIRO as a axenic liquid culture or an agar slide which must be resuspended in growth media. New Zealand law requires that an importing permit be obtained from Ministry of Agriculture (MAF) for bringing biological products into the country.

2.3 Culturing

Note: All steps involved in transferring algae must take place in a sterile environment. If a laminar flow cabinet is used, it must have been sterilized using UV light and operating for at least 30 minutes prior to use.

2.3.1 Starter Culture

The “starter” culture must be aseptically transferred from the ampule or slope and resuspended in a defined growth medium to maintain a stock culture of algae for the toxicity tests.

2.3.2 Stock Culture

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

Aseptically transfer 2 mL of the “starter” algal culture using a disposable sterile pipette into a 250 mL Erlenmeyer flask containing 50 mL liquid growth medium (Section 2.3.3.). Incubate the algal cultures at 20 ± 2 °C under continuous “cool white” fluorescent light with an intensity of $200 \mu\text{mol m}^{-2} \text{s}^{-1}$ PAR at the surface of the flask. 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 3 to 5 days to reach the exponential growth phase. When this occurs, the culture is green in colour.

The culture should be renewed once or twice a week to ensure a regular supply of exponentially growing algal cells. This is the stock culture. Renewal of the stock culture is achieved by aseptically transferring 2.0 mL of a stock algal culture that is, on average, between 3 and 5 days postinoculation, to a 250 mL Erlenmeyer flask containing 50 mL of fresh liquid growth medium.

2.3.3 Liquid Growth Medium for Stock Algae Culture

F/2 marine algae culture medium (Guillard and Ryther, 1962; Guillard, 1975) modified with the addition of triple the original vitamin concentration is used as the growth medium for the stock algae culture. This consists of a trace metals solution, a vitamins solution, two

macronutrient solutions and 35 ppt oceanic water. Prepare the stock nutrient solutions (1 - 6) in screw top glass bottles using reagent grade chemicals and deionised water (Table 1).

To prepare the F/2 liquid growth medium for the stock algal cultures, add 1mL of macronutrient solutions 5, 6 and 1mL of the trace metals solution 2 and 3 mL of the vitamins solution to approximately 800 mL of oceanic water (35 ppt) and then complete to 1 L in a 1000 mL volumetric flask. Mix well between each addition.

The growth medium should be filter-sterilized at a vacuum not exceeding 50.7 kPa (380mmHg), using a sterile filtration apparatus and a sterile 0.2 μm millipore filter membrane. The filter is pre-rinsed by passing 50 mL of deionised water through the filter to waste. Repeat this step three times. This is the rinse procedure for all filter sterilizing carried out in this protocol. Sterilization of the growth medium by autoclaving is not recommended as this process reduces algal growth and can result in precipitation of the media. Filter sterilize the F/2 medium into a sterile 1 L glass bottle that has been pre-rinsed 3 times with the filtered material. The sterile liquid growth medium can be stored in the dark at 4 °C for up to 1 month.

Dispense liquid growth medium aseptically into a sterile Erlenmeyer flask. 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 (USEPA, 1989).

Table 1: Preparation of Working Stock Nutrient Solutions for F/2 Liquid Growth Medium

Trace Metals Solution

	Compound	weight (mg)	Preparation
Solution 1	ZnSO ₄ .7H ₂ O	230	Make up to 1 L in a volumetric flask using deionised water.
	(NH ₄) ₆ Mo ₇ O ₂₄ .4H ₂ O	52.9	
	CoCl ₂ .6H ₂ O	119	
	CuSO ₄ .5H ₂ O	100	
Solution 2	MnCl ₂ .4H ₂ O	178	Add to a 1 L volumetric flask containing 600 mL of deionised water. Add 100 mL of solution 1 and complete to volume.
	FeCl ₃ .6H ₂ O	3162	
	EDTA (292.25)	3419	

Vitamin Solution

	Compound	weight (mg)	Preparation
Solution 3	Cyanocobalamin	50	Dissolve in 100 mL of deionised water.
	Biotin	50	
Solution 4	Thiamine.HCl	100	Dissolve in 800 mL deionised water in a 1 L volumetric flask. Add 1 mL of solution 3 and complete to volume.

Macronutrients Solutions

	Compound	weight (mg)	Preparation
Solution 5	NaNO ₃	7,480	Add to 100 mL deionised water
Solution 6	NaH ₂ PO ₄ .2H ₂ O	566	Add to 100 mL deionised water

2.4 Quality of Organism

Purity of the stock should be verified at each transfer by examining a subsample under a microscope (x400) for contamination by micro-organisms. Purity is also checked monthly by transferring 0.2 mL of algal stock culture to petri dishes containing solid bacterial nutrient agar (Appendix 9.1), and incubating at 20 °C for 14 days.

This procedure should reveal the presence of contaminating bacteria that cannot be detected microscopically, even at high magnifications. Sensitivity of the algae must also be evaluated by routinely monitoring the growth within the cultures and the

relative sensitivity of the algae to a reference toxicant (Section 3.6.2).

3.0 Test System

3.1 Summary of test system

Three microplates are prepared for each test namely a test plate, reference toxicant plate and control plate. The test plate is used to determine the inhibition of algal growth in response to a whole effluent and the reference toxicant and control plates allow conditions for the validity of the test to be assessed. The reference toxicant plate is used to assess the reproducibility and reliability (as precision and consistency) and results are compared with historical test results to identify whether they fall within an acceptable range of variability. The control plate is a standard for optimum growth of the algae under the test conditions and significant differences suggest a problem with volatility or with the experimental procedures.

3.2 Facilities

The microplate algal growth inhibition test should be conducted in a facility where the temperature and lighting can be controlled and monitored continuously. The test preparation area should be well ventilated, free of toxic dust and vapors, be protected

from unnecessary external perturbations and be a sterile environment (e.g., laminar flow cabinet). This area should be isolated from the algal culturing facility. The environmental chamber for algal culturing must meet the recommended specifications for agitation, temperature, and light quality and intensity (Section 3.4).

3.3 Equipment

All instruments for routine measurements of the basic chemical, physical, and biological variables must be properly maintained and calibrated regularly. Any equipment that contacts the test organisms, oceanic water, stock solutions, growth media, 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. Equipment must not be made of copper, zinc, brass, galvanized metal, lead, or natural rubber. Table 2 lists the non consumable equipment, the consumable equipment, and the reagents required to execute the algal growth inhibition test using the microplate technique.

