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Method Development and Applications Section
Environmental Technology Centre
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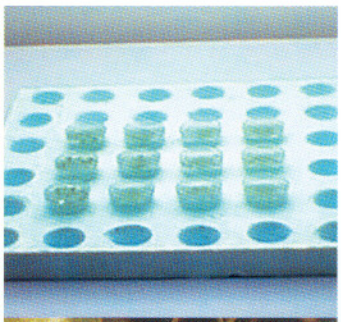

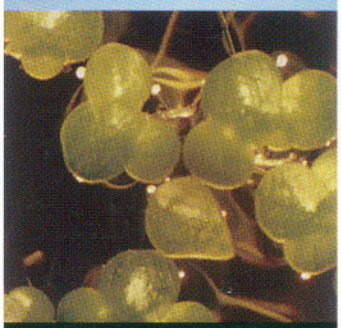

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Biological Test Method: Test for Measuring the Inhibition of Growth Using the Freshwater Macrophyte, *Lemna minor*

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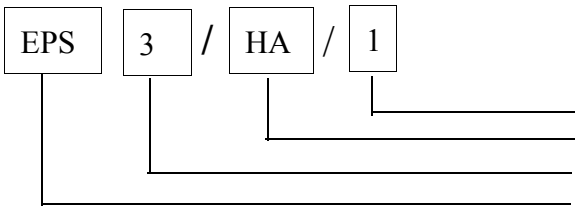

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PF Paper and Fibres
PG Power Generation
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TX Textiles
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Biological Test Method:
Test for Measuring the Inhibition of Growth Using the Freshwater Macrophyte, *Lemna minor*

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Environmental Technology Centre
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Abstract

A biological test method recommended by Environment Canada for performing toxicity tests that measure the inhibition of growth using the aquatic macrophyte, Lemna minor*, is described in this report. This second edition of EPS 1/RM/37, published in 2007 supersedes the first edition that was published in 1999. It includes numerous procedural modifications as well as updated guidance and instructions to assist in performing the biological test method.*

The test is conducted at 25 ± 2°C in test vessels containing a minimum of 100 mL of test solution and two, 3-frond plants. The test may be run as a multi-concentration assay to determine the threshold of effect, or with only one concentration as a regulatory or pass/fail test. This test uses ≥3 replicated test vessels/treatment for a single-concentration test, and ≥4 replicated test vessels/treatment for a multi-concentration test. A second option for test design in a multi-concentration test includes unequal replicates per treatment (i.e., six per treatment for control(s); four replicates for each of the lowest 3-5 test concentrations; and three replicates for each of the highest 4-5 test concentrations).

The test may be performed either as a static (i.e., no renewal) assay or as a static-renewal toxicity test. The static option is recommended as the standard procedure, whereas the static-renewal option is recommended for test solutions where the concentration of the test substance (or a biologically active component) can be expected to decrease significantly (i.e., >20%) during the test period. If the static-renewal option is chosen, test solutions are replaced at least every three days during the test. The endpoints for the test are frond number and frond dry weight at the end of a 7-day toxicity test.

Procedures are given for culturing L. minor *in the laboratory. General or universal conditions and procedures are outlined for testing a variety of materials or substances for their effects on* Lemna *growth. Additional conditions and procedures are stipulated, which are specific for testing samples of chemical, effluent, elutriate, leachate, or receiving water. Instructions and requirements are included on apparatus, facilities, handling and storing samples, preparing test solutions and initiating tests, specific test conditions, appropriate observations and measurements, endpoints, methods of calculation, validation, and the use of reference toxicants.*

Résumé

Le présent rapport décrit la méthode d'essai biologique recommandée par Environnement Canada pour les essais toxicologiques mesurant l'inhibition de la croissance de la plante macroscopique aquatique Lemna minor. *Cette deuxième édition de la méthode SPE 1/RM/37 remplace la première édition, publiée en 1999. Elle comporte de nombreuses modifications procédurales, de même que des conseils et des instructions à jour concernant la conduite de la méthode d'essai biologique.*

L'essai se déroule à 25 ± 2 °C; les récipients d'essai renferment au moins 100 mL de la solution expérimentale et deux plantes à trois thalles. On peut utiliser des concentrations multiples s'il s'agit de déterminer le seuil à partir duquel s'exerce un effet, ou une seule concentration s'il s'agit d'un essai réglementaire à résultat unique (satisfaisant ou non satisfaisant). Au moins trois récipients d'essai de répétition sont utilisés par traitement pour un essai à une seule concentration, et au moins quatre récipients d'essai de répétition par traitement pour un essai à concentrations multiples. Dans le cas d'un essai à concentrations multiples, on peut également avoir recours à un nombre inégal de répétitions par traitement (soit six par traitement pour le ou les témoins, quatre pour chacune des trois à cinq concentrations les plus basses et trois pour chacune des quatre ou cinq concentrations les plus élevées).

L'essai peut se dérouler dans des conditions statiques (sans renouvellement de la solution d'essai) ou dans des conditions de renouvellement intermittent. On recommande comme mode opératoire normalisé l'essai en conditions statiques, le renouvellement intermittent étant recommandé quand la concentration de la substance d'essai (ou d'un ingrédient biologiquement actif) risque de diminuer notablement (>20 %) au cours de l'essai, auquel cas il faut remplacer les solutions au moins tous les trois jours pendant l'essai. Les paramètres à mesurer sont le nombre de thalles et la masse sèche de ces dernières au terme d'un essai toxicologique de 7 jours.

Le présent document décrit la méthode de culture de L. minor *en laboratoire, de même que les conditions et les modes opératoires généraux ou universels pour mesurer les effets de diverses matières ou substances sur la croissance de cette macrophyte. Le lecteur y trouvera la description des conditions et des modes opératoires propres à la nature des échantillons (produit chimique, effluent, élutriat, lixiviat ou eau réceptrice), de même que des instructions et des exigences sur les éléments suivants : l'appareillage, les installations, la manipulation et l'entreposage des échantillons, la préparation des solutions expérimentales et la mise en route des essais, les conditions précises dans lesquelles se déroulent ces derniers, les observations à faire et les mesures à prendre, les paramètres à mesurer, les méthodes de calcul, la validation des essais, l'emploi de toxiques de référence.*

Foreword

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

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

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

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

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List of Abbreviations and Chemical Formulae

ANOVA . . . . analysis of variance
°C . . . . . degree(s) Celsius
$CaCl_2$ . . . . . calcium chloride
$Ca(NO_3)_2$ . . . calcium nitrate
$CoCl_2$ . . . . . cobalt chloride
$Co(NO_3)_2$ . . . cobalt nitrate
cm . . . . . centimetre(s)
$CuCl_2$ . . . . . copper chloride
$CuSO_4$ . . . . . copper sulphate
CV . . . . . coefficient of variation
d . . . . . day(s)
EC50 . . . . . median effective concentration
EDTA . . . . . ethylenediamine tetraacetic acid ($C_{10}H_{16}O_8N_2$)
$FeCl_3$ . . . . . ferric chloride
g . . . . . gram(s)
g/kg . . . . . gram(s) per kilogram
g/L . . . . . gram(s) per litre
h . . . . . hour(s)
H_3BO_3 . . . . . boric acid
HCl . . . . . hydrochloric acid
H_2O . . . . . water
%I . . . . . percent growth inhibition
ICp . . . . . inhibiting concentration for a (specified) percent effect
ID . . . . . inside diameter
KCl . . . . . potassium chloride
kg . . . . . kilogram(s)
KH_2PO_4 . . . . potassium dihydrogen phosphate anhydride
K_2HPO_4 . . . . potassium phosphate
KNO_3 . . . . . potassium nitrate
KOH . . . . . potassium hydroxide
kPa . . . . . kilopascal
L . . . . . litre(s)
LOEC . . . . . lowest-observed-effect concentration
m . . . . . metre(s)
mg . . . . . milligram(s)
$MgCl_2$ . . . . . magnesium chloride
$MgSO_4$ . . . . . magnesium sulphate
min . . . . . minute(s)
mL . . . . . millilitre(s)
mm . . . . . millimetre(s)
mS . . . . . millisiemens

$MnCl_2$ . . . . . manganese chloride
MOPS . . . . . 4-morpholinepropane sulphonic acid
N . . . . . Normal
NaCl . . . . . sodium chloride
Na_2CO_3 . . . . . sodium carbonate
Na_2EDTA . . . disodium ethylenediamine tetraacetic acid ($C_{10}H_{14}N_2O_8 \cdot 2H_2O$)
Na_4EDTA . . . tetrasodium ethylenediamine tetraacetic acid ($C_{10}H_{12}N_2O_8 \cdot 2H_2O$)
$NaHCO_3$ . . . . sodium bicarbonate
Na_2MoO_4 . . . sodium molybdate
$NaNO_3$ . . . . . sodium nitrate
NaOH . . . . . sodium hydroxide
nm . . . . . nanometer
NOEC . . . . . no-observed-effect concentration
SD . . . . . standard deviation
s . . . . . second
spp. . . . . species (plural)
SRC. . . . . Saskatchewan Research Council
TIE . . . . . Toxicity Identification Evaluation
TM (^{TM}) . . . . Trade Mark
µg . . . . . microgram(s)
µm . . . . . micrometre(s)
µmhos/cm . . micromhos per centimetre
µmol/($m^2 \cdot s$) micromole per metre squared per second
UTCC . . . . . University of Toronto Culture Collection
v:v . . . . . volume-to-volume
$ZnCl_2$ . . . . . zinc chloride
$ZnSO_4$ . . . . . zinc sulphate
> . . . . . greater than
< . . . . . less than
≥ . . . . . greater than or equal to
≤ . . . . . less than or equal to
± . . . . . plus or minus
/ . . . . . per, alternatively, “or” (e.g., control/dilution water)
~ . . . . . approximately
≅ . . . . . approximately equal to
% . . . . . percentage or percent
‰ . . . . . parts per thousand

Terminology

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

Grammatical Terms

Must is used to express an absolute requirement.

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

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

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

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

Technical Terms

Acclimation is physiological adjustment to a particular level of one or more environmental factors such as temperature. The term usually refers to the adjustment to controlled laboratory conditions.

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

Biomass is the total dry weight (mass) of a group of plants or animals.

Chlorosis is the loss of chlorophyll (yellowing) in frond tissue.

Clone is a group of individuals reproducing vegetatively (by mitosis) from a single ancestor (i.e., frond).

Colony means an aggregate of mother and daughter fronds (usually 2 to 4) attached to each other. Sometimes referred to as a plant.

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

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 on the solution's temperature. Conductivity is measured at 25°C, and is reported as millisiemens/metre (mS/m), or as micromhos/centimetre (µmhos/cm); 1 mS/m = 10 µmhos/cm.

Culture, as a noun, is the stock of organisms raised in the laboratory under defined and controlled conditions through one or more generations, to produce healthy test organisms. As a verb, it means to carry out the procedure of raising healthy test organisms from one or more generations, under defined and controlled conditions.

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

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

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

Frond is the individual leaf-like structure of a duckweed plant. It is the smallest unit (i.e., individual) capable of reproducing.

Gibbosity means fronds exhibiting a humped or swollen appearance.

Growth is the increase in size or weight as the result of proliferation of new tissues. In this test, it refers to an increase in frond number over the test period as well as the dry weight of fronds at the end of the test.

Growth rate is the rate at which the *biomass* increases.

Lux is a unit of illumination based on units per square metre. One lux = 0.0929 foot-candles and one foot-candle = 10.76 lux. For conversion of lux to quantal flux [$\mu mol/(m^2 \cdot s)$], the spectral quality for the light source must be known. Light conditions or irradiance are properly described in terms of quantal flux (photon fluence rate) in the photosynthetically effective wavelength range of approximately 400 to 700 nm. The relationship between quantal flux and lux or foot-candle is highly variable and depends on the light source, the light meter used, the geometrical arrangement, and the possibilities of reflections (see ASTM, 1995). Approximate conversion between quantal flux and lux, however, for full spectrum fluorescent light, is 1 lux $\cong$ 0.016 $\mu mol/(m^2 \cdot s)$ (Deitzer, 1994; Sager and McFarlane, 1997).

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

Necrosis indicates dead (i.e., with brown or white spots) frond tissue.

Percentage (%) is a concentration expressed in parts per hundred parts. One percentage represents one unit or part of material or substance (e.g., chemical, effluent, elutriate, leachate, or receiving water) diluted with water or medium to a total of 100 parts. 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 substance or 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 indicating increasingly greater acidic reactions, and numbers greater than 7 indicating increasingly basic or alkaline reactions.

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

Precipitation means the formation of a solid (i.e., precipitate) from some or all of the dissolved components of a solution.

Pretreatment means treatment of a sample, or dilution thereof, before exposure of test organisms.

Protocol is an explicit set of procedures for a test, formally agreed upon by the parties involved, and described precisely in a written document.

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

Root is that part of the *Lemna* plant that assumes a root-like structure.

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 bromide and iodide have been replaced by chloride, and all organic matter has been oxidized. Salinity can also be measured directly using a salinity/conductivity meter or other means (see APHA *et al.,* 1989). It is usually reported in grams per kilogram (g/kg) or parts per thousand (‰).

Stock culture is an ongoing laboratory culture of a specific test organism from which individuals are selected and used to set up separate test cultures.

Strain is a variant group within a species maintained in culture, with more or less distinct morphological, physiological, or cultural characteristics.

Subculture is a laboratory culture of a specific test organism that has been prepared from a pre-existing culture, such as the stock culture. As a verb, it means to conduct the procedure of preparing a subculture.

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

Test culture means the culture established from organisms isolated from the stock culture to provide plants for use in a toxicity test. Here, it refers to the 7- to 10-day old *Lemna* cultures maintained in modified Hoagland's medium that are then transferred to control/dilution water for an 18- to 24-h *acclimation* period.

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

Terms for Test Materials or Substances

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

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

Control/dilution water is the water, or in this report, the test medium used for the control treatment, for diluting the test material or substance, or for both.

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

Dilution water is the water, or in this report, the test medium used to dilute a test substance or material to prepare different concentrations for a toxicity test.

Dilution factor is the quotient between two adjacent concentration levels (e.g., 0.32 mg/L ÷ 0.1 mg/L = 3.2 dilution factor).

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

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

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

Material is the *substance* or substances from which something is made. A material would have more or less uniform characteristics. Effluent, leachate, elutriate, or surface water are materials. Usually, the material would contain several or many substances.

Medium is deionized or glass-distilled water (ASTM Type-1 water) to which reagent-grade chemicals have been added. The resultant synthetic fresh water is free from contaminants.

Nutrient-spiked wastewater is a wastewater sample to which the same nutrients that are used to make up the test medium have been added at the same concentrations (e.g., effluent is spiked with the modified APHA nutrient stock solutions A, B, and C, at a ratio of 10 mL of each per 1000 mL of effluent) before test solutions are prepared.

Nutrient-spiked receiving water is a sample of receiving water to which the same nutrients that are used to make up the test medium have been added at the same concentrations (e.g., receiving water that is to be used as control/dilution water for effluent testing is spiked with the modified APHA nutrient stock solutions A, B, and C, at a ratio of 10 mL of each per 1000 mL of receiving water) before test solutions are prepared.

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 immediately "upstream" or up-current from the discharge point). Further descriptive information must be provided to indicate which meaning is intended.

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

Reference toxicity test is a test conducted using a reference toxicant in conjunction with a toxicity test, to appraise the sensitivity of the organisms and/or the precision and reliability of results obtained by the laboratory for that chemical at the time the test material or substance is evaluated. Deviations outside an established normal range indicate that the sensitivity of the test organisms, and the performance and precision of the test, are suspect.

Stock solution is a concentrated aqueous solution of the substance or material to be tested or the chemicals used to prepare growth/test media. Measured volumes of a stock solution are added to dilution water to prepare the required strengths of test solutions or media.

Substance is a particular kind of material having more or less uniform properties. The word substance has a narrower scope than *material*, and might refer to a particular chemical (e.g., an element) or chemical product.

Test medium is the complete synthetic culture medium (in this case modified APHA, SIS, or modified Steinberg medium) that enables the growth of test plants during exposure to the test substance. The test substance will normally be mixed with, or dissolved in, the test medium.

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 particular concentration of prepared test sample. In the case of this test, the test substance/wastewater is dissolved in test medium or spiked upstream receiving water, which is then subjected to testing.

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

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

Statistical and Toxicological Terms

Acute means within a short period of exposure (seconds, minutes, hours, or a few days) in relation to the life span of the test organism. An acute toxic effect would be induced and observable within a short period of time.

Chronic means occurring within a relatively long period of exposure (weeks, months, or years), usually a significant portion of the life span of the organism such as 10% or more. A chronic toxic effect might take a significant portion of the life span to become observable, although it could be induced by an exposure to a toxic substance that was either acute or chronic.

Chronic toxicity implies long-term effects that are usually related to changes in such things as metabolism, growth, reproduction, or ability to survive.

Coefficient of Variation (CV) is the standard deviation (SD) of a set of data divided by the mean of the data-set, expressed as a percentage. It is calculated according to the following formula:

$$CV\ (\%) = 100\ (SD \div mean).$$

EC50 is the *median effective concentration*. It is the concentration of material in water (e.g., mg/L), soil or sediment (e.g., mg/kg) that is estimated to cause a specified toxic effect to 50% of the test organisms. In most instances the EC50 and its 95% confidence limits are statistically derived by analyzing the percentages of organisms showing the specified effect at various test concentrations, after a fixed period of exposure. The duration of exposure must be specified (e.g., 72-h EC50). The EC50 describes quantal effects, lethal or sublethal, and is not applicable to quantitative effects (see *ICp*). Other percentages could be used, see *ECp*.

ECp has the same meaning as *EC50*, except that "p" can represent any percentage, and is to be specified for any particular test or circumstance. Some investigators and agencies, particularly European and international, have mistakenly used ECp to mean ICp, but the distinction is important and should be maintained.

Endpoint means the measurement(s) or value(s) that characterize the results of the test (e.g., ICp). It also means the response of the test organisms that is being measured (e.g., number of fronds or frond dry weight).

Geometric mean is the mean of repeated measurements, calculated on a logarithmic basis. It has the advantage that extreme values do not have as great an influence on the mean as is the case for an arithmetic mean. The *geometric mean* can be calculated as the n^{th} root of the product of the "n" values, and it can also be calculated as the antilogarithm of the mean of the logarithms of the "n" values.

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

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

ICp is the inhibiting concentration for a (specified) percent effect. It represents a point estimate of the concentration of test substance or material that causes a designated percent impairment in a quantitative biological function such as growth. For example, an IC25 could be the concentration estimated to cause fronds to attain a dry weight that is 25% lower than that attained by control fronds at the end of the test. This term should be used for any toxicological test which measures a quantitative effect or change in rate, such as dry weight at test end. (The term EC50 or *median effective concentration* is not appropriate in tests of this kind since it is limited to *quantal* measurements, i.e., number of exposed individuals which show a particular effect.)

LOEC is the lowest-observed-effect concentration. This is the lowest concentration of a test material or substance to which organisms are exposed, that causes adverse effects on the organism which are detected by the observer and are statistically significant. For example, the LOEC might be the lowest test concentration at which the dry weight of exposed organisms at test end differed significantly from that in the control.

NOEC is the no-observed-effect concentration. This is the highest concentration of a test material or substance to which organisms are exposed, that does not cause any observed and statistically significant adverse effects on the organism. For example, the NOEC might be the highest test concentration at which an observed variable such as dry weight or frond number at test end does not differ significantly from that in the control.

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

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

Quantal is an adjective, as in quantal data, quantal test, etc. A quantal effect is one for which each test organism either shows the effect of interest or does not show it. For example, an animal might either live or die, or it might develop normally or abnormally. Quantal effects are typically expressed as numerical counts or percentages thereof.

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

Replicate (*treatment, test vessels*) refers to a single test chamber containing a prescribed number of organisms in either one concentration of test material or substance, or in the control or reference treatment(s). A *replicate* of a treatment must be an independent test unit; therefore any transfer of organisms or test material from one test vessel to another would invalidate a statistical analysis based on replication.

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

Static-renewal describes a toxicity test in which test solutions are renewed (replaced) periodically (e.g., at specific intervals) during the test period. Synonymous terms are batch replacement, renewed static, renewal, intermittent renewal, static replacement, and semi-static.

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

Sublethal (*toxicity*) means detrimental to the organism, but below the concentration or level of contamination that directly causes death within the test period.

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

Toxicant is a toxic substance or material.

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

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

Toxicity test is a determination of the effect of a substance or material on a group of selected organisms (e.g., *Lemna minor*), under defined conditions. An aquatic toxicity test usually measures: (a) the proportions of organisms affected (*quantal*); and/or (b) the degree of effect shown (*quantitative*), after exposure to specific concentrations of chemical, effluent, elutriate, leachate, or receiving water.

Toxicology is a branch of science that studies the toxicity of substances, materials, or conditions. There is no limitation on the use of various scientific disciplines, field or laboratory tools, or studies at various levels of organization, whether molecular, single species, populations, or communities. Applied toxicology would normally have a goal of defining the limits of safety of chemical or other agents.

Treatment is, in general, an intervention or procedure whose effect is to be measured. More specifically, in toxicity testing, it is a condition or procedure applied to the test organisms by an investigator, with the intention of measuring the effects on those organisms. The treatment could be a specific concentration of a potentially toxic material or substance. Alternatively, a treatment might be a particular test material (e.g., a particular sample of effluent, elutriate, leachate, receiving water, or control water).

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

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

Acknowledgements

The first edition of this biological test method, printed in March 1999 was written by Jennifer A. Miller (Miller Environmental Sciences Inc., Stroud, ON). It was based on a method developed by Ms. Mary Moody and Dr. Hans Peterson at the Saskatchewan Research Council (1997), which was a modification of the "8211 Duckweed (Proposed)" toxicity test (APHA et al., 1995). The report also incorporated test modifications from existing guidance documents and reports (published or otherwise) that describe procedures and conditions used in the United States, Canada, and Europe for culturing *Lemna minor*, and for conducting toxicity tests using this species of freshwater aquatic macrophyte.

Mr. R. P. Scroggins (Method Development and Applications Section, Environmental Technology Centre, Environment Canada, Ottawa, ON) acted as Scientific Authority and provided technical input and guidance throughout the work. Special acknowledgement is made of the many useful comments and suggestions provided by each member of the Environment Canada (EC) committee of scientific experts responsible for the initial and final reviews of the first edition of this report: G. Gilron (ESG International Inc., Guelph, ON); E. Jonczyk, Beak International Inc., Brampton, ON); D.J. McLeay (McLeay Environmental Ltd., Victoria, BC); M. Moody (Saskatchewan Research Council, Saskatoon, SK); J. Rathbun (AScI Corporation, Livonia MI); J. Staveley (ARCADIS Geraghty & Miller, Inc., Raleigh, NC); L. Taylor (McMaster University, Hamilton, ON); and P. Whitehouse (WRc, Marlow, Bucks, U.K.).

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This (second) edition was prepared by J. A. Miller (Miller Environmental Sciences Inc., King, ON), with assistance and guidance from L. Taylor (Manager, Method Development and Applications Section), R. Scroggins (Chief, Biological Methods Division) and L. Van der Vliet (Method Development and Applications Section) of the Environmental Technology Centre, Environment Canada, Ottawa, Ontario, as well as M. Moody (Saskatchewan Research Council, Saskatoon, SK). The second edition includes numerous updates such as modified culture and test media and the use of regression analyses for quantitative endpoint data. Numerous comments and suggestions for change to the first edition, which were forwarded to Environment Canada's Method Development and Applications Section by Canadian laboratory personnel performing this toxicity test method were considered when preparing this second edition of Report EPS 1/RM/37. Procedures outlined in the 2005 International Organization for Standardization (ISO) draft *Lemna minor* growth inhibition test have also been incorporated. Many procedural modifications were provided through research carried out by M. Moody.

Photographs for the method were supplied by M. Moody (SRC, Saskatoon, SK).

Section 1

Introduction

1.1 Background

Aquatic *toxicity tests* are used within Canada and elsewhere to determine and monitor the *toxic* effects of discrete *substances* or *materials* that *might* be harmful to aquatic life in the environment. The results of toxicity tests *can* be used to determine the need for control of discharges, to set effluent standards, for research, and for other purposes. Recognizing that no single test method or test organism can be expected to satisfy a comprehensive approach to environmental conservation and protection, Environment Canada and the Inter-Governmental Environmental Toxicity Group (IGETG) (Appendix A) proposed that a set of standardized aquatic toxicity tests be developed, that would be broadly acceptable for use in Canada. It was decided that a battery of federally approved biological test methods was required that would measure different *acute* and *chronic* toxic effects using different test substances or materials and organisms representing different trophic levels and taxonomic groups (Sergy, 1987). As part of this ongoing undertaking, a toxicity test for determining the effect of contaminants on the inhibition of *growth* of the aquatic macrophyte, *Lemna minor,* was recommended for standardization. The first edition of this method was used in Environment Canada's regional laboratories (Appendix B), as well as in provincial and private laboratories, to help meet Environment Canada's metal mining effluent regulations and other testing requirements. The current (second) edition includes numerous procedural improvements, updated and more explicit guidance, and instructions for the use of revised statistics (i.e., regression analyses) when calculating the test *endpoint* for growth inhibition.

Universal procedures and conditions for conducting aquatic toxicity tests that measure growth inhibition of the aquatic macrophyte, *L. minor*, are described in this second edition. Also presented are specific sets of conditions and procedures required or recommended when using the test to evaluate different types of substances or materials (e.g., samples of one or more *chemicals*, *effluents*, *receiving waters*, *leachates*, or *elutriates*) (see Figure 1). Some details of methodology are discussed in explanatory footnotes.

This biological test method has been developed following a review of variations in specific culturing and test procedures indicated in existing Canadian, American, and European methodology documents[1] that describe how to prepare for and conduct phytotoxicity tests using *Lemna* spp. A summary of these culturing and testing procedures is found in Appendix C. A summary of various media used for culturing and testing *Lemna* spp. in existing or past procedures is found in Appendix D. The biological endpoints for this method are: (a) increased number of *fronds* during the 7-day test; and (b) dry weight (as an indication of growth) at the

[1] Documents used to prepare listings of the variations in specific culturing and test procedures (see Appendices C and D) include published "how-to" references, unpublished Standard Operating Procedures used by testing facilities, and draft reports. Citations of source documents are listed in these appendices by originating agency and then by author(s), and formal citations are identified in the appendices.

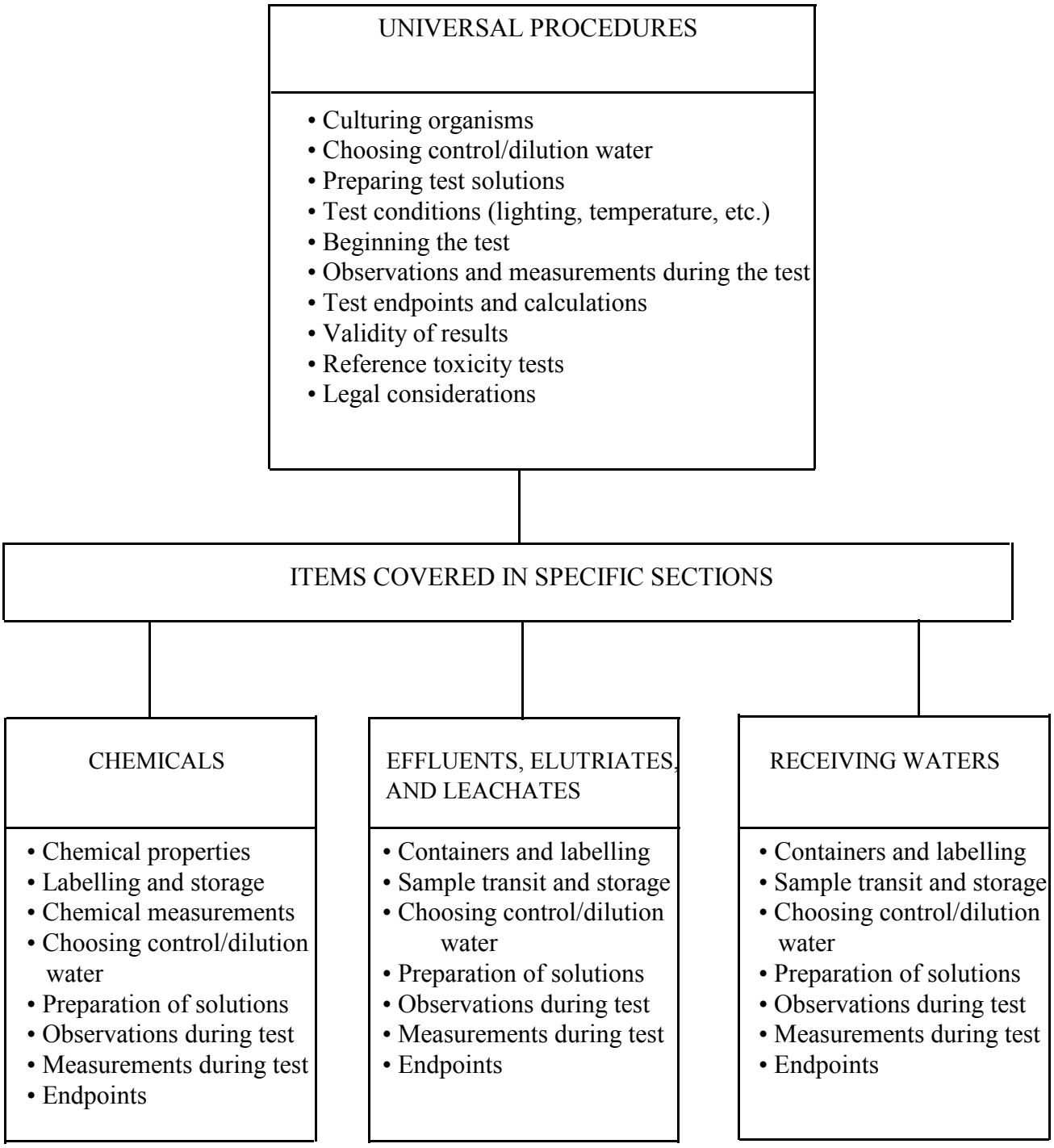

Figure 1 Considerations for Preparing and Performing Toxicity Tests Using *Lemna minor* with Various Types of Test Materials or Substances

end of the test.[2] The test method is intended for use in evaluating samples of:

(1) single chemicals, commercial products, or known mixtures of chemicals;

(2) freshwater industrial or urban effluents, elutriates, or leachates; and

(3) freshwater surface or receiving waters.

In formulating these procedures, an attempt was made to balance scientific, practical, and cost considerations, and to ensure that the results would be accurate and precise enough for most situations in which they would be applied. It is assumed that the user has a certain degree of familiarity with aquatic toxicity tests. Guidance regarding test options and applications is provided here. Explicit instructions that *might* be required in a regulatory *protocol* are not provided, although this report is intended as a guidance document useful for this and other applications.

For guidance on the implementation of this and other biological test methods and on the interpretation and application of the endpoint data, consult the Environment Canada report (EC, 1999a).

1.2 Species Description and Historical Use in Tests

Lemna minor, commonly referred to as lesser duckweed or common duckweed, is a small, vascular, aquatic macrophyte belonging to the family Lemnaceae. Members of the family Lemnaceae are free-floating, monocotyledonous angiosperms which are found at, or just below, the surface of quiescent water (Hillman, 1961). There are four genera (*Spirodela, Lemna, Wolffiella,* and *Wolffia*) and approximately 40 *Lemna* (i.e., duckweed) species world wide (Wang, 1990). The two species commonly used in toxicity tests, *L. minor* and *L. gibba*, are well represented in temperate areas (OECD, 1998, 2002).