Table 2: Equipment and reagents required to perform 72 h *D. tertiolecta* microplate toxicity test

Non consumable equipment required to execute a microplate algal toxicity test

- electronic particle counter (e.g., Coulter Counter Z1) and sampling cups or haemocytometer
- controlled temperature room or incubator set at 20 °C
- deionising or distilled water system
- refrigerator
- microscope with 100 to 400 X magnification
- calculator
- autoclave
- laminar flow cabinet
- vacuum pump
- refractometer or conductivity meter and standards
- filtering apparatus
- tweezers
- multichannel pipette 20 - 200 µL
- adjustable pipettes, 200 - 1000 µL and 1- 5 mL
- waste container with at least 600 mL capacity (beaker or similar)
- 6 x 250 mL glass beaker
- Borosilicate glass bottles; 100 mL, 500 mL and 1000 mL
- tube racks
- analytical balance and weighing spatula
- volumetric flasks; 100, 500 and 1000 mL capacities
- pH meter
- glass Erlenmeyer flasks; 250 mL

Consumable materials required to execute a microplate algal toxicity test

- microplates; sterile, disposable, rigid, polystyrene, 96-well, non treated for cell culture and without surface stippling
- sterile disposable pipette tips; 100 µl, 200 - 1000 µl and 1- 5 mL
- sterile disposable plastic reservoirs
- sterile disposable polypropylene centrifuge tubes with lids; 12 and 50 mL
- sealable transparent plastic bags
- sterile disposable 100 x15 mm petri dishes
- sterile filtration membranes; 0.20 and 0.45 µm porosity
- aluminium foil
- weighing dishes

Reagents required to execute the algal microplate toxicity test

- reagent grade chemicals are to be used in all tests
- reagent water: deionised water or equivalent (e.g., must be free of ions, organic molecules, particles, and micro-organisms)
- reference toxicant
- cleaning reagents; commercially available nonphosphate detergent and Nitric acid
- isotonic diluent for use in particle counter (1% W/V NaCl)
- certified buffer solutions of pH 4, 7, and 10 for calibration of pH meter
- stock nutrient solutions (section 2.3.3; Table 1)
- liquid growth medium (section 2.3.3)
- bacterial nutrient agar (Appendix 9.1)
- NaOH and HCl solutions, ≤1N
- inoculum of *D. tertiolecta* from stock algal culture that is 3 - 5 days old and in exponential growth phase

3.3.1 Selection of a Microplate Configuration

The microplate configuration recommended for reference and test plates of this toxicity test is illustrated in Figure 2.

Peripheral wells on the microplate are excluded from the test because of an “edge-effect” phenomenon associated with microplates. Evaporation 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 evaporation 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. Evidence of the effect of volatile toxicity will be apparent in the reduction of algal growth along the control row from the surrounding high to low sample concentrations. An additional control row is used (e.g. row E) when a receiving water is used as the control/dilution water. The concentration gradient in the dilution series is present across the plate with the lowest concentration being C10 and the highest concentration being C1. Four replicates of each concentration are used.

A control plate would contain sterile oceanic seawater (26 ppt) in all wells. If a diluent water was supplied, then rows B, C and D would contain sterile oceanic seawater (26 ppt) and rows E, F and G would contain sterile diluent water (26 ppt).

A	SW	SW										SW
B	SW	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	SW
C	SW	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	SW
D	SW	Cont	Cont	Cont	Cont	Cont	Cont	Cont	Cont	Cont	Cont	SW
E	SW	Dil C1	Dil C2	Dil C3	Dil C4	Dil C5	Dil C6	Dil C7	Dil C8	Dil C9	Dil C10	SW
F	SW	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	SW
G	SW	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	SW
H	SW	SW										SW

Figure 1: Layout of dilution series used in microplates. C = concentration; Cont = control (sterile Oceanic water (26ppt)); Dil = control in diluent water if applicable. SW = sterile sea water only

3.4 Test Conditions

Test conditions (Table 3) must be constant and monitored continuously throughout the test.

Table 3: Test Conditions for the Algal Microplate Toxicity Test

Temperature:	20 ± 2 °C
Lighting:	continuous “cool-white” fluorescent PAR should be approximately 200 μmol m ⁻² s ⁻¹

3.5 Washing of Glassware

All contaminated glassware and non disposable plastic must undergo a complete wash according to the following method:

- Wash with a non-phosphate and non-ionic detergent solution
- Rinse ten times with tap water
- *Rinse with acid solution (10 % HNO₃ v/v)
- *Rinse three times with tap water
- *Rinse with acetone
- *Rinse three times with tap water
- Rinse three times with deionized water
- Place in oven to dry
- Cover openings of glassware with cling wrap or other cap as necessary, and store

*.Note: Those steps marked with * need only be completed if the glassware has been in contact with effluent or zinc contaminants.*

Flasks used for algal culturing require a foam bung before being covered with aluminum foil. Bungs must also be cleaned by washing in detergent solution and rinsing in tap water and deionised water and left to dry at room temperature. Any glassware that is required to be sterile is autoclaved at 121 °C for 30 minutes.

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

3.6 Preparation of Reagents

Note: All steps involved in preparing solutions and setting up the test must take place in a sterile environment. If a laminar flow cabinet is used, it must have been sterilized using UV light and operating for at least 30 minutes before use.

3.6.1 Preparation of Oceanic Sea Water and Diluent Water

Tests conducted with samples of effluent or leachates should use oceanic water at 26 ppt as the control/dilution water if the objective is to assess the toxicity of the particular sample. If the objective is to assess the potential impact of a sample on a particular receiving water, then the receiving water itself should be used as the dilution water. When a receiving water is used as the dilution water, a standard control with oceanic water 26 ppt must also be included in the test.

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.

3.6.1.1 Sterile Oceanic Seawater (35 ppt)

To be used for:

- diluting *D. tertiolecta* culture to the required 220,000 cells/mL.
- tube 1 of test sample dilution series where salinity of the test sample must be adjusted to 26 ppt.

Oceanic water is collected at sea in areas preferably out past coastal water boundaries to ensure that the water is not contaminated by land based discharges. Where this is not possible, collection may occur in coastal waters at a non-contaminated site with no discharges and minimal influence from land run off. A salinity check must be done to ensure the water is at 35 ppt. Filter 100 mL of 35 ppt oceanic water into a sterile glass bottle, using a pre rinsed 0.2 μm sterile filter.

3.6.1.2 Sterile Oceanic Seawater (26 ppt)

To be used in:

- rows B, C and D of control microplate
- rows E, F and G of control microplate, when a diluent water is not provided
- row D of the test sample plate/s and reference toxicant microplate
- tubes 2 -10 of the test sample dilution series, where a diluent water is not provided
- tubes 2 - 10 of the reference toxicant microplate
- peripheral wells of microplate
- preparation of the reference toxicant solution

26 ppt oceanic water is prepared by a 25% (v/v) dilution of 35 ppt oceanic water using deionised water. For example add 250 mL of deionised water to 750 mL of 35 ppt Oceanic water. Check salinity and adjust until oceanic water 26 ppt is reached. Filter sterilize using a 0.2 µm sterile filter.

3.6.1.3 Sterile Diluent Water (35 ppt)

To be used in:

- tube 1 of test sample dilution series

This may be provided with the effluent or leachates and is used to dilute the test solution instead of the 35 ppt oceanic water collected by the laboratory. This water should be collected and stored in the same manner as the test samples (section 3.7.1). Prepared for use in the same manner as sterile 35 ppt oceanic water (section 3.6.1.1).

3.6.1.4 Sterile Diluent Water (26 ppt)

To be used in:

- rows E, F and G of control plate
- row E of test sample plate/s
- tubes 2 - 10 of test sample dilution series

26 ppt diluent water is prepared in the same manner as the sterile 26 ppt oceanic water (section 3.6.1.2).

3.6.2 Preparation of Reference Toxicant

Reference toxicant tests are used to assess the reproducibility and reliability of results using a given test organism and test procedure over a specific period of time.

Zinc sulphate ($ZnSO_4 \cdot 7H_2O$) is the recommended reference toxicant. It is stable in aqueous form, has a good shelf life and is easy to measure analytically. The reference toxicant dilution series is prepared from a 100 mg/L Zn^{2+} stock solution (219.8 mg $ZnSO_4 \cdot 7H_2O$ made up in 500 mL of deionised water) which can be stored in the dark at fridge temperature (4 °C). Chemically verify the stock solution concentration every six months by analysing a sub-sample preserved with 0.2% HNO_3 (v/v). The source and purity of the reference toxicant must be reported.

Toxicity testing with a zinc reference toxicant should occur each time a test is performed. The test results should be plotted according to a mean chart where the vertical axis represents the endpoint concentration (e.g., IC_{50} 72 h), and the horizontal axis represents the test date or test number (Figure 1). With a sufficiently large data set (i.e. more than 20 data points) the chart can be used to assess the validity of results from subsequent tests with that reference toxicant. If the IC_{50} for a recently completed test does not fall within the $\pm 2SD$ range of the mean, it is highly probable that the test is unacceptable. It may indicate a change in test organism health or genetic sensitivity, a procedural inconsistency, or a combination of these factors. In this situation the test should be repeated with all aspects of the test being carefully scrutinized (Environment Canada, 1990).

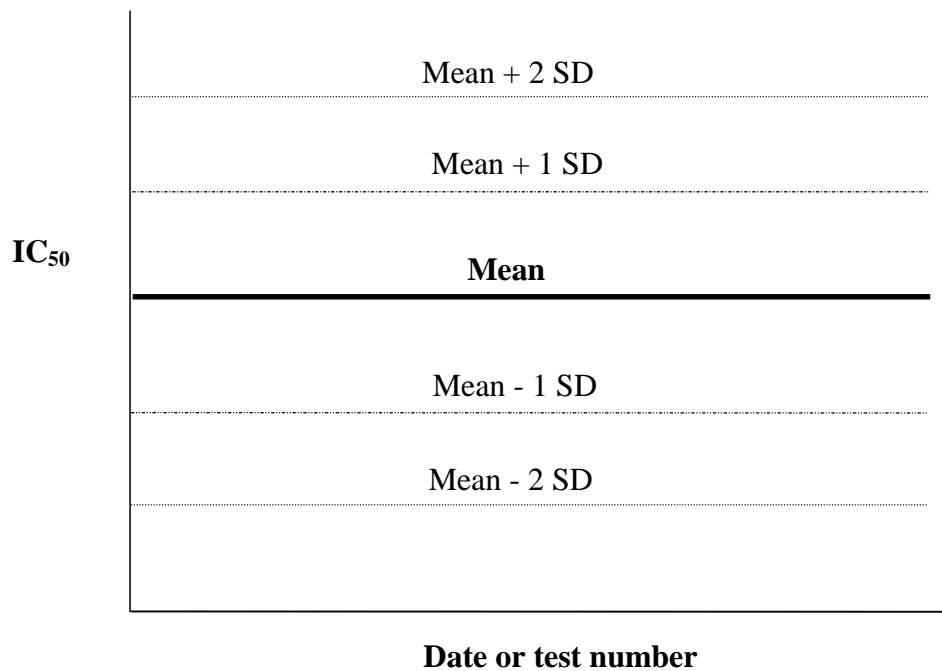


Figure 2: Analytical quality control chart with mean \pm 2 standard deviations (taken from Environment Canada, 1990)

3.6.2.1 Preparation of ZnSO₄ (1 mg/L) Solution

To be used in:

- tube 1 of the reference toxicant dilution series.

Place 1 mL of the 100 mg/L ZnSO₄ stock solution in a 100 mL volumetric flask and complete to volume with oceanic water (26 ppt). Filter sterilize into a 100 mL glass bottle using a 0.2 μ m sterile. This is the 1 mg/L reference toxicant stock solution. This stock solution may be kept for 5 days if held at 4 °C in the dark.

3.6.2.2 Preparation of Reference Toxicant Dilution Series

A geometric series of test concentrations is recommended. The sterile oceanic water (26 ppt) routinely used in the algal toxicity tests should be used as the control/dilution water in tests with the reference toxicant.

1. In a sterile environment, set up a row of 10 sterile 10 mL disposable centrifuge tubes.
2. Add 10 mL of Zn stock solution at 1 mg/L into tube 1.

3. Add 5 mL of oceanic water (26 ppt) into tubes 2 - 10.
4. Complete a serial dilution of 50% to give the following concentrations: 1, 0.5, 0.25, 0.15, 0.0625, 0.0313, 0.015, 0.0078, 0.0039, 0.002 mg/L. Shake the tubes well between dilution's to ensure a homogenous sample with each transfer.

3.6.3 Preparation of Hypernutrient Solution (HPN)

To be used for:

- addition to algal culture to provide nutrient requirements for growth.

This is made using stock solutions from the F/2 marine algae culture medium (section 2.3.3 - Table 1)

1. To a 100 mL volumetric flask containing approximately 80 mL of oceanic water (35 ppt), add:
 - 900 μ L Macronutrient Solution 5 (NaNO₃)
 - 200 μ L Macronutrient Solution 6 (NaH₂PO₄·2H₂O)

- 1.375 mL Vitamin Solution 4
2. Add 190 μ L of Trace Metal Solution prepared exactly the same as Solution 2 (section 2.3.3) but without any EDTA and using FeSO_4 instead of FeCl_3 . Adjust the stock solution to a pH of 2. EDTA reduces algal sensitivity to some heavy metals due to its chelating properties and for this reason is excluded from solution 2 for the use of preparing the HPN solution in the toxicity test stage
 3. Mix well after each addition. And complete to volume with oceanic water (35 ppt).
 4. Filter sterilize using a 0.2 μ m sterile filter into a sterile glass bottle.