L. minor is ubiquitous in nature, inhabiting relatively still fresh water (ponds, lakes, stagnant waters, and quiet streams) and estuaries ranging from tropical to temperate zones (APHA *et al.*, 1992). It is a cosmopolitan species whose distribution extends nearly world wide (Godfrey and Wooten, 1979). In North America, *L. minor* is one of the most common and widespread of the duckweed species (Arber, 1963; APHA *et al*., 1992). The fronds of *L. minor* occur singly or in small clusters (3 to 5) and are flat, broadly obovate to almost ovate, 2- to 4-mm long, green to lime green, and have a single *root* that emanates from the centre of the lower surface (Hillman, 1961; Godfrey and Wooten, 1979; Newmaster *et al*., 1997). Vegetative growth in *Lemna* spp. is by lateral branching, and is rapid compared with other vascular and flowering plants (Hillman, 1961; APHA *et al*., 1992). Further details on the taxonomy, description, distribution and ecology, and reproductive biology of this species are provided in Appendix E.

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[2] Various methods can be used to measure or estimate growth. The most common and simplest indirect measurement of growth is the determination of the number of plants or number of fronds (ASTM, 1991). Frond count is simple, rapid, and nondestructive (and therefore can be observed during the test); however, frond count alone is irrelevant to frond size or *biomass* (Wang, 1990). Wang (1990) notes that under toxic stress, small buds might form and be counted as individual fronds. A small bud might be < 5% of the biomass of a healthy frond in a control group; however, they are considered equal in a frond count. Therefore, *toxicity* might be greatly underestimated with frond counts alone. Also, frond count does not differentiate definitively between live and dead fronds. Cowgill and Milazzo (1989) found that dry weight is the most objective and reproducible of the endpoints when compared to other endpoints (e.g., number of fronds, number of plants, number of roots, total root elongation, % Kjeldahl N, and chlorophyll a and b).

Duckweeds have been used as test organisms for the detection of phytotoxicity since the 1930s. They were among the species used to define the effects of the earliest phenoxy herbicides on plants (Blackman and Robertson-Cumminghame, 1955). In 1979, the United States Environmental Protection Agency (USEPA) proposed that *L. minor* be classified as a "representative" aquatic macrophyte, useful in the environmental safety assessment of chemicals (Federal Register, 1979 in Bishop and Perry, 1981). In the past several years, there has been increasing interest in the use of vascular plants for environmental *monitoring* and assessment, including laboratory phytotoxicity tests (Wang and Freemark, 1995). Besides being an essential component of aquatic ecosystems[3], aquatic macrophytes have a key role in assessing the effects of herbicides on vegetation in aquatic environments through phytotoxicity testing (Wang and Freemark, 1995).

Many important environmental legislation and guidelines developed under different authorities have included phytotoxicity testing as part of environmental monitoring and assessment (Wang and Freemark, 1995). The USEPA requires phytotoxicity testing under the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA), including a duckweed growth test. Duckweed testing can also be required in the USEPA's Toxic Substances Control Act (TSCA) and is optional for National Pollution Discharge Elimination System (NPDES) permits under the U.S. Water Quality Act, 1987 (Wang and Freemark, 1995).

A duckweed growth inhibition test developed for the Organization for Economic Cooperation and Development (OECD, 1998, 2002) underwent interlaboratory validation (Sims *et al.,* 1999). The international ring test included the participation of 37 testing laboratories from Europe, North America, and the Far East. The key performance characteristics of the draft test method that were assessed included *compliance* with the critical quality criteria, repeatability of the method within laboratories, and reproducibility between laboratories. The results of the ring test, which included testing of two *Lemna* species (*Lemna minor* and *Lemna gibba*), indicate that the requirements of the draft OECD *Lemna* growth inhibition guideline were successfully met by most of the data sets submitted (Sims *et al*., 1999). Other findings from the ring test apply to the use of 3,5-dichlorophenol and potassium dichromate as *reference toxicants*.

Duckweed test methods currently available and used in North America and abroad include those by: the American Public Health Association *et al.* (APHA *et al.,* 1995); the American Society for Testing and Materials (ASTM, 1991); the United States Environmental Protection Agency (USEPA, 1996); the Association Française de Normalisation (AFNOR, 1996); the Swedish Standards Institute (SIS, 1995); and the Institute of Applied Environmental Research (ITM, 1990). More recently, the International Organization for Standardization (ISO) has also developed a *Lemna minor* growth inhibition test method (2005).

Duckweed species have many attributes that make them advantageous for use in laboratory toxicity tests and assessments of freshwater systems. These include their:

- small size[4];

[3] Macrophytes as well as phytoplankton constitute a major fraction of the total biomass of photosynthetic organisms in aquatic environments. Characterized and standardized higher plants need representation in studies of aquatic ecosystem health, and are needed to complement the developing animal and microbial studies (Wang, 1990; Greenberg *et al*., 1992).

[4] Duckweeds are small enough that large laboratory facilities are not necessary, but duckweeds are large enough that effects can be observed visually (ASTM, 1991).

- relative structural simplicity; and
- rapid growth[5] (Hillman, 1961; Smith and Kwan, 1989).

Duckweeds also have several characteristics that make them uniquely useful for toxicity tests:

- their vegetative reproduction and genetically homogenous populations enable clonal colonies to be used for all experiments, and eliminate effects due to genetic variability (Hillman, 1961; Bishop and Perry, 1981; Smith and Kwan, 1989);

- duckweeds can be disinfected and grown in a liquid *medium* as well as on agar, autotrophically or heterotrophically (Hillman, 1961);

- duckweeds cultured in the laboratory can grow indefinitely and controlled conditions of temperature, light, and nutrition are far easier to maintain than for most other angiosperms (Hillman, 1961; Wang, 1987);

- they have a high surface area to volume ratio, and little, if any, cuticle present on the underside of the frond that is in contact with the *test solution* (Bishop and Perry, 1981);

- they are excellent accumulators of a number of metallic elements, making them good candidates for use in water quality monitoring and in laboratory tests for toxicity and uptake studies (Jenner and Janssen-Mommen, 1989; Smith and Kwan, 1989);

- duckweeds are especially susceptible to surface-active substances, hydrophobic compounds, and similar substances that concentrate at the air-water interface (Taraldsen and Norberg-King, 1990; ASTM, 1991); and

- unlike algal toxicity tests, test solutions can be renewed, and coloured or turbid *wastewater* or receiving-water samples can be tested (Taraldsen and Norberg-King, 1990; Forrow, 1999).

Since *Lemna* spp. were first used for comparative phytotoxicity studies, a number of test procedures have been described. Plant growth has been quantified by various procedures including frond count, dry weight, *growth rate*, doubling time, percent inhibition, frond area, root length, chlorophyll content, and photosynthesis (Lockhart and Blouw, 1979; Bishop and Perry, 1981; Cowgill and Milazzo, 1989; Wang, 1990; Greenberg *et al*., 1992; Huang *et al*., 1997). Examples of *Lemna* species that have been used for testing include: *Lemna aequinoctialis, Lemna major, Lemna minor, Lemna gibba, Lemna paucicostata, Lemna perpusilla, Lemna trisulca,* and *Lemna valdiviana* (OECD, 1998, 2002). Numerous test options, including test duration, type (*static*, *static-renewal*, flow-through), test and culture media, light intensity, and temperature have been investigated and reviewed (see Appendices C and D).

The *Lemna minor* growth inhibition test, developed by the Saskatchewan Research Council (SRC) Water Quality Section (SRC,

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[5] When cultivated under well-controlled laboratory conditions favourable for growth, the amount of *L. minor* biomass doubles every two days (ITM, 1990). This is in agreement with the results of an 18-month study (Wang, 1987), where the doubling time for *L. minor* fronds ranged from 1.3 to 2.8 days. The mean value and standard deviation were 1.9 and 0.36 days, respectively (Wang, 1987). The SRC (1997) reports that its maximum rate of growth is close to one doubling every 24 hours.

1997) is a modification of the "8211 Duckweed (Proposed)" toxicity test procedure published by APHA *et al*. (1995). The major modifications include changes to the medium composition (potassium added, *pH* stabilized, and EDTA removed), pre-cultivation methods, and the use of *axenic cultures*, as well as the establishment of a requirement for a greater *biomass* increase during the test. The method developed by the SRC has been used successfully in assessing single-metal solutions, as well as metal mine wastewaters (SRC, 1997).

Precision of the test appears to be satisfactory. The SRC has demonstrated within-laboratory coefficients of variation (CVs) for mean percent inhibition of biomass, using chromium (Cr) as a reference toxicant, of <10% (Moody, 1998).

The purpose of the biological test method herein is to provide a "standardized" Canadian methodology for estimating the toxicity of various substances or materials in fresh water using *L. minor*. Whereas the application of other published methods (see Appendix C) for performing this test might have been restricted to certain types of substances or materials, this report is intended for use in evaluating the *sublethal* toxicity of chemicals, effluents, leachates, elutriates, and receiving waters. The generic culture and test conditions and procedures herein are largely those developed by SRC (1997), with incorporation of useful test modifications and harmonization with OECD (1998, 2002), ISO (2005) and elsewhere. The rationale for selecting certain approaches is provided in the document.

This method is intended for use with freshwater-acclimated *L. minor*, with fresh water as the dilution and control water, and with effluents, leachates, or elutriates that are essentially fresh water (i.e., *salinity* $\leq$10 g/kg) or are saline but are destined for discharge to fresh water. Its application *may* be diverse but includes instances where the effect(s) or potential effect(s) of substances or materials on the freshwater environment is under investigation. Other tests, using other species acclimated to seawater, may be used to assess the effect(s) or potential effect(s) of substances or materials in estuarine or marine environments, or to evaluate wastewaters having a salinity >10 g/kg.

Section 2

Test Organisms

2.1 Species and Life Stage

Lemna minor Linnaeus (Arales:Lemnaceae) is the species that *must* be used in this biological test method. Landolt *clones* 8434 and 7730 are recommended for use in this test.[6] A general description of *L. minor* and features that distinguish it from similar species are provided in Appendix E.

The test *culture*, comprised of plants isolated exclusively for obtaining test organisms, must be axenic and must be used to inoculate all vessels used in a given test.[7] Inocula from these cultures must be 7- to 10-days old and consist of young, rapidly growing colonies[8] without visible lesions before being used to set up a given test (see Figure 2).[9]

2.2 Source

All organisms used in a test must be from the same *strain*. Sources of plants required to establish cultures may be culture collections, government or private laboratories that culture *L. minor* for toxicity tests, or commercial biological suppliers. Upon initiating cultures using organisms from outside sources, species identification must be confirmed and documented by a qualified taxonomist, experienced in identifying aquatic macrophytes.[10] It is also important to identify the *L. minor* clone being used (if possible), because it has been shown that different clones of the same species can have different sensitivities (Cowgill and

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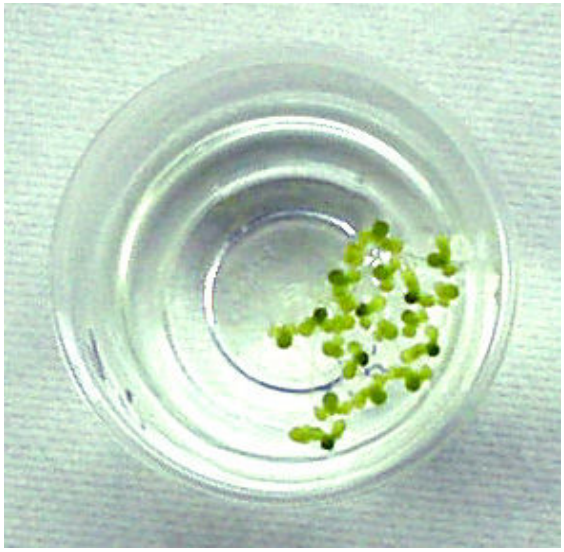
6 The Landolt 8434 *Lemna minor* clone was collected from the Niagara Peninsula, Ontario in 1977, and isolated in axenic cultures in Zürich, Switzerland. The Landolt 7730 *Lemna minor* clone was collected from Elk Lake, British Columbia in 1973 and isolated in axenic cultures in Zürich, Switzerland. Both *L. minor* clones are available from the University of Toronto Culture Collection (see Section 2.2).

7 For greater standardization, a culture grown from a single isolated plant can be used to inoculate all the flasks used in a given test (USEPA, 1992; 1996).

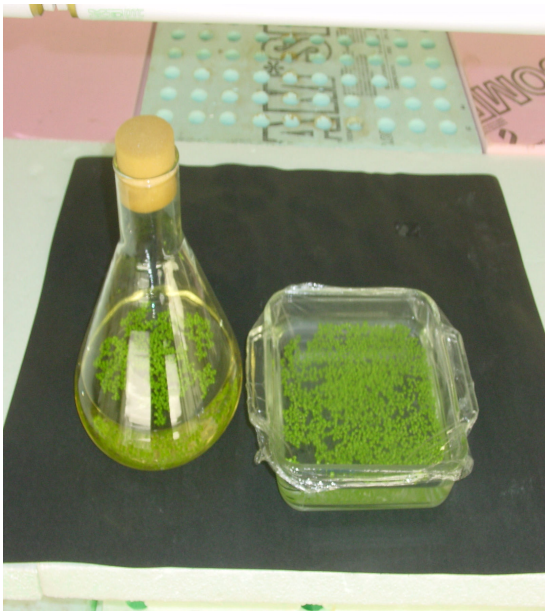
8 Good quality cultures are indicated by a high incidence of colonies comprised of at least two fronds (2–4 fronds). A large number of relatively small single fronds (with or without two unsatisfactorily developed fronds) is indicative of environmental stress, e.g., nutrient limitation, and plant material from such cultures must not be used for testing. *L. minor* in its most intensive growth phase (younger plants) are lighter in colour, have shorter roots, and consist of two to three fronds of different size (ITM, 1990; OECD, 1998, 2002).

9 SRC (1995) growth curves indicate that the most intensive growth phase for *L. minor* in modified Hoagland's E+ medium is between Days 7 and 10. USEPA (1992; 1996) and AFNOR (1996) recommend cultures < 2 weeks old be used as test inocula.

10 The taxonomy of *Lemna* species is complicated by the existence of numerous phenotypes. Also, taxonomic keys are based mainly on the flowering and fruiting characteristics of *Lemna* and contain relatively few diagnostic vegetative characteristics. Since flowering and fruiting are rarely observed in *Lemna* species, positive taxonomic identification can be extremely challenging. *L. minor*, for example, can only be positively differentiated from another closely related species *Lemna turionifera* by the lack of overwintering turions and the lack of reddish anthocyanin blotches on the ventral side of *L. minor*. These characteristics are produced only under culturing conditions that differ substantially from those commonly used to culture *Lemna* in laboratories.



(a)



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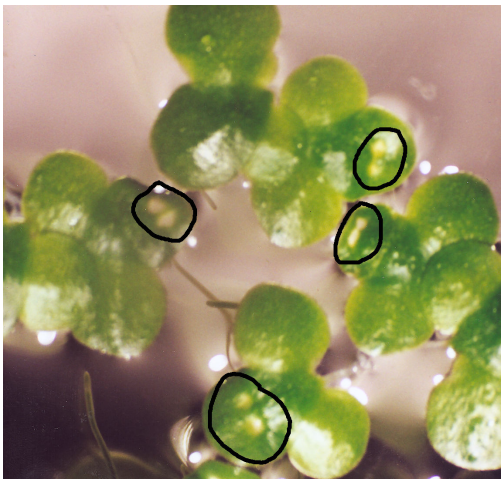

(c)

(d)

Figure 2 **General Appearance of Healthy and Unhealthy *Lemna minor***
(a) Normal control growth in plastic test cup containing modified APHA medium, showing fronds with variable shades of green. (b) *Test culture* in Hoagland's medium (left) and acclimation culture in modified APHA medium (right), both "uncrowded". (c) Colonies with "snake-bite" lesions from long-term iron deficiencies when cultured in the original (EC, 1999b) Hoagland's E+ medium. (d) Cultures showing *chlorosis* (loss of chlorophyll/yellowing) of fronds in plastic test cup.

Milazzo, 1989; SRC, 2003, 2005)[11]. Periodic (e.g., annual) taxonomic checks of the laboratory's culture, or replacements (i.e., renewal) of the culture from a recognized culture collection, are also advisable to ensure that the laboratories *L. minor* culture hasn't been contaminated with other *Lemna* species or clones, especially if the laboratory maintains several different *Lemna* cultures.

Axenic and non-axenic cultures of *L. minor* can be obtained from the following Canadian source:

University of Toronto Culture Collection[12]
Dept. of Botany, University of Toronto
25 Willcocks St., Toronto, Ontario
Canada, M5S 3B2

Telephone:(416) 978-3641
Facsimile: (416) 978-5878
e-mail: jacreman@eeb.utoronto.ca

Web site: http://www.botany.utoronto.ca/utcc

Lemna minor: UTCC 490[13] and 492[14].

2.3 Culturing

2.3.1 General

Recommended or required conditions and procedures for culturing *L. minor* are discussed here and summarized in Table 1. These are intended to allow some degree of inter-laboratory flexibility while standardizing those conditions which, if uncontrolled, might affect the health and performance of the test organisms. A large portion of Section 2.3 is derived from SRC (1997) and OECD (1998, 2002).

If organisms are obtained from an outside laboratory or culture collection, plants must be cultured in the laboratory for a minimum of 3 weeks before being used.

[11] Cowgill and Milazzo (1989) tested four different clones of *L. minor* in modified Hoagland's medium with various concentrations of selenium (Se), vanadium (V), cobalt (Co), and tin (Sn), to determine the optimum levels of these elements in culture medium for plant growth. They found that the clones varied in their responses. Clone 6591 showed no increase in growth with Sn and Co added to the Hoagland's medium and their biomass (dry weight) peaked with 8.4 µg/L of Se and 12.8 µg/L of V. Clone 7102 achieved peak biomass at 8.4 µg/L of Se, 685 µg/L of Sn, and 10.2 µg/L of Co added to the medium. Clone 7101 also achieved peak biomass at 8.4 µg/L of Se and 685 µg/L of Sn added to the medium, but showed no increase in growth on addition of V and Co. Clone 7136, however, performed best with no Sn, V, Se, or Co added to the modified Hoagland's medium.

In more recent studies (SRC, 2003, 2005), the sensitivity of various *L. minor* clones (UTCC 490, 492, and 620) differed, depending on the *toxicant* to which they were exposed and the methodology followed. IC25 values for zinc (Zn), cadmium (Cd), copper (Cu), and nickel (Ni) were not significantly different between UTCC strains 490 and 492 (SRC, 2003). In addition, there was no significant difference in IC25s for UTCC strains 490, 492, and 620 exposed to potassium chloride (KCl) (SRC, 2005). IC25s for Ni based on frond count, however, were 4 times higher for UTCC 620 compared to UTCC 492 using the Environment Canada method (EPS 1/RM/37), whereas the IC25s for Ni were not significantly different between UTCC clones 492 and 620 using the methodologies outlined in the ISO draft standard (SRC 2005).

[12] Certificates of taxonomic confirmation should be obtained upon acquisition of the *Lemna* culture for future reference and evidence of culture integrity.

[13] UTCC 490: Axenic culture; Landolt clone 8434; Niagara Peninsula, Ontario, Canada.

[14] UTCC 492: Axenic culture; Landolt clone 7730; Elk Lake, British Columbia, Canada.

Table 1 Checklist of Recommended Conditions and Procedures for Culturing *Lemna minor*

Source	- culture collection, biological supply house, government laboratories, or private laboratories; species confirmed taxonomically and clone identified (if possible)
Culture medium	- modified Hoagland's E+ medium (see Table 2); subcultured weekly in fresh medium
Temperature	- within the range 25 ± 2°C
pH	- 4.4 to 4.8
Lighting	- continuous, full-spectrum fluorescent or equivalent; 64 to 90 μmol/(m^2 · s) at surface of culture media; within 15% of the selected light fluence rate throughout culture area
Test culture	- 5 to 10 plants transferred from a week-old test tube culture to sterile, modified Hoagland's E+ medium and incubated for 7 to 10 days under test conditions
Acclimation	- 7- to 10-day old plants from test culture transferred to fresh test medium for 18 to 24 hours before testing
Health criteria	- in order for the test culture to be acceptable for use in the test, the frond number must increase to ≥8-times (i.e., ≥24 fronds) the original frond number in 7 days in a culture set up for monitoring organism health; plants in test culture must appear healthy

Axenic *stock cultures* can be maintained by the weekly *subculture* of 1 plant into approximately 25 mL of sterile modified Hoagland's E+ medium (SRC, 2003) in 25 × 150 mm test tubes with Kimcaps™. *Lemna* is aseptically transferred into test tubes containing fresh modified Hoagland's E+ medium and incubated on an angle under controlled light and temperature.

Cloudy medium in a *Lemna* stock culture indicates bacterial contamination, whereas contamination with mould may not be clearly evident until large colonies appear in the medium or a slime layer develops on the vessel. Contaminated *Lemna* cultures (e.g., with algae, protozoa, fungi, or bacteria) must be discarded or sterilized (see Section 2.3.7).

Cultures used for toxicity tests (i.e., *test cultures*) should be initiated 7 to 10 days before starting the test. For best harvest of plants having 2 to 3 fronds, prepare one or more test cultures. Aseptically transfer 5 to 10 plants from a week-old test tube culture into a 150 mm diameter petri dish (or other sterile, shallow containers) filled with sterile Hoagland's E+ medium to a depth of at least 1 to 1.5 cm (≥100 mL), and incubate

under test conditions. Test cultures should not be crowded at the end of the 7- to 10-day incubation. Cultures are considered crowded if plants cover more than two thirds of the medium surface.

For determining whether the test culture meets the health criteria outlined in Section 2.3.8, one or more vessels containing approximately 100 mL of test medium (modified APHA, SIS, or modified Steinberg medium, whichever will be used in the test), is prepared each time a test culture is initiated.

Multiple subcultures of an axenic *Lemna* culture *should* be made to ensure the availability of at least one sterile culture, in case of contamination.[15] The maintenance of a clean laboratory, good sterile technique, and the proper use of a laminar flow hood are all essential for axenic culturing of *Lemna minor* (Acreman 2006; see Appendix F).

A single, three-frond *Lemna* plant is placed into each vessel. Assuming that the cultures appear healthy (see footnote 8 and Figure 2), the culture is considered acceptable for use in the test if the number of fronds (or mean number of fronds if several vessels are used) in the vessel(s) set up for monitoring the health of the culture has increased to ≥8-times the original number of fronds in the test vessel(s), in 7 days (i.e., ≥24 fronds) (Section 2.3.8).

Cultures older than 10 days become crowded and the plants are smaller in size; such cultures should not be used for testing. The *test culture* is easily contaminated if exposed to non-sterile air or equipment. If the medium becomes cloudy, indicating bacterial contamination, the *Lemna* cannot be used and must be replaced with an uncontaminated culture (see Section 2.3.7).

The day before the test is to be set up, sufficient *L. minor* (7- to 10-day old uncrowded culture in modified Hoagland's E+ medium) are rinsed twice in *test medium* (see Section 3.4) by replacing the spent modified Hoagland's E+ medium with fresh test medium (modified APHA medium, SIS medium, or modified Steinberg medium). The *Lemna* should then be transferred into a shallow container containing ≥2 cm fresh test medium.[16] *Lemna* should not be crowded (i.e., *Lemna* should not be overlapping and at least one third of the surface area of the medium should be free of *Lemna* fronds). Incubate these acclimation cultures under test conditions for 18 to 24 hours before being used. Although the *Lemna* stock culture is maintained under aseptic conditions, *acclimation* and testing are not carried out in sterile medium. Reasonable care should be taken to avoid algal contamination of the culture and therefore, it is recommended that *Lemna* be handled in a laminar flow cabinet (see Appendix F).

2.3.2 Facilities and Apparatus

Lemna are to be cultured in facilities with controlled temperature and lighting (constant-temperature room, incubator, or environmental chamber).[17] The culture area should be well ventilated to prevent the occurrence of a local temperature increase underneath the illumination

[15] Larger vessels (e.g., 250-mL or 125-mL Erlenmeyer flasks containing 100 mL or 50 mL of modified Hoagland's E+ medium) can be used to sustain well-growing healthy cultures as long as sterility is maintained.

[16] The SRC (1995) attempted a longer acclimation in modified APHA medium (test medium); however, they observed increasing deterioration of control growth with longer cultivation in the medium, particularly at test loading. Good quality plants could be obtained up to 7 days, but thereafter the plants deteriorated and grew poorly in the test. The SRC (1995) concluded that it is better to culture *Lemna* in "rich" media, such as modified Hoagland's E+, followed by a defined pre-cultivation period in the test medium before testing in "lean" medium is carried out.

[17] Water baths are not acceptable because they prevent proper illumination of the culture vessels (ASTM, 1991).

equipment (ITM, 1990), and the air supply should be free of odours and dust. Ideally, the culturing facility should be isolated from the test facility to reduce the possibility of culture contamination by test substances or materials. Cultures should also be isolated from regions of the laboratory where stock or test solutions are prepared, effluent or other test material or substance is stored, or equipment is cleaned.

Vessels and accessories in contact with the *Lemna* cultures and culture media must be made of nontoxic, chemically inert material, and where necessary, should be sterile. Materials such as borosilicate glass (e.g., Pyrex™), stainless steel, porcelain, nylon, high density polystyrene, or perfluorocarbon polyethylene plastics (e.g., Teflon™), may be used to minimize leaching and sorption. Plastic vessels may be used only if duckweeds do not adhere to the walls[18] and the test substance does not sorb to the plastic more than it does to the glass (ASTM, 1991). Materials or substances such as copper, brass, galvanized metal, lead, and natural rubber must not contact the culture vessels or media, *test samples*, test vessels, *dilution water*, or test solutions.

Items made of materials or substances other than those mentioned herein should not be used unless it has been shown that their use does not adversely affect the quality of the *Lemna* cultures. All culture vessels and accessories should be thoroughly cleaned and rinsed with culture water between uses. New and previously used glassware must be chemically cleaned and sterilized before use (EC, 1992a). All culture and test vessels should be covered with appropriate transparent covers to exclude dust and minimize evaporation (see Section 3.3).

Equipment recommended for the maintenance of axenic *Lemna* cultures includes: disposable inoculating loops, for the aseptic transfer of *Lemna*; an autoclave, for sterilizing glassware and media; and a sterile transfer hood (laminar flow hood) for maintaining axenic conditions (see Appendix F).[19]

2.3.3 Growth Medium

Modified Hoagland's E+ (SRC, 2003) is the medium required for culturing *L. minor* that are to be used for tests involving wastewater (e.g. effluents, elutriates, leachates) or receiving water.[20] The chemical composition of modified Hoagland's E+ medium is presented in Table 2.

[18] Plastic cups may be soaked in clean water before use to reduce the static charge and therefore the possibility of plants sticking to the sides of the vessels.

[19] The following procedures are recommended for laboratories that are not equipped with a laminar flow cabinet. A small pre-sterilized space with minimal air flow is recommended for handling and/or transferring *Lemna*. This can be done by building an opaque Plexiglass™ hood, equipped with a UV light for pre-sterilization of the work space within the hood. The light can be left on when the hood or transfer room is not in use but must be turned off when the hood is in use (exposure to UV light is highly dangerous to skin and eyes). A bunsen burner and a gas source (or a portable, gas bunsen burner) is needed to conduct aseptic culturing techniques (i.e., for flaming the mouths of culture test tubes and media vessels, etc.). Handling of the plant should be minimal and transfers should be carried out as quickly as possible (Acreman, 1998).

[20] The SRC (1995) found that the highest quality *Lemna* plants can be obtained from fast growing cultures in Cowgill and Milazzo's (1989) Hoagland's E+ medium. This medium contains high levels of organic and inorganic nutrients and trace metals. Subsequent research at SRC (2003), resulted in further modifications of Hoagland's E+ medium (now recommended herein) for improved long-term health of *L. minor* cultures. These modifications included the replacement of separate iron (Fe) and EDTA solutions with a combined solution (Stock C) containing increased amounts of Fe and EDTA (SRC, 2003). Modified APHA medium is required only as a test medium since it produces fronds of excellent quality in the short-term; however, it is unsuitable for long-term cultivation of *Lemna* (SRC, 1995).

Table 2 Chemical Composition of Nutrient Stock Solutions for Preparing Modified Hoagland's E+ Medium (SRC, 2003), Used for Culturing *Lemna minor*

Stock	Substance	Concentration	
		Stock Solution (g/L)	Medium[a] (mg/L)
A[b]	$Ca(NO_3)_2 \cdot 4H_2O$	59.00	1180.0
	KNO_3	75.76	1515.2
	KH_2PO_4	34.00	680.0
B	Tartaric Acid	3.00	3.00
C[c]	$FeCl_3 \cdot 6H_2O$	1.21	24.20
	$Na_2EDTA \cdot 2H_2O$[d]	3.35	67.00
D	$MgSO_4 \cdot 7H_2O$	50.00	500.0
E	H_3BO_3	2.86	2.86
	$ZnSO_4 \cdot 7H_2O$	0.22	0.22
	$Na_2MoO_4 \cdot 2H_2O$	0.12	0.12
	$CuSO_4 \cdot 5H_2O$	0.08	0.08
	$MnCl_2 \cdot 4H_2O$	3.62	3.62
----	Sucrose	-----	10.00 g/L
----	Yeast extract	-----	0.10 g/L
----	Bactotryptone	-----	0.60 g/L

[a] Concentration of substance in medium
[b] Add 6 mL of 6*N* HCl to stock solution A
[c] Add 1.2 mL of 6*N* KOH to stock solution C
[d] $Na_4EDTA \cdot 2H_2O$ can be used instead of $Na_2EDTA \cdot 2H_2O$. If $Na_4EDTA \cdot 2H_2O$ is used, the concentrations in the stock solution and the test medium are 3.75 g/L and 75 mg/L, respectively, and KOH should not be added to stock solution C (see footnote C above)

To prepare 1 L of modified Hoagland's E+ medium, the following are added to 900 mL of glass-distilled, *deionized water* (or equivalent):

Solution A	20 mL
Solution B	1 mL
Solution C	20 mL
Solution D	10 mL
Solution E	1 mL
Sucrose	10 g
Yeast Extract	0.10 g
Tryptone (Bactotryptone)[21]	0.6 g

Chemicals must be reagent-grade. The medium is stirred until all the contents are dissolved. Adjust the pH to within the range of 4.4 to 4.8 with NaOH or HCl and bring the volume up to 1 L with *distilled water*. Autoclave for 20 minutes at 121°C and 124.2 kPa (1.1 kg/cm^2). *Stock solutions* should be stored in the dark (i.e., dark amber or covered bottles) due to potential photosensitivity. Individual stock solutions (i.e., A, B, C, etc.) may be stored in the refrigerator (4°C) for up to one month, provided they are isolated from solvents or other potential contaminants. Once autoclaved, prepared modified Hoagland's E+ medium can be stored for up to one month at room temperature in the dark.[22]

Other nutrient-rich media (i.e., SIS medium or Steinberg medium) can be used for maintaining cultures of *L. minor* to be used for chemical tests only, as long as the *Lemna* cultures meet the health criteria of organisms to be used in the test (Section 2.3.8).