Note:

All reagents must be at room temperature prior to all testing.

3.7 Preparation of Test Solutions

3.7.1 Collection and Handling of Effluent and Leachates

Aqueous samples must be collected in a manner that ensures that they adequately reflect the true nature of the effluent or leachates. Generally, a sample volume of 1 L is sufficient for testing. The containers for transport and storage should be new or thoroughly cleaned (section 3.5). Rinse the container with the sample prior to filling. Fill to the brim to minimise headspace to prevent volatiles escaping into the air 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. Transport the sample in a chilled but unfrozen condition by placing the container on ice in a chillybin.

Once the sample has reached the laboratory, the sample should be stored in the dark and at 4 °C. Samples should be tested as soon as possible i.e. within 36 h of the last sample being collected and must be tested within 72 h of collection (USEPA, 1993). The temperature, DO, pH and salinity of the

sample must be recorded before testing commences.

It may be desirable to conduct chemical analyses of the sample or measure total suspended solids and total settled solids in effluents characterized with appreciable amounts. Removal of these fractions of the effluent could influence the results of the toxicity tests.

All safety precautions associated with effluent and leachates must be taken when handling and working with these samples. This includes wearing gloves, a laboratory coat and safety glasses and using a face mask or fume hood if the sample is particularly volatile. Any of the test solution that comes in contact with the skin should be washed off immediately.

3.7.2 Preparation of Effluent Test Solution

Agitate the sample thoroughly to ensure homogeneity. Sub-samples of the effluent (i.e., a sample divided between two or more containers) must be mixed together to ensure their homogeneity.

The pH of the sample should be between 6 and 9. If it does not read within these limits, adjust by using either NaOH or HCl solutions. Adjust to 6.5 or 8.5, whichever is closest to the initial pH of the sample (USEPA, 1993). A pH adjusted test may have to be performed concurrently. If the DO level in the undiluted sample is < 4.0 mg/L the sample should be aerated for a few minutes until the DO level is at an acceptable level (USEPA, 1993).

Remove a subsample of approximately 20 mL and filter through a pre-rinsed 0.45 μ m sterile filter, into a 50 mL sterile centrifuge tube. This will remove most of the bacteria that could grow and interfere with algal growth.

3.7.3 Preparation of Test Solution Dilution Series

Ideally, a test should include a concentration that has no effect on algal growth, and two concentrations each above and below the IC_{50} value. If the toxicity of a substance to *D.*

tertiolecta is known, prepare concentrations of the test solutions to encompass a response range that includes no inhibition of growth (NOEC) and no growth (IC₉₀-IC₁₀₀). A geometric series is recommended. If the toxicity of a sample is unknown, a preliminary range-finding test can be used.

1. Set up a row of 10 sterile 10 mL disposable centrifuge tubes.
2. Add 3.2 mL of the test sample and 6.8 mL of sterile diluent water (35 ppt) or oceanic water (35 ppt) if a diluent water is not provided. This results in an initial concentration of 32% at 26 ppt in tube 1.
3. Add 5 mL of sterile diluent water (26 ppt) or oceanic water (26 ppt) if a diluent water is not provided into tubes 2 - 10.
4. Complete a serial dilution of 50% starting at tube 1 to give you the following concentrations: 32%, 16%, 8%, 4%, 2%, 1%, 0.5%, 0.25%, 0.125% and 0.0625%. If the sample is suspected to be highly toxic then a range of lower concentrations should be included as well as the general range of concentrations.

Note:

You may also use a 33% serial dilution and/or adjust your initial concentration depending on the toxicity of your test sample and salinity.

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.

3.8 Preparation of Algal Inoculum

The algal inoculum must be prepared no more than 2 - 3 hours before incubation of the microplate. The inoculum is composed of *D. tertiolecta* cells harvested from a liquid stock algal culture that is 3 to 5 days old and in an exponential phase of growth. The initial cell density for the microplate algal growth inhibition test should be 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.

The use of an electronic particle counter allows a rapid determination of the cell concentration (cells/mL). The counter must be calibrated according to standard operating procedures before use. If a Coulter counter model Z1 is used, the settings are:

- upper size limit 8.0 µm
- lower size limit 3.7 µm
- metered volume 0.5 mL
- aperture size 100 µm

An alternative to using an electronic particle counter is to count the cells using a haemocytometer slide under a microscope (400 X magnification).

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

1. Take 20 mL of algal culture into a sterile 50 mL centrifuge tube.
2. Visually check the stock algal culture for clumping and impurities such as other algae species using the microscope (400X magnification).
3. Count a sample of isotonic solution designed for the particle counter to be used (e.g., Isoton II coulter balance electrolyte solution, if a coulter counter model Z1 is used) as a blank control until the readings appear stable.
4. Take 1.0 mL of algal culture and dilute it with 9.0 mL isotonic solution in a counter sample cup. Determine cell concentration using the coulter counter at the appropriate settings.

5. Calculate cell concentration (cells/mL)

$$\text{i.e. cells/mL} = \frac{\text{mean algal count} - \text{mean blank count}}{\text{amount of algae (1.0 mL)}} \times \frac{\text{total volume of sample (10 mL)}}{\text{volume sampled by particle counter (0.5 mL)}}$$

6. Calculate dilution of culture required to get a concentration of $220,000 \pm 22,000$ cells/mL. Do this by dividing the initial concentration of algae (cell/mL) by 220,000 to find the dilution factor (DF).

e.g., if the initial cell concentration is

1,000,000 cells/mL

$$\text{DF} = 1,000,000 \div 220,000 = 4.55$$

Subtract 1.0 from the DF to find the amount of oceanic water (35 ppt) that must be added to every 1.0 mL of the initial stock culture of algae to get 220,000 cells/mL, e.g., Add 3.55 mL oceanic water to every 1.0 mL of initial algal stock culture.

7. Confirm cell counts to ensure inoculum culture contains 220,000 cells/mL by using the particle counter as previously described. Cell concentration should be $220,000 \text{ cells/mL} \pm 22,000$.
8. If the concentration is not correct, recalculate the DF and adjust using oceanic sea water (35 ppt) until correct.
9. Combine 1:1 volumes of algal inoculum solution (220,000 cells/mL) and the sterile HPN solution into a sterile 50 mL centrifuge tube, i.e., 5 mLs of algae plus 5 mLs of HPN. This results in a final concentration of $110,000 \text{ cells/mL} \pm 11,000 \text{ cells/mL}$, which is the algae concentration necessary to conduct the test. Twenty μL of this inoculum will contain 2,200 algae cells, resulting in a final concentration of 10,000 cells/mL in the microplate wells, as required at test initiation.

4.0 Test Procedures

4.1 Summary of Test Procedure

Table 4: Summary of Recommended Test Conditions for the 72 h *D. tertiolecta* Toxicity Test

Test Parameter	Test Condition
Test Organism:	<i>Dunaliella tertiolecta</i> (marine algae)
Source:	CSIRO culture strain CS-175
Test Type:	Static non-renewal 72h duration
Temperature:	20 ± 2 °C
Light intensity/quality:	Continuous overhead “cool-white” fluorescent illumination PAR should be approximately 200 μmol m ⁻² s ⁻¹
Test chambers:	96 well polystyrene non tissue cultured microplate, round bottomed
Test solution volume:	200 μL per well
Dilution water:	Sea water or receiving water (26ppt) filtered through 0.2μm sterile filter
Renewal of test concentrations:	None
Age of test organisms:	3 - 5 days old in exponential growth phase
Number of test organisms per chamber:	10,000 cells/mL in each plate well
Number of replicate chambers per treatment:	4 per treatment, 10 per control
Aeration:	None
Observation:	Algal cell concentration after 72h
Effect:	Growth
Endpoint:	IC ₅₀
Test acceptability criteria:	Algal growth in controls must have increased by a factor of 16 and CV% in the controls must be less than 20%

4.2 Preparation for the test

exponential growth phase at the start of the test (3-5 days old).

Note:

All steps must be undertaken in sterile conditions.

3. Prepare all glassware required as outlined in section 3.5.

Three days before test starts

1. Prepare sterile oceanic sea water and dilution water as outlined in section 3.6.1.
2. Prepare algal stock culture as outlined in section 2.3.2. This is done on a weekly basis and the culture should be in an

T₀ i.e., day test commences

1. Prepare reference toxicant (ZnSO₄) as outlined in section 3.6.2.1.
2. Prepare HPN solution as outlined in section 3.6.3.
3. Prepare test solution as outlined in section 3.7.2.

4.3 Beginning the test

1. Prepare reference toxicant dilution series as outlined in section 3.6.2.2.
2. Prepare test solution dilution series as outlined in section 3.7.3.
3. Prepare algal inoculum as outlined in section 3.8.
4. An algal control plate, a reference toxicant plate and a test plate must all be clearly labeled with the type of plate, job number and start date of the test.
5. Add 200 µL of sterile oceanic sea water (26 ppt) from a plastic reagent reservoir to each of the 36 peripheral wells of a microplate. These wells are not inoculated with any algae or toxicants. Use a multichannel pipette for pipetting into microplate wells.

4.3.1 Inoculating control plate

1. Pipette 200 µL of sterile oceanic water (26 ppt) into each of the wells on rows B, C and D.
2. Pipette 200 µL of sterile dilution water (26 ppt) into each of the wells on rows E, F and G. If a dilution water is not provided inoculate all wells with sterile oceanic water (26 ppt).
3. Add 20 µL of algal inoculum to each of the wells except for the 36 peripheral wells.

4.3.2 Inoculating reference toxicant plate

1. Pipette 200 µL of sterile oceanic water (26 ppt) into each of the wells in row D.
2. Begin with the lowest concentration in the dilution series and pipette 200 µL into the appropriate microplate wells beginning at row 11 (figure 2). Take care not to contaminate other wells of the microplate plate. A single plastic reservoir may be used for all concentrations provided that the lowest concentration is dispensed first with a progression towards the highest concentration, and that the reservoir is adequately emptied between each addition of test solution.
3. Add 20 µL of algal inoculum to each of the wells except for the 36 peripheral wells. Start with the control rows and lowest concentration wells first. The algal inoculum should be kept homogenous within the sampling reservoir during the inoculation steps.

4.3.3 Inoculating the test plate

1. Pipette 200 µL of sterile oceanic water (26 ppt) into each of the wells in row D (Control row).
2. If a diluent is used, pipette 200 µL of sterile diluent water (26 ppt) into each of the wells in row E.
3. Begin with the lowest concentration of the sample dilution series and pipette 200 µL into the appropriate microplate wells beginning at row 11 (figure 2). Take care not to contaminate other wells of the microplate. A single plastic reservoir may be used for all concentrations provided that the lowest concentration is dispensed first with a progression towards the highest concentration, and that the reservoir is adequately emptied between each addition of test solution.
4. Add 20 µL of algae/HPN inoculum to each of the wells except for the 36 peripheral wells. Start with the control rows and lowest concentration wells first. The algal inoculum must be kept homogenous within

the sampling reservoir during the inoculation steps.

Record the time of algal inoculation as this is the official start time for the test. Record this on Form 1 (Appendix 9.2).

4.4 During the Test

Ensure that each microplate has a lid and is sealed individually in a transparent plastic bag. This minimizes evaporation during the exposure period and prevents volatile substances contaminating other microplates.

Incubate all microplates in an environmental chamber for $72 \text{ h} \pm 2 \text{ h}$. This chamber must meet the test conditions as outlined in section 3.3 (Table 5).

4.5 Ending the Test and Recording Data and Observations (72 h)

1. Remove the microplates from their plastic bags.
2. Record whether condensation is present on the lid or in the bag and record its location (Form 2, Appendix 9.2). Place the microplate on a white background and visually examine the plate for algal growth in the test treatments. Algal growth is indicated by green colour. The presence of a white growth could indicate dead algal cells or bacterial growth during incubation and should also be recorded (Form 2, Appendix 9.2).

4.5.1 Measuring cell concentration in the wells

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 a 200 μL pipette and expel the contents back into the wells. Repeat at least 5 times.

Withdraw 200 μL from each well and dispense into individual particle counter

sample cups each containing 9.8 mL of isotonic diluent.

Enumerate algae from the control, reference toxicant and test plate(s) using the particle counter and record results. Alternatively the algal cells can be counted using a microscope and haemocytometer. Results are tabulated according to the microplate configuration used in the test (Form 3, Appendix 9.2). Each sample should be counted two to three times, and the mean of the results from the two or three counts should be calculated prior to statistical analysis.

5.0 Acceptability of Test Data

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 inoculum culture must have reached at least 2×10^6 cells/mL by the time of test initiation.
- Algal growth in the control row of the test plate must not differ significantly ($p < 0.05$) from that in the quality control microplate. Significant differences suggest a problem with volatility or with the experimental procedures.
- The number of algal cells in the standard controls must have increased by a factor > 16 in 72 h.
- Culture sensitivity assessment with a reference toxicant must satisfy the criteria for acceptability.
- The coefficient of variation for the control replicate should be $< 20\%$ (USEPA, 1989).

If the conditions of validity are not satisfied, the reasons why should be investigated and the test should be repeated.