2.3.4 Lighting

Organisms being cultured should be illuminated using continuous full-spectrum fluorescent or equivalent lighting.[23] The light fluence rate, measured at the level of the culture medium, should be 64 to 90 µmol/(m^2 · s) (approximately 4000 to 5600 *lux*).[24] Since light intensity tends to vary in a given space, it should be measured at several points within the culture area (at the level of the culture medium) and should not vary by more than ± 15% of the selected light fluence rate.

2.3.5 Temperature

L. minor should be cultured at a temperature of 25 ± 2°C.[25] If cultures are maintained outside this temperature range, temperature must be adjusted gradually (≤ 3°C/day) to within the range of 25 ± 2°C, and held there for a minimum

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[21] The use of BDH #7213 Peptone from casein trypsin-digested is an acceptable alternative to Bacto-tryptone (SRC, 1997).

[22] A large batch of modified Hoagland's E+ can be prepared, autoclaved as smaller aliquots (i.e., in 1 L bottles), and stored for future use. Each aliquot of medium should be used up within a short period of time after opening (i.e., not re-stored for future use), in order to reduce the risk of contamination of the medium. Any stocks or prepared media that contain precipitates or algae, or that show any signs of deterioration should not be used.

[23] Both warm- and cool-white fluorescent lights have been used for culturing *L. minor* (Appendix C). Full-spectrum light, which is recommended for both culturing and testing in this method, is more representative of natural light conditions than cool-white light, and is being used with increased frequency for photosynthetic plant testing (SRC, 1995).

[24] This conversion of µmol/(m^2 · s) to lux assumes an average wavelength of 550 nm, which is the average wavelength of many common laboratory light sources for visible light (e.g., cool-white fluorescent). However, if the light source has a spectral quality that is not centred at 550 nm (e.g., outside the 400 to 700 nm range), the assumed wavelength for conversion of µmol/(m^2 · s) to lux will have to be adjusted (see ASTM, 1995).

[25] To reduce the frequency of culture maintenance, e.g., when no *Lemna* tests are planned for a period, plants can be held under reduced illumination and temperature (4 to 10°C). Under these conditions, subculturing may be conducted less frequently. Intervals of up to three months have been found to be acceptable (OECD, 1998, 2002). According to the Swedish method (ITM, 1990), stock cultures can be stored at a temperature of 8 to 10°C in subdued lighting (e.g., 2 × 10 Watt warm-white fluorescent tubes).

of two weeks before the test is initiated. If temperature in the culture vessels (or in one or two extra vessels set up for the purpose of monitoring water temperature) is based on measurements other than those in the vessels themselves (e.g., in the incubator or controlled temperature room within the vicinity of the culture vessels) the relationship between the readings and the temperature within the culture vessels must be established and periodically checked to ensure that the plants are being cultured within the desired temperature range.

2.3.6 pH

Lemna cultures should be at a pH range of 4.4 to 4.8. The pH of modified Hoagland's E+ medium is around 4.6 and therefore *Lemna* plants will be at that pH when transferred into fresh medium. The pH, however, drifts up towards a pH of 7 to 8 as the culture ages for 7 to 10 days in modified Hoagland's E+ medium. (Moody, 1998). The pH of *Lemna* cultures should not be adjusted.

2.3.7 Culture Maintenance

Several stock cultures should be prepared each week in modified Hoagland's E+ medium, to maintain the laboratory's stock culture in a rapidly growing state (see Section 2.3.1). *Lemna* that has not been subcultured on a weekly basis must be subcultured in fresh medium at least twice during the 14 days immediately preceding the test, to allow the recovery to its fast growth rate. *Lemna* should be subcultured each time a test is set up so that an adequate number of test organisms will be available and acclimated.

Sterilization of *Lemna* cultures in the event of culture contamination (e.g., with algae, protozoa, fungi, or bacteria) should be avoided if possible. It is strongly recommended that cultures showing signs of contamination be discarded rather than treated. This might be a feasible approach if several cultures are held separately. If the use of cultures having undergone sterilization cannot be avoided, a minimum 8-week period must follow sterilization before use in tests. Records (including date of sterilization, sterilization procedure applied, chemicals and quantity applied, and reason for treatment) must be kept for any cultures treated for contamination.[26]

2.3.8 Health Criteria

Individual test cultures of *L. minor* to be used in toxicity tests must meet the following health criteria:

- the number of fronds in the vessel(s) set up for monitoring culture health must have increased to $\geq$8-times the original number of fronds by the end of 7 days in order for the test cultures to be valid for use in setting up a test (i.e., mean number of fronds in the vessel(s) set up for the purpose of determining culture health must be $\geq$24 per vessel at the end of 7 days).

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[26] Surface sterilization can be used to eliminate contaminating organisms (e.g., algae) from a stock culture. A sample of contaminated plant material is taken, and the clonal clusters separated from each other. The individual plants should then be dipped into a 0.5% (v:v) sodium hypochlorite solution for at least 1 min. The plants may be treated with bleach for varying amounts of time to ensure that at least one culture is both sterile and alive. The plant material is then rinsed several times with sterile water or medium and transferred into fresh culture medium. Many fronds will die as a result of this treatment, especially if longer exposure periods are used, but some of those surviving will usually be free of contamination. Properly sterilized plants will have a small green area in the bud zone along the center of the frond. Surviving plants can then be used to re-inoculate new cultures (see Appendix F) (AFNOR, 1996; OECD, 1998, 2002; Acreman, 2006).

This can be determined by preparing individual test vessels[27] containing 100 mL of the test medium (modified APHA, SIS, or modified Steinberg medium) that will be used in a given test, each time a test culture is initiated (see Section 2.3.1). A single 3-frond *Lemna* plant is transferred from the stock culture into each vessel and incubated for 7 days. The number of *Lemna* fronds in each vessel are counted at the end of 7 days and if the mean number of fronds per vessel have increased to ≥8-times the original number of fronds (i.e., ≥24 fronds), then the test culture is considered acceptable for use in the test. *Lemna* plants from the vessels set up for monitoring culture health must not be used in the toxicity test.

The general appearance of the test culture (in modified Hoagland's E+) must also be taken into consideration. The culture must consist of young, rapidly growing colonies without visible lesions (see Section 2.1, footnote 8, and Figure 2). Plants that appear in good condition must be used to set up the test. Characteristics indicative of good plant health include: bright green fronds with no discoloured areas.

Reference toxicity tests should be conducted monthly with the *Lemna* culture(s), when toxicity tests are being conducted on a regular basis in the laboratory, using the conditions and procedures outlined in Section 4.6. Alternatively, a reference toxicity test should be performed in conjunction with the toxicity test. Related criteria used to judge the health and sensitivity of the culture, according to the findings of this and earlier reference toxicity tests, are given in Section 4.6.

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[27] Different types of test vessels (i.e., plastic cups, Erlenmeyer flasks, beakers) produce significantly different performance in the controls. Laboratories can assess the suitability of their choice of test vessel as well has the health of the culture if the test vessel used to set up the health test is the same as that to be used for substance/material testing.

Test System

3.1 Facilities and Apparatus

The *Lemna minor* growth inhibition test must be conducted in a constant-temperature room, incubator, environmental chamber, or equivalent facility with good temperature control and acceptable lighting (see Section 3.2). The test facility must be capable of maintaining the daily mean temperature of all test solutions at 25 ± 2°C (see Section 4.3). Test conditions (e.g., light quality, light fluence rate, and temperature) should be uniform throughout the environmental chamber. The facility should be well ventilated, and isolated from physical disturbances or any contaminants that could affect the test organisms. The test facility should also be isolated from the area where *Lemna* are cultured. Dust and fumes should be minimized within the test and culturing facilities.

Any construction materials and equipment that might contact the test material, test solutions, or *control/dilution water* must not contain any substances or materials that can be leached into the solutions at concentrations that could cause toxic effects, or that increase sorption of the test substances or materials (see Section 2.3.2). The laboratory must have the instruments to measure the basic variables of water quality (temperature, *conductivity*, dissolved oxygen, pH), and it should be prepared to undertake prompt and accurate analysis of other variables such as hardness, alkalinity, ammonia, and residual chlorine.

All instruments used for routine measurements of the basic chemical, physical, and biological variables must be maintained properly and calibrated regularly.

Disposal facilities should be adequate to accommodate laboratory-generated waste, as well as any bench covering, lab clothing, or other contaminated materials (USEPA, 1996).

3.2 Lighting

Lighting conditions to which test organisms are subjected should be the same as those defined in Section 2.3.4 . Full-spectrum fluorescent or equivalent lighting is recommended (see footnote 23). Continuous light is required for the duration of the test, and the light fluence rate must be 64 to 90 μmol/(m^2 · s) (approximately 4000 to 5600 lux; see footnote 24) at the level of the *Lemna* in the test.[28] The light fluence rate measured at several points in the test area, at the level of the medium, should not vary by more than ±15% of the selected light fluence rate.[29]

3.3 Test Vessels

Test vessels recommended for use in the test include disposable polystyrene cups or Erlenmeyer flasks. Crystallizing dishes, petri

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[28] The type of photo-receiver (collector) used to measure the light fluence rate can influence the measured value. Spherical photo-receivers (which respond to diffuse and reflected light of all angles below and above the measured plane) and hemispherical receivers (which respond to light of all angles only above the measured plane) are preferable to unidirectional receivers and give a higher value for non-punctual light sources (AFNOR, 1996).

[29] Light intensity, and the control thereof, can be as important, if not more so, than pH and temperature for plant testing. The light fluence rate in the entire test area should be checked before initiating the test. Cheesecloth can be used to reduce the lighting in specific areas of the test facility in order to achieve the appropriate light conditions (Staveley, 1998). Alternatively, the portion of the test area that is within 15% of the selected light fluence rate can be "mapped out" to designate the boundaries of adequate versus inadequate light fluence rate (Moody, 1998).

dishes, or glass beakers may also be used[30]; however, a standard type and size should be selected and used within a laboratory.[31] Glass vessels should be used for chemical testing (Section 5). The test vessels must be wide enough for the fronds in the control vessels to grow without overlapping at the end of the test. It does not matter if the roots reach the bottoms of the test vessels; however, a minimum depth of 4 cm of test solution is recommended. The vessel must contain at least 100 mL of solution during the test and 150 mL is recommended.[32]

The test vessels should be covered to avoid potential contamination from the air and loss of volatile components. Polystyrene lids that fit plastic test cups, or petri dish lids or bottoms placed on top of Erlenmeyer flasks are recommended; however, other suitable covers may be used.[33] For a given test, all test vessels and covers (i.e., type, size, shape) as well as solution depth and volume must be identical.

Test vessels should be placed on a non-reflective dark background (e.g., black poster board) for the duration of the test.[34] Any new test system (e.g., vessel, cover, lighting and temperature conditions) should be tested by conducting a non-toxicant test in which all test vessels contain only test medium. The *coefficient of variation* (CV) for frond number and dry weight at the end of test should be < 20%.

3.4 Control/Dilution Water

For a given test, the same water must be used to prepare sample dilutions and controls. The choice of control/dilution water will depend on the test substance or material and objectives, and on the logistics, practicality, and costs of sample collection (see Sections 5 to 7). Accordingly, these factors might lead to the selection of a specific type of control/dilution water that is best suited for a particular situation. The control/dilution water recommended for use is test medium which is deionized or glass-distilled water to which reagent-grade chemicals (i.e., nutrients for growing *Lemna*) have been added.

Three different test media are recommended for use in this biological test method, and the selection will depend on the type of substance being tested. For wastewaters (see Section 6.3) and receiving waters (see Section 7.3), a modified APHA growth medium (SRC, 1997)[35]

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[30] The use of beakers should be approached with caution as frond numbers and health were reduced significantly in tests carried out using beakers (SRC, 2003).

[31] Variations in size of test vessel might affect the results of the test through changes in relative depths, relative surface area of the test solution, and other variables, in ways that are as yet unrecognized.

[32] Jonczyk and Gilron (1996) determined that larger test vessels (100 mL) yielded improved growth over smaller test vessels (50 mL).

[33] Transparent covers will allow the illumination of test organisms, while minimizing evaporation of test solutions and reducing their contamination. However, the use of watch glasses as covers for tests vessels is not recommended due to excessive losses of test medium via evaporation and the possible increase in light reflection (SRC, 2003).

[34] In a series of studies carried out to determine the impact of methodology differences between the draft ISO standard and the Environment Canada *L. minor* test method, Moody determined that frond appearance and general health (i.e., number, colour and uniformity) were improved when test vessels were placed on a black background for the duration of the test. The black background reduced the amount of reflective light to which the fronds were exposed, thereby enabling frond exposure to higher light intensities (i.e., those recommended by ISO) (SRC, 2003).

[35] The modified APHA medium differs from the medium described in the American Public Health Association (APHA *et al*., 1992) *L. minor* test method (SRC, 1995). The modifications include the addition of potassium chloride (KCl), the omission of EDTA, and the stabilization of medium pH by aeration (SRC, 1995)

must be used as control/dilution water. For chemicals, commercial products, or known mixtures (see Section 5.3), a modification of the Swedish Standard (SIS) growth medium (OECD, 1998, 2002), or a modified Steinberg medium (ISO, 2005) should be used.[36]

A sample of receiving water or *upstream water* (collected adjacent to the source of contamination but removed from it, or upstream from the source), spiked with the same reagent-grade nutrients and at the same concentration as those used to make up the modified APHA growth medium (nutrient-spiked receiving water), may also be used as control/dilution water for testing effluents (see Section 6.3) or receiving waters (see Section 7.3).[37] In instances where the toxic effect of a specific chemical or chemical compound in a particular receiving water is to be appraised, receiving water spiked with the same concentration of nutrients as those used to prepare the SIS medium or the modified Steinberg medium may be used as control/dilution water (see Section 5.3). In either case, if nutrient-spiked receiving water is used, it must first be filtered through glass fibre filters (approximately 1µm, e.g., Whatman GF/C filters) to reduce the possibility of contamination of the test by algae, and may be further filtered through 0.22µm filters to eliminate any remaining potential for algal or bacterial contamination (SRC, 1997). Conditions for collection, transport, and storage of surface water should be as described in Section 6.1.

The test medium or nutrient-spiked receiving water (used for control and dilution water) must be prepared as outlined in Sections 5, 6, and 7, and adjusted to 25 ± 2°C before use (see Section 4.1).

(see Appendix D, Table 9).

The addition of KCl roughly doubled the potassium content of the original APHA medium, resulting in increased rate and reproducibility of frond growth and reproducibility of reference toxicant results. EDTA was omitted since it can potentially interact with certain substances or materials (e.g., metals) in the test sample resulting in altered toxicity. The pH drift, observed in the original APHA medium, was eliminated (pH stabilized) by including a 1- to 2-hour aeration period following medium preparation (SRC, 1995).

[36] The Swedish Standard (SIS) growth medium is recommended for substance testing with *L. minor* in the draft OECD *Lemna* growth inhibition test (OECD, 1998, 2002). The modified Steinberg medium is recommended for testing substances or materials that do not contain predominantly metals in the draft ISO *Lemna minor* growth inhibition test (ISO, 2005).

[37] Receiving water may be used as the control/dilution water in certain instances where site-specific information is required regarding the toxic effect(s) of an effluent, elutriate, or leachate on a particular receiving water. "Upstream" water may be used as control/dilution water for receiving-water samples collected in the vicinity of a wastewater discharge, chemical spill, or other point-source of possible contamination.

Universal Test Procedures

Procedures described in this section apply to each of the toxicity tests for samples of chemical, wastewater, and receiving water described in Sections 5, 6, and 7. All aspects of the test system described in Section 3 must be incorporated into these universal test procedures. A summary checklist in Table 3 describes recommended universal procedures for performing growth inhibition tests with *Lemna minor*, as well as conditions and procedures for testing specific types of substances or materials.

Universal procedures are described herein for performing a 7-day toxicity test. They include the following two test options:

(1) a static test, where the test solutions are not renewed during the test; and

(2) a static-renewal test, where the test solutions are replaced at least every three days during the test.

The static-renewal option is recommended for test solutions where the concentration of the test substance (or a biologically active component) can be expected to decrease significantly during the test period[38] due to factors such as volatilization, photodegradation, *precipitation*, or biodegradation (ITM, 1990; OECD, 1998, 2002).[39]

Biological endpoints measured are the increase in frond number during the test, as well as the dry weight of fronds at the end of the test.

4.1 Preparing Test Solutions

All vessels, measurement and stirring devices, *Lemna* transfer apparatus (e.g., inoculating loops), and other equipment must be thoroughly cleaned and rinsed in accordance with standard operating procedures (see EC [1992a] for glassware cleaning procedures). Distilled or deionized water should be used as the final rinse for items that are to be used immediately in setting up the test. If items are to be stored, they should be rinsed in distilled or deionized water, oven dried, and covered to avoid contamination before use.

For a given test, the same control/dilution water (test medium) must be used for preparing the control and all test concentrations. Fresh control/dilution water should be prepared as outlined in Section 5.3 if testing chemicals, Section 6.3 if testing wastewaters, and Section 7.3 if testing receiving waters.

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[38] Water solubility and vapour pressure, along with other useful information gathered on the test substance (see Section 5.1), will help to indicate if significant losses of the test substance during the test period are likely and whether steps to control such losses should be taken (OECD, 1998, 2002). Historical data (i.e., on samples of wastewater) may also give some indication as to whether the static-renewal option should be chosen for a given test.

[39] Wang (1991) demonstrated the value and suitability of using the static-renewal option with *L. minor* for testing unstable substances. In his study, Wang found that unionized ammonia-N did not inhibit duckweed growth up to 8.85 mg/L using the static option; however, in daily renewal tests, concentrations of > 3.0 mg/L depressed duckweed growth by ≥20%, and a concentration of 7.16 mg/L of unionized ammonia-N caused a 50% reduction in *Lemna* growth (IC50).

Table 3 Checklist of Recommended Test Conditions and Procedures for Conducting Toxicity Tests Using *Lemna minor*

Universal

Test type	- static or static-renewal; 7-day test
Solution renewal	- at least every three days for static-renewal option; none for static option
Control/dilution water	- test medium (modified APHA medium, SIS medium, or modified Steinberg medium); nutrient-spiked receiving water (spiked with the same nutrients used in test medium) to assess toxic effect at a specific location (for this option, there must be an additional control comprised of the test medium)
Test organisms	- *Lemna minor* from 7- to 10-day old culture (test culture), acclimated for 18 to 24 hours in test medium; two, 3-frond plants/replicate
Number of concentrations	- minimum of 7, plus control(s); recommend more (i.e., >8), plus control(s)

Number of replicates	- For single-concentration test: ≥3 replicates/treatment
	- For multi-concentration test:
	  - ≥4 replicates/treatment for equal replicate test design; or
	  - regression design; unequal replicates among test treatments:
	  - 6 replicates for control(s)
	  - 4 replicates for lowest 3-5 test concentrations
	  - 3 replicates for highest 4-5 test concentrations

Vessel/solution	- test vessels should be disposable polystyrene cups or Erlenmeyer flasks; may be crystallizing dishes, petri dishes, or glass beakers; require no overlapping of *Lemna* fronds in controls at test end; volume ≥100 mL, preferably 150 mL; covered - test vessels should be placed on a non-reflective dark background for test duration
Temperature	- daily mean of 25 ± 2°C throughout the test
Filtration	- none for wastewater samples, unless algae present; receiving-water samples must be filtered through glass fibre filters (pore size ~1μm); additional filtration through 0.22 μm filters is optional.
Nutrient spiking	- test samples are spiked with the same nutrients, at the same concentrations as those in the test medium; receiving-water samples or wastewater samples containing algae are spiked following sample filtration (if sample filtration is required)
Aeration	- wastewater and receiving-water samples must be gently pre-aerated for 20 minutes at a minimal rate (e.g., 100 bubbles per min.) before test initiation or renewal of test solutions
pH	- no adjustment if pH of test solution is in the range 6.5 to 9.5 for tests with modified APHA medium, 6.0 to 8.0 for SIS medium and 5.0 to 8.0 for modified Steinberg medium; a second (pH-adjusted) test might be required or appropriate, for pH beyond this range
Lighting	- Full-spectrum (fluorescent or equivalent); light must be continuous, and selected light fluence rate must be 64 to 90 μmol/($m^2 \cdot s$) at surface of test solution; fluence rate in the entire test area should be within ±15% of the selected light intensity

Table 3 - Continued

Observations	- number of fronds and appearance at test start and test end (Day 7); dry weight at test end; optional counting of fronds on two other occasions during the test for growth rate calculation
Measurements	- temperature measured daily in representative vessels; for static test, pH measured at start and end of the test in representative concentrations; for static-renewal test, pH measured at start and end of test and before and after each test solution renewal in representative concentrations; light fluence rate measured at several locations in the test area once during the test
Endpoints	- growth based on increase in the number of fronds during the test and dry weight at the end of the test; if multi-concentration test, ICp
Reference toxicant	- Ni or KCl; 7-day test for ICp (growth) started within 14 days of test-period, following the same procedure (modified APHA, SIS, or modified Steinberg) as the definitive test
Test validity	- invalid if the number of fronds in controls at the end of the 7-day test period is <8-times the original number of fronds (i.e., the mean number of fronds per control vessel is <48 at test end)
Chemicals	
Solvents	- only in special circumstances; maximum concentration 0.1 mL/L; a second control with solvent is required
Concentration	- recommended measurements are at the beginning and end of exposure, in high, medium, and low strengths and in the control(s) for the static option; and at the beginning and end of each renewal period, in high, medium, and low strengths and in the control(s) for the static-renewal option
Control/dilution water	- SIS or modified Steinberg medium; APHA medium if metals are being tested; nutrient-spiked receiving water can be used if the objective is to assess local toxic effect(s)
Effluents, Elutriates, and Leachates	
Sample requirement	- for static tests performed off-site, a single sample is collected (or prepared, if elutriate); for static-renewal tests performed off-site, either 3 subsamples from a single sampling or ≥3 separate samples are collected (or prepared, if elutriate) and handled as indicated in Section 6.1; for on-site tests, samples are collected every 3 days and used within 24 h; volumes of ≥1 L (single concentration test) or ≥4 L (multiple-concentration test)
Transport and storage	- If warm (> 7°C), must cool to 1 to 7°C with regular ice (not dry ice) or frozen gel packs upon collection; transport in the dark at 1 to 7°C (preferably 4 ± 2°C) using regular ice or frozen gel packs as necessary; sample must not freeze during transit or storage; store in the dark at 4 ± 2°C; use in testing should begin as soon as possible after collection and must start within 3 days of sample collection or elutriate extraction
Control/dilution water	- modified APHA medium; nutrient-spiked receiving water may be used for monitoring and compliance

Table 3 - Continued

Receiving water

Sample requirement	- as for effluents, leachates, and elutriates
Transport and storage	- as for effluents, leachates, and elutriates
Control/dilution water	- modified APHA medium; nutrient-spiked “upstream” receiving water for estimating local effect(s)

The characteristics of the control/dilution water used throughout the test period should be uniform. If the static-renewal option is used, uniformity is improved in a sample if a volume of control/dilution water sufficient to complete the test is properly stored and aliquots used for the periodic renewal of test solutions (Section 4.3).

If receiving or upstream water is used as control/dilution water to simulate local situations such as effluent discharge, a chemical spill, or pesticide spraying, a second control solution must be prepared using test medium (modified APHA medium, SIS medium, or modified Steinberg medium; see Sections 5.3, 5.6, 6.3, and 6.6). Upstream or receiving water cannot be used, however, if it is clearly toxic and produces an invalid result in the control according to the criteria of this growth test.[40] In such a case, test medium should be used as control/dilution water.

The temperature of the control/dilution water and the sample or each test solution must be adjusted as necessary to within ± 2°C of the test temperature, before starting the test. Sample or test solutions may be adjusted to the test temperature by heating or chilling in a water bath, but must not be heated by immersion heaters, since this could alter chemical constituents and toxicity.

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[40] Contaminants already in the receiving water, might not affect the controls by themselves, but could alter the toxicity of the substance or material being tested. In such cases, uncontaminated dilution water (test medium) would give a more accurate estimate of the individual toxicity of the substance or material being tested, but not necessarily of the total toxic effect at the site of interest.

If the intent of the test is to determine the effect of a substance or material on a specific receiving water, the receiving water should be used for control/dilution water regardless of whether it mitigates (e.g., through the presence of humic acids) or enhances toxicity (e.g., through additive effects of toxicant in the receiving water). In the case of toxicity being added by the receiving water, it would be appropriate to include in the test, as a minimum, a second control of laboratory test medium and, as a maximum, another series of concentrations using such "clean" test medium as dilution water.

If the intent of the test is to measure the extent to which a particular receiving water might modify the toxicity of the test material or substance due to its physicochemical characteristics (e.g., hardness, pH, *turbidity*, humic or fulvic acid content) and/or the presence of other contaminants, the investigator might choose to use the upstream water to prepare the test concentrations and as one of the control solutions. A comparison of results for this water with those for the controls held in laboratory water will identify toxic responses that might be contributed by the upstream water. A clearer understanding of the differing influence of each type of control/dilution water on the toxicity of the test material or substance can be achieved by undertaking a side-by-side comparison for toxic effects using each control/dilution water to prepare a series of test concentrations.

If a sample requires filtration (i.e., receiving-water sample or wastewater sample containing algae), then it is filtered through a glass fibre filter (pore size ~ 1µm, e.g., Whatman GF/C filters) before testing (see Sections 6.2 and 7.2). The pH of the sample is then recorded. An aliquot of each of the same nutrient stock solutions used to prepare the modified APHA medium (i.e., stock solutions A, B, and C) is then added to the wastewater or receiving-water sample at a ratio of 10 mL aliquot per 1000-mL sample. This dilutes the sample to 97%, which is the maximum concentration of wastewater or receiving water (or any sample that requires a v:v dilution) that can be tested. The nominal concentrations of the solutions corrected for the volume of nutrient stock (or for chemicals, measured concentrations; see Section 5.4) are adopted as the test concentrations.

Samples of effluent, elutriate, leachate, and receiving water must then be pre-aerated before they are used to set up test solutions. Pre-aeration of spiked wastewater and receiving-water samples serves to equilibrate the sample with the added nutrients and stabilize the sample pH after the addition of the nutrient stock solutions. Oil-free compressed air should be dispensed through airline tubing and a disposable plastic or glass tube (e.g., capillary tubing or a pipette with an Eppendorf tip) with a small aperture (e.g., 0.5 mm ID). The rate of aeration should not exceed 100 bubbles/min[41], and the duration of pre-aeration must be 20 minutes.[42]

Adjustment of sample/solution pH might be necessary (see Section 4.3.1). Solutions of hydrochloric acid (HCl) or sodium hydroxide (NaOH) at strengths ≤ 1 *N* should normally be used for all pH adjustments. Some situations (e.g., effluent samples with highly buffered pH) could require the use of higher strengths of acid or base.

For any test that is intended to estimate the *ICp* (see Section 4.5), at least seven concentrations plus a control solution (100% test medium) must be prepared, and more (>8 plus a control) are recommended to improve the likelihood of bracketing each endpoint sought. An appropriate geometric series may be used in which each successive concentration is about a factor of 0.5 of the previous one (e.g., 100, 50, 25, 12.5, 6.3, 3.1, 1.6 or, in the case of wastewater and receiving-water samples, 97, 48.5, 24.3, 12.1, 6.1, 3.0, 1.5). Test concentrations may be selected from other appropriate dilution series (e.g., 100, 75, 56, 42, 32, 24, 18, 13, 10, 7.5; see column 7 in Appendix G). Usually, there is not a great improvement in precision of the test from the use of concentrations closer together than those obtained with the 50% dilution. In routine tests, concentrations should not be more widely spaced than those obtained using a factor of 0.3, because this leads to poor precision of the toxicity endpoint estimate. If there is considerable uncertainty about the toxic levels, more concentrations should be used to obtain a greater spread, rather than using a lower *dilution factor* for wider spacing.

Test dilutions can be prepared directly in the test vessels. First, the appropriate volumes of control/dilution water are pipetted into the individual test vessels. Nutrient-spiked, pre-aerated test sample is then added to each test vessel, and the mixtures thoroughly mixed to achieve the desired test concentrations. Alternatively, test dilutions can be prepared in volumetric flasks and then distributed to the *replicate* test vessels. Test vessels are left at

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[41] More vigorous aeration might strip volatile chemicals from the sample, or might increase their rate of oxidation and degradation to other substances or materials. Therefore, minimal rates (i.e., 100 bubbles/min) and duration (i.e., 20 min) are used for pre-aeration of wastewater and receiving-water samples.

[42] Pre-aeration rate and duration are consistent with procedures used in other Environment Canada biological test methods (EC 1992b; 1992c).

room temperature for 1 h to allow equilibration of the medium and toxicant.

In cases of appreciable uncertainty about sample toxicity, it is beneficial to run a range-finding (or screening) test for the sole purpose of choosing concentrations for the definitive test. Conditions and procedures for running the screening test should be identical to the definitive test; however, the experimental design might differ. A wide range of concentrations (e.g., ≥2 orders of magnitude) should assist in selection of the concentrations for the definitive test.

Single-concentration tests used for regulatory purposes (e.g., pass/fail), would normally use full-strength (or 97% in the case of this method) effluent, leachate, receiving water, elutriate, or an arbitrary or prescribed concentration of chemical. Use of controls would follow the same rationale as multi-concentration tests. Single-concentration tests are not specifically described herein, but procedures are evident, and all items apply except for testing only a single concentration and a control.

For a single-concentration test, a minimum of three replicate test vessels and three replicate control vessels must be set up. For a multi-concentration test, either equal or unequal replication across *treatments* can be used. If replication is equal across treatments, at least four replicate test vessels must be set up for each treatment. Alternatively, unequal replication across treatments (i.e., regression design) may be used when historical data is available and/or the laboratory has experience with the dose response.[43] If replication is unequal across treatments, six replicate vessels should be prepared for the control(s), four replicate vessels should be prepared for the lowest 3-5 test concentrations, and three replicate vessels should be prepared for the highest 4-5 test concentrations.

4.2 Beginning the Test

Once the test solutions have been prepared and any permitted and/or required adjustments made for temperature, pH, and filtration (see Sections 4.1, 6.2, and 7.2), the test should be initiated.

Lemna fronds used in the test must be from cultures that satisfy the requirements indicated in Section 2.3 and the health criteria given in Section 2.3.8. For multi-concentration tests, 3-frond plants, of identical (or as identical as possible) size and condition,[44] are selected from the acclimated culture for use in setting up the test. The plants may be transferred directly from the acclimated culture into the test cups. Alternatively, 3-frond plants may be selected from the acclimated culture and transferred to a shallow dish containing fresh test medium before being transferred to the test cups. This latter procedure is particularly useful, since the investigator can ensure that there are an adequate number of *Lemna* plants, of identical quality, before initiating the test (Moody, 1998).

An identical number of fronds must be added to each test vessel. To begin the test, two, 3-frond *Lemna* plants are randomly assigned or transferred to each test vessel (for a total of 6 fronds per test vessel) using a disposable plastic sterile inoculating loop. The plants are submersed briefly in the test solution. Care must be taken to not contaminate the *Lemna* designated for use in the test while transferring the plants to their individual test cups. If the plants are being selected directly from the acclimated culture or from a single dish of washed *Lemna* allocated for use in the test (see

[43] The unbalanced nature of the regression design (i.e., unequal replicates among treatments) allows for the same level of effort but increased focus where needed in the dose-response curve.