6.0 Data Analysis

6.1 Test Endpoints and calculations

The endpoint of the microplate algal toxicity test with *D. tertiolecta* is expressed as an IC_{50} . This is an estimate of the sample concentration causing a 50% reduction in the growth of the algal population compared to a control.

Various computer programs for calculating the IC_{50} and confidence limits are available. The software used by NIWA is ToxcalcTM version 5.0 from Tidepool Scientific Software (1994). This software is used to produce a database for all toxicity test results and offers a full suite of parametric and non parametric statistical methods of analysis that meet with standards required by United States Environmental Protection Agency. The procedure of which statistical test to use is outlined in the flow chart (figure 3).

Once the raw data has been entered as the mean cell count per replicate after 72 h, the readings taken from blank samples (i.e. isotonic solution only) are subtracted and cell enumeration is calculated as cells/mL. Hypothesis testing is then used to detect statistically significant differences between treatments. This requires that the basic assumptions of hypothesis testing, i.e. that the observations within treatments are independent and normally distributed and that the variance is homogenous across all concentrations and controls, be validated. Normality is tested using the Shapiro-Wilks test and homogeneity of variances is tested with the Bartlett's test. If these two assumptions are violated then the data must be transformed using an $\log x+1$ transformation and the assumptions tested again. If the data pass the normality and homoscedasticity tests, a parametric multiple comparisons test, e.g., Dunnett's test, can be applied. If the data fail the normality and homoscedasticity tests with original and transformed data then a non-parametric method such as Steel's Many One Rank test or Wilcoxon Rank Sum test should be performed. A one tailed hypothesis test with

$P > 0.05$ is conducted. From this, the no observed effect concentration (NOEC) and lowest observed effect concentration (LOEC) can be calculated.

Toxcalc will automatically conduct a two tailed t-test when testing for significant differences between the algal growth in the control plate (B-control, Figure 3) and the control row in the sample or reference plate (D-control, Figure 3). If no significant difference occurs between the two types of control, the algal counts from the control row on the sample or reference plate (D-control) should be used for comparison with algal growth in the toxicant solutions.

A dose response curve should be constructed to provide a graphical interpretation of the toxicant effect and assist in deriving the endpoint. A typical dose response curve is shown in Appendix 9.3. When a stimulation response in algal growth is observed, the dose response curve shows a positive relationship with increasing toxicant concentration. This shifts the concentration of the IC_{50} and can make it ambiguous for interpretation. This stimulation response must be noted in the report.

The IC_{50} is calculated using the linear interpolation method which assumes a linear response from one concentration to the next and provides confidence limits of this linear relationship. It also assumes that the data are monotonically decreasing whereby the mean response for each higher concentration is less than or equal to the mean response from the previous concentration. If this is not the case then the data must be smoothed by pooling adjacent means and the confidence limits calculated using bootstrapping techniques. An example of the standard printout from ToxcalcTM is given in Appendix 9.3.

NOEC and LOEC concentrations are based on hypothesis testing of the organisms response at the concentrations used in the toxicity test. Therefore an *a priori* determinant of the NOEC and LOEC is the experimenter's

choice of test concentrations (Grothe *et al.*, 1996). Caution must be exercised when using NOEC and LOEC values and they must be viewed in conjunction with another endpoint e.g. IC₁₀.

To identify the degree of test variability, the minimum significant difference (MSD), or amount of effect “allowable” at the NOEC, has been introduced by USEPA (USEPA, 1995, Grothe *et al.*, 1996). The MSD is a measure of the within-test variability and represents the amount of difference from the control that can be detected significantly. It incorporates a level of significance (e.g. $\alpha = 0.05$), number of experimental units, as well as an estimate of test variability (within-test mean square error). The MSD is often expressed as a percentage of the effect in the control response ($\%MSD = MSD / \text{control mean} \times 100$) (Grothe *et al.*, 1996). Toxcalc^{TN} calculates the MSD in transformed units if the data has been transformed (Appendix 9.4). It also calculates the MSD in untransformed units (MSDu) and as a percentage of the control response (MSDp).

The MSD should be presented with the endpoint and calculated as a proportion of the mean control response.