[44] Plants that appear in good condition must be used to set up the test. Characteristics indicative of good plant health include bright green fronds, no discoloured areas, and no extra small frond buds (see Figure 2).

above), a separate inoculating loop for each plant should be used or the inoculating loop should be rinsed in distilled/deionized water before it is returned into the dish of washed *Lemna*. Alternatively, enough *Lemna* plants can be placed into a shallow dish filled with test medium, designated for division between the replicates in a single test concentration. A single inoculating loop can then be used to transfer the *Lemna* plants into each test cup at a given test concentration. Care must be taken to ensure that the plant does not adhere to the side of the cup and that the roots are inside the cup. Any plants that break apart during the transfer process must be replaced.

In carrying out these procedures, there must be formal random assignment of organisms to test vessels. The group of replicate vessels representing a particular treatment (e.g., a specific test concentration) must also be in randomized positions in the environmental chamber or test area. The test vessels must be coded or labelled to enable proper identification of the sample and its concentration. The date and time that the test is started must be recorded on separate data sheets dedicated to the test.

Lemna transfers should be done in a clean, draft-free area, as quickly as possible, to minimize contamination of the colonies. Once the plants have been placed into the test vessels, care should be taken not to swirl or agitate the vessels as plants may adhere to the sides of the vessel. The day the *Lemna* plants are initially exposed to solutions of test substance is designated Day 0. Day 7, therefore, is the day the test is terminated.

4.3 Test Conditions

The duration of the *L. minor* growth inhibition test is 7 days. The test can be a static type, or, in the case of degradable test substances or materials or chemicals, a static-renewal test. The test solutions are not changed for the duration of the test if a static test is done.

If the static-renewal option is chosen, each test solution must be replaced every 3 days (i.e., on Days 3 and 5), or more frequently, during the test (see Sections 5.2 and 6.1).[45] Replacement solutions and test vessels should be prepared, as described in Section 4.1. *Lemna* colonies must be transferred carefully, with an effort to minimize contamination, to respective vessels containing fresh test solutions. The transfer of *Lemna* to new test solutions must be done in random order across the replicates within a concentration and should follow procedures for handling the plants (see Section 4.2). The physical/chemical characteristics of the old solutions should be determined (see Section 4.4) and then the test solutions should be discarded (following provincial and federal regulations) or stored if additional chemical determinations are required (see Section 5.4).

Tests are initiated using two *Lemna* plants per 100-mL (or 150-mL) volume of test solution in each replicate test vessel (see Sections 3.3 and 4.1).

The test must be conducted at a daily mean temperature of 25 ± 2°C. Light conditions must be as described in Section 3.2. Test solutions must not be aerated during the test, and the test must end seven days after initiation.

The test must be considered invalid if the mean number of fronds in the controls has not increased to ≥8-times the original number fronds by the end of the test (i.e., the mean number of fronds per control test vessel must be ≥48 at the end of the test, for the test to be valid).

4.3.1 pH

Toxicity tests should normally be conducted without adjustment of pH. However, if the sample of test substance causes the pH of any test

[45] More frequent renewal of test solutions may be required in chemical testing to maintain 80% of the initial concentration of the test substance (USEPA, 1996; OECD, 1998, 2002).

solution to be outside the range 6.5 to 9.5 for tests with modified APHA medium, 6.0 to 8.0 for SIS medium and 5.0 to 8.0 for modified Steinberg medium, and the toxicity of the test substance rather than the deleterious or modifying effects of pH is being assessed, the pH of the test solutions or sample should be adjusted, or a second, pH-adjusted test should be conducted concurrently. For this second test, the initial pH of the sample, the test solutions, or of each fresh solution before renewal (static-renewal tests) may, depending on the objectives, be neutralized (adjusted to pH 7.0) or adjusted to within ± 0.5 pH units of that of the control/dilution water, before *Lemna* exposure. Another acceptable approach for this second test is to adjust the pH of the sample upwards to pH 5.0 to 7.0 (if the sample has/causes a pH <5.0), or downward to pH 9.0 to 9.5 (if the sample has/causes a pH > 9.5). Solutions of hydrochloric acid (HCl) or sodium hydroxide (NaOH) at strengths ≤1 *N* should normally be used for all pH adjustments. Some situations (e.g., effluent samples with highly buffered pH) might require higher strengths of acid or base.

If sample pH is to be adjusted, it is done so after the addition of the nutrient stock solutions and pre-aeration (see Section 4.1). If adjustment of the pH by more than 0.5 units is required, a further 30-minute period of aeration followed by another pH adjustment is recommended (SRC, 1997). Abernethy and Westlake (1989) provide useful guidelines for adjusting pH. Aliquots of samples or test solutions receiving pH-adjustment should be allowed to equilibrate after each incremental addition of acid or base. The amount of time required for equilibration will depend on the buffering capacity of the solution/sample. For effluent samples, a period of 30 to 60 minutes is recommended for pH adjustment (Abernethy and Westlake, 1989). Once the test is initiated, the pH of each solution is monitored, but not adjusted. Volumes of nutrient spikes, and NaOH and HCl used in pH adjustment, must be recorded and used to calculate the nominal concentration of the test substance at the beginning of the test.

If the purpose of the toxicity test is to gain an understanding of the nature of the toxicants in the test substance, pH adjustment is frequently used as one of a number of techniques (e.g., oxidation, filtration, air stripping, addition of chelating agent) for characterizing and identifying sample toxicity. These "*Toxicity Identification Evaluation*" (TIE) techniques provide the investigator with useful procedures for assessing the physical/chemical nature of the toxicant(s) and their susceptibility to detoxification (USEPA, 1991a; 1991b).

4.4 Test Observations and Measurements

The fronds in each vessel must be observed and counted at the beginning and end of the test (Day 0 and Day 7).[46] Control solutions must receive identical treatment. Observation is improved if a magnifying glass, dissecting microscope, or other magnifying device is used to observe plants and a light is directed into the side or bottom of the cup.

The number of fronds in each test vessel must be counted and recorded at each observation. The count must include every frond[47] and every visible protruding bud. Observations of the following should also be made and recorded for

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[46] Two more observations of frond number in each test vessel should be made during the test (e.g., Days 3 and 5) if an investigator wishes to calculate the average specific growth rate (also known as relative growth rate; based on changes in frond number determined during the course of the seven-day exposure period in controls and in each treatment group) and/or area under the curve (based on frond number in the controls and each treatment group, as integrated with exposure period) (Section 4.5.5).

[47] All fronds, regardless of their colour or condition, are counted and included in the endpoint calculation.

each test vessel: *chlorosis* (loss of pigment); *necrosis* (localized dead tissue on fronds, which appears brown or white); yellow or abnormally sized fronds; *gibbosity* (humped or swollen appearance); *colony* destruction (single fronds); root destruction; and loss of buoyancy (see Figure 2).

Temperature must be monitored throughout the test. As a minimum, temperature must be measured daily in representative test vessels (i.e., in at least the high, medium, and low concentrations plus the control solutions in a multi-concentration test). Extra test vessels may be prepared for the purpose of measuring water temperature during the test. If temperature records are based on measurements other than in the test vessels (e.g., in the incubator or controlled-temperature room within the vicinity of the test vessels), the relationship between these readings and temperature within the vessels must be established. Continuous recordings or daily measurement of the maximum and minimum temperatures are acceptable options.

For both static and static-renewal exposures, the pH must be measured at the beginning of the test, before the *Lemna* plants are added and at the end of the test, in at least the high, medium, and low test concentrations and in the control(s). For static-renewal exposures, the pH must also be measured immediately before and immediately after each test solution renewal (i.e., in fresh solutions and those to be discarded) in at least the high, medium, and low test concentrations and in the control(s).

Light fluence rate must be measured at least once during the test period at points approximately the same distance from the light source as the *Lemna* fronds and at several locations in the test area.

The general appearance of test samples and any changes that occur during the preparation of the test solutions should be noted and recorded as well as any changes in the appearance of test solutions observed during the test period (see Sections 5.4, 6.4, and 7.4).

The number of fronds are recorded for each replicate of the control and the various concentrations of the test substance at the beginning and end of the 7-day exposure. Vessels that have fronds or colonies accidentally removed or stuck (and dried) to their sides during the test should be removed from the test and that replicate should be eliminated from endpoint calculations.

Once the *Lemna* fronds are counted, they are dried and weighed. For each vessel of test solution, dry weight is determined for the *Lemna* fronds as a group. Colonies in the respective vessels (including the roots) are collected, blotted dry[48], and dried immediately in a drying oven in small tared and numbered weighing boats, at either 100°C for six hours or at 60°C for 24 hours. Upon removal from the oven, the boats must be moved immediately to a desiccator. Thereafter, the boats should be individually and randomly removed from the desiccator, and weighed on a balance that measures consistently to 0.01 mg. To avoid excessive and inconsistent absorption of water vapour, rapid weighing and standard timing among boats is necessary. Trays should be removed in random order for weighing, and the first one weighed should be replaced in the desiccator and weighed again at the end as a check on gain of water by the last trays weighed. The change should not be >5%. If it is, the trays should be re-dried for 1 to 2 hours and then re-weighed. A few weighing boats should be tared, dried, and weighed without plants, and results should conform to the

[48] Plants can be collected in a petri dish covered with fine netting or with a fine-mesh bottom. Plants should then be rinsed with deionized water (using a spray bottle). Excess water is blotted by pressing absorbent paper against the net or mesh petri dish. Plants can then be transferred to weigh boats by inverting the petri dish over the weigh boat (ITM, 1990).

laboratory's quality control standards. The total dry weight of fronds in each test vessel (i.e., in each replicate of each test concentration and the control) must be determined.

4.5 Test Endpoints and Calculations

The endpoints of the test are based on the adverse effects of test materials or substances on the growth of *L. minor*, assessed by comparison with the controls. There are two biological endpoints for the test, the first is based on the reduction of the increase in the number of fronds compared to the control, and the second is based on a decrease in the final dry weight of the fronds compared to the control. The increase in frond number is calculated by subtracting the initial number of fronds in a given test vessel from the final number of fronds in the same test vessel. The biological endpoint for frond dry weight measures the total dry weight of *Lemna* fronds compared to the control at the end of the test (Day 7). This is essentially a measurement of growth, except that no determination of initial weight is made.

4.5.1 Validity of Test

Assuming that all the recommended procedures and conditions were followed[49], the mean number of fronds in the controls must have increased to ≥8-times the original number of fronds by the end of the 7-day test period in order for the test to be valid (i.e., mean number of fronds in the controls must be ≥48 per test vessel at the end of the test, for the test to be valid).

4.5.2 Multi-Concentration Tests

In a multi-concentration test, the required statistical endpoint for growth data (frond number, frond dry weight) is an ICp[50,51] and its 95% confidence limits. A separate ICp and its 95% confidence limits must be calculated for each of the two biological endpoints (i.e., one for reduction of increase in frond number and one for reduction of total dry weight). For derivation of ICp and the 95% confidence limits, the *quantitative* measurement endpoints are used directly (i.e., increase in frond number and total dry weight). Environment Canada (2005) provides direction and advice for calculating the ICp, including decision flowcharts to guide the selection of appropriate statistical tests. All statistical tests used to derive endpoints require that concentrations be entered as logarithms and if applicable, that concentrations be corrected for the volume of nutrient stock (i.e., 97% dilution)..

An initial plot of the raw data (increase in frond number, dry weight) against the logarithm of concentration is highly recommended, both for a visual representation of the data, and to check for reasonable results by comparison with later statistical computations.[52] Any major disparity

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[49] More specifically, it is assumed that all items of apparatus and all test materials or substances were identical in each replicate; all concentrations were assigned randomly to replicates; all organisms were assigned randomly to replicates; the test was not terminated prematurely; all required physicochemical variables were monitored as prescribed; and all required biological variables were monitored as prescribed.

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

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

[52] As an alternative to plotting the raw data, investigators might choose to calculate and plot the percent inhibition for each test concentration; this calculation is the difference

between the approximate graphic ICp and the subsequent computer-derived ICp must be resolved. The graph would also show whether a logical relationship was obtained between log concentration (or, in certain instances, concentration) and effect, a desirable feature of a valid test (EC, 2005).

Regression analysis is the principal statistical technique to be used for calculation of the ICp. A number of models are available to assess growth data (using a quantitative statistical test) via regression analysis. Use of regression techniques requires that the data meet assumptions of *normality* and *homoscedasticity*. Weighting techniques may be applied to achieve the assumption of *homoscedasticity*. The data are also assessed for outliers using one of the recommended techniques (see Section 10.2 in EC, 2005). Any statistical analyses conducted without outliers should also be conducted with the outliers. Any outliers and the justification for their removal must be reported. Finally, the model with the best fit[53] must be chosen as the most appropriate for generation of the ICp and associated 95% confidence limits. Endpoints generated by regression analysis must be bracketed by test concentrations; extrapolation of endpoints beyond the highest test concentration is not an acceptable practice.

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

In the event that the data do not lend themselves to regression analysis, linear interpolation (e.g., ICPIN; see Section 6.4.3 in EC, 2005) can be used in an attempt to derive an ICp. The same decision-making for statistical analysis must be followed for each of the two *Lemna minor* test endpoints (i.e., frond increase and frond dry weight) independently. For example, if frond increase data cannot be analyzed by regression, and the analyst defaults to ICPIN, regression analysis must still be attempted on the frond dry weight data. The fact that the first endpoint examined is analyzed by ICPIN does not preclude regression analysis for the second endpoint.

For each test concentration including the control treatment(s), the following calculations must be performed and reported: (i) the mean (± SD) of the increase in frond number in each treatment, including control(s) as determined at test end, and (ii) the mean ± SD for dry weight of *Lemna*

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between the average control response and the treatment response (average control response minus average treatment response in the numerator), divided by the average control response (denominator), expressed as a percentage (multiplied by 100%). The value for each treatment is graphed against the concentration; see ASTM (1991) for more details. The x-axis represents log concentration or, in some instances, concentration, depending on the preferences and purpose of the investigator. For example, using a log scale will match the regression data scales, but concentration might be clearer in the final report. To improve the use of a graph as a visual representation of the data, the investigator might choose to include the regression line as well as the raw data.

[53] As described in Section 6.5.8 of EC (2005), Environment Canada's current guidance on statistical methods for environmental toxicity tests specifies the use of the following five models for regression analysis, when estimating the ICp: linear, logistic, Gompertz, exponential and *hormesis* (logistic adapted for hormetic effect at low doses). Specific mathematical expressions of the model, including worked examples for a common statistics package, are also provided in that guidance document (Section 6.5.8 and Appendix O in EC, 2005). More than one model must be fit to the data. The lowest residual mean square error is recommended to determine best fit; it is available in the ANOVA table for any of the models.

fronds in each treatment, including control(s) as determined at test end.

4.5.3 Single-Concentration Tests

In single-concentration tests, the response in the test concentration is compared with the control response[54]. If frond number and dry weight (quantitative data) are assessed at a single test site and control site, a t-test[55] is normally the appropriate method of comparing the data from the test concentration with that for the control. In situations where more than one test site is under study, and the investigator wishes to compare multiple sites with the control, or compare sites with each other, a variety of ANOVA (or non-parametric equivalent) tests exist (Section 3.3. in EC, 2005). Choice of the test to use depends on:

(i) the type of comparison that is sought (e.g. complete a series of pairwise comparisons between all sites or compare the data for each location with that for the control only);
(ii) if a chemical and/or biological response gradient is expected, and
(iii) if the assumptions of *normality* and *homoscedasticity* are met.

As with multi-concentration tests, other calculations which must be performed and reported when performing a single-concentration test include: (i) the mean (± SD) of the increase in frond number in each treatment, including control(s) as determined at test end, and (ii) the mean ± SD for dry weight of *Lemna* fronds in each treatment, including control(s), as determined at test end.

4.5.4 Stimulatory Effects

A *stimulatory effect* (increased response at all concentrations or at high concentrations) must be reported for all concentrations in which significant stimulation was observed. If a stimulatory effect was observed, statistical comparison with controls is performed using ANOVA analysis, followed by appropriate pairwise comparisons with control (see Section 3.3 and 7.5 of EC, 2005). This analysis will identify which concentrations show a stimulatory effect that is significantly different from controls. The percent stimulation for these concentrations must be reported, using the following calculation (USEPA, 2002)[56]:

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

Where:

S(%) = percent stimulation
T = average increase in frond number, or average total dry weight of fronds at test end in test vessel
C = average increase in frond number; or average total dry weight of fronds in the controls

[54] See Sections 4.1, 5.3, 6.3 and 7.3 for a description of the type(s) of control/dilution water that could be used in a single-concentration test.

[55] Strictly speaking, the t-test assumes a t-distribution and equal variances in the two groups. Tests for distribution and equal variances have been outlined, and alternatives in the case of unequal variances are recommended (EC, 2005).

[56] USEPA (2002) specifies T = mean effluent or surface water response and C = mean control response; these values have been further specified to those listed in the equation above.

4.5.5 Other Test Designs and Purposes

Average specific growth rate (or relative growth rate)[57] and/or area under the curve[58] can also be

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[57] To determine the average specific growth rate for each test concentration and control, frond numbers for each replicate in the controls and each treatment at each observation time are plotted against time as a semilogarithmic graph to produce growth curves. The average specific growth rate for a specific period is calculated as the slope of the logarithmic growth curve from the equation (OECD, 2002):

$$\mu_{i-j} = \frac{\ln(N_j) - \ln(N_i)}{t_j - t_i}$$

where: μ_{i-j} is the average specific growth rate from moment time i to j;
N_i is the number of fronds observed in the test or control vessel at time i;
N_j is the number of fronds observed in the test or control vessel at time j;
t_i is the moment time for the start of the period; and
t_j is the moment time for the end of the period.

The average specific growth rate for exponentially growing cultures (or where growth is closer to an exponential pattern than a linear one) and if no significant periods of lag or stagnancy are observed, and if the course of the growth curve is monotonous, the average specific growth rate can be derived from the slope of the regression line in a plot of ln N versus time.

Percent inhibition of growth rate, I_r can then be calculated for each test concentration according to the following formula:

$$\%I_r = \frac{(\mu_C - \mu_T)}{\mu_C} \times 100$$

where: $\%I_r$ is the percent inhibition in average specific growth rate;
μ_C is the mean value for μ in the control; and
μ_T is the mean value for μ in the treatment group.

[58] The area under the growth curves can be calculated for each control and treatment replicate according to the following equation (OECD, 2002):

$$A = \frac{\ln N_1 - \ln N_0}{2} t_1 + \frac{\ln N_1 + \ln N_2 - 2\ln N_0}{2} (t_2 - t_1) + \ldots + \frac{\ln N_{n-1} + \ln N_n - 2\ln N_0}{2} (t_n - t_{n-1})$$

where: A is the area under the growth curve;
N_0 is the number of fronds observed in the test or control vessel at the start of the test (t_0);
N_1 is the number of fronds observed in the test or control vessel at time t_1;
N_n is the number of fronds observed in the test or control vessel at time t_n;
t_1 is the time of first measurement after beginning of test; and
t_n is the time of the n^{th} measurement after beginning the test.

The area should be calculated for the entire test period, or a rationale for selecting only a portion of the growth curve provided. For each test concentration and control, a mean area is calculated, with variance estimates.

Percent inhibition of area under the curve, I_a, can then be calculated for each test concentration according to the following formula:

$$\%I_a = \frac{(A_C - A_T)}{A_C} \times 100$$

where: A_C is the mean value for area under the curve in the control group; and
A_T is the mean value for area under the curve in the treatment group.

calculated based on frond numbers in each replicate; however, measurements at intervals during the test (e.g., Days 3 and 5) are required for both average specific growth rate and area under the curve estimate (ASTM, 1997; OECD, 1998, 2002).[59]

4.6 Reference Toxicant

The routine use of a reference toxicant or toxicants is practical and necessary to assess, under standardized conditions, the relative sensitivity of the culture of *Lemna* being used, and the precision and reliability of data produced by the laboratory for the selected reference toxicant (EC, 1990). Sensitivity of *Lemna* to reference toxicant(s) must be evaluated within 14 days of the toxicity test (i.e., the reference toxicity test must be started within 14 days of the period over which the test was conducted). The same test culture (7- to 10-days old) may be used for tests with both the reference toxicant and sample(s). The reference toxicity test must be performed under the same experimental conditions as those used with the test sample(s).

Criteria used in recommending the appropriate reference toxicants for this test include:

- chemical readily available in pure form;
- stable (long) shelf life of chemical;
- highly soluble in water;
- stable in aqueous solution;
- minimal hazard posed to user;
- easily analyzed with precision;
- good dose-response curve for *L. minor*; and
- knowledge of the degree and type of any influence of pH on toxicity of chemical to test organism.

Reagent-grade nickel (Ni)[60] and/or potassium chloride (KCl)[61] are recommended for use as

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[59] Estimates of toxicity expressed in terms of final biomass are generally more sensitive than those based on average specific growth rate (Sims *et al*., 1999). The average specific growth rate, however, is advantageous for comparing data from tests having different exposure times since the average specific growth rate or relative growth rate is less dependent on the time of exposure than endpoints based on final biomass (e.g., frond number or dry weight) (Nyholm, 1990). Also, the intrinsic growth rates of duckweeds are not constant over time, even under controlled laboratory conditions (Huebert and Shay, 1993). Calculation of the average specific growth rate requires measurements of effect at intervals during the test and requires that growth in the controls is exponential. If growth in the controls is not exponential, then it is preferable to base estimates of toxicity on area under the curve rather than average specific growth rate (OECD, 1998).

Another advantage of examining the growth rate or area under the growth curve is that valuable information can be gained by looking at the time of toxic effect on growth. For example, the growth curve might show an immediate toxic effect that does not change over time, an initial toxic effect that decreases over time, or a toxic reaction where toxicity is not displayed until several days after test initiation (ASTM, 1997).

[60] Several problems related to the use of chromium (Cr) as a reference toxicant for *L. minor* lead to the investigation of several metals (Zn, Cd, Cu, and Ni) as potential alternatives. Ni was considered favourable due to the relatively steep dose response curve produced (i.e., little flattening at higher concentrations as seen for Zn, Cu, and Cd; SRC, 2003). Further reference toxicity testing with Ni and UTCC *L. minor* strain 492 produced a mean IC25 for frond increase, based on nominal concentrations of Ni, of 13.2 µg/L (SRC, 2005).

[61] Potassium chloride (KCl) has been used successfully as a reference toxicant for *L. minor* tests. The mean IC25 for KCl was 4840 mg/L (n = 20) and

the reference toxicant(s) for this test. If Ni is used as the reference toxicant(s), it is recommended that the appropriate Material Safety Data Sheets be carefully consulted, and all necessary safety precautions be followed.

Lemna sensitivity must be evaluated by standard tests following the procedures and conditions given herein to determine the ICp for the reference toxicant(s) chosen. If nickel is chosen, nickel sulphate ($NiSO_4 \cdot 6H_2O$) should be used to prepare the stock solutions. Fresh stock solutions should be prepared for each reference toxicity test. The concentration of nickel should be expressed as mg Ni/L. Stock solutions of KCl should be prepared on the day of testing. The control/dilution water should be appropriate for the reference toxicant used (i.e., modified APHA medium for tests with Ni and modified APHA, SIS, or modified Steinberg medium for KCl).

Concentrations of reference toxicant in all stock solutions should be measured chemically using appropriate methods (e.g., APHA *et al*., 1995). Upon preparation of test solutions, aliquots should be taken from at least the control, low, middle, and high concentrations, and analyzed directly or stored for future analysis, in case the ICp is outside the *warning limits*. If stored, sample aliquots must be held in the dark at 4 ± 2°C and preserved if necessary (see APHA *et al*., 1995). Stored aliquots requiring chemical measurement should be analyzed promptly upon completion of the toxicity test. It is desirable to measure concentrations in the same solutions at the end of the test after completing biological observations. Calculations of ICp should be based on measured concentrations if they are appreciably (i.e., ≥20%) different from nominal ones, and if the accuracy of the chemical analyses is satisfactory.

Once sufficient data are available, a *warning chart*, which plots ICp values for frond number must be prepared and updated for each reference toxicant used (EC, 1990; 2005). A separate warning chart must be prepared for each *L. minor* clone used in toxicity testing since the clones can differ in their sensitivity to toxicants (see Section 2.2; footnote 11). A separate warning chart must also be prepared for each medium used in reference toxicant testing (i.e., a separate chart for testing in each of modified APHA, SIS, and modified Steinberg medium). Successive ICps are plotted on this chart and examined to determine whether the results are within ± 2 SD (= warning limits) of values obtained in previous tests using the same reference toxicant and test procedure. The mean and standard deviation of available log ICps are recalculated with each successive test until the statistics stabilize (EC, 1990; 2005). The warning chart should plot logarithm of ICp on the vertical axis against date of the test (or test number) on the horizontal axis.

The logarithm of concentration (log ICp) must be used in all calculations of mean and standard deviation. This simply represents continued adherence to the assumption by which each ICp was estimated on the basis of logarithms of concentrations. The warning chart may be constructed by plotting the logarithms of the mean and its limits on arithmetic paper, or by plotting arithmetic values on the logarithmic scale of semi-log paper. If it were definitely shown that the ICps failed to fit a log-normal distribution, an arithmetic mean and limits might prove to be more suitable.

Coefficients of Variation (CV) ranged from 21.3 to 28.3 (Jonczyk, 1998). Further KCl data showed mean IC50 for KCl of 4770 mg/L (n = 18) and a %CV of 15.9% (Stantec, 2005). Advantages of using KCl as a reference toxicant are that it is stable in solution and unaffected by water quality characteristics and it is much safer to use.

Each new ICp for the reference toxicant should be compared with the established warning limits for frond number. The ICp is considered to be acceptable if it falls within the warning limits. If a particular ICp falls outside the warning limits, the sensitivity of the *Lemna* culture and the performance and precision of the test are suspect. Since this might occur 5% of the time due to chance alone, an outlying value does not necessarily mean that the sensitivity of the *Lemna* culture or the precision of the toxicity data produced by the laboratory are in question. Rather, it provides a warning that this might be the case. A thorough check by laboratory personnel of all culturing and test conditions and procedures is required at this time. Depending on the findings, it might be necessary to repeat the reference toxicity test, and/or to prepare a new *Lemna* culture before undertaking further toxicity tests with the test organisms.

Results that remained within the warning limits would not necessarily indicate that a laboratory was generating consistent results. Extremely variable data for a reference toxicant would produce wide warning limits; a new data point could be within the warning limits but still represent undesirable variability. For guidance on reasonable variation among reference toxicant data (i.e., warning limits for a warning chart), please refer to Section 2.8.1 and Appendix F in EC, 2005.

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

4.7 Legal Considerations

Care must be taken to ensure that samples collected and tested with a view to prosecution will be admissible in court. For this purpose, legal samples must be: representative of the substance or material being sampled; uncontaminated by foreign substances or materials; identifiable as to date, time, and location of origin; clearly documented as to the chain of custody; and analyzed as soon as possible after collection. Persons responsible for conducting the test and reporting the findings must maintain continuity of evidence for court proceedings (McCaffrey, 1979), and ensure the integrity of the test results.

Specific Procedures for Testing Chemicals

This section gives particular instructions for testing chemicals, in addition to the procedures outlined in Section 4.

5.1 Properties, Labelling, and Storage of Sample

Information should be obtained on the properties of the chemical, formulated product, or chemical mixture to be tested, including concentration of the major ingredients and impurities, water solubility, vapour pressure, chemical stability, dissociation constants, toxicity to humans and aquatic organisms, and biodegradability. Data sheets on safety aspects of the substance(s) (e.g., Material Safety Data Sheets) should be consulted, if available. Where aqueous solubility is in doubt or problematic, acceptable procedures previously used for preparing aqueous solutions of the chemical(s) should be obtained and reported, and/or chemical solubility in test water should be determined experimentally. Other available information such as structural formulae, nature and *percentage* of significant impurities, presence and amounts of additives, and n-octanol:water partition coefficient, should be obtained and recorded.[62] Water solubility and vapour pressure can be used to calculate Henry's Law Constant, which will indicate if significant losses of the test substance during the test period are likely. This will help signify whether steps to control such losses should be taken (OECD, 1998, 2002) (see Section 5.2). An acceptable analytical method should be available for the chemical in water at concentrations intended for the test, together with data indicating the precision and accuracy of the analysis.

Chemical containers must be sealed and coded or labelled upon receipt. Required information (chemical name, supplier, date received, grade or purity, person responsible for testing, etc.) must be indicated on the label and/or recorded on a separate data sheet dedicated to the sample, as appropriate. Storage conditions (e.g., temperature, protection from light) are frequently dictated by the nature of the chemical. Standard operating procedures of the laboratory, or else those recommended by manufacturers, by a Material Safety Data Sheet, or by similar advisory information should be followed for handling and storage of a chemical.

5.2 Preparing Test Solutions

Solutions of the test chemical are usually prepared by adding aliquots of a stock solution made up in control/dilution water (Swedish Standard [SIS] growth medium, modified Steinberg medium, or modified APHA medium; see Section 5.3). Volumetric flasks should be used to prepare stock and test solutions. Stock solutions should normally be prepared by dissolving the test substance(s) in test medium. For some substances or materials (e.g., pesticides), a foliar application (spray) of the test substance directly onto the fronds might be applicable, if this is considered to be the most likely exposure scenario (Lockhart *et al*., 1989; Boutin *et al*., 1993; OECD, 1998, 2002). Alternatively, for strong solutions or large volumes, weighed (analytical balance) quantities of chemical may be added to control/dilution water (e.g., SIS or modified Steinberg medium) to give the nominal strengths for testing. Regardless of how test solutions are prepared, the

[62] Knowledge of the properties of the chemical will assist in determining any special precautions and requirements necessary for handling and testing it (e.g., testing in a specially vented facility, or the need to use a solvent).

concentration, solubility, and stability of the chemical in the test medium under test conditions should be determined before the test is initiated. Stock solutions subject to photolysis should be shielded from light, and unstable solutions must be prepared as frequently as necessary to maintain concentrations for each test solution renewal.

The water solubility of the test substance should not be exceeded in any test concentration (OECD, 1998, 2002).[63] For chemicals that do not dissolve readily in water, stock solutions may be prepared using the generator column technique (Billington *et al*., 1988; Shiu *et al*., 1988) or, less desirably, by ultrasonic dispersion.[64] Organic solvents, *emulsifiers*, or *dispersants* should not be used to assist chemical solubility except in instances where they might be formulated with the test chemical for its normal commercial purposes. If used, an additional control solution must be prepared containing the highest concentration of the agent used in the test. Solubilizing agents should be used sparingly, and should not exceed 0.1 mL/L in any test solution; the type and final concentration used must be reported. If solvents are used, the preferred ones are triethylene glycol and dimethyl formamide (ASTM, 1991; OECD, 1998).[65] Methanol, ethanol, and acetone could also be used but are more volatile and can stimulate the undesirable growth of microorganisms (ASTM, 1991).

The static test is recommended for use with stable chemicals, commercial products, and mixtures of known substances. However, for tests where the concentration of the test substance is not expected to remain within ±20% of the nominal concentration (or a preliminary stability test shows that the concentration of the test substance or one or more of its biologically active ingredients falls below 80% of the measured initial concentration) over the duration of the test (7 days), the static-renewal procedure must be followed (OECD, 1998, 2002). In the static-renewal test, *Lemna minor* colonies must be transferred to new test solutions on at least two occasions during the test (e.g., Days 3 and 5) (see Section 4.3). More frequent renewals might be necessary to maintain concentrations (≥80%) of highly unstable or volatile substances (USEPA, 1996; OECD, 1998, 2002).

5.3 Control/Dilution Water

For tests designed to assess toxicity of a chemical to *L. minor*, either the modified Swedish Standard (SIS) medium (OECD, 1998, 2002), the modified Steinberg medium (ISO, 2005), or receiving water spiked with SIS or modified Steinberg nutrient stock solutions (nutrient-spiked receiving water) should be used as the control/dilution water.[66] Where appropriate (e.g., for testing metals), modified APHA medium, which contains no EDTA, or receiving water spiked with modified APHA nutrient stock solutions, may be used as control/dilution water (see Section 6.3).

The control/dilution water recommended for standard use for tests with chemical samples is either the SIS medium or the modified Steinberg medium. The SIS medium consists of seven

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[63] In some cases the targeted nominal concentration should be slightly above solubility to achieve 100% solubility (as a measured concentration) in the full strength test solution.

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

[65] Dimethylformamide and triethylene glycol are commonly used solvents that do not cause phytotoxicity at concentrations up to 100 mg/L.

[66] If the purpose of the test is to harmonize with OECD's draft *Lemna* growth inhibition test (OECD, 1998, 2002), then SIS medium should be used, whereas to harmonize with the ISO draft standard (ISO, 2005), then modified Steinberg medium should be used.

stock solutions, as outlined in Table 4. Stock solutions are prepared in distilled water, or equivalent, using reagent grade chemicals. Stock solution VII (MOPS buffer) is only used for testing substances or materials in which additional pH control is required.[67] Stock solutions I to V are sterilized by autoclaving at 120°C for 15 minutes or by membrane filtration (0.2 µm pore size). Stock solutions VI and VII (optional) are sterilized by membrane filtration (0.2 µm pore size) only (they should not be autoclaved), and then they are aseptically added to the remaining stock solutions.

To prepare 1 L of SIS test medium, the following are added to 900 mL of glass-distilled, deionized water (or equivalent):

10 mL of stock solution I,
5 mL of stock solution II,
5 mL of stock solution III,
5 mL of stock solution IV,
1 mL of stock solution V, and
5 mL of stock solution VI.

If buffer is required, 1 mL of stock solution VII (optional) is also added. The pH is adjusted to 6.5 ± 0.2 with either 0.1 or 1 *N* HCl or NaOH, and adjusted to 1 L with distilled water (OECD, 1998, 2002).

Sterile stock solutions should be stored under cool and dark conditions. Stock solutions I to V have a shelf life of 6 months, whereas stock solutions VI and VII should be discarded after 1 month. The medium is stored in the dark to preclude possible (unknown) photochemical changes. Under these conditions, the prepared medium has a shelf-life of approximately 6 to 8 weeks; however, it is recommended that fresh medium be prepared for use in a test. The SIS medium should be prepared 1 to 2 days before use to allow the pH to stabilize, although it is advisable to check the pH of the medium before use. If the pH lies outside the specified range (6.5 ± 0.2), it may be readjusted by adding NaOH or HCl as previously described (OECD, 1998, 2002).

The modified Steinberg medium can also be used as control/dilution water for tests with chemical samples, as recommended in the draft ISO *L. minor* growth inhibition test (ISO, 2005). This medium consists of eight stock solutions as outlined in Table 11 of Appendix D. Details on how to prepare the medium are found in the draft ISO standard (ISO, 2005).

In instances where the toxic effect of a chemical in a particular receiving water is to be appraised, the recommended control/dilution water is the receiving water itself, spiked with the same nutrients as those used to prepare the SIS medium or the modified Steinberg medium (nutrient-spiked receiving water), whichever is being used (see footnote 40 and Table 4). Examples of such situations would include appraisals of the toxic effect of chemical spills or intentional applications of chemicals (e.g., pesticide) on a water body.

If a sample of upstream receiving water is to be used as dilution and control water, a separate control solution must be prepared using the SIS medium or the modified Steinberg medium, depending on which medium is used in the test (see Section 4.1).[68] Test medium might be used for all dilutions and the control when a high degree of standardization is required for testing

[67] When pH control of the test medium is particularly important (e.g., when testing metals or substances or materials that are hydrolytically unstable), the addition of MOPS buffer to the test medium is recommended (OECD, 1998, 2002).

[68] A comparison of *Lemna* growth rates in the SIS medium or the modified Steinberg medium versus the nutrient-spiked receiving-water sample collected upstream might distinguish demonstrable toxic responses attributable to contaminants within the upstream water.

Table 4 Chemical Composition of Nutrient Stock Solutions for Preparing SIS Medium and Nutrient-Spiked Receiving Water, for Testing Chemical Samples Using *Lemna minor*

Stock	Substance	Concentration	
		Stock Solution (g/L)	Medium[a] (mg/L)
I	$NaNO_3$	8.50	85
	KH_2PO_4	1.34	13.4
II	$MgSO_4 \cdot 7H_2O$	15.0	75
III	$CaCl_2 \cdot 2H_2O$	7.20	36
IV	Na_2CO_3	4.00	20
V	H_3BO_3	1.00	1.00
	$MnCl_2 \cdot 4H_2O$	0.200	0.200
	$Na_2MoO_4 \cdot 2H_2O$	0.010	0.010
	$ZnSO_4 \cdot 7H_2O$	0.050	0.050
	$CuSO_4 \cdot 5H_2O$	0.005	0.005
	$Co(NO_3)_2 \cdot 6H_2O$	0.010	0.010
VI	$FeCl_3 \cdot 6H_2O$	0.168	0.84
	$Na_2EDTA \cdot 2H_2O$	0.280	1.40
VII	MOPS (buffer)[b]	488	488

[a] Concentration of substance in prepared SIS medium.
[b] The free acid of MOPS is recommended since it is easily dissolved; pH adjustment may be necessary.

(e.g., if the toxicity of a chemical is to be determined and compared at a number of test facilities), or when the collection and use of receiving water is impractical (e.g., too expensive).

5.4 Test Observations and Measurements

In addition to the observations on toxicity described in Section 4.4, there are certain additional observations and measurements to be made while testing with chemicals.

During the preparation of solutions and at each of the prescribed observation times during the test, each solution should be examined for evidence of chemical presence and change (e.g., odour, colour, opacity, precipitation, or *flocculation* of chemical). Any observations should be recorded.

It is desirable and recommended that test solutions be analyzed to determine the chemical

concentrations to which *L. minor* are exposed.[69] If chemicals are to be measured in a static test, sample aliquots should be taken from all replicates in at least the high, medium, and low test concentrations, and the control(s). Separate analyses of the aliquots should be performed on samples taken immediately before the start of the initial exposure and at the end of the test, as a minimum. If chemicals are to be measured in a static-renewal test, sample aliquots should be taken from at least the high, medium, and low test concentrations, and the control(s). As a minimum, separate analyses should be performed with samples taken at the beginning and end of each renewal period and on the first and last days of the test.

All samples should be preserved, stored, and analyzed according to proven methods with acceptable detection limits for determining the concentration of the particular chemical in aqueous solution. Toxicity results for any tests in which concentrations are measured should be calculated and expressed in terms of those measured concentrations, unless there is a good reason to believe that the chemical measurements are not accurate.[70] In making calculations, each test solution should be characterized by the geometric average of the measured concentration to which the organisms are exposed.

At the start of the test, frond and colony numbers in the test vessels are recorded. Frond numbers and the appearance of the colonies must be observed at the beginning and at the end of the test. Two additional observations of frond number (e.g., on Days 3 and 5) should be made if the average specific growth rate or area under the curve is the preferred statistical endpoint (see footnote 46 and Section 4.5.5). Any changes in plant development, frond size, appearance, necrosis, or chlorosis should be noted as well as additional observations of root length, atypical appearance of the test media (e.g., presence of undissolved material), or any other abnormalities.

5.5 Test Endpoints and Calculations

The ICp is the statistical endpoint recommended for a multi-concentration test performed using a chemical (see Section 4.5.2).

If a solvent control is used to maintain the test substance in solution, there must be assurance that the solvent itself does not cause undue effects. Such a test is rendered invalid if *Lemna*

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[69] Such analyses need not be undertaken in all instances, due to analytical limitations, cost, or previous technical data indicating chemical stability in solution under conditions similar to those in the test. Chemical analyses are recommended if the test substance or one or more of its biologically active ingredients is volatile, insoluble, or precipitates out of solution, or if the test chemical is known to sorb the material(s) from which the test vessels are constructed. Some situations (e.g., testing of pesticides for purposes of registration) could require the measurement of chemical concentrations in test solutions.

The OECD requires chemical analyses, if the test substance is not expected to remain within ±20% of the nominal concentration. For tests in which the measured initial concentration of the test substance is not within ±20% of nominal but where sufficient evidence can be provided to show that the initial concentrations can be repeatedly prepared and are stable (i.e., range within 80–120% of the initial concentrations), chemical determinations can be carried out on only the highest and lowest test concentrations. In all cases, determination of the concentrations of test substance before renewal need only be performed on one replicate vessel at each test concentration (or the contents of the vessels pooled by replicate) (OECD, 1998, 2002).

[70] The OECD test guideline (1998, 2002) indicates that the analysis of the results can be based on the nominal or measured initial concentration if there is evidence that the concentration of the substance being tested has been maintained within ± 20% of the nominal or measured initial concentration throughout the test. If the deviation is greater than ± 20%, analysis of the results should be based on the time-weighted mean.

growth in the solvent control (or untreated control) does not meet the criteria for test validity specified in Section 4.5.1.

When a solvent or other chemical is used, it becomes the control for assessing the effect of the toxicant. Data for the solvent control must not be pooled with those for the control/dilution water. Pooling the controls could bias endpoint calculations; the control/dilution water lacks an influence that could act on organisms in the other concentrations (i.e., the solvent).

Average specific growth rate (i.e., relative growth rate) and/or area under the curve[71] can also be calculated based on frond number data. Calculation of either of these two optional endpoints requires additional observations at intervals (e.g., Days 3 and 5) during the test (see Sections 4.5.5 and 5.4).

5.6 Interpretation of Results

For any test which uses a water source other than SIS medium, modified Steinberg medium or, where appropriate, modified APHA medium as the control/dilution water, particular attention should be given to a comparison of *Lemna* growth in the control/dilution water with that in the standard controls using test medium (SIS, Steinberg, or APHA). This comparison is necessary to determine whether the control/dilution water is phytotoxic. Any enhanced growth in test solutions, relative to that in the control solutions, must be reported and considered when interpreting the findings (see Sections 4.5.2 and 4.5.4).

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[71] The OECD *Lemna* test guideline requires the calculation of average specific growth rate or area under the curve based on frond number data (collected at four different observation times during the test), as well as final biomass using one other growth parameter (dry weight, fresh weight, or total frond area). Results of the ring test of the draft OECD *Lemna* test guideline showed that estimates of toxicity based on final biomass were more sensitive than those based on average specific growth rate (Sims *et al*., 1999). The advantages of expressing toxicity in terms of average specific growth rate, however, are that the effect of exposure time is minimized, and data from tests having different exposure times may be compared (Huebert and Shay, 1993; Nyholm, 1990).

The test validity criterion in the OECD test guideline is based on the doubling time of frond number in the control [must be <2.5d (60h)]. This corresponds to approximately a minimum 8-fold increase in 7 days (OECD, 1998), which is the test validity criterion outlined herein (Section 4.5.1). Results of the OECD ring test indicate that most laboratories met the test acceptability criterion for control doubling time. Failure to comply with the doubling time criterion was often associated with low light intensities, low temperatures, or excessive pH values (Sims *et al*., 1999).

Section 6

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

This section gives specific instructions for collecting, preparing, and testing samples of effluent, elutriate, and leachate, in addition to the procedures described in Section 4.

6.1 Sample Collection, Labelling, Transport, and Storage

Containers for transporting and storing samples or subsamples of effluent, elutriate, or leachate must be made of nontoxic material. Collapsible polyethylene or polypropylene containers manufactured for transporting drinking water (e.g., Reliance™) are recommended. The volume of these containers can be reduced to fit into a cooler for transport, and the air space within can be minimized or eliminated if possible, when portions are removed in the laboratory for toxicity testing or chemical analyses. The containers must either be new or thoroughly cleaned, and rinsed with uncontaminated water. They should also be rinsed with the sample to be collected. Containers should be filled to eliminate any air space.

The requirements for volume of wastewater sample should be given serious consideration before undertaking the program. Generally, a 4-L sample of effluent or leachate is adequate for an off-site multi-concentration test and the associated routine sample analysis. Smaller amounts are required for single-concentration tests (see Section 4.5.3). Upon collection, each sample container must be filled, sealed, and labelled or coded. Labelling should include at least sample type, source, date and time of collection, and name of sampler(s). Unlabelled or uncoded containers arriving at the laboratory should not be tested nor should samples arriving in partially filled or unsealed containers be routinely tested, since volatile toxicants can escape into the air space. However, if it is known that volatility is not a factor, such samples might be tested at the discretion of the investigator. The chain-of-custody during sample collection, transport, and storage should be recorded along with any sample conditions (anomalies) that could effect test results.

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

Upon arrival at the laboratory, the temperature of the sample or, if collected, one of the subsamples (with the remaining subsamples left unopened and sealed), must be measured and recorded. An aliquot of effluent or leachate required at that time may be adjusted immediately or overnight to the test temperature and used in the test. The remaining portion(s) of sample or subsamples required for subsequent solution renewal or held for possible additional testing must be stored in darkness, in sealed containers, without headspace, at 4±2°C. For elutriates, as well as for samples intended for aqueous extraction and subsequent testing of elutriate, transport and storage conditions should be as indicated for effluents and leachates.

Tests with effluent, leachate, or elutriate may be performed "off-site" in a controlled laboratory facility. The static test option is recommended for standard use with samples of effluent, elutriate, and leachate. If, however, the active component in the wastewater can be expected to decrease significantly during the test period, the static-renewal test option is recommended (see Section 4.3).

If the static test option is followed, a single sample of wastewater must be collected and used to prepare the test solutions at the beginning of the test. If the static-renewal test option is followed, samples must be collected using one of the following procedures and approaches:

1. A single sample of wastewater may be used throughout the test, provided it is divided into three separate containers (i.e., three subsamples) upon collection.[72]

2. In instances where the toxicity of the wastewater is known or anticipated to change significantly if stored for up to 7 to 10 days before use, fresh samples must be collected on at least three separate occasions using sampling intervals of 2 to 3 days or less. These samples must be used consecutively during the test.[73]

An alternative approach for unstable wastewater is to perform these tests on-site, using fresh wastewater and static-renewal conditions (see Section 4.3).

Testing of effluent and leachate samples should commence as soon as possible after collection. Use of any sample in a test should begin within 1 day whenever possible, and must begin no later than 3 days after sampling. If effluents or leachates are tested at on-site laboratories, samples should be used in the test within 1 day or less following their collection [74] (USEPA, 1989, 2002).

Samples of sediment or other solid material collected for aqueous extraction and subsequent testing of the elutriate should also be tested as soon as possible, following their collection and no later than 10 days following receipt in the laboratory. Procedures provided by Environment Canada (EC, 1994) for the preparation of elutriates should be followed. For the derived elutriates, aliquots of the prepared sample should be used on the same schedule as indicated for samples of effluent or leachate, if possible. The prolonged storage of elutriate samples is undesirable because the toxicity of the sample might not be stable. Testing of elutriates must commence within 3 days of their preparation, unless specified otherwise in a regulation or prescribed method.

6.2 Preparing Test Solutions

Each sample or subsample in a collection or storage container must be agitated thoroughly just before pouring to ensure the re-suspension of settleable solids. The pH of each sample or subsample must be measured just before being used.

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[72] For example, the first subsample could be used for test initiation (Day 0), the second subsample for renewal on Day 3, and the third subsample for renewal on Day 5.

[73] For example, if three samples are collected at 2- to 3-day intervals (e.g., on Monday, Wednesday, and Friday), the first must be used for test initiation (Day 0), the second for renewal on Day 3, and the third for renewal on Day 5. Wastewaters known or anticipated to be particularly unstable could, if tested off-site, be sampled at daily intervals for seven consecutive days, and each sample used in order of sampling for daily (or more frequent) renewal of the test solutions.

[74] On-site testing might use the schedule and procedures described herein for off-site tests. Alternatively, certain on-site tests might require fresh wastewater that is renewed continuously (flow-through test) or at intervals of $\leq$12 h into each test vessel.

Filtration of samples or subsamples is normally not required nor recommended. However, if the wastewater samples are mixed with, or contain receiving water (e.g., effluent collected from a mixing zone in a lake, stream, river, etc.) they may contain algae and sample filtration may be required to reduce the possibility of contamination (i.e., excessive algae growth) during the test. All wastewater samples should be checked under the microscope for the presence of algae. If algae is present then the sample should be filtered through glass fibre filters (pore size of approximately 1 µm; e.g., Whatman GF/C filters) to reduce the risk of algal contamination. Samples may be subsequently filtered through 0.22 µm filters to eliminate any remaining potential for algal contamination (SRC, 1997). Such filtration could remove some suspended solids that are characteristic of the sample and might otherwise contribute part of the toxicity or modify the toxicity. In instances where there is concern about the effect of this filtration on sample toxicity, a second (concurrent) test should be conducted using portions of the unfiltered sample/subsample, but procedures should otherwise be identical.

A sample of wastewater must then be spiked with the same nutrients as those used to prepare the modified APHA growth medium (*nutrient-spiked wastewater*) (see Section 6.3; Table 5). An aliquot of each of three nutrient stock solutions (A, B, and C) are added to the wastewater sample in the ratio of 10 mL aliquot per 1000 mL sample diluting the samples to 97%. The spiked wastewater sample is then gently pre-aerated for 20 minutes (see Section 4.1) before being distributed to replicate test vessels.

6.3 Control/Dilution Water

Tests conducted with samples of effluent or leachate, intended to assess compliance with regulations, must use modified APHA medium (Table 5) or a sample of the receiving water spiked with modified APHA nutrient stock solutions (nutrient-spiked receiving water) as the control/dilution water. The objectives of the test must be defined before selecting the appropriate control/dilution water because the results could be different for the two sources of water. Difficulties and costs associated with the collection and shipment of receiving-water samples for use as control/dilution water should also be considered.

The APHA (modified) test medium is prepared with 3 stock solutions, as outlined in Table 5. The stock solutions are prepared using reagent-grade chemicals in glass-distilled, deionized water, or equivalent. To prepare 1 L of medium, 10 mL of each stock solution (A, B, and C) are added to 970 mL of distilled water in a 1 L media bottle. The medium is aerated vigorously for at least 1 to 2 hours. If a larger volume (>4 L) of media is prepared, overnight aeration of the medium is recommended to stabilize the pH of the medium. Immediately before testing, the pH of the test medium is adjusted to 8.3 ± 0.1 using 0.5*N* NaOH and 0.5*N* HCl.[75] The medium is not sterilized.Stock solutions A, B, and C can be stored as separate solutions in a refrigerator (4 ± 2°C) for up to one month.

Modified APHA medium is the control/dilution water required for standard use with samples of effluent, elutriate, and leachate. The use of receiving water as the control/dilution water, however, might be desirable in certain instances

[75] The pH naturally stabilizes at approximately 8.3 with aeration (Moody, 1998)

Table 5 Chemical Composition of Nutrient Stock Solutions for Preparing Modified APHA Medium, Nutrient-Spiked Wastewater, and Nutrient-Spiked Receiving Water, for Testing Samples of Effluent, Elutriate, Leachate, or Receiving Water, Using *Lemna minor*

Stock	Substance	Concentration	
		Stock Solution (g/L)	Medium[a] (mg/L)
A	$NaNO_3$	25.5	255
	$NaHCO_3$	15.0	150
	K_2HPO_4	1.04	10.4
	KCl	1.01	10.1
B[b]	$CaCl_2 \cdot 2H_2O$	4.41	44.1
	$MgCl_2 \cdot 6H_2O$	12.17	121.7
	$MnCl_2 \cdot 4H_2O$	0.4149	4.149
	$FeCl_3 \cdot 6H_2O$	0.16	1.6
C[c]	$MgSO_4 \cdot 7H_2O$	14.7	147
	H_3BO_3	0.186	1.86
	$Na_2MoO_4 \cdot 2H_2O$	0.00726	0.0726
	$ZnCl_2$	0.00327	0.0327
	$CoCl_2 \cdot 6H_2O$	0.0014	0.014
	$CuCl_2 \cdot 2H_2O$	1.5×10^{-5}	1.5×10^{-4}

[a] Concentration of substance in prepared medium.

[b] Acidify solution B to pH 2.0 to prevent precipitation. Protect solution B from the light by storing in a dark amber bottle.

[c] For greater accuracy, stock C can also be prepared using more concentrated stocks for each of the trace metals, as follows. Weigh and dissolve 14.7g/L of $MgSO_4 \cdot 2H_2O$ and 0.186 g/L of H_3BO_3 in 900 mL of glass-distilled or deionized water, or equivalent. Prepare individual stocks for each of the remaining trace metals in stock solution C as follows: 0.363g/50mL for $NaMoO_4 \cdot 2H_2O$, 0.1635 g/50 mL for $ZnCl_2$, 0.0714 g/50mL for $CoCl_2 \cdot 6H_2O$, and 0.0057 g/50mL for $CuCl_2 \cdot 2H_2O$. Add 1mL of each of the Na, Zn, and Co stocks, and 0.1 mL of the Cu stock to the $MgSO_4$ and H_3BO_3 solution and bring the volume up to 1 L.

where site-specific information is required on the potential toxic effect of an effluent, leachate, or elutriate on a particular receiving water (see footnote 40 and Section 4.1). An important example of such a situation would be testing for sublethal effect at the edge of a mixing zone, under site-specific regulatory requirements. Conditions for the collection, transport, and storage of such receiving-water samples should be as described in Section 6.1.

An aliquot of the receiving water, to be used as control/dilution water, is filtered through glass fibre filters (approximate pore size of 1 μm, e.g., Whatman GF/C filters), before being used, to reduce the possibility of the test being contaminated by algae. Receiving waters may

be subsequently filtered through 0.22 µm filters to prevent the growth of algae (SRC, 1997). The receiving-water sample must then be enriched with the same levels of nutrients as the modified APHA medium (10 mL of each stock solution (A, B, and C) per 1000 mL of receiving water). Once enriched, the receiving-water samples should be aerated vigorously for 1 to 2 hours (or longer for larger volumes), without pH adjustment, to stabilize the pH of the nutrient-spiked receiving water.[76] The pH of the aerated, spiked, receiving water is recorded before testing.

If a sample of upstream receiving water is to be used for control/dilution water, a separate control solution must be prepared using the modified APHA medium. Test conditions and procedures for evaluating each control solution should be identical and as described in Sections 4 and 5.3.

If a high degree of standardization is required, modified APHA medium should be used for all dilutions and as the control water, since the use of a specific medium increases the probability of reducing the modifying influences attributable to different chemical compositions of dilution water. Situations where such use is appropriate include investigative studies intended to interrelate toxicity data for various effluent, leachate, or elutriate types and sources, derived from a number of test facilities. In such instances, it is desirable to minimize any modifying influence of dilution-water chemistry.

6.4 Test Observations and Measurements

There are certain observations and measurements that should be made during tests with effluents, elutriates, and leachates in addition to those described in Section 4.4.

Colour, *turbidity*, odour, and homogeneity (the presence of floating or settled solids) of the effluent, leachate, or elutriate sample should be observed and recorded before and after the sample is filtered. Any changes that occur during the preparation of the test sample should be recorded (e.g., precipitation, flocculation, change in colour or odour, release of volatiles, etc.), as well as any changes in the appearance of test solutions during the test period (e.g., foaming, settling, flocculation, increase or decrease in turbidity, colour change, etc.).

For effluent samples with appreciable solids content, it is desirable to measure total suspended and settleable solids (APHA *et al*., 1995) upon receipt, as part of the overall description of the effluent, and as sample characteristics that might influence the results of the toxicity test. Additional measurements that would help characterize each sample of effluent, leachate, or elutriate should also be made. These could include pH, conductivity, hardness, alkalinity, colour, chemical oxygen demand, biological oxygen demand, dissolved oxygen, and concentrations of specific toxic contaminants (e.g., resin acids, chlorophenolic compounds, dissolved metals, chlorine, chloramine, ammonia, etc.).

6.5 Test Endpoints and Calculations

The endpoints for tests performed with samples of wastewater will normally be IC25s based on increase in frond number during the test and frond dry weight attained at test end, as indications of growth. Tests for monitoring or regulating effluents, leachates, or elutriates must use the standard options and endpoints defined in Section 4.

Tests for monitoring and compliance with regulatory requirements should normally include, as a minimum, three or more replicate

[76] The pH might be considered stable when it does not vary by more than 0.1 units during a 30-minute period of aeration.

solutions of the undiluted sample/subsamples (or a specified dilution thereof), and three or more replicate control solutions. Depending on the specified regulatory requirements, tests for compliance might be restricted to a single concentration (e.g., "full-strength" sample, which is 97% using this test method, unless otherwise specified) or might require a series of concentrations (i.e., a multi-concentration test) (see Section 4.5.2). Single-concentration tests are often cost-effective for determining the presence of measurable toxicity, and also for screening a large number of samples.

Specific adaptations of the standard toxicity test could be adopted for special purposes such as locating in-plant sources of toxicity, or assessing the effectiveness of in-plant process changes or of effluent treatment. The tests could be multi-concentration or single-concentration (97% or an appropriate dilution, plus a control). Endpoints would depend on the objectives of the undertaking, but could include arbitrary "pass/fail" limits or percent reduction in growth at a specified concentration (Section 4.5.3).

Section 4.5.3 provides relevant instructions on statistical analysis and reporting for sets of tests with different samples, each tested at only one concentration.

6.6 Interpretation of Results

For any test that uses a water source other than modified APHA medium for the control/dilution water, particular attention should be given to a comparison of *Lemna* growth in the control/dilution water with that in the standard controls using modified APHA medium. A statistical comparison is necessary to determine whether the control/dilution water is phytotoxic (see Section 4.5.3). Any enhanced growth in test solutions, relative to that in the control solutions, must be reported and considered when interpreting the findings (see Sections 4.5.2 and 4.5.4).

Specific Procedures for Testing Receiving-Water Samples

Instructions for testing samples of receiving water, in addition to those provided in Section 4, are provided in this section.

7.1 Sample Collection, Labelling, Transport, and Storage

Procedures for collecting, labelling, transportation, and storing samples are found in Section 6.1. Testing of receiving-water samples/subsamples should commence as soon as possible after collection, preferably within 24 hours of sampling, but no later than 3 days after sampling.

7.2 Preparing Test Solutions

Samples in the collection container(s) should be agitated before pouring to ensure their homogeneity.

Each receiving-water test sample must be filtered through a glass fibre filter (approximate pore size of 1 µm, e.g., Whatman GF/C filters) before being used, to reduce the possibility of test contamination by algae. Receiving waters may be subsequently filtered through 0.22 µm filters to prevent the growth of algae (SRC, 1997). A second, unfiltered test should be run concurrently if there is concern about the effect of filtration on toxicity (see Section 6.2).

Receiving-water test samples are then spiked with modified APHA nutrient stock solutions and gently pre-aerated for 20 minutes (see Sections 4.1 and 6.2).

7.3 Control/Dilution Water

For samples of surface water collected in the vicinity of a wastewater discharge, chemical spill, or other point-source of contamination, "upstream" water may be sampled concurrently and used as control/dilution water for the downstream sample (see footnote 37 and Section 6.3). This control/dilution water should be collected as close as possible to the contaminant source(s) of concern, but upstream or outside of the zone of influence. Such surface water must be filtered to remove organisms, as described in Section 7.2.

If "upstream" water is used as control/dilution water, a separate control solution must be prepared using the modified APHA medium that is normally used for testing *L. minor*. Test conditions and procedures for preparing and evaluating each control solution should be identical, and as described in Sections 4, 5.3, and 6.3. Results of test exposures must be statistically compared with those for the control that used receiving water (see Section 4.5).

Logistic constraints, expected toxic effects, or other site-specific practicalities might prevent or rule against the use of upstream water as the control/dilution water. In such cases, modified APHA medium should be used as the control water and for all dilutions (see Section 6.3).

7.4 Test Observations and Measurements

The primary observations on test organisms should be as described in Section 4.4. In

addition, there should be observations of sample and solution colour, turbidity, foaming, precipitation, etc., as described in Section 6.4, both during the preparation of test solutions and during the tests.

Each receiving-water sample should be characterized chemically. Depending on the suspected nature of the toxicants, measurements might include pH, conductivity, hardness, alkalinity, colour, chemical oxygen demand, biochemical oxygen demand, and concentrations of specific toxicants (e.g., resin acids, chlorophenolic compounds, dissolved metals, chlorine, chloramine, ammonia, etc).

7.5 Test Endpoints and Calculations

Endpoints for tests with samples of receiving water should be consistent with the options and approaches identified in Sections 4.5, 6.5, and 6.6.

Tests with receiving water could be multi-concentration or single concentration. Tests of regulatory compliance would normally include three or more replicates containing "full-strength" (or 97%, in the case of this test) sample and three or more replicate control solutions to determine the growth inhibition obtained for *L. minor* exposed to 97% receiving water for 7 days (Section 4.5). Single-concentration tests are often cost-effective for determining the presence of measurable toxicity, and also for screening a large number of samples (e.g., from various locations within the receiving water). Statistical testing and reporting of results for such tests should follow the procedures outlined in Section 4.5.3.

If receiving-water samples are predicted to be toxic, and information is desired concerning the degree of dilution necessary to permit normal duckweed growth, a multi-concentration test to determine the IC25 for growth should be conducted, as outlined in Section 4. Any multi-concentration test should include the "full strength", nutrient-spiked receiving water (97%) as the highest concentration in the series tested.

Certain sets of tests might use a series of samples such as surface waters from a number of locations, each tested at "full strength" (97%) only. Statistical testing and reporting of results for such tests should follow the procedures outlined in Section 4.5.3.

Reporting Requirements

Each test-specific report must indicate if there has been any deviation(s) from any of the *must* requirements delineated in Sections 2 to 7 of this biological test method, and, if so, provide details of the deviation(s). The reader must be able to establish from the test-specific report whether the conditions and procedures preceding and during the test rendered the results valid and acceptable for the use intended.

Section 8.1 provides a list of items that must be included in each test-specific report. Section 8.2 lists items that must either be included in the test-specific report, provided separately in a general report, or held on file for a minimum of five years. Specific monitoring programs, related test protocols, or regulations might require selected test-specific items listed in Section 8.2 (e.g., details about the test substance or material and/or explicit procedures and conditions during sample/subsample collection, handling, transport, and storage) to be included in the test-specific report, or might relegate certain test-specific information as "data to be held on file".

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

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

- a record of the chain-of-continuity for samples tested for regulatory or monitoring purposes;
- a copy of the record of acquisition for the sample(s);
- chemical analytical data on the sample(s) not included in the test-specific report;
- bench sheets for the observations and measurements recorded during the test;
- bench sheets and warning chart(s) for the reference toxicity tests;
- detailed records of the source of the test organisms, their taxonomic confirmation, and all pertinent information regarding their culturing and health; and
- information on the calibration of equipment and instruments.