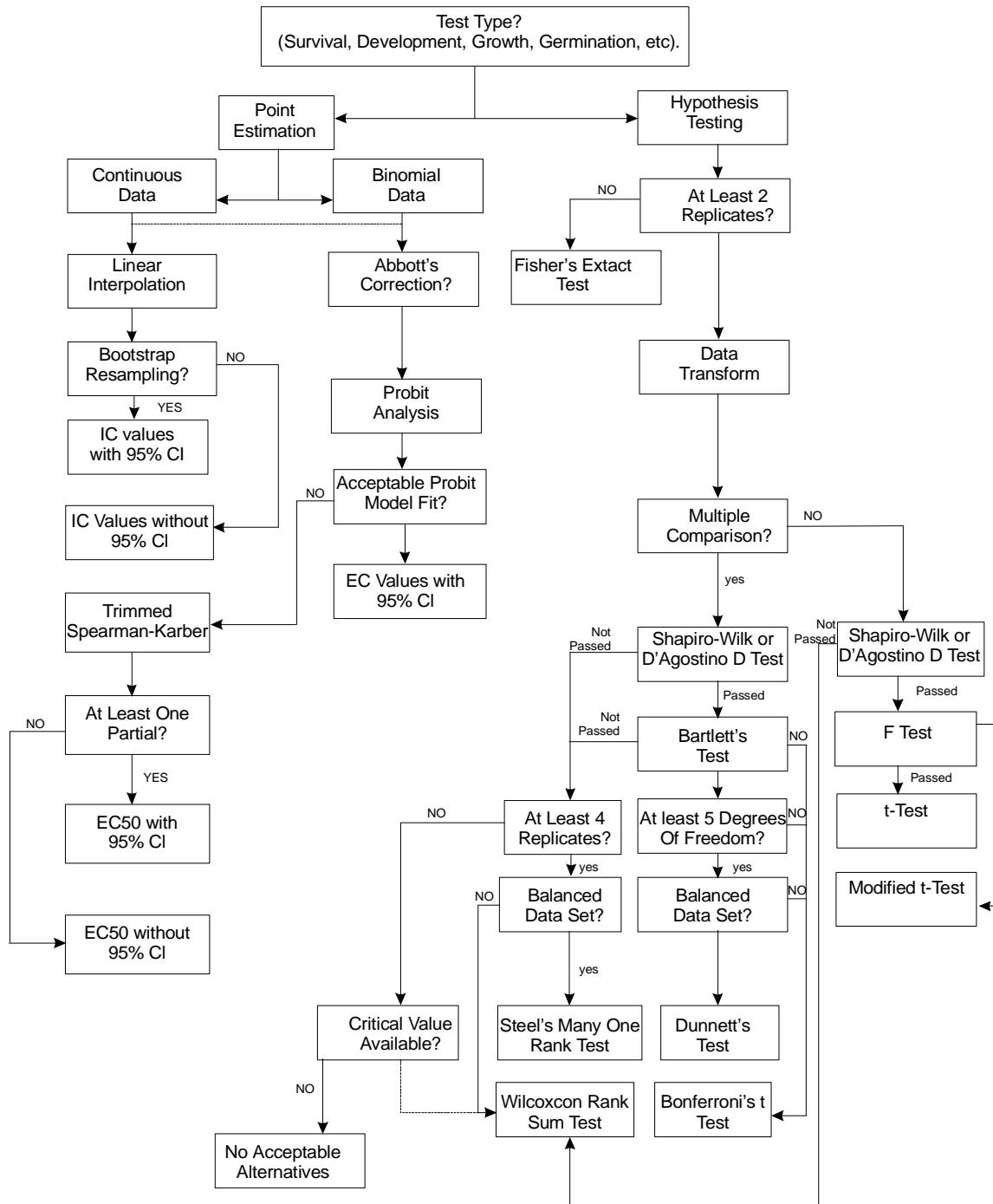


Figure 3: Flow diagram of USEPA approved statistical methods performed by Toxcalc^{TN}. (Tidepool, 1994).

7.0 Reporting of Results

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

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

The following should be included in the report:

7.1 Test Material

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

7.2 Test Organisms

- species and source;
- composition of growth medium;
- culturing apparatus and incubation procedure;
- light intensity; and
- temperature.

7.3 Test Facilities and Apparatus

- name and address of test laboratory;
- name of person(s) performing each stage of the sample handling and testing;
- description of culture facilities, including light, aeration and temperature regulating systems; and
- description of testing containers.

7.4 Control/Dilution Water

- type(s) and source(s) of water used as control and dilution water;
- measured water quality variable before and/or at the time of commencement of toxicity test;
- type and quantity of any chemical(s) added to the control/dilution water;
- sampling location and storage details if the control/dilution water was receiving water from an area not affected by the effluent or leachate discharge; and
- water pre-treatment (adjustment of temperature, pH, DO and salinity).

7.5 Test Method

- if a standard method is used, cite the document;
- describe procedure if modifications or changes to specific experimental design occur;
- method of preparing and storing stock and test solution(s);

- description of pH or salinity adjustment procedure, if applicable;
- any chemical and physical analyses of test solutions and reference to analytical method(s) used;
- composition of the test medium;
- use of preliminary or range-finding test; and
- method for measuring cell concentration.

7.6 Test Conditions

- date, times, and duration of tests;
- concentrations tested;
- number of concentrations and volume of test solutions including controls, number of replicates per treatment;
- initial cell density of the inoculum
- number of algal cells per solution;
- photoperiod, light source, and intensity at surface of test solutions;
- description of any test solutions receiving pH or salinity adjustment, including procedure and timing;
- any chemical measurements on test solutions (e.g., chemical concentration, suspended solids content);
- temperature, pH, dissolved oxygen (mg/L and % saturation) and salinity as measured/monitored in each test solution; and
- conditions and procedures for measuring the 72h IC₅₀ of the reference toxicant(s).

7.7 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;
- report a stimulation of algal growth response (section 6.1) or any unusual growth patterns;
- 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);

- report the MSD value as a proportion of the control for untransformed data for any analyses done;
- 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;
- if the IC₅₀ is greater than the highest concentration tested it should be reported as > X% test substance where X is the concentration tested; and
- anything unusual about the test, any problems encountered and remedial measures taken.

8.0 References

- APHA et al., (1989). "Toxicity Test Methods for Aquatic Organisms", in: Standard Methods for the Examination of Water and Wastewater, 17th ed., American Public Health Association, American Water Works Association, and Water Pollution Control Federation, Washington, DC, Part 8000, p 8-1 to 8-143.
- Blaise, C. (1986). "Micromethod for acute aquatic toxicity assessment using the green alga *Selenastrum capricornutum*," *Tox Assess.*, 1 : 377 - 385.
- Blaise, C. (1991). "Microbiotests in aquatic ecotoxicity: Characteristics, Utility and Prospects," *Environ - Toxicol. Water Quality*, 6 : 145 - 155.
- Environment Canada. (1990). Guidance document on control of toxicity test precision using reference toxicants. Environmental Protection Conservation and Protection Environment Canada, Report EPS 1/RM/12, August 1990.
- Environment Canada. (1992). Biological test method: Growth inhibition test using the freshwater alga *Selenastrum capricornutum*. Environmental Protection Conservation and Protection Environment Canada, Report EPS 3/HA/1.
- Grothe, D.R.; Dickson, K.L.; Reed-Judkins, D.K. editors. (1996). Whole effluent toxicity testing: an evaluation of methods and prediction of receiving system impacts. Society of Environmental Toxicology and Chemistry (SETAC) Special Publication Series, Pensacola, Florida.
- Guillard, R.R.L. (1975) Culture of phytoplankton for feeding marine invertebrates. In "Culture of Marine Invertebrate Animals". Editors Smith, W.L. and Chanley M.H. Plenum Press, New York, USA. pp26-60.
- Guillard, R.R.L. Ryther, J.H., (1962) Studies of marine planktonic diatoms. *I. Cyclotella nana* Hustedt and *Detonula confervacea*. *Cleve. Can. J. Microbiol.* 8:229-239.
- Miller, W.E.; Greene, J.C.; Shiroyama, T. (1978). "The *Selenastrum capricornutum* Printz Algal Assay Bottle Test," United States Environmental Protection Agency-600/9-78-018, Corvallis, Oregon, p 126.
- St-Laurent, D.; Blaise, C.; MacQuarrie, P.; Scroggins, R.; Trottier, B. (1992). Comparative assessment of herbicide phytotoxicity to *Selenastrum capricornutum* using microplate and flask bioassay procedures. *Environ. Toxicol. Water Quality*, 7:35-48.
- Thellen, C.; Blaise, C.; Roy, Y.; Hickey, C. (1989). "Round Robin Testing with *Selenastrum capricornutum* Microplate Toxicity Assay," *Hydrobiologia*, 188/189: 259-268.
- Tidepool Scientific Software. (1994). Toxcalc user's guide. Comprehensive toxicity data analysis and database software version 5.0.
- USEPA. (1987). *Methods for toxicity tests of single substances and liquid complex wastes with marine unicellular algae*. US Environmental Protection Agency. Cincinnati, Ohio. EPA-600-8/87/043.
- USEPA. (1989). "Algal (*Selenastrum capricornutum*) Growth Test," in: *Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms*, Environmental Monitoring Systems Laboratory, Environmental Protection Agency, Cincinnati, Ohio, 147-174.
- USEPA. (1993). *Methods for measuring the acute toxicity of effluents and receiving waters to freshwater and marine organisms*. Fourth edition. EPA/600/4-90/027F.