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

8.1 Minimum Requirements for a Test-Specific Report

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

8.1.1 Test Substance or Material

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

- information on labelling or coding for each sample/subsample;
- date of sample/subsample collection;
- date and time sample(s)/subsample(s) are received at the test facility;
- dates or days during the test when individual sample(s) or subsample(s) were used;
- measurement of the temperature of wastewater or receiving-water sample or, for multiple subsamples, measurement of the temperature for one (only) of the subsamples upon receipt at the test facility;
- measurement of the pH of sample(s) or subsample(s) of wastewater or receiving water just before it is prepared and used in the toxicity test; and
- date of elutriate generation and description of procedure for preparation; dates or days during an elutriate test when individual samples or subsamples were used.

8.1.2 Test Organisms

- species, clone identification code (if known), and origin of culture;
- age (i.e., 7 to 10 days) of test culture used to provide inocula of test organisms at start of test;
- indication as to whether test culture is axenic;
- growth medium used for culturing *Lemna minor*;
- test medium in which *Lemna* were acclimated for the 18 to 24 hours before test start;
- data showing increase in frond number in vessels setup to monitor health; and
- any unusual appearance or treatment of the test culture, before it is used in the test.

8.1.3 Test Facilities and Apparatus

- name and address of test laboratory;
- name of person(s) performing the test; and
- brief description of test vessels (size, shape, and type of material).

8.1.4 Control/Dilution Water

- type of test medium used as control and dilution water;
- type and source of water used to prepare test medium; and
- type and quantity of chemical(s) used to prepare control/dilution water.

8.1.5 Test Method

- citation of the biological test method used (i.e., as per this document);
- indication as to whether test is performed with or without renewal of test solutions and, if static-renewal test, frequency of renewals;
- design and description if specialized procedure (e.g., test performed with and without filtration of sample; test performed with and without adjustment of sample pH; preparation and use of elutriate; preparation and use of solvent and, if so, solvent control) or modification of standard test method;

- brief description of frequency and type of observations and measurements made during test; and

- name and citation of program(s) and methods used for calculating statistical endpoints.

8.1.6 Test Conditions and Procedures

- design and description of any deviation(s) from or exclusion(s) of any of the procedures and conditions specified in this document;

- number, concentration, volume, and depth of solutions in test vessels including controls;

- number of fronds per plant and number of plants per test vessel at start of test;

- number of replicates per treatment;

- brief statement (including procedure, rate, and duration) of any pre-aeration of samples or test solutions before starting the test;

- description of the procedure for sample filtration (i.e., pore size of filters, number of filtrations, type of filter paper, etc.), if applicable;

- type and quantity of chemicals added to test sample before starting the test (i.e., nutrient-spiking);

- brief description of any sample or test solutions receiving pH adjustment, including procedures;

- all required (see Section 4.4) measurements of temperature and pH in test solutions (including controls), and measurements of light fluence rate made during the test; and

- dates and times when test was started and ended;

- brief statement indicating whether the reference toxicity test was performed under the same experimental conditions as those used with the test sample(s); and description of any deviation(s) from or exclusion(s) of any of the procedures and conditions specified for the reference toxicity test in this document.

8.1.7 Test Results

- number of fronds and frond appearance in each test vessel as noted during each observation period over the 7-day exposure;

- for each treatment including the control treatment(s): the mean ± SD for the increase in frond number, as determined at test end;

- for each treatment including the control treatment(s): the mean ± SD for the dry weight of *Lemna* fronds determined at test end;

- any ICp (together with its 95% confidence limits) determined for the growth (i.e., increase in frond number during the test and frond dry weight attained at test end) using concentrations corrected for the volume of nutrient stock; details regarding any weighting techniques applied to the data; and indication of quantitative statistic used;

- any outliers and the justification for their removal;

- the results and duration of any toxicity tests with the reference toxicant(s) performed within 14 days of the test, together with the *geometric mean* value (± 2 SD) for the same reference toxicant(s), test species and clone, and test medium as derived at the test facility in previous tests using the procedures and conditions herein;

- any findings of significant growth stimulation, expressed as % stimulation, at any concentration(s); and

- anything unusual about the test, any problems encountered, any remedial measures taken.

8.2 Additional Reporting Requirements

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

8.2.1 Test Substance or Material

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

8.2.2 Test Organisms

- name of person(s) who identified the organisms and the taxonomic guidelines used to confirm species;
- history of laboratory culture;
- description of culture conditions and procedures including: lighting (fluence rate, quality, and *photoperiod*) and temperature conditions; composition of culture medium; and procedures and conditions for preparation and storage of culture medium;
- frequency of culture renewal;
- procedures, observations, and records related to the purity of stock cultures; and
- records of all *Lemna* growth curves performed to monitor culture health and performance.

8.2.3 Test Facilities and Apparatus

- description of system for regulating light and temperature within the culturing and test facilities; and
- description of procedures used to clean, rinse, and sterilize test apparatus.
- records of maintenance and performance checks conducted on apparatus (e.g., laminar air flow hoods, growth cabinets, meters, scales, pipettes).

8.2.4 Control/Dilution Water

- sampling and storage details if the control/dilution water was “upstream” receiving water;
- details regarding any water *pretreatment* (i.e., procedures and conditions for filtration, sterilization, aeration; adjustment of temperature and/or pH);
- any ancillary water-quality variables measured before and/or during the toxicity test; and
- storage conditions and duration before use.

8.2.5 Test Method

- description of previous experience the laboratory has had with this biological test method for measuring toxicity using *L. minor*;
- procedure used in preparing and storing stock and/or test solutions of chemicals; description and concentration(s) of any solvent used;
- methods used (with citations) for chemical analyses of sample or test solutions (including details on sampling, sample/solution preparation and storage, before chemical analyses); and

- use and description of preliminary or range-finding test.

8.2.6 Test Conditions and Procedures

- photoperiod, light source, and fluence rate adjacent to the surface of test solutions;
- appearance of sample and test solutions before and after sample filtration and any change in appearance noted during test;
- water quality measurements for culture/control/dilution water;
- any other physical or chemical measurements on sample, stock solutions, or test solutions (e.g., concentrations of one or more specific chemicals before and/or at time of the test);
- conditions, procedures, frequency, dates, and times for toxicity tests with reference toxicant(s) using *L. minor*; and
- chemical analyses of concentrations of chemical in test solutions of reference toxicant.

8.2.7 Test Results

- results for any range-finding test(s) conducted;
- results for any statistical analyses conducted both with outliers and with outliers removed; for regression analyses, hold on file information indicating sample size (e.g, number of replicates per treatment), parameter estimates with variance or standard error, any ANOVA table(s) generated, plots of fitted and observed values of any models used, results of outlier tests, and results of tests for normality and homoscedasticity;
- growth curves, if generated;
- control/warning chart showing the most recent and historic results for toxicity tests with the reference toxicant(s);
- graphical presentation of toxicity data; and
- original bench sheets and other data sheets, signed and dated by the laboratory personnel performing the test and related analyses.

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Appendix A

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Appendix C

Procedural Variations for Culturing *Lemna* spp. and for Undertaking Growth Inhibition Tests Using *Lemna* spp., as Described in Canadian, American, and European Methodology Documents

Source documents are listed chronologically by originating agency in the following order: (1) major committees and government agencies, and (2) major authors.

ITM, 1990 represents the Institutet för tillämpad miljöforskning. This publication gives culturing and toxicity test procedures for *Lemna minor* compiled and used by the Swedish National Environmental Protection Board in collaboration with the National Chemicals Inspectorate (Institutet för tillämpad miljöforskning), Solna, Sweden.

ASTM, 1991 is the standard guide published by the American Society for Testing and Materials for conducting static toxicity tests with *Lemna gibba* G3.

APHA, 1992 represents the American Public Health Association, the American Water Works Association, and the Water Environment Federation, 1992. The publication (in Standard Methods for the Examination of Water and Wastewater - 18th ed.) gives culturing and testing procedures for *L. minor* which was included as a monitoring tool under the Environmental Effects Monitoring component of the Canadian Federal Pulp and Paper Effluent Regulations. This guideline document was revised in 1996.

USEPA, 1992 is the standard guide published by the Office of Pollution Prevention and Toxics (OPPT), United States Environmental Protection Agency, for conducting toxicity tests using *L. gibba* G3 to develop data on the phytotoxicity of chemicals [under the Toxic Substances Control Act (TSCA)]. It appeared in Title 40, Chapter I, Subchapter R of the Code of Federal Regulations. This guideline document was revised, harmonized with other publications, and re-published (draft) in 1996 (see following citation).

USEPA, 1996 is the draft (April, 1996) standard guideline (OPPTS 850.4400) developed by the Office of Pollution Prevention and Toxics (OPPT), United States Environmental Protection Agency, for conducting toxicity tests using *L. gibba* G3 and *L. minor* to develop data on the phytotoxicity of chemicals [under the Toxic Substances Control Act (TSCA), and Federal Insecticide, Fungicide and Rodenticide Act (FIFRA)]. This guideline blends testing guidance and requirements that existed in OPPT and appeared in Title 40, Chapter I, Subchapter R of the Code of Federal Regulations (CFR); the Office of Pesticide Programs (OPP) which appeared in the publications of the National Technical Information Service (NTIS) and the guidelines published by the Organization for Economic Cooperation and Development (OECD). It represents the harmonization of two documents: 40 CFR 797.1160 *Lemna* Acute Toxicity Test, and OPP 122-2 Growth and Reproduction of Aquatic Plants (Tier I) and 123-2 Growth and

Reproduction of Aquatic Plants (Tier 2) (Pesticide Assessment Guidelines, Subdivision J--Hazard Evaluation; Nontarget Plants) EPA report 540/09-82-020, 1982.

AFNOR, 1996 is the standard guide published by the Association française de normalisation (test method XP T 90-337,1996). This document gives culturing and toxicity test procedures using *L. minor*.

OECD, 1998 is the draft (June, 1998) standard procedure published by the Organization for Economic Cooperation and Development. The guideline is designed to assess the toxicity of substances to *L. gibba* and *L. minor* and is based on existing guidelines and standards published by ASTM (1991), USEPA (1996), AFNOR (1996), and the Swedish Standards Institute (SIS) (1995).

SRC, 1997 is the (unpublished) standard operating procedures developed in 1997 by H. Peterson and M. Moody of the Saskatchewan Research Council, Water Quality Section Laboratory, for culturing and testing *L. minor*. It is based on research conducted by Peterson and Moody (1994–1997) and is a modification of the APHA, 1995–8211 Duckweed (proposed) toxicity test procedure.

DFO, 1979 represents Lockhart and Blouw, 1979. This method, published in a document entitled Toxicity Tests for Freshwater Organisms, E. Scherer (ed.), describes procedures for testing herbicides and sediments with *L. minor*.

B & P, 1981 represents Bishop and Perry, 1981. This publication describes a standard flow-through growth inhibition test for *L. minor*. It also compares the relative sensitivity of duckweeds with that of fish and invertebrate species for various test materials.

C & M, 1989 represents Cowgill and Milazzo, 1989. This publication develops rearing conditions and a successful long-term culture medium for maintaining *L. gibba* G3 and several clones of *L. minor*. A number of endpoints are examined and compared, and the relative sensitivity of the two duckweed species and various clones to various test materials is investigated.

T & N-K, 1990 represents Taraldsen and Norberg-King, 1990. This publication describes a method for culturing and testing *L. minor*, primarily for testing effluents. The relative sensitivity of duckweed, *Ceriodaphnia dubia*, and fathead minnows *(Pimephales promelas)* to various chemicals and effluents is also discussed.

1. Test Substance and Type of Test

Document[a]	Test Substance	Test Type	Test Duration (days)
ITM, 1990	individual substances, wastewaters	static, static-renewal[b]	7
ASTM, 1991	chemicals, commercial products, known mixtures[c]	static	7
APHA, 1992	metals, organic compounds, industrial effluents, leachates, receiving waters	static, static-renewal, flow-through[b]	4
USEPA, 1992	chemicals (under TSCA)	static-renewal	7
USEPA, 1996	chemicals (under TSCA & FIFRA)	static-renewal	7
AFNOR, 1996	chemicals, surface or water samples, industrial or urban effluents, subterraneous waters	static, static-renewal[b]	4
OECD, 1998	substances	static, static-renewal[b]	7
SRC, 1997	effluents, elutriates, leachates receiving waters, chemicals[d]	static	7
DFO, 1979	herbicides, sediments	NI[e]	14
B & P, 1981	heavy metals, surfactants, herbicides	flow-through	7
C & M, 1989	sodium selenate (Na_2SeO_4) cobalt nitrate $(CoNO_3)_2 \cdot 6H_2O$ stannic chloride ($SnCl_4$) vanadyl sulphate ($VOSO_4$) $\cdot 2H_2O$	NI	7
T & N-K, 1990	effluents, single toxicants	static-renewal	4

[a] See preceding pages for complete citation information.
[b] If test solutions are unstable (e.g., high microbial activity, high volatility, photodegradation, or biodegradation), the test solutions should be renewed.
[c] Effluents, leachates, oils, particulate matter sediments, and surface waters can also be tested with modification to the test procedure.
[d] Effluents and receiving waters are filtered through glass fibre filters (1µm poresize) to reduce algal growth.
[e] NI = Not indicated.

2. Test Species

Document	Species	Strain/Clone	Life Stage	Confirmed Taxanomically?
ITM, 1990	*L. minor*	NI[a]	most intensive growth phase (light colour and short root)	NI
ASTM, 1991	*L. gibba*	G3	NI	Yes
APHA, 1992	*L. minor*	NI	NI	Yes
USEPA, 1992	*L. gibba*	G3	culture < 2 weeks old; plants grown from a single isolated frond should be used in a given test	Yes
USEPA, 1996	*L. gibba* *L. minor*	G3 NI	culture < 2 weeks old; plants grown from a single isolated plant should be used in a given test	Yes
AFNOR, 1996	*L. minor*	NI	~ 2-week old culture	NI
OECD, 1998	*L. gibba* *L. minor*	identified (if known)	young, rapidly growing colonies without visible lesions[b]	Yes
SRC, 1997	*L. minor*	C4	≤7–10 days old	NI
DFO, 1979	*L. minor*	NI	< 1-month old	NI
B & P, 1981	*L. minor*	#6	NI	Yes
C & M, 1989	*L. gibba* *L. minor*	G3 6591(CA)[c] 7102(=LMS)(KS) 7101(LMY)(CT) 7136(46)(IL)	NI	Yes
T & N-K, 1990	*L. minor*	NI	NI	NI

[a] NI = Not indicated.

[b] Good quality cultures are indicated by a high incidence of colonies comprising of at least two fronds. A large number of single fronds is indicative of environmental stress and plant material from such cultures should not be used for testing.

[c] CA = California; KS = Kansas; CT = Connecticut; IL = Illinois.

3. Stock Culture Maintenance

Document	Medium	Transfer	Container	Depth/Vol.	Axenic?
ITM, 1990	Stock Culture Medium	monthly, 10 young green plants	300 mL Erlenmeyer Flasks	5–6 cm	Yes
ASTM, 1991	Hoagland's E+, M-Hoagland's, or 20X-AAP[a]	weekly	NI[b]	NI	Yes
APHA, 1992	Duckweed Nutrient Medium	monthly; nutrients added weekly	15 L aquarium or stainless steel basin	≥40 mm	No
USEPA, 1992	Hoagland's	as necessary	aquaria	NI	Yes
USEPA, 1996	M-Hoagland's	as necessary	aquaria	NI	Yes
AFNOR, 1996	Culture Medium	once per 14 days, ten 2-frond plants	NI	150 mL	Yes
OECD, 1998	L.g.– 20X-AAP[a,c] *L.m.*–SIS medium[d,e]	monthly[f]	glass	NI	Yes
SRC, 1997	Hoagland's E+	weekly	25 × 150 mm test tubes with Kimcaps®	25 mL	Yes
DFO, 1979	Hillman's-M Medium	NI	250 mL Erlenmeyer flasks	100 mL	Yes
B & P, 1981	0.01× Hutner's Solution	NI	NI	NI	NI
C & M, 1989	M-Hoagland's	*L.g.*–5 plants (15 fronds) weekly[c] *L.m.*–10 plants (30 fronds) weekly[d]	250 mL glass Erlenmeyer flasks Shimadzu closure	100 mL	Yes
T & N-K, 1990	Nutrient Enriched Water (NEW)	NI	10 L aquaria	4 L	NI

[a] M-Hoagland's = Modified Hoagland's E+ medium; 20X-AAP = twenty times the strength of AAP (the medium used for microalgae testing).
[b] NI = Not indicated.
[c] *L.g. = Lemna gibba.*
[d] *L.m. = Lemna minor.*
[e] SIS medium is similar to the inoculum medium used in Swedish Standards (ITM, 1990), see Table 1 in Appendix D.
[f] Monthly transfers of cultures can be extended up to once every three months if cultures are maintained at lower temperatures (4–10°C).

4. Type of Culture Medium

Document	Medium	Chemical Modification(s) of Medium	Type of Water	Preparation
ITM, 1990	Stock Culture Medium	culture and inoculation (acclimation) media have more nitrogen (N) and phosphorous (P) to prevent shortage during the last part of the growth phase. MOPS recommended as pH buffer	deionized or equiv.	6 of 8 stock solutions mixed with water; pH adjust to 6.5; make up to 1L; autoclave or filter sterilize; add solutions 7 and 8.
ASTM, 1991	Hoagland's E+[a]	None	deionized or distilled	9 stock solutions; make up to 1L; pH adjust to 4.6; autoclave
	or			
	Modified Hoagland's	same as Hoagland's E+ except no sucrose, EDTA, bacto-tryptone, and yeast	deionized or distilled	2 stock solutions; make up to 1L; autoclave; pH adjust to 4.9– 5.1
	or			
	20X-AAP	same nutrients as AAP medium (used for micro-algae testing) but at 20× the concentration; pH 7.5.	deionized or distilled	7 stock solutions; make up to 1L; pH adjust to 7.4–7.6; sterilize with 0.22 μm pore filter.
APHA, 1992	Duckweed Nutrient Solution	omit EDTA if test samples contain toxic metals (acidify to pH 2 to prevent precipitation if EDTA omitted)	deionized	3 stock solutions; pH adjust to 7.5–8.0.
USEPA, 1992	Hoagland's Nutrient Medium	no EDTA, other chelating agents, or organic metabolites such as sucrose	deionized or distilled	pH adjust to 4.8–5.2
USEPA, 1996	Modified Hoagland's Nutrient Medium	no EDTA, no organic metabolites such as sucrose	high quality (e.g., distilled, deionized, or ASTM Type I)	pH adjust to 4.8–5.2
	or			
	20X- AAP	EDTA present to ensure that trace nutrients are available to the fronds; no organic metabolites such as sucrose	high quality	pH adjust to 7.4–7.6
AFNOR, 1996	Concentrated Medium	culture medium is 10% concentrated medium and 90% water	distilled or equivalent	7 stock solutions; make up to 1L; pH adjust to 5.0–6.0; sterilize with 0.22 μm pore filter
OECD, 1998	*L.g.*–20X-AAP[b,c]	None	distilled	pH adjust to 7.4–7.6
	L.m.–SIS medium[c,d]	$FeCl_3 \cdot 6H_2O$ (0.84 mg/L) instead of Fe (III) ammonium citrate; no citric acid[e]	distilled	pH adjust to 6.3–6.7
SRC, 1997	Hoagland's E+ Medium	None	NI[f]	NI

4. Type of Culture Medium (continued)

Document	Medium	Chemical Modification(s) of Medium	Type of Water	Preparation
DFO, 1979	Hillman's M Medium	None	distilled	10 of 11 stock solutions are mixed; made up to 1L; autoclave; add $FeCl_3$ stock (autoclaved separately)
B & P, 1981	0.01× Hutner's solution	None	filtered [g]	flow-through diluters
C & M, 1989	Hoagland's E+ Medium	None	distilled	9 stock solutions; make up to 1L; pH adjust to 4.6; autoclave
T & N-K, 1990	Nutrient Enriched water (NEW)	reconstituted water (APHA, 1985) and commercial soil; no EDTA	NI	filtered (1.2 μm filter)

[a] Any medium which demonstrated a ≥5× increase in biomass in the controls within 7 days is acceptable.
[b] *Lemna gibba*.
[c] Other nutrient rich media can be used for stock cultures.
[d] *Lemna minor*.
[e] These are modifications of an earlier version (ITM, 1990) of the Swedish Standard medium.
[f] NI = Not indicated.
[g] Carbon- and reverse-osmosis-filtered well water.

5. Culture Conditions

Document	Temperature (°C)	Photoperiod	Light Type	Light Intensity[a]
ITM, 1990	8–10	constant	fluorescent (warm white)	2 × 10 W
ASTM, 1991	25 ± 2	constant	fluorescent (warm white)	6200–6700 lux
APHA, 1992	25 ± 2	constant	fluorescent (cool white)	4300 or 2150 lux
USEPA, 1992	NI[b]	NI	NI	NI
USEPA, 1996	NI	NI	NI	NI
AFNOR, 1996	25 ± 1	16 h:8 h (light:dark)	NI	3500 ± 500 lux
OECD, 1998	24 ± 2 (4–10, optional)	continuous	fluorescent (warm- or cool-white)	6500–10 000 lux[c]
SRC, 1997	25 ± 2	continuous	fluorescent (full-spectrum)	4000–4500 lux
DFO, 1979	25	16 h:8h (light: dark)	Sylvanic Gro-Lux (plant growth lights)	60 μE/m^2·s^{-1}
B & P, 1981	NI	NI	NI	NI
C & M, 1989	25 ± 2	NI	NI	*L.g.*–6461 ± 323[d] *L.m.*–5385 ± 323[e]
T & N-K, 1990	25	NI	NI	NI

[a] Light intensity is measured at the level of the test solution.
[b] NI = Not indicated.
[c] Plants can be held under reduced illumination.
[d] *L.g. = Lemna gibba*.
[e] *L.m. = Lemna minor*.

6. Acclimation and Selection of Test Organisms

Document	Medium	Acclimation Conditions	Acclimation Period
ITM, 1990	inoculum medium[a]	10–12 plants initiated; same light and temperature conditions as test; medium not changed during acclimation	10–14 days or when 100–200 fronds in each flask
ASTM, 1991	Hoagland's E+, Hoaglands, or 20X-AAP	same light and temperature conditions as test	8 weeks
APHA, 1992	Duckweed Nutrient Solution	same as test environment	2 weeks
USEPA, 1992	Hoagland's	NI[b]	< 2 weeks
USEPA, 1996	M-Hoagland's or 20X-AAP	NI	< 2 weeks
AFNOR, 1996	Culture Medium	select 2-frond plants from 14-day old culture and subculture under culture conditions for use in test	5–18 hours
OECD, 1998	*L.g.*–20X-AAP[c] *L.m.*–SIS medium[d]	sufficient colonies are transferred into fresh sterile medium and cultured under test conditions	7–10 days[e]
SRC, 1997	APHA (Modified) Medium	150 × 25 mm petri dishes; under test conditions[f]	18–24 hours
DFO, 1979	Hillman's M Medium	test organisms selected from stock culture	< 1 month
B & P, 1981	NI	NI	NI
C & M, 1989	Hoagland's E+	test organisms selected from stock culture	8 weeks
T & N-K, 1990	NI	NI	NI

[a] Inoculum medium is the same as the basic medium (see Appendix D, Table 1) except the dosage of stock solutions II (nitrogen) and V (phosphorus) are increased two-fold to prevent shortage during the last part of the growth phase.
[b] NI = Not indicated.
[c] *L.g. = Lemna gibba*.
[d] *L.m. = Lemna minor*.
[e] If plant material is collected from the field, plants should be maintained in culture for a minimum of eight weeks before use. If obtained from another laboratory or a culture collection, they should be similarly maintained for a minimum of three weeks.
[f] Plants for the test are selected from a test culture where 10 to 20 plants are aseptically transferred from a week-old test tube culture and maintained in 100 mL of Hoagland's E+ for 7–10 days.

7. Type of Test Medium

Document	Medium	Chemical Modification(s) of Medium	Type of Water	Preparation
ITM, 1990	Basic Medium	same compositions as stock culture medium (See Appendix C, Table 4) but contains less N and P	deionized or equiv.	8 stock solutions added to water; pH adjusted, made up to 1L; not autoclaved
ASTM, 1991	Same as Culture Medium (Appendix C, Table 4)			
APHA, 1992	Same as Culture Medium (Appendix C, Table 4)			
USEPA, 1992	Same as Culture Medium (Appendix C, Table 4)			
USEPA, 1996	Same as Culture Medium (Appendix C, Table 4) [a]			
AFNOR, 1996	Same as Culture Medium (Appendix C, Table 4)			
OECD, 1998	Same as Culture Medium: 20X-AAP for *L.gibba*; and SIS medium for *L.minor* (Appendix C, Table 4)			
SRC, 1997	APHA (Modified) Medium[b]	addition of KCl; omission of EDTA	Milli-Q	3 stock solutions; make up to 1L; aerate 1–2 h; pH adjust to 8.3; not autoclaved
DFO, 1979	Same as Culture Medium (Appendix C, Table 4)			
B & P, 1981	Same as Culture Medium (Appendix C, Table 4)			
C & M, 1989	Same as Culture Medium (Appendix C, Table 4)			
T & N-K, 1990	Nutrient-enriched Water	Same as Culture Medium (Appendix C, Table 4)		
	or Modified APHA (1985)	no EDTA; $MgCl_2$ = 12.16 mg/L	NI[c]	NI

[a] M-Hoagland's medium should be used for test solution preparation if it is suspected that the chelator will interact with the test chemical.

[b] Receiving water can be used as test medium to evaluate the effect of wastewater on its immediate environment.

[c] NI = Not indicated

8. Test System[a]

Document	Test Vessel	Test Concentrations	Design
ITM, 1990	300 mL Erlenmeyer flask or large enough for frond growth without overlapping; sealed with air permeable cellulose plugs	geometric series; 0.83–0.5 dilution factor[b,c]	randomization of test vessels; vessels moved daily
ASTM, 1991	glass: 250 mL beakers, 200 mL flat-bottomed test tubes, 250 mL fruit jars, 250 or 500 mL Erlenmeyer flasks; 5:2 test vessel:test volume ratio; plastic may be used if *Lemna* does not adhere and material does not sorb; covered[e]	≥5 plus control(s); geometric series; ≥0.6 dilution factor[c]	randomization of test vessels (RBD[d])
APHA, 1992	60 × 15 mm glass petri dishes; plastic may be used if *Lemna* does not adhere; covered	≥6 plus control(s); 0.5 dilution factor	NI[f]
USEPA, 1992	glass beakers or Erlenmeyer flasks large enough to allow frond growth without crowding (250 mL recommended)[e]	≥5 plus control(s)[c]	RCBD[g], or randomization within chambers
USEPA, 1996	glass beakers or Erlenmeyer flasks large enough to allow *Lemna* growth without crowding (250 mL recommended); 5:2 test vessel:test volume ratio[e]	≥5 plus control(s); geometric series; 0.67–0.5 dilution factor[c]	RCBD, or randomization within chambers
AFNOR, 1996	250 mL conical flasks, crystallizing dishes or other, allowing ≥4cm ht. and ≥35 cm^2 surface area; air permeable stoppers	3–4 within those causing 10–90% growth inhibition; geometric series; dilution factor: 0.1 for substances, 0.5 for water samples	NI
OECD, 1998	Erlenmeyer flasks, crystallizing dishes, or glass petri dishes, ≥20 mm deep, ≥100 mL volume, large enough for frond growth without overlapping; covered	≥5 plus control(s); geometric series; ≥0.3 dilution factor	randomization of test vessels; blocked design or reposition test vessels after observations
SRC, 1997	1 oz (30 mL) polystyrene cup; polystyrene petri lid cover	10 plus control(s); geometric series[h,i]	NI
DFO, 1979	125 mL Erlenmeyer flasks	NI	NI
B & P, 1981	7.5 × 10.8 × 6.8 cm glass test chambers	5 plus control(s)	NI
C & M, 1989	250 mL glass Erlenmeyer flask; Shimadzu closure	6 plus control(s); 0.1 dilution factor	NI
T & N-K, 1990	30 mL polystyrene plastic cups	0.5, 0.3, and 0.25 dilution series	RBD ; test boards rotated daily

[a] Testing and culturing are conducted in an environmental chamber, incubator, thermostat room, or cupboard with appropriate illumination and constant temperature control.
[b] Due to the addition of stock solutions and pH adjustments, the possibility of testing wastewater concentrations > 90–95% are limited.
[c] Selected concentrations should bracket the predicted effect levels (e.g., IC10, IC50, NOEC).
[d] RBD = Randomized Block Design.
[e] All test chambers and covers in a test must be identical.
[f] NI = Not indicated.
[g] RCBD = Randomized Complete Block Design.
[h] The highest possible test concentration of effluent is 97% due to the addition of stock solution.
[i] Test concentrations should include concentrations that inhibit biomass < 10% and > 90%. Other concentrations, that range between these, will bracket the IC_{25} and IC_{50}.

9. Test Conditions

Document	Test Volume	Number of Plants per Vessel[a]	Number of Fronds per Plant	Total Number of Fronds Inoculated	Number of Replicate Vessels	Test Solution Renewal
ITM, 1990	250 mL	3	3	9	5	Days 2 and 4[b]
ASTM, 1991	5:2 (vessel:volume)	3–5[c]	3–4[c]	12–16[c]	≥3	None
APHA, 1992	15 mL	≥6[d]	2	≥12	4	daily if assessing effluent toxicity in receiving environ.
USEPA, 1992	150 mL	3	4	12	7	Days 3 and 6 or more[e]
USEPA, 1996	150 mL	3–5[c]	3–4[c]	12–16[c]	3	Days 3 and 5 or more[e]
AFNOR, 1996	4-cm deep	8	2	16	3	daily
OECD, 1998	NI[f]	NI	2–4[c]	9–12[c]	≥3	≥ 2 × (e.g., Days 3 and 5)[g,h]
SRC, 1997	25 mL	1	3	3	8	None
DFO, 1979	50 mL	NI	NI	10	5	NI
B & P, 1981	400 mL	7 (- root)[i]	2	14	4	flow-through; 14 volume replacements/day
C & M, 1989	NI	NI	NI	NI	NI	NI
T & N-K, 1990	15 mL	6 (- root)	2	12	4	daily

[a] Care should be taken to ensure that plants and fronds are approximately the same size and quality in each test chamber at the beginning of the test.
[b] Test solutions are renewed if: the concentration of the tested substance (or active component in the wastewater) can be expected to decrease remarkably during the test period; if there are considerable changes in the pH value; or high microbial activity.
[c] The number of plants and fronds must be identical or as nearly identical as possible in each test chamber.
[d] Cutting the roots before test initiation is optional.
[e] Colonies should be transferred more frequently for highly volatile test substances to maintain 80% of the initial test substance concentration.
[f] NI = Not indicated.
[g] A static-renewal test should be used if a preliminary stability test shows that the test substance concentration cannot be maintained over the test period (i.e., the measured concentration falls below 80% of the measured initial concentration. In some circumstances, a flow-through procedure might be required.
[h] More frequent renewals might be necessary to maintain concentrations of unstable or volatile substances.
[i] Roots are removed with scissors before beginning the test.

10. Light, Temperature, and pH Conditions During Test

Document	Photoperiod	Light Intensity	Light Type[a]	Temperature (°C)	pH Range
ITM, 1990	continuous	4000–6000 lux[b]	fluorescent (warm-white)	25 ± 1	5.5–7.5
ASTM, 1991	continuous	6200–6700 lux [b,c]	fluorescent (warm-white)	25 ± 2	NI[d]
APHA, 1992	continuous	4300 or 2150 lux	fluorescent (cool-white)	25 ± 2	7.5–9.0
USEPA, 1992	continuous	350–450 µE/m^2·s^{-1}[c]	NI	25 ± 2	4.8–5.2[e]
USEPA, 1996	continuous	4200 and 6700 lux[b,c]	fluorescent (warm-white)	25 ± 2	4.8–5.2 or 7.4–7.6[e,f]
AFNOR, 1996	continuous	3000–4000 lux[g]	fluorescent (universal-white; natural)	25 ± 1	6.5–8.5[e]
OECD, 1998	continuous	6500–10 000 lux[b,c]	fluorescent (warm- or cool-white)	24 ± 2	6.0–8.0[h]
SRC, 1997	continuous	4000–4500 lux[b]	fluorescent (full-spectrum)	25 ± 2	8.3–9.0
DFO, 1979	16 h:8h light:dark	60µE/m^2·s^{-1}	Sylvanic Gro-Lux (plant growth lights)	25	NI
B & P, 1981	continuous	3875 lux	fluorescent (Gro & Sho and cool-white)	22 ± 1	NI
C & M, 1989	NI	*L.g.*–6461 ± 323 lux[i]; *L.m.*–5385 ± 323 lux[j]	NI	25 ± 2	4.8–5.2
T & N-K, 1990	continuous	1505–1725 lux[k]	fluorescent (warm-white)	25	NI

[a] Even distribution of light above the entire exposure area.
[b] Light intensity should not vary more than ± 15% from the selected light intensity throughout the incubation area.
[c] Light intensity is measured at the surface of the test solution.
[d] NI = Not indicated.
[e] No pH adjustment.
[f] pH of 4.8–5.2 for Modified Hoagland's medium and 7.4–7.6 for 20X-AAP.
[g] Light intensity is as measured at the level of the test vessels.
[h] The pH of the control medium should not increase by more than 1.5 units during the test.
[i] *L.g. = Lemna gibba*.
[j] *L.m. = Lemna minor*.
[k] Light was diffused using a 66 × 50 cm piece of 0.32 cm translucent plastic.

11. Monitoring Water Quality During Test

Document	Variable[a]	Frequency (days)
ITM, 1990	cond., pH	- test start, before and after each test solution renewal, test end
	conc.	- before renewal, test end
	T	- regularly
ASTM, 1991	pH, conc.	- test start and end; in controls and high, medium, and low concentrations
	T	- hourly or daily maximum and minimum
APHA, 1992	pH, DO, cond., T	- test start and end; in all test concentrations and control(s)
USEPA, 1992	pH, conc.	- before and after test solution renewal on Days 3, 6, and 7
USEPA, 1996	pH, conc.	- before and after test solution renewal on Days 3, 5, and 7
AFNOR, 1996	NI[b]	NI
OECD, 1998	pH	- test start and end and ≥2 other occasions, for static test; before and after each test solution renewal, for static-renewal test
	light intensity	- once during test
	T	- at least daily
	conc.	- all freshly prepared solutions or highest and lowest test conc.[c]
SRC, 1997	pH	- test end; in controls and high and low concentrations
	T	- continuously or daily mean maximum and minimum
DFO, 1979	NI	NI
B & P, 1981	NI	NI
C & M, 1989	NI	NI
T & N-K, 1990	pH, T	- test start (before frond addition) and after each test solution renewal
	cond.	- test start (before frond addition)

[a] conc. = test substance concentration
cond. = specific conductivity
DO = dissolved oxygen
pH = hydrogen ion concentration
T = temperature

[b] NI = Not indicated.

[c] For tests where the concentration of the test substance is not expected to remain within ±20% of the nominal concentration, it is necessary to analyze all freshly prepared test solutions and the same solutions at each renewal. However for those tests where the measured initial concentration of the test substance is not within ±20% of nominal, but where sufficient evidence can be provided to show that the initial concentrations can be repeatedly prepared and are stable (i.e., within 80–120% of the initial concentrations), chemical determinations may be conducted on only the highest and lowest test concentrations. In all cases, determination of test substance concentrations before renewal needs to be performed on one replicate vessel only, at each test concentration (or the contents of the vessels pooled by replicate). If there is evidence that the concentration of the substance being tested has been satisfactorily maintained within ±20% of the nominal or measured initial concentration throughout the test, analysis of the results can be based on nominal or measured initial values. If the deviation from the nominal or measured initial concentration is greater than ±20%, analysis of the results should be based on the time-weighted mean.

12. Biological Observations During Test and Biological Endpoints

Document	Variable	Frequency (days)	Special Equipment	Biological Endpoint(s)	Other Observations
ITM, 1990	No. of fronds[a] dry weight (105°C; 24 h)	2, 4, 7[b] 7	mag. glass	growth growth	NI[c]
ASTM, 1991	No. of fronds[a,d] or No. of plants dry weight (constant at 60°C)[e]	NI	NI	growth growth	change in colour, colony breakup, root destruction
APHA, 1992	No. of fronds[a,e]	daily	≥2× scope	growth	chlorosis, necrosis, colony break-up, root destruction, loss of buoyancy, gibbosity
USEPA, 1992	No. of fronds[a,f]	start, 3, 6, end	hand lens or dissecting scope	growth, mortality	necrosis, chlorosis (chorophyll content), loss of buoyancy
USEPA, 1996	No. of fronds[a,e,f]	start, 3, 5, end	hand lens or dissecting scope	growth, mortality	necrosis, chlorosis, frond size, loss of buoyancy
AFNOR, 1996	No. fronds[a]	end, (daily - optional)	NI	growth	colour, chlorosis, frond size, necrosis, dissociation of fronds, loss of buoyancy, root loss
OECD, 1998	No. of fronds dry weight (constant at 60°C); fresh weight; or total frond area	start, every 3 days start[g], end	NI	growth growth	frond size, appearance, necrosis or mortality, root length
SRC, 1997	No. of fronds[a,h]	end	NI	growth	chlorosis, necrosis, colour, frond size, gibbosity, colony breakup
DFO, 1979	No. of fronds % chlorosis,	daily daily	NI	growth chlorosis	NI
B & P, 1981	No. of fronds[a] dry weight (103°C; 3 h) root length	daily end end	NI	growth	NI

12. Biological Observations During Test and Biological Endpoints (continued)

Document	Variable	Frequency (days)	Special Equipment	Biological Endpoint(s)	Other Observations
C & M, 1989	No. of fronds, No. of plants root length dry weight (constant 60°C) chlorophyll a, b; Kjeldahl nitrogen	NI	dissecting scope HPLC	growth	NI
T & N-K, 1990	No. of fronds[a] chlorophyll a, b, c; pheophytin a	daily end	 spectrophot.	growth chlorophyll content	NI

[a] Every frond that visibly projects beyond the edge of the parent frond should be counted as a separate frond.
[b] The same replicates should be used for all counts.
[c] NI = Not indicated.
[d] Fronds that have lost their pigmentation should not be counted
[e] Other parameters (e.g., frond area, plant colony counts, root number, root length, fresh biomass, C-14 uptake, total chlorophyll concentration, chlorophyll a, b, c content, Kjeldahl nitrogen, and pheophytin pigment) can be measured.
[f] Both living and dead fronds are counted.
[g] Mean dry weight of inoculum plants is determined at the beginning of the test by collecting representative samples at test initiation.
[h] Frond counts in each cup include those fronds that are yellow and green, but not those that are white, brown, or black.

13. Statistical Test Endpoint

Document	Endpoint(s)	Calculation
ITM, 1990	EC50, EC10	graphical; statistical computer program
	NOEC, LOEC	ANOVA or Dunnett's
ASTM, 1991	IC50	graphical; statistical interpolation.
	NOEC	hypothesis test, test of heterogeneity, and pairwise comparison; contingency table test; ANOVA; multiple comparison
APHA, 1992	IC10, IC50, IC90	graphical; statistical methods
USEPA, 1992	EC10, EC50, EC90	graphical; statistical methods (goodness-of-fit) for concentration-response curves
USEPA, 1996	EC5, EC50, EC90, NOEC, LOEC	graphical; statistical methods (goodness-of-fit) for concentration-response curves
AFNOR, 1996	IC50	graphical; statistical methods
OECD, 1998	EC50	graphical; non-linear regression using appropriate function (logistic curve, cumulative normal model, or linear interpolation with bootstrapping (ICp); statistical interpolation
	NOEC, LOEC	ANOVA, multiple comparison method (e.g., Dunnett's or Williams), and non-parametric analysis (Wilcoxon Rank Sum test) if tests for normality (Shapiro-Wilk's) and homogeneity (e.g., Bartlett's or Levene's) are severely violated.
SRC, 1997	ICx values (e.g., IC25 and IC50)	non-linear regression model
DFO, 1979	NI[a]	NI
B & P, 1981	EC50	non-linear regression model
C & M, 1989	mean comparisons	Chi-square, linear correlations coefficients
T & N-K, 1990	numerical data	ANOVA
	LOEC, NOEC	Dunnett's
	chronic value	geometric mean of NOEC and LOEC

[a] NI = Not indicated.

14. Validity of Test

Document	Acceptable Growth in Control	T[a] (°C)	pH[b]	Other (Test invalid if...)
ITM, 1990	frond doubling time ≤50 h ≥8 mg mean dry weight per replicate 0.1–0.2 mg mean frond weight	NI[c]	1.0	inoculum not from a monoculture; concentration of test substance <70% nominal value (not relevant for wastewaters)
ASTM, 1991	≥5 × increase in frond number	NI	4	test chambers and covers not identical; treatments and/or plants not randomly assigned; growth medium solvent controls not included; and/or acclimation did not follow procedure; test lasted < 7 days; temp. not measured; light intensity differed by >15% from selected intensity; # of plants and the # of fronds was not identical in all test chambers at the start of test
APHA, 1992	≥2 × increase in frond number in 4 days	NI	NI	>10% mortality, disease or stress in controls
USEPA, 1992	NI	NI	NI	NI
USEPA, 1996	NI	NI	NI	NI
AFNOR, 1996	daily growth rate (μ)[d] = 0.25–0.35/d	NI	NI	IC50 of potassium dichromate (ref. tox.) <10 mg/L or > 30 mg/L
OECD, 1998	frond number doubling time <2.5 days (60 h) ≅8× increase in biomass in 7 days	24 ± 2°C	6.0–8.0	NI
SRC, 1997	≥8 × increase in frond number in 7 days	25 ± 2°C	NI	exhibition of algae growth; *Lemna* not maintained in fast growing axenic condition in Hoagland's E+ medium by weekly subculture; light and temperature conditions not maintained for duration of test; testing of effluent did not begin within 72 h of collection; mean control growth rate and mean % inhibition of biomass by the ref. tox. does not lie within the cumulative 95% confidence limits of ≥5 tests
DFO, 1979	NI	NI	NI	NI
B & P, 1981	NI	NI	NI	NI

14. Validity of Test (continued)

Document	Acceptable Growth in Control	T[a] (°C)	pH[b]	Other (Test invalid if...)
C & M, 1989	3 × increase in plant # and 3 × increase in frond # in 7 days	NI	NI	NI
T & N-K, 1990	NI	NI	NI	NI

[a] Maximum temperature (T) variation allowed in test vessels during a test.
[b] Maximum pH variation allowed in control vessels during a test.
[c] NI = Not indicated.
[d]

$$\mu = \frac{\text{Ln } N_4 - \text{Ln } N_0,}{4}$$

where: N_4 = number of fronds observed in the control vessel after 4 days; and
N_0 = number of fronds observed in the control vessel at the beginning of the test.

15. Reference Toxicant

Document	Chemical	Concentration (mg/L)	Frequency
ITM, 1990	NI[a]	NI	NI
ASTM, 1991	NI	NI	NI
APHA, 1992	potassium chromate	20 or 35 (as Cr)	every test as +ve control
USEPA, 1992	NI	NI	NI
USEPA, 1996	zinc chloride ($ZnCl_2$)	NI	periodically
AFNOR, 1996	potassium dichromate ($K_2Cr_2O_7$)	10–30[b] (as Cr)	depends on test frequency
OECD, 1998	to be resolved	to be resolved	to be resolved
SRC, 1997	potassium chromate	1 (as Cr)	each time testing is done
DFO, 1979	NI	NI	NI
B & P, 1981	NI	NI	NI
C & M, 1989	NI	NI	NI
T & N-K, 1990	sodium chloride (NaCl)	15 000, 4000	6 tests

[a] NI = Not indicated.
[b] 4-day IC50 of potassium dichromate to *L. minor*.

Appendix D

Review of Culture and Test Media Used in *Lemna* spp. Growth Inhibition Tests, as Described in Canadian, American, and European Methodology Documents

Source documents are listed chronologically by originating agency.

ITM, 1990 represents the Institutet för tillämpad miljöforskning. This publication gives culturing and toxicity test procedures for *Lemna minor* compiled and used by the Swedish National Protection Environmental Board in collaboration with the National Chemicals Inspectorate (Institutet för tillämpad miljöforskning), Solna, Sweden.

ASTM, 1991 is the standard guide published by the American Society for Testing and Materials for conducting static toxicity tests with *Lemna gibba* G3.

APHA, 1992 represents the American Public Health Association, the American Water Works Association, and the Water Environment Federation, 1992. The publication (in Standard Methods for the Examination of Water and Wastewater - 18th ed.) gives culturing and testing procedures for *L. minor* which was included as a monitoring tool under the Environmental Effects Monitoring component of the Canadian Federal Pulp and Paper Effluent Regulations. This guideline document was revised in 1996.

USEPA, 1992 is the standard guide published by the Office of Pollution Prevention and Toxics (OPPT), United States Environmental Protection Agency, for conducting toxicity tests using *L. gibba* G3 to develop data on the phytotoxicity of chemicals [under the Toxic Substances Control Act (TSCA)]. It appeared in Title 40, Chapter I, Subchapter R of the Code of Federal Regulations. This guideline document was revised, harmonized with other publications, and re-published (draft) in 1996 (see following citation).

USEPA, 1996 is the draft (April, 1996) standard guideline (OPPTS 850.4400) developed by the Office of Pollution Prevention and Toxics (OPPT), United States Environmental Protection Agency, for conducting toxicity tests using *L. gibba* G3 and *L. minor* to develop data on the phytotoxicity of chemicals [under the Toxic Substances Control Act (TSCA), and Federal Insecticide, Fungicide and Rodenticide Act (FIFRA)]. This guideline blends testing guidance and requirements that existed in OPPT and appeared in Title 40, Chapter I, Subchapter R of the Code of Federal Regulations (CFR); the Office of Pesticide Programs (OPP) that appeared in the publications of the National Technical Information Service (NTIS) and the guidelines published by the Organization for Economic Cooperation and Development (OECD). It represents the harmonization of two documents: 40 CFR 797.1160 *Lemna* Acute Toxicity Test, and OPP 122-2 Growth and Reproduction of Aquatic Plants (Tier I), and 123-2 Growth and Reproduction of Aquatic Plants (Tier 2) (Pesticide Assessment Guidelines, Subdivision J--Hazard Evaluation; Nontarget Plants) EPA report 540/09-82-020, 1982.

AFNOR, 1996 is the standard guide published by the Association française de normalisation (test method XP T 90-337,1996). This document gives culturing and toxicity test procedures using *L. minor*.

OECD, 1998 is the draft (June, 1998) standard procedure published by the Organization for Economic Cooperation and Development. The guideline is designed to assess the toxicity of substances to *L.*

gibba and *L. minor* and is based on existing guidelines and standards published by ASTM (1991), USEPA (1996), AFNOR (1996), and the Swedish Standards Institute (SIS) (1995).

SRC, 1997 is the (unpublished) standard operating procedures developed in 1997 by H. Peterson and M. Moody of the Saskatchewan Research Council, Water Quality Section Laboratory, for culturing and testing *L. minor*. It is based on research conducted by Peterson and Moody (1994–1997) and is a modification of the APHA, 1995–8211 Duckweed (proposed) toxicity test procedure.

SRC, 2003 is the (unpublished) report prepared by M. Moody of the Saskatchewan Research Council, Water Quality Section Laboratory, describing the development of a modified Hoagland's E+ medium for culturing *L. minor*. The modified Hoagland's E+ medium is based on research conducted by Moody and is a modification of the Hoagland's E+ medium described in the first edition of Environment Canada's *Lemna minor* test method document.

ISO, 2005 is the draft international standard test method for testing the effects of water constituents and wastewater on the growth of *L. minor*, published by the International Organization for Standardization in Geneva, Switzerland.

DFO, 1979 represents Lockhart and Blouw, 1979. This method, published in a document entitled Toxicity Tests for Freshwater Organisms, E. Scherer (ed.), describes procedures for testing herbicides and sediments with *L. minor*.

1. ITM, 1990—Culture and Test Media for *Lemna minor*

Substance	Concentration				Element	Stock Solution
	Stock Solution (g/L)	Medium[a] (mg/L)				
		Basic[b]	Cult.[c,d]	Inoc.[d,e]		
$MgSO_4 \cdot H_2O$	15	75	75	75	NI[f]	I
$NaNO_3$	8.5	42.5	425	85	NI	II
$CaCl_2 \cdot 2H_2O$	7.2	36	36	36	NI	III
Na_2CO_3	4.0	20	20	20	NI	IV
K_2HPO_4	1.34	6.7	67	13.4	NI	V
H_3BO_3	1.0	1.0	1.0	1.0	NI	VI
$MnCl_2 \cdot 4H_2O$	0.2	0.2	0.2	0.2	NI	VI
$Na_2MoO_4 \cdot 2H_2O$	0.010	0.010	0.010	0.010	NI	VI
$ZnSO_4 \cdot 7H_2O$	0.050	0.050	0.050	0.050	NI	VI
$CuSO_4 \cdot 5H_2O$	0.005	0.005	0.005	0.005	NI	VI
$Co(NO_3)_2 \cdot 6H_2O$	0.010	0.010	0.010	0.010	NI	VI
Na_2EDTA	0.28	1.4	1.4	1.4	NI	VII[g]
citric acid	0.12	0.6	0.6	0.6	NI	VII[g]
Fe(III) ammonium citrate	0.12	0.6	0.6	0.6	NI	VII[g]
MOPS (buffer)[h]	488[i]	488	488	488	NI	VIII[g]
pH Adjustment	pH adjust to 6.5 by addition of NaOH or HCl					
Sterilization	Stock solutions are sterilized by use of sterilizing filters (pore diameter 0.2μm) or by autoclaving					

[a] Concentration of substance in medium.
[b] The complete synthetic culture medium used for dilution of the test substance/wastewater.
[c] The complete synthetic culture medium used for maintenance of *Lemna* stock cultures.
[d] Dosage of stock solutions II (nitrogen) and V (phosphorus) has been increased to prevent the inoculum plants from suffering from lack of nutrition during the last part of the growth phase.
[e] The complete synthetic culture medium used for the acclimation of *Lemna* 10–12 days before the test.
[f] NI = Not indicated.
[g] Added after autoclaving.
[h] pH adjust to 6.5 with NaOH.
[i] If the change in pH is expected to be considerable, the buffer added should be increased to 2.0 mL per litre of test solution.

2. ASTM, 1991—Hoagland's E+ Medium for Culturing and Testing *Lemna gibba* G3

Substance [a]	Concentration		Element	Stock Solution
	Stock Solution (g/L)	Medium [b] (mg/L)		
$MgSO_4 \cdot 7H_2O$	50.00	500.0	NI [c]	E [d]
KNO_3	75.76	1515.2	NI	A
$Ca(NO_3)_2 \cdot 4H_2O$	59.00	1180.0	NI	A
KH_2PO_4	34.00	680.0	NI	A
H_3BO_3	2.86	2.86	NI	F
$MnCl_2 \cdot 4H_2O$	3.62	3.62	NI	F
$Na_2MoO_4 \cdot 2H_2O$	0.12	0.12	NI	F
$ZnSO_4 \cdot 7H_2O$	0.22	0.22	NI	F
$CuSO_4 \cdot 5H_2O$	0.08	0.08	NI	F [e]
EDTA	9.00	9.00	NI	D
Sucrose	-----	1×10^4	NI	G
$FeCl_3 \cdot 6H_2O$	5.40	5.40	NI	C
Yeast extract	-----	100	NI	H
Bactotryptone	-----	600	NI	I
Tartaric Acid	3.00	3.00	NI	B
pH Adjustment	Adjust the pH to 4.60 with KOH or HCl			
Sterilization	Autoclave 20 min at 121°C and 1.1 kg/cm^2			

[a] It has been shown that growth of *Lemna gibba* G3 is enhanced by the addition of the following to the growth medium: Se 4.2 µg/L, V 25.6 µg/L, Co 20.3 µg/L, and Sn 457µg/L (Cowgill and Milazzo, 1989).
[b] Concentration of substance in prepared medium.
[c] NI = Not indicated.
[d] Add 6 mL of 6*N* HCl to stock solution A.
[e] Add 8 mL of 6*N* KOH to stock solution D.

3. ASTM, 1991—Modified Hoagland's Medium[a] (no Sucrose or EDTA) for Culturing and Testing *Lemna gibba* G3

Substance	Concentration		Element	Stock Solution
	Stock Solution (g/L)	Medium[b] (mg/L)		
$MgSO_4 \cdot 7H_2O$	NI[c]	492	NI	A[d]
KNO_3	NI	1515	NI	A
$Ca(NO_3)_2 \cdot 4H_2O$	NI	1180	NI	A
KH_2PO_4	NI	680	NI	A
H_3BO_3	NI	2.86	NI	B[e]
$MnCl_2 \cdot 4H_2O$	NI	3.62	NI	B
$Na_2MoO_4 \cdot 2H_2O$	NI	0.12	NI	B
$ZnSO_4 \cdot 7H_2O$	NI	0.22	NI	B
$CuSO_4 \cdot 5H_2O$	NI	0.08	NI	B
$FeCl_3 \cdot 6H_2O$	NI	5.40	NI	A
Tartaric Acid	NI	3.00	NI	A
pH Adjustment	Adjust the pH to 5.0 ± 0.1 with 0.1*N* KOH or HCl, after autoclaving			
Sterilization	Autoclave 20 min at 121°C and 1.1 kg/cm^2			

[a] This medium is the same as Hoagland's E+ medium (Table 2) except the sucrose, bacto-tryptone, yeast, and EDTA have been excluded.
[b] Concentration of substance in prepared medium.
[c] NI = Not indicated.
[d] Add each chemical (A) to distilled or deionized water.
[e] Add 1 mL of micronutrient stock solution (solution B).

4. ASTM, 1991—20X-AAP Medium[a] for Culturing and Testing *Lemna gibba*

Substance	Concentration: Stock Solution[b] (g/L)	Concentration: Medium[c] (mg/L)	Element	Stock Solution
$MgSO_4 \cdot 7H_2O$	14.70	38.22	S	D
$NaNO_3$	25.50	84.00	N	A
$CaCl_2 \cdot 2H_2O$	4.410	24.04	Ca	F
$NaHCO_3$	15.00	220.02	Na	B
	-----	42.86	C	B
K_2HPO_4	1.044	9.38	K	C
	-----	3.72	P	C
H_3BO_3	0.18552	0.64920	B	G
$MnCl_2 \cdot 4H_2O$	0.41561	2.30748	Mn	G
$MgCl_2 \cdot 6H_2O$	12.164	58.08	Mg	E
$Na_2MoO_4 \cdot 2H_2O$	0.00726	0.05756	Mo	G
$ZnCl_2$	0.00327	0.0314	Zn	G
$CuCl_2 \cdot 2H_2O$	1.2×10^{-5}	8×10^{-5}	Cu	G
$CoCl_2 \cdot 6H_2O$	0.00143	0.00708	Co	G
$Na_2EDTA \cdot 2H_2O$	0.300	-------	----	G
$FeCl_3 \cdot 6H_2O$	0.160	0.66102	Fe	G
pH Adjustment	Adjust to pH 7.5 ± 0.1 with 0.1*N* NaOH or HCl			
Sterilization	Filter medium through a 0.22µm pore size membrane filter into a sterile container			

[a] Ionic strength is much less than Hoagland's medium.

[b] Add 20 mL of each of the six macronutrient stock solutions (solutions A - F) and 20 mL of the micronutrient stock solution (solution G) to approximately 800 mL of deionized or distilled water. Bring the volume to 1L.

[c] Concentration of element in medium.

5. APHA, 1992—Duckweed Nutrient Solution for Culturing and Testing *Lemna minor*

Substance	Concentration: Stock Solution[a] (g/L)	Concentration: Medium[b] (mg/L)	Element	Stock Solution
$MgSO_4 \cdot 7H_2O$	14.7	19.1	S	C
$NaNO_3$	25.5	42.0	N	A
	-----	110.0	Na	A
$CaCl_2 \cdot 2H_2O$	4.41	12.0	Ca	B
$NaHCO_3$	15.0	21.4	C	A
K_2HPO_4	1.04	4.69	K	A
	-----	1.86	P	A
H_3BO_3	0.186	0.325	B	C
$MnCl_2$	0.264	1.15	Mn	B
$MgCl_2$	5.7	29.0	Mg	B
$Na_2MoO_4 \cdot 2H_2O$	0.00726	0.0288	Mo	C
$ZnCl_2$	0.00327	0.0157	Zn	C
$CuCl_2$	9×10^{-6}	4×10^{-5}	Cu	C
$CoCl_2$	0.00078	0.00354	Co	C
$Na_2EDTA \cdot 2H_2O$[c]	0.3	-----	-----	B
$FeCl_3$	0.096	0.33	Fe	B
pH Adjustment	Adjust to pH 7.5–8.0			
Sterilization	None			

[a] To prepare duckweed nutrient solution, add 1 mL of each stock solution to 100 mL deionized water.
[b] Concentration of element in medium.
[c] Omit $Na_2EDTA \cdot 2H_2O$ in solution B if test samples contain toxic metals. In that case, acidify solution B to pH 2 to prevent precipitation.

6. USEPA, 1992 and 1996[a]—Modified Hoagland's Medium[b] (no Sucrose or EDTA) for Culturing and Testing *Lemna gibba*

Substance	Concentration		Element	Stock Solution
	Stock Solution (g/L)	Medium[c] (mg/L)		
$MgSO_4 \cdot 7H_2O$	NI[d]	492	NI	A[e]
KNO_3	NI	1515	NI	A
$Ca(NO_3)_2 \cdot 4H_2O$	NI	1180	NI	A
KH_2PO_4	NI	680	NI	A[f]
H_3BO_3	NI	2.86	NI	B
$MnCl_2 \cdot 4H_2O$	NI	3.62	NI	B
$Na_2MoO_4 \cdot 2H_2O$	NI	0.12	NI	B
$ZnSO_4 \cdot 7H_2O$	NI	0.22	NI	B
$CuSO_4 \cdot 5H_2O$	NI	0.08	NI	B
$FeCl_3 \cdot 6H_2O$	NI	5.40	NI	A
Tartaric Acid	NI	3.00	NI	A
pH Adjustment	Adjust the pH to 5.0 ± 0.2 with 0.1*N* NaOH[g]			
Sterilization	Autoclave			

[a] USEPA, 1996 recommends Modified Hoagland's or 20X-AAP nutrient media.

[b] This medium is the same as Hoagland's E+ medium (Table 2) except the sucrose, bacto-tryptone, yeast and EDTA have been excluded. Chelating agents, such as EDTA are present in the 20X-AAP medium to ensure that trace nutrients will be available to the *Lemna* fronds. Modified Hoagland's medium , which contains no EDTA, should therefore be used for test solution preparation if it is suspected that the chelator will interact with the test chemical.

[c] Concentration of substance in prepared medium.

[d] NI = Not indicated.

[e] Add each chemical (A) to distilled or deionized water.

[f] Add 1 mL of micronutrient stock solution (solution B).

[g] pH of Modified Hoagland's medium should be adjusted to 4.8–5.2 with 0.1*N* or 1*N* NaOH. If 20X-AAP is used, the pH should be adjusted to 7.4–7.6 with 0.1*N* NaOH or HCl.

7. AFNOR, 1996—Culture and Test Media for *Lemna minor*

Substance	Concentration: Stock Solution (g/L)	Concentration: Medium[a] (mg/L) Conc.[b]	Concentration: Medium[a] (mg/L) Cult. and Test[c]	Element	Stock Solution
$MgSO_4 \cdot 7H_2O$	123.3	4932	493.2	NI[d]	3
KNO_3	101.1	5055	505.5	NI	2
$Ca(NO_3) \cdot 4H_2O$	118	11800	1180.0	NI	1
KH_2PO_4	68	680	68.0	NI	4
FeEDTA	3.46	34.6	3.46	NI	5
H_3BO_3	28.6	28.6	2.86	NI	6
$MnSO_4 \cdot 7H_2O$	15.5	15.5	1.55	NI	6
$ZnSO_4 \cdot 7H_2O$	2.2	2.2	0.22	NI	6
$CuSO_4 \cdot 5H_2O$	0.79	0.79	0.079	NI	6
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	1.28	1.28	0.128	NI	7
NH_4VO_3	2.296	2.296	0.2296	NI	7
$CrK(SO_4)_2 \cdot 12H_2O$	0.96	0.96	0.096	NI	7
$NiSO_4 \cdot 7H_2O$	0.4785	0.4785	0.0479	NI	7
$Co(NO_3)_2 \cdot 6H_2O$	0.493	0.493	0.0493	NI	7
$Na_2MoO_4 \cdot 2H_2O$	0.1794	0.1794	0.01794	NI	7
$TiOSO_4 \cdot 4H_2O$	0.2416	0.2416	0.02416	NI	7
pH Adjustment	Adjust the pH of the culture and test media to 5.5 ± 0.5 with NaOH or HCl[e]				
Sterilization	Filtration through 0.22 µm filter				

[a]
[b] Concentration of substance in prepared medium.
[c] Concentrated nutrient medium– prepared just before use.
[d] The culture and test media are composed of 10% of the concentrated nutrient medium and 90% distilled water or water of equivalent quality.
[e] NI = Not indicated.
pH of concentrated nutrient medium is adjusted to 3.8 ± 0.3 with HCl and NaOH.

8. OECD, 1998—Culture and Test Media for *Lemna minor* (SIS growth medium)

Substance	Concentration		Element	Stock Solution
	Stock Solution (g/L)	Medium[a] (mg/L)		
$MgSO_4 \cdot 7H_2O$	15	75	NI[b]	II
$NaNO_3$	8.5	85	NI	I
$CaCl_2 \cdot 2H_2O$	7.2	36	NI	III
Na_2CO_3	4.0	20	NI	IV
KH_2PO_4	1.34	13.4	NI	I
H_3BO_3	1.0	1.0	NI	V
$MnCl_2 \cdot 4H_2O$	0.2	0.2	NI	V
$Na_2MoO_4 \cdot 2H_2O$	0.010	0.010	NI	V
$ZnSO_4 \cdot 7H_2O$	0.050	0.050	NI	V
$CuSO_4 \cdot 5H_2O$	0.005	0.005	NI	V
$Co(NO_3)_2 \cdot 6H_2O$	0.010	0.010	NI	V
Na_2EDTA	0.28	1.4	NI	VI[c]
$FeCl_3 \cdot 6H_2O$	0.168	0.84	NI	VI[c]
MOPS (buffer)[d]	488	488	NI	VII[c]
pH Adjustment	Adjust the pH to 6.5 ± 0.2 by addition of NaOH or HCl.			
Sterilization	Stock solutions I to V are sterilized by autoclaving (120°C, 15 min.) or by membrane filtration (pore diameter 0.2μm); stock solutions VI (and optional VII) are sterilized by membrane filtration only (i.e., these should not be autoclaved).			