USEPA. (1995). Short-term methods for estimating the chronic toxicity of effluent and receiving waters to west coast marine and estuarine organisms. EPA/600/R-95/136.



9.0 Appendices

Appendix 9.1 - Preparation of Bacterial Nutrient Agar

Add 11.5 g of Nutrient Agar (DIFCO Bacto Nutrient Agar dehydrated) to a 500mL glass bottle containing 500mL of F/2 liquid growth media. Heat to dissolve completely and autoclave at 121 °C for 15 min. Allow to cool to 46 °C and under aseptic sterile conditions (i.e. laminar flow hood) pour into sterile petri dishes. Allow plates to set. Plates can be stored in a closed container or bag at 4°C for 2 weeks.

Appendix 9.2 - Report Forms

Job Number: _____

Page: ___ of ____

Form 1.

Microplate Test

Species: _____

Project # _____

Sample type and number: _____

Dilution water: _____

Date and time of initiation: _____

Date and time of termination: _____

Operators: _____

Calculations:

1. Initial algal concentration (1.00 ml sample, 9.0 ml isoton)

Counts _____

Mean _____

Cells/mL = mean x 20

2. Dilution factor = cells/mL / 220,000 =

3. Check of cell concentration (1 ml sample, 9 ml isoton)

Counts _____

mean _____

Cells/mL = mean x 20=

Operator Signature _____

Date _____

Job Number: _____

Page: ___ of ___

Form 2.

Microplate Test

Visual Observations of wells

Type of Plate: _____

G = green growth representing algal growth

C = condensation

W = white growth (probably bacterial) O = others (specify)

Operator Signature _____

Date _____

Job Number: _____
 Page: __ of _____

Form 3

Microplate Test

Type of Plate _____

ROW 2	3	4	5	6	7	8	9	10	11
B									
C									
D									
E									
F									
G									

Cells / mL = mean count x 100
 Blank =

Operator Signature _____

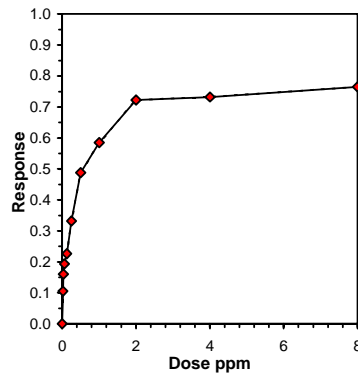
Date _____

Appendix 9.3 - Example of Toxcalc^{TN} results

Phytoplankton Test-cells/ml														
Start Date:	9/12/97	Test ID:	AQC395	Sample ID:	REF-Ref Toxicant									
End Date:	12/12/97	Lab ID:	PM	Sample Type:	ZNSO-Zinc sulfate									
Sample Date:	9/12/97	Protocol:	NIWA	Test Species:	DUNALIELLA									
Comments: B-control is control plate, D-control is the control row on the reference plate														
Conc-ppm	1	2	3	4	5	6	7	8	9	10				
B-Control	340600	374850	371750	367350	268600	363650	286100	301250	360850	357500				
D-Control	194300	260600	221250	247450	220300	226050	248750	220550	203050	206000				
0.016	198600	206000	232550	167850										
0.032	200800	181050	181600	191550										
0.063	193350	174850	191200	165250										
0.125	166300	177650	175750	175150										
0.25	160250	153650	150150	136250										
0.5	118650	138550	97800	105400										
1	96950	95950	94900	85400										
2	64150	61100	57250	66400										
4	61000	56950	61250	62250										
8	56750	45450	48650	60500										
Transform: Log (X + 1)														
Conc-ppm	Mean	N-Mean	Mean	Min	Max	CV%	N	t-Stat	1-Tailed Critical	MSD	Isotonic Mean	N-Mean		
B-Control	339250	1.5089	5.5277	5.4291	5.5739	0.956	10							
D-Control	224830	1.0000	5.3501	5.2885	5.4160	0.772	10				224830	1.0000		
0.016	201250	0.8951	5.3008	5.2249	5.3665	1.103	4	2.096	2.708	0.0636	201250	0.8951		
*0.032	188750	0.8395	5.2755	5.2578	5.3028	0.405	4	3.174	2.708	0.0636	188750	0.8395		
*0.063	181162.5	0.8058	5.2572	5.2181	5.2863	0.619	4	3.954	2.708	0.0636	181163	0.8058		
*0.125	173712.5	0.7726	5.2397	5.2209	5.2496	0.244	4	4.698	2.708	0.0636	173713	0.7726		
*0.25	150075	0.6675	5.1756	5.1343	5.2048	0.577	4	7.427	2.708	0.0636	150075	0.6675		
*0.5	115100	0.5119	5.0573	4.9903	5.1416	1.305	4	12.461	2.708	0.0636	115100	0.5119		
*1	93300	0.4150	4.9693	4.9315	4.9866	0.514	4	16.204	2.708	0.0636	93300	0.4150		
*2	62225	0.2768	4.7933	4.7578	4.8222	0.583	4	23.695	2.708	0.0636	62225	0.2768		
*4	60362.5	0.2685	4.7805	4.7555	4.7941	0.358	4	24.239	2.708	0.0636	60362.5	0.2685		
*8	52837.5	0.2350	4.7201	4.6575	4.7818	1.220	4	26.811	2.708	0.0636	52837.5	0.2350		
Auxiliary Tests								Statistic	Critical	Skew	Kurt			
Shapiro-Wilk's Test indicates normal distribution (p > 0.01)								0.97861	0.93	0.03385	-0.04297			
Bartlett's Test indicates equal variances (p = 0.20)								13.4004	23.2093					
The control means are significantly different (p = 1.27E-07)								8.37377	2.10092					
Hypothesis Test (1-tail, 0.05)														
			NOEC	LOEC	ChV	TU	MSDu	MSDp	MSB	MSE	F-Prob	df		
Bonferroni t Test			0.016	0.032	0.02263		30516.1	0.13629	0.25349	0.00158	1.8E-28	10, 39		

Linear Interpolation (80 Resamples)				
Point	ppm	SD	95% CL(Exp)	Skew
IC05*	0.0076	0.0045	0.0036	0.0240
IC10*	0.0153	0.0054	0.0072	0.0287
IC15	0.0290	0.0139	0.0090	0.0853
IC20	0.0738	0.0305	0.0181	0.1543
IC25	0.1519	0.0297	0.0363	0.2116
IC40	0.3585	0.0476	0.2540	0.5015
IC50	0.5616	0.1017	0.3962	0.8023

* indicates IC estimate less than the lowest concentration



Dose-Response Plot