[a] Concentration of substance in prepared medium.
[b] NI = Not indicated.
[c] Added after autoclaving.
[d] MOPS buffer is only required when pH control of the test medium is particularly important (e.g., when testing metals or substances that are hydrolytically unstable).

9. SRC, 1997—Modified APHA Medium for Testing *Lemna minor*

Substance	Concentration		Element	Stock Solution
	Stock Solution[a] (g/L)	Medium[b,c] (mg/L)		
$MgSO_4 \cdot 7H_2O$	14.7	147	NI[d]	C
$NaNO_3$	25.5	255	NI	A
$CaCl_2 \cdot 2H_2O$	4.41	44.1	NI	B[e]
KCl[f]	1.01	10.1	NI	A
$NaHCO_3$	15.0	150	NI	A
K_2HPO_4	1.04	10.4	NI	A
H_3BO_3	0.186	1.86	NI	C
$MnCl_2 \cdot 4H_2O$	0.4149	4.149	NI	B
$MgCl_2 \cdot 6H_2O$	12.17	121.7	NI	B
$Na_2MoO_4 \cdot 2H_2O$	0.00726	0.0726	NI	C
$ZnCl_2$	0.00327	0.0327	NI	C
$CuCl_2$	9.0×10^{-6}	9.0×10^{-5}	NI	C
$CoCl_2$	0.00078	0.0078	NI	C
$FeCl_3 \cdot 6H_2O$	0.16	1.6	NI	B
pH Adjustment	Adjust to pH 8.30 ± 0.05 immediately before testing			
Sterilization	None			

[a] To prepare medium, add 10 mL of each stock solution to 970 mL Milli-Q water and aerate vigorously at least 1 to 2 hours.
[b] *Lemna* stock cultures are maintained in sterile Hoagland's E+ medium (Cowgill and Milazzo, 1989). *Lemna* to be used for testing are acclimated for 18–24 hours in modified APHA medium under test conditions.
[c] Concentration of substance in medium.
[d] NI = Not indicated.
[e] Acidify solution B to pH 2.0 to prevent precipitation. Protect the solution from light by storing in a dark amber bottle.
[f] Underlined text indicates modifications from the original APHA medium (APHA, 1992).

10. SRC, 2003— Modified Hoagland's E+ Medium for Culturing *Lemna minor*

Substance	Concentration		Element	Stock Solution
	Stock Solution[a] (g/L)	Medium[b] (mg/L)		
$MgSO_4 \cdot 7H_2O$	50.00	500.0	NI[c]	D[d]
KNO_3	75.76	1515.2	NI	A
$Ca(NO_3)_2 \cdot 4H_2O$	59.00	1180.0	NI	A
KH_2PO_4	34.00	680.0	NI	A
H_3BO_3	2.86	2.86	NI	E
$MnCl_2 \cdot 4H_2O$	3.62	3.62	NI	E
$Na_2MoO_4 \cdot 2H_2O$	0.12	0.12	NI	E
$ZnSO_4 \cdot 7H_2O$	0.22	0.22	NI	E
$CuSO_4 \cdot 5H_2O$	0.08	0.08	NI	E[f]
$Na_2EDTA \cdot 2H_2O$[e]	3.35	67.00	NI	C
Sucrose	-----	1×10^4	NI	-
$FeCl_3 \cdot 6H_2O$	1.21	24.20	NI	C
Yeast extract	-----	100	NI	-
Bactotryptone	-----	600	NI	-
Tartaric Acid	3.00	3.00	NI	B
pH Adjustment	Adjust the pH to 4.6 ± 0.2 with NaOH or HCl			
Sterilization	Autoclave 20 min at 121°C and 124.2 kPa (1.1 kg/cm^2)			

[a] To prepare 1 L of modified Hoagland's E+ medium, add 20 mL of solution A, 1 mL of solution B, 20 mL of solution C, 10 mL of solution D, 1 mL of solution E, 10 g of sucrose, 0.10 g of yeast extract, and 0.6 g of Bactotryptone to 900 mL of glass-distilled, deionized water (or equivalent). The medium is stirred until all the contents are dissolved.. The pH is adjusted, the volume is brought up to 1 L with distilled water, and the medium is autoclaved.

[b] Concentration of substance in prepared medium.

[c] NI = Not indicated.

[d] Add 6 mL of 6*N* HCl to stock solution A.

[e] $Na_4EDTA \cdot 2H_2O$ can be used instead of $Na_2EDTA \cdot 2H_2O$. If $Na_4EDTA \cdot 2H_2O$ is used, the concentrations in the stock solution and the test medium are 3.75 g/L and 75 mg/L, respectively, and KOH should not be added to stock solution C (see footnote f below)

[f] Add 1.2 mL of 6*N* KOH to stock solution C.

11. ISO, 2005— Modified Steinberg Medium for Culturing and Testing *Lemna minor*

Substance	Concentration		Element	Stock Solution[c]
	Stock Solution[a] (g/L)	Medium[b] (mg/L)		
$MgSO_4 \cdot 7H_2O$	5.00	100.0	NI[d]	2
KNO_3	17.5	350.0	NI	1
$Ca(NO_3)_2 \cdot 4H_2O$	14.75	295.0	NI	3
KH_2PO_4	4.50	90.0	NI	1
K_2HPO_4	0.63	12.6	NI	1
H_3BO_3	0.12	120.00	NI	4
$MnCl_2 \cdot 4H_2O$	0.18	180.00	NI	7
$Na_2MoO_4 \cdot 2H_2O$	0.044	44.00	NI	6
$ZnSO_4 \cdot 7H_2O$	0.18	180.00	NI	5
$Na_2EDTA \cdot 2H_2O$	1.50	1500.00	NI	8
$FeCl_3 \cdot 6H_2O$	0.76	760.00	NI	8
pH Adjustment	Adjust the pH to 5.5 ± 0.2 with NaOH or HCl, if necessary			
Sterilization	Autoclave 20 min at 121°C or filter (0.2µm) for longer shelf life			

[a] To prepare 1 L of modified Steinberg medium, add 20 mL of each of stock solutions 1, 2, and 3 to about 30 mL of distilled or deionised water. Then add 1.0 mL of each of stock solutions 4, 5, 6, 7, and 8. The pH should be 5.5 ± 0.2 (adjust by addition of a minimal amount of NaOH or HCl). The volume is brought up to 1 L with distilled or deionised water. If stock solutions are sterilized and appropriate water is used, no further sterilisation is necessary. If sterilisation is done with the final medium, stock solution 8 should be added after autoclaving (at 121°C for 20 min).

[b] Concentration of substance in prepared medium.

[c] Stock solutions 2 and 3 and 4 to 7 may be pooled (taking into account the required concentrations). For longer shelf life, treat stock solutions in an autoclave at 121°C for 20 min or alternatively carry out a sterile filtration (0.2 µm).

[d] NI = Not indicated.

12. DFO, 1979—Hillman's M Medium for Culturing and Testing *Lemna minor*

Substance	Concentration		Element	Stock Solution
	Stock Solution (g/L)	Medium[a,b] (mg/L)		
$MgSO_4 \cdot 7H_2O$	0.492	4.92×10^{-4}	NI[c]	A
KNO_3	0.100	1.52	NI	B
$Ca(NO_3)_2 \cdot 4H_2O$	1.180	1.18	NI	C
KH_2PO_4	0.170	0.680	NI	D
H_3BO_3	0.0286	2.86×10^{-3}	NI	E
$MnCl_2 \cdot 4H_2O$	0.0362	3.62×10^{-3}	NI	F
$Na_2MoO_4 \cdot 2H_2O$	0.012	1.2×10^{-4}	NI	G
$ZnSO_4 \cdot 7H_2O$	0.022	2.2×10^{-4}	NI	H
$Cu(SO_4) \cdot 5H_2O$	0.008	8.0×10^{-5}	NI	I
$FeCl_3 \cdot 6H_2O$	0.054	5.40×10^{-3}	NI	J[d]
Tartaric Acid	0.003	3.00×10^{-3}	NI	K
pH Adjustment	NI			
Sterilization	NI			

[a] Medium is prepared by diluting stock solutions with distilled water. All components except $FeCl_3$ are added to distilled water before autoclave sterilization.
[b] Concentration of substance in prepared medium.
[c] NI = Not indicated.
[d] The $FeCl_3$ stock solution is autoclaved separately and the appropriate quantity transferred to the working medium after cooling.

Appendix E

General Description of *Lemna minor*

Taxonomy and Phyletic Relationships

Lemna minor Linnaeus (Arales:Lemnaceae) is a small, vascular, aquatic macrophyte belonging to the family Lemnaceae. Members of the family Lemnaceae are structurally the simplest and the smallest, flowering plants in the world, likely by reduction from more complex ancestors (Godfrey and Wooten, 1979). Most investigators place Lemnaceae in the order Spathiflorae (Arales), relating them to the Araceae through the water-lettuce Pistia (Hillman, 1961).

Four genera are usually recognized: *Spirodela, Lemna, Wolffiella, and Wolffia* (Hillman, 1961). The fronds (or thalli) of *Spirodela* and *Lemna* are flat, more or less oval, in outline and leaf-like. *Spirodela* bears two or more thread-like roots on each frond, whereas *Lemna* has only one. The two genera have been grouped in a tribe (Lemneae) (Hegelmaier - 1895) or subfamily (Lemnoideae) (Lawalrée - 1945) (Hillman, 1961). *Spirodela* has also been considered a subgenus of *Lemna* (Hutchison, 1934, in Hillman, 1961). *Wolffiella* and *Wolffia* have no roots and have been grouped in a tribe (Wolffieae, Hegelmaier) or subfamily (Wolffioideae, Lawalrée) (Hillman, 1961). *Wolffia* consists of almost microscopic meal-like bodies, whereas *Wolffiella* is made up of strap-shaped bodies, occurring singly or radiating from a point (Fassett, 1957).

The taxonomy of *Lemna* spp. (also known as duckweeds) is difficult, being complicated by the existence of a wide range of phenotypes (OECD, 1998). In 1957, Landolt reported the existence of at least two distinct strains of *L. minor* in the United States that differed in size and in ability to flower in culture (Hillman, 1961). *L. perpusilla* and non-gibbous forms of *L. gibba* might easily be mistaken for *L. minor* (cf. Mason, 1957 in Hillman, 1961). *L. gibba* differs from *L. minor* in that the fronds of *L. gibba* are broadly elliptic to round, its upper surface often has red blotches, and its lower surface is generally swollen (gibbous). *L. perpusilla* can be distinguished from *L. minor* by its wing-like appendages at the base of the root sheath and sometimes by its prominent apical and central papillae which are lacking in *L. minor* (Hillman, 1961; Godfrey and Wooten, 1979). The lack of overwintering turions (dark green or brownish daughter plants), lack of prominent dorsal papules, and of reddish anthocyanin blotches on the ventral side separate *L. minor* from another closely related species *Lemna turionifera* Landolt. Taxonomic descriptions and photographs of many Lemnaceae species can be found on the Internet at Wayne P. Armstrong's Key to the Lemnaceae of western North America (Palomar College/Oregon State University) (http://waynesword.palomar.edu/1wayindx.htm).

Species Description

L. minor is a small, colonial plant with a single, flat, sub-orbicular to elliptic-obovate, leaf-like frond (discoid stem). Each plant is 2- to 4-mm long and consists of a solitary or, in the case of a colony, several (3 to 5) fronds (Hillman, 1961; ITM, 1990). The frond (or thallus) is a complex structure representing both leaf and stem (Hillman, 1961) with the distal end of the frond being foliar and the proximal end being axial (Arber, 1963). The frond is composed largely of chlorenchymatous cells, separated by large intercellular spaces, which are filled with air or other gases and provide buoyancy (Hillman, 1961).

L. minor fronds are obscurely 3-veined (or 3-nerved) and have a smooth convex or somewhat flattened dorsal surface. Although not prominent (Hillman, 1961; Britton and Brown, 1970), the dorsal surface has a small central papilla and usually, a median line of smaller papillae extending near the apex (Godfrey and Wooten, 1979). The lower surface of the frond is convex (or rarely concave when growing in insufficient light or nutrients) (Godfrey and Wooten, 1979). They are green to lime green, glossy when fresh (Godfrey and Wooten, 1979).

The plant has a single root or rootlet that emanates from a deep root furrow in the centre of the lower surface of each frond (Hillman, 1961). The root arises at the node just beneath the lower epidermis and is usually <0.5 mm in diameter, devoid of vascular tissue, and provided with an obtuse or sub-truncate rootcap (Hillman, 1961; Britton and Brown, 1970). Since the entire lower surface of *Lemna* fronds can absorb nutrients from the medium, and plants can grow well under conditions which entirely prevent root elongation, the functional importance of the root is difficult to evaluate (Hillman, 1961). It has been suggested (cf. Arber, 1920; in Hillman, 1966) that they serve chiefly as anchors to keep the fronds right side up, and to form the tangled masses that aid in dispersal and protection from water motion (Hillman, 1961).

Distribution and Ecology

L. minor is a cosmopolitan species whose distribution extends nearly worldwide (Godfrey and Wooten, 1979). It is widely distributed throughout North America, except the extreme north and in the Bahamas and, is also found in Europe, Asia, Africa, and Australia (Britton and Brown, 1970). In North America, it is found from Newfoundland to Alaska and south to California, Texas, and Florida (Newmaster *et al.*, 1997). In Canada, its distribution extends as far north as Great Slave Lake in the Northwest Territories; Lake Athabasca in Alberta and Saskatchewan; Churchill, Manitoba; James Bay, Ontario; Côte-Nord and Anticosti Island in Québec; and Newfoundland, New Brunswick, Prince Edward Island, and Nova Scotia (Scoggan, 1978).

Duckweeds inhabit lentic environments from tropical to temperate zones, from fresh water to brackish estuaries, and throughout a wide range of trophic conditions (Hillman and Culley, 1978). They can be found in still or slightly moving water of freshwater ponds, marshes, lakes, and quiet streams. Flourishing growth can be found in nutrient-rich, stagnant marshes, bogs, small ponds, or ditches rich in organic matter. Duckweeds are also found commonly near sewer outlets (ITM, 1990).

Duckweeds form an essential component of the ecosystem in shallow, stagnant waters. They are an integral portion of the food chain, providing food for waterfowl and marsh birds such as coots, black ducks, mallards, teals, wood ducks, buffleheads, and rails, and are occasionally eaten by small mammals such as muskrats and beavers. They also provide food, shelter, shade, and physical support for fish and aquatic invertebrates (Jenner and Janssen-Mommen, 1989; Taraldsen and Norberg-King, 1990; APHA *et al*., 1992; Newmaster *et al*., 1997). Under conditions favourable for growth, they can multiply quickly and form a dense mat, dominated by a single species (Wang, 1987; ASTM, 1991) made up of mixed genera and species (Riemer, 1993).

Reproductive Biology

Lemna spp. are fast growing, and reproduce rapidly compared with other vascular and flowering plants (Hillman, 1961; APHA *et al*., 1992). Reproduction of *L. minor* is usually vegetative (i.e., asexual). New “daughter” fronds are produced from two pockets on each side of the narrower end of an older

"mother" frond, very near the point at which the root arises (Hillman, 1961). This end of the frond is usually designated as "basal" or "proximal" since, in an attached daughter frond, it is the portion closest to the mother. The wider end of the frond is denoted as "distal" (Hillman, 1961). Each daughter frond becomes a mother in turn, usually while still attached to its own mother. Groups of attached fronds are called colonies (Hillman, 1961). In *Lemna*, daughter fronds are produced alternately from each side, developing earlier in one pocket than in the other. Clones of the same species differ as to which pocket produces the first daughter, but this normally remains constant within a clone (Hillman, 1961).

Flowering (i.e., sexual reproduction) in *L. minor* is rare and occurs only under changing environmental conditions. Photoperiod and high temperatures have been associated with flowering (Landolt, 1957 in Hillman, 1961). Current knowledge indicates that a frond produces only one flower in its lifetime. The flower arises in or near the same meristematic area that produces daughter fronds (Hillman, 1961). Each flower consists of a single flask-shaped pistil (which matures first) and 1 or 2 stamens (which mature at different rates) (Hillman, 1961; Newmaster *et al*., 1997). These organs are surrounded during development by a membranous sack-like "spathe" open at the top (Hillman, 1961).

The fruit of *L. minor* is symmetrical, ovoid or ellipsoid, and wingless, and the seed is deeply and unequally 12- to 15-ribbed, with a prominent protruding hilum (Britton and Brown, 1970; Godfrey and Wooten, 1979).

Appendix F

Axenic Culture Techniques for *Lemna* (Acreman, 2006)

Various species of *Lemna* (duckweed), vascular, aquatic macrophytes belonging to the Lemnaceae family, can be grown under axenic conditions in liquid media or on nutrient agar using methods similar to those for plant tissue culture. Axenic cultures are free of any contaminants and are literally "without strangers". Good sterile technique and the proper use of a laminar flow hood are essential for axenic culturing of *Lemna.* Careful monitoring of the cultures and regular testing for contamination is crucial. *A basic rule when working with all axenic cultures is to treat the workspace for manipulation of the cultures as you would a surgical operating area.* An axenic culture is valuable and if it becomes contaminated, the contamination is not always easy to eliminate. Always make multiple subcultures of the plants to help ensure that at least one or more of them will remain sterile. Tips provided here should help to reduce the potential for contamination of the cultures.

Maintaining a Clean Laboratory

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

Laminar Flow Hood: Operation and Maintenance

The use of a laminar flow hood is very important to maintaining axenic cultures and good maintenance procedures are critical to the performance of the hood. Handling axenic cultures without such a hood means risking contamination in the long term. Inexpensive hoods costing in the range of $1000-$3000 are available from Envirco Corporation, 1185 Mt. Aetna Road, Hagerstown, MD 21740, USA, (Tel: 1-800-645-1610).

The most important part of a laminar flow hood is a High Efficiency Particulate Air filter (HEPA). Room air is taken into the unit and passed through a pre-filter to remove gross contaminants (lint, dust etc). The air is then compressed and channeled up behind and through the HEPA filter in a laminar flow fashion. The purified air flows out over the entire work surface in parallel lines at a uniform velocity. The HEPA filter is about 99% efficient in removing bacteria and fungal spores of > 22 microns from the air. HEPA filters should be replaced approximately every 7 years for best performance. Routinely check the filter for cracks or damage by sharp instruments. The flow velocity patterns should also be checked annually by a filter service company professional (e.g. H.E.P.A. Filter Services Inc. Tel: 1(800) 669-0037) for any blocked or damaged areas.

If no testing service is available or your budget cannot accommodate the cost of testing, the hood can also be checked for efficiency by using sterility test agar plates (for description of plate preparation see the section below "Testing *Lemna* for sterility"). It is good practice to periodically check the hood efficiency using this method in between checks by a filter specialist. Spread the plates across the center of the bench and leave them open for at least 24 hours with the hood running. Note the position of each numbered plate. Close the plates, seal them with a double layer of Parafilm and leave in a warm dark location for at least 5 days to monitor for bacterial or fungal growth. If your test indicates that some areas of the HEPA filter are defective, it is possible to repair the filter by injecting silicone sealant if the damaged areas are small. Large patches will cause some air turbulence in the workspace. Ideally the repairs should be done by a company that specializes in HEPA filtered equipment.

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

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

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

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

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

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

Sterilization of Loops and Other Instruments

Bunsen burners and other continuous flame gas burners are effective but can produce turbulence, which disturbs the protective airflow patterns of the laminar flow cabinet, and additionally, the heat produced by the continuous flame may damage the HEPA filter. If a gas burner must be used, one with a pilot light should be selected and the burner should not be closer than 20 cm from the HEPA filter. Electric sterilizers may also be considered. Alternatively, disposable plastic loops and needles may be used for culture work where electric incinerators or gas flames are not available

Hand Cleaning

Before performing any manipulations or subculturing, remove any rings and wash hands thoroughly with an antibacterial soap followed by a cleanser e.g. One-Step, Endure or 70 % ethanol. Pay attention particularly to the areas of your hands that may come in contact with the culture vessels or transfer loops. Examination gloves (e.g. Nitrile) may be used and sprayed with ethanol before handling cultures.

Preparation and Sterilization of Media

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

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

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

Bottles and tubes containing media should be no more than 2/3 full to prevent boiling over. If using screw capped media bottles leave the caps slightly unscrewed. Flasks can be loosely plugged with a bung

made of non-absorbent cotton wool covered with cheesecloth and with a square "skirt" of either Bio-Shield Wrap (VWR 59100 -234) or aluminum foil over the top. After autoclaving, the pressure release valve on the autoclave should not be opened until the temperature has cooled to below 80˚C. As the pH of media rises during autoclaving, allow at least one day before using the media in order for the pH to readjust to the level set prior to sterilization.

Autoclaving is a process that may have negative effects on media as components may be broken down on prolonged exposure to heat. Precipitates of phosphate (white) or iron (yellow) may occur at times. To avoid this problem the iron and phosphate solutions can be sterilized separately and added aseptically after autoclaving. Precipitates in media may also be avoided by filter-sterilizing using filters of pore size 0.22 microns or smaller.

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

Transfer Techniques

The following procedures should always be used when transferring cultures:

- All culture vessels, transfer tools, cotton-plugged pipettes and media must be sterilized and ready to use. Media should be at room temperature.
- Loops should be first dipped in 95% Ethanol and then sterilized in a flame or electric sterilizer for 15 seconds until they are red-hot before use. Cool the loop by touching it to sterile agar or liquid before using it to pick up the plants. The flame from a gas burner effectively sterilizes small glass or metal objects, such as inoculating loops, but one must avoid "frying" the plants by contact with objects heated in a flame.
- Clear the laminar flow hood so that nothing is between the path of the airflow coming from the HEPA filter and the area where the subculture is being done. Do not allow anything to come in contact with the HEPA filter.
- Clean the bench of the laminar flow hood thoroughly just before use but avoid spraying any solutions on the HEPA filter.
- Wash hands thoroughly or put on gloves (see above) immediately prior to subculturing.
- Flame all openings of glass culture vessels for 15 seconds before and after transferring the new culture material to them.
- To minimize contamination, always carry out the transfers at least 6 inches (15 cm) from the front of the hood to ensure that the area is not contaminated by room air. Where possible, perform the operation at eye level.
- Don't touch anything that will come in contact with the culture and if you do touch it, sterilize it again before using it.
- When subculturing to screw-capped tubes, loosen the caps slightly before picking up the plants to be transferred to prevent the plants from falling from the loop while opening tightly sealed tubes.

- Avoid talking, singing, whistling, coughing or sneezing in the direction of things that should be sterile. Long hair, if not tied back, may be a source of contamination.
- Work quickly to minimize the time that the culture vessels are open.
- Try not to touch the edges of the Petri plate covers. Hold the cover by the top.
- Seal all Petri plates with a double layer of Parafilm or Duraseal. Monitor carefully for cracks. (Dust mites are attracted to the smell of the media and may crawl into the sterile plates.)
- Monitor plates every 2-3 days for presence of contaminants.
- Transfer the cultures every 2-3 weeks for best results.

Testing Lemna for Sterility

Contaminants such as bacteria and fungi are readily apparent when *Lemna* is cultured in a medium enriched with organic components e.g. Hoagland's E+. If the plants are not cultured routinely in such medium they should be periodically tested for sterility by removing a few plants and placing them in Hoagland's E+, which contains 1% sucrose, 0.6% Bacto-tryptone (or peptone) and 0.1% yeast extract. This can be done in liquid culture or on agar plates. Contamination by fungi and bacteria will usually show up in solutions or on agar plates within several days. If the solution becomes cloudy or colonies of bacteria or fungi grow on the plates you can try the cleaning technique described below or obtain a new *axenic culture* from an outside source.

Cleaning Lemna Plants

If *Lemna* plants become contaminated they can be made sterile again but the techniques require time and patience. In order to do this, plants connected in clonal clusters should be separated from each other. Individual plants should be dipped in a 0.5% solution of sodium hypochlorite (10% Clorox® or Purex® bleach solution) for at least one minute. Treat plants with bleach for varying amounts of time to ensure that you have at least one living culture that is sterile. Be sure to rinse the plants in several changes of sterile medium or sterile water before transferring to dilute growth medium (e.g. modified Hoagland's medium containing 1% sucrose). Examine your plants after rinsing them in fresh medium. Properly sterilized plants will have a small green area in the bud zone along the center of the frond. If there is no green bud remaining, the plant was treated too long and is dead. Since only a small bud is left to re-grow after surface sterilization, it may take some time before sufficient plant material is available to do experiments.

According to Landolt (1987), about 1-10% of the plants normally survives this treatment and becomes axenic. Plants that do survive this sterilization technique (and are not contaminated or infected by fungal molds or bacteria) can be transferred to an enriched medium such as Hoagland's E+ in liquid form or solidified with 1.25 % Difco-Bacto agar in Petri plates or tubes.

Long Term Preservation of Lemna by Cryopreservation

Cryopreservation is a technology to store living cells at ultra-low temperatures indefinitely. Valuable strains of *Lemna* can be maintained at ultra-low temperatures in the liquid or vapour phase of liquid nitrogen to preserve their genetics and to maintain the cultures over long terms without maintenance through subculturing (Day 1995; Kartha 1985).

The techniques used must minimize the formation of destructive intracellular ice crystals which damage cell membranes and walls. The basic procedures of cryopreservation involve removal of the free water

by osmotic agents followed by addition of cryoprotectants such as sucrose and glycerol. Cultures are stored in cryovials and then may be slowly cooled in a –80ºC freezer to minimize ice crystal formation, followed by immersion directly into liquid nitrogen at –196º C. Cultures are regenerated by rapid thawing in a water bath at 45ºC and subcultured to fresh medium.

For further information, please refer to the following websites:

Armstrong, Wayne. Treatment of the Lemnaceae. Palomar University
http://waynesword.palomar.edu/1wayindx.htm

Cross, John. The Charms of Duckweed.
http://www.mobot.org/jwcross/duckweed/duckweed-charms.htm

McCauley, D. Aseptic technique. GlobalRPh Inc.
http://www.globalrph.com/aseptic.htm

APPENDIX 1

Solutions for Disinfecting Surfaces

1 % sodium hypochlorite solution (0.5 L)

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

Chlorinated solution from powder

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

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

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

Appendix G

Logarithmic Series of Concentrations Suitable for Toxicity Tests[a]

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

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

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

Appendix H

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

Title of Biological Test Method or Guidance Document	Report Number	Publication Date	Applicable Amendments
A. Generic (Universal) Biological Test Methods			
Acute Lethality Test Using Rainbow Trout	EPS 1/RM/9	July 1990	May 1996
Acute Lethality Test Using Threespine Stickleback (*Gasterosteus aculeatus)*	EPS 1/RM/10	July 1990	March 2000
Acute Lethality Test Using *Daphnia* spp.	EPS 1/RM/11	July 1990	May 1996
Test of Reproduction and Survival Using the Cladoceran *Ceriodaphnia dubia*	EPS 1/RM/21 2nd Edition	February 2007	—
Test of Larval Growth and Survival Using Fathead Minnows	EPS 1/RM/22	February 1992	November 1997
Toxicity Test Using Luminescent Bacteria (*Photobacterium phosphoreum*)	EPS 1/RM/24	November 1992	—
Growth Inhibition Test Using a Freshwater Alga	EPS 1/RM/25 2nd Edition	March 2007	—
Acute Test for Sediment Toxicity Using Marine or Estuarine Amphipods	EPS 1/RM/26	December 1992	October 1998
Fertilization Assay Using Echinoids (Sea Urchins and Sand Dollars)	EPS 1/RM/27	December 1992	November 1997
Toxicity Tests Using Early Life Stages of Salmonid Fish (Rainbow Trout, Coho Salmon, or Atlantic Salmon)	EPS 1/RM/28 1st Edition	December 1992	January 1995
Toxicity Tests Using Early Life Stages of Salmonid Fish (Rainbow Trout)	EPS 1/RM/28 2nd Edition	July 1998	—
Test for Survival and Growth in Sediment Using the Larvae of Freshwater Midges (*Chironomus tentans* or *Chironomus riparius*)	EPS 1/RM/32	December 1997	—

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

Title of Biological Test Method or Guidance Document	Report Number	Publication Date	Applicable Amendments
A. Generic (Universal) Biological Test Methods (cont'd.)			
Test for Survival and Growth in Sediment Using the Freshwater Amphipod *Hyalella azteca*	EPS 1/RM/33	December 1997	—
Test for Measuring the Inhibition of Growth Using the Freshwater Macrophyte, *Lemna minor*	EPS 1/RM/37 2nd Edition	January 2007	—
Test for Survival and Growth in Sediment Using Spionid Polychaete Worms (*Polydora cornuta*)	EPS 1/RM/41	December 2001	—
Tests for Toxicity of Contaminated Soil to Earthworms (*Eisenia andrei, Eisenia fetida*, or *Lumbricus terrestris*)	EPS 1/RM/43	June 2004	—
Tests for Measuring Emergence and Growth of Terrestrial Plants Exposed to Contaminants in Soil	EPS 1/RM/45	February 2005	—
Test for Measuring Survival and Reproduction of Springtails Exposed to Contaminants in Soil	EPS 1/RM/47	2006	—
B. Reference Methods[b]			
Reference Method for Determining Acute Lethality of Effluents to Rainbow Trout	EPS 1/RM/13 1st Edition	July 1990	May 1996, December 2000
Reference Method for Determining Acute Lethality of Effluents to Rainbow Trout	EPS 1/RM/13 2nd Edition	December 2000	—
Reference Method for Determining Acute Lethality of Effluents to *Daphnia magna*	EPS 1/RM/14 1st Edition	July 1990	May 1996, December 2000
Reference Method for Determining Acute Lethality of Effluents to *Daphnia magna*	EPS 1/RM/14 2nd Edition	December 2000	—
Reference Method for Determining Acute Lethality of Sediment to Marine or Estuarine Amphipods	EPS 1/RM/35	December 1998	—
Reference Method for Determining the Toxicity of Sediment Using Luminescent Bacteria in a Solid-Phase Test	EPS 1/RM/42	April 2002	—

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

Title of Biological Test Method or Guidance Document	Report Number	Publication Date	Applicable Amendments
C. Supporting Guidance Documents			
Guidance Document on Control of Toxicity Test Precision Using Reference Toxicants	EPS 1/RM/12	August 1990	—
Guidance Document on Collection and Preparation of Sediment for Physicochemical Characterization and Biological Testing	EPS 1/RM/29	December 1994	—
Guidance Document on Measurement of Toxicity Test Precision Using Control Sediments Spiked with a Reference Toxicant	EPS 1/RM/30	September 1995	—
Guidance Document on Application and Interpretation of Single-Species Tests in Environmental Toxicology	EPS 1/RM/34	December 1999	—
Guidance Document for Testing the Pathogenicity and Toxicity of New Microbial Substances to Aquatic and Terrestrial Organisms	EPS 1/RM/44	March 2004	—
Guidance Document on Statistical Methods for Environmental Toxicity Tests	EPS 1/RM/46	March 2005	—